ASGCT 28th Annual Meeting Abstracts

Oral Abstracts

Presidential Symposium

1 CRISPR-hfCas12Max Genome Editing Therapy Demonstrates Preclinical Efficacy and Early Clinical Benefit in Duchenne Muscular Dystrophy

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Background: Duchenne muscular dystrophy (DMD) is a severe X-linked neuromuscular disease caused by mutations in the *DMD* gene, resulting in progressive muscle degeneration and premature mortality. Current therapies, including antisense oligonucleotides such as Casimersen (targeting exon 45) and Eteplirsen (targeting exon 51), achieve dystrophin restoration to < 2% of normal levels after 1-year with the burden of weekly administration. Similarly, AAV-micro-dystrophin gene therapies require high doses, often leading to adverse events such as liver toxicity, immune-related myositis, and myocarditis. To address these limitations, we developed HG302, a one-time, CRISPR-high-fidelity(hf)Cas12Max-based single-cut genome editing therapy designed to induce exon skipping and restore dystrophin expression with minimal off-target effects. Preclinical studies demonstrated robust safety and efficacy, and HG302 is now being evaluated in the MUSCLE clinical trial to assess its therapeutic potential in DMD patients.

Methods: A novel humanized DMD mouse model carrying human exon 51 was generated to evaluate single-cut genome editing using human sgRNAs and Cas variants in vivo. HG302, a single adeno-associated virus (AAV) vector delivering hfCas12Max and a guide RNA targeting the splice donor (SD) site of human exon 51, was evaluated for in vitro and in vivo editing efficiency, dystrophin restoration, and muscle function. Preclinical studies were conducted in both *DMD* mice (**Fig.1A**) and wild-type non-human primates (NHPs) (**Fig.1G**) to assess safety and efficacy. The ongoing **MUSCLE** trial (an open-label, **M**ultidose, dose-escalation study to **U**nderstand the **S**afety of **C**RISPR gene-editing therapy and its long-**L**asting **E**ffects in DMD patients; NCT06594094) is enrolling ambulant DMD boys aged ≥4 years with impaired muscle function to evaluate the safety, tolerability, and clinical efficacy of HG302 (**Fig. 2**).

Results: The humanized *DMD* mouse model exhibited severe DMD disease phenotypes, making it suitable for in vivo evaluation of CRISPR-based therapies. HG302 achieved efficient exon skipping in vitro, targeting the SD site of human DMD exons 51. Systemic AAV delivery of HG302 in DMD mice restored dystrophin protein levels to up to 70% of wild-type levels (**Figs.1C** and **1D**) and significantly improved motor functions, as evidenced by enhanced rotarod performance and grip strength (**Figs.1E** and **1F**). In NHPs, low-dose systemic HG302 administration confirmed efficient genome editing across muscle tissues (**Figs.1H** and **1I**). Safety evaluations revealed no abnormalities in liver enzymes (ALT, AST) or kidney function in either DMD mice or NHPs. In the **MUSCLE** trial (**Fig.2**), the first DMD boy dosed with HG302 demonstrated favorable safety and tolerability, with no observed elevations in ALT, AST, total bilirubin, or thrombotic microangiopathy. Early clinical data at a low AAV dose (E13

vg/kg) demonstrated improved motor function post-injection, outperforming reported outcomes for AAV micro-dystrophin gene therapies.

Conclusions: CRISPR/hfCas12Max-mediated single-cut genome editing therapy offers a transformative, "one-and-done" therapeutic approach for DMD. It provides significant advantages over existing treatments in safety, efficacy, and durability. The U.S. FDA has recognized HG302 with Orphan Drug Designation and Rare Pediatric Disease Designation, underscoring its potential to revolutionize DMD therapy and improve patient outcomes.

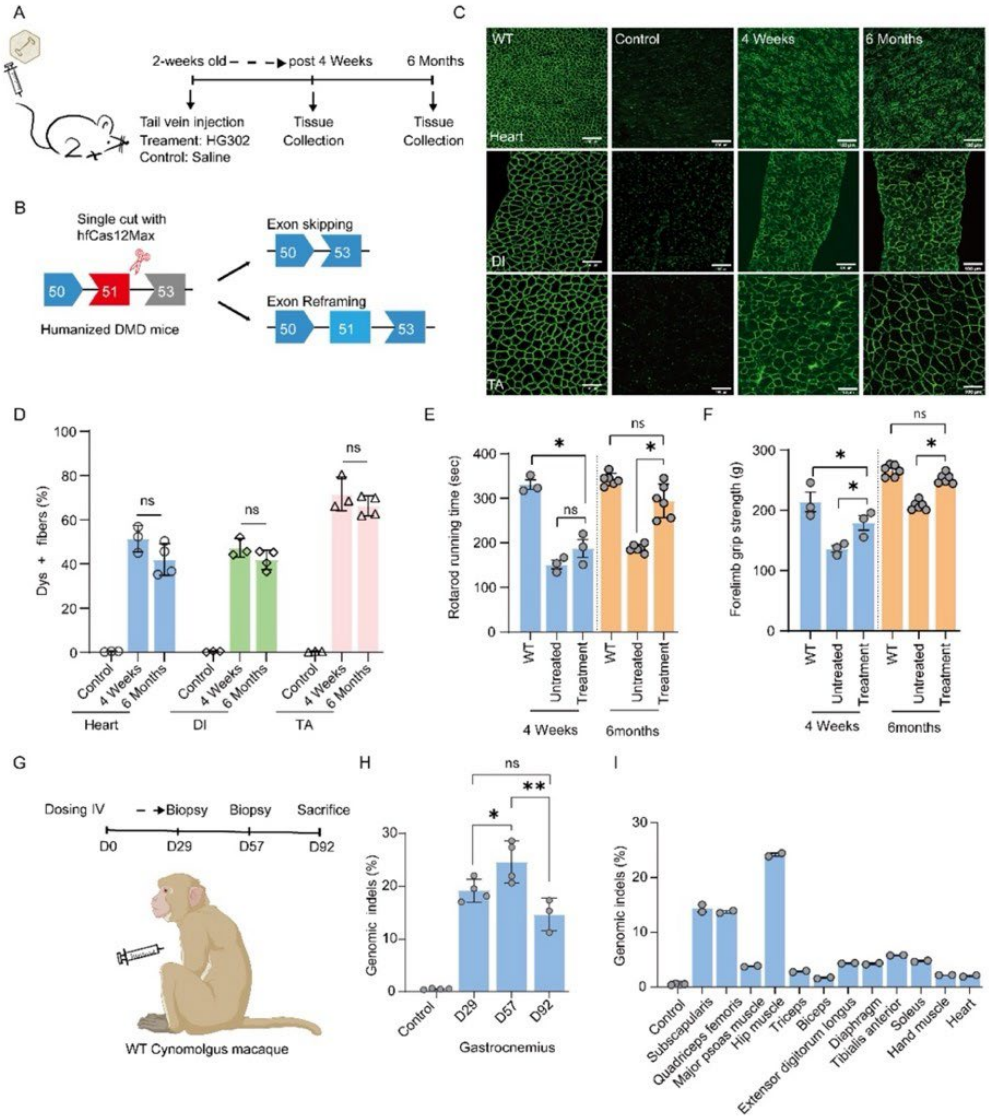

Figure 1. Intravenous delivery of the hfCas12Max system in humanized *DMD* mice and NHP.
(**A**) Schematic of intraperitoneal injection of AAV particles (HG302). HG302 was injected intravenously into 2-week-old $DMD^{\Delta mE51E52,hE51KI}$ mice. (**B**) Schematic of the therapeutic single-cut strategy. (**C**) Immunohistochemistry for dystrophin in TA, DI, and heart of $DMD^{\Delta mE51E52,hE51KI}$ mice was performed at indicated time points post-treatment. Dystrophin is shown in green. (**D**) Quantification of Dys+ in cross sections of TA, DI, and heart muscles (n=3-6). Rotarod rod (**E**) and forelimb grip strength (**F**) were measured in WT, $DMD^{\Delta mE51E52,hE51KI}$ mice, and $DMD^{\Delta mE51E52,hE51KI}$ mice treated with all-in-one AAV particles (n=3-6). (**G**) Systemic delivery of low dose HG302 on 1-year-old wild-type cynomolgus macaque. Black nodes indicate time points for tissue collection after intravenous injection. (**H**) Gastrocnemius muscle was processed D29, D57and D92 later by biopsy for editing efficiency analysis. D, Day. (**I**) Editing efficiency analysis (D92) in different tissues of wild-type cynomolgus macaque.

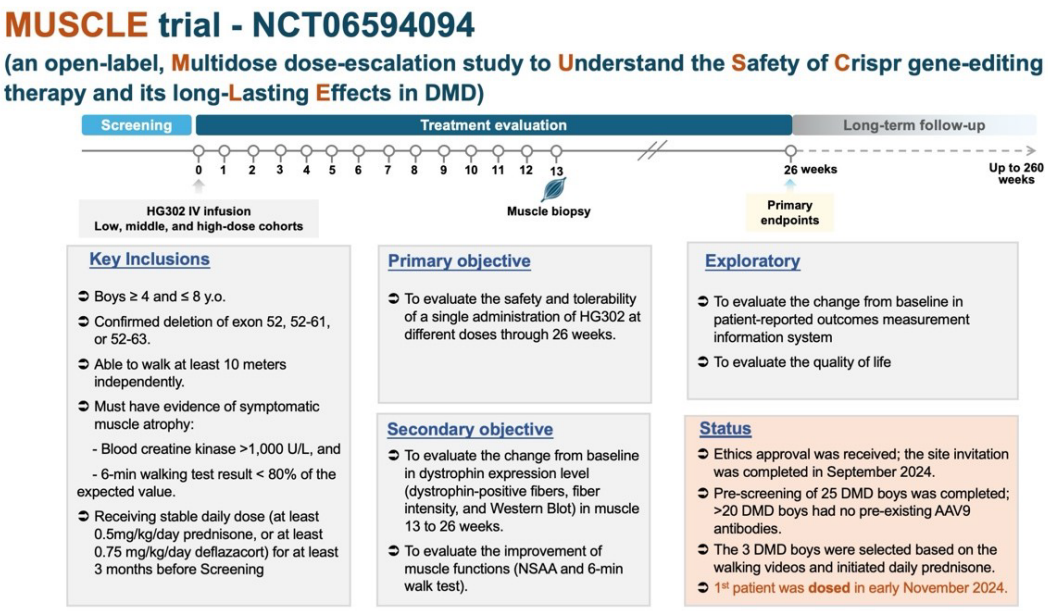

Figure 2. MUSCLE clinical trial study design and status.

2 Sustained Brain-wide Reduction of Prion via Zinc Finger Repressors in Mice and Nonhuman Primates as a Potential One-Time Treatment for Prion Disease

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Prion disease is a progressive and fatal neurodegenerative disorder arising from misfolding of the normal cellular prion protein (PrP^{C}) into a pathogenic "Scrapie" form (PrP^{Sc}). There are at least 1300 new cases each year in the US and Europe. While there is currently no approved treatment for prion disease, the histopathological, biochemical, and genetic evidence all converge on PrP^{C} as the primary drug target for treating prion disease. We are developing a one-time, intravenously (IV) administered AAV-based therapy using a zinc finger repressor (ZFR) epigenetic regulator to achieve sustained lowering of prion protein (PrP^{C}) in neurons throughout the brain as a potential therapeutic for prion disease. We conducted pharmacology and efficacy studies to investigate the dose-response relationship between prion repression and survival across a 10-fold AAV dose range (1E+13 to 1E+14 vg/kg) and used a combination of bulk and single-cell measurements to assess the relationship between pharmacology and efficacy. In C57BL/6 mice, brain-wide neuron-selective ZFR expression mediated up to 50% bulk level mRNA repression of mouse prion gene, *Prnp*, and 45% PrP protein reduction in a dose-dependent manner. Moreover, ZFRs were stably expressed and lowered neuronal *Prnp* expression throughout the central nervous system for at least 17 months with no diminution of effect. At the single-cell level, near complete repression was observed in multiple brain regions in ZFR+ cells at all AAV doses tested, indicating that the dose-dependent repression observed at the bulk level is primarily driven by the fraction of neurons transduced. In a parallel efficacy study, C57BL/6 mice were

inoculated with Rocky Mountain Laboratory (RML) prions and dosed with an AAV-ZFR at these same doses and at different timepoints during the disease course. A significant and dose-dependent increase in median survival was observed for all AAV-ZFR treatment groups, even in mice exhibiting clinical signs of prion disease at the dosing time, with many mice surviving until the planned endpoint of the study (500 days post RML inoculation).

We next used a single IV dose of a novel BBB-penetrant AAV capsid (STAC-BBB) to deliver a single construct expressing green fluorescent protein (GFP) and a human *PRNP*-targeted ZFR to nonhuman primates (NHPs). Neuronal transduction efficiency ranged from ~26-65% across a range of cortical and subcortical regions. We detected ZFR expression and *PRNP* mRNA reduction in all 35 brain regions analyzed. At the single-cell level, STAC-BBB mediated brain-wide ZFR expression and significant *PRNP* repression in transduced neurons across prion disease relevant brain regions, including the postcentral gyrus (82.5%), precentral gyrus (90%), supramarginal gyrus (80%), hippocampus (90.5%), midbrain (72%), putamen (95%), caudate (95%), and thalamus (82%). Critically, AAV transduction and *PRNP* repression levels observed in the NHP study were similar to those associated with profound survival benefits in RML disease mice, supporting the translational potential of STAC-BBB delivered ZFRs for the potential treatment of prion disease.

3 Comet LV: a lentiviral vector-based mRNA co-packaging technology for enhanced *ex vivo* and *in vivo* gene therapy

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Background

Lentiviral vectors (LVs) are versatile tools for genetic engineering, capable of stably transducing both dividing and non-dividing cells. LVs have been traditionally used to integrate foreign genetic material into the host's genome, enabling its stable expression. However, persistent transgene expression may be undesirable in some applications, such as those involving toxic payloads or selectors of transduced cells. To address this limitation, we developed Comet LV, a novel LV-based platform which enables the simultaneous delivery of transiently and stably expressed genetic payloads to targeted cell populations.

Materials and Methods

Comet LVs incorporate RNA-binding proteins localized to the viral envelope via a membrane-targeting domain. In turn, these proteins bind mRNAs of interest thanks to specific aptamer-protein interactions, promoting their packaging into the viral particle. Thus, Comet LVs can be employed to co-deliver mRNAs of interest in vitro to cell lines and primary cells, or in vivo to mice by intravenous injection. The expressions of the transgenes encoded by either the integrated viral vector or the co-packaged mRNA was evaluated by flow cytometry or ELISA.

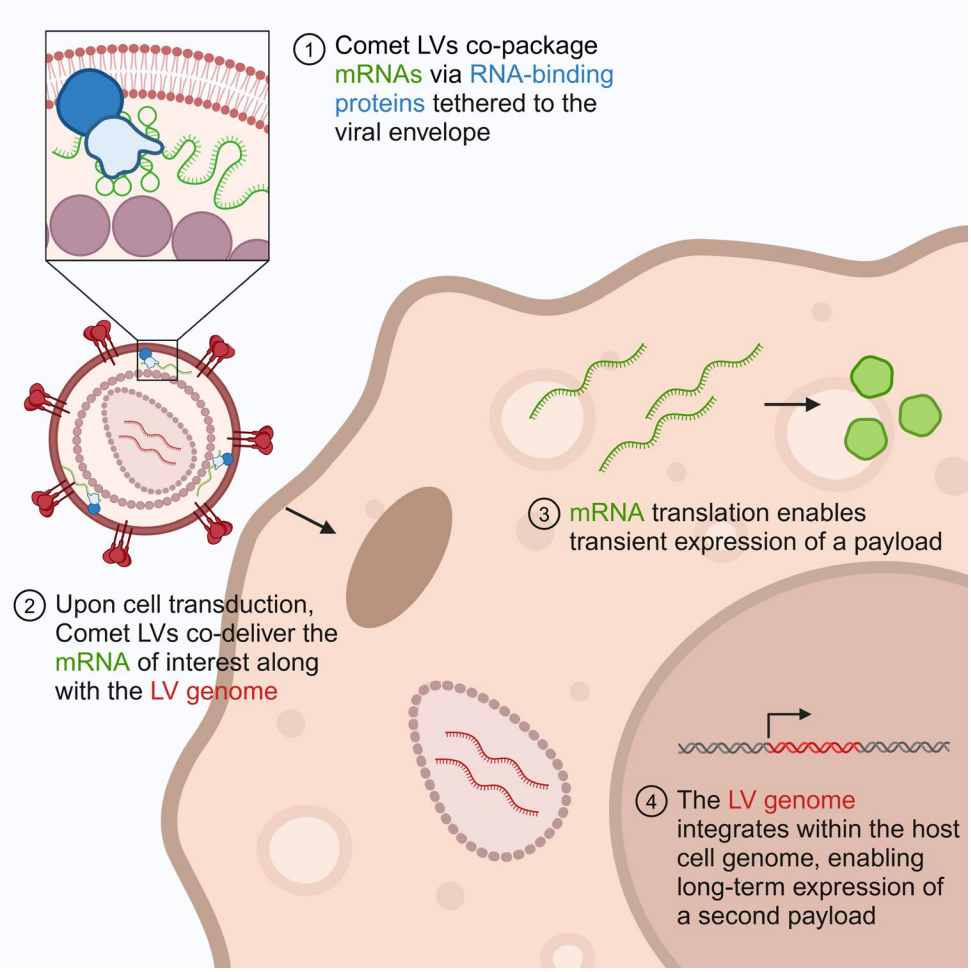
Results

We employed Comet LVs for in vivo antitumor vaccination, delivering a tumor antigen (TA) mRNA alongside an integrating cassette encoding IFNa, an immune-stimulating cytokine. Mice injected with these vectors presented elevated circulating IFNa levels comparable with standard vectors and developed TA-specific T cells.

Additionally, we employed Comet LVs to deliver CXCR4 mRNA to hematopoietic stem and progenitor cells (HSPCs) while transducing them with a corrective integrating payload. Of note, transient CXCR4 expression enhances HSPC engraftment, enabling a chemotherapy-free, mobilization-based conditioning regiment. HSPCs transduced with Comet LVs displayed transient CXCR4 overexpression compared to untreated cells.

Conclusions

Our system couples the benefits of mRNA transient expression with LV potential for stable transduction and favorable safety profile. This approach offers a modular, customizable tool for cell engineering that can be especially useful in applications requiring joint short and long-term transgene expression. For instance, Comet LV could prove useful for antitumor vaccination and enhanced engraftment of edited HSPCs. Furthermore, this platform could improve genome editing strategies by enabling temporary expression of gene editors delivered alongside corrective IDLV templates. For these reasons, Comet LVs represent an innovative tool that could significantly advance both therapeutic and research applications in genetic engineering.



Clinical Trial Spotlight Symposium

4 Treatment effect of atidarsagene autotemcel (arsa-cel) in age-matched treated vs. untreated sibling pairs with early-onset metachromatic leukodystrophy (MLD)

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INTRODUCTION

Metachromatic leukodystrophy (MLD) is a rare neurodegenerative lysosomal storage disease caused by a deficiency of arylsulfatase A (ARSA) enzyme that leads to rapid motor and cognitive decline and premature death in untreated patients. Atidarsagene autotemcel (arsa-cel) is a hematopoietic stem cell gene therapy consisting of autologous CD34+ cells transduced *ex vivo* with a lentiviral vector encoding the human *ARSA* gene. Primary efficacy and safety data have previously been presented for 39 early-onset MLD patients (consisting of late infantile [LI] and early juvenile [EJ] subtypes) treated with arsa-cel, compared to 49 patients from a natural history (NHx) cohort; however, no specific analysis of paired treated and untreated siblings has been presented. Sibling pair analyses undertaken to estimate treatment effect in patients with any genetic disease remove some of the inherent uncertainty related to variability in disease progression due to different genotypes or environmental influences. This analysis compares clinical outcomes for age-matched arsa-cel treated vs. untreated NHx sibling pairs.

METHODS

Twenty-five patients treated pre-symptomatically in the arsa-cel clinical development program (CDP) had a sibling in the NHx cohort (16 LI pairs; 9 EJ pairs); all were identified because of their older untreated symptomatic sibling's diagnosis. Pairs of siblings were included in the analysis if a gross motor function assessment (GMFC-MLD or GMFM-88) for the NHx siblings was performed at an age of assessment within +/- 3 months of the treated sibling, and the treated sibling had been followed up for at least 2 years post gene therapy. The latest matched assessments were used to achieve the longest follow-up (range: age at assessment 30.6-106.7 months).

RESULTS

Of the age-matched sibling pairs (N=22), 86.4% (n=19) had a difference of at least 4 levels in the GMFC-MLD scale in favor of arsa-cel (Wilcoxon signed rank test for difference in GMFC-MLD levels $p<0.001$) at the last age-matched assessment. Specifically, 86.4% (n=19) of the treated siblings retained the ability to walk independently (GMFC-MLD level 0 or 1), while 81.8% (n=18) of NHx LI and EJ siblings had lost all gross motor function (GMFC-MLD level 6).The mean (95% CI) absolute difference in total GMFM-88 score for all sibling pairs (N=11) was 76.3 (58.4, 94.3) in favor of arsa-cel ($p<0.001$). This difference was more pronounced in the LI subtype where the mean absolute difference was 84.9. Table 1 presents gross motor function outcomes for the sibling pairs by MLD subtype.

CONCLUSION

The results for the subset of sibling pairs align with the previously presented data from the presymptomatic LI and EJ CDP population vs. the NHx cohort. Given the stark difference in motor outcomes between the treated and untreated sibling pairs, this analysis reinforces the importance of early diagnosis through newborn screening to enable all early-onset patients to have the opportunity for pre-symptomatic treatment, not just the younger siblings of symptomatic patients.

Clinical trial registration: NCT01560182, NCT03392987, EudraCT 2009-017349-77, EudraCT 2017-001730-26

Table 1: Gross motor function outcomes for LI and EJ MLD sibling pairs by MLD subtype

Subtype	Proportion with difference in GMFC-MLD level of ≥ 4			Absolute difference in total GMFM-88 score		
	N	n (%)	P value [a]	N	Score (95% CI)	P value [b]
Late infantile	15	14 (93.3%)	p<0.001	8	84.9 (77.3, 92.5)	p<0.001
Early juvenile	7	5 (71.4%)	p=0.016	3	53.6 (-62.9, 100)	p=NS

[a] Wilcoxon signed rank test; [b] paired t-test; N = number of sibling pairs with age-matched data

5 RESET-Myositis™: Clinical Trial Evaluating Rese-cel (Resecabtagene Autoleucel), A Fully Human, Autologous 4-1BB Anti-CD19 CAR T Cell Therapy in Idiopathic Inflammatory Myopathies: Correlative Findings

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Background:
Current therapies for idiopathic inflammatory myopathy (IIM) aim to control inflammation and disease activity and decrease long-term morbidity and mortality but many do not provide durable clinical responses without requiring chronic immunosuppressive drugs. CD19-targeting chimeric antigen receptor (CAR) T cells have achieved durable, drug-free responses in IIM patients in an academic program. Rese-cel (formerly referred to as CABA-201) is a fully human, autologous 4-1BB anti-CD19-CAR T cell therapy, designed to deeply and transiently deplete CD19 positive B cells following a one-time weight-based infusion of $1x10^6$ CAR T cells/kg. This approach may enable durable responses in patients without the need for chronic immunosuppression. RESET-Myositis™ (NCT06154252) is an ongoing Phase 1/2 trial evaluating rese-cel in three independent cohorts of adults with IIM (immune mediated necrotizing myopathy (IMNM), dermatomyositis (DM), and antisynthetase syndrome (ASyS)), as well as a fourth cohort of patients with juvenile IIM (JIIM).

Results:
Here we report on the first three patients with IIM treated with rese-cel that have completed at least 1 month of follow-up in the RESET-Myositis™ trial (IMNM-1, IMNM-2 and DM-1). Rese-cel has been well-tolerated with no dose-limiting toxicity, cytokine release syndrome (CRS) or immune effector cell-associated neurotoxicity syndrome (ICANS) in these patients with at least 1 month of follow-up.

Infusion products consisted of predominantly CD4+ CAR T cells and exhibited in vitro cytolytic activity towards a NALM6 target cell line. Leukocyte and lymphocyte counts dropped quickly and transiently after a standard preconditioning regimen of fludarabine ($25mg/m^2$/day x 3) and cyclophosphamide ($1000mg/m^2$) which inversely corresponded with a transient elevations in serum IL-15 levels in all patients. Following infusion of rese-cel, CAR T cells peaked in circulation between days 8 and 15 (C_{max}). CAR T cells were undetectable peripherally by day 29 for

IMNM-1 and IMNM-2 and remained detectable at low levels for up to 12 weeks for DM-1. Serum IFN-γ levels increased prior to or in parallel with rese-cel C_{max} and CAR T cells exhibited a predominantly CD8+ phenotype at C_{max}. B cells were rapidly depleted by 15 days post rese-cel infusion and remained undetectable through the first month in all 3 patients. Repopulation with transitional naïve B cells ($CD19^{+}CD20^{+}CD38^{++}CD24^{++}$) occurred at 8 weeks post-infusion in IMNM-1 and DM-1. B cells were detected at low levels in IMNM-2 at 12 weeks following rese-cel infusion. Antibodies associated with infectious diseases and vaccinations remained generally stable in patients, whereas serum levels of myositis-specific autoantibodies, including SRP-54, SAE1/2, and HMGCR decreased over the follow-up period in IMNM-1, DM-1, and IMNM-2, respectively. To date, all patients have had increases in their total improvement scores despite discontinuation of all immunosuppressants at the time of infusion, a clinically validated scoring system used to assess improvements in IIM patients.

Conclusions:
These emerging data detail the pharmacokinetics and pharmacodynamics of rese-cel in adult patients with IIM, supporting the potential for rese-cel to induce durable, drug-free remissions. Evaluation of the safety and efficacy of rese-cel continues in the ongoing Phase 1/2 RESET-Myositis™ trial.

6 Extensive detoxification and favorable effects on systemic clinical outcomes after Hematopoietic Stem Cell Gene Therapy for Mucopolysaccharidosis Type I-Hurler (OTL-203)

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Preliminary results of a phase I/II study of OTL-203, an autologous hematopoietic stem cell gene therapy (HSC-GT, NCT03488394), in 8 patients (6M, 2F; mean ±SD age at treatment: 1.9±0.5 years) with mucopolysaccharidosis type I-Hurler (MPS-IH) suggest superior metabolic correction compared to an external cohort treated with allogeneic hematopoietic stem cell transplantation (allo-HSCT) and initial clinical response in several organs (Gentner *et al. N Engl J Med* 2021; *Consiglieri et al. Sci Transl Med 2024*). Here we report interim multi-system clinical outcomes at a median follow-up of 5 years post-HSC-GT (range 4-5 years).

IDUA activity was measured in blood and cerebrospinal fluid (CSF) and glycosaminoglycans (GAGs) in urine and CSF at baseline and regular timepoints up to 5-years post-treatment. Skeletal, neurological, corneal clouding (CC), carpal tunnel syndrome (CTS) and cardiac score were assessed at baseline and up to 5-years post-treatment. For specific outcomes OTL-203 patients were compared with retrospectively collected data from two external cohorts of MPS-IH patients who underwent allo-HSCT in two experienced transplant centres (N=9 and 14, respectively).

All OTL-203 treated patients are alive after a median follow-up of 5 years, without any evidence of anti-IDUA IgG antibody response or vector integration site-driven clonal dominance. At last evaluation, all patients continued to show sustained engraftment of gene-corrected cells and supraphysiologic IDUA activity in blood, resulting in extensive urinary GAG substrate clearance (dermatan sulfate (DS) to normal/near normal levels). In CSF, IDUA activity became detectable as early as 3 months post-OTL-203 and persisted over time, accompanied by extensive reduction of GAGs in CSF. All patients exhibited longitudinal growth within expected reference ranges according to age and gender, and experienced a complete and earlier normalization of joint mobility compared with allo-HSCT patients. Both hip X-ray and MRI parameters improved after OTL-203 treatment, indicating amelioration of hip dysplasia up to last follow-up. A specific spine MRI score showed stabilization of the typical spine MRI features at last follow-up. After OTL-203 treatment, 7/8 patients continued to gain motor and cognitive skills in line with

typically developing children and experienced an improvement/stabilization of typical brain MRI abnormalities. At baseline, all patients showed mild to moderate CC. Of the OTL-203 treated patients, 4/8 achieved CC resolution and 4/8 had mild CC at last follow-up. In the allo-HSCT cohort, no patient experienced CC resolution with all patients showing moderate CC at 5-years post-treatment. One OTL-203 patient underwent carpal tunnel surgery pre-treatment and another 6 months after OTL-203 due to pre-existing severe damage pre-treatment. The remaining patients did not develop CTS. In the allo-HSCT cohort 7/9 patients developed CTS, requiring surgery at a median of 3.7 years post-allo-HSCT. A specific echocardiac score showed worsening of cardiac involvement in allo-HSCT patients compared to OTL-203 patients who stabilized or improved their score.

Our results indicate sustained engraftment of gene-corrected cells, extensive detoxification of GAGs and favorable effects of OTL-203 on MPS-IH multi-systemic clinical manifestations up to 5-years after treatment. Long-term comparative studies are warranted for definitive conclusions.

7 Phase I Clinical Trial of Safety and Efficacy of the dAd5GNE Anti-Cocaine Vaccine

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Cocaine addiction is a major health issue for which there is no effective therapy. We have developed a vaccine (dAd5GNE) to treat cocaine addiction, based on the cocaine analog GNE linked to the capsid proteins of a disrupted serotype 5 adenovirus. The vaccine is designed to evoke anti-cocaine antibodies that sequester cocaine in the blood, preventing access to the CNS and blocking access to the cocaine cognate receptors, abrogating the reward mechanism. The dAd5GNE vaccine is a disrupted $E1^{-}E3^{-}$ serotype 5 Ad with GNE, a stable cocaine analog covalently linked to the Ad capsid proteins. In rodents and nonhuman primates, dAd5GNE evoked persistent, specific, high titer and high affinity anti-cocaine antibodies that prevent cocaine from reaching the brain, blocking cocaine-induced hyperactivity and limiting cocaine self-administration behavior with an acceptable safety profile for a clinical trial. Based on this data, we initiated a phase I, blinded, placebo controlled clinical trial where chronic cocaine addicts (average use of cocaine 3.9 gm/week) were immunized with dAd5GNE together with the Adjuplex® adjuvant. This interim report summarizes the assessment of dose 1 (100 μg intramuscular (IM) monthly x 6 months dAd5GNE n=7, placebo n=3) and dose 2 (316 μg IM monthly x 6 months dAd5GNE n=3, placebo n=3). Drug-related adverse events included minor arm soreness, transient fever and headache. Both doses evoked highly stable levels of anti-cocaine specific antibodies (100 μg dose, titer $2.4x10^5 \pm 1.2x10^5$, 316 μg dose, titer $3.6x10^5 \pm 2.1x10^5$). Assessment of the 2 dose cohorts demonstrated that for dAd5GNE vaccination *vs* placebo: (1) higher levels of anti-cocaine titers were associated with less craving ($p<0.02$); (2) cohort 1 had less craving and fewer days of cocaine use ($p<0.05$); (3) cohort 2 used less cocaine ($p<0.02$); and (4) cohort 2 was associated with more days in a row of no cocaine use ($p<0.02$). In summary, dAd5GNE has an excellent safety profile, high titer antibody response and initial measures of efficacy, suggesting it may be a useful vaccine for treatment of cocaine addiction.

8 Phase I/II Trial of Adipose Tissue Derived Mesenchymal Stem Cell Delivery of a Measles Virus Strain Engineered to Express the Sodium Iodine Symporter (MV-NIS) in Ovarian Cancer Patients

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Ovarian cancer is the most lethal gynecologic malignancy with an urgent need for development of novel therapeutics. Overexpression of the measles virus (MV) receptor CD46 and nectin-4 in ovarian cancer makes the latter an ideal target for MV based therapeutics. We conducted first in human trials of intraperitoneal administration

of the MV-NIS strain, and we demonstrated excellent safety and evidence of biologic activity as indicated by decreases in tumor marker CA125, dose dependent disease stabilization and promising median overall survival of 26.6 mo or higher in a heavily pretreated patient (pt) population (Galanis et al, Cancer Res). We hypothesized that MV delivery using adipose tissue derived mesenchymal stem cells (MSC) as carriers can further improve efficacy by facilitating viral homing in tumor sites and protecting the virus from antibody neutralization. Following proof-of-principle studies supporting efficacy in orthotopic ovarian cancer models and FDA guided toxicology and biodistribution studies demonstrating safety, we activated a phase I/II trial. Primary endpoints were to determine the maximum tolerated dose of MV infected MSC/characterize safety (Ph I) and evaluate efficacy (Ph II). Eligible ovarian cancer pts had platinum resistant or refractory disease, were immune to MV and had acceptable, hematologic liver and kidney function. A total of 11 pts were enrolled in the phase I trial, 4 at dose level 0 (10^7 MSC infected with 10^9 TCID50 MV-NIS) and 7 pts at dose level 1 (10^8 MSC infected with 10^9 TCID50 MV-NIS). Abdominal fat was harvested at the time of intraperitoneal catheter implantation and MSC were produced by the Mayo Clinic's Center for Regenerative Biotherapeutics. Pts received 10^9 TCID50 of MV-NIS in cycle 1; MSC at the corresponding dose level were thawed and infected with MV prior to intraperitoneal administration in cycles 2-6. There was no dose-limiting toxicity in Phase I and no patient experienced ≥ grade 3 toxicity. A total of 25 patients were evaluable for efficacy in phase II: the study met its primary efficacy endpoint: 18/25 pts (72%) were alive at 1 year post treatment initiation, despite heavy pretreatment (median of 3 chemotherapy regimens for recurrent disease). Median overall survival was 37.4 months (95%CI 16.6-50.5). Treatment was well tolerated: most common toxicities were grade 1, including fever (20 %,), chills (20%) and thrombocytopenia (16%). To further investigate the ability of this approach to elicit an antitumor immune response, we tested for the development of humoral and cellular immunity (using IFN-g, IL-4 and IL-17 ELISPOTs) against the ovarian cancer antigens folate receptor alpha, HER2/neu and IGFBP2: 81.0% (17/21) of patients developed a T cell response to at least one of the tested antigens, while 70.8% (17/24) of patients developed an antibody response to at least one of the tested antigens. **Conclusions:** Adipose tissue derived mesenchymal stem cells can safely deliver measles virus strains engineered to express sodium iodine symporter gene without dose limiting toxicity being encountered. This form of cell-based virus delivery led to efficacy that was significantly better than virus alone or contemporary data on standard of care treatments in pretreated recurrent ovarian cancer and to the development of antitumor cellular and humoral immune responses. Further clinical development is warranted. Supported by R01 CA200507, P50 CA136393.

9 Safety and Efficacy of DYNE-101 in Adults with DM1 in the Phase 1/2 ACHIEVE Trial

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Myotonic dystrophy type 1 (DM1) is rare, progressive, genetic disease caused by the expansion of an unstable CTG trinucleotide repeat in the 3' noncoding region of the gene encoding *DMPK*. RNA transcribed from the mutated *DMPK* gene forms stable hairpin-loop structures that are retained in the nucleus where they form toxic foci and sequester RNA-binding proteins including splicing factors. This results in dysregulated alternative splicing, called spliceopathy, in hundreds of genes and leads to multisystem clinical manifestations. Individuals with DM1 experience a wide range of symptoms related to skeletal and non-skeletal muscle involvement such as myotonia and muscle weakness, cardiac and pulmonary dysfunction, gastrointestinal issues, and CNS-related symptoms such as fatigue and cognitive impairment. There are no approved therapies for the treatment of DM1.

DYNE-101, an investigational therapeutic for the treatment of DM1, consists of a transferrin receptor 1-binding Fab conjugated to an antisense oligonucleotide designed to target mutant nuclear *DMPK* RNA to correct splicing. The

safety and efficacy of DYNE-101 in adults with DM1 are being investigated in the Phase 1/2 ACHIEVE trial (NCT05481879). In the 24-week placebo-controlled multiple ascending dose (MAD) portion of ACHIEVE, 56 participants received one of 5 dose/dose regimens of DYNE-101. All 56 participants have completed the MAD.

At the dose selected for the registrational expansion cohort of ACHIEVE (6.8 mg/kg Q8W), DYNE-101 demonstrated robust total *DMPK* knockdown (33% mean change from baseline) and splicing correction (25% mean change from baseline in the composite alternative splicing index [CASI]) at 3 months. Correction in splicing was associated with early and robust functional benefit across multiple measures of muscle strength and function at 6 months, including video hand opening time (vHOT, -38% mean change from baseline vs. +5% for placebo), Quantitative Muscle Testing total score (+10.1% mean change from baseline vs. -3.9% for placebo), 10-meter walk/run test (-7.2% mean change from baseline vs. +4.3% for placebo), and 5 times sit-to-stand (-12.4% mean change from baseline vs. +5.2% for placebo). Importantly, regression analyses show that CASI improvement at 3 months was well correlated with functional improvements at 6 months, suggesting that CASI is a reliable biomarker predictive of functional outcomes. Trends in improvement with 6.8 mg/kg Q8W DYNE-101 were also noted in the myotonic dystrophy health index (MDHI) total score, a patient reported outcome, including in the CNS-related subscales of fatigue, cognitive impairment, sleep, communication, emotional issues, and pain. DYNE-101 had a favorable safety profile as of the data cut date, with mostly mild/moderate TEAEs, and no serious related TEAEs.

Data from the ACHIEVE trial suggest that DYNE-101 has a favorable safety profile and results in improvement in molecular biomarkers, such as CASI, that may have the ability to predict clinical outcomes in DM1.

10 Hemophagocytic Lymphohistiocytosis (HLH)/hyperinflammatory syndrome following high dose AAV9 therapy

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Background:
Hemophagocytic Lymphohistiocytosis (HLH) is a rare, severe hyperinflammatory syndrome characterized by excessive activation of the immune system, widespread inflammation, and tissue damage. Most cases are triggered by strongly immunogenic stimuli such as infection, malignancy, or CAR-T therapy. Key features of HLH include fever, elevated ferritin, cytopenia, and evidence of hepatic injury. Cases of HLH reported after high dose adeno-associated virus (AAV) gene therapy, have been reversible with prompt recognition and treatment. Galletta et al described a case of HLH symptoms at 36-hours with elevated ferritin of 2959 ng/mL (normal <150 ng/mL) at 48-hours post treatment with Zolgensma (1.1 E14 vg/kg dose), resolving with high-dose corticosteroids. Byrne et al noted HLH cases, with a systemically administered AAV9 product at a similar dose, resolving with anakinra, an IL-1 receptor antagonist. The FDA Adverse Event Reporting system database for Zolgensma reveals serious adverse events with symptoms consistent with HLH, including elevated ferritin and fever. Early recognition and aggressive treatment of HLH with immunosuppression and immunomodulation are critical for favorable outcomes.

Methods:
Rett syndrome is a severe X-linked neurodevelopmental disorder caused by mutations in the MECP2 gene leading to deficiency of MeCP2 protein. NGN-401 is an AAV9 gene therapy containing full-length human MECP2 gene given via a one-time intracerebroventricular (ICV) administration. In an ongoing clinical trial of NGN-401, as of January 21, 2025 five subjects were dosed with 1E15 vg (estimated peripheral exposure: 3.6-7E13 vg/kg) and 3 subjects were dosed with 3E15 vg (1.4-1.5E14 vg/kg). The 1E15 vg dose was well-tolerated with no treatment-related serious adverse events and only Grade 1 treatment-related AEs. The first two subjects treated with 3E15 vg experienced no treatment-related SAEs. Most TEAEs have resolved or are resolving. The case report below focuses on the 3rd subject dosed with 3E15 vg.

Case summary:
A 7-year-old female with classic RTT was treated with 3E15 vg (1.5E14 vg/kg) of NGN-401 via ICV administration with no procedure-related complications. Prophylactic immunosuppression included rituximab, sirolimus, and corticosteroids. On Day 5 post dosing, she developed fever, vomiting, and lethargy and was admitted for dehydration. On Day 6, she rapidly declined, and ferritin was markedly elevated at 18,850 ng/mL, along with elevated liver enzymes, coagulopathy, and thrombocytopenia. She developed respiratory failure, acute kidney injury, and hypotension and was intubated, admitted to intensive care, and received Continuous Veno-Venous Hemofiltration and inotropic support. Due to suspected HLH, she was given high dose corticosteroids and anakinra. A single dose of eculizumab was given as a precaution, without signs of thrombotic microangiopathy based on negative blood smear. Ferritin improved to 6439 ng/mL; however, multi-system failure was too far advanced on presentation and despite intensive supportive care, her condition declined, resulting in death 2 weeks post dosing.

Conclusions:
High dose AAV has been linked to rare cases of HLH. Monitoring ferritin, complete blood count, and liver function frequently in the early post-dose period is warranted. Prompt treatment for suspected HLH should include high-dose corticosteroids, immunomodulation, and management by a multidisiplinary team.

AAV Biology and Mechanisms

11 Epigenetic Blueprint: Understanding how the AAV Capsid influences the Vector Epigenome

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We recently showed that the AAV capsid is directly involved in setting up the AAV-vector epigenome (Gonzalez-Sandoval 2023) and that the primate selective tropism of AAV-LK03, which does not transduce rodents well (Lisowski et al., 2014) is not related to differences in cell or nuclear vector DNA entry. We found that the species specific vector-mediated transgene expression was correlated with the prevalence of transcriptionally permissive histone H3 epigenetic modifications (H3K4me3 and H3K27ac) on the vector genome. Interestingly, insertion of a single glycine into position 266 of the LK03 capsid, (266iG) named AAV-AM, enhanced the concentration of permissive histone marks on delivered genomes in mice allowing for efficient and similar transduction in both mice and human cells. Structural simulations suggest that this additional glycine in position 266 establishes a more flexible local structure that could have an influence on interactions between the capsid and/or AAV genome with key host factor(s). To evaluate the structural effect of the 266iG on the capsid, we are currently performing cryo-EM on both LK03 and AM capsids.

Our data taken together had suggested that post nuclear entry events are responsible for setting up the vector epigenome. Therefore, we set out to understand how the two capsids interact with host proteins in the nucleus to achieve different epigenetic structures. To do so, we performed anti-A20 immunoprecipitation and mass spectrometry, followed by siRNA silencing evaluation of candidate hits on AAV-LK03 and AAV-AM transduction. Our siRNA screen identified the replication protein (RPA) complex as a novel host restriction factor required for robust transgene expression. Consistently, further analysis demonstrated that the improvement of transduction efficiency facilitated by RPA inhibition was unrelated to cell and nuclear genome entry but rather due to an increase in the vector genome transcriptional activity. Further supporting the importance of the RPA complex, we performed dCas9-Apex labelling to identify host factors that directly interact with the delivered vector genomes. Several of the identified candidates are proteins that have been shown to directly interact with RPA and are involved in chromatin remodeling.

Furthermore, to investigate the direct effect of RPA on epigenetic regulation, we performed Cut&Tag to determine the relative abundance of 4 modified histone proteins associated with vector genomes delivered by either the LK03 and AM capsids in cells after siRNA-mediated RPA knockdown. RPA knockdown increased transgene expression by 60x and significantly increased the abundance of H3K4me3/H3K27ac active histone modifications while the restrictive epigenetic marks H3K9me3/H3K27me3 remained unchanged on the genomes delivered both with AAV-LK03 and AAV-AM. Because RPA is not only involved in chromatin remodeling but also DNA processing, we are employing Nanopore sequencing to investigate whether differences in AAV vector genome structures may be affected and influenced based on which capsid is used for transduction.

Our study establishes the interplay between the AAV capsid, RPA (a member of the DNA damage repair pathway) and epigenetic regulation. These studies will improve our ability to correlate outcomes in humans based on preclinical testing in animals and offer new approaches to enhance efficacy and improve safety in humans.

12 Vector assembly factories in recombinant adeno-associated virus type 2 producing cells

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The assembly process of recombinant adeno-associated virus type 2 (rAAV2) is associated with an intranuclear condensate called the nucleolus, where capsid formation and genome encapsidation have been shown to depend on the functions of the viral assembly-activating protein (AAP) and Rep proteins. Better understanding the role of the nucleolus in rAAV2 assembly is crucial for optimizing the production of AAV2-based vectors and further improving their gene therapy applications. The ultrastructural aspects of the AAV assembly process and the mechanism of virion morphogenesis are currently unknown, however. Here, we combined 3D correlative-light-and-electron-microscopy (CLEM) with a trans-complementation assay to capture production of complete rAAV2 capsids in suspension HEK293 cells. Using high-precision on-section CLEM we were able to correlate the signal of fluorescently tagged AAP to positions in the nucleus at early stages of the vector production, and to acquire electron tomograms from these regions. Tomograms revealed that rAAV2 assembly sites did not completely overlap with AAP localization and capsid reminiscent structures were found only inside the nucleolus fibrillar centers (FC) or in perinucleolar chromatin. Accumulations of rAAV2 particles in these two functionally interconnected regions facilitating transcription and processing of rRNA precursors were thus reminiscent of 'virus factories' utilized by rAAV2 as a platform for assembly in association to the nucleolus. In early assembly deposits, only a low number of capsid-like structures contained a dense material suggested to correspond to the viral genome. No significant difference in empty:full ratio was observed between assembly in FC and perinucleolar chromatin, indicating that encapsidation of the viral genome inside the FC is not restricted. Interestingly, structures resembling individual circularizing capsid lattices were also found in sites of assembled particles, suggesting presence of forming capsid intermediates. Later (48 h) post-transfection, accumulation of intact full particles was observed around the nucleolus by CLEM and confocal microscopy. Our data provides high-resolution analysis of the AAV assembly process, indicating that AAP driven formation of AAV2 capsid is initiated in specific sites of the nucleolus or in adjacent nucleoplasm, where formation of empty capsid shell precedes the encapsidation of the viral genome. Further, immunocytochemistry analyses to confirm involvement of the factors in assembly factories, as well as testing the effect of re-targeting rAAV2 assembly into different subcellular locations on vector production by using heterologous AAP, are currently ongoing.

13 Capsid-mediated differences in epigenetic transgene modifications between AAV2 and AAV9

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Vectors based on Adeno-Associated Viruses (AAV) are widely used in clinical trials and are the basis for multiple approved therapies, yet knowledge on epigenetic modifications on the delivered transgenes over time remains sparse. Typically, AAV capsids are selected for their cell binding and transgene expression capabilities, however, the capsid can demonstrably affect transduction beyond mere transgene delivery. This complex influence on the trajectory of the transgene after nuclear entry has recently been demonstrated by others investigating changing histone modification levels that depend on the used capsid serotype and/or VP1 N-Terminus. Here, we specifically investigated AAV9 and AAV2 because both of them are widely studied and because AAV9 is the basis for the majority of variants that are currently in clinical testing. Interestingly, by combining nanopore sequencing with Cut-and-Tag experiments, we find a notable difference in epigenetic modifications between AAV2- and AAV9-delivered transgenes in adult mice. Congruent with the literature, we find that in the liver, AAV9 initially yields higher levels of transgene transcription compared to AAV2. However, over a 12-week time span, AAV9-delivered transgenes continually declined in their transcriptional activity, while AAV2 transgenes remained more stable. While direct nanopore sequencing of transgene episomes only showed a biologically insignificant increase over time in DNA methylation frequency, we found a distinct difference in histone modification levels. In Cut-and-Tag experiments targeting H3K27me3 and H3K27ac, we found the inactivating modification H3K27me3 to accumulate more on AAV9-delivered transgenes compared to AAV2-delivered ones. This occurs predominantly at later time points, corresponding with the decline in transcription from AAV9-delivered transgenes. Histones with H3K27me3 are primarily associated with the gene body of the AAV9 transgenes and less so with the promoter region. Our results show that differences between the AAV2 and AAV9 capsid do not end after successful transduction but reverberate long-term in the form of differential epigenetic transgene modifications. These findings underscore the notion that AAV capsids and vectors derived thereof should be assessed and optimized for their capability to yield episomes that are epigenetically primed to mediate long-term expression.

14 Inverted Terminal Repeat (ITR) Degeneracy Promotes AAV Genomic Integration

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Infectious adeno-associated viruses (AAVs) can be rescued from plasmids that harbor a transgene flanked by two ITRs, the only genomic element for viral packaging in *cis*. ITR instability was first observed in the original AAV2 plasmid isolates, which are ancestral to virtually all recombinant AAV (rAAV) vectors currently in use. Propagation of these plasmids commonly results in the expansion of molecular subpopulations that contain a variety of ITR mutations, including deletions.

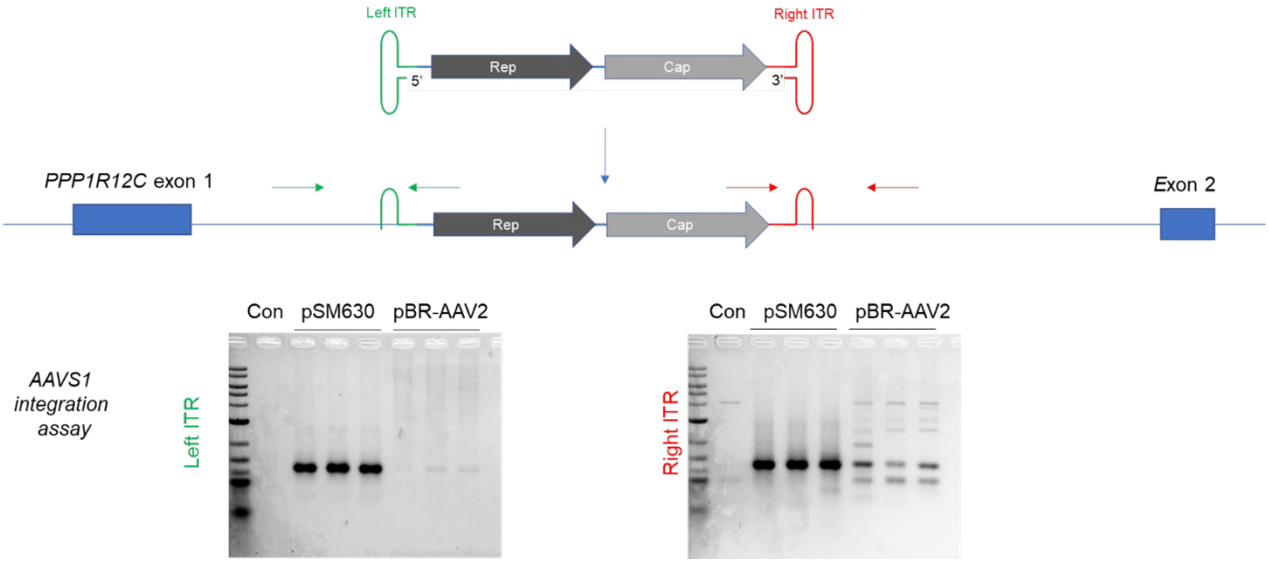
We previously employed long-read DNA sequencing methodology to investigate the basis for ITR instability, and observed that the pattern of instability in the founding AAV2 plasmids was asymmetric. While deletions could arise in either ITR, intermolecular heterogeneity was most prominent in the ITR that was positioned in closest proximity to the origin of replication (*ori*). By repositioning the ITR-transgene-ITR cassette so that both ITRs were equally distant from the *ori* element, we were able to mitigate ITR instability and thereby produce homogenous *cis* plasmid preparations.

Having comparable stocks of ITR-heterogenous and ITR-homogenous plasmids provided us with a unique opportunity to investigate the functional consequences of ITR instability/heterogeneity. A focus of this work has been on genomic integration. It is currently believed that integration into a “safe-harbor” site known as AAVS1 on chromosome 19 is a distinct stage of the natural AAV life cycle that facilitates durable latency. AAV would be the only known DNA-based virus that uses such an integration strategy. We investigated whether ITR instability could influence integration at AAVS1.

AAV2 stocks, containing the endogenous viral genome, were derived from the ITR-unstable and the ITR-stable plasmid vectors. Both viruses were used to infect A549 cells, a human cell line derived from a non-small cell lung adenocarcinoma. Following expansion of infected cells, genomic DNAs were harvested and assessed for genomic

integration. This analysis revealed striking differences between the two stocks. The heterogenous virus stock derived from the unstable vector integrated at a frequency that was >10-fold higher than that observed with the homogenous preparation. We are currently using Oxford Nanopore sequencing technology to quantitatively assess integration at AAVS1 and at other sites around the human genome.

Genomic integration by rAAV vectors used for human therapy has long been considered a potential safety hazard. Our studies suggest that the risk of integration can be mitigated by implementing simple changes in the design of *cis* plasmids. While our studies are still ongoing, our results thus far suggests that it is possible to engineer rAAV vectors with minimal potential for insertional mutagenesis.



15 Genome Wide rAAV Integration Analysis in a Tyrosinemia Mouse Model Revealed Preference for R-loops in Actively Transcribed Genes and in the Absence of Homology Arms.

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Recombinant adeno associated virus (rAAV) integration has been a topic of special interest and discussion in the field of gene therapy in the last few years. Although rAAV vectors are mainly maintained as episomes and concatemers in transduced cells, several reports have demonstrated that rAAVs can also integrate into the host cell's genome. rAAV integration has been described to be random, however, a preference for integration happening in transcriptionally active sites, more prone to DNA strand breaks, has been reported. Interestingly, a recent study by Puzzo et al addressed the association between integration sites of an rAAV, containing homology arms targeting the 3' locus of the murine Albumin gene, and regions containing R-loops both in vitro and in vivo by performing DNA-RNA immunoprecipitation sequencing (DRIP-Seq). R-loops are described as three-stranded nucleic acid structures, consisting of a DNA-RNA hybrid and a displaced DNA strand, that are formed during transcription and are linked to transcriptionally active sites and to increased genome instability.
In our work, we describe the integration profile of a hFAH expressing AAV8 vector (AAV-FAH) in the livers of Fah1RTyrc/RJ mice (#018129, Jackson), a model of hereditary tyrosinemia type I (HT1). These mice recapitulate the liver damage caused by accumulation of toxic metabolites, that are also linked to DNA damage and

hepatocellular carcinoma (HCC) promotion, due to mutations affecting the fumarylacetoacetate hydrolase (FAH) gene. Standard of care consists on a diet restrictive in tyrosine and phenylalanine and nitisinone (or NTBC) treatment, which results in the cease of toxic metabolite accumulation. Interestingly, a positive selection for hepatocytes that express the proper FAH gene for liver repopulation has been described. Thus, to assess the therapeutic potential of our therapeutic rAAV, 7-week-old female mice received an intravenous injection of an AAV-FAH vector at a dose of 1E11 vg/kg. Mice were maintained on NTBC for two weeks to allow for rAAV genome stabilization before NTBC removal. 10-weeks-post injection (8-weeks after NTBC removal), mice were sacrificed, livers were extracted and genome wide integration analyses were performed using two different techniques: shearing extension primer tag selection mediated PCR (S-EPTS/LM-PCR) and target enrichment sequencing (TES). Results obtained from both techniques were equivalent and showed preferential integration of rAAVs in actively transcribed genes. Furthermore, a comparison between the integration sites observed in our samples and the previously described DRIP-Seq results identifying R-loops, showed statistically significant association between rAAV integration and R-loops present in transcriptionally active genes. Moreover, our construct carries only one sequence, the promoter, with homology to the mouse genome and we did not find preferential integration in this locus. These results suggest that rAAV integration happens mainly through non-homologous end joining (NHEJ). It is important to note that the observed levels of rAAV integration are favoured by the disease model, in which FAH expressing hepatocytes are selected for liver repopulation and in which considerable levels of DNA damage are observed.

16 An alternate receptor for adeno-associated viruses

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We report the identification and characterization of an alternate receptor for adeno-associated viruses (AAV), distinct from the universal AAV receptor KIAA0319L or AAVR. This receptor, referred to as AAVR2 hereafter, enables the transduction of Clade E AAVs, including AAV8. AAVR2 is also essential for a previously undescribed AAVR-independent transduction pathway for AAV11 and AAV12. We characterised a direct binding between the AAV8 capsid and AAVR2 and identified contact residues using single-particle cryo-electron microscopy. We then observed that AAV8 directly binds to the carboxypeptidase-like domain 1 of AAVR2 via its variable region-VIII and demonstrated that AAV capsids lacking AAVR2 binding can be bioengineered to engage with AAVR2. Finally, we overexpressed AAVR2 to enhance AAV transduction allowing for efficient gene transfer in vivo.

17 Identification of KIAA0319L-interacting host factors during intracellular trafficking of AAV

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In 2016, KIAA0319L was identified as a proteinaceous receptor for multiple serotypes of adeno-associated viruses (AAVs). It is a type I transmembrane protein containing a N-terminal MANSC domain, five extracellular polycystic kidney disease (PKD) domains, and a C6 motif in the transmembrane region at the C tail. KIAA0319L is transiently expressed on the cell surface but primarily locates to the perinuclear trans-Golgi network (TGN), and recycles from cell surface to the TGN. While the PKD domains interact with AAV capsids, what host factors KIAA0319L interact with and how it mediates AAV entry from cell surface and intracellular traffic to the TGN are largely unknown. In this study, using Ascorbate peroxidase (APEX)-based proximity labeling and quantitative mass spectrometry, we identified carboxypeptidase D (CPD) and TBC1D10A (TBC1 Domain Family Member 10A) were closely associated with KIAA0319L. Both in vivo and in vitro interaction assays, as well as confocal microscopy, confirmed

their interactions, highlighting their relevance to AAV intracellular transport. As TBC1D10A is a homolog of TBC1D23, we further explored the involvement of the TBC1D23 in promoting AAV endosome-to-TGN trafficking. CRISPR/Cas9-mediated depletions of CPD, TBC1D10A, or TBC1D23 revealed reduced AAV transduction by over 80% in HEK293 cells and polarized human airway epithelium. Mutagenesis studies identify the critical motif of threonine-leucine-tyrosine (TYL) KIAA0319L in its interaction with TBC1D proteins, underscoring an essential role of KIAA0319L in mediating the endosome-to-TGN trafficking of recombinant AAV vectors. Thus, our work offers valuable insights into promising targets to enhance the efficacy of recombinant AAV-based gene therapies.

AAV Vector Manufacturing: Process Development

18 Engineering Protein Carrier AAV to Rescue Intracellular Trafficking | Selective Purification of AAV Based on Capsid Composition

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Adeno-associated virus (AAV) is widely used for the efficient delivery of DNA payloads to target cells in both therapy and research. In prior work, we engineered the ability to package protein payloads into AAV capsids through the insertion of a domain in the interior of the capsid that facilitates packaging of a target protein during the capsid assembly process, a technology we termed Protein Carrier AAV (pcAAV). Protein packaging efficiency depended on the location of the domain insertion, the VP isoform into which the domain was inserted, and the intracellular localization of the target protein during pcAAV production. We demonstrated that pcAAV mediates intracellular delivery of target proteins using flow cytometry and further investigated the subcellular localization of the delivered protein by confocal live cell imaging which revealed that a portion of the delivered protein payload localizes to lysosomes with no nuclear delivery being observed. We hypothesize the lack of nuclear delivery is the result of an inability to either internalize or expose VP1u, required for endosomal escape and nuclear import. We are pursuing several strategies to rescue pcAAV trafficking on which we will present initial results characterizing key AAV capsid properties (e.g., capsid morphology and engineered VP subunit incorporation) focused on validating individual system components.

The AAV capsid assembles stochastically from 60 copies of three structural proteins named VP1, VP2, and VP3 in an average ratio of 1:1:10. Insertion of domains or peptides into individual VP isoforms can affect the rate of incorporation and potentially influence both particle yield and infectivity. To explore how VP1 incorporation into assembled capsids affects infectivity and develop a strategy to purify capsids containing domain insertions, we developed a system capable of separating capsids based on their composition. This system works through the insertion of the small, rationally designed ALFA tag into a target VP subunit. By incubating crude AAV productions with magnetic beads conjugated to the ALFA nanobody, which recognizes the ALFA tag with high affinity and specificity, AAV capsids incorporating the target VP subunit can be selectively purified. We demonstrate that our purification strategy can purify AAV capsids containing a retargeting domain insertion and that purified capsids retain their infectivity towards target cells. Further, we explore if our purification strategy can be used to separate capsids based on the extent of VP1 incorporation by modulating the elution temperature. Our preliminary results suggest that capsid populations eluted at distinct temperatures exhibit differing infectivity, which we hypothesize may be due to differences in VP1 incorporation between these populations.

19 Post-Processing Conjugation System for Manufacturing of Retargeted AAV-SpyT-SpyC-Ab Molecules

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Adeno-associated viral vectors (AAV) have emerged as a leading platform for gene therapy due to their safety

profile and ability to deliver therapeutic genetic payloads *in vivo*. However, a challenge facing the field is delivery of AAV genetic payloads to specific tissues or organ targets, as even AAV serotypes with tissue-specific tropism are mostly directed to the liver for clearance. Thus, higher patient doses are required to achieve therapeutic activity in tissues outside the liver. An approach for delivering AAV vectors at a lower dose to specific tissue types includes (i) detargeting AAV from the liver using capsid point mutations and (ii) covalently linking antibodies (Abs) to AAV to retarget it to specific tissue types. The AAV-Ab conjugates are manufactured using a SpyTag-SpyCatcher ("SpyT-SpyC") Post Processing Conjugation System, where a SpyT peptide inserted into AAV capsid proteins spontaneously forms a covalent bond with a SpyC domain expressed on the antibody light or heavy chain.

The post-processing conjugation system enables development of a scalable and robust manufacturing process as both the AAV-SpyT and SpyC-Ab molecules can be produced separately, meeting efficacy, yield and purity targets for the individual components. Once manufactured, the two components are combined in an *in-vitro* conjugation reaction that is well-characterized, resulting in a consistent number of conjugated antibodies for each conjugation reaction before removal of unconjugated species and final formulation. In this presentation, we present the AAV9-SpyT manufacturing process, development of optimized conditions for the *in-vitro* post-processing conjugation reaction, approaches for at-line monitoring of reaction kinetics, and techniques for removal of unconjugated species. We also showcase successful application of the platform for a 500 L scale production of an AAV9-SpyT-SpyC-Ab molecule carrying a therapeutic transgene.

20 DMSO Enhances Recombinant AAV Production via Downregulation of the Expression of the KAT5/TIP60 Acetyltransferase

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Vectors based on adeno-associated virus (AAV) are a promising delivery platform for gene therapies. To manufacture AAV vectors, producer cell lines (PCL) can be employed, in which the AAV *rep/cap* genes and the recombinant AAV vector genome are integrated into the genome and vector is produced following Adenovirus (wtAd5) infection. However, a primary challenge remains in producing sufficient AAV vector to permit accessible and affordable treatments. Here we report that supplementing the culture media with DMSO can improve AAV vector production in a PCL by two to three-fold. DMSO enhanced AAV production without impacting wtAd5 infection efficiency or adenoviral titer; however, an increase in Adenovirus E1A gene expression was observed. To identify the mechanism of enhanced AAV production, we conducted RNA-Seq analysis and found numerous biological processes significantly impacted by DMSO, including reduction in histone acetyltransferase KAT5 expression and differential regulation of genes regulated by KAT5. Knockdown of KAT5 via siRNA simulated the DMSO effect on AAV productivity and on the expression of the viral Rep, Cap, and E1A proteins. Thus, DMSO seems to act at least in part through KAT5 to increase viral gene expression and AAV vector production. Though the DMSO effect on AAV vector production was also observed in the context of transient transfection of HEK293 cells, evaluation of DMSO supplementation in additional producer cell lines revealed heterogeneity in the response to KAT5 knockdown, suggesting that there may be multiple factors contributing to the DMSO mechanism of action. Nevertheless, KAT5 mRNA downregulation by DMSO was observed in all producer cell lines tested and KAT5 knockdown may provide an additional low-cost approach to increase AAV vector production.

21 Development of a Commercial Manufacturing Process for RGX-202, a Systemically Delivered AAV for the Treatment of Duchenne Muscular Dystrophy

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RGX-202 is REGENXBIO's investigational gene therapy for the treatment of Duchenne Muscular Dystrophy (DMD). It uses an AAV8 vector, packaging a novel, next-generation microdystrophin expression cassette encoding functional elements of the C-terminal domain found in full-length dystrophin.

Gene therapies for the treatment of DMD typically require large doses of systemically delivered AAV. This creates several critical manufacturing-related challenges:

1. Product quality profile – To minimize patient safety risk, reducing impurities is key, specifically empty and partially filled capsids. This is reflected in informal regulatory guidance suggesting at least a "majority" of full capsids for systemic administration. The currently approved DMD gene therapy reportedly contains ~50-60% full capsids (Memo from FDA Division of Pharmacology/Toxicology dated June 2023).
2. Batch yield – The amount of vector required for clinical development and commercialization can be significant and early-stage processes may be low yielding and costly. It is important that batch yields are sufficiently high to supply the patient population.
3. Robustness – The manufacturing process should be robust to ensure a high level of consistency.

The initial proof-of-concept (POC) batches utilized a first-generation process with transient transfection of suspension HEK293 cells, designed for programs with lower doses. It had low batch yields meaning that supply would be constrained during clinical development and was nonviable for eventual commercial manufacturing. The capsid population from this preclinical POC manufacturing process was ~35% full with high levels of empty and partial capsids and did not meet the regulatory expectation.

REGENXBIO undertook a project to develop a new, enhanced manufacturing platform that would improve the product quality profile to enable high systemic doses and improve batch yields to allow efficient clinical development and eventual commercial supply. This was a cross-functional effort by the entire development team to further characterize, optimize, and improve upon the process used for the POC batches. A comprehensive effort examined the entire manufacturing process including expression system, cell culture conditions, plasmid design, transfection, and chromatography resins and conditions. In addition, an extensive set of analytical tools was used to characterize the product and capsid population to provide a deeper understanding of the product profile and how it may be improved.

These efforts were successful. The scale independent batch yield was improved 56-fold from the POC process to the commercial-ready process driven by a 28-fold improvement in the cell culture productivity and 2-fold improvement to the processing yields. The capsid population was improved from ~35% full to >80% full. The relative *in vitro* potency of the product was unchanged. Other product attributes such as mispackaged host cell DNA were significantly improved. The resultant NAVXpress® manufacturing process is an industry leading platform in terms of productivity and purity. With these improvements, the commercial manufacturing process for RGX-202 is more robust, more scalable and operationally simpler. The cost per dose has been greatly reduced and the overall supply chain has been simplified and is much more efficient. The RGX-202 commercial manufacturing process has been successfully executed at the REGENXBIO Manufacturing Innovation Center.

22 Nutritional Counselling at the Cellular Level – Combining Ambr® 15 and Orbitrap Mass Spectrometry to Analyze the Metabolome of HEK293 During AAV Production

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Despite significant advancements in the field, the production of adeno-associated viruses (AAVs) for in vivo gene therapy remains insufficient for widespread use, necessitating further research to optimize HEK293 suspension

processes and address the current supply-demand gap. Omics provides a powerful tool for analyzing viral vector production at different cellular levels, including DNA, RNA, and metabolism. The analysis of metabolites, both secreted and retained, can facilitate the identification of cellular changes that impact AAV production, thereby offering insights into both favorable and detrimental processes, particularly in comparisons between HEK293 processes exhibiting different yields.

In light of the aforementioned premise, a comparative analysis of the metabolome and secretome alterations in HEK293 cells during the production of AAV2 and AAV8 was undertaken. Furthermore, the production of AAV2 was evaluated after subjecting the cells to a commercially available enhancer, which has previously resulted in titer increases of 50 to 100%. To achieve this, a commercially available HEK293 cell line was cultivated and transfected using a proprietary three-plasmid system and FectoVIR (Polyplus), with the Ambr® 15 (Sartorius) serving as the cultivation platform. Samples of biological duplicates were taken at 6, 24, and 48 hours after transfection for analysis of the metabolome and secretome. Following the preparation of the samples and the extraction of the metabolites, an analysis was conducted using an Orbitrap mass spectrometer (Thermo Fisher Scientific). All samples were compared to a mock-transfection process, which only received the ITR-containing plasmid and FectoVIR, in order to control for any effects related to GFP expression and impact of the transfection reagent.

Following a thorough data curation process, 1,500 compounds present within the intra- and extracellular space were considered and analyzed using SIMCA® (Umetrics), a sophisticated multivariate data analysis (MVDA) tool. Principal component and orthogonal partial least squares analysis revealed a pronounced separation between sample groups, which reflected the tested conditions, including AAV2 and AAV8 production, enhancer addition, and temporal changes.

The analysis yielded the identification of several metabolic pathways and mechanisms that were deemed to be of interest for the purposes of AAV production, including nucleotide metabolism. In accordance with these findings, the process was sought to be enhanced by activating or inhibiting each of the identified mechanisms. This was achieved by subjecting the cells to active compounds, where feasible, or by supplementing biological precursors.

This approach utilizes untargeted analytical techniques, such as high-resolution mass spectrometry in conjunction with MVDA, to investigate alterations in the metabolic profile of HEK293 cells during viral vector production. It is further demonstrated how the generated data can be utilized to further optimize AAV production processes, thereby contributing to a broader objective of enhancing the accessibility of in vivo gene therapy applications.

23 Small-Scale Manufacturing of Innovative rAAV Capsid Variants for Enhanced Retinal Gene Therapy

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Recombinant adeno-associated viral vectors (rAAV) have emerged as promising tools for gene therapy, offering potential "one-and-done" treatments that surpass conventional therapies. Recent advancements in AAV capsid engineering have focused on enhancing vector tropism and transduction efficiency, including for retinal applications. To accelerate AAV capsid development, we established a robust small-scale production and purification strategy for manufacturing a diverse array of rAAV capsid variants with process compatibility for rapid scale up of promising candidates.

In this example, we aimed to generate a rAAV pool to identify novel capsids with improved retinal transduction capabilities in non-human primates (NHP). We packaged a comprehensive collection of 105 recombinant rAAV capsid variants – each individually barcoded – using a HEK293 suspension cell line. The variants were engineered using two primary methods: peptide display and DNA shuffling. The collection also includes parental controls for comparison. The engineered capsids were pre-selected based on their performance in previous NHP biodistribution

studies. To prevent selection bias from affinity chromatography, purification was conducted exclusively via iodixanol gradient ultracentrifugation.

Our goal was to purify $1.3x10^{10}$ vector genomes (VG) of each variant. We found that the GFP transgene, fused with KASH tag and barcodes, showed poor packaging efficiency, so we produced the particles at a 30 mL scale. The cells were either centrifuged and lysed with detergent while the supernatant was PEG-precipitated and resuspended with the lysate, or detergent lysis was performed directly in the culture medium. Both lysis methods proved equally effective, however, because the maximum sample volume for Quick-Seal ultracentrifugation tubes is 19 mL, we opted to use the former method to ensure the entire sample could be processed in bulk. By leveraging this method, we successfully purified up to 36 vectors concurrently. Each purified vector was quantified by qPCR using ITR-specific primers. The final yields ranged from $6.48x10^{9}$ to $1.4x10^{12}$ VG, with only three vectors being below the $1.3x10^{10}$ VG target yield. The vectors were analyzed on an SDS-PAGE gel to examine the VP1, VP2, and VP3 capsid proteins, confirming the integrity and purity of the viral particles. All 105 vectors were generated by a single individual over a period of four weeks, including a few repeat productions.

The rAAVs were then pooled in equal amounts to create a comprehensive collection of vectors for ocular research purposes. Three vectors were underrepresented in the pool due to their lower than target yield. The pool was administered via subretinal injection to two NHPs, ultimately leading to the identification of novel capsids with enhanced tropism for specific retinal cell types.

Iodixanol gradient ultracentrifugation, a method used for over 20 years in rAAV manufacturing, remains a cornerstone technique, even as the field shifts towards more scalable methods like chromatography. Our approach combines this classical method with higher-throughput capabilities for the rapid production and purification of >100 rAAVs, as used in generating rAAV libraries. By processing multiple samples in parallel and applying economies of scale, we streamline small-scale rAAV manufacturing in suspension, saving time and reducing costs associated with the discovery of next-generation gene therapy vectors.

24 Harnessing protein trans-splicing by combining inteins and SpyTag systems

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Approximately 15% of the gene coding regions are longer than 4 Kb, making difficult or impossible to package them into AAVs for gene therapy applications. A transformative system to overcome this limitation is based on inteins, which promote their own excision from a precursor protein and the subsequent ligation of the flanking peptides chains to reconstitute the complete protein. Intein-based trans-splicing (ITS) can occur when the two separate parts are cloned in independent AAVs and the complete protein is fully reconstituted in co-infected cells. ITS-mediated protein reconstitution has been successfully applied to express long genes by dual AAV delivery for gene therapy applications. However, in some cases, particularly for very long genes, its efficiency may not be high enough and additional improvements are needed. We have combined the SpyTag/SpyCatcher module together with the inteins and have shown that the two systems work synergically to enhance protein trans-splicing. Initially, using the GFP protein reconstitution assay, we showed that the SpyTag/SpyCatcher module does not interfere with the intein self-processing, but significantly increases the reconstitution rate of the functional full protein. We named this approach STRING, for SpyTag-assisted tRans-splicING. We then selected ATM, a very long protein of 3056 amino acids, and compared ITS with STRING for efficient protein reconstitution. In HEK293 cells, fibroblasts and human fibroblasts STRING outperformed ITS for full reconstitution of ATM. To assess the functionality of the complete protein, ATM was reconstituted in mutant fibroblasts and the active forms of key ATM downstream targets were evaluated. Interestingly, pKAP1, pCHK2 and p53 were more upregulated after ATM protein reconstitution by STRING compared to ITS. ATM loss-of-function mutations cause ataxia telangiectasia (A-T), a neurological disease associated with immunodeficiency and high cancer predisposition. Due to its excessive length, ATM gene replacement therapy using viral vectors has not been feasible. We therefore generated a dual lentiviral system expressing the two halves of the gene associated with the STRING modules. After dual lentiviral infection fibroblasts from A-T patients reconstituted high levels of the full ATM protein and the phosphorylated forms of its

direct protein targets. Altogether, the STRING methodology ensures high efficiency of protein trans-splicing and may represent a suitable methodology for ATM gene replacement therapy, a long-sought translational opportunity for A-T.

Advances in Gene Therapy of Neurological Diseases in Small Animal Models

25 Gene Therapy for Megalencephalic Leukoencephalopathy with Subcortical Cysts: Restoring Brain Homeostasis in Preclinical Models

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Megalencephalic leukoencephalopathy with subcortical cysts (MLC, or Van der Knaap disease) is an ultrarare, infantile onset, and orphan leukodystrophy characterized by white matter swelling and vacuolation, macrocephaly, cerebellar ataxia, spasticity and muscle stiffness, epileptic seizures, and mild cognitive decline. Most patients carry biallelic loss-of-function mutations in the astrocyte-specific gene *MLC1* (~75%), or *GLIALCAM* (~7%). MLC1 and GlialCAM oligomerize to form a functional unit at glial cell junctions, where they regulate the activity and membrane localization of key players involved in brain water and ion homeostasis, including aquaporins (AQP4), ion channels and transporters (Kir4.1, ClC-2, Na^+/K^+-ATPase), and G protein-coupled receptors (GPRC5B), among others. Disease-associated mutations in *MLC1* or *GLIALCAM* disrupt this essential interaction network, leading to pathological brain edema and neurological dysfunction.

To address the underlying molecular basis of MLC, we developed two preclinical gene therapy strategies using adeno-associated viral vectors (AAVs) in *Mlc1*$^{-/-}$ and *Glialcam*$^{-/-}$ mice, two knockout models of the disease. First, we intravenously injected 3.2 x 10^{13} vg/kg of an AAV9P31 vector encoding the human *MLC1* cDNA under the control of the full-length human glial fibrillary protein (GFAP) promoter, gfa2, to 10-month-old *Mlc1*$^{-/-}$ mice, seven months after the onset of white matter vacuolation. This noninvasive treatment provided long-term, astrocyte-driven expression of MLC1 for over one year, and reversed interstitial and intramyelinic oedema, as demonstrated by histological analyses and magnetic resonance imaging (MRI; T_2 relaxometry, and diffusion tensor imaging). Most importantly, motor tests used to assess cerebellar ataxia-like behaviors in mice (hanging wire and hindlimb clasping tests), revealed that treated *Mlc1*$^{-/-}$ mice exhibited a complete normalization of motor performance.

GLIALCAM is expressed in astrocytes and oligodendrocytes, so we implemented a dual therapy based on the administration of AAV9P31 vectors encoding the human *GLIALCAM* cDNA under the gfa2 (astrocyte-specific) or the human myelin basic protein (MBP, oligodendrocyte-specific) promoters. To achieve simultaneous expression from both cell types, we delivered 5 x 10^{10} vg via intra-cisterna magna injection (2.5 x 10^{10} vg/vector) to 15-month-old *Glialcam*$^{-/-}$ mice. Preliminary data suggest that this alternative administration route resulted in molecular and neuroimaging correction comparable to our previous approach for *Mlc1*$^{-/-}$ mice, despite the later and lower-dose intervention, six months after viral vector administration. Taken together, our results not only demonstrate the longevity of astrocyte-driven therapeutic gene expression at safe AAV dosages, but also the reversibility of two key hallmarks of MLC, white matter edema and motor impairment, establishing a foundation for potential clinical translation.

26 In Vivo Systemic Genome Editing to Correct Mecp2 Mutations Improves Symptoms and Extends Lifespan in a Severe Model of Rett Syndrome

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Rett Syndrome is a debilitating neurodevelopmental disorder associated with de novo mutations in the X-linked MECP2 gene. Due to random X chromosome inactivation, females with Rett Syndrome display a spectrum of severities, while affected hemizygous males rarely survive. Mecp2 is a multifunctional methyl CpG binding protein that functions as transcription regulator, chromatin modifier and mRNA processor. Mecp2 is expressed in all tissues with the highest levels observed in the brain. Expression of Mecp2 requires stringent regulation as both under- and over-expression are pathogenic. Two early-stage gene therapy clinical trials for Rett Syndrome utilizing vectors that incorporate some of the regulatory elements are underway. Here we describe a genome editing strategy that corrects pathogenic MECP2 mutations directly in the genome while retaining all native regulatory elements and resulting in physiologic expression. Importantly, this approach avoids both over-expression-related toxicity and under-expression-related pathogenesis. Correction of MECP2 mutations was achieved using the nuclease-free, BRCA2-dependent, homologous recombination-based AAVHSC mediated editing platform. Since AAVHSC vectors cross the blood brain barrier and transduce both peripheral tissues and the central nervous system (CNS), we hypothesized that systemic administration of the AAVHSC Mecp2 editing vector would result in the permanent systemic correction of Rett-mutations. Recent studies suggest that MECP2 mutations in the periphery contribute significantly to disease progression in addition to the primary sequelae of Rett in the CNS, emphasizing the importance of systemic editing. Here, we evaluated the in vivo efficacy of AAVHSC mediated systemic genome editing in the Bird Mecp2 knockout mouse model, which exhibits a severe disease phenotype. Mecp2-deficient hemizygous male mice have a shortened lifespan of approximately 8 weeks and display severe Rett symptoms including hindlimb clasping, abnormal gait, uneven breathing, tremors and mobility problems. We designed an AAVHSC genome editing vector to insert the missing portion of the murine Mecp2 gene in the genome, restoring Exons 3, 4 and the 3' untranslated (UTR) region. The murine Mecp2 editing vector was packaged in AAVHSC15 and injected intravenously in hemizygous Bird mice at 5E+13 to 6E+13 vg/kg. Mice were scored weekly for Rett symptoms and compared with untreated Bird mice and wild-type controls. The AAVHSC-treated mice exhibited significant symptomatic improvement including mobility, breathing and exploratory behavior in open field tests. Immunofluorescence analysis revealed that symptomatic improvement correlated with restoration of Mecp2 expression in CNS neurons. Notably, we observed a significant extension in lifespan as compared with untreated Bird mice. We conclude that systemic in vivo genome editing to permanently correct Mecp2 mutations offers an attractive platform for the treatment of Rett syndrome while maintaining durable and physiologic levels of expression. Thus, supporting the therapeutic potential of AAVHSC to mediate efficient, safe, nontoxic, durable in vivo genome editing of the Mecp2 gene to treat Rett syndrome.

27 Preclinical Study for the Treatment of Hereditary Spastic Paraplegia Type 52 (SPG52)

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Hereditary spastic paraplegia type 52 (SPG52) is an ultra-rare inherited neurological disorder characterized by weakness and spasticity in the lower limbs, global developmental delay, intellectual disability, and seizures. SPG52 is caused by loss-of-function mutations in the *AP4s1* gene, which encodes the sigma subunit of the adaptor protein complex 4 (AP-4). Disruptions in any subunit of the AP-4 complex lead to its destabilization and degradation, resulting in a shared pathophysiology and symptomatology. While the exact function of AP-4 remains unclear, it is believed to have a role in the biogenesis of the autophagosome. Currently, there is no cure or treatment available. Therefore, a gene therapy approach to restore *AP4s1* expression represents a rational therapeutic approach for SPG52.

To develop a suitable human model to test the efficacy of our viral vector gene therapy strategy, we have generated and characterized induced pluripotent stem cells (iPSCs) from skin biopsies of an SPG52 patient and two controls. The patient's iPSC-derived cortical neurons showed an accumulation of the main cargo of the AP-4 complex at the trans-Golgi level, reduced neuronal arborization, impaired autophagy flux, as well as decreased neuronal activation when compared to both controls. Upon overexpression of the *AP4s1* gene, we are able to completely rescue distinct cellular disease phenotypes.

In parallel, we have developed and characterized the first murine model for SPG52 carrying a complete knock-out (KO) of the *AP4s1* gene. This animal model recapitulates one of the main hallmarks described in patients of the disease, a thinning of the corpus callosum. In addition, KO mice display a decrease in body weight, muscle strength, motor coordination, and lifespan when compared to wild-type (WT) mice. Neuromuscular deficits in the hindlimb muscle of KO mice were corroborated by neuromuscular functionality assays showing a decreased compound muscle action potential (CMAP). The compromised sensory function of these animals was also confirmed by nerve conduction tests. KO mice also display behavioral differences when compared to WT mice such as increased exploratory behavior without signs of anxiety, and short and long-term memory deficiencies. To test the efficacy of our gene therapy approach we have treated two cohorts of animals. One cohort of animals has been treated at the neonatal stage and the other one at the young-adult stage. Both cohorts of animals were administered with a neurotropic adeno-associated viral vector carrying a correct copy of the *AP4s1* gene. Phenotype rescue was assessed in both cohorts showing a complete rescue of the CMAP and sensory nerve, partial rescue of the thinning of the corpus callosum, motor coordination, and force, but no effect on the animals' body weight.

Collectively, these promising results pave the way for the final safety trials, bringing us closer to a clinical study that could offer a treatment option for children with SPG52, who currently have no other treatment available.

28 Targeting MSH3 for Huntington's Disease: Preclinical Validation of AAV-DB-3-miRNA to Prevent Somatic CAG Repeat Expansion

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The identification of genetic modifiers of Huntington's disease (HD) onset and progression were landmark findings; the data provided insight into key pathogenic pathways and revealed novel, potentially more druggable, and genetically validated therapeutic targets (e.g. MSH3) to treat HD (GeM-HD Consortium, 2015, 2019; Hensman-Moss et al., 2017). Preclinical studies in HD models have shown that reducing MSH3 protein expression through genetic (Dragileva et al., 2009, Ferguson et al., 2023, Mouro-Pinto et al., 2024) or pharmacological manipulation (O'Reilly et al., 2023, Driscoll et al., 2024) effectively prevents somatic instability of the CAG trinucleotide repeat expansions in the *HTT* gene.

Latus Bio is developing a gene therapy to reduce MSH3 as a potential treatment for HD. The LTS-201 candidate is an engineered AAV capsid (AAV-DB-3) designed to deliver an artificial microRNA to reduce human MSH3

(miMSH3) and halt somatic CAG repeat expansion in medium spiny neurons of the caudate nucleus and putamen and in striatal projection neurons in layer 5 of the motor cortex. miMSH3 also shows potency against murine Msh3.

To establish preclinical proof-of-concept for AAV-DB-3.miMSH3, LTS-201 was evaluated in Q111 knock-in mice, a HD model that undergoes progressive disease phenotypes including somatic repeat expansion in striatal neurons. Following bilateral intrastriatal injections of AAV-DB-3.miMSH3 to 8 week old Q111 mice, AAV biodistribution (vector genome copy number) measured by ddPCR was high in the striatum and cortex, and undetectable in peripheral and off-target tissues. *Msh3* mRNA levels were reduced by 20% in bulk striatal tissue resulting in a 51% reduction in Msh3 protein levels. Somatic instability in the striatum was reduced by 57% at 24 weeks old, demonstrating proof-of-concept for the potential clinical efficacy of LTS-201 and warranting its advancement for further translational development.

In conclusion, human genetics and preclinical proof-of concept data in HD mouse models support the therapeutic hypothesis that depletion of MSH3 protein using a miRNA expressed by Latus' novel AAV-DB-3 delivery vector, may significantly delay symptom onset in HD through prevention of somatic CAG repeat expansions in the *HTT* gene.

29 Achieving 'Just Right': Preclinical Development of A Gene Therapy for *SURF1* Leigh Syndrome Using Two Disease Models

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SURF1 (surfeit locus protein 1) Leigh syndrome is an early onset neurodegenerative disorder, characterized by a reduction in complex IV activity that disrupts mitochondrial function. Currently, there are over 150 patients identified worldwide, and no disease-modifying treatments available. Previously, we reported that a gene replacement therapy for *SURF1* Leigh syndrome showed improved complex IV activity, restored exercise-induced lactate acidosis in a *Surf1* KO mouse model, and displayed a high safety profile in wildtype (WT) mice. However, further investigations of this original SURF1 vector design uncovered cytotoxicity in multiple tissues of WT rats despite having minimal immune responses. We hypothesized that this cytotoxicity was elicited by SURF1 protein overexpression driven by the strong ubiquitous promoter, CBh. Thus, we designed 4 new vectors by modifying the promoter and poly(A) sequence, along with revising the transgene codon optimization. Of the 4 candidate vector constructs, one vector showed both short-term and long-term therapeutic efficacy in the *Surf1* KO mouse model, including improved complex IV activity, improved endurance capacity, and reduced exercise-induced lactate acidosis after the treatment, to an extent that is comparable to the original CBh version. Additionally, we performed studies to compare the short-term efficacy using three different doses and two treatment ages. Our data indicates that earlier treatment with a higher dose of vector is necessary to achieve maximum treatment benefits. To further support the efficacy of the gene therapy treatment, we also developed a *Surf1* KO rat disease model using CRISPR/Cas9 technology. In this model, female *Surf1* KO rats showed reduced locomotor activity and grip strength at 4 months old, as well as reduced body weight starting from 6 months old, compared with WT and HET littermates. Our treatment was able to partially restore their grip strength and body weight. More importantly, this vector did not show toxicity when tested in normal rats at two ages (young and adult). Taken together, our revised vector design was able to achieve similar preclinical efficacy for *SURF1* Leigh syndrome as our original strong expression design, but with minimal toxicity. This therapeutic candidate warrants further translational evaluations to benefit *SURF1* Leigh syndrome patients.

30 Therapeutic and Safe *GFAP* Silencing in Astrocytes Rescues Alexander Disease in a Rat Model

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Alexander disease (AxD) is a rare, devastating neurodegenerative disease known as leukodystrophies. AxD is caused by dominant gain-of-function mutations in the glial fibrillary acidic protein (GFAP) gene and occurs in approximately one in one million births. The disease is progressive and fatal, ranging from infancy to adulthood. GFAP is mainly present in astrocytes of the central nervous system (CNS) and plays an essential role in controlling myelination. Mutations in *GFAP* result in the overproduction and accumulation of the mutant form of GFAP, which causes the formation of Rosenthal fibers, the histopathological hallmark of AxD throughout the CNS. Currently, there is no therapy available to treat AxD. Thus, silencing of *GFAP* expression could be an attractive therapeutic strategy in AxD.

We developed a scAAV9 vector (scAAV9-GfaABC1D-amiR-*GFAP*) expressing an artificial microRNA (amiR) to target the conserved *GFAP* sequences among mice, rats, NHPs, and humans. The amiR-*GFAP* is driven by the endogenous promoter of *the GFAP gene,* which restricts the amiR-*GFAP* expression within the GFAP-expressing cells. We delivered the scAAV9-GfaABC1D-amiR-*GFAP* into three-week-old AxD rats by intracerebroventricular (i.c.v.) administration at a dose of 2.0 x e13 vector genome/kg. We found that the treated AxD rats started gaining body weight 3 weeks after the vector treatment as compared to the untreated AxD rats. Four weeks later, the treated AxD rats showed significant motor function improvements, including the rotarod test, grip strength, and horizontal ladder test, when compared to the untreated animals. In addition, histopathologic analysis showed a significant reduction of Rosenthal fibers in the brain of treated AxD rat. Reduced hyperintense signals (i.e. signs of brain edema) on T2 MRI imaging were observed in the treated AxD rat brains as well. Recently, dorsal root ganglion (DRG) toxicity was observed in non-human primates and mice after AAV intra-CSF delivery of RNAi expression constructs, evidenced by increased neurofilament-light in plasma. We analyzed the neurofilament-light in the plasma from AxD rats dosing with vector after one month and observed no elevation compared to the untreated disease rats, indicating no treatment-associated DRG toxicity. Furthermore, the treated AxD rats continue to show improved performances in motor function tests at 13 weeks post-injection. Taking together, our data suggests that astrocyte-restricted gene silencing is a promising strategy for efficient and safe treatment of AxD and potentially other gain-of-function diseases that affect astrocytes. GG and JX are co-corresponding authors.

31 Assessing the Cognitive Benefit of a Vectorized Tau Reduction Therapy

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Tauopathies are a broad group of neurodegenerative diseases (ND) defined by the aberrant misfolding, fibrillization, and spread of microtubule associated protein tau (tau). There are no approved therapies targeting misfolded tau. Clinical trials of antisense oligonucleotides (ASOs) targeting tau provide evidence that tau reduction is a promising therapeutic option. However, ASOs require multiple doses which confer financial and temporal burdens on patients and their caregivers. Our lab has developed an artificial miRNA targeting human tau (hTaui), but not mouse tau, which reduces toxic tau for up to three months in the brains of a mouse model of tauopathy (PS19) following a single dose. PS19 mice develop severe neuromuscular impairment and social cognitive deficits due to overexpression of human tau. We hypothesized that AAV9-hTaui provides long-term reduction of tau and prevents cognitive deficits associated with the spread of toxic tau. To test our hypothesis, we packaged our previously reported hTau5i candidate and a newly designed candidate predicted to improve selectivity for tau (hTau13i) into separate recombinant AAV9 capsids for delivery to the brain. AAV9-hTau5i, AAV9-hTau13i, AAV9-Scri (scrambled miRNA control) or vehicle control was administered to 6-month-old male and female PS19 mice via a single bolus injection into the intra-cisterna magna. Wild-type littermates received AAV9-hTau13i or vehicle to

serve as controls. Mice were monitored for activity, body weight, and disease progression for six months. Neuromuscular outcomes were evaluated via hindlimb clasping and social cognition was assessed in the 3-Chamber Social Approach Task at 8.5 months (early-stage ND) and 11 months (late-stage ND) of age. Brains were collected at 12 months of age and evaluated for tau expression via qPCR and ELISA and for tau seeding.

In agreement with our prior studies, PS19 mice that received AAV9-hTau5i exhibited improved survival and reduced disease severity by 40-50%. Mice that received AAV9-hTau13i also had improved survival and reduced disease severity but to a lesser extent (12-15%). Biochemical analysis confirmed tau was significantly reduced in the brains of both AAV9-hTaui treated groups. At early-stage ND, moderate to severe hindlimb clasping was already evident in 10% of PS19 control mice, whereas AAV9/hTaui treated mice had no to up to 5% moderate hindlimb clasping. At late-stage ND, only 19% of PS19 control mice had no or mild hindlimb clasping, which was improved to 40% for AAV9-h13i treated mice and 35% for AAV9-hTau5i treatment. No deficits in social cognition were observed in early-stage ND PS19 mice compared to WT controls while there was significant social cognitive impairment in late-stage ND PS19 control mice. Importantly, tau reduction by either AAV9-hTau5i and AAV9-hTau13i prevented social cognitive impairment at late-stage ND. Together, this data provides evidence that a vectorized tau reduction gene therapy using 2 different tau miRNA targeting sequences can provide lasting neurocognitive benefit.

In summary, both hTaui candidates significantly reduced tau levels in the brain, reduced disease severity and progression, and prevented cognitive deficits when assessed at the late stage timepoint. Our study provides novel evidence that reducing tau can preserve cognitive function. These findings support further development of this therapeutic approach for human use.

Gene and Cell Therapy for Ophthalmic and Auditory Diseases

32 HG004 Gene Therapy Reduces Chorioretinal Atrophy and Demonstrates Superior Retinal Safety Over Luxturna Clinical Settings

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Background:
Chorioretinal atrophy is a significant complication associated with Luxturna therapy, reported in up to 80% of treated eyes in real-world studies, with progressive atrophy affecting therapeutic outcomes in patients with *RPE65*-mediated inherited retinal dystrophy (IRD). This study compares the retinal safety profiles of HG004 (AAV9-*hRPE65*), developed with an AAV9 vector showing enhanced retinal pigment epithelium (RPE) tropism, lower total vector doses, and reduced injection volumes, against real-world Luxturna data. The **LIGHT** trial evaluates HG004's potential to mitigate chorioretinal atrophy while maintaining efficacy.

Methods:
Nine participants with Leber's congenital amaurosis type 2 (LCA2) received subretinal injection of HG004 (6×10^9 to 2×10^{10} vg/eye) in **LIGHT** trial (NCT06088992). Chorioretinal atrophy was assessed using optical coherence tomography (OCT), fundus autofluorescence, and fundus photography, defined by loss of autofluorescence, pigmentary changes, and thinning or loss of the outer retina on OCT. HG004-treated eyes were compared to real-world Luxturna-associated chorioretinal atrophy, as documented in the literature (Gange 2022; Reichel 2023; Stingl 2023).

Results:
Over a 52-week follow-up, localized retinal atrophy was observed in 33.3% (3/9) of cases in the low- and middle-dose cohorts, confined to the retinotomy sites. OCT revealed stable, non-progressive thinning of the outer nuclear layer and RPE at retinotomies (**Figs. 1a** and **1b**) without structural damage. In the high-dose cohort, no retinal atrophy was observed during the 38-week follow up. One subject (#009, **Fig. 1c**) exhibited pigment displacement around blebs, with no retinal structural damage. Importantly, no progressive chorioretinal atrophy was detected in any dose cohort.
By contrast, real-world data of Luxturna reported chorioretinal atrophy in up to 80% of treated eyes, with progressive perimacular atrophy affecting up to 55.5% cases (published example in **Fig. 2**). The differences are attributed to HG004's AAV9 vector, featuring superior RPE transduction efficiency, lower total vector dose (1/25 to 1/7.5th of Luxturna), reduced injection volume (200 µL vs. 300 µL), and optimized surgical techniques that minimize inflammation and mechanical retinal stretching.
Conclusions:
The **LIGHT** trial demonstrates that HG004 significantly mitigates the risk of progressive chorioretinal atrophy commonly observed with Luxturna. Retinal atrophy with HG004 is confined to retinotomy sites and remains non-progressive, emphasizing the benefits of enhanced RPE tropism, lower dosing, and optimized surgical techniques to provide a safer alternative for LCA2 patients. These findings underscore the critical role of vector design and delivery optimization in improving gene therapy safety profiles for IRDs. HG004 is being evaluated in an ongoing multi-national trial (NCT05906953) and has received Orphan Drug and Rare Pediatric Disease Designations by the U.S. FDA and Orphan Drug Designation by the EMA's COMP (a total of three designations).

Figure 1: Fundus images of the injected eyes before and after HG004 administration

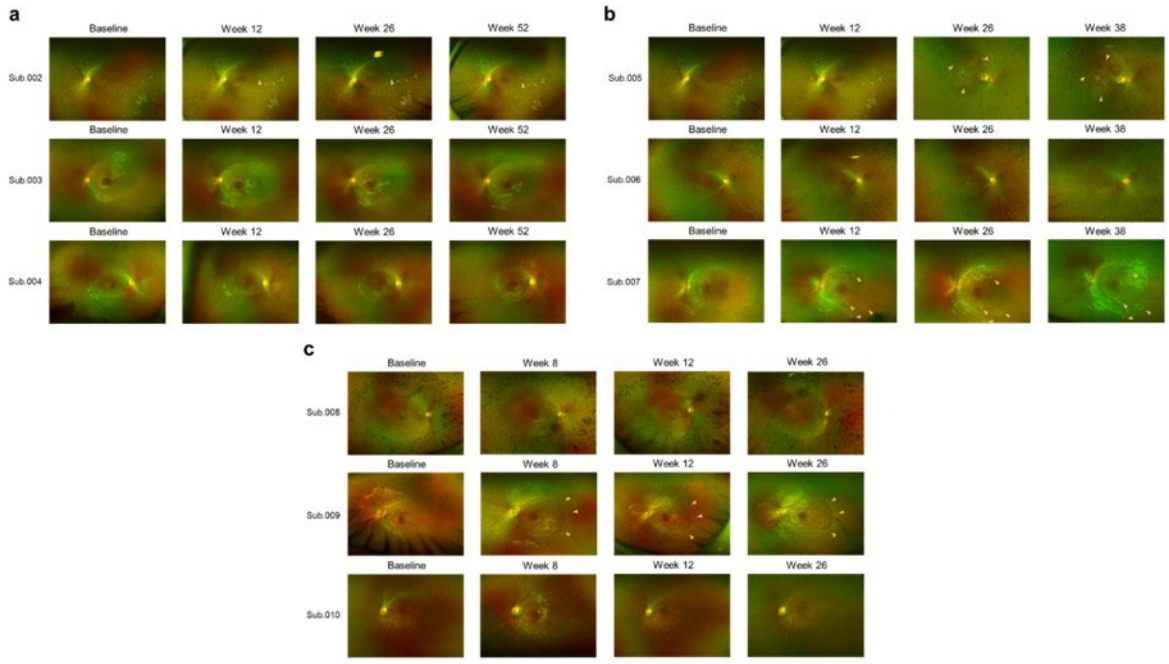
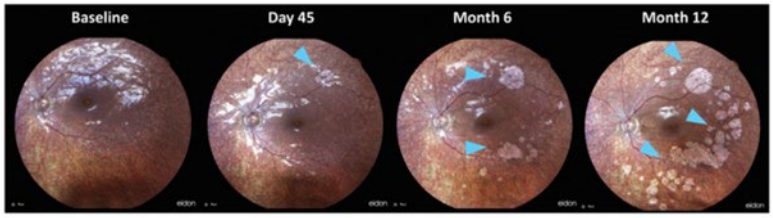


Fig 1a, **1b** and **1c** show fundus images of low-dose, middle-dose and high-dose cohorts. Yellow arrows (Sub#002, #005 and #007) indicate localized retinal atrophy at the injection sites and the pigment displacement (Sub#009).

Figure 2: Progressive atrophy developed after Luxturna in real world study



Growth of progressive atrophy (blue arrows) occurred within 1 year post-injection of Luxturna.
Progressive atrophy was observed as early as 45 days after injection of Luxturna*.

* Melillo P, Testa F, Di Iorio V, et al. Objective outcomes to evaluate voretigene neparvovec treatment effects in clinical practice. Ophthalmol Retina 2024.

33 CRISPR-Cas13 Gene Editing Therapy Targeting VEGFA Demonstrated Early Efficacy in Neovascular Age-Related Macular Degeneration

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Background:
Neovascular age-related macular degeneration (nAMD) is a leading cause of vision loss worldwide, driven by abnormal choroidal neovascularization primarily mediated by vascular endothelial growth factor (VEGF) overexpression. While anti-VEGF therapies have become the first line of treatment and significantly improved visual outcomes, their dependence on long-term repeated intravitreal injections and frequent clinic monitoring poses substantial burdens on patients and healthcare systems, often resulting in suboptimal treatment adherence and under-treatment. To address these challenges, the SIGHT-I clinical trial (NCT06031727) was initiated to evaluate HG202, a novel CRISPR-Cas13 RNA-targeting therapy designed to partially reduce VEGF-A mRNA expression and mitigate disease progression.

Methods:
The SIGHT-I trial is a multicenter, open-label, dose-escalation study evaluating the safety, tolerability, and preliminary efficacy of HG202, a one-time CRISPR-Cas13 RNA-editing treatment delivered via a single adeno-associated virus (AAV) vector, in patients with nAMD. Nine patients aged 50-80 years with active choroidal neovascularization, a best-corrected visual acuity (BCVA) between 73 and 23 letters, and either hard-to-treat or responsive to prior anti-VEGF therapy were enrolled. Participants were assigned to receive one of three doses of HG202 (1×10^{10}, 6×10^{10}, or 1×10^{11} vg/200 µL/eye) via subretinal injection. The primary endpoints were safety and tolerability, assessed through ocular and systemic adverse events (AEs), serious adverse events (SAEs), and immunogenicity. Secondary endpoints included changes in BCVA, central retinal thickness (CRT) measured via spectral domain optical coherence tomography (SD-OCT), and the annualized frequency of supplemental anti-VEGF injections.

Results:
As of October 2024, 6 subjects have been enrolled across the low- and middle-dose cohorts. Two subjects in the low-dose cohort have completed their 52-week follow-up, while the last subject in the mid-dose cohort has completed the 4-week follow-up. A total of 2 serious adverse events (SAEs) occurred: one case of subretinal hemorrhage secondary to polypoidal choroidal vasculopathy (PCV) and another case of rapid cataract progression post-surgery. Both SAEs resolved following surgical intervention, and no drug-related AEs, including retinal detachment, inflammation, or acute retinal toxicity, were observed.
For the first 5 patients (3 in the low-dose cohort and 2 in the mid-dose cohort), the CRT decrease ranged from 13 to 88 µm. The annualized frequency of supplemental anti-VEGF injections was reduced by 45.8% to 100%. In the low-

dose cohort, two phakic subjects experienced approximately a 10-letter BCVA decline due to cataract progression. In contrast, one subject in the mid-dose cohort exhibited a 7-letter improvement in BCVA following cataract surgery.

Conclusion:
Subretinal injection of HG202, a CRISPR-Cas13 RNA-targeting gene therapy, demonstrates a favorable safety profile with no retinal detachment, drug-related inflammation, or retinal toxicity. Early efficacy data reveal significant reductions in CRT and the frequency of anti-VEGF injections. These results suggest that HG202 has the potential to provide a durable, one-time therapeutic option for nAMD, addressing critical unmet needs in the management of this chronic disease.

34 Development of an AAV-Based Gene Therapy for the Ocular Phenotype of Friedreich's Ataxia

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Purpose:
Friedreich's ataxia (FA) is an autosomal recessive neurodegenerative disorder caused by mutations in the FXN gene that affects multiple systems. Up to a third of patients exhibit ophthalmic abnormalities by the second decade of life, including nystagmus, saccadic dysmetria, and optic nerve atrophy, along with a reduction in both retinal ganglion cells (RGCs) and retinal nerve fiber layer (RNFL) thickness. The purpose of this study is to generate and characterize a novel mouse model wherein FXN is preferentially ablated in RGCs, and to design an adeno-associated virus (AAV)-mediated gene therapy for subsequent evaluation in this model.

Approach:
Mice expressing Cre recombinase under the *Pou4f2* promoter, a transcription factor predominantly expressed in RGCs (Pou4f2Cre mice), were bred with $Fxn^{+/-}$ mice to generate $Pou4f2^{Cre+/-}$*;flFxn+/Fxn-* (Pou4f2-FXN KO) and $Pou4f2^{Cre-/-}$*;flFxn+/Fxn-* (Pou4f2-FXN control) mice. A natural history study lasting six months was performed to characterize retinal structure and function. Retinal nerve fiber layer (RNFL), ganglion cell layer + inner plexiform layer (RGC+IPL), inner nuclear layer (INL), and outer nuclear layer (ONL) thicknesses were measured via optical coherence tomography (OCT) and full-field (ffERG) and pattern (pERG) electroretinography assessed retinal function. Optokinetic tracking (OKT) was conducted to characterize visual function. Immunohistochemistry (IHC) was performed on retinal sections postmortem to evaluate transgene expression and to quantify RGC abundance. For gene therapy, an engineered AAV capsid, AAV.P2-V1(Y-F+T-V), was used to deliver AAV-hSyn1-FXN-HA (AAV-FXN), while AAV-hSyn1-GFP (AAV-GFP) served as a control. Intravitreal injections were performed in Pou4f2-FXN KO mice (one eye receiving AAV-FXN and the contralateral eye receiving AAV-GFP). Retinal structure and function were assessed by the in-life and post-mortem methods mentioned above.

Results:
Pou4f2-FXN KO mice remained healthy throughout the study, displaying no ataxia or other systemic signs of FXN deficiency. By postnatal day 30 (P30), OCT revealed a progressive thinning of the RNFL and GCL+IPL, with INL thinning becoming evident by P120. pERG showed a significant reduction in P1 amplitudes in KO mice relative to controls at P30; by P90, both P1 and N2 waveforms were significantly reduced. OKT assessments corroborated a reduced visual response in KO mice compared to controls as early as P30. Retina wholemount staining at P180 with RBPMS, an RGC marker, confirmed a loss of RGCs in KO mice. In the gene therapy arm, AAV-FXN treatment conferred significant protection of retinal structure (thickness of the GCL+IPL and INL) at 1 MPI (P60) compared

to AAV-GFP–treated eyes, and retinal function (pERG amplitudes) by 3 MPI (P120). RBPMS staining further showed that RGC loss was partially mitigated in AAV-FXN–treated eyes relative to AAV-GFP–treated controls.

Conclusions:
We successfully generated and characterized an RGC-specific FXN knockout mouse model that exhibits marked and progressive retinal dystrophy and mirrors the ocular phenotype of FA. Preliminary findings indicate that AAV-mediated FXN supplementation preserves retinal structure and function in Pou4f2-FXN KO mice, establishing proof-of-concept for this therapeutic strategy. Ongoing work will explore enhanced vector designs and extended evaluations of AAV-FXN in the Pou4f2-FXN KO model.

35 Safety and efficacy of Multi-characteristic opsin gene therapy in improving vision in NHP model of geographic atrophy

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Intravitreally delivered AAV2 packaged ambient light activatable multi-characteristic opsin (MCO) has demonstrated durable safety and efficacy in clinical trials on patients with severe vision loss due to inherited retinal diseases. Here, we report an evaluation of the safety, expression, and functional efficacy of MCO treatment in a non-human primate (NHP) model of geographic atrophy (GA) created using optical coherence tomography (OCT)-guided laser micro-irradiation.

The GA model was created in African green monkeys using OCT-guided scanning green (532 nm, 120 mW) laser micro-irradiation of the retinal pigment epithelium (RPE) and photoreceptor layers between the temporal vascular arcades leading to progressive RPE and outer retinal atrophy, confirmed by OCT, color fundus photography, and confocal scanning laser ophthalmoscopy (cSLO) imaging. Two weeks after laser injury, unilateral intravitreal AAV-MCO (0.5E11 gc/eye) injection was carried out in the treatment group to match the human experience (~1E11 gc/eye), while the control group was monitored to evaluate the progression of the atrophy. Evaluation of safety included monitoring of body weight, clinical observations, and pathological analysis. Further, measurement of intraocular pressure, and slit lamp examinations was carried out. OCT imaging was conducted to monitor any post-injection changes in retina structure, and multifocal electroretinography (mfERG) was performed at baseline and up to 13 weeks post-treatment to evaluate functional changes. Upon euthanization at the end of the 13-week study period, immunohistochemical (IHC) analysis was conducted to determine gene expression in retina.

The MCO treatment was well-tolerated, with only mild, transient inflammation observed in treated eyes two days post-injection. The treated animals maintained stable body weights and showed no significant treatment-emergent adverse effects throughout the study period. OCT analysis showed attenuation of retinal degeneration in MCO-treated eyes compared to untreated control eyes. At the end of the study, mfERG signal was significantly higher in MCO-treated eyes with photoreceptor degeneration compared to controls ($p<0.05$). IHC analysis confirmed robust expression of MCO in the higher-order neurons across the retina.

This study demonstrated the therapeutic potential of AAV-MCO gene therapy in an NHP model of GA. Intravitreal AAV-MCO was well-tolerated, with only transient mild inflammation observed shortly after administration. The treated eyes showed attenuation of further retinal degeneration and enhanced retinal functioning as compared to untreated controls. The favorable safety profile and observed efficacy in NHP support further development of MCO as a potential therapeutic approach for patients with GA.

36 Gene Therapy vs Cochlear Implantation in Restoring Hearing Function and Speech Perception for Congenital Deafness Individuals: An Observational Cohort Study

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IMPORTANCE *OTOF* Gene therapy (GT), a novel therapeutic strategy for congenital deafness, has been shown to improve hearing and speech. The efficacy of GT remains to be compared against the performance of cochlear implantation (CI), which have been the gold standard for congenital deafness in the last half-century.

OBJECTIVE To comprehensively evaluate the treatment outcomes in auditory and speech perception between congenitally deaf patients treated with GT, CI, or both.

DESIGN This observational non-blind cohort study was conducted between 28 December 2022 and 30 November 2024. The GT patients received follow-up at 3 (T1), 6 (T2), and 12 (T3) months; CI patients received one-time evaluation at the corresponding time intervals or longer (T1, T2, or T3).

SETTING Single-center, Class A tertiary hospital in China.

PARTICIPANTS Participants with congenital severe-to-complete hearing loss, aged 1-18 years, who received GT or CI were enrolled. They were matched on duration of deafness, hearing thresholds, and speech ability at the pre-surgical baseline. There were 1568 participants screened and 72 participants enrolled.

EXPOSURE Based on the hearing modality: 1) GT-only patients (n=8) vs matched CI patients (n=43); 2) Bimodal patients (unilateral GT + contralateral CI): GT+CI (n=3) vs bilateral CI (n=12), GT (CI-off) (n=3) vs unilateral CI (n=6).

MAIN OUTCOMES AND MEASURES The primary outcomes were the auditory and speech perception evaluated by questionnaires including the Infant-Toddler Meaningful Auditory Integration Scale/Meaningful Auditory Integration Scale (IT-MAIS/MAIS), Categories of Auditory Performance (CAP), Speech Intelligibility Rating (SIR), and Speech, Spatial, and Other Qualities of Hearing Scale for Parents (SSQ-P) and tests including audiometry, speech in quiet and noise, and music tests, etc. The main secondary outcomes was auditory information processing ability assessed by mismatch negativity (MMN).

RESULTS A total of 11 GT patients (6 males, 55%) and 61 CI patients (34 males, 56%) were enrolled. The average auditory brainstem response thresholds were restored from >95.0 (SD, 0.0) dB nHL to 54.8 (SD, 15.9) dB nHL in nine GT patients at 12 months. For GT-only vs CI in auditory and speech perception, GT patients performed better in IT-MAIS/MAIS (median [IQR], 31.0 [30.0-32.0] vs 23.5 [19.0-26.3], P=0.01), SIR (2.0 [2.0-2.0] vs 1.0 [1.0-2.0], P=0.001), SSQP-speech (7.3 [6.4-7.8] vs 6.1 [4.9-6.7], P=0.03), SSQP-other qualities (7.6 [6.6-8.5] vs 5.5 [3.4-6.7], P=0.01), and SSQP noise related (8.3 [7.5-9.2] vs 6.3 [5.0-7.4], P=0.01) at T2; In IT-MAIS/MAIS (32.0 [31.0-32.0] vs 28.0 [24.5-30.5], P=0.007), and CAP (6.5 [6.0-7.0] vs 6.0 [5.0-6.0], P=0.04) at T3. GT patients showed shorter latencies of MMN at T2 (0.20 s [0.05 to 0.21] vs 0.23 s [0.22 to 0.25], P=0.006). For bimodal patients at T3, GT (CI-off) patients performed better than unilateral CI patients in speech in noise (disyllable, -1.0 [-3.0 to 2.4] dB SPL vs 5.3 [3.1 to 12.1] dB SPL, P=0.03); GT+CI patients performed better than bilateral CI patients in singing in-tune rates (66.6% [53.7 to 83.9] vs 37.1% [30.3 to 56.3], P=0.04); GT + CI patients showed shorter latencies of MMN at T3 (0.08 [0.07-0.10] s vs 0.21 [0.15 to 0.23] s, P=0.01).

CONCLUSIONS AND RELEVANCE GT patients show stable hearing recovery and exhibit more rapid improvements in auditory and speech performance than in CI patients, while outperforming CI in speech in noise

37 Prevention of Vision Loss using Subretinal Gene Therapy in a Mouse Model of Bardet-Biedl Syndrome Type 10

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Introduction:
The Bardet-Biedl Syndrome 10 (BBS10) protein is essential for the assembly of the BBSome and ciliary trafficking, particularly in photoreceptor cells in the eye. Biallelic mutations in BBS10 are the second most common cause of BBS, leading to obesity, polydactyly, and vision loss. Human-grade gene therapy vector candidates, *AAV8-RK-hBBS10* and *AAV8-CAG-hBBS10*, were developed for clinical translation, and *AAV8-RK-hBBS10* was designated as the lead candidate. In this study, we will report the safety and efficacy of subretinal gene delivery using *AAV8-RK-hBBS10* in a murine model of BBS10.

Methods:
The human BBS10 gene driven by either the rhodopsin kinase (RK) promoter or the CMV enhancer/chicken β-actin promoter (CAG) was cloned into a shuttle plasmid, packaged into AAV2/8, and titered by quantitative PCR for in vivo gene delivery. For toxicity testing, 2.5×10^{11} vg (high dose) of either vector was subretinally injected into unaffected wild-type (WT) or heterozygous (HET) mice in a 2 µL volume, and optical coherence tomography (OCT) was performed. For efficacy testing, *Bbs10*$^{-/-}$ mice were subretinally injected with 2.5×10^{11} (high dose), 2.5×10^{10} (medium dose), 2.5×10^{9} (low dose) vg/eye of *AAV8-RK-hBBS10*, and outcome measures including electroretinography (ERG) and OCT were conducted at 1-, 2-, 3-, 5-, and 7-months post injection (MPI). ERG tests for cone- and rod-dependent functions were conducted. A visually-guided swim assay for rodent vision was conducted at 5- to 7-MPI. Results are shown as averages ± standard error of the mean, and p values were derived from two-way ANOVA with post-hoc Tukey's test.

Results:
WT or HET unaffected mice injected with *AAV8-RK-hBBS10* did not show signs of toxicity, in contrast to those receiving *AAV8-CAG-hBBS10*. Efficacy testing was conducted using *AAV8-RK-hBBS10* in a knockout mouse model of BBS10 (*Bbs10*$^{-/-}$). First, cone-dependent electrical function was tested using the 5 Hz flicker ERG test. At 5-MPI, eyes of *Bbs10*$^{-/-}$ mice that received the high dose of *AAV8-RK-hBBS10* had greater ERG amplitudes when exposed to a 5 Hz flicker compared to signals from those receiving the medium dose ($p = 0.0856$), the low dose ($p = 0.0006$), or signals from untreated contralateral eyes ($p < 0.0001$) (high dose: 17.79 ± 5.28 µV; medium dose: 9.596 ± 1.11 µV: low dose: 5.61 ± 1.01 µV; untreated *Bbs10*$^{-/-}$: 4.97 ± 0.68 µV). Flicker amplitudes in eyes that received low dose were not different than those that were left untreated ($p = 0.9972$), indicating that dosing significantly impacts rescue of cone-dependent electrical function. Then, rod electrical function was measured by the 0.01 cd·s/m2 dim flash ERG test. At 5-MPI, ERG amplitudes were greater in eyes injected with the high ($p < 0.0001$) and medium doses ($p = 0.0416$), but not in those injected with the low dose ($p = 0.8763$) when compared to amplitudes in untreated contralateral eyes (high dose: 111.83 ± 10.30 µV; medium: 76.91 ± 13.32 µV; low: 31.04 ± 12.17 µV; untreated: 26.61 ± 5.826 µV). *Bbs10*$^{-/-}$ mice receiving the high dose took less time to find the platform in a visually guided swim assay compared to those that did not, indicating rescue of functional vision.

Conclusions:
Efficacy of subretinal gene therapy using *AAV8-RK-hBBS10* depends on dosing, and toxicity was not observed in any of the dose groups. Rescue in both rod-dependent and cone-dependent function was observed. Subretinal gene therapy using *AAV8-RK-hBBS10* has the potential to prevent vision loss.

38 Sustained Drug Delivery of Ciliary Neurotrophic Factor by Encapsulated Cell Technology: NT-501 Implants:

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Purpose:
Neurotech's novel encapsulated cell therapy (ECT) drug product NT-501 utilizes a genetically engineered cell line based on retinal pigment epithelial cells to deliver ciliary neurotrophic factor (CNTF) to the retina. CNTF is a potent neuroprotective protein that promotes the survival and maintenance of photoreceptors and has shown a therapeutic benefit in Macular Telangiectasia type II patients. This study is a retrospective analysis of CNTF release over drug product stability and shelf life. Additionally, the long-term durability and function of the NT-501 implant was evaluated by collecting CNTF delivery data from explanted devices that were implanted from 6 months up to 14.5 years.

Methods:
In-house and commercial methods were used to characterize CNTF including direct Western Blot, ELISA and bioassays. These methods were used to characterize the stability of the implant over shelf life, the rate of CNTF production, and the potency of NT-501 secreted CNTF. NT-501 implants from multiple clinical trials were recovered from participants who underwent explantation over a wide duration. All trials were approved by the relevant institutional review board/ethics committee, and all studies adhered to the tenets of the Declaration of Helsinki.

Results:
The CNTF secretion over product release and shelf-life was shown to deliver CNTF with comparable levels and specific potency to reference protein up to 14 weeks. With regards to the explanted devices, the data from the study participants demonstrate that the NT-501 implants continued to produce steady levels of bioactive CNTF (1.75 ng/day), again with extremely close specific activity with a geometric mean potency of 1.27 (Upper 95% CI 1.38 & Lower 95% CI 1.18) of patients implanted for durations of six months to 14.5 years.

Conclusions:
Neurotech's ECT was developed to overcome the challenge of long-term therapeutic delivery to the retina. NT-501 consists of an encapsulated cell line that has been genetically engineered to produce a novel secreted form of human CNTF. Extensive characterization was conducted across product shelf life, stability, and on explanted devices which were implanted for up to 14.5 years. This retrospective analysis suggests that a single intraocular administration of NT-501 provides sustained, bioactive CNTF to the vitreous for a significant period of time.

Gene and Cell Therapy for Metabolic Diseases

39 Systemically delivered mRNA therapy crosses blood brain barrier and shows neurological benefit in mouse model of Argininosuccinic Aciduria

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Argininosuccinic aciduria (ASA) is an autosomal recessive metabolic disorder caused by deficiency of cytosolic enzyme argininosuccinate lyase (ASL). ASL converts argininosuccinate into arginine and fumarate, key step in the urea cycle for ammonia detoxification and in the nitric oxide (NO) cycle for NO synthesis. ASA is the second most common urea cycle defect. Patients can develop hyperammonemic decompensations and a systemic disease with neurological symptoms, i.e. intellectual difficulties, epilepsy and movement disorder. The neurological pathophysiology is likely multifactorial, caused by ammonia and argininosuccinate toxicity, nitro-oxidative stress, central catecholamine deficiency and blood-brain barrier (BBB) leakage. Standard of care aims to normalise ammonia levels but limited efficacy, especially on the neurological phenotype, asserts the need for novel therapies. We previously showed proof of concept of intravenously (IV) delivered liver-targeting lipid nanoparticles (LNPs) encapsulating human ASL (hASL) mRNA (ASL-LNP) in a hypomorphic ASA mouse ($Asl^{Neo/Neo}$) in correcting the liver disease. Here, we investigate the efficacy of IV ASL-LNP therapy on the neurological phenotype of AslNeo/Neo mice. $Asl^{Neo/Neo}$ mice were IV-treated weekly with ASL-LNP from birth (neonatally-treated) or from juvenile age (adult-treated). They were compared to wild-type (WT), untreated or Luciferase mRNA (Luc-LNP) neonatally IV-treated weekly $Asl^{Neo/Neo}$ littermates, or ASL-LNP IV-treated weekly AslNeo/Neo mice from birth with therapy stopped at 6-weeks of age (withdrawn group). Surviving animals were harvested at 3 months of age. Alongside confirmed correction of ureagenesis and restoration of hASL liver expression, ASL-LNP neonatally- and adult-treated mice showed cerebral hASL protein expression, demonstrating significant LNP-mRNA crossing of BBB. Behavioural tests showed decreased anxiety and increased locomotion in ASL-LNP treated AslNeo/Neo mice versus withdrawn mice, and comparable to WT. Cerebral biochemistry showed normalisation of toxic metabolites (arginosuccinate, guanidinosuccinate), arginine, citrulline, creatine and creatinine levels, compared to untreated controls. Cerebral Magnetic Resonance Imaging (MRI) showed reduced neurite density index in grey matter areas but preserved white matter in AslNeo/Neo withdrawn versus WT mice. This finding was corrected in ASL-LNP neonatally-treated littermates. Cerebral [18]Fluorodeoxyglucose positron emission tomography (FDG-PET) showed significantly reduced glucose consumption in withdrawn versus WT mice, corrected in ASL-LNP neonatally-treated littermates. We assessed epileptogenesis in withdrawn AslNeo/Neo mice compared to WT and ASL-LNP neonatally-treated mice following injection with seizure-inducing GABA-A antagonist pentylenetetrazole. Withdrawn mutants showed higher seizure probability and severity, compared to WT, which were normalised in ASL-LNP treated mice. Assessment of oxidative stress in brain slices showed glutathione deficiency, and increased levels and altered kinetics of cytosolic and mitochondrial reactive oxygen species in untreated mutants versus WT. These findings were normalised in ASL-LNP neonatally-treated mice at 2-weeks of age. This therapy provides in situ cerebral ASL expression correcting neuropathology and supporting clinical translation in ASA. ASA is a unique genetic disease model enabling first proof of concept of BBB crossing by systemically delivered LNP-mRNA

40 Rescue of Glutaric Aciduria Type I by GalNAC conjugated siRNA against Aminoadipate Semialdehyde Synthase

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Glutaric Aciduria Type 1 is a serious metabolic disorder that occurs with a frequency of 1:100,000 live births. It is caused by a deficiency in Glutaryl-CoA Dehydrogenase (GCDH), a crucial enzyme in the lysine degradation pathway. The deficiency results in accumulation of toxic metabolites such as glutaric acid (GA) and 3-OH-glutaric acid (3-OH-GA), causing encephalopathic crisis and debilitating movement disorders if untreated. Early detection, strict dietary control and emergency interventions reduce the risk of severe cerebral injury. Nevertheless, complex movement disorders are still encountered in one-third of patients.

We previously showed that restoring a normal lysine catabolism in the liver of adult *Gcdh-/-* mice via hepatocyte transplant or AAV gene therapy is sufficient to rescue the lethality and the neurological phenotype. We also identified Aminoadipic Semialdehyde Synthase (AASS), an enzyme upstream of GCDH in the lysine degradation pathway, as a novel therapeutic target by deleting the gene using a liver-specific CRISPR approach (Barzi et al. *Sci Transl Med* 2023).

Here, we explored the efficacy of an N-acetylgalactosamine (GalNAC) conjugated small interfering RNA (siRNA) targeting *Aass* (siRNA-*Aass*) in *Gcdh-/-* mice. In both adult and neonatal mice treated with siRNA-*Aass*, we observed a dose dependent survival and weight gain. The rescued mice showed no obvious clinical phenotypes. Toxic catabolites GA and 3-OH-GA were reduced in the liver, brain, kidney, urine and plasma. Importantly, siRNA treated mice had fewer vacuolations in the hippocampus, indicating a reduction in brain associated pathologies. In summary, a liver-targeted siRNA-*Aass* therapy is an effective treatment for the GA-I mouse model. These findings hold promise for an improved therapeutic management of GA-I patients, with the potential to prevent debilitating movement disorders.

41 Encapsulated cell therapy demonstrated therapeutic efficacy in metachromatic leukodystrophy and sustained ARSA production in NHP

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Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder caused by an inherited deficiency of arylsulfatase A (ARSA) and characterized by accumulation of sulfatides in both glial cells and neurons resulting from myelin degeneration in the central and peripheral nervous systems. Currently, no effective treatment is available for patients once symptoms are already present. Libmeldy©, an Ex vivo gene therapy, is the gold standard for pre-symptomatic MLD patients. Here, we investigated an innovative approach to deliver the recombinant ARSA enzyme by encapsulated-cell therapy. A cell-based device is capable of continuously delivering human ARSA compared to weekly intrathecal delivery of rARSA which is under clinical evaluation. Two constructs of genetically engineered cells have been tested to determine which one could target more efficiently the central nervous system. Moreover, a dose response evaluation has been done with 2 doses (cells number loaded) to evaluate the best therapeutic effect. MLD mice were implanted at 6 months once disease is well established. The device biocompatibility as well as the therapeutic efficacy have been evaluated 3 months after implantation. The device is well tolerated in animals. Moreover, we demonstrate a correction of sulfatide storage and significant improvement of neuroinflammation in all treated mice. Encapsulated, immortalized human myoblast genetically engineered to produce ARSA can deliver controlled, sustained, stable ARSA over months. After a proof of concept demonstrating therapeutic effect if subcutaneous capsule implantation in MLD mice we present here safety and biological activity of prolonged intracranial implantation for 5 months in non-human primates in MLD mice

MyoP devices, loaded with ARSA producing human myoblast cells were successfully implanted subdurally in 2 NHP. Over 5 months the tolerance was perfect with no adverse effect especially no neurological or behavioral changes.

NHP were sacrificed after 5 months and extensive biological/anatomical analysis were performed.

- Supernatants from all explanted capsules showed stable ARSA activity
- ARSA activity in neuronal tissues was doubled in both NHP compared to non-treated historical controls and or baseline (CSF)
- Hemagglutinin A, a tag attached to ARSA produced by the myoblast cell line, was detected in most area of the brain at necropsy; confirming the good release and biological processing of ARSA delivered by the encapsulated cells.

Beyond MLD treatment, these findings establish proof-of-concept for subdural protein delivery, unlocking therapeutic potential for CNS diseases where proteins face challenges crossing the blood-brain barrier. To advance towards clinical translation, we will:

- Establish GMP manufacturing process for clinical-grade Myo-P encapsulated-cell products,
- Conduct first-in-human phase I/II study in early symptomatic late infantile and juvenile MLD patients.

42 Definition of a Minimal Therapeutic Dose of *In Vivo* Liver-Directed Lentiviral Gene Therapy for Methylmalonic Acidemia

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Methylmalonic acidemia (MMA) is a severe metabolic disorder caused by mutations in the *MMUT* gene, resulting in a build-up of toxic methylmalonic acid (*MMAc*). Immune-shielded lentiviral vector (ISLV)-based liver gene therapy may provide a stable benefit in young children at the initial disease stage by virtue of vector integration into the genome of target cells. We have shown that administration of an ISLV expressing the wild-type *MMUT* transgene (ISLV.*MMUT*wt) into the MCK-*Mmut*-/- mice, an intermediate MMA mouse model, resulted in long-term (>1 year) amelioration of disease-related biomarkers, hepatic and extrahepatic detoxification from *MMAc*, and global rescue of the metabolomic profile. To increase the transgene expression per transduced cell and reduce the therapeutic ISLV dose, we tested a codon-optimized *MMUT* transgene under the control of a hepatocyte-specific expression cassette (ISLV.*MMUT*co), showing higher transgene expression and therapeutic efficacy when using it at similar doses compared to ISLV.*MMUT*wt. Reducing the effective LV doses facilitates manufacturing and alleviates potential dose-dependent toxicities. We administered ISLV.*MMUT*co to 2-week-old MCK-*Mmut*-/- mice at three different doses (1, 2, and 4×10^{10} TU/kg). We showed dose-dependent amelioration of disease biomarkers and hepatic and extrahepatic detoxification from *MMAc* with different kinetics depending on the ISLV dose. End-point RNA in situ hybridization analyses on the livers showed dose-dependent hepatocyte transduction efficiency, ranging from 20% to 80% of transgene-positive cells. We observed a substantial increase in transgene-expressing hepatocytes in livers collected at late (6 months) compared to early (6 weeks) time points after vector administration. This indicates a selective advantage of the transduced hepatocytes at the lowest tested ISLV dose. The progressive amelioration of the MMA-related biomarkers over time confirmed the selective advantage in this experimental group, with slower kinetics compared to mice administered at the two higher ISLV doses. These data highlight the potential for long-term therapeutic benefit, even when starting from sub-therapeutic doses. On the other hand, at the two higher doses, we found a rapid and sustained up to 8-fold reduction of circulating *MMAc* concentrations (mean of 276 µM and 1608 µM in treated and untreated MCK-*Mmut*-/- mice 2 weeks post-ISLV; mean of 157 µM and 1313 µM in treated and untreated MCK-*Mmut*-/- mice 20 weeks post-ISLV). To determine the ISLV dose required to achieve comparable transduction levels in human hepatocytes for clinical translation, we administered ISLV expressing a reporter transgene to liver-humanized Fah-/-Rag2-/-Il2rg-/- NOD mice (FRGN) with ≥70% human hepatocyte repopulation. We showed efficient and dose-dependent human hepatocyte transduction, achieving levels comparable to those observed in the MMA mouse model but at substantially lower doses (e.g., $1.9–7.5\times10^{9}$ TU/kg). The higher transduction efficiency achieved in this setting is due to the enrichment of human CD47, a phagocytosis inhibitor, on the surface of ISLV. This signal is recognized in FRGN mice because of the NOD background, protecting ISLV from macrophage uptake and thus increasing gene transfer into hepatocytes. These data provide critical insight into the minimal therapeutic ISLV dose required for first-in-human in vivo liver-directed ISLV gene therapy, supporting progression toward a clinical trial in MMA patients.

43 Neonatal AAV gene therapy provides long term rescue in mouse models of combined methylmalonic acidemia and homocystinuria, *cblC* type

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Combined methylmalonic acidemia and homocystinuria, *cblC* type (*cblC*) is the most common inborn error of intracellular cobalamin (vitamin B_{12}) metabolism caused by pathogenic variants in *MMACHC*. The protein encoded by *MMACHC* is responsible for the transport and processing of vitamin B_{12}. Early onset disease can be severe with symptoms including growth failure, anemia, heart defects, neurocognitive impairment, and progressive vision loss despite treatment. In order to further characterize manifestations of the disease and investigate AAV gene addition therapy as a possible treatment, we generated and characterized three *Mmachc* mutant alleles using gene editing: *Mmachc*^{del2} c.163_164delAC p.(Pro56CysfsTer4) , *Mmachc*^{del3} c.162_164delCAC p.(Ser54_Thr55delinsArg) and *Mmachc*^{del23} c.163_185del p.(Thr55ProfsTer42). *Mmachc*$^{-/-}$ mutants recapitulated the clinical phenotypes of *cblC* including embryonic and neonatal lethality, poor survival (median 5 days, with complete lethality by 1 month), growth impairment, neuropathology (hydrocephalus and thinning of the corpus callosum) and the hallmark biochemical features of elevated plasma methylmalonic acid, increased homocysteine and decreased methionine. We tested three AAV gene therapy vectors: rAAVrh10-CBA-*Mmachc* (AAVrh10), rAAV9-CBA-co*MMACHC* (AAV9) and rAAV9.PHP.eB-CBA-co*MMACHC* (PHP.eB). AAVrh10 and AAV9 were utilized to target liver, brain and other tissues and PHP.eB to target the central nervous system. The AAVs were delivered by a single intrahepatic injection at 1 x 10^{11} GC/pup P0-2. Neonatal treatment with all three AAV vectors [*Mmachc*^{del2} AAV9, PHP.eB; *Mmachc*^{del3} AAVrh10, AAV9; *Mmachc*^{del23} PHP.eB] resulted in long-term survival of mutants (n=7-11 per group, median >250 days) in all studies which was statistically significant compared to untreated mutants ($p<0.01$). Disease related metabolites (methylmalonic acid, homocysteine and cystathionine) remained elevated in mutants compared to wildtype and heterozygous controls: methylmalonic acid was greatly reduced in AAVrh10 (159.7 ± 7.5 uM; N = 3) and AAV9 treated *Mmachc*$^{del3/del3}$ mice (136.9 ± 47.5 uM; N = 3) vs untreated (503.3 ± 279.5 uM) $p<0.01$. Pathological examination of *Mmachc*$^{del3/del3}$ mutant mice treated with AAV9, one year after vector administration, indicated a mitigation of neuropathology. These *Mmachc* mouse models recapitulate key phenotypic and metabolic disease manifestations of severe cobalamin C deficiency and can be used to assess the preclinical efficacy studies of new genomic therapies. A single intrahepatic injection of an AAV gene addition vector, carrying either the mouse *Mmachc* or human *MMACHC* gene and pseudoserotyped with various capsids, led to increased survival and long-term rescue of *Mmachc*$^{-/-}$ mice. Systemic AAV gene therapy therefore holds promise as a potential therapeutic strategy to treat individuals with cobalamin C deficiency, the most common disorder of intracellular vitamin B12 metabolism.

44 ABO-101, a Novel Gene Editing Therapy for Primary Hyperoxaluria Type 1, is Efficacious and Well Tolerated in NHPs and Results in High Fidelity Editing in Primary Hepatocytes

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Primary hyperoxaluria, or PH, is a group of rare genetic metabolic disorders characterized by the overproduction of oxalate by the liver. Oxalate builds up and accumulates in the kidneys and other organ systems, causing kidney damage and progression towards renal failure. PH1, the most common and clinically severe subtype of PH, results from a mutation of the *AGXT* gene that is expressed primarily in the liver. Here we present preclinical mouse and NHP *in vivo* data as well as genotoxicity data supporting ABO-101 as a novel CRISPR-Cas-mediated gene editing therapy for the treatment of PH1.

The current standard of care for PH1 is siRNA therapy that requires repeated lifelong treatments and targets *HAO1*, which encodes the glycolate oxidase enzyme (GO) and is upstream of the mutant *AGXT*. ABO-101 was designed to specifically inactivate the *HAO1* gene in liver hepatocytes and provide a durable reduction of oxalate levels through a single course of treatment. Here, we demonstrate durable reduction of hepatic GO protein expression in a rodent hepatectomy model (~88% GO knockdown pre- and post-hepatectomy) as well as long-term reduction of urinary oxalate to therapeutically meaningful levels (>30% reduction) for >6 months in a PH1 disease model. In addition to mouse pharmacology, we show that in NHPs treated with ABO-101, we achieved *HAO1* editing of approximately 60% in whole liver tissue, which indicates editing of the majority of hepatocytes and drives a greater than 2-fold increase in serum glycolate, a biomarker of knockdown of the pathway, all with a well-tolerated safety profile. Lastly, we highlight our genotoxicity and reproductive toxicity data packages demonstrating high fidelity of ABO-

101 across >1,500 candidate off-target sites; the maintenance of the chromosomal structure; and lack of germline transmission in vivo. Taken together, these results demonstrate signals of efficacy in mice and NHPs and safety at the genome-wide and organismal level, further supporting the advancement of ABO-101 into the clinic as a potential treatment for PH1.

45 RNA Switch-Regulated Biofactory Gene Therapies for Rare and Common Metabolic Disorders

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AAV-transduced skeletal muscle cells can function as long-lived biofactories to continuously produce and secrete AAV-encoded biologics into the bloodstream. This approach has been demonstrated promising and is particularly attractive for biologics with short blood half-lives, such as protein/peptide hormones, which typically have blood half-lives ranging from minutes to hours. Genetic switches that enable precise temporal and dose control of transgene expression may improve safety and efficacy, and broaden the use of this approach, particularly for therapeutics with narrow therapeutic windows and/or major contraindications.

We recently developed a class of ultra-efficient RNA ON-switch systems (Z^{on}) that allow for switch regulator dose-dependent, monthly or less frequent repeated inductions of AAV transgene expression for more than a year, with regulatory ranges spanning five logs or more in mice. Here, we describe the utilization of Z^{on} to tightly regulate the expression of two short-lived protein/peptide hormones in two POC studies aimed at developing controllable biofactory gene therapies for (i) a rare genetic metabolic disease, congenital leptin deficiency (CGL), and (ii) multiple related common metabolic conditions, including obesity, insulin resistance, and metabolic dysfunction-associated steatohepatitis (MASH).

In the POC study for the treatment of CGL, administration of a switch regulator every six weeks to induce Z^{on}-controlled AAV-*leptin* resulted in (i) leptin protein production at physiological levels in circulation, (ii) a profound body weight-lowering effect, and (iii) normalization of hyperglycemia in leptin-deficient ob/ob mice. In the other POC study targeting obesity, insulin resistance, and MASH, Z^{on}-controlled expression of another short-lived protein/peptide hormone in genetically obese ob/ob mice led to (i) significant reductions in body weight, (ii) normalization of blood glucose and insulin levels, and (iii) substantial improvement in MASH-associated markers of liver injury and inflammation. These data demonstrate that Z^{on} RNA switch-controlled biofactory gene therapy is a promising approach for the treatment of certain rare genetic and common metabolic disorders.

Physical Delivery Methods and DNA/RNA Drug Development

46 The Tethosome Platform is a Non-Viral, Dual DNA/mRNA System That Facilitates Potent, Re-Dosable, and Durable Therapeutic Gene Expression

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Genetic medicine offers the potential to treat many currently incurable heritable or acquired diseases. Numerous viral DNA delivery systems have resulted in life-changing therapies, but challenges relating to immunogenicity, redosability, and manufacturability currently limit the utility of viral vectors. While non-viral gene delivery systems possess favorable manufacturability and redosability properties, several key challenges remain. Historically, gene expression from non-viral DNA vectors has been several orders of magnitude below therapeutic levels for most indications; this is attributed largely to the limited efficiency of DNA transport into the nucleus. In addition,

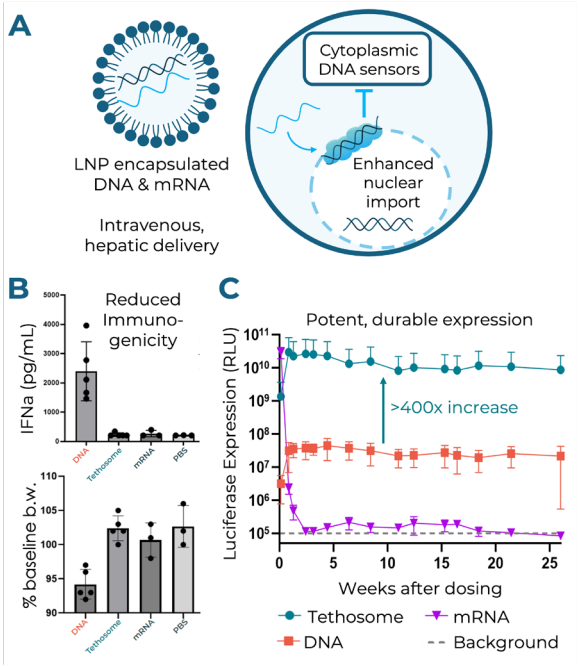
significant acute toxicity has been observed in animal models as a result of innate immune sensing of cytoplasmic DNA. The combination of low expression and immunogenicity has hampered the advancement of non-viral DNA medicines.

To enable DNA medicines to reach their significant therapeutic potential, Engage Bio has developed the Tethosome platform. Here, we co-encapsulate a modified DNA encoding one or more transgenes and an mRNA in lipid nanoparticles. The mRNA encodes our proprietary, engineered Tethosome protein which actively shuttles the DNA expression vector into the nucleus, while evading cytoplasmic DNA sensors including cGAS-STING and TLR9 (Fig. 1a).

To demonstrate these advantages, nucleic acids encoding firefly luciferase were delivered intravenously to the murine liver. Non-viral delivery of DNA resulted in pronounced increases in proinflammatory cytokines such as IFNα, as well as significant body-weight loss at day 1; in contrast, Tethosomes show no significant differences from nucleoside modified mRNA or PBS (Fig. 1b). By facilitating nuclear entry and abrogating innate immune activation, the Tethosome platform enables significantly higher and longer-lasting transgene expression, here leading to >400-fold increases in firefly luciferase expression relative to traditional DNA, and six months of durable expression (compared to <1 week with mRNA) (Fig. 1c).

We will also present data from several preclinical programs for monogenic gene replacement, biologics, and oncology. As a case study for monogenic gene replacement, a single dose of Tethosomes encoding full-length human Factor VIII (FVIII) was able to generate 50-100% of normal FVIII serum levels in mice, remaining consistent over the duration of the study (26 weeks). Crucially, the ability for Tethosomes to be safely redosed permits both titration to optimal levels, and "topping up" if expression wanes, ensuring that expression does not fall below therapeutic levels. As a case study for biologic drugs, a single dose of Tethosomes encoding a therapeutic GLP-1 receptor agonist peptide was able to elicit pronounced weight-loss in a mouse model of diet induced obesity, with months of durability and effect sizes comparable to clinically-approved GLP-1 drugs. Finally, in murine models of hepatocellular carcinoma (HCC), Tethosomes encoding a cocktail of clinically-validated, pro-inflammatory cytokines under the control of a HCC-specific promoter elicited >80% tumor growth inhibition and >250% median survival increase.

The Tethosome system offers a novel solution to the limitations that have held back non-viral DNA medicines, potentially enabling a new class of durable, redosable therapies to treat a wide range of diseases.



47 Next-Generation DNA-Based Delivery of Therapeutic Proteins Using MYO Technology: Preclinical Results on a Broadly Neutralizing Anti-Zika Virus Antibody

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Therapeutic proteins, a large class of drugs including antibodies, hormones, and enzymes, have seen a substantial increase in clinical development and use across many disease areas. Despite the broad applicability of these drugs, their more widespread use is often limited by high manufacturing costs and hurdles to delivery. Many therapeutic proteins are administered via time-consuming intravenous infusions. Others, regardless of administration route, need to be dosed frequently. Another drawback of many biologics is the requirement for maintenance in a low temperature environment prior to use. Such cold-chain dependencies can significantly hinder the ability to deliver therapeutics to large numbers of people, a problem that is amplified in resource-poor settings.

MYO Technology™, a DNA-based platform for the delivery of therapeutic proteins, was developed to overcome these barriers. The MYO Technology platform consists of therapeutic-encoding plasmid DNA (pDNA) and a proprietary medical device for the intramuscular injection of pDNA, followed by the delivery of very short electrical pulses to the muscle tissue surrounding the injection site. These pulses promote the in vivo electroporation of muscle cells and efficient uptake of pDNA, leading to production and secretion of the therapeutic protein, and ultimately release into peripheral circulation.

MYO Technology offers several advantages over standard delivery of therapeutic proteins, in part because pDNA manufacturing is a simpler and less specialized process compared to protein manufacturing, and pDNA is very stable and lacks most cold chain requirements. Furthermore, administration using MYO Technology takes only a few minutes, and the serum level of a therapeutic protein can potentially be maintained for many months without the need for redosing.

Zika virus (ZIKV), an arthropod-transmitted flavivirus, has been linked to severe neurological complications in humans, and prenatal exposure can cause congenital malformations including microcephaly. Although it remains a public health concern, no vaccines or specific treatments for ZIKV are approved for clinical use. ZIKV-117, a potent, broadly neutralizing human monoclonal antibody against ZIKV, was isolated from convalescent blood (Sapparapu et al., 2016, *Nature*), and has been demonstrated to prevent and treat ZIKV infection in animal models, including non-human primates (NHP) (Sapparapu et al., 2016, *Nature*; Nkolola et al., 2024, *J. Virol*).

Here, we present our preclinical studies on the DNA-based delivery of ZIKV-117 with MYO Technology. Proof of concept studies in mice show a single treatment driving antibody expression for longer than one year. ZIKV-117 protein produced in mice and rabbits in vivo following MYO Technology delivery shows comparable neutralizing activity to recombinant ZIKV-117 protein. Finally, we demonstrate that ZIKV-117 delivered by MYO Technology is protective against ZIKV infection in NHPs.

Through improvements in administration frequency and freedom from cold-chain requirements, MYO Technology has the potential to provide long-lasting protection from ZIKV, particularly in resource-poor areas. Furthermore, MYO Technology has the potential to confer the same advantages to a range of biologics, from other monoclonal antibodies to small therapeutic proteins.

48 Optimizing Focused Ultrasound Parameters for Enhanced AAV Delivery Across the Blood-Brain Barrier for the Treatment of Neurodegenerative Diseases

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Introduction:
Rapid advancements in nuclease technology and gene-editing tools have created new opportunities for the treatment of neurodegenerative diseases. However, despite these developments in gene therapy technology, gene delivery to the brain remains a significant challenge due to the blood-brain barrier (BBB). Current methods of overcoming the BBB include intrathecal and intraparenchymal injections which are invasive and often result in limited diffusion beyond the injection site. Adeno- associated virus (AAV) vectors are widely used as delivery vehicles for in vivo gene therapy but BBB crossing ability of different capsids varies depending on AAV serotype and across species. Focused ultrasound (FUS) combined with microbubbles (MB) has emerged as a promising technique that can be used to temporarily disrupt the BBB to deliver AAV's to specific brain regions. FUS-mediated AAV delivery has been demonstrated in mice and non-human primates. There have not yet been any clinical trials to investigate the safety of using FUS for AAV delivery in patients. The efficiency of FUS-mediated AAV delivery can be enhanced by optimizing a range of treatment parameters including microbubble dose and FUS pressure. Here, we investigated the optimal FUS parameters for efficient FUS-mediated delivery of AAV9 to the striatum.

Methods:
FUS-BBB opening was targeted to the right striatum in C57BL/6J mice (N=4 per group). Treatment parameters were varied over microbubble dose (20, 40, 80 µL/kg) and harmonic dose, an integrated measure of microbubble response that is correlated with FUS pressure (51, 74,76, 89 a.u.). Other FUS parameters were fixed at 10 ms bursts and 1Hz repetition frequency for 120s, with resulting pressures ranging from 0.22-0.33 MPa (Table 1). Following sonications, mice were injected intravenously with 5.2×10^{11} vg/mouse (2.1×10^{10} vg/g) of scAAV9-hSYN-GFP. Gadolinium enhanced T1-weighted MRI images were acquired to confirm BBB opening in the striatum. Mice were euthanized three weeks after treatment and the brains were harvested for immunohistochemical analysis, with cell counting done based on confocal microscopy.

Results:
We were able to achieve successful BBB-opening in the striatum over the range of FUS-pressures used in this study (Figure 1A-C). BBB permeability was significantly higher in high pressure group compared to the low and medium pressure groups (Figure 1D). We did not observe any tissue damage using the more aggressive FUS parameters. In all groups, the percentage of neurons transduced was significantly higher in the FUS-treated striatum compared to the untreated side. There were no significant differences in neuronal transduction across the low, medium and high-pressure groups or across low, medium and high microbubble doses (Figure 2F and 2G).

Conclusion:
All parameters resulted in safe BBB opening with enhanced AAV delivery in the treated compared to untreated hemisphere. These findings suggest that the correlation between BBB opening volume as measured by gadolinium-based MRI and neuronal transduction following FUS-mediated AAV delivery is not direct. Further work needs to be done to elucidate the mechanism of FUS-mediated AAV delivery to improve transduction efficiency in FUS gene therapy studies.

Table 1. FUS parameters

	Gd/AAV Injection time from treatment	Harmonic Dose (a.u.)	Microbubble dose
FUS Pressure	0 hrs	51, 74*, 89	20 µL/kg
Bubble Dose	0 hrs	76	20*, 40, 80 µL/kg

**Indicates same group*

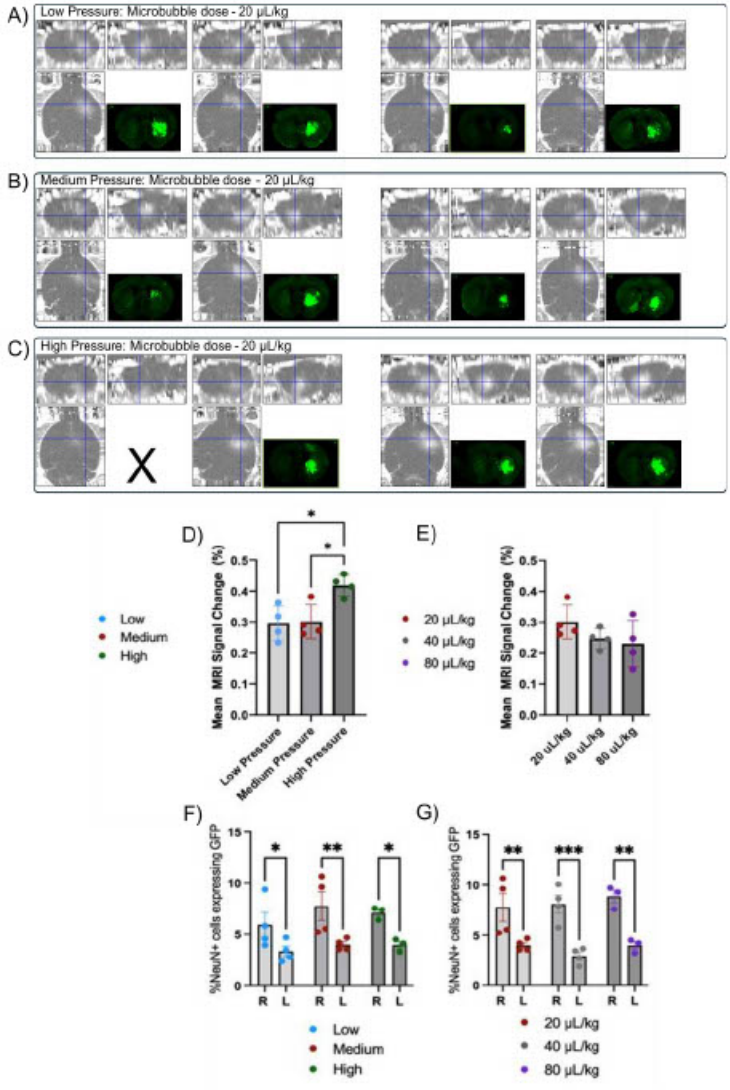

Figure 1. AAV delivery and neuronal transduction following FUS-BBB opening. Gadolinium enhanced T1-weighted MRI images and immunofluorescence images for GFP (green). Demonstrating BBB opening and GFP expression in the right striatum for the A) Low pressure, B) Medium pressure and C) High pressure groups receiving a microbubble dose of 20 µL/kg. MRI images were acquired using a preclinical T7 MRI scanner. D) Percent signal change in the striatum of the low, medium and high-pressure groups. E) Percent signal change in the striatum with a microbubble dose of 20 µL/kg, 40 µL/kg and 80 µL/kg. F) Percentage of Hoechst cells that are GFP+ and NeuN+ in the striatum of low, medium and high-pressure groups and G) Low (20 µL/kg), medium (40 µL/kg and high (80 µL/kg) microbubble dose groups. Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$. **X- This animal was excluded from GFP analysis.*

49 Non-Viral Transcutaneous Fluoroscopy Guided Ultrasound-Mediated FVIII Delivery as a Promising Therapeutic Approach for Hemophilia A Treatment

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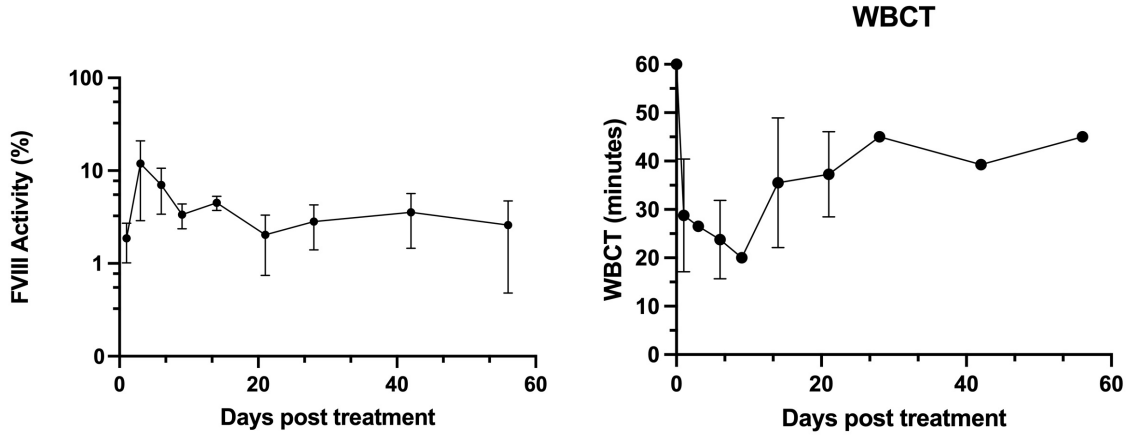
Hemophilia A (HA) is an X-linked clotting disorder characterized by a deficiency of functional factor VIII (FVIII) protein. Viral gene therapy is promising for treating hemophilia but currently there are safety concerns and challenges brought by the pre-existing antibodies against viral vectors. Ultrasound-mediated gene delivery (UMGD) has emerged as a promising non-viral method that utilizes therapeutic ultrasound (US) and microbubbles (MBs) to facilitate gene transfer.

Previously, we successfully performed UMGD of a human FVIII (hFVIII) plasmid in normal canines, achieving sustained hFVIII expression of approximately 20% over 60 days in two treated canines. In this study, we evaluated FVIII expression following UMGD with a canine FVIII (cFVIII) plasmid in HA canine models. A balloon catheter was inserted into the hepatic vein via jugular vein access of canines. A mixture of a high-expressing FVIII plasmid driven by the CAG promoter and RN18 microbubbles (MBs) was injected. Simultaneously, the ultrasound transducer, H114, was placed transcutaneously over the liver and administered pulsed US treatment. Each dog received treatment in two lobes, the left lateral lobe (LLL) and right lateral lobe (RLL), with four injections of the pDNA and MBs per lobe. Each lobe was treated for 2 minutes with the US transducer per injection, resulting in a total treatment time of 8 minutes per dog.

HA dogs (n=2) treated with the FVIII plasmid under UMGD, initially achieved approximately 10% activity, sustaining around 2.5% for two months. (Fig 1a). To confirm the improvement in coagulation function in the HA dogs, samples were measure by whole blood clotting time (WBCT) and Thromboelastography (TEG). The WBCT of non-treated HA dogs exceeded 60 minutes. In contrast, the treated HA dogs maintained a WBCT of less than 40 minutes within a month, (Fig 1b). TEG results also showed improvement. All dogs exhibited slight elevations in AST/ALT levels on the first day, but these levels returned to normal by day seven. Additionally, no significant changes were observed in any other parameters from the CBC and whole blood chemistry analysis in either dog.

The results of this study demonstrate that UMGD is a viable and effective method for delivering FVIII plasmids in HA canine models, achieving sustained FVIII activity with minimal adverse effects. This approach holds significant potential for advancing gene therapy for hemophilia A, offering a safer alternative to viral vector-based methods.



50 Safety, Tolerability, Biodistribution, Transgene Expression and Effectiveness of Image-Guided Laser-based Non-Viral Targeted Delivery of Multi-Characteristic Opsin in Retinae of African Green Monkeys

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Multi-characteristic opsin (MCO) is a trimeric fusion protein, containing three different light-sensitive proteins, which is responsive to ambient white light. Virally-delivered MCO is being evaluated in multiple clinical trials for retinal diseases causing photoreceptor degenerations. With the combined use of gold nanorods (GNRs), and near-infrared (NIR) laser wavelength tuned to the GNRs, non-viral delivery of MCO-020 plasmids is accomplished in a spatially-targeted manner. Upon intravitreal injection and laser delivery, MCO-020 expression in retinal neurons downstream to the dysfunctional/degenerated photoreceptors leads to visually-evoked signal. Here, we report the results of a GLP study evaluating the safety, tolerability, biodistribution, transgene expression, and effectiveness of MCO-020 delivery using an optical coherence tomography (OCT)-guided NIR laser procedure.

Specifically, high and low doses of MCO-020 plasmids mixed with GNRs were intravitreally injected into one eye of the sedated monkeys in 2 different groups (4-10 years old; both sexes; N = 3 / group), followed by OCT-guided scanning NIR laser irradiation in a 4 x 4 mm^2 area covering the macula. Another group of 3 animals received laser delivery of low doses of MCO-020 plasmids (and GNRs), which underwent laser redosing of a high dose of MCO-020 plasmids in the presence of a high dose of GNRs after 8 weeks of initial dosing. Control group eyes just received the vehicle buffer and the NIR laser irradiation. Several physiological, functional, and structural parameters were recorded through the 13-week study period, including measurements of intraocular pressure, slit lamp microscopy, fundus imaging, specular microscopy, OCT, confocal scanning laser ophthalmoscopy, and multifocal electroretinography (mfERG).

Regular ophthalmic exams did not show any adverse treatment-related toxicity or behavioral changes in any of the monkeys in any group. Mild, transient intraocular inflammation (attributed to the intravitreal injection procedure) was observed in all treatment groups but resolved on its own. No differences in IOP or central retinal thickness were observed between the MCO-020 treated and control groups over the 13-week study. Fundus autofluorescence imaging indicated higher levels of red fluorescence (attributable to mCherry, an active component of MCO-020) in the macula of the high-dose MCO-020 treatment group compared to the peripheral retinal areas. Similarly, there was a small but progressive increase in the mfERG amplitudes in monkeys that received the MCO-020 treatment. The increase in mfERG amplitudes above baseline values began at day-14 and continued through to day-28 and day-84.

In conclusion, the non-viral method described herein to deliver MCO-020 plasmids to NHP retinae was safe and well tolerated, and it effectively permitted transfection of the higher-order retinal neurons. Therefore, this OCT-guided NIR laser-based gene delivery procedure in targeted retinal cells offers an effective alternative to AAV-mediated transduction. Such a non-viral plasmid delivery platform can be used to help restore vision in patients suffering from degenerative retinal diseases, including geographic atrophy.

51 Single-dose electroporation delivery of plasmid-encoded incretin mimetics supports extended weight and blood glucose control in mouse models of diet-induced obesity

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Obesity and obesity-associated diabetes affect millions of patients worldwide. Incretin mimetic drugs including glucagon-like peptide-1 (**GLP-1**) and glucose-dependent insulinotropic polypeptide (**GIP**), have advanced the treatment of diabetes and obesity. However, challenges such as short half-lives, protein production constraints, and increasing costs continue to limit the availability of these drugs. We developed plasmid-encoded long-acting incretin analogues (**pLincretin**s) by fusion of native and novel incretin molecules to IgG heavy chains. We evaluated the efficacy of these constructs in mouse models of diet-induced obesity (**DIO**). pGLP1-mFc and pGIP-mFc were detectable in mouse sera for four weeks. Treated mice exhibited significant weight loss, increased glucose tolerance, and decreased food intake compared to controls. Lipidomic analysis of serum identified differentially represented

lipid species between naïve animals and those treated with plasmid-encoded incretins. Similarly, Histological analyses revealed a reduction in liver fat droplets. A benefit of the nucleic acid platform is the ability to support the design of synthetic consensus molecules. We aligned several native and synthetic incretin molecules to design a plasmid-launched novel synthetic consensus incretin mimetic (**pSynCretin**) which also supported decreased body weight and food intake in DIO mice for four weeks following a single administration. These studies demonstrate that DNA-delivered incretin therapy can support weight and blood glucose control following a single *in vivo* administration and support the translation of nucleic acid approaches for incretin mimetic delivery.

52 Expanded Analysis of in vivo-delivered SARS-CoV-2 Plasmid DNA-encoded Monoclonal Antibodies (DMAb) in a Phase 1 Clinical Trial in Healthy Adults

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Background:
dMAbs for Prevention of COVID-19 is an ongoing Phase 1 clinical trial in healthy human adults evaluating safety, tolerability, and pharmacokinetic expression of a synthetic plasmid DNA-encoded monoclonal antibody (DMAb) cocktail (NCT05293249). DMAbs are skeletal muscle expressed monoclonal antibodies, achieved through pDNA gene delivery of immunoglobulin genes and facilitated by CELLECTRA® *in vivo* electroporation technology. In this study, we evaluated in vivo gene delivery of a DMAb cocktail encoding AZD5396 and AZD8076, modified versions of the SARS-CoV-2 neutralizing mAb cocktail Evusheld. The most notable outcome of this study is the detection of durable in vivo DMAb expression >72 weeks, with further demonstration of a dose response and consistency within treatment groups. Ongoing efforts are therefore focused on the functional profile of DMAbs.

Methods:
The universal SARS-CoV-2 seropositivity in study participants (either by vaccination or natural infection) necessitated the development of unique assays that distinguish human IgG1 DMAb resulting from pDNA delivery from naturally occurring antibodies. In addition to development of a sensitive ELISA designed to specifically detect DMAb, bead-based isolation was optimized to maximize recovery from sera for additional functional analyses.

Results:
DMAbs expressed in serum demonstrated binding to the SARS-CoV-2 (RBD), including ancestral, Delta, and Omicron BA.2 and BA4/5 variants with a comparable profile to the parent Evusheld cocktail. Binding activity was detected longitudinally over >72 weeks, indicating consistent production of DMAb that functionally binds to target antigen. Serum-purified DMAbs potently neutralized in a sensitive, ancestral SARS-CoV-2 pseudovirus neutralization assay, with low ng/mL IC50 values, consistent with those observed with Evusheld. In-depth profiling of in vivo, muscle-produced DMAbs is underway and these protocols are being further optimized for large-scale purification from plasma apheresis.

Conclusions:
In parallel with the durable in vivo expression profile, these data highlight the biologic activity of an in vivo-expressed synthetic DNA-delivered mAb cocktail and are supportive of further development as a strategy for delivery of antibody-based gene-encoded biologic drugs.

Novel CAR-T Engineering Strategies for Hematological Malignancies

53 Efficacy, safety, and biological properties of Sleeping Beauty-engineered CAR T cells in patients with B-cell acute lymphoblastic leukemia

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CAR T-cell therapy targeting CD19 has revolutionized the treatment landscape for B-cell acute lymphoblastic leukemia (B-ALL). To offer a more streamlined and cost-effective approach to achieve stable gene transfer, we utilized Sleeping Beauty transposon to engineer donor-derived anti-CD19 CAR T cells (CARCIK-CD19) for B-ALL patients relapsed after allogeneic hematopoietic stem cell transplantation (alloHSCT). Results from 36 patients in a pooled analysis of Phase 1/2, compassionate base use, and Phase 2 trials, sponsored by Tettamanti Center, Monza demonstrated encouraging safety and efficacy, with 83% remission rate and improved overall survival, associated with expansion, persistence, and high clonal diversity by integration site analysis of SB-engineered T cells. Despite these promising results, only 40-50% of B-ALL patients treated with any type of CAR T cells maintained a durable remission long term. In a considerable portion of treated patients, relapse occurs in the presence of CD19-expressing tumors, mainly because of CAR T-cell dysfunction. We hypothesized that the immunological niche of the BM reacts to CAR T cell-mediated inflammation by modulating the immune response through the activation of inhibitory pathways and molecules. To test our hypothesis, we performed transcriptional analysis of CAR T cells and the BM microenvironment from patients treated with commercial and our investigational CAR T-cell products and findings were validated by flow cytometry. We observed profound changes in the composition of BM after CAR T-cell infusion compared to pre-treatment samples, with an increase in the myeloid cells, and exhausted CD8+ T cells. Interestingly, myeloid cells displayed a higher resemblance to myeloid-derived suppressor cells (MDSCs) post-infusion. Significant enrichment in Interferon response, Hypoxia, and TGF-β signaling was associated with the expansion of MDSC, and exhausted T cells, detected by performing spectral flow cytometry of additional 20 matched BM samples pre and post CAR T-cell treatment. By modeling intercellular communications, we revealed that HIF1-α, VEGF and TGFβR2 are key players in the crosstalk between CAR T cells and the immune niche, leading to general immune suppression. These data were also validated in hematopoietic stem progenitor cell (HSPC)- humanized NSG mice engrafted with a human tumor, which recapitulated the human tumor microenvironment. PD1 expression in endogenous T cells post CAR-T treatment was associated with a lack of durable response. In conclusion, CAR T cells-mediated myeloid activation is associated with pathways of immune dysregulation that may dampen CAR T cell expansion and antagonize the effects of the therapy.

54 In Vivo Persistence and Function of CAR19 T-Cells for Pediatric B-Cell Acute Lymphoblastic Leukemia Are Impacted by Media, Cytokine, and Metabolite Bioavailability During Ex Vivo Expansion

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Chimeric antigen receptor (CAR) T-cell therapy targeting CD19 has been highly effective in treating pediatric B-cell acute lymphoblastic leukemia (B-ALL). However, responses are not durable in all patients, and up to 50% will relapse within two years of infusion. Relapse of CD19+ disease is likely due to inhibition or loss of functional CAR T-cells. There is interest in developing strategies to enhance persistence and function of CAR T-cells, but the exact mechanisms that drive functional persistence in patients are poorly understood. In two recent clinical trials performed at the Children's Hospital of Philadelphia evaluating a murine (NCT04276870) and humanized (NCT03792633) CD19/41BBz CAR for pediatric patients with B-ALL, we observed that two modifications made to the manufacturing process appeared to impact long-term functional persistence of CAR T-cells: base media formulation and cytokine supplementation. There are four manufacturing conditions across these trials—X-VIVO™-15 IL-2, X-VIVO™-15 IL-7/IL-15, OpTmizer™ IL-2, and OpTmizer™ IL-7/IL-15. Initial clinical response and expansion of CAR T-cells are similar across manufacturing conditions when compared to X-VIVO™-15 IL-2 manufacturing conditions used in prior murine (NCT02906371) and humanized (NCT02374333) pediatric CD19/41BBz CAR T-cell studies; however, cells produced in X-VIVO™-15 IL-2 demonstrate superior functional persistence in patients with 71% (95% CI: 64%,79%) of patients maintaining CAR T function—defined by B-cell aplasia—6-months post-infusion compared to 11% (1.8%, 71%), 43% (23%, 78%), and 36% (18%, 72%) of patients in other manufacturing groups. Patients treated with X-VIVO™-15 IL-2 CAR-T cells had a 2.7 to 6.7-fold increase in circulating CAR T-cells 3-months post-infusion compared to other manufacturing groups. Bulk and single-cell RNA-sequencing reveals that media formulation, but not cytokine stimulation, drives major transcriptional differences in patient CAR T-cells both pre-infusion and at peak expansion post-infusion. T-cells grown in X-VIVO™-15 have increased effector/effector memory signatures, larger cell volume, upregulation of cholesterol biosynthesis pathway, and increased CD8+ T-cells, suggesting an increased activation status. Deeper investigation into activation pathways reveals increased NF-kB activity in CAR T-cells grown in X-VIVO™-15 media compared to those grown in OpTmizer™. X-VIVO™-15 grown CAR T-cells demonstrate increased sensitivity to cytokines and a superior ability to grow and persist in the absence of target antigen in vitro. In vivo evaluation of donor-matched CAR T-cells cultured in X-VIVO™-15 shows increased CAR T-cell numbers and tumor control in a xenograft model of leukemia compared to OpTmizer™ cultured cells, resulting in increased survival. In a targeted metabolomics screen, we identified several differences in metabolite abundance across formulations. We note that X-VIVO™-15 was enriched with metabolites involved in TCA cycle activity, one carbon metabolism, as well as arginine, ornithine, and inosine metabolism, several of which have been implicated in T-cell stemness and mitochondrial function. Based on this data, we propose that metabolite bioavailability during manufacturing of CD19/41BBz CAR T-cells has a significant impact on the phenotype, transcriptional signature, and persistence of infused cells, which can determine long-term durability of responses in pediatric B-ALL patients.

55 Optimized anti-CD70 CAR-T cells for high-risk multiple myeloma

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Emilio Ramos[1], Anjanaa Vijayanarayanan[1], Fernando Salangsang[1], Paul Phojanakong[1], Juan Camara Serrano[1], Ons Zakraoui[1], Isa Tariq[1], Veronica Steri[1], Mala Shanmugam[6], Lawrence Boise[7], Tanja Kortemme[3], Elliot Stieglitz[8], Jonathan Licht[9], William Karlon[5], Benjamin Barwick[10], Arun Wiita[1]*
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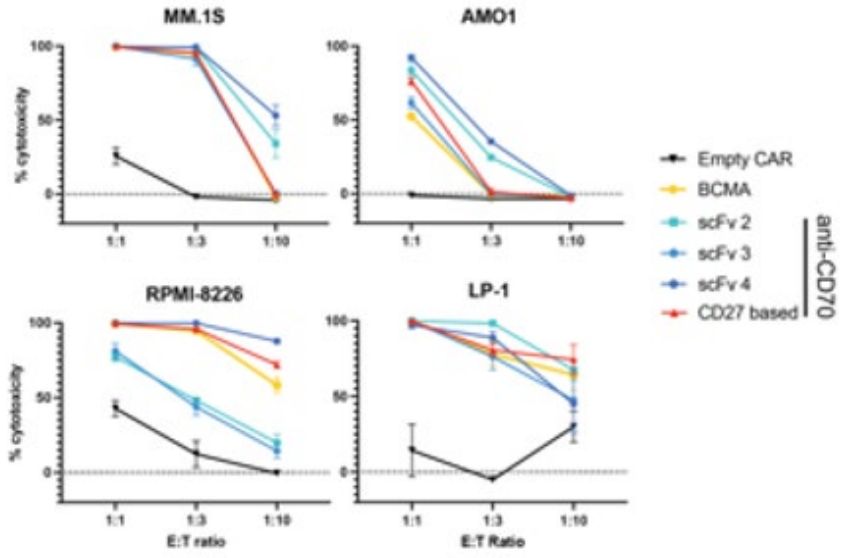
Background:
Despite the success of BCMA-targeting therapies in multiple myeloma (MM), patients with high-risk features are more likely to suffer early relapse and need novel therapies. Using the MMRF CoMMpass database, we identified CD70 as a cell surface unpregulated in high-risk MM. Though CD70 was previously identified in MM (McEarchern et al, Clin Cancer Res (2008)), heterogeneous expression in newly diagnosed samples led to its later deprioritization as an immunotherapy target. In the post-BCMA CAR T era, anti-CD70 cellular therapies could be highly beneficial in high-risk MM patients at the highest risk of relapse.

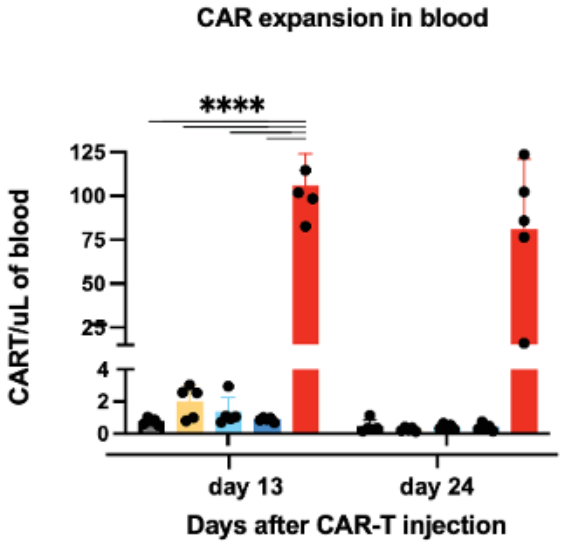
Methods:
Primary patient samples were obtained under an IRB-approved protocol. CAR-T lentiviral transduction was performed on paired CD4/CD8 human T cells from at least 3 healthy donors. Effector function of CAR-Ts was assessed by cytotoxicity, cytokine secretion, and memory/exhaustion marker profiling. In vivo murine xenograft studies were assessed using survival and bioluminescence. ATAC-seq peaks were called using Promo and HOMER (data from Jin et al. Blood (2018)). Machine learning was performed using XGBoost approach applied to CoMMpass RNA-seq data with 80% of samples for training and 20% as test set.

Results:
We analyzed CoMMpass mRNA data (release IA19) for surface protein encoding-genes upregulated in tumors at relapse versus newly diagnosed. We focused on CD70 given its low or absent expression in normal tissues. CD70 was markedly elevated in high risk genotypes such as t(4;14) and gain 1q, as well as R-ISS 3 at diagnosis. Flow cytometry profiling of 8 myeloma cell lines, including t(4;14) models KHM11, KMS26, and LP1, as well as relapsed primary MM samples revealed increased CD70 protein expression with antigen density similar to BCMA. We engineered 10 anti-CD70 CAR-T binders, 9 of which used scFv-based formats, in to both 4-1BB and CD28-zeta CAR frameworks. For the tenth design, we used a structure-guided design based on the CD70:CD27 co-crystal structure (PDB: 7KX0) to identify a CD27-based fragment, which outperformed all tested scFv-based CARs in both CAR frameworks. All CD70 CARs were efficacious in in vitro cytotoxicity assays against MM cell lines (Fig. 1), with similar cytokine as well as memory and exhaustion cell marker profiling. Murine xenograft studies comparing scFv based-CARs versus our natural ligand (CD27) CAR-T demonstrated prolonged survival compared to empty CAR (n=5 mice/arm, $p<0.005$ by log-rank) in both MM.1S and LP-1 xenografts, similar to BCMA CAR-T controls. Our CD27-based CAR showed superior expansion (50-85x, $p< 0.0001$ by ANOVA) and persistence compared to scFv- and BCMA-based CAR in both the CD28z and 4-1BB frameworks (Fig. 2). We further investigated mechanisms of CD70 upregulation in high risk MM. We showed that knockout of NSD2 in t(4;14) cell line models led to significantly decreased CD70 surface expression, implicating epigenetic regulation of CD70 overexpression in this high-risk MM subtype. Analysis of ChIP-seq in ENCODE and primary MM tumor ATAC-seq was used to nominate a set of transcription factors that bind the CD70 locus. Extending from CoMMpass data, we built a predictive machine learning model for CD70 expression as a function of transcription factor gene expression, nominating key transcription factors TFAP2A, LEF1, MYB, and PAX5 as CD70 regulators.

Conclusion:
Together, these results highlight the promise of targeting CD70 with optimized CAR-Ts in myeloma

Figure 1





56 Mutated Ligand-Based CAR-T Cells Targeting the CD123/CD131 Complex Demonstrate Enhanced Cytotoxicity and Reduced Hematopoietic Toxicity Against CD123^{+} AML

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Introduction
CD123 is expressed in over 90% of acute myeloid leukemia (AML) cases, making it an ideal target antigen.

However, clinical trials have shown that CD123-specific chimeric antigen receptor (CAR)-T cells are associated with on-target, off-tumor toxicities, including severe myelosuppression and cytokine release syndrome, alongside limited clinical responses. To address these issues, we developed ligand-based CAR-T cells targeting the interleukin-3 receptor (IL3R CAR-T), leveraging IL-3's differential affinity for normal myeloid cells expressing CD123 alone versus AML cells co-expressing CD123/CD131. Using protein structure prediction, we extensively modified the CAR binder to create mutated ligand-based CD123 CAR-T cells. These IL3R CAR-T cells demonstrated improved tumor control *in vivo* and reduced toxicity toward neutrophils and myeloid precursor cells compared to scFv-based CD123 CAR-T cells.

Method

A ligand-based CD123 CAR plasmid was constructed by linking IL-3 to a spacer, a CD28 endodomain, and CD3ζ. Six IL-3 mutants (A–F) with varying affinities for IL3R were designed using biophysical calculations. CAR-T cells were generated via *piggyBac* gene modification. The efficacy and safety of these modified IL3R CAR-T cells were evaluated in co-culture experiments with AML cell lines (MV4-11, THP-1, and Kasumi-1) and neutrophils. Colony formation assays were conducted to assess hematopoietic toxicity following co-culture with bone marrow mononuclear cells. *In vivo* efficacy was tested using NSG mice intravenously injected with THP-1 cells expressing firefly luciferase (THP-ffLuc). Seven days post-inoculation, CAR-T cells were injected intravenously, and anti-tumor activity was monitored via an *in vivo* imaging system.

Results

Six CAR constructs with mutated IL-3 ligands were generated and evaluated. Three mutants (A, B, and E) supported successful generation of IL3R CAR-T cells, enabling favorable *ex vivo* expansion and CAR expression by day 16. CAR-T cells using high-affinity ligand E exhibited superior cytotoxicity against MV4-11 cells compared to those with wild-type (WT) IL-3. Consequently, ligand E-based IL3R (mutated IL3R) and WT IL3R CAR-T cells were selected for further experiments. Both mutated IL3R and scFv-based CD123 CAR-T cells exhibited similar *ex vivo* expansion, CAR expression levels, CD4/CD8 ratios, and exhaustion marker expression (PD1, LAG3). Both also showed comparable cytotoxicity against AML cell lines MV4-11, THP-1, and Kasumi-1. In a THP-1-ffLuc xenograft model, mutated IL3R CAR-T cells achieved better tumor control than scFv-based CD123 CAR-T cells, with significantly lower luminescence levels (1390 ± 22.5 vs. 2218 ± 177 p/s/cm²/sr, $p = 0.0017$) on day 35. Safety assessments revealed that mutated IL3R CAR-T cells caused less neutrophil toxicity compared to scFv-based CD123 CAR-T cells. Colony formation assays also demonstrated reduced hematopoietic toxicity for mutated IL3R CAR-T cells, with an improved safety margin compared to scFv-based CD123 CAR-T cells (1.72 ± 0.1885 vs. 1.00, $p = 0.019$).

Conclusions

We successfully developed ligand-based mutated IL3R CAR-T cells targeting $CD123^+$ AML, demonstrating enhanced safety and efficacy compared to scFv-based CD123 CAR-T cells. These findings highlight the potential of mutated IL3R CAR-T cells as a promising therapeutic approach for AML. A clinical trial for relapsed/refractory myeloid malignancies using these CAR-T cells is currently being planned.

Figure 1

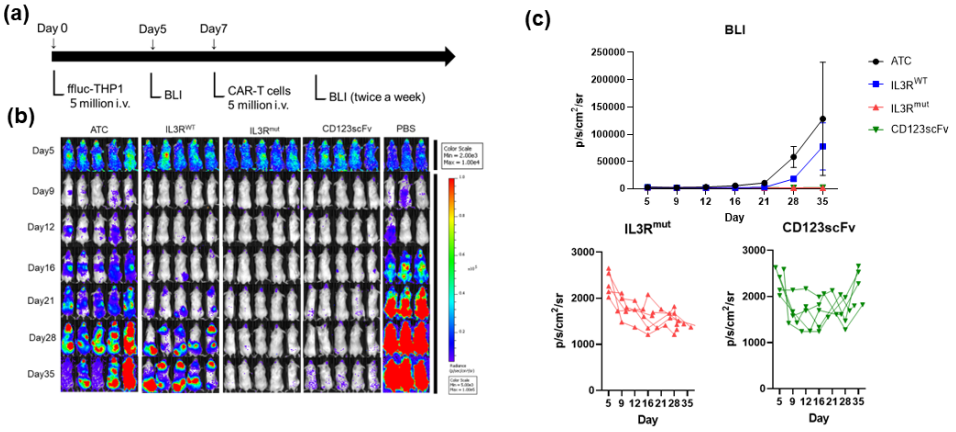

Figure 1: Anti-tumor effects of IL3R CAR-T cells in THP-1 xenograft model.
(a) Schematic explanation of experimental plan.
(b-c) Sequential analysis of BLI in the treated mice (n=3−5).

Figure 2

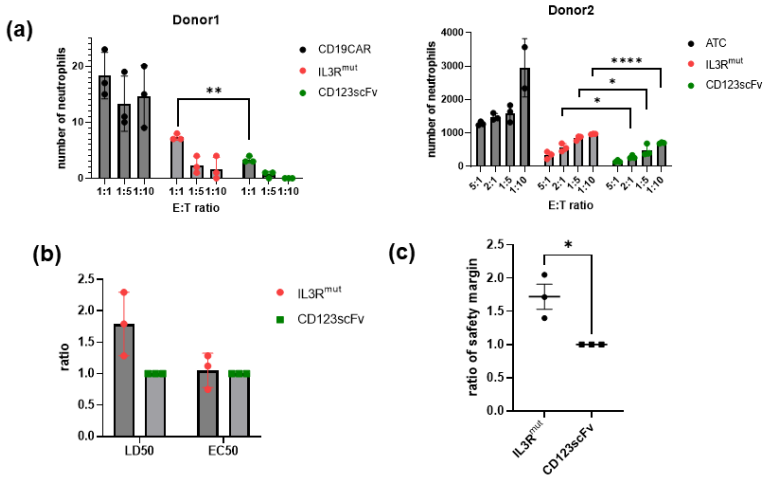

Figure 2: Hematopoietic toxicity profile of IL3R CAR-T cells
(a) Cytotoxicity of IL3R or scFv-based CD123 CAR-T cells are shown.
(b-c) Comparison of toxicities between IL3R or scFv-base CD123 CAR-T cells against bone marrow precursor cells .

57 Cell-free DNA Profiling as a Non-Invasive Approach for Assessing CAR-T Therapy Outcomes and Toxicity

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Chimeric Antigen Receptor T-cell (CAR-T) therapy has achieved significant success in cancer treatment, particularly for patients with relapsed/refractory B-cell malignancies. However, about half of patients receiving anti-CD19 CAR-T ultimately experience disease progression. The effectiveness of this therapy depends on the intrinsic features of the drug product and individual factors present at or after infusion. Significant gaps remain in understanding the in vivo behavior of CAR-T and the absence of reliable biomarkers to address treatment efficacy and toxicity. These unmet needs hinder our ability to predict outcomes and mitigate treatment risks. For this reason, we developed a strategy combining clonal tracking and epigenomic monitoring via liquid biopsies, which are cell-free DNA (cfDNA) fragments released in body fluids from dying cells capturing dynamics changes in peripheral organs. Here, we profiled cfDNA in 16 patients who received g-retroviral or lentiviral-based drug products encoding an anti-CD19 CAR targeting B-cell tumors. All patients were relapsed/refractory to prior therapies, with half achieving a complete response (CR) 90 days after infusion, while the others experienced disease progression (NR). State-of-the-art methods for both qualitative and quantitative evaluation of engineered T-cells allowed the retrieval of integration sites (IS) from PBMC and cfDNA samples collected up to one year after infusion. A significantly higher number of IS ($p<0.024$) was found in CR patients (98,482 IS) compared to NR patients (2,906 IS) in both blood cells and cfDNA. Longitudinal analysis revealed higher clonality ($p<0.01$) and persistence of engineered cells in CR versus NR patients, both in cells and cfDNA, indicating greater CAR-T-cell activity and engraftment even in peripheral tissues. Beyond vector IS, cfDNA levels and dynamics significantly differed between the two cohorts ($p<0.02$). In CR patients, high cfDNA levels detected before treatment progressively decreased from the early days post-infusion, whereas in NR patients, these levels remained high or even increased. Methylation studies can identify the cell of origin of these fragments, as cfDNA retains the epigenetic signatures specific to its source cell. In healthy donors (HD) at steady state, leukocytes—as granulocytes, monocytes/macrophages, and megakaryocytes—account for the majority of cfDNA production (80%) while in diseased patients a shift in cfDNA composition is expected depending on the extent of tissue/cell damage. In CAR-T patients, a higher fraction of cfDNA fragments derived from B-cells (80-fold increase) was observed compared to HD, both before and for two weeks post-infusion, reflecting tumor burden and the cytotoxic activity of engineered cells. Furthermore, neuronal and liver damage were also detected in patients who developed cytokine release syndrome, immune effector cell-associated neurotoxicity syndrome, or other inflammatory responses, demonstrating that this epigenetic approach can capture systemic response after treatment, which may differ in CR and NR patients. Overall, our data suggest that cfDNA holds significant potential for monitoring CAR-T therapy's effectiveness and safety at both clonal levels by IS and at the organismal level through cfDNA-methylation, providing valuable insights into disease progression and treatment-related events post-infusion and uncovering mechanisms of therapeutic success and failure that have yet to be explored.

58 Increased Potency, Persistence, and Stem Cell Memory/Naïve Phenotype is Achieved with PRGN-3008 UltraCAR-T, an Overnight-Manufactured CD19 CAR-T Cell Expressing membrane-bound IL-15 and miRNA-based PD-1 Blockade from a Single Non-Viral Transposon

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Despite advances in CAR-T therapy for B-cell malignancies, challenges of durability, manufacturing complexity, high treatment cost, and treatment-related toxicities remain. Recent in-depth analyses across clinical studies agree that CAR-T with a Naïve or Stem-Cell Memory phenotype (Tscm) are the most likely to engraft, expand, and provide persistent anti-tumor activity, thus spurring the development of several approaches to address these challenges. A best-in-class therapy will need to provide several modifications to the therapeutic cell in order to maintain stemness and ensure product safety, all while keeping manufacturing time and cost as low as possible to ensure accessibility of CAR-T therapy to patients who need it.

Here, we describe preclinical development of PRGN-3008, a CD19-targeted cell therapy built on the NextGen UltraCAR-T platform. PRGN-3008 is manufactured by engineering of autologous T cells to express CD19 CAR, membrane-bound IL-15 (mbIL15) for enhanced persistence and maintenance of Tscm phenotype, a miRNA-based PD-1 blockade to avoid exhaustion, and a safety switch to allow cell depletion, all from a single non-viral transposon via an overnight, decentralized manufacturing process using the UltraPorator™ device.

In vitro analyses confirmed the co-expression of all transgenes and appropriate processing of the miRNA module for highly specific targeting of the PD1 transcript without significant impact on other genes or cellular miRNA processing machinery. In a repetitive antigen challenge assay designed to mimic the highly suppressive tumor microenvironment, PRGN-3008 demonstrated durable tumor killing in contrast to conventional CAR-T cells lacking mbIL15 and PD-1 blockade. Single-cell proteomics assay demonstrated up to 7-fold improvement in CAR-T polyfunctionality, a trait linked to improved clinical outcomes, with PRGN-3008 compared to conventional CAR-T cells. Transcriptome profiling demonstrated a robust impact of mbIL15 expression on genes linked to T cell functionality.

In *in vivo* tumor models, a single administration of PRGN-3008 manufactured overnight, without activation or *ex vivo* expansion, showed enhanced expansion and persistence, robust antitumor efficacy, and significantly longer survival compared to conventional CD19 CAR-T cells. Moreover, long-term antitumor immunity was observed when the resulting tumor-free mice were rechallenged 76 days later to simulate tumor relapse, which led to significant efficacy and overall survival without additional PRGN-3008 treatment. PRGN-3008 treatment resulted in a significant enrichment of Tscm/naïve cell population, associated with durable T cell memory and prevention of tumor relapse, compared to conventional CAR-T cells.

In summary, PRGN-3008 is engineered to address the limitations of conventional CAR-T, with the combination of intrinsic PD-1 blockade and mbIL15 potentiating CAR-T functions and providing a phenotype associated with clinical effectiveness. The decentralized overnight manufacturing process, where cells are delivered back to patients the day post-electroporation, is expected to contribute to the less-differentiated cell phenotype as well as reduce manufacturing costs and increase accessibility of the treatment. These results demonstrate that the PRGN-3008 has potential to be the best-in-class treatment.

59 Development of anti-CD21 Chimeric Antigen Receptor (CAR)-T Cells for T-Cell Acute Lymphoblastic Leukemia (T-ALL) - CAR engineering for a complex antigen.

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Background:
Relapsed/refractory T-cell acute lymphoblastic leukemia (T-ALL) has a poor prognosis. While CD19-targeting CAR-T have revolutionized the treatment of B-ALL, progress in T-ALL has been slower. CD7-targeting CAR-T trials have shown promising results, proving the potential of CAR-T for T-ALL. However, targeting pan-T-cell antigens is limited by T cell aplasia and fratricide, requiring rescue stem cell transplantation. One alternative approach is to target T-lymphoblast restricted antigens. Here, we explore CD21, a pan-B-cell marker with a complex structure (Figure 1), as a novel CAR target in T-ALL.

Methods:
We validated CD21 expression in T-ALL and normal tissues using cell lines, patient samples, and healthy donors. We developed anti-CD21 CAR-T, optimized for potent activity against CD21-positive T-ALL cells. This involved testing various CAR architectures to address antigen-specific challenges.

Results:
CD21 RNA was expressed in 80% of T-ALL in a published dataset, in all molecular subtypes. Half (29/58) of newly diagnosed T-ALL samples expressed CD21, (mean ABC 1265). At relapse, 45% (5/11) of samples were positive for CD21, (mean ABC 820). CD21 in normal donors was restricted to B cells and ~10% T cells, with no expression on non-lymphoid tissues. Anti-CD21 single-chain variable fragments (scFvs) were derived via rat vaccination and immune phage library screening. Identified scFvs bound to CD21 membrane-distal epitopes. Initial 8-8-41BBz CAR-T cells did not show CD21-specific cytokine release or cytotoxicity in vitro. Further testing using an engineered cell line expressing only five distal CD21 epitopes, significantly improved cytotoxicity and cytokine release. A second binder campaign identified anti-CD21 scFvs that bound membrane-proximal epitopes. These CAR-T cells were active against CD21+ cells but exhibited high tonic signalling in CD21-KO Jurkat reporter cells. To address this, scFvs were reformatted into a 41BBz Fab CAR construct, which reduced tonic signalling significantly. Fab binders showed equivalent binding kinetics to scFvs but with increased thermal stability. One Fab binder, NM2, demonstrated particularly high thermal stability. NM2 Fab CAR additionally had lower surface expression. NM2 Fab CAR-T exhibited potent cytotoxicity against T-ALL cell lines and patient samples, even at low antigen density. In proliferation assays, NM2 Fab CAR-T expanded without fratricide. In a Jurkat Xenograft model, NM2 Fab CAR-T significantly improved tumor control and survival (median 41 vs 22 d). In two T-ALL patient-derived xenograft (PDX) models with low CD21 expression (<1000 ABC), NM2 Fab CAR-T prolonged survival and controlled tumor growth. To investigate enhancing efficacy in CD21 low density cases, we explored pharmacological upregulation of CD21. The dual αδ PI3K inhibitor copanlisib upregulated CD21 in T-ALL cell lines and PDX both in vitro and in vivo. In a T-ALL PDX with CD21 expression below a threshold anticipated to allow CAR-T activation, (ABC ~200), copanlisib pre-treatment before NM2 Fab CAR-T resulted in significant T-cell expansion and tumor control, which was not seen in the CAR alone group (Figure 2).

Conclusion:
Anti-CD21 Fab-CAR-T cells, targeting membrane-proximal epitopes, showed potent anti-CD21 activity. Pharmacological upregulation of CD21 can enhance efficacy in low antigen density. This novel approach avoids fratricide and T-cell aplasia, offering promise for treating T-ALL

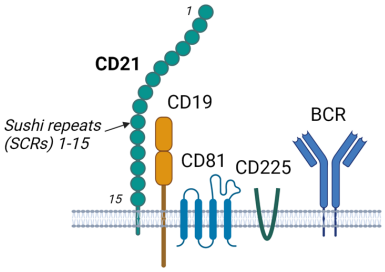

Figure 1: Structure of CD21. CD21 is a type 1 transmembrane glycoprotein found on the surface of B cells and follicular dendritic cells (FDCs) as part of the B cell co-receptor complex. CD21 has a large and heavily glycosylated extracellular domain consisting of 15/16 highly conserved short consensus repeats (SCRs).

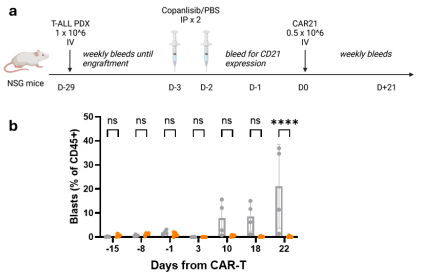

Figure 2: Pharmacological upregulation of CD21 enhances anti-CD21 CAR-T. (a) NSG mice were engrafted with very low CD21 expressing T-ALL PDX then treated with two doses of the Pi3K inhibitor copanlisib/control prior to anti-CD21 CAR-T. (b) % of T-lymphoblasts in peripheral blood at timepoints after CAR-T injection. Grey = CAR21 alone. Orange = copanlisib + CAR21.

474 CRG-023, A Tri-specific CAR T-cell Product Candidate, Demonstrates Strong and Durable Anti-Tumor Activity *In Vivo* Across Lymphoma Models

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Introduction
CRG-023 is a novel tri-specific CAR T-cell product candidate that targets three B-cell surface proteins (CD19, CD20, and CD22). The construct design is intended to address factors that may limit CAR T-cell response against B-cell malignancies, such as tumor antigen escape and CAR T-cell exhaustion. Iterative engineering resulted in new scFv binders for CD19 and CD20 CARs that delivered optimized CAR performance, the selection of distinct costimulatory domains CD2, CD28, and 4-1BB for each of the three CARs, and the assemblage of the three CAR transgenes into a unique tri-cistronic vector format. Previous studies demonstrated that CRG-023 CAR T cells sustained durable anti-tumor activity despite repeated tumor challenge and had superior tumor control as compared to monospecific CAR T cells. Here we assessed the anti-tumor activity of CRG-023 CAR T against several disseminated and sub-cutaneous (s.c.) *in vivo* lymphoma models with varied antigen expression, different lymphoma histology, and in conditions that may require T-cell infiltration.

Results
A Burkitt lymphoma cell line (Raji) and two large B-cell lymphoma (LBCL) cell lines (SU-DHL-6 and SU-DHL-10), expressed all three target antigens at varying levels and were implanted into NCG mice. SU-DHL-10 and SU-DHL-6 cells expressed the highest and lowest levels of CD22 antigen respectively, and Raji cells expressed the lowest level of CD20 antigen, with the highest level of CD19 antigen. In a dose-response study, CRG-023 CAR T cells were able to clear Raji tumor cells in a dose-dependent manner and down to the lowest dose tested ($1x10^5$ tri-CAR^+ cells/mouse), despite an initial 100X increase in tumor burden at this lowest dose. The tri-specific CAR T cells also exhibited strong activity against both disseminated and s.c. models using SU-DHL-6 and SU-DHL-10 ($5x10^6$ tumor cells/mouse), demonstrating high levels of anti-tumor activity across histologies and capacity to traffic to the tumor site. CRG-023 CAR T cells exhibited superior anti-tumor activity as compared to single antigen (CD19, CD20 or CD22) targeting CAR T cells in a disseminated high tumor burden model (Raji 10^6 cells/mouse, day -4). Pharmacokinetic (PK) analysis of mouse blood in the dose-response disseminated Raji model and in both SU-DHL-6 and SU-DHL-10 s.c. models revealed measurable CAR T expansion at 2 weeks post-CAR T cell injection. CAR T cells were persistently detectable in spleen and bone marrow by the end of the study (Day 52).

Conclusion
I*n vivo* pharmacology studies reported here demonstrated effective and sustained anti-tumor activity for CRG-023 CAR T across several lymphoma models representing a variety of lymphoma histologies and expressing a variety of antigen levels. Additionally, CRG-023 CAR T cells were able to durably clear tumor burden in both disseminated and s.c. models, demonstrating CRG-023 CAR T cell ability to effectively traffic to tumor site in s.c. models. The significant, durable anti-tumor activity shown here suggests that CRG-023 offers a promising approach for the treatment of B-cell malignancies. The FDA recently cleared an Investigational New Drug (IND) application for this product candidate. A Phase 1 dose-finding study will enroll R/R LBCL patients who have received at least two prior lines of therapy, including both CAR T-naïve and CAR T-exposed patients.

Insights from AAV Engineering

60 Evolving Synthetic Membrane Associated Accessory Proteins (synMAAPs) for Enhanced AAV Vector Egress

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The infectious biology of Adeno-associated virus (AAV) has been widely studied with particular attention to receptors and intracellular trafficking, however unanswered questions surrounding capsid assembly, genome packaging, and viral egress remain. Our lab previously demonstrated that the membrane associated accessory protein (MAAP) functions as a viral egress factor. Structural analysis of different serotype-derived MAAPs revealed functional domains that are essential for protein expression, linker domains, membrane binding. Subsequent studies by other groups with natural and modified MAAP derived from AAV2 have indicated potential functional implications for vector genome packaging and yield. Despite these advances, one particularly striking aspect of MAAP / AAV biology are the structure-function correlates as evidenced by the serotype-specific sequence variability and distinct capsid secretion kinetics. This observation has remained unexplored to date.

Here, we employed a structure-guided library approach to evolve new and synthetic AAV egress factors through infectious cycling. We report newly engineered synthetic MAAPs (synMAAPs) with significantly improved secretion profiles, achieving up to an 8-fold increase in AAV secretion from HEK293 producer cells independent of serotype. SynMAAPs display remarkable structural convergence, as predicted by Alphafold3, showing improved amphipathic membrane binding domains. Compared to natural MAAPs, synMAAPs display strikingly distinct subcellular localization patterns characterized by nuclear exclusion and concentrated within the Golgi as evidenced by confocal microscopy. Proximity labeling experiments identified a cluster of membrane trafficking and fusion proteins mediated by SNAREs that are predicted to interact with MAAP, corroborating recent reports and providing new insights into how MAAP-host interactions facilitate AAV egress. Specific functional interactions of cellular host proteins hijacked preferentially by synMAAPs over natural MAAPs using a panel of knockout cell lines will be highlighted. In addition to dissecting the biology of AAV egress, we integrate these findings into engineered AAV vector production workflows, demonstrating the potential for improved secretion and streamlined manufacturing eliminating the need for cell lysis, reducing host cell contaminants and potentially enabling continuous perfusion systems.

61 CBN-1100: A Novel Recombinant Parvovirus Gene Delivery Platform with Enhanced Cargo Capacity, Immune Evasion, and Natural Liver Detargeting Properties

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Gene delivery technology has advanced significantly over the past decade, with adeno-associated virus (AAV)-based vectors leading the field due to their favorable safety and durability profiles. However, traditional AAV vectors face substantial challenges, including significant off-target biodistribution and a high prevalence of anti-capsid antibodies in the human population, limiting their applicability in a large percentage of patients. To address these limitations, Carbon Biosciences has developed CBN-1100, a novel capsid derived from an autonomous parvovirus isolated from a non-primate mammal. CBN-1100 offers several key advantages over conventional AAV vectors, including an expanded cargo capacity of ~1kb greater than AAV), a pronounced lack of neutralizing antibodies in humans, and a unique biodistribution profile characterized by near-complete liver de-targeting. These attributes enable the efficient delivery of larger therapeutic transgenes and robust, specific promoters, surpassing the capacity and specificity limitations of existing AAV technologies. In this study, we show that CBN-1100 capsid supports encapsidation of AAV2 ITR-flanked cassettes of up to 5.9 kb while maintaining robust manufacturability. Vector production yields are consistent with industry standards across various scales, including up to a 40-liter bioreactor, achieving 85% filled capsid formation. Serological analyses showed significantly lower anti-drug antibody titers against CBN-1100 capsids compared to AAV9, consistent with the lack of exposure to the parental virus in humans. Systemic administration in mice and cynomolgus macaques revealed broad transduction efficiency and high transgene expression in therapeutically relevant tissues, including the heart, and the CNS, with over

10,000-fold lower liver biodistribution and gene expression compared to conventional AAV vectors. This liver de-targeting property substantially improved tolerability, as dose-escalation studies showed negligible elevations in liver transaminases, cytokines, and complement factors. Additionally, CBN-1100 was successfully enhanced through peptide insertions in two different variable regions (VRs) of its capsid, leading to a significant increase in potency in primary skeletal muscle cells and iPSC-derived cardiomyocytes. These modifications highlight its potential for improved tissue selectivity and potency. These unique pharmacological properties position CBN-1100 as a transformative platform for gene therapy, offering solutions for previously undruggable targets and indications. The combination of enhanced cargo capacity, immune evasion, and improved tissue specificity provide a new benchmark for next-generation gene therapy technologies.

62 Synthetic RNA Switches for Temporal and Dose Control of *In Vivo* Gene Therapies

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In vivo gene therapy technologies, based on adeno-associated viral (AAV) vector delivery of genes encoding therapeutic proteins, noncoding RNAs, or genome/epigenome editors, could provide long-lasting (years of or even lifelong) treatments for various human diseases. Genetic switches that enable precise temporal and dose control of transgene expression may improve safety and efficacy, and broaden the use of these therapies, particularly for payloads with narrow therapeutic windows and/or major contraindications. However, to date, only a few genetic switches developed for this purpose function in animals, yet they exhibit significant leakiness and are typically controlled by switch regulators that have undesirable side effects and require frequent dosing (e.g., QD or BID).

Here, we describe a class of ultra-efficient RNA ON-switch systems (Z^{on}) that can be built in an AAV vector and allow for switch-regulator-dependent precise control of transgene expression. Z^{on} exhibits negligible to zero leakiness and regulatory ranges spanning five logs or more in cultured mammalian cells as well as in mice. The switch regulator is part of a class of oligonucleotide drugs safe for chronic use in humans; it can be administered once every four weeks (Q4W) or even less frequently in mice to maintain Z^{on}-controlled transgene expression. In a long-term repeated dosing study in mice, monthly administration of the regulator has consistently induced Z^{on}-controlled AAV transgene expression with near-flat expression kinetics for more than a year.

Z^{on} has broad potential applications. We have utilized Z^{on} in multiple POC studies in mice to develop temporal and dose-controllable biofactory gene therapies. These therapies leverage AAV-transduced skeletal muscle cells as biofactories to produce and secrete protein therapeutics into the bloodstream, targeting autoimmune diseases and metabolic disorders, respectively. Furthermore, since Z^{on} exhibits negligible to zero leakiness, it offers a unique opportunity to develop a 'suicide switch'-controlled, terminable AAV vector platform, as well as a leak-free, inducible AAV-genome-editor platform. This ultra-efficient, broadly applicable RNA switch technology has the potential to enable safer and broader use of AAV gene therapy in a variety of human diseases, including some common chronic disorders.

63 *In Vivo* High-Throughput Screening of Regulatory Elements to Distinguish AAV Transgene Expression Between Cardiac and Skeletal Muscle

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Engineering robust and safe gene delivery vehicles is critical to clinical translation of gene therapies. Adeno-associated virus (AAV) is well-established for *in vivo* delivery of gene therapies due to its widespread biodistribution and natural tissue tropism. However, transgene expression in unwanted tissues can result in toxicities

that can lead to adverse consequences in many studies. Tropism conferred by natural or engineered viral capsids provides some control over tissue expression, but greater specificity is needed for many applications. Regulatory elements within AAV vectors can be engineered to increase specificity of expression downstream of cell entry. Massively parallel reporter assays (MPRAs) have been used to screen for tissue-specific candidate regulatory elements (cREs). AAV vectors containing a library of cREs can be screened via MPRAs to identify elements that drive tissue-specific expression. In this study, we develop an *in vivo* AAV-MPRA to identify muscle-type-specific cREs that distinguish between striated muscle types and have low hepatic expression.

We designed a library of 1031 cardiac muscle-specific cREs, 668 skeletal muscle-specific cREs, 200 dinucleotide shuffled negative controls, and 30 fragments of muscle-specific gene promoters. Each 250 bp cRE was designed using human ENCODE data with the following criteria: 1) DNase-seq signal enriched in the target tissue, 2) presence of H3K27ac, H3K4me3, or H3K4me1 signal enrichment in the target tissue, and 3) proximity to a gene preferentially expressed in the target tissue. We then applied a binary classification CNN model to select cREs with the highest probability of tissue specificity. The AAV9-cRE library was delivered systemically into adult C57Bl6 mice. The hamstring, heart, and liver tissues were harvested for DNA and RNA isolation, followed by next-generation sequencing to determine relative transcription rates of each cRE.

In the hamstring, we identified 185 sequences with significant differential expression over both non-target tissues. Of these, 138 were designed to be skeletal muscle-specific, 37 were designed to cardiac muscle-specific, and 8 were shuffled controls. Additionally, 90 of those cREs had significant activity over shuffled controls. In the heart, 294 sequences had significant differential activity over both non-target tissues. Of these, 240 were designed to be heart muscle-specific, 39 were designed to be skeletal muscle-specific, and 8 were shuffled controls. 117 of those sequences had significant activity over shuffled controls. Significantly active cREs generally showed higher sequence conservation compared to the non-significant cREs. Motif analysis revealed enrichment for GATA motifs in heart-specific cREs and FOS/JUN and SIX motifs in hamstring-specific cREs.

In summary, we performed an *in vivo* AAV-MPRA that identified cREs driving muscle-type-specific transgene expression. Many identified cREs exhibited tissue-specific and high expression compared to shuffled controls, both of which are desirable qualities for use in a gene therapy vector and highlight their potential for therapeutic use in treating diseases that require distinction between muscle types, including certain muscular dystrophies. While our study focused on muscle tissues, this approach can be adapted for other tissues to advance gene therapy applications.

64 Understanding subcellular trafficking of natural and engineered AAV capsids by novel imaging techniques in skeletal muscle cells

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Adeno-Associated Viral (AAV) vector mediated gene therapies are at the forefront of novel treatments for muscular dystrophies. In recent years, the number of clinical trials and approved AAV-based treatments have increased. Nonetheless, certain roadblocks still arise during clinical trials, such as immune responses due to the high doses administered. While efforts to reduce the dose by capsid engineering are being made, investigating subcellular AAV trafficking would enable us to better understand the differences in transduction efficiency between natural and novel capsids to further develop efficient AAV capsids. Using 2D and 3D confocal imaging on either fixed sample or live-cell fluorescent microscopy, we aimed to investigate subcellular trafficking of a natural AAV9 and an engineered LICA1 capsid in immortalized human myotubes. The objective was to identify key difference between these two capsid variants. In fixed immortalized human myotubes, we saw that AAVs followed a time-dependent kinetic in known subcellular compartments and host factors such as early endosomes, AAVR, the Golgi, and the nucleolus. Surprisingly, despite the cellular monolayer, we observed significant differences in capsid accumulation between neighboring fibers, as well as myonuclei from the same fibers though no differences were observed in receptor presence. These findings were used to develop live-imaging techniques where AAVs were fluorescently labelled using covalently bound dyes. Differences in entry and movement between the two capsid variants were observed, and in combination with endo-lysosomal tracking, we were able to follow the cellular trafficking along the

myotubes. The development of novel methods to study subcellular trafficking paves the way to better characterize and understand the transduction efficiency of novel capsids without the need for specific antibodies. These techniques can be used to study AAV movement not only in immortalized cells lines, but also in isolated muscle fibers, which represent the targeted cell type in muscular dystrophies gene therapy much closer. This will allow us to have a better understanding of possible bottlenecks within a muscle fibers for efficient gene transfer, and could be further optimized for other applications.

65 Development of a Platform for Vectorized Expression of Secretory IgA

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Most infections initiate at mucosal surfaces including the respiratory, gastrointestinal, and genitourinary tracts, where secretory IgA (sIgA) serves as a critical component of immune defense. Comprised of J-chain-containing IgA dimers plus the secretory component (SC) of the polymeric Ig receptor (pIgR), sIgA effectively neutralizes pathogens at mucosal surfaces through high-avidity antigen binding and agglutination. Despite its well-documented role in preventing and clearing infections, recombinant sIgA has yet to be translated to the clinic due to the complexity of its production, requiring plasma cells to synthesize IgA subunits and epithelial cells to add SC during transcytosis. One way to overcome these manufacturing hurdles is to use a gene therapy vector to deliver antibody genes to the host - an approach termed vectored immunoprophylaxis (VIP).

Adeno-associated virus (AAV) vectors represent a leading platform for safe and efficient in vivo gene transfer. AAV-mediated expression of pathogen-specific monoclonal IgG antibodies has provided durable protection against infectious diseases like Marburg, RSV, Ebola, and influenza in various animal models. The objective of this research is to develop a novel AAV platform that will promote prolonged in vivo expression of mAbs of the sIgA isotype at therapeutic levels within mucosal surfaces of the upper and lower respiratory tract and to validate the efficacy of vectorized sIgA expression using an influenza virus challenge model.

We have constructed five AAV genomes: two that express IgA with or without the J chain, one that expresses full-length pIgR and two that express different versions of the SC. Due to our use of influenza as a model pathogen for these studies, the IgA antibody was designed using the heavy and light chain variable domain sequences from FluA-20, a pan-influenza mAb that protects against all major influenza A subtypes that infect humans, and synthesized as a human IgA2 as it is more resistant to proteolysis than IgA1. The AAV6.2FF capsid, a triple mutant with enhanced tropism for lung and muscle tissues, enabling rapid and robust monoclonal antibody expression, was utilized as the AAV vector. In vitro expression of IgA, J chain, SC, and pIgR has been confirmed by western blot and antibodies secreted into the supernatant were confirmed to bind to the hemagglutinin (HA) target antigen by indirect ELISA. Human IgA was detected in the serum of Balb/c mice administered with different AAV vector combinations via either intranasal or intramuscular routes and the concentration of sIgA in lavage fluid will be quantified by ELISA. Next steps are to assess the therapeutic efficacy of vectorized sIgA in a PR8 influenza challenge model.

This study aims to establish a novel gene therapy platform for in vivo production of sIgA in the respiratory mucosa, offering protection against respiratory infections, reduced transmission, and the potential for sterilizing immunity- an achievement elusive with conventional vaccines for respiratory viruses.

66 Novel adeno-associated virus (AAV) vector production system is useful for gene therapy and analyzing the molecular mechanism of viral replication

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Adeno-associated virus (AAV) vector is a promising tool for gene therapy, and various clinical trials are ongoing in the world. However, there are many issues which should be improved for further development. Among various issues, a number of empty capsids during AAV vector production are one of the big problems, meaning that costly multistep purification is required to remove empty capsids. It leads to increased price for AAV vector-based drugs.

To improve the efficiency of vector production, we have established new AAV vector production system controlling capsid (Cap) expression timing after separating replicase (Rep)/ Cap genes in conventional AAV vector production system. Our new AAV vector production system controlling Cap expression timing by the tetracycline(Tet)-dependent promoter significantly increased the yield of AAV2 vector and improved the empty/full capsid ratio. It is also applicable for various serotypes to improve the quantity and quality of viral vectors. Additionally, our system can show the same improvement of vector productivity even in scaled up culture sizes such as T225 and Cell Factory multi-layer flasks. Moreover, no difference in infectivity was observed between AAV vectors produced by conventional and new system in various serotypes, and there was no difference of tissue tropism in mice using AAV9 vectors. These indicate that new AAV vector production system can generally improve AAV vector productivity in various serotypes without changing the nature of AAV vectors. Further analyses showed that the improvement of AAV vector productivity was due to additive effect of the change of Rep expression pattern by individual expression of Rep and the Cap expression timing control.

As the other aspect, Rep and Cap expression is individually regulated by CMV or Tet promoters in our AAV vector system, so that Adeno helper genes are no longer required for these proteins expression. This suggests a possibility that our AAV production system can be used for screening of new essential host factors regulated by helper genes, which may be mainly involved in the late steps of viral production. We have identified about 60 genes from over 100 RNAs upregulated by helper genes. The expression of some genes, instead of helper genes, can increase vector yield in our AAV production system.

Our newly developed AAV vector production system has the potential to shed light on the reduction of cost for gene therapy and to identify essential host factors for AAV vector productions. Further analyses based on these findings would help not only elucidating the molecular biology of AAV but also solving various existing issues of gene therapy using AAV vector.

Cell Therapy Product Engineering I

67 Comparative Analysis of Lentiviral and CRISPR-Cas12i-Generated CAR T Cells: In Vitro and In Vivo Functional Outcomes

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Chimeric antigen receptor (CAR) T-cell therapies have demonstrated remarkable clinical success, but the impact of the engineering method used for CAR integration has not been thoroughly investigated across different CAR receptors.

In this study, we compared CD318-CAR T cells generated via lentiviral transduction (random integration) with those generated using CRISPR-Cas12i-mediated targeted integration into the constant chain of the TCR beta receptor (*TRBC*). For both gene integration methods, an identical CD318 (CDCP1)-targeting CAR receptor was

used, and CAR T-cell functionality was validated using CD318-expressing AsPC-1 cells. Lentiviral- and Cas12i-generated CAR T cells were evaluated both *in vitro* and *in vivo* to assess efficacy, exhaustion, and persistence.

In vitro, CRISPR-generated CAR T cells exhibited reduced killing at lower effector-to-target (E:T) ratios, higher expression of exhaustion markers (e.g., PD-1, LAG-3), and reduced expansion following a tumor-killing assay compared to lentiviral-generated CAR T cells. *In vivo*, CRISPR-generated CAR T cells demonstrated slower tumor clearance and a 50-fold reduction in total CAR T-cell numbers in the blood two weeks after injection. The total CAR T-cell count in the blood of CRISPR-generated CAR T cells remained low for the entire duration of the study. Interestingly, CRISPR-generated CAR T cells isolated from the bone marrow and spleen exhibited reduced exhaustion markers, suggesting a potential reservoir of less-differentiated cells. Graft-versus-host disease (GVHD) was detected in NSG mice receiving lentivirus-generated CAR T cells. However, CRISPR-generated CAR T cells, which lack a functional *TRBC* gene, did not induce measurable GVHD until the study endpoint.

These findings highlight critical differences in CAR T-cell behavior based on the integration method, as well as the additional knockout of the T-cell receptor. While CRISPR-targeted integration provides precise genomic editing, it leads to functional differences in tumor clearance and persistence. Further research is needed to determine whether these differences present limitations or advantages for clinical translation. Collectively, this study provides insights into the trade-offs between lentiviral and CRISPR-based CAR T-cell generation, indicating that TCR gene disruption may affect the kinetics and potency of CAR T cells.

68 Development of a 1XX-Enhanced and Fully Non-Viral, CRISPR-edited BCMA CAR T Cell Therapy for Relapsed and Refractory Multiple Myeloma (UCCT-BCMA-1)

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B-cell maturation antigen (BCMA) targeting chimeric antigen receptor (CAR) T cell therapies have shown promising treatment outcomes for relapsed or refractory multiple myeloma (RRMM) patients with two approved therapies now commercially available. Currently, all of the approved CAR T cell therapies, as well as most others in clinical trials, utilize viral vectors to semi-randomly insert effector genes during the manufacturing process, leading to heterogeneous genomic composition and potential efficacy and safety variability. Lastly, shortages in lentiviral supply and high treatment costs have led to challenges with patient access, with only a fraction of eligible RRMM patients ultimately receiving commercial BCMA CAR products.

To address these issues at UCSF, we have internally developed a fully non-viral CRISPR/Cas9 engineering platform, enabling precise and efficient CAR integration at the T Cell Receptor Alpha Chain (TRAC) locus through hybrid ssDNA-mediated homology-directed-repair (HDR). The BCMA CAR construct includes the CD28 costimulatory domain and the modified 1XX CD3z intracellular signaling domain, which significantly enhances the product's potency and durability. Our Process Development team has developed a robust manufacturing process and demonstrated GMP compatibility across multiple engineering runs with a shortened manufacturing time, while maintaining consistently high CAR integration rates and yield, exceeding all proposed release criteria for purity, safety, potency, and quality (Figure 1).

Knock-in efficiencies from 7 full scale process development and engineering runs ranged from 38.2 to 72.7%, generating yields of 2.4e8 to 1.9e9 CAR+ cells, which is well above the maximum dose for the proposed clinical trial. Off-target analysis by GUIDE-seq and *in silico* nomination, followed by targeted amplicon sequencing identified two non-pathogenic off-target sites with low editing frequency (<0.2%). Residual Cas9 levels were monitored throughout the manufacturing process, which showed a rapid decay with minimal to absent levels in the final drug substance. Cryopreservation was optimized to improve post-thaw recovery and potency of the final drug products, demonstrating robust and durable tumor clearance *in vitro* and *in vivo*, with particular improvement for a subset of donors whose cells are more sensitive to cryopreservation.

Altogether, these results demonstrate a fully non-viral manufacturing process for generation of advanced 1XX BCMA CAR T cell therapies. We plan to make the manufacturing SOPs and critical study reports publicly available to enable widespread use of this platform and improve access to cutting edge therapies in the academic setting.

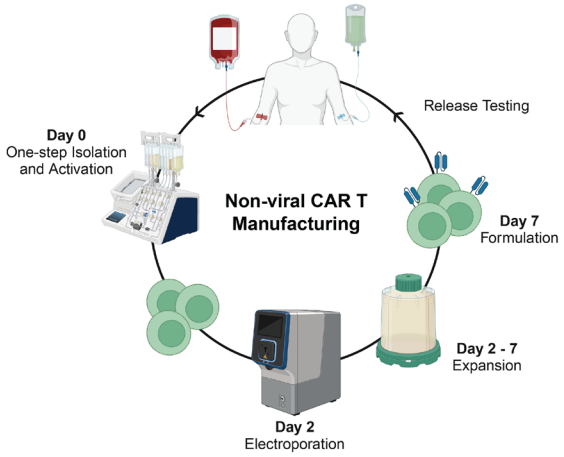

Figure 1: Overview of the UCCT-BCMA-1 cGMP manufacturing process
Created with BioRender.com

69 Establishing Microporous Particle Scaffolds as a Scalable, 3D Platform for Continuous Production of Immunomodulatory Mesenchymal Stromal Cell Extracellular Vesicles

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Introduction:
Due to their immunomodulatory function, mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) are a promising therapeutic with the potential to treat neuroinflammation associated with neurodegenerative diseases. However, clinical translation has yet to be achieved due in part to the critical need for developing scalable manufacturing systems capable of producing consistent EV products. Existing large-scale systems provide limited control over key aspects of the cellular microenvironment, such as hydrodynamic forces, cell-cell communication, and substrate stiffness, that impact MSC growth and secretory function leading to poor reproducibility[1]. Three-dimensional (3D) hydrogel-based microporous particle (MP) scaffolds, in contrast, provide precise control over microenvironmental parameters, which has been shown to influence MSC growth and function[2,3]. Therefore, we aimed to investigate the ability of MP scaffolds to serve as a scalable, tunable platform for large-scale MSC-EV manufacturing.

Methods:
Scaffold void fraction was assessed by quantifying fluorescent dextran (2000kDa) using confocal microscopy (Zeiss LSM900). For expansion, cryopreserved adipose-derived MSCs (AD98, RoosterBio) were thawed and cultured in chemically defined media (CDM)[4] for 72 hours. For growth studies, MSCs were seeded in triplicate 25µL scaffolds

at three seeding densities (63, 125, and 250 cells/µL), and DNA content was assessed using PicoGreen assay. For EV manufacturing, MSCs were seeded into triplicate 75µL MP scaffolds (125 cells/µL), primed with pro-inflammatory cytokines (50ng/mL IFNϒ+TNFα) under hypoxia (2% O2) for 48 hours, and conditioned media was harvested for EV isolation at 72-hour intervals. EVs were isolated via ultracentrifugation and stored at -80°C, while particle count, CD63 expression, and lipid bilayer confirmation (MemGlow) were conducted using NanoFCM. Finally, EV bioactivity was assessed using our microglia morphology assay, as described previously[5]. *Results*: We observed consistent void fraction (33%) and cell seeding across replicate MP scaffolds (Figure 1A & 1B). MP scaffolds supported MSC expansion over a 15-day period, with a seeding density of 125 cells/µL exhibiting the greatest overall fold change (Figure 1C). Variability in MSC growth across scaffold replicates at each timepoint was minimal (CV < 20%), indicating that consistent scaffold preparation and the controlled 3D microenvironment provided by MP hydrogels support reliable MSC expansion (Figure 1D). MPs also supported production of MSC-EVs at each of the four cycles with positive tetraspanin CD63 expression and the presence of a lipid bilayer. MSC-EV yield increased over time and plateaued after the third EV harvest cycle (Figure 2A), agreeing with the trend of increased cell growth over time in Figure 1. Furthermore, EV preparations from each cycle differentially modulated microglia morphology, with preparations from later cycles showing higher bioactivity as evidenced by a significant shift in microglia morphology away from the stimulated positive control (Figure 2B).

Conclusions:
These findings establish MP scaffolds as a promising manufacturing platform for MSC expansion and production of bioactive MSC-EVs for treatment of neuroinflammation. *References*: [1] Thompson, W., Biotechnol. Adv., (2023). [2] Pfaff, B. N. Adv. Biol. (2023). [3] Caldwell, A. S., Biomaterials, (2020). [4] Spoerer, T.M., bioRxiv. (2024). [5] Daga K., J. Biol. Eng. (2024)

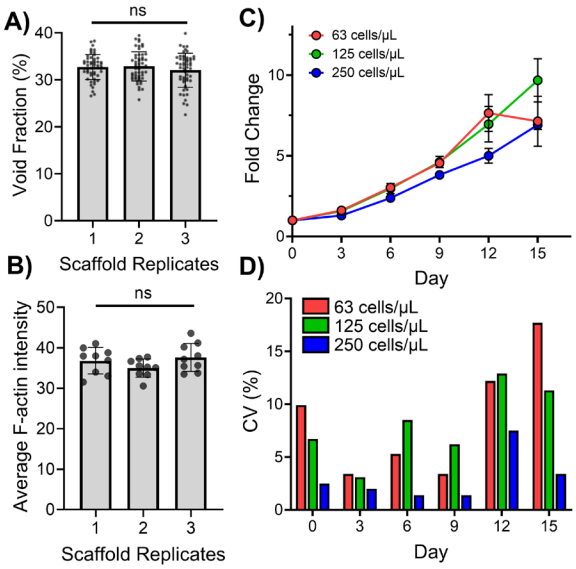

Figure 1: Consistent MP scaffold preparation allows for reliable MSC growth overtime. A) Mean VF across replicate MP scaffolds. n=54 images from z-stacks (18 slices, 13.5µm intervals) collected from three ROIs per replicate. B) Mean F-actin intensity across replicate MP scaffolds. n=9 ROI z-stack averages (20 slices, 40µm intervals) per replicate. C) Fold change in cell count over 15-day period for MSCs cultured at three different seeding densities in MP scaffolds. (n=3 scaffolds per seeding density group at each timepoint) D) CV (%) of fold change per group at each timepoint. ns = p>0.05, via one-way ANOVA with Tukey's multiple comparison test.

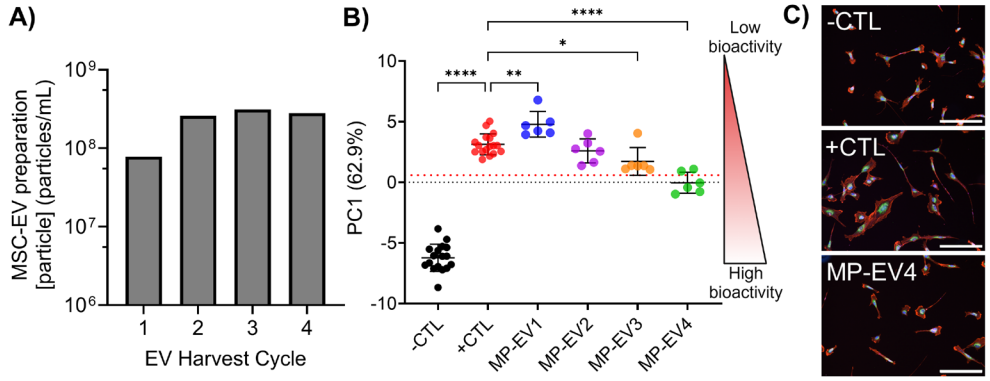

Figure 2: MP scaffolds support production of bioactive MSC-EVs across multiple harvest cycles. A) MSC-EV preparation concentration at each harvest cycle. B) Principal component 1 (PC1, 62.9% of variance) calculated using 21 morphological features of microglia treated with inflammatory signals and/or MSC-EVs *in vitro*. Each point represents a median of ~700-1000 cells/well with mean and standard deviation plotted for n=6 wells/group. Red line = historical data of microglia morphological response to treatment with EVs from 2D flask cultures. C) Representative images of microglia morphology (red=actin and Golgi apparatus, blue=nucleus, green=endoplasmic reticulum, magenta=mitochondria). Scale bar = 200µm. One-way ANOVA calculated using Dunnett's method where ****=p<0.0001, **=p<0.01, *=p<0.05.

70 Transcriptomic and Epigenomic Analysis of CAR Treg Stability

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Treg (regulatory T cell) cell therapy shows promise for treating autoimmune diseases, but these might acquire effector T (Teff) phenotype upon destabilization by improper signaling. Here, we investigated this phenomenon using a tonic chimeric antigen receptor (CAR) Treg ex vivo model, subjecting cells from Treg production (D14) to a destabilizing protocol that included repetitive stimulation to induce destabilization defined by FOXP3^{lo}Helios^{-} expression with flow cytometry.

Flow cytometry analysis revealed an increased fraction of destabilized Tregs (FOXP3^{lo}Helios^{-}) after repetitive stimulation. In vitro suppression assay showed a decreased capability to suppress CD4+ and CD8+ Teff cell proliferation compared to D14 control samples, though the suppressive function remained distinct from that of Teff cells.

To assess the molecular changes beyond FOXP3 and HELIOS, underpinning the phenotypic alterations due to destabilizing conditions, we performed NGS molecular profiling using both bulk RNA sequencing (RNA-seq) and Enzymatic Methyl-seq (EM-seq). Key Treg genes (FOXP3, IKZF2 and IL2RA) showed consistently reduced levels of expression accompanied by increased DNA methylation at their respective promoter regions in destabilized cells. While D14 Tregs exhibited significant differences in gene expression, their methylome showed minimal changes as compared to D0 Tregs, indicating that the Treg product is epigenetic stable. However, under destabilization conditions, the molecular profiles of Treg and Teff became more similar, with reduced differences observed in both transcription and methylation. Scoring samples based on pre-defined Treg/Teff identity gene panels reflect the stability of the product. Additionally, we identified specific CpG regions that could serve as markers for tracking the stability of the Treg products after exposure to CAR target.

Together, our results demonstrate that Treg stability can be efficiently monitored through molecular profiling using expression or methylation panels, providing valuable insights for optimizing Treg-based cell therapies.

71 HDR gene correction of CD4$^+$ T cells approaching the clinic for the treatment of Hyper IgM1

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Gene editing for Hyper IgM1
Mutations in the *CD40LG* gene impair the crosstalk between activated CD4+ T cells and effector immune cells, preventing the development of a robust immune response against pathogens. This defect underlies Hyper IgM 1 (HIGM1), an X-linked combined immunodeficiency with a median survival of 25 years. Furthermore, constitutive *CD40LG* expression leads to lymphoproliferation, emphasizing the need for a gene-correction strategy. We designed a knock-in gene editing (GE) strategy based on Cas9 and integrase-defective lentiviral vector (IDLV) template delivery, and developed a scalable good manufacturing practice (GMP)-compliant process for correction, selection, and expansion of CD4$^+$ T cells.

Tech transfer to academic GMP facility and biodistribution
The manufacturing process was transferred to the academic GMP facility of IRCCS San Gerardo. Three independent full-scale runs with healthy donors' cells were performed during the tech transfer, with a selection yield of 85% and a CD4+ purity >93%. *CD40LG* correction in the final product was around 90%, and dose requirements were met. Expecting graft-versus-host disease upon xenotransplantation of the drug product, in accordance with local regulatory authorities, a GLP biodistribution (but not toxicology) study was performed. The cell product engrafted similarly across groups, with preserved human CD4+ purity. Progressive maturation towards an effector phenotype was observed, in both treated and untreated groups, and editing efficiency remained stable.

Methods validation
We developed a panel of assays addressing identity, purity, potency and safety (cytokine-independent growth, genome integrity, RCL and process related impurities), and validated them according to EMA/CHMP/ICH/172948/2019 ICH guideline M10 and to FDA Bioanalytical Method Validation Guidance for Industry. We did not observe signs of exhaustion, cytokine-independent growth, or concerning genotoxic events. Edited patient cells restored regulated *CD40LG* expression, binding to CD40, and downstream signal transduction. No process related impurities were present.

Process performance with patient cells
Given differences in white blood cell counts and immunophenotype of HIGM1 patients as compared to the general population, we addressed potential concerns of reproducibility of our process with patient-derived cells from a cohort of 7 patients aged from 7 months to 54 years. Informed consent was provided to the attending physician by

patients or legal guardians and samples were pseudoanonymized. Patient cells were edited with high efficiency; purity and clonal diversity were maintained until the end of the process. Interestingly, the proportion of T stem cell memory cells was higher upon GE of patient cells as compared to the healthy donor counterpart. Moreover, patient cells displayed a slightly different proliferation kinetic, respectively faster in the early phases of culture and slower in the latter ones as compared to healthy donor cells. For this reason, a concomitant verification strategy for the clinical trial was agreed upon with Italian regulatory authorities (AIFA/ISS).

Outlook
We report on scientific, manufacturing, and regulatory challenges and success of academic development of an innovative drug product for the treatment of HIGM1, a rare pediatric disease with limited life expectancy and treatment options. Clinical trial enrollment is expected to start in the near future.

72 Comparative Analysis of Motixafortide versus Plerixafor for Autologous Stem Cell Mobilization and Collection in Multiple Myeloma: A Single Center Real-World Experience

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Background:
Stem cell collection after 4-8 cycles of induction therapy remains standard for multiple myeloma (MM). These cells are used after high-dose melphalan as autologous stem cell rescue/transplant (ASCT). Growing demand for apheresis services upstream of chimeric antigen receptor and other cell therapies emphasizes a need for efficiency. FDA approval of Motixafortide (Aphexda®) and a phase III publication (Crees, Nature Med 2023) suggest this newer CXCR4 antagonist with longer-acting kinetics may be superior to plerixafor in MM stem cell collection.

Methods:
In this quality initiative, we compared a prospective cohort of 30 consecutive MM patients mobilized with motixafortide+G-CSF to 30 contemporary MM patients receiving plerixafor+G-CSF. Cohorts were matched for age, gender, race, and exposure to daratumumab and lenalidomide/pomalidomide (IMiDs). Primary outcome analyses included # of apheresis/collection days (enable more/other collections), shortening of apheresis sessions (allow same-day apheresis catheter removal), and adverse events requiring intervention. Local practice patterns included plerixafor (0.16- 0.24 mg/kg sc per renal function) for pCD34 <30 cells/mL at 6pm on day prior to collection (Day +4 of 5 G-CSF) or administration of motixafortide (1.25 mg/kg sc) regardless of pCD34 around 4-5pm, based on clinical logistics. Premeds for motixafortide were cetirizine, famotidine, montelukast, acetaminophen, prednisone 20mg, and lidocaine cream, with ice during a 1-hour monitoring period. Target is 4.0x10^6 CD34 cells/kg with minimum 12L collection for peripheral CD34 count (pCD34) $\geq$ 50 cells/mL and min 4 blood volumes for pCD34 <50 cells/mL. Historically, over 80% of MM patients target in one day with plerixafor+G-CSF.

Results: 29 of 30 patients were able to receive motixafortide. Fold-increase in pCD34 was not different after receiving either agent, though 3 super responders to motixafortide were noted. (Figure 1). We found a trend to superior increase in pCD34 after motixafortide in patients with Day 4 pCD34 $\leq$ 5, but sample size was limiting. Product CD34 yield did not differ. 27 motixafortide and 27 plerixafor patients completed collection in 1 day/procedure. Only 1 of 22 patients receiving motixafortide had his catheter removed the same day. Volume of blood processed and CD34 % product collection efficiencies were the same.

48% of motixafortide recipients required intervention (additional acetaminophen or famotidine or new administration of methylprednisolone IV, diphenhydramine or ketorolac) for pruritis (45%), tingling/burning or pain (34 and 31%), and erythema/rash or facial flushing (24% each). (Figure 2) No nursing or pharmacologic interventions were required after plerixafor. One motixafortide patient experienced painful nodules at injection sites for up to 3 months.

Conclusions: This initial real-world evaluation of motixafortide administration on feasibility, safety, and stem cell mobilization in MM patients at a single center (without changes in practice patterns) did not show superiority over plerixafor in pCD34, stem cells collected, or time/days required to target 4x10^6 CD34/kg. Our institution continues using plerixafor in initial ASCT mobilization procedures for MM. Ongoing investigations include cumulative daratumumab and IMiD exposure, financial and resource analyses, further observation of poor mobilizers with pCD34 of 1-2 cells/mL, and evaluation of post-transplant engraftment.

Figure 1. Comparison of Motixafortide versus Plerixafor as Auto Myeloma Mobilization Agents

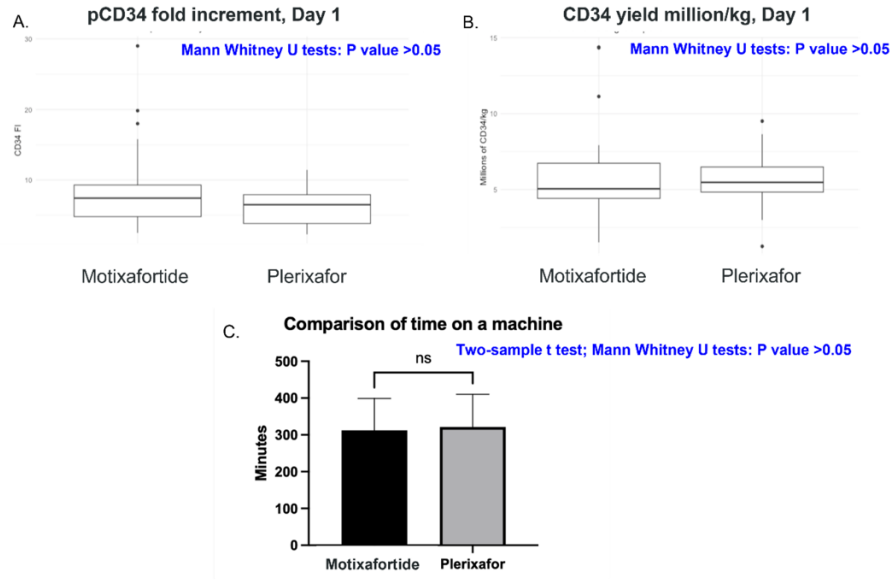

Figure 2. Comparison of Immediate Adverse Reactions to Motixafortide versus Plerixafor

A. Immediate adverse reactions to motixafortide

Characteristic	N (%)
Pruritis	13 (45%)
Numbness/tingling/burning	10 (34%)
Pain (mild to severe)	9 (31%)
Erythema/Rash	7 (24%)
Facial flushing	7 (24%)
Hives	4 (14%)
Nausea/Vomiting	1 (3%)

	Yes N (%)
Intervention made	14 (48%)

*Interventions included: Methylprednisolone, Famotidine, Diphenhydramine, Acetaminophen, Ketorolac, Ondansetron

B. Immediate adverse reactions to plerixafor

Characteristic	N (%)
Tingling	1 (3%)
Nausea	1 (3%)
No information	3 (10%)

No Interventions required

73 Use of IL-12, IL-15, and IL-18 Prior To Cryopreservation Confers a CIML Phenotype To CAR-NK Cells That Maintains Post Thaw Viability and CAR Functionality After Infusion

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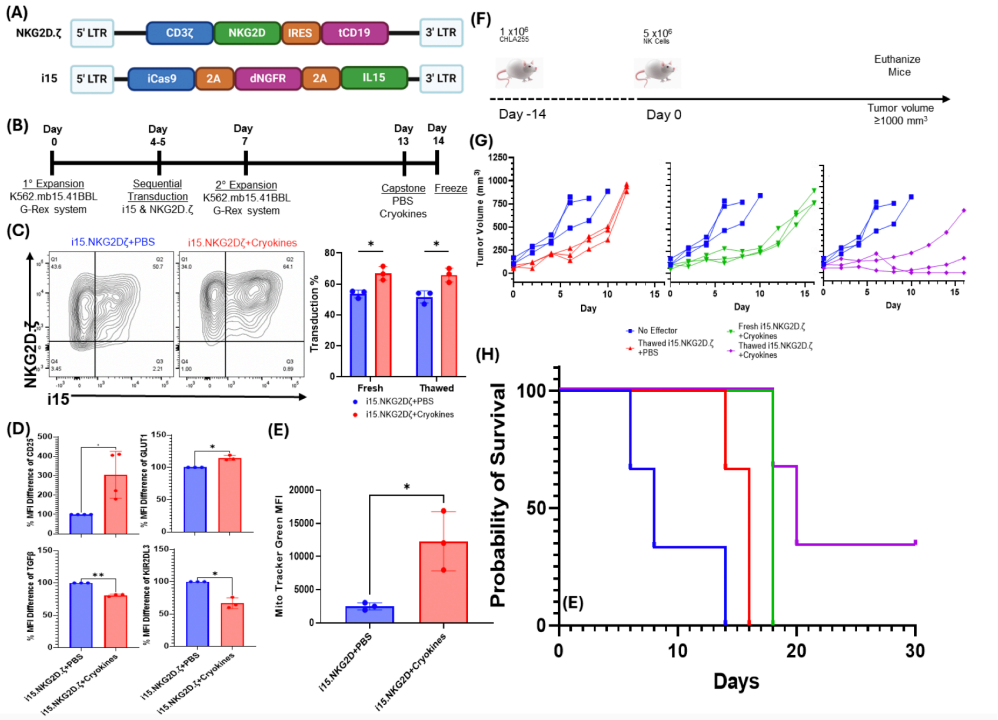
BACKGROUND AND AIM:
Cryopreservation of primary human NK cells modified with chimeric antigen receptors (CARs) has been problematic due to poor long-term viability and function post thaw. Recent methods to preserve activity of non-modified NK cells post thaw do not adequately apply to the unique features of peripheral blood-derived CAR-NK

cells. This barrier hinders use of CAR-NK cell products as truly “off-the-shelf” and may result from CAR-NK metabolic vulnerability during cryopreservation and thaw. Approaches that enhance metabolic fitness in CAR-NK cells during manufacture to decrease vulnerability and improve function post-thaw remain untested. Activation of NK cells with IL-12, IL-15, and IL-18 results in cytokine-induced, memory-like (CIML) NK cells with enhanced effector functions and metabolic fitness. We hypothesized that utilizing CIML cytokines in the terminal step of CAR-NK cell manufacture prior to cryopreservation could shift CAR-NK cell metabolism to a phenotype that maintains functional CAR-based killing post thaw.

METHODS:
Peripheral blood mononuclear cells obtained from healthy donors were cocultured with irradiated K562-mb15-41BB-L feeder cells at a 1:10 (NK cell:irradiated K562) ratio in G-Rex® culture devices for 6 days in medium supplemented with 10% FBS and 100 IU/mL IL-2. Cell suspensions on day 6 (containing 90-95% expanded/activated NK cells) underwent CD3 depletion, followed by sequential transduction with two separate retroviral vectors encoding IL-15 with an iCaspase9 safety-switch and an NKG2D.ζ CAR (Fig. 1A). The dual transduced cell population was secondarily expanded in G-Rex® devices at the same NK cell:irradiated K562 ratio with 100 IU/mL IL-2 for 7 additional days. 10 ng/mL IL-12, 10 ng/mL IL-15, and 50 ng/mL IL-18 (termed here as “cryokines” in this protocol) were added for the final 24 hours of secondary expansion (Fig. 1B). This 16-day human gene-modified NK cell production protocol resulted in >99% pure CD56+/CD3− NK cell population with avg. 62% dual-transduced NK cells (Fig. 1C). We examined post-thaw cell phenotypes, immediate and long-term CAR-NK viability, maintenance of transgene expression, mitochondrial mass as a marker of metabolic activity, and anti-tumor activity in a solid tumor xenograft model of CAR-NK cells manufactured and frozen with or without cryokines.

RESULTS:
Phenotypic evaluation post-thaw revealed maintenance of CAR and IL-15 transgene expression in cryokine treated CAR-NK cells compared to non-treated. Cryokine treatment increased expression of CD25, GLUT1, and decreased expression of TGFβ and KIR2DL3 on CAR-NK cells, consistent with CIML phenotype (Fig. 1D). Cryokine treated CAR-NK cells exhibited increased mitochondrial mass compared with non-cryokine treated CAR-NK (Fig 1E). This shift to a CIML-like phenotype corresponded with enhanced tumor control in vivo and improved survival in thawed cryokine treated CAR-NK group compared to controls, resulting in superior anti-tumor outcomes to even freshly infused CAR-NK cells (Fig. 1H).

CONCLUSION:
Cryokine treated CAR-NK cells share some properties consistent with a CIML-NK phenotype. Preserved functionality of cryokine treated CAR-NK cells is associated with increased mitochondrial mass, an indicator of increased metabolic activity. Addition of cryokines during late stages of CAR-NK cell manufacture represents an attractive option to maintain functionality in cryopreserved CAR-NK cells manufactured from peripheral blood.



Neurologic Diseases - Vectorology and Gene Therapy

74 CRISPR-hfCas12Max-Mediated Therapy HG303 Improves Neuromuscular Function and Survival in Amyotrophic Lateral Sclerosis Models

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Background:
Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by the loss of motor neurons, resulting in progressive muscular weakness, paralysis, and ultimately death within 2 to 5 years of symptom onset. Over 90% of ALS patients exhibit toxic aggregation of the mutated nuclear transactive response DNA-binding protein 43 (TDP-43) protein, a hallmark of the disease. Targeting *ATXN2*, a key gene associated with TDP-43 aggregation, has shown promise in reducing these toxic protein clusters. HG303, a novel CRISPR-hfCas12Max-based therapy, was developed to knock down *ATXN2* expression, providing a potential new treatment for ALS.

Methods:
A single AAV vector delivering hfCas12Max and *ATXN2/Atxn2* gRNA (gATXN2) was injected into the neonatal ALS mouse models (*TDP-43$^{Tg/Tg}$*) via intracerebroventricular (ICV) administration. Motor function (gait, tremor, and kyphosis) was assessed tri-daily between P15 and P21, and survival was monitored daily. The editing efficiency and Atxin-2 and p-TDP43 protein levels in the cortex (Ctx) and spinal cord (SC) of *TDP-43$^{Tg/Tg}$* mice were evaluated using next-generation sequencing (NGS), western blot (WB), and immunofluorescence (IF) 21 days after treatment (Fig. 1A). Additionally, HG303 was tested in wild-type (WT) non-human primates (NHPs) via intrathecal

(IT) administration to assess hfCas12Max DNA and mRNA biodistribution, safety, and editing efficiency of *ATXN2* 4 weeks after dosing in the central nervous system (CNS) (Fig. 2A).

Results:
Compared to PBS-treated *TDP-43*$^{Tg/Tg}$ mice, HG303 significantly improved motor coordination and neuromuscular function in HG303-treated *TDP-43*$^{Tg/Tg}$ mice in a dose-dependent manner, reducing gait impairment ($p<0.05$), tremors ($p<0.05$), and kyphosis ($p<0.05$) (Fig. 1B–D). Notably, middle- and high-dose HG303 treatments extended survival in *TDP-43*$^{Tg/Tg}$ mice, with 61% (8 out of 13) of high-dose-treated mice surviving beyond 160 days (Fig. 1E). qPCR results showed that hfCas12Max DNA and mRNA were distributed in the Ctx and SC (Fig. 1F and G). NGS results showed that the editing efficiency of *Atxn2* increased in *TDP-43*$^{Tg/Tg}$ mice from low-, to middle-, and high-doses, with 22.50±2.70%, 37.40±3.51%, 37.63±1.07% in Ctx and 2.92±0.44%, 8.18±1.06%, 14.70±0.42% in SC, respectively (Fig. 1H). After treating *TDP-43*$^{Tg/Tg}$ mice with low-, middle-, and high-doses, the Ataxin-2 protein in the Ctx and SC significantly decreased compared to the control mice (Fig. 1I and J). IF result also showed a significant knockdown of Ataxin-2 protein in the Ctx and SC (Fig. 1K and L). In WT NHPs, IT administration of HG303 (Fig. 2A) demonstrated efficient biodistribution of hfCas12Max DNA and mRNA across the SC and dorsal root ganglion (DRG) 4 weeks after injection (Fig. 2B and C), with *ATXN2* editing efficiency in SC ranging from 2.04-11.47% (4.82±2.28 %, mean±SD) (Fig. 2D). IF confirmed significant Ataxin-2 protein knockdown in the spinal cord (Fig. 2E). No adverse effects were observed in the CNS or peripheral tissues.

Conclusions:
HG303 offers a promising new approach to treating ALS by improving motor function, reducing toxic protein aggregation, and extending lifespan in preclinical models. Its successful application in NHPs supports the potential for translation into clinical trials, paving the way for transformative ALS therapy.

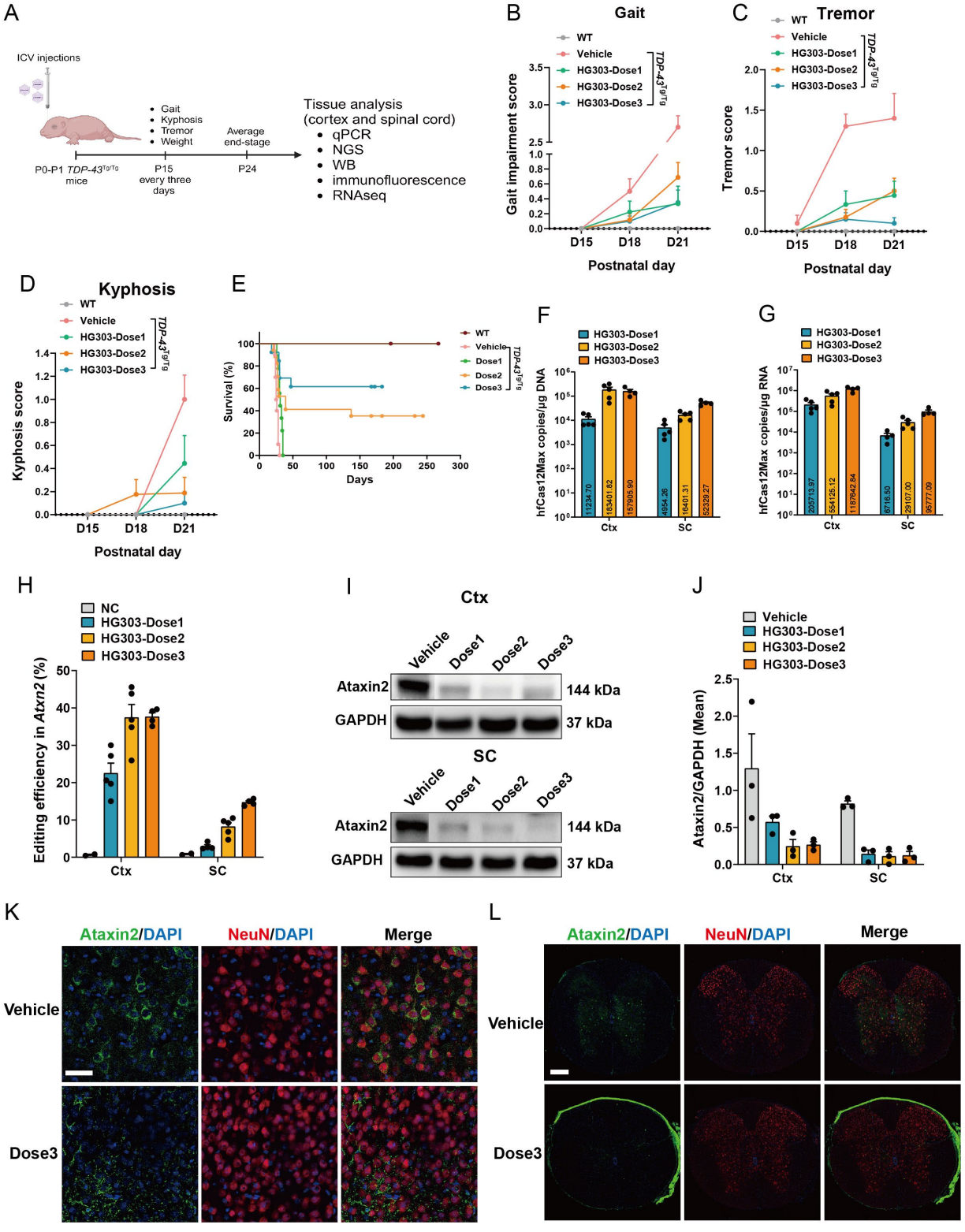

Figure 1. AAV-hfCas12Max-g*ATXN2* improves motor coordination, neuromuscular function, and lifespan of *TDP-43*$^{Tg/Tg}$ mice

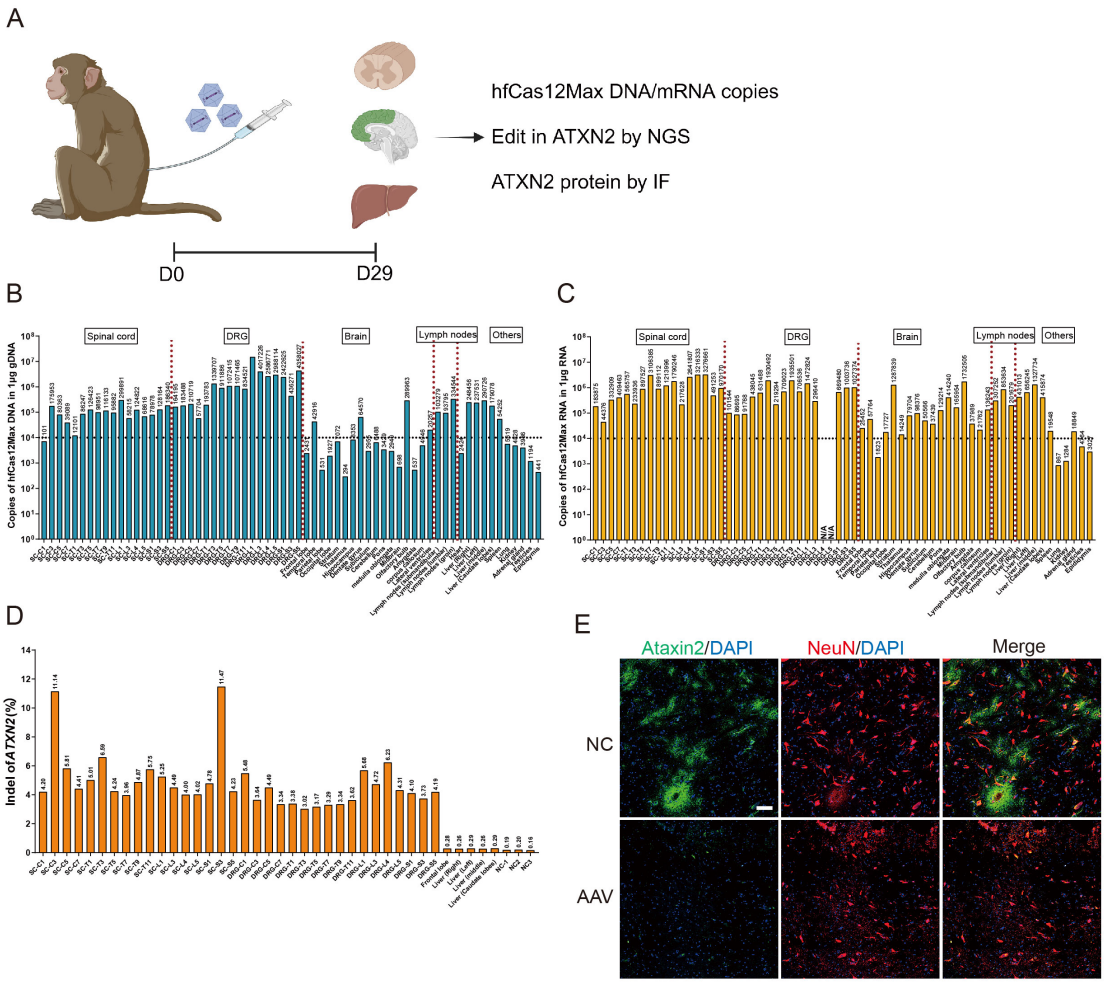

Figure 2. The editing efficiency of *ATXN2* in the spinal cord of WT NHPs with AAV-hfCas12Max-g*ATXN2* IT injection

75 Systemic AAV Gene Therapy with Next Generation Engineered Capsid Demonstrates Expression Levels Supporting Potential Therapeutic Benefit for CNS, Cardiac, and Sensory Symptoms in Friedreich's Ataxia

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Friedreich's Ataxia (FA) is a rare, hereditary, form of ataxia caused by an intronic trinucleotide repeat expansion that diminishes the expression of the frataxin (FXN) protein. Low FXN expression levels cause progressive deficits in cellular respiration and cell death, with pronounced impact to non-regenerative cell types (neurons, cardiomyocytes). There are no current treatments that directly address FXN deficiency, and investigational gene therapies to date do not cross the blood brain barrier to address CNS pathologies. Leveraging our high throughput directed evolution engineering platform, Capsida has identified a systemically administered (IV) capsid that achieves NHP brain-wide biodistribution, transducing >80% of neurons in key FA-related brain areas in addition to

delivering meaningful cardiac transduction. Moreover, identification of a novel human receptor for CAP-004 with complete homology between humans and macaques in the predicted binding pocket increases confidence in the clinical translatability of CAP-004 performance from NHP to humans.

In an NHP study dosing a small pool of capsids simultaneously (N=3 NHP), the engineered capsid drives RNA expression levels ~100x higher than AAV9 in CNS, while maintaining similar RNA expression in cardiac tissue, and ~10x de-targeting in the liver. In addition to evaluating the performance of engineered capsids relative to AAV9 in the pool study, multiple promoters were tested to establish their ability to limit overexpression of FXN, thus mitigating the risk for cargo-induced cytotoxicity. Capsida's proprietary endogenous human FXN promoter, miniFXN10, maintained steady levels of RNA enrichment across a range of DNA biodistribution levels suggesting that it successfully regulates FXN expression. When the drug candidate, CAP-004, was administered IV as a single variant at a low to moderate dose, the engineered capsid delivering hFXN transduced more than 80% of cerebellar Purkinje cells, dentate nucleus neurons, motor neurons in the cortex and spinal cord, and nearly 30% of cardiac left ventricle tissue area. Bulk protein levels in treated NHPs were 8.2x higher than endogenous levels in the motor cortex, and 1.7x higher in the left ventricle. Furthermore, substantial FXN RNA expression was detected in the retina (~1E6 copies/ug RNA) suggesting a potential benefit for sensory vision loss experienced by FA patients. Significant de-targeting of the liver and other non-target tissues contributed to the favorable safety profile characterized by no adverse immunogenicity, clinical pathology, and histopathology findings. Together, these data demonstrate that CAP-004 has the potential to become a best-in-class targeted gene therapy for the treatment of FA by providing therapeutic benefit for CNS and sensory manifestations of the disease that are not targeted by other approaches in addition to impacting cardiac symptoms with a single intervention.

76 Hematopoietic Stem Cell Lentiviral Gene Therapy for the Treatment of Angelman Syndrome

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Introduction

Angelman syndrome (AS) is a rare non-degenerative neurogenetic disorder characterized by a universal lack of speech, impaired motor function and developmental delay. AS is caused by loss of function of the maternal copy of the Ubiquitin Ligase E3A (*UBE3A*) gene in neurons of the brain, where the paternal allele is silenced by a long noncoding RNA antisense transcript. A potential one-time treatment for AS is hematopoietic stem and progenitor cells (HSPCs) therapy overexpressing functional UBE3A via lentiviral vectors (LV) in the differentiated macrophage progeny of the bone marrow that traffic to the brain. UBE3A-overexpressing in myeloid cells in the brain secrete the protein into the extracellular space allowing for cross-correction, where UBE3A is taken up by neighboring neurons.

Methods

A preliminary in-vitro screen was conducted in both human and murine cells to identify candidate LV with high vector titer, gene transfer, UBE3A expression, secretion, and bioactivity. Upon identification of two lead candidate LV, murine lineage-depleted "Lin-" cells were transduced with these vectors then transplanted into busulfan-conditioned adult AS mice. 10-12 weeks post-transplant, AS mice underwent behavioral analysis to assess movement, balance, motor, anxiety and cognitive neurobehavior. Following behavioral analysis, mice underwent electroencephalogram (EEG) analysis to measure neurological electrical activity, specifically delta brainwave power, a robust translational biomarker in both AS patients and mice. AS mice were then euthanized and bone marrow, spleen, peripheral blood, and brain tissues were harvested to measure engraftment, vector copy number (VCN), UBE3A expression, and biodistribution.

Results

HSPC transplanted AS mice transduced with either of two lead candidate LV's showed statistically significant improvements in all neurobehavioral tests conducted: open field, novel object recognition, balance beam, and rotarod assays (Figure 1). Additionally, vector-containing mice showed detectable long-term donor-cell engraftment and VCN in all tissues up to 20-weeks post-transplant. EEG showed significant improvement in delta power in LV transplanted mice relative to non-transplanted mice. Confocal microscopy showed diffuse UBE3A protein expression in both engrafted immune cells of the brain and throughout neurons of the brain. This confirmed that the UBE3A protein as being secreted from the immune cells and taken-up by neighboring neuronal cells (Figure 2). Protein samples from each tissue are currently undergoing UBE3A bioactivity evaluation to confirm proper ubiquitin ligase functional activity, which has previously been documented after in vitro transduction.

Conclusions

Collectively, we have designed two lead candidates of UBE3A-expressing LV with high titers, effective gene transfer, and high UBE3A protein expression in-vitro. We were then able to validate the lentiviral gene therapy strategy in vivo in a robust adult mouse model of AS and showed comprehensive improvement of all well characterized neurobehavioral, electrophysiological, and biochemical AS phenotypes. These promising results serve as the critical preclinical experiments required to advance this program into IND-enabling studies and engage with the FDA to potentially bring the first LV gene therapy forward for a monogenic non-degenerative, non-lysosomal neurodevelopmental disorder, with a large clinical unmet need.

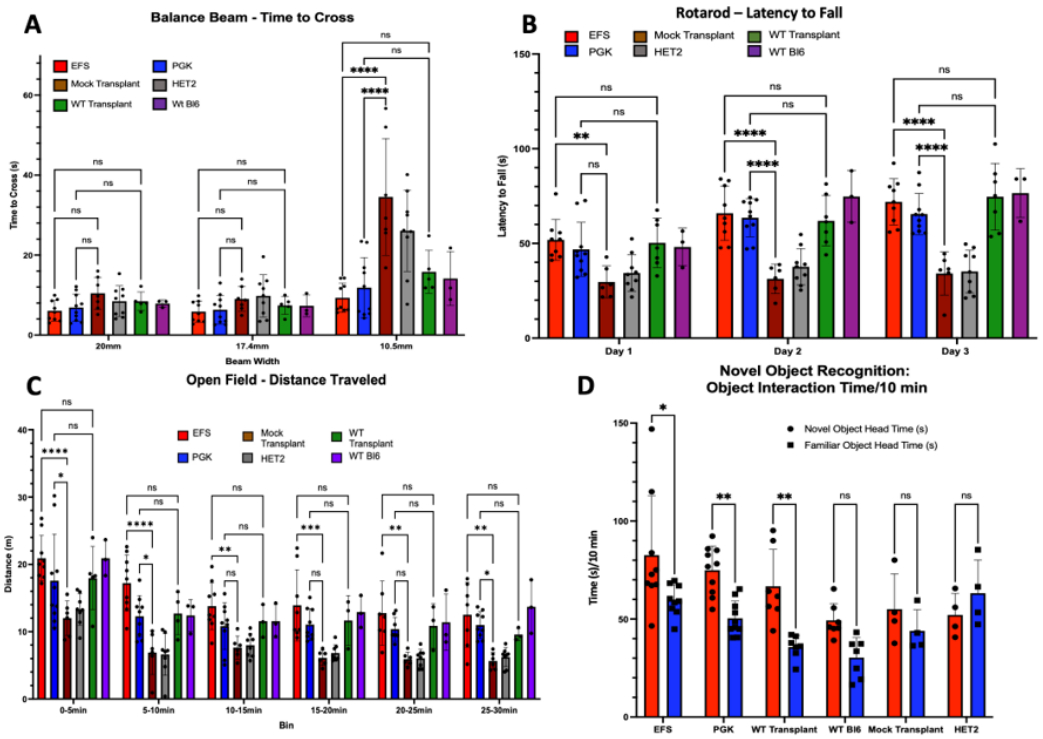

Figure 1. AS Mice which received Ube3a vectors show improvement in various behavioral phenotypes. (A) Mice underwent a balance beam assay in which time to cross beams of 3 different widths is measured. (B) Mice underwent a rotarod assay in which mice are placed on a rod with consistently increasing RPM's – data is displayed a latency to fall off of beam. (C) Mice underwent an open field assay in which mice are placed in a open field and allowed to roam – data is displayed as distance travelled in given 5 minute bins. (D) Mice underwent a novel object recognition in which they are trained with two familiar objects followed by replacement of one of the familiar objects with a novel object - data displayed is number of seconds each mouse spent interacting with the novel vs familiar object in a 10 minute window. **EFS/PGK** = AS mice transplanted with EFS/PGK-Ube3a vectors, **HET2** = AS Mice, **Mock transplant** = AS mice transplanted with unedited AS Lin- cells, **WT transplant** = AS mice transplanted with WT Bl6 Lin- cells. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001.

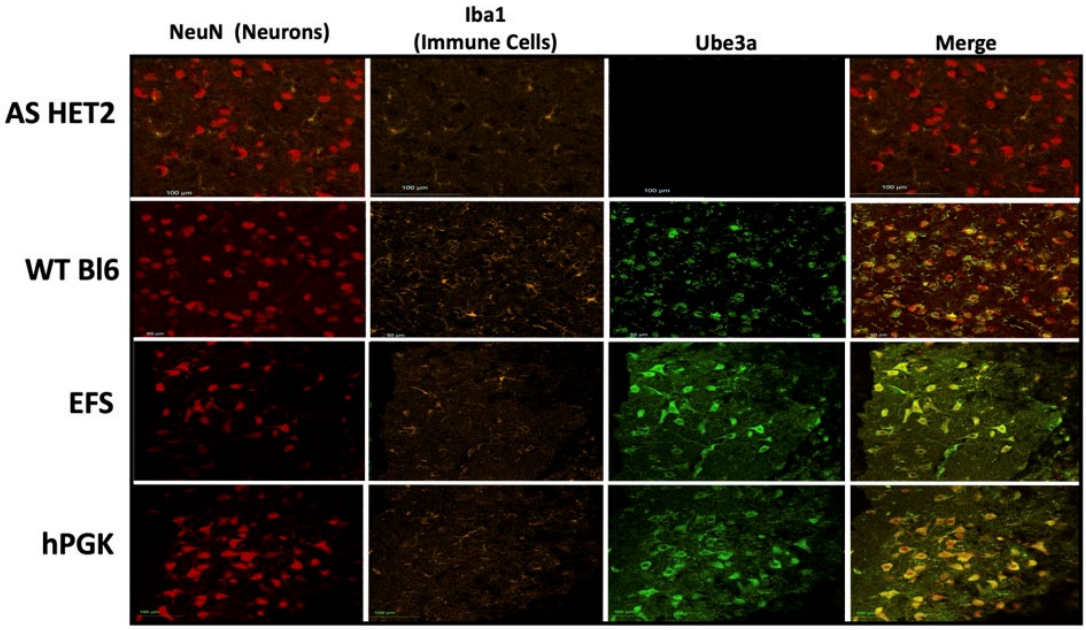

Figure 2. AS mice which received Ube3a vectors show restoration of Ube3a expression in immune cells and neurons. Mice were euthanized and brains were harvested, fixed, cryopreserved, and 30uM slices were generated. Brain slices were then stained with neuronal marker NeuN (red), Immune cell marker Iba1 (orange), and Ube3a (green). Stained brain slices were then imaged using super-resolution confocal microscopy. Images above show Neurons, Immune cells, Ube3a, and a merge of all three channels – displaying both a restoration of Ube3a expression in transplanted mice as well as Ube3a expression present in the neurons of edited mice.

77 ASPA Gene Therapy Increases Expression of Oligodendrocyte Transcription Factors, Correlating with Remyelination in Canavan Disease Animal Model

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Gene therapy using recombinant adeno-associated viruses (rAAVs) has shown great promise in promoting therapeutic response and disease correction for CNS diseases, particularly for dysmyelinating diseases. Strategies such as gene addition are an effective means of restoring the wildtype (WT) gene for otherwise absent or mutated gene(s) of interest. Previously our group has demonstrated that a gene addition approach for Canavan disease (CD), a white matter disease, is efficacious in disease prevention and reversal in CD mouse models; this therapy is currently being evaluated in a clinical trial: NCT04998396. CD is caused by a loss-of-function mutation in aspartoacylase (ASPA), the metabolizing enzyme of N-acetylaspartate (NAA). CD mouse models lacking functional Aspa expression ($Aspa^{-/-}$) have elevated NAA levels, severe dysmyelination, and neurodegeneration, analogous to observed phenotypes in the developing CNS of CD patients. Systemic delivery of rAAV9.CB6-hASPA in neonatal and juvenile $Aspa^{-/-}$ mice can prevent and/or reverse the disease to a wildtype state, as determined by normalized ASPA expression, decreased NAA levels, and remyelination in the CNS. While these findings are encouraging, the underlying mechanism of how ubiquitous hASPA gene addition drives remyelination and corrects neurodegeneration in CD is not well understood.

Oligodendrocytes (OLs) play a critical and indispensable role in promoting efficient neuronal communication and health through the myelination of axons. The capacity for OLs to myelinate and/or remyelinate is contingent upon the proliferation of oligodendrocyte precursor cells (OPCs) and their lineage progression into mature myelinating oligodendrocytes (mOLs). In dysmyelinating diseases such as Multiple Sclerosis and Canavan disease, OPCs are abundant but fail to differentiate into mOLs. With our Canavan disease gene therapy we previously showed that

hASPA expression in Aspa$^{-/-}$ mice can surmount this differentiation block, leading to an increase of mOL cell populations which interestingly couples with increased NeuN^{+} neuronal cells. We hypothesized that this therapeutic potential is achieved through downstream effects of hASPA gene therapy on transcriptional factors (TFs) which drive OL and neural cell fate recovery. We found that protein expression of Sox2 and Olig1 transcriptional factors regulating cell proliferation and OL maturation decrease in Aspa$^{-/-}$ mice, but significantly increase post-hASPA reconstitution; this correlates with increased myelin basic protein (MBP) expression in hASPA-treated but not in untreated animals. These findings suggest a prospective avenue of using gene addition of transcriptional factors as a means to promote disease correction. We additionally discovered that phosphorylated Stat3 (pStat3), the active form of the Stat3 transcription factor, is upregulated in Aspa$^{-/-}$ brains but is normalized to wildtype levels after hASPA gene therapy. pStat3 activates transcription of cell survival and immune-related genes in response to cytokine and growth factor signaling. Therefore, the downregulation of pStat3 after hASPA gene replacement highlights the potential to either dampen immune response and/or promote cellular proliferation post-gene therapy via transcriptional factor activity. We are currently determining the cell-type distribution of these TFs before and after hASPA expression and how gene therapy could be leveraged to promote disease reversal through TF activity.

78 Preclinical Advances for the Translation of an AAV-Mediated Gene Therapy for CTNNB1 Syndrome

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CTNNB1 Syndrome is a severe and rare neurodevelopmental disorder caused by loss-of-function variants in the *CTNNB1* gene, which encodes for beta-catenin, a protein with essential functions in cellular properties, neurogenesis, and synapse formation. Consequently, loss of one functional copy of *CTNNB1* leads to developmental delay in 97% of cases, with patients mainly presenting intellectual and motor disabilities among other phenotypes. While there is no treatment for CTNNB1 Syndrome, the genetic cause could be addressed by delivering a functional copy of the gene to the affected cells. Our group developed a gene augmentation strategy mediated by adeno-associated viral (AAV) vectors.

For this purpose, we designed six AAV-CTNNB1(C1-6) therapeutic candidates, composed of the *CTNNB1* coding sequence accompanied by unique combinations of regulatory elements. Expression cassettes were individually packaged into AAV vectors and used for transduction of patient-derived cortical brain organoids. Based on comparative mRNA and protein expression analyses, we identified one construct, AAV-CTNNB1(C4) as the most promising candidate to significantly increase beta-catenin expression and function in patient-derived organoids without leading to the overexpression of Wnt-regulated genes associated with tumorigenesis, such as *MYC*. RNA sequencing analysis also confirmed the lack of activation of other oncogenic pathways.

We advanced the AAV-CTNNB1(C4) cassette for preclinical *in vivo* proof-of-concept studies in a *Ctnnb1*$^{+/-}$ murine model presenting motor and anxiety phenotypes, two symptoms frequently associated with CTNNB1 Syndrome. Four-week-old wild-type (WT) and *Ctnnb1*$^{+/-}$ mice were intracerebroventricularly (ICV) infused with a dose range comprised of 4.24e9, 2.12e10, and 1.06e11 vector genomes (vg) of test or control vector, and phenotypic outcomes were evaluated at 12 and 20 weeks of life (N=10-16 mice/genotype/dose level or control). Tests revealed normalization of anxiety-like behaviour and locomotor function in *Ctnnb1*$^{+/-}$ mice ICV-infused with the highest dose level. No differences were observed in general health, appearance and weight of mice across test cohorts through five months of life, and endpoint molecular analyses revealed normalization of mRNA and protein beta-catenin expression levels, consistent with patient-derived organoid findings.

To evaluate safety of AAV-CTNNB1(C4), WT mice were ICV-infused with 1.06e11, 2.25e11 vg of test or control vector and monitored weekly for changes in behaviour, appearance, and weight through 8 months of life. Biochemical markers of liver, kidney, and bile duct function measured from blood samples were normal in control and test vector infused groups, and comparable to non-infused mice. Brain and peripheral tissues revealed only age-related histopathological findings, independent of treatment. Together, these studies confirmed a lack of treatment-related toxicity.

Overall, this project demonstrated that AAV-CTNNB1(C4) could serve as an effective and safe therapeutic approach for CTNNB1 Syndrome. The vector, produced under current Good Manufacturing Principles (cGMP), is now in use for biodistribution studies in mice, as well as Good Laboratory Principles (GLP) toxicology studies in mice and non-human primates. These ongoing studies aim to support an investigational gene therapy clinical trial for CTNNB1 Syndrome, anticipated to commence in Europe and Australia in Q3 2025.

79 Treatment of Patients with Spinal Muscular Atrophy Using Covalently closed-end AAV

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Several clinical gene therapies employ self-complementary adeno-associated viral (scAAV) vectors for improved efficacy by eliminating the need for second-strand formation that exists for natural AAV genomes. We recently developed an alternative self-complementary genome with a covalently closed-end inverted terminal repeat. Importantly, packaged genomes of these cceAAV vectors are markedly more intact than traditional scAAVs, which typically contain a large fraction of incomplete genomes, including many that lost their self-complementary configuration. Here, we report first-in-human experience with a cceAAV vector that was systemically administered to two pediatric patients with spinal muscular atrophy (SMA). In preclinical studies, cceAAV32 SMN1 achieved superior therapeutic outcomes than scAAV-SMN1 in SMA Δ7 mice, including substantially extended survival, higher body weight gain, and enhanced motor activity. Studies in non-human primates supported safety of clinical-grade cceAAV-SMN1 (NKG001). Clinical trial in two SMA patients at 12-24 months of age revealed no treatment-associated severe adverse events. Our strategy of initial treatment with RNA-splicing modifying drug during infancy enabled gene therapy in older SMA patients. Both patients showed rapid improvements in motor capabilities after gene therapy, as evidenced by substantial gains in motor function and electrophysiological parameters and capacity for independent mobility. These results strongly support clinical use of cceAAV vectors.

80 Endogenous MicroRNA Feedback for Dosage-Regulated AAV/SLC6A1 Gene Therapy

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Introduction:
SLC6A1-related neurodevelopmental disorder (SLC6A1-NDD) is characterized by a spectrum of symptoms including seizures and intellectual disability. SLC6A1-NDD is caused by haploinsufficiency of the SLC6A1 gene, encoding a neuronal transporter for GABA (GAT1). Preclinical testing of an AAV9/SLC6A1 gene replacement therapy was capable of rescuing disease phenotypes, but revealed adverse consequences from mis-expression of

SLC6A1, by which too much or too little of SLC6A1 can cause an epileptic phenotype (Guo W et al, J Clin Invest, 2024). Therefore, we aim to develop a self-regulated gene therapy, to maintain SLC6A1 within its 'Goldilocks zone' of expression.

Objective:
In this work, we investigate how SLC6A1 expression is naturally tuned in the brain, to test the implementation of these features into an AAV/SLC6A1 gene therapy. Similar to a previous approach to utilize miRNAs to create a feedback regulation loop for the transgene (Sinnett S et al, Brain, 2021), we hypothesized that miRNAs might respond to varying levels of SLC6A1 expression, enabling a similar regulation scheme.

Methods:
MicroRNA (miRNA) responses to SLC6A1 were determined by small RNA-sequencing of the brain cortices of (n=3) wildtype, $Slc6a1^{+/-}$, $Slc6a1^{-/-}$, and AAV/SLC6A1-treated mice. Candidate SLC6A1-repressor miRNAs were identified by cross-referencing public HITS-CLIP datasets of miRNA-SLC6A1 mRNA binding in the brain with *in silico* predicted repressors of SLC6A1. A short genetic element containing the binding sites for putative feedback-regulator miRNAs was generated and appended to the AAV/SLC6A1 transgene. Functionality of the element was validated by a luciferase reporter assay.

Results:
We found that global miRNA expression levels adjust in response to the gene dosage of SLC6A1 in the cortex. Some of the top SLC6A1-binding miRNAs were up-regulated by SLC6A1. Gene ontology terms for these miRNAs were related to SLC6A1 function, including neurotransmitter regulation in GABAergic neurons. These miRNAs were confirmed to repress gene expression via elements in the SLC6A1 3'UTR. A compact minimal 3'UTR, comprised of these elements, was generated and incorporated into the transgene of AAV/SLC6A1.

Conclusions:
We have identified putative negative feedback loops between SLC6A1 and miRNAs, that may be exploitable for the dynamic fine-tuning of AAV/SLC6A1 gene therapy. A novel AAV/SLC6A1 variant, regulated by these miRNA binding sites, is next being tested in mice for improved safety and efficacy profiles for the treatment of SLC6A1-NDD.

Gene Therapy Clinical Trials

81 Revolutionizing Wilson's Disease Treatment: Clinical Safety and Efficacy of LY-M003, A Copper-Responsive AAV Gene Therapy Vector

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Introduction:
Wilson disease (WD) is a rare autosomal recessive metabolic disorder primarily caused by mutations in the *ATP7B* gene. These mutations result in reduced ATP7B activity, leading to the progressive accumulation of copper in critical organs such as the liver, brain, and kidneys. LY-M003, an investigational adeno-associated virus serotype 8 (AAV8)-based gene therapy, was designed to produce a modified miniATP7B^{LY} protein, which retains comparable functionality to the wild-type ATP7B, primarily in the liver. The LY-M003 injection employs the Copper Responsive Engineering of ATP7B Expression (CREATE™) platform, which modulates protein expression based on hepatic copper levels. This innovative approach ensures both efficacy and safety in the treatment of Wilson's disease.

Method:
A clinical study (NCT06650319) to evaluate the safety and efficacy of a single intravenous infusion of LY-M003 in patients with WD is ongoing in First Affiliated Hospital of Zhejiang University, China. Stable treated patients with WD are eligible for inclusion. Patients are permitted to continue standard of care (SoC) therapy following LY-M003 administration until clinical symptom improvement is observed. A prophylactic immune management regimen including both steroid and immunomodulating agent is administered to all participants. The primary objectives of the study are to assess the safety and efficacy of escalating doses of LY-M003. Participants will be monitored for 52 weeks post-treatment, and subsequently enrolled in a long-term follow-up phase lasting five years.

Results:
Since September 2024, seven participants have received LY-M003 injections, with the treatment demonstrating a favorable tolerability profile across all dosage cohorts. No anti-drug antibodies (ADA) against ATP7B were detected, and no serious adverse events (SAEs) were reported. Instances of elevated liver enzyme were promptly managed and resolved through immune modulation therapy. Most participants demonstrated significantly elevated ceruloplasmin levels, with the peak values surpassing 50% of the normal range for one participant, suggesting reestablished hepatic copper transport functionality by ATP7B. Furthermore, several participants have either discontinued or reduced the standard of care (SoC) with normalized urinary copper levels. Notably, most participants began SoC reduction within one month post gene therapy administration. One individual from the high-dose cohort achieved complete discontinuation of SoC within six weeks of receiving LY-M003. For individuals exhibiting neurological symptoms improvement was observed with the administration of LY-M003, as evidenced by a reduction in UWDRS scores related to writing ability, language skills, and other functional capabilities.

Conclusion:
LY-M003 is a novel AAV8-based therapeutic approach for Wilson's disease currently under clinical investigation. Preliminary clinical data from various dosage groups thus far indicated a favorable safety profile and therapeutic efficacy on both peripheral and neurological symptoms, which underscores LY-M003's profound potential in revolutionizing the therapeutic landscape for Wilson disease, heralding a new era of treatment possibilities.

82 Updated Safety and Efficacy Results of RM-001, Autologous HBG1/2 Promoter-modified CD34+ Hematopoietic Stem and Progenitor Cells, in Treating Transfusion-Dependent β-Thalassemia

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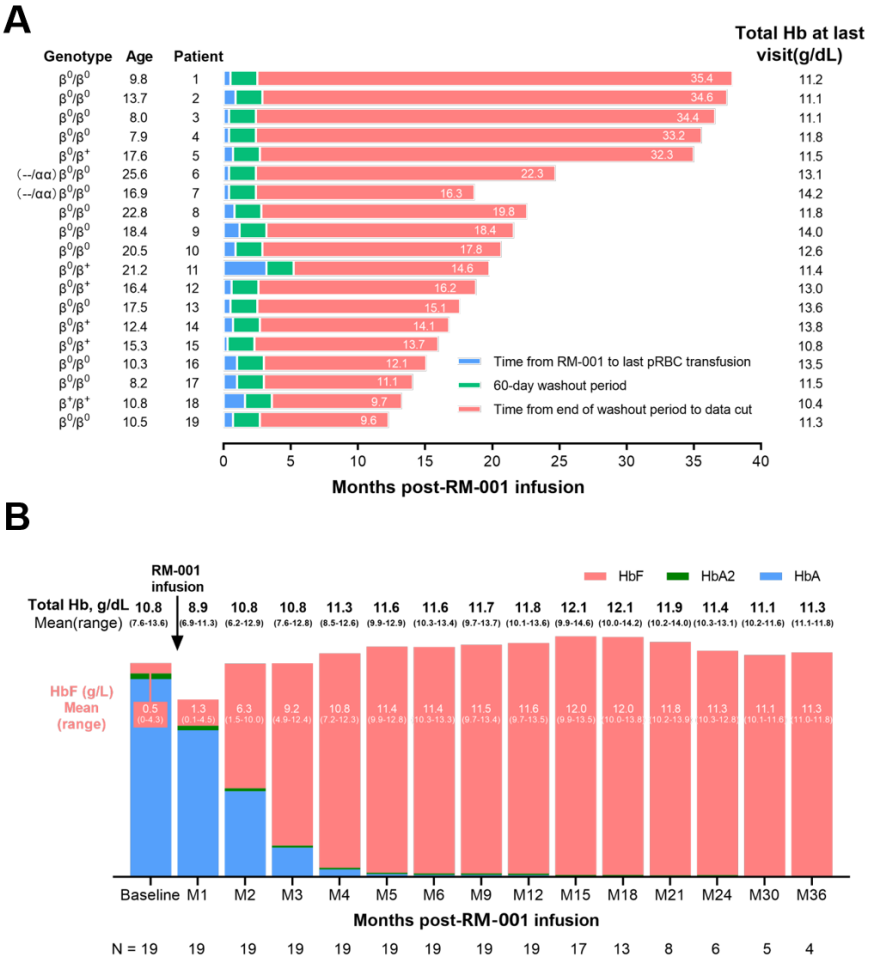
Background:
RM-001 is a novel non-viral cell therapy for β-hemoglobinopathies, which is designed to reactivate fetal hemoglobin (HbF) via ex vivo CRISPR-Cas9 gene-editing of autologous CD34+ hematopoietic stem and progenitor cells (HSPCs) at the binding site of BCL11A on the promoter of the γ-globin genes (HBG1/2). Here we report that in both investigator initiated trial (IIT) and phase I trial, all the patients (pts) with transfusion-dependent β-thalassemia (TDT) achieved transfusion-free after RM-001 treatment.

Methods:
Both IIT (ChiCTR2100053406 and ChiCTR2100052858, n =7) and phase I trial (ChiCTR2300069244, n =12) have been conducted to evaluate the safety and efficacy of RM-001 in treating TDT. Patients (6–35 y of age) with TDT and a history of ≥100 mL/kg/y or ≥10 units/y packed red blood cell (pRBC) transfusions in the 2 years before screening were eligible. Primary efficacy endpoint is transfusion independence defined as proportion of pts maintaining a weighted average hemoglobin (Hb) ≥9 g/dL without pRBC transfusion for ≥12 consecutive months (TI12). Key secondary endpoint is proportion of pts maintaining a weighted average Hb ≥ 9 g/dL without RBC

transfusion for ≥ 6 consecutive months (TI6). Evaluation of TI12 and TI6 started 60 days after last pRBC transfusion. Pts completing the 24-month trial will be enrolled in a long-term follow-up study.

Results:
As of Dec 31, 2024, 19 pts (mean age 15.9; 5 pts aged ≥18, 7 pts aged ≥12 to <18y and 7 pts aged ≥6 to <12y) have received RM-001, with a median follow-up of 19.8 (12.3-37.9) months. Pts received a mean of 53.0 (35.3-106.3) units/y pRBC transfusions before enrollment; 13 pts (68.4%) have the most severe genotype (β0/β0), 5 pts (26.3%) have β0/β+ genotype and the remaining one (5.3%) has a β+/β+ genotype. Following infusion, all pts engrafted neutrophils and platelets (median 15 and 21 days, respectively). All of 19 (100%) pts stopped transfusions and maintained transfusion-free ≥ 11 (11.6-37.4) months. Pts stopped transfusions at a median of 22 (10-95) days post-RM-001 infusion and achieved stable Hb ≥9 g/dL at a median of 31 (14-127) days. Of the 16 pts had ≥15 months of follow-up after RM-001 infusion, all of them (100%) achieved TI12. For all pts, the mean total Hb and HbF were 10.8 g/dL and 9.2 g/dL at Month 3, 11.6 g/dL and 11.4 g/dL at Month 6, 11.8 g/dL and 11.6 g/dL at Month 12, respectively. Proportion of edited HBG1/2 alleles was stable over time in bone marrow cells. The first 6 pts have finished 24-month follow-up and the first 3 pts have finished 36-month follow-up. No RM-001-related serious adverse event report. All of adverse events have been resolved. There were no deaths, discontinuations, or malignancies.

Conclusion:
The data from 19 TDT pts infused with RM-001 demonstrated clinically meaningful and sustained increases in total Hb and HbF, leading to transfusion-free in all of subjects. The safety profile of RM-001 is very well and no product-related serious adverse event was reported during the study. These results indicate that RM-001 has the potential to cure TDT with one-time treatment.



83 Phase 1/2 clinical trial of autologous hematopoietic stem and progenitor cell (HSPC) gene therapy for cystinosis

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Cystinosis is a lysosomal disorder characterized by cystine accumulation within the lysosomes of all organs caused by mutations in the *CTNS* gene encoding the transmembrane lysosomal cystine transporter, cystinosin. Major complications of cystinosis include early renal Fanconi syndrome, chronic kidney disease, renal failure, and ocular pathology that can lead to blindness. Cystinosis also affects the heart, thyroid, skeletal muscle, pancreas, and CNS, eventually causing premature death in early adulthood. Cysteamine delays, but does not stop, disease progression. Here we report results from the phase 1/2 open-label clinical trial (NCT03897361) evaluating safety and efficacy of CTNS-RD-04, consisting of autologous $CD34^+$ cells transduced with a lentiviral vector (LV) carrying the *CTNS* cDNA (CCL-EFS-CTNS-WPRE). Oral and topical cysteamine were withdrawn prior to infusion.

The trial is fully enrolled, and six participants (ages 20 to 46 years) have been treated with CTNS-RD-04 with follow-up ranging from 24 to 63 months. CTNS-RD-04 cell doses ranged from 3.63×10^6 to 9.59×10^6 $CD34^+$ cells/kg with vector copy numbers (VCNs) ranging from 0.59 to 2.5 copies/diploid genome. No adverse events related to drug product have been reported to date. Sustained and highly polyclonal hematopoietic reconstitution was observed in all patients with VCNs at 24 months ranging between 0.51 to 2.67. Patient 4 and 5 restarted low does cysteamine due to low level of VCN (<1). White blood cell cystine and tissue cystine crystals in skin and rectal mucosa decreased compared to baseline. Overall stabilization of the disease and improvement of muscle strength and motor coordination have been observed for patients 1 and 3 who have the longest follow up periods and VCNs $\geq$1. Updated data will be presented on patients infused to date. This is the first *ex vivo* gene therapy approach for a multisystemic disease due to a transmembrane lysosomal protein. The results in adults are encouraging with a good safety profile

84 First-in-Human Study of Autologous HBA2-Edited CD34+ Hematopoietic Stem and Progenitor Cells in Alpha-Thalassemia with Constant Spring Mutation

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Background:
Hemoglobin H Constant Spring (HbH-CS) (--/αCSα) is the most common non-deletion α-thalassemia that can lead to severe health problems. The clinical severity of HbH-CS is widely variable and a considerable proportion of patients with HbH-CS need regular or irregular transfusion to support a normal growth, development and life. Currently, there is no cure for this type of disease worldwide. Because patients with HbH-CS has only a single base mutation in the Hemoglobin Subunit Alpha 2 (HBA2: c.427T>C) compared to that of asymptomatic carriers (--/αα), we speculate that repairing of the mutation by base editing may provide a potential cure for this type of disease.

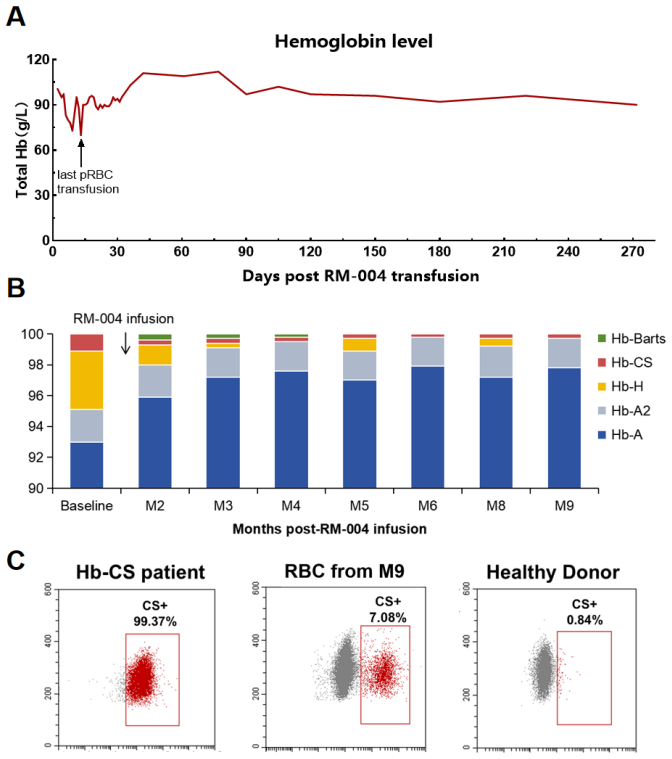
Aims:
To repair the CS mutation of HBA2 gene and increase the expression of normal α-globin chain in erythrocytes, we used ex vivo Cytosine Base Editor (CBE)–based gene editing to modify HBA2 gene in hematopoietic stem and progenitor cells (HSPCs), producing RM-004. NCT06107400 is an investigator initiated first-in-human study of

edited patient cells (RM-004) in transfusion-dependent HbH-CS. Here, we present the initial results from the first patient treated with RM-004.

Methods:
Patients (12–35 y of age) with HbH-CS receiving packed red blood cell (pRBC) transfusions of ≥100 mL/kg/y or ≥10 units/y in the previous 2 y were eligible. Peripheral CD34+ HSPCs were collected by apheresis after mobilization with G-CSF and plerixafor. CD34+ cells were edited with CBEs using a guide RNA specific for the CS mutation. Prior to RM-004 infusion, patient received myeloablative conditioning with busulfan form day-7 to day-3. Patient were monitored for stem cell engraftment/hematopoietic recovery, adverse events (AEs), Hb production, expression of HbA/HbH/Hb-CS, proportion of CS negative red blood cells and requirements for packed red blood cell (pRBC) transfusions.

Results:
The first subject was a 14-y female patient with HbH-CS, who received an annualized 29 units/y pRBC transfusions before enrollment. Patient received a single dose of RM-004 cells and engrafted neutrophils and platelets at Day14 and Day20 post-RM-004 infusion, respectively. The last pRBC transfusion was on Day13 after RM-004 treatment; and the patient has been transfusion-free over 8.5 months. The total Hb stablely over 9 g/dL on Day26 and maintained between 9~11 g/dL currently. Following the infusion of RM-004, the proportion of HbH and Hb-CS were dropped from 3.8% and 1.1% to 0 and 0.3%, respectively. Concurrently, the proportion of HbA1 increased from 93% to 97.8%.The proportion of CS positive RBCs significantly decreased from 100% to about 7%. The safety profile was generally consistent with busulfan myeloablation and autologous hematopoietic stem cell transplantation. No serious adverse event reported.

Conclusion:
The first patient treated with RM-004 demonstrated successful engraftment and clinical meaningful increase in Hb level. Although the follow-up time is relatively short, available data have shown that the patient has entered a transfusion-independence state. This is the first-ever clinical report of patients with α-thalassemia treated successfully with gene editing and indicates that RM-004 is a promising approach for the treatment of HbH-CS. Data will be updated for the presentation.



85 Use of Expanded Non-genetically Modified Natural Killer Cells (Troculeucel) with Enhanced Cytotoxicity in Patients with Alzheimer's Disease. Preliminary Clinical and Biomarker Results.

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Background:
Misfolded protein deposits whether it be amyloid in Alzheimer's, α-synuclein in Parkinson's, and Lewy Body dementia, or tau proteins in Frontotemporal dementia are known to illicit a cascade of autoreactive neuroinflammation and damage. Recent clinical studies have shown that removing these proteins by themselves will not fully address this complex process. The importance of Natural Killer (NK) cells of the innate immune system in neurodegenerative disease has largely been overlooked despite studies demonstrating their ability to reduce neuroinflammation (by eliminating activated T cells, degrading protein aggregates and secreting anti-inflammatory cytokines). Troculeucel is a first in-kind autologous non-genetically modified NK cell product with significantly increased cytotoxicity and over 90% activating receptor expression that can be consistently produced from any donor.

Method:
In this Phase 1 dose escalation study (NCT04678453), SNK01 was administered intravenously (IV) every three weeks for a total of 4 treatments using a 3+3 design in pts with mild, moderate or severe Alzheimer's Disease (AD) confirmed by complete evaluation including MRI and PET scans. The starting dose was 1 x 10^9 cells, and the highest dose was 4 x 10^9 cells. The primary endpoint was safety and secondary endpoints included changes in cognitive assessments, immune cytokines, and CSF protein levels. A second Phase I/IIa trial (NCT06189963), administering a dose of 6 x 10^9 every three weeks for one year, has completed the initial phase I portion.

Results:
In the initial dose escalation Phase I study, troculeucel was found to cross the blood brain barrier via a simple IV via CXCR3 and to identify and eliminate autoreactive T cells via NKG2D and DNAM-1. It was found to be very safe with no drug related adverse reactions. Despite 70% of subjects being treated at relatively low doses of troculeucel, 90% of all evaluable subjects had either stable or improved (±0.1) composite ADCOMS scores at Week 11 (one-week after the final dose). One-week post-final dose, improvement in CSF biomarkers were observed in 70% of subjects for p-Tau181, 60% for AB42/40 ratio, 60% for α-synuclein, 60% for GFAP, 40% for GDF-15, 30% for LTBP2, and 30% for NfL. Early data from a new phase I/IIa trial shows that after treatment with a dose of 6 x 10^9 cells every 3 weeks for three months, 2 of the 3 phase I subjects had already improved from a moderate to a mild AD rating on the CDR-SB scale and after six months, all three subjects had stable or improved CDR-SB, ADAS-Cog 11 and ADCOMS scores.

Conclusions:
Troculeucel has been shown to be very safe and well tolerated. It appears to have clinical activity in AD while also improving CSF protein levels and reducing neuroinflammation. A randomized phase II trial in moderate stage Alzheimer's patients is currently underway, and troculeucel is planned to be studied in Parkinson's Disease, Frontotemporal Dementia, and Lewy Body Dementia.

86 Lentiviral hematopoietic stem cell gene therapy (atidarsagene autotemcel) for late juvenile Metachromatic leukodystrophy (MLD): Interim analysis of a Phase III trial

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Background
Metachromatic Leukodystrophy (MLD) is a rare, severely disabling lysosomal storage disease. Atidarsagene autotemcel ("arsa-cel") is a hematopoietic stem cell (HSC) gene therapy (GT) consisting of autologous CD34+ cells transduced ex vivo with a lentiviral vector encoding the human ARSA gene. These cells are infused following intravenous myeloablative busulfan conditioning. Arsa-cel has shown long-term favorable outcomes in early-onset MLD.

Methods
A phase III trial (NCT04283227) evaluates the safety and efficacy of "arsa-cel" in pre-symptomatic or early-symptomatic Late Juvenile MLD (LJ-MLD) patients. This report presents preliminary engraftment, pharmacodynamic and safety data from an ad hoc interim analysis

Results
As of January 2025, six patients enrolled in the study have been treated. Four were pre-symptomatic, and two were early-symptomatic. One patient had a prior allogeneic HSC transplant with myeloablative busulfan-based conditioning one year before enrollment, followed by autologous reconstitution. This patient met eligibility for HSC-GT under the protocol due to confirmed full-host chimerism and normal bone marrow (BM) findings. Among treated patients, the median age at GT was 10.4 years (range: 2.7 to 15.5 years). Stem cell source was mobilized peripheral blood collected after 6-8 doses of G-CSF and administration of Plerixafor 6-8 hours before leukapheresis. Median yield was 34.3×10^6 CD34+ cells/kg (range: 30.3-37.7×10^6 CD34+ cells/kg). The infused drug product median dose was 23.1×10^6 CD34+ cells/kg (range: 16.0-28.9×10^6 CD34+ cells/kg), with a vector copy number (VCN)/cell range of 2-5. The patient with prior allogeneic HSC transplant had CD34+ yield and graft characteristics comparable to other patients. All patients received myeloablative conditioning with busulfan as a single agent, with a cumulative target area under the curve of 85 mg×h/L. After a median follow-up of 27.8 months (range: 12.6 to 34.3 months), all patients are alive. Five patients remained neurologically stable, while one early-symptomatic patient experienced disease progression shortly after GT, followed by stabilization up to 30 months post-treatment. Short-term adverse events were consistent with busulfan's safety profile. Two patients required therapy to normalize platelet counts. No malignancies, abnormal clonal expansion, replication-competent lentivirus, immune responses against ARSA enzyme, or treatment-related serious adverse events were reported. Median neutrophil and platelet engraftment times were 33 days (range: 25-41) and 27.5 days (range: 24-48), respectively. All 6 treated patients showed rapid engraftment of transduced cells. In the five patients with available data at one year post-GT, the median VCN was 0.3 (range 0.2-0.9) and 0.8 copies/cell (range 0.3-2.5) in peripheral blood mononuclear cells (PBMCs) and CD15+ cells, respectively; the percentage of transduced BM progenitors ranged from 18.6% to 85.4%. ARSA activity increased to supranormal levels in PBMCs and normal levels in cerebrospinal fluid (indirect measure of functional ARSA enzyme in the CNS) at the latest follow-up.

Conclusion
This interim analysis suggests that the drug product characteristics, short-term safety profile, and pharmacodynamic efficacy of arsa-cel in LJ-MLD align with previous studies in early-onset MLD. Further follow-up is ongoing to assess brain imaging, clinical efficacy endpoints, and long-term safety

87 Autologous human peripheral blood B cells genetically engineered to express human iduronidase: Results from a first-in-human clinical trial in subjects with mucopolysaccharidosis type I (MPS I)

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We report clinical outcomes from the first administration of genetically engineered B cells in a human subject. MPS I is a rare ($1/10^5$) lysosomal disease caused by a deficiency of a-L-iduronidase (IDUA), leading to systemic accumulation of glycosaminoglycans, organomegaly, skeletal dysplasias, cardiopulmonary complications and in severe cases neurologic impairment. Current treatments by enzyme replacement or by allogeneic hematopoietic stem cell transplant address some but not all of these manifestations. ISP-001 is an autologous B cell product engineered to express human IDUA for the treatment of MPS I. ISP-001 is generated from a non-mobilized peripheral blood apheresis product, enriched for the CD19+ fraction, expanded in culture and genetically engineered for human IDUA expression using the *Sleeping Beauty*transposon system. The first subject is a 25-year-old male who has been on enzyme replacement therapy (ERT; laronidase) for 13 years. He was infused with a low dose of ISP-001 (2.29×10^7 CD19/kg) in November 2023 while continuing ERT. No chemotherapy was administered. At 12 months post infusion no safety concerns have been identified, including a review of available data at 6 months by the FDA and an independent data and safety monitoring committee. Transposed cells were detected by quantitative PCR in marrow samples at 3 and 6 months. Urine glycosaminoglycans (GAGs) decreased into the normal range for the first time during the first six months post-treatment. Surprisingly, cerebrospinal fluid heparin sulfate levels also decreased post-treatment. Range of motion (shoulder flexion) improvement was noted through 48 weeks post treatment versus baseline. There was also continuous improvement in the subject's six-minute walk test, reaching the normal range at 12 months post-treatment. In summary, dosing of the first patient with genetically engineered autologous human B cells (ISP-001) demonstrated excellent safety to date and encouraging early pharmacodynamic and clinical activity in combination with ERT. These early results support both the dose escalation of ISP-001 to treat MPS I and the expansion of this new engineered B cell platform to treat other human diseases.

AAV Gene Transfer (A): Crossing the Blood-Brain Barrier

88 Widespread CNS Delivery With Best-In-Class Liver Detargeting Following Intravenous Injection of a Novel AAV

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Intravenous (IV) administration of AAV gene therapies is limited by the high burden of AAV to off-target tissues and the high doses required to achieve sufficient on-target delivery. Minimizing liver tropism while improving blood-brain barrier (BBB) crossing allows for the use of lower IV doses to achieve widespread CNS delivery, thus improving the safety and lowering the cost of CNS-targeted therapies. Using large datasets from multiplexed libraries tested in non-human primates (NHPs), AI model-based design enables the simultaneous optimization of on-target CNS and off-target liver delivery. Here we present characterization of a novel capsid designed with this AI-based approach showing best-in-class liver detargeting combined with broad delivery across the CNS after IV administration.

From a high-throughput library of ~100,000 synthetically designed capsids tested in two cynomolgus NHPs (M. fascicularis), we identified a capsid which achieved 335x greater aggregate brain transduction and 32x improved liver detargeting when compared with AAV9. Next, we evaluated the performance of this capsid individually in adult cynomolgus NHPs with a self-complementary transgene administered via IV delivery at doses of 4e12 and 3e13 vg/kg. At 3e13 vg/kg, this capsid showed widespread transgene delivery across the CNS, combined with strikingly improved liver detargeting, reaching vector genome biodistribution as low as 0.75 vg/dg in the liver. In

the CNS, we observed transduction in 14% of neurons in the motor cortex, in 19% of neurons in the premotor cortex and in 14-17% of neurons in the basal ganglia using RNAscope. In the liver, transgene protein IHC revealed transduction in 0.76% of hepatocytes at the 3e13 vg/kg dose, even though the payload used a strong ubiquitous promoter, thus highlighting the greatly improved safety profile. As compared to IV-administered AAV9 in NHP studies where the liver receives roughly ~1000X higher vector genome copies than the CNS, this capsid has a dramatic shift in biodistribution to only ~4X higher vector genomes in the liver compared to the motor cortex. In addition to characterizing in vivo properties in NHPs, we confirmed the interaction of this capsid with a candidate receptor that is expressed in the human BBB and whose human and NHP orthologs are highly conserved.

Together these data present an engineered AAV capsid that achieves substantial liver detargeting combined with improved blood-brain barrier crossing, making it an excellent candidate for clinical development of low dose IV-administered gene therapies targeting neurological diseases.

89 Rationally Designed Receptor-targeting AAV Variants Achieved Efficient CNS Transduction in both Mice and NHPs

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**Corresponding Author*

Intravenous administration of recombinant adeno-associated virus (rAAV) vectors delivering therapeutic payloads with broad distribution in the central nervous system (CNS) provides a non-invasive and promising treatment for many neurological diseases. Several engineered AAV9 variants have been demonstrated to penetrate blood-brain barrier (BBB) in mice but not in non-human primates (NHPs). We developed a Receptor-targeting AAV (RtAAV®) capsid engineering platform for efficient CNS distribution and cross-species translatability. A polypeptide library was incorporated into AAV capsids and screened for binding conserved transferrin receptor 1 (TfR1). Top-performing AAV capsids incorporated with different TfR1-targeting polypeptides were individually packaged with cynomolgus frataxin (FXN) transgene with tags driven by ubiquitous promoter. The capsids were intravenously injected into adult cynomolgus macaque at the dose of 2E13 vg/kg/capsid. Three weeks after injection, the animal was euthanatized, and tissues were collected for vector genome (vg) copy and transgene expression analysis by using ddPCR. One variant, RtAAV001 exhibited up to 14-fold and 12-fold improvements over AAV9 at vector genomic DNA (gDNA) level in the brain and spial cord, respectively. In an individual validation study, RtAAV001 capsid packaged with CMV-GFP-HA transgene expression cassette was intravenously administrated into adult cynomolgus macaque at dose of 5E13 vg/kg. Three weeks after administration, tissues were harvested and processed for vector gDNA and transgene mRNA analysis, as well as immunohistochemical (IHC) staining for protein expression characterization. High levels (6 – 25 vg/diploid genome) of vector gDNA were observed in all CNS regions examined. IHC analysis and immunofluorescent (IF) co-staining of NeuN and GFP-HA demonstrated efficient and broad neuron transduction by RtAAV001 in the CNS of NHP (Fig. 1A and 1B). About 17%, 30%, 42%, 58%, 61%, and 36% of neurons were transduced in the putamen, thalamus, cerebral cortex, hippocampus, lateral geniculate nucleus (LGN) and cerebellum, respectively. To test if RtAAV001 can penetrate BBB and transduce CNS across species, adult C57BL/6 mice were intravenously injected with RtAAV001 at dose of 1E12 vg/mouse. Compared to AAV9 control, RtAAV001 showed about 20-fold improvements at gDNA level in the CNS. About 42-fold and 62-fold enhancements over AAV9 at transgene mRNA levels were observed in the brain and spinal cord, respectively. The efficient and broad neuronal transduction of RtAAV001 in mice was also confirmed by co-staining of NeuN and GFP-HA (Fig.2). About 50%~90% of neurons in the mouse brains were transduced after intravenous delivery of RtAAV001. Interestingly, RtAAV001 showed diminished transduction in the liver. In conclusion, by rationally designing AAV capsids with incorporated TfR1 binding peptides, we successfully generated and validated novel AAV variants that can efficiently penetrate the BBB and transduce the CNS in both mice and NHPs following intravenous administration. These novel RtAAV® capsids represent more efficient and translatable delivery tools for AAV-based gene therapy for neurological diseases.

Fig.1A IHC of NHP brain samples

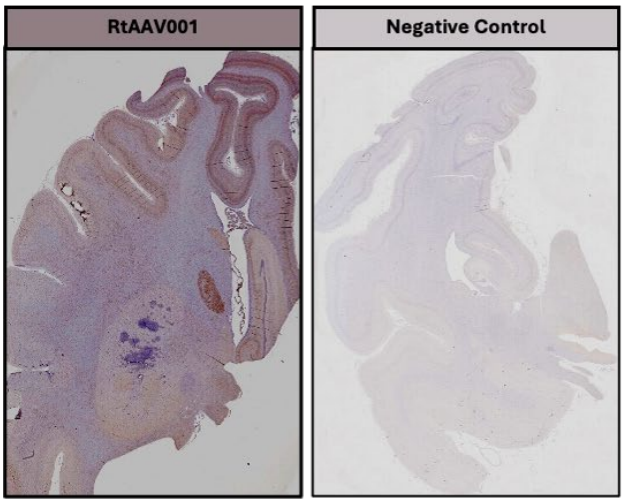


Fig.1B IF of NHP brain transduced by RtAAV001

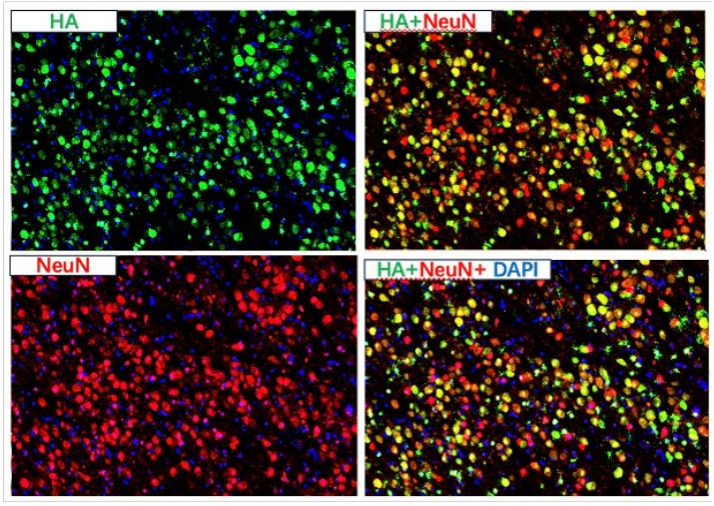
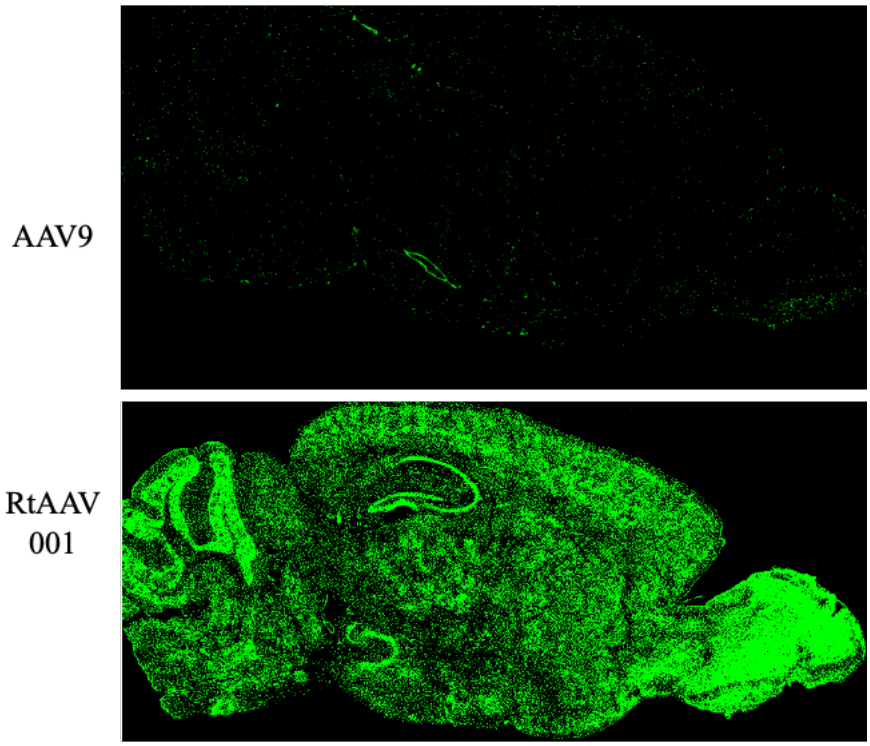


Fig.2 C57BL/6



90 Cryo-EM unveils molecular mechanism of liver detargeting and neuronal tropism of CNS-enhanced AAV capsids

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Developing adeno-associated virus (AAV) vectors capable of crossing the blood-brain barrier (BBB) while minimizing liver transduction remains a key challenge in gene therapy for neurological disorders. The highly brain-enhanced capsid AAV-PHP.eB was developed by inserting a 7-amino acid (7-mer) sequence into variable region (VR)-VIII. Further refinement with an additional 7-mer substitution in VR-IV resulted in a highly liver-detargeted variant, AAV.CAP-B10.

Here, we report single-particle cryo-electron microscopy (cryo-EM) structures of CAP-B10 alone and in complex with AAV receptor (AAVR) domain PKD2 at resolutions of 2.03 Å and 2.30 Å, respectively. Our structural analysis revealed that CAP-B10 retains a VR-VIII configuration similar to PHP.eB, which facilitates binding to the BBB-transducing receptor LY6A while introducing steric hindrance that reduces AAVR affinity. Additionally, sequence changes in VR-IV alter the conformation of residues 454 and 456, further decreasing the affinity of CAP-B10 to AAVR-PKD2. Biochemical assays, including pull-down experiments and biolayer interferometry (BLI), confirmed that CAP-B10 exhibits significantly reduced AAVR binding compared to PHP.eB. Notably, substituting residues 454 and 456 in PHP.eB to match those in CAP-B10 resulted in a substantial reduction in AAVR interaction and liver transduction efficiency. These findings suggest that evolutionary modifications favoring liver detargeting are closely linked to the disruption of AAVR binding.

Reducing AAVR affinity also led to diminished astrocytic transduction, thereby shifting CAP-B10's cell tropism toward neurons. This indicates that altering AAVR interactions can modulate capsid tropism. We solved additional cryo-EM structures of AAV9-X1, AAV9-X1.1, and the AAV9-X1.1-PKD2 complex at resolutions of 3.10 Å, 2.40 Å, and 2.25 Å, respectively. AAV9-X1.1, which features the same VR-IV 7-mer substitution as CAP-B10, showed reduced AAVR binding relative to AAV9-X1, though the reduction was less than that observed for CAP-B10. This implies that effective liver detargeting results from synergistic steric effects of modifications in both VR-VIII and VR-IV.

Our findings suggest a dual-pronged approach for engineering brain-specific AAV capsids: reducing AAVR binding to minimize off-target transduction and introducing central nervous system (CNS)-specific receptor interactions to ensure efficient trafficking and transduction. This strategy provides a framework for tailoring AAV capsids to treat CNS disorders.

91 Discovery of AAV9-derived CNS capsids evading pre-existing neutralizing antibodies

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Adeno-Associated Virus (AAV) gene therapy holds significant potential for treating a broad range of human genetic disorders. However, its application is often constrained by the presence of pre-existing anti-AAV antibodies, which arise from previous exposure to natural AAVs. These antibodies hinder AAV biodistribution and tissue transduction, especially for systemically administered AAVs. Recent advancements in capsid engineering have not only enhanced AAV tissue tropism but also opened avenues for improving other key AAV characteristics.

In this study, we leveraged our proprietary RNA-driven TRACER evolution platform to detarget Voyager's 2nd generation AAV9-derived CNS-tropic capsids from neutralizing antibodies (NAbs). We evolved potential epitopes displayed on the capsid surface and screened the resulting libraries *in vitro* and *in vivo* in the presence of monoclonal NAbs and/or human intravenous immunoglobulin (IVIG) to maximize the clinical relevance.

We identified several capsid families with specific amino acids enriched by IVIG selection pressure at several mutation hotspots. The top capsid candidate was able to evade neutralization by 30% of NAb-positive human sera, while retaining mouse brain transduction properties of its CNS-tropic parent. Most importantly, this capsid was still capable of transducing the brain of Cynomologus macaque with pre-existing anti-AAV9 neutralizing antibodies,

whereas transduction by its parent was completely eliminated. Moreover, the mutations present in this capsid did not alter brain cellular tropism.

Additional mutations in a different capsid region further enhanced NAb evasion, achieving resistance to up to 43% of AAV9-neutralizing human sera; however these mutations adversely affected NHP brain transduction. Remarkably, a second generation of mutations in this region, identified using Machine Learning capsid modeling, enhanced both antibody evasion and brain transduction in pooled capsid library assays.

Overall, this study identified a 3rd generation of bioengineered AAV capsids with mutations conferring evasion from pre-existing neutralizing antibodies while retaining the improved CNS tropism of their 2nd generation parent. These novel capsids have the potential to significantly improve a key bottleneck in AAV treatments by increasing patient eligibility to receive AAV gene therapies.

92 Incorporation of Transferrin receptor binder and surface mutations into AAV enables efficient brain delivery and reduced liver tropism

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Efficient intravenous delivery of adeno-associated virus (AAV) gene therapy for CNS indications in humans has been limited by a poor penetration of any AAV serotype across the blood-brain barrier (BBB) and is further restraint by frequently observed liver toxicity. To overcome these liabilities, we created AAVs with the following modifications: Firstly, we incorporated an expression cassette in the production plasmid for an AAV that encodes the transferrin receptor (TfR) binder previously used successfully to transport protein therapeutics into the CNS via receptor-mediated transcytosis. The TfR binder cross-reacts with cynomolgus monkey and human TfR, thereby easing the validation across species. Secondly, we modified several capsid domain encoding sequences in the production plasmid to investigate their influence of decreasing liver tropism.
For the initial validation in mice, we compared the efficacy of AAV9 or TfR-binder tagged AAV encoding green fluorescent protein (GFP) in the CNS transduction efficiency in human-TfR knock-in mice.
A 77-fold higher expression of GFP was observed in mice intravenously injected with the TfR-binder tagged and liver-detargeted AAV. Analysis of VG in liver revealed a 99% reduced tropism to the liver compared with AAV vectors that did not contain capsid modifications to reduce liver tropism. Immunohistochemistry against GFP revealed a homogenous expression of GFP in mice injected with the tagged virus.
Next, in an attempt to further increase brain transduction, we combined TfR-binder and another tag which targets a second receptor, and compared brain uptake in mice. We observed more than 2-fold increased transduction for the AAV containing binders for two separate receptors compared to TfR-binder tagged AAV.
We then validated the technology in non-human primates (NHP) and intravenously injected a cynomolgus monkey with untagged AAV9 encoding mCherry and dual tagged, liver-detargeted AAV encoding mRuby2. Tens to hundreds-fold increase of transduction was observed across regions of the brain in NHPs injected with the modified dual tagged AAV. Analysis of VG in liver revealed more than 90% reduced tropism of the modified dual tagged AAV compared with AAV9. Transduction of dorsal root ganglia, which can be a safety concern, was also reduced more than 90% compared with AAV9.
We finally validated the technology on a mouse model of CLN2 (Batten's type 2). In this model, mice lack the enzyme TPP1 which leads to the accumulation of toxic substrate lipofuscin in several parts of the body, including the CNS. Untreated TPP1 KO mice had a life span of ~160 days, which was barely increased with TPP1 enzyme replacement therapy. Infection of TPP1 KO mice with tagged AAV encoding TPP1 led to a functional cure as indicated by survival and absence of tremor.
We conclude that our modified AAV capsids may provide an invaluable tool for the safe and efficient delivery of gene therapy to the CNS.

93 Identification of Multiple Novel Blood-Brain-Barrier Receptors for CNS Gene Therapy and Other Drug Modalities via an Integrated AAV Capsid Engineering Platform

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Significant progress has been made in recent years targeting novel therapeutics to the CNS by leveraging known blood-brain-barrier (BBB) shuttling mechanisms like the transferrin receptor. Building upon this work, we have leveraged our high-throughput AAV directed evolution platform to screen for novel shuttling proteins localized to the BBB that enable more efficient and specific CNS delivery. Leveraging Capsida's high-throughput capsid engineering platform, we designed viral libraries with targeted peptide insertions and substitutions across multiple surface-exposed loops that converge to form a binding pocket. We then systematically screened capsid libraries derived from multiple AAV serotypes across >100 non-human primates (NHPs) and identified at least 5 novel binding proteins that mediate CNS shuttling, to date. Notably, these receptors stand out for their diverse advantages in CNS delivery, demonstrating higher expression specificity in brain vasculature compared to known BBB receptors, and demonstrating the ability to bias delivery towards certain cell types within the CNS like neurons or glial cells.

In characterization studies, the novel BBB shuttling mechanisms have demonstrated the ability for their AAV binders to transduce >80% of neurons throughout the CNS, with >99% specificity for neurons relative to other cell types, showcasing the capability of these novel BBB shuttling proteins to mediate greater efficacy and specificity for CNS-targeted therapies. Sequence analysis has revealed high sequence identity across rodents, NHPs, and humans, and in vitro assays have confirmed cross-species capsid protein binding, enabling a more predictive path from discovery to the clinic. Taken together, these findings demonstrate the capability of our integrated capsid engineering platform to identify both CNS-tropic AAVs and an unmatched portfolio of BBB shuttling mechanisms, paving the way for not only highly targeted AAV variants but also the development of non-viral CNS drug modalities that mediate greater CNS efficacy and specificity.

94 Engineering Second-Generation Human Transferrin Receptor 1 (TfR1)-Targeting Capsids with Enhanced Brain Transduction via Differential Directed Evolution and Site Saturation Mutagenesis

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Achieving safe and efficient gene delivery remains a significant challenge for central nervous system (CNS)-targeted gene therapy. Recombinant adeno-associated viruses (rAAVs) developed through directed evolution have shown potential as CNS-targeting vectors. However, these novel capsids may face limitations in human translatability due to genetic differences between humans and model organisms. To address this, we have developed Differential Directed Evolution, a method designed to engineer capsids that can penetrate the blood-brain barrier (BBB) by binding to human receptors. This technique involves screening peptide-insertion AAV libraries in parallel using a humanized knock-in (KI) mouse model and wild-type (WT) mice, allowing us to identify human transferrin receptor 1 (TfR1)-dependent CNS capsids through bioinformatic analysis. Utilizing this approach, we successfully identified our first generation (Gen1) of hTfR1-targeted capsids, which demonstrate enhanced CNS targeting in hTfR1 KI mice.

To identify additional hTfR1-targeting capsids, we designed an optimization library from two separate initial libraries. The resultant optimization library was then screened in WT and hTfR1-KI mice. Several promising capsid variants exhibit strong hTfR1-dependent CNS transduction. A subset of these were selected for further validation and characterization. Top second-generation (Gen2) capsids show hTfR1-dependent transduction in a cell-based assay, confirming their interaction with the human TfR1 homolog. When evaluated in hTfR1 KI and WT mice following intravenous injection, Gen2 capsids exhibit hTfR1-dependent widespread CNS transduction in KI mice.

In summary, we identified additional hTfR1-targeting capsids through optimized Differential Directed Evolution (Gen2 capsids) with enhanced CNS transduction. These capsids may provide new options as gene delivery vehicles for neurological disorders.

Translational Applications of Base and Prime Editors

95 *In Vivo* Delivery of HBG1/2 Promoter Editing Cargo to HSC of Humanized Mouse and Non-Human Primate with Lipid Nanoparticle

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Introduction
Induction of fetal hemoglobin (HbF) by editing DNA regulatory elements to reactivate γ-globin gene (HBG1/2) expression is a clinically validated therapeutic strategy for sickle cell disease (SCD) and β-thalassemia. Ex vivo–based autologous genome editing of the HBG1/2 promoter of hematopoietic stem and progenitor cells (HSPCs) for hematopoietic stem cell transplantation (HSCT) has shown promising results in clinical trials of renizgamglogene autogedtemcel (reni-cel). Patients receiving reni-cel in these trials were observed to have rapid and sustained increases in HbF, early correction of anemia, and durable normalization of total hemoglobin. The patient journey for ex vivo genome-edited HSCT, requiring both HSPC mobilization and collection and myeloablative conditioning, is burdensome and limits patient access to the therapy. Thus, an in vivo–based genome editing approach using the clinically validated HBG1/2 promoter–targeted gene editing strategy offers potential to significantly decrease patient burden and provides an opportunity for global access.

Methods
We developed targeted lipid nanoparticles (tLNPs) that deliver green fluorescent protein (GFP) mRNA and editing cargos in vivo to HSPCs and/or hematopoietic stem cells (HSCs) in bone marrow (BM) of NBSGW mice engrafted with human CD34+ HSPCs (hNBSGW mice). The potency of the editing machinery was improved with a multi-pronged approach, including targeting moiety and lipid composition optimization. We tested a leading tLNP carrying GFP mRNA and HBG1/2 editing cargo in an hNBSGW mouse model and non-human primates (NHPs; Cynomolgus macaques).

Results
Switching targeting moiety on the base LNP formulation increased GFP expression and HBG1/2 editing in HSPCs in BM of hNBSGW mice after a single intravenous (IV) administration. This targeting moiety enabled higher editing of HSCs (Lin-CD34+CD38-CD45RA-CD90+). With B2M gRNA editing cargo, B2M knockout was achieved in 16% of HSPCs and 72% of HSCs based on flow cytometry analysis of human leukocyte antigen 1 week after dosing. Systemic delivery of this tLNP containing GFP mRNA resulted in GFP expression in ~90% of HSPCs (Lin-CD34+CD38-CD45RA-CD90+) in hNBSGW mice. Further optimization of the tLNP composition described

above led to B2M knockout in 42% of HSPCs and 84% of HSCs in hNBSGW mice. Editing levels for the HBG1/2 promoters with this optimized tLNP were 29% in HSPCs after 1 week and ~40% in HSCs after 4 weeks. Corresponding HbF induction was observed upon editing the HBG1/2 promoters; HbF-positive red blood cells in BM increased from 2% in controls to ~23% (13.1%–41.2%) in the treatment group at 4 weeks. Using the tLNP, delivery and editing were evaluated in NHPs after a single IV injection. tLNP containing GFP mRNA showed ~75% of the HSCs (CD34+CD90+CD45RA-) were GFP+. A single IV injection of this tLNP carrying surrogate HBG1/2 gRNA editing cargo resulted in editing levels of up to 26% in HSCs 1 week after tLNP treatment. Evaluation of the more optimized tLNP composition in NHPs is ongoing.

Conclusion
Our data from humanized mouse and NHP models represent a significant advancement in developing in vivo gene editing therapies for treating β-hemoglobinopathies. As ~30% donor chimerism can significantly ameliorate disease symptoms in patients with SCD, the editing level and percentage of HbF-positive cells achieved with in vivo editing of HBG1/2 promoters may be therapeutically relevant and supports further

96 In Vivo Prime Editing Rescues Alternating Hemiplegia of Childhood in Mice

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Alternating hemiplegia of childhood (AHC) is a neurodevelopmental disorder that presents within the first 18 months of life with recurrent paroxysmal attacks that include hemiplegia (paralysis affecting one side of the body), dystonia (painful involuntary muscle contractions), abnormal eye movements, and seizures. Patients with AHC also exhibit non-paroxysmal hypotonia (low muscle tone), developmental delay, and intellectual disability. AHC is exceptionally rare, with an estimated incidence of 1 in 1,000,000, and no disease-modifying treatments have been reported. Approximately 75% of AHC cases are associated with pathogenic variants in *ATP1A3*, which encodes the catalytic α_3 subunit of the neuronal Na^+/K^+ ATPase protein complex.

Here, we report prime editing (PE) and base editing (BE) correction of AHC mutations in human cells and in two mouse models of AHC. In engineered HEK293T cells and iPSCs derived from patients with AHC, we developed PE and BE strategies that efficiently correct (40-90% in treated cells) five *ATP1A3* mutations that together cause more than 65% of *ATP1A3*-linked AHC cases (Figure 1). To investigate how gene editing can mitigate the pathology of AHC *in vivo*, we engineered PE strategies to efficiently correct the mouse *Atp1a3* D801N and E815K mutations—orthologous to the two most prevalent human pathogenic variants—in two AHC mouse models. Dual AAV-mediated delivery to the mouse CNS as a P0 intracerebroventricular injection efficiently corrects the *Atp1a3* D801N and E815K mutations (up to 48% efficiency in cortex), significantly rescues a survival defect in D801N mice (Figure 2), and rescues clinically-relevant behavioral phenotypes, including stress-induced paroxysmal episodes, in D801N and E815K mice. Collectively, these findings establish efficient and precise genome editing strategies that correct the most prevalent AHC mutations and the first durable multi-phenotype rescue of AHC in animals. Our work provides a proof-of-concept for in vivo prime editing in the CNS of an animal model to rescue a neurological disorder, and suggests the feasibility of a one-time gene editing treatment that can mitigate the pathology of AHC through direct, efficient correction of AHC-causing mutations.

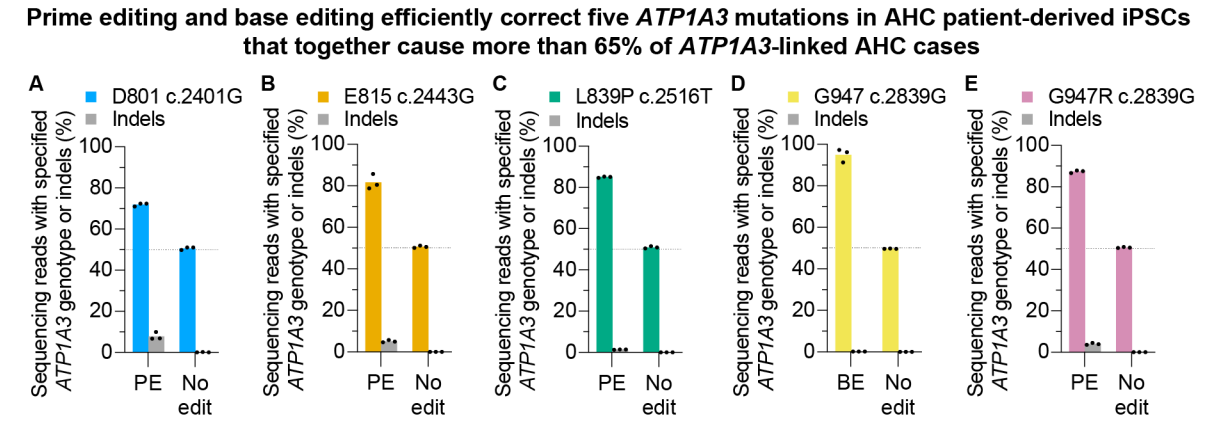

Figure 1. Monoclonal iPSCs derived from AHC patients with heterozygous *ATP1A3* mutations were used to evaluate correction with prime editing (PE) and base editing (BE). X-axis labels identify conditions treated with PE, BE, or control conditions in which cells were not edited ("No edit"). Because iPSCs derived from AHC patients are heterozygous for pathogenic *ATP1A3* mutations, all samples start from approximately 50% wild type genotype. Data represent the mean of n = 3 independent biological replicates and dots show individual replicate values. **(A)** PE correction of D801N c.2401A to D801 c.2401G. **(B)** PE correction of E815K c.2443A to E815 c.2443G. **(C)** PE correction of L839P c.2516C to L839 c.2516T. **(D)** BE correction of G947R c.2839A to G947 c.2839G. **(E)** PE correction of G947R c.2839C to G947 c.2839G.

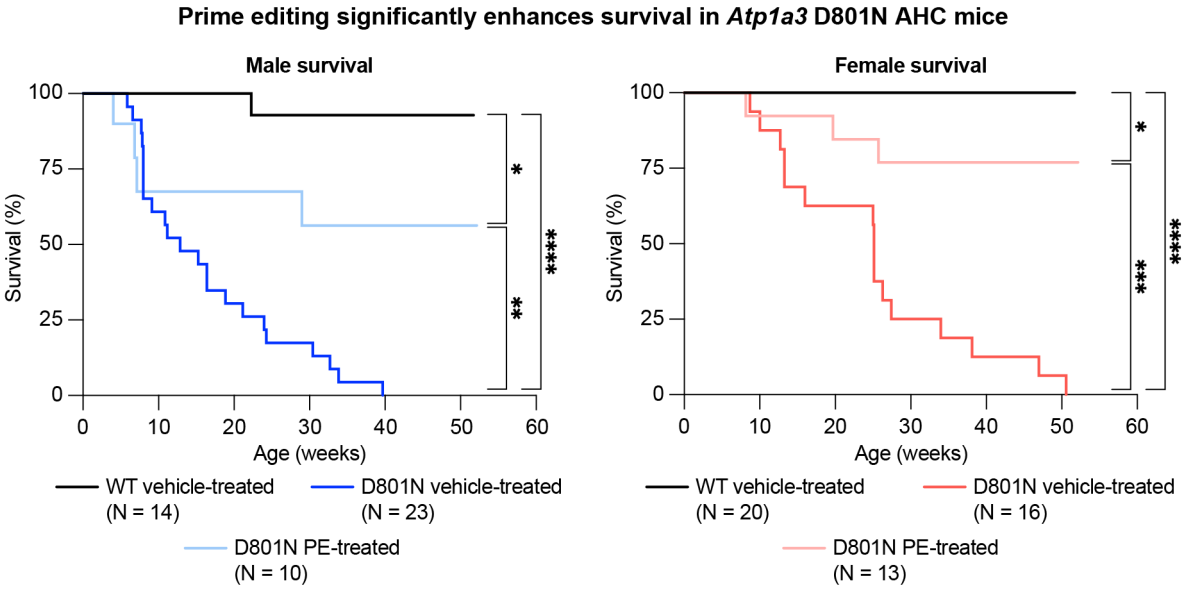

Figure 2. (A) Kaplan-Meier survival curve for male wild type (WT) and *Atp1a3* D801N (D801N) mice treated with phosphate-buffered saline (vehicle) or AAV-mediated prime editing (PE-treated). **(B)** Kaplan-Meier survival curve for female WT and D801N mice treated with vehicle or AAV-mediated prime editing (PE-treated). For (A and B), the size of each cohort is displayed in the plot's legend and two-way ANOVA followed by Tukey's multiple comparisons test was used to determine P-values between groups. ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

97 Long-term Brain Editing and Disease Mitigation After Intracerebroventricular Administration of Base Editor in a Murine Model of Mucopolysaccharidoses Type I

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Introduction:
Mucopolysaccharidosis Type 1 (MPS-1H, Hurler Syndrome) is a progressive, multisystem disorder caused by the alpha-L-iduronidase (IDUA) G◊A (W402X) point mutation, resulting in IDUA deficiency and lysosomal accumulation of glycosaminoglycans (GAGs). Previous studies have demonstrated efficient liver and heart editing in a murine model of MPS-1H (Idua-W392X) following adeno-associated virus serotype 9 (AAV9) mediated delivery of an adenine base editor (ABE) *in utero* via intravenous (IV) injections, but brain editing was minimal. This study aims to evaluate *in vivo* AAV9/ABE delivery via intracerebroventricular (ICV) injection for treatment of MPS-1H.

Methods:
Idua-W392X mice were injected at postnatal day 0 (P0) with split-intein AAV9 vectors containing ABE 8.8 and *Idua*-W392X-gRNA via IV, ICV, or dual IV/ICV injections. Age-matched, uninjected Idua-W392X mice and age-matched healthy mice (B6) served as controls. Terminal analysis was performed at 6 months, at which time mice underwent neurobehavioral testing. Mice were then euthanized and organs were harvested for DNA sequencing, IDUA enzyme activity and GAG analysis.

Results:
All injected mice survived until terminal analysis at 6 months (n=31). Survival rate of uninjected W392 mice was 80%. Liver editing was on average 39.9% in the IV group (n=11; $p<0.0001$), 25.7% in the ICV group (n=10; $p<0.0001$), and 42.5% in the IV/ICV group (n=10; $p<0.0001$) greater than the W392X control (n=7). In the heart, average editing was 9.0% in the IV group ($p=0.0006$), 6.6% in the ICV group ($p<0.03$), and 9.9% in the IV/ICV group ($p=0.0002$) greater than the W392X control. Average editing in the left cortex was 14.2% in the ICV group and 13.9% in the IV/ICV group compared to 0.4% in the IV group ($p<0.001$). The degree of editing correlated with increased tissue IDUA enzyme activity; higher IDUA activity was demonstrated in the livers and hearts of mice in the IV and IV/ICV groups compared to ICV injected mice and W392X controls. Correspondingly, IDUA enzyme activity was highest in the brain in ICV and dual IV/ICV injected groups compared to the IV group and W392X control. GAG analysis revealed a similar trend. GAGs were significantly lower in the IV and IV/ICV groups in the liver and heart compared to ICV injected mice and W392X controls, while the brain had significantly lower GAGs in the ICV and IV/ICV groups compared to the IV and W392X control groups. IDUA activity and GAG levels were improved in the liver and heart in the ICV group. Rotarod testing for sensorimotor coordination and motor learning demonstrated significant improvement in ICV and dual IV/ICV groups compared to the IV and W392X control groups.

Conclusions:
ICV-mediated injections result in greater brain editing efficiency compared to IV injections. Both IV and ICV injections lead to liver and heart editing. Higher editing correlates with improved survival, increased IDUA activity, decreased GAG accumulation, and neurobehavioral improvements. Our findings demonstrate the potential for ICV-mediated base editing as a therapeutic model for MPS-1H and other neurologic disorders.

98 A base editor for the long-term restoration of auditory function in mice with recessive profound deafness

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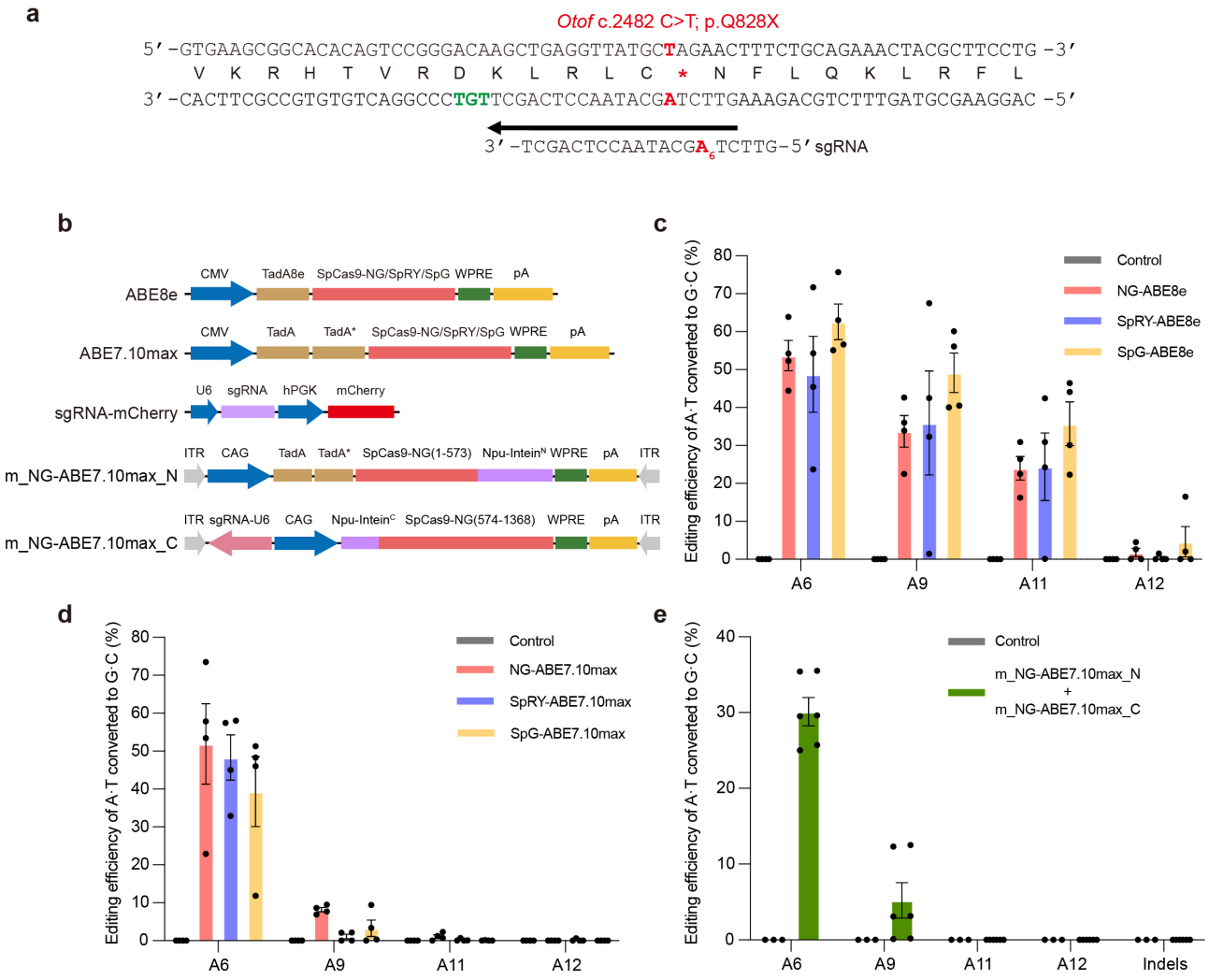
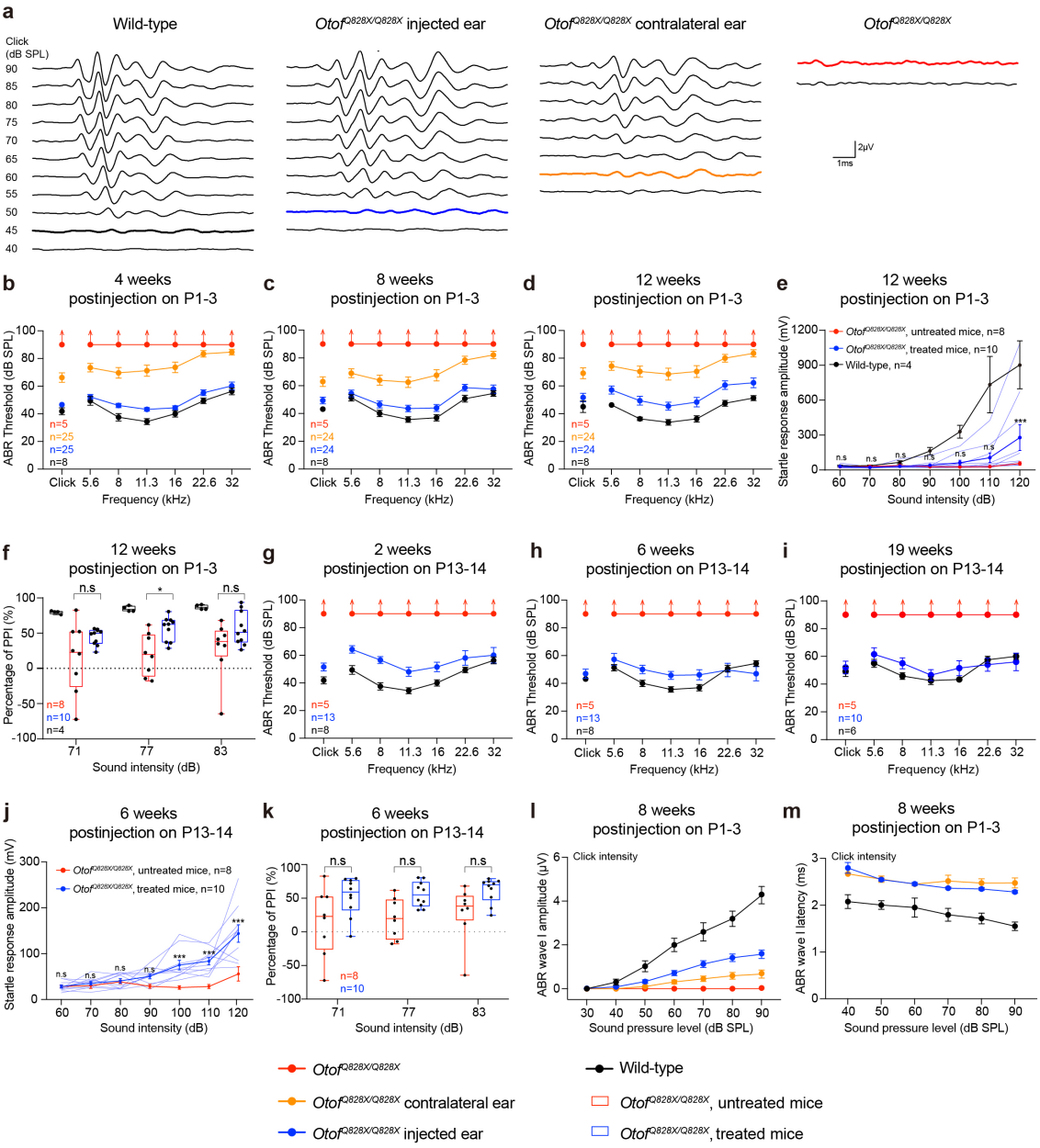
A prevalent recessive mutation (c.2485C>T, p.Q829X) within the OTOF gene leads to profound prelingual hearing loss. Here we show that in Otof mice harbouring a mutation (c.2482C>T, p.Q828X) homozygous to human OTOF that faithfully mimics the hearing-loss phenotype, a base editor (consisting of the deaminase ABE7.10max and the Cas9 variant SpCas9-NG) packaged in adeno-associated viruses and injected into the inner ear of the mice via the round-window membrane effectively corrected the pathogenic mutation, with no apparent off-target effects. The

treatment restored the levels of the otoferlin protein in 88% of the inner hair cells and stably rescued the auditory function of the mice to near-wild-type levels for over 1.5 years while improving synaptic exocytosis in the inner hair cells. We also show that an adenine base editor that targets the prevalent human OTOF mutation restored hearing in humanized mice to levels comparable to those of the wild-type counterparts. Base editors may be effective for the treatment of hereditary deafness.





99 Rescue of Zellweger Spectrum Disorder in Mice and in Patient Cells by Base Editing

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Zellweger spectrum disorder (ZSD) is a disease continuum caused by biallelic loss-of-function variants in *PEX* genes required for peroxisome biogenesis and critical for cellular metabolism and signaling. The *PEX1*-p.G843D (c.2528G>A) allele, present in approximately 30% of ZSD patients, frequently results in chronic liver disease that can progress to cirrhosis and hepatocellular carcinoma and also impact neurological health. Here, we report the development and application of an adenine base editing strategy to correct metabolic abnormalities in an established homozygous *Pex1*-p.G844D ZSD mouse model that manifests liver pathologies and metabolic dysfunction found in patients. Through intravenous delivery of AAV9 encoding ABE8e-V106W into both neonatal and four-week-old mice, we achieved up to 60% allele correction in the bulk liver, consistent with the correction of most hepatocytes. By restoring peroxisome function, base editing eliminated the accumulation of very long-chain fatty acids and potentially neurotoxic C27-bile acid intermediates. Elevated levels of phytanic acid, a neurotoxic branched-chain fatty acid substrate of peroxisomal catabolism, were normalized in blood, liver, and brain tissue. Treated mice showed rescue of liver pathologies, normalized liver transcriptomes, and normalized body weights in a dose- and time-dependent manner. Non-viral lipid nanoparticle (LNP) delivery of ABE8e-V106W mRNA to four-week-old mice also yielded correction of the *Pex1*-p.G844D allele correction in 27% of bulk liver cells. In patient-derived fibroblasts, base editing corrected >80% of *PEX1*-p.G843D alleles and restored peroxisome biogenesis. Genome-wide experimental and computational off-target analysis found minimal off-target editing in either mouse or human genomes. Collectively, these findings suggest that liver base editing over a range of ages may benefit a significant proportion of ZSD patients, and provide a foundation for developing precision gene correction treatments that address the root cause of a wide range of peroxisomal disorders.

100 A Type I CRISPR adenine base editor with a guide-length-tunable editing window and its application in correcting CFTR-G542X null mutation

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Base editors typically use a DNA deaminase fused to a Cas9 nickase to install single nucleotide conversions without causing double-stranded genomic breaks. They are emerging as a promising platform in human gene therapy, due to their high efficiency in correcting single nucleotide variants and better safety profile. However, the targeting scope of a base editor is confined by an editing window that covers a specific range of nucleotides within the R-loop, and measured from a nearby suitable 2-3-nt PAM.

To overcome this editing window rigidity, we developed a sliding-window type I CRISPR adenine base editor (T1ABE) through extensive protein and crRNA engineering. We first fused a TadA-8e deaminase to the Cas11 subunit of *N. lactamica* (Nla) I-C Cascade and engineered the NlaCas3 nuclease into a ssDNA nickase. The resulting first-generation T1ABE v1 introduces up to 45% A•T-to-G•C edits across multiple endogenous genomic sites and in various human cell lines. T1ABE displayed an unconventional 8-nt activity window on the PAM-distal, target-strand DNA that lies outside the R-loop region.

Exploiting the “Cascade remodeling” property inherent to type I CRISPR, we achieved editing window tunability over a 40-nt range (relative to a fixed PAM) through spacer/guide length extensions. Moreover, through structure-guided engineering and TadA8e-Cas linker optimization, we arrived at a hyper-activity variant T1ABE v3, which can access hard-to-edit sites in the human genome. The prominent features of T1ABE v3, including high-efficiency, a guide-length-tunable window, and T/C-rich PAM preference, should facilitate targeting to a wide range of most genomic sites. Indeed, our computational analysis of the ClinVar database revealed that approximately 95% of the all pathogenic and likely pathogenic G•C-to-A•T mutations in ClinVar would in theory be targetable by T1ABE.

To demonstrate the therapeutic potential of T1ABE's tunable window, we pursued a preclinical application to correct the cystic fibrosis *CFTR(G542X)* null mutation, which lacks small molecule drugs or a current base edit strategy. In an induced pluripotent stem cell (iPSC) model, all-RNA delivery of T1ABE v3 successfully corrected the pathogenic adenine into guanine with a ~32% editing efficiency. The T1ABE-installed edit (resulting in a G542R variant) can restore robust CFTR channel function in our CFTR-dependent organoid swelling assay.

Lastly, we showed that T1ABE exhibited minimal off-target effects at the transcriptome level, and genome level. Our results highlight the potential of type I CRISPR-Cas system to support precise gene correction applications. These findings advance Type I CRISPR as a platform for precision base editing applications.

101 AI-Assisted Development of Compact Base Editors for Enhanced Efficiency and Specificity with Broad Targeting Range

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Background:
Base editors provide a revolutionary approach to correcting genetic mutations without causing double-strand breaks (DSBs), minimizing possible adverse effects associated with traditional genome editing tools. While SpCas9-based base editors achieved excellent efficiency and fidelity, their large size hinders their use in the in vivo single AAV delivery systems. Conversely, previous small IscB-delivered base editors enable single AAV delivery but suffer from limitations such as low efficiency, strict TAM requirements, and increased off-target risks due to short spacer lengths (14–16 bp) (Fig. 1C). To address these challenges, we applied artificial intelligence (AI) to develop eMM101ABE8e, a compact and efficient adenine base editor optimized for single AAV delivery.

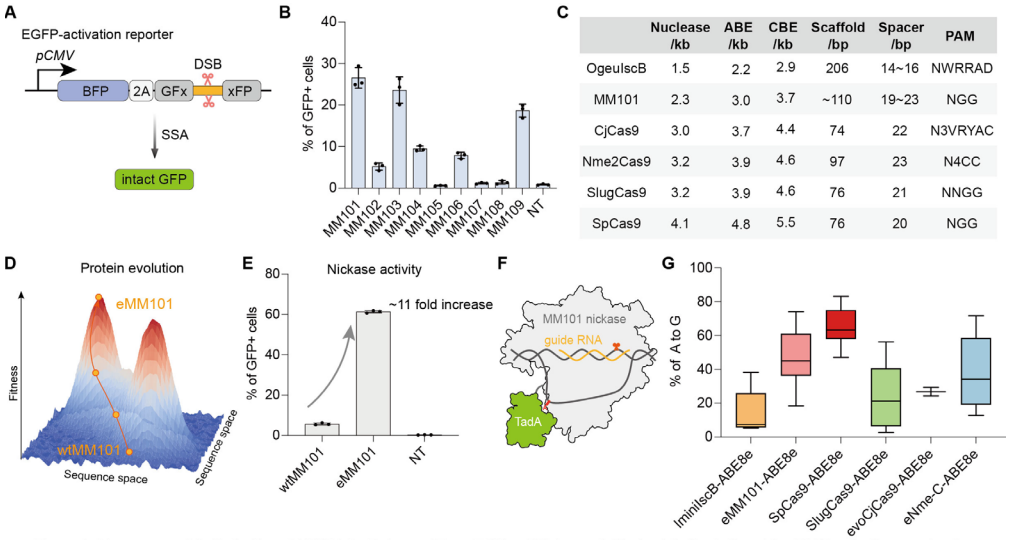
Methods:
We utilized a computational AI pipeline to analyze bacterial genomes and identified a group of novel Cas proteins, designated MM101 to MM109 (600-800 amino acids). Functional screening using an EGFP-reporter system (Fig. 1A) identified MM101 as a strong candidate. AI-assisted protein engineering enhanced MM101, resulting in eMM101, with 11-fold higher activity (Fig. 1D and E). Based on eMM101, we developed eMM101ABE8e, an adenine base editor (Fig. 1F), and compared its editing efficiency with other base editors derived from IscB, SlugCas9, CjCas9, Nme2Cas9, and SpCas9 across multiple therapeutic targets in HEK293T cells. Additionally, we assessed the efficiency of eMM101ABE8e in induced pluripotent stem cells (iPSCs) through mRNA electroporation. We also evaluated its in vivo performance in a humanized Duchenne muscular dystrophy (DMD) mouse model. Editing efficiency, dystrophin recovery, and therapeutic potential were evaluated using qPCR, immunofluorescence staining, and Western blot analysis. This DMD model was created by deleting mouse exon 51 and replacing mouse exon 50 with its human counterpart (Fig. 2B and C).

Results:
Our study identified MM101 as a promising candidate due to its compact size (2.3 kb), suitable spacer length (19–23

bp), and broad NGG PAM targeting range (Fig. 1B and C). Through protein engineering, we developed eMM101, which exhibited approximately 11-fold higher activity than wild-type MM101 (Fig. 1E). The eMM101ABE8e base editor demonstrated significantly higher editing efficiency compared to IscB-, SlugCas9-, CjCas9-, and Nme2Cas9-based base editors, with performance close to SpCas9ABE8e (Fig. 1G). In iPSCs, eMM101ABE8e achieved comparable editing efficiency to SpCas9ABE8e across therapeutic target loci such as PDCD1 and CD52 (Fig. 2A). In the DMD mouse model, eMM101ABE8e achieved efficient single AAV delivery, modified the splice donor site, and restored dystrophin protein expression to functional levels (Fig. 2D–G).

Conclusions:

The AI-engineered eMM101ABE8e is a compact and efficient base editor with a broad target range and high specificity. Its compatibility with single AAV delivery and successful application in iPSCs and in vivo DMD model underscore its transformative potential for both research and therapeutic applications, paving the way for next-generation precision medicine.



	Nuclease /kb	ABE /kb	CBE /kb	Scaffold /bp	Spacer /bp	PAM
OgeuIscB	1.5	2.2	2.9	206	14~16	NWRRAD
MM101	2.3	3.0	3.7	~110	19~23	NGG
CjCas9	3.0	3.7	4.4	74	22	N3VRYAC
Nme2Cas9	3.2	3.9	4.6	97	23	N4CC
SlugCas9	3.2	3.9	4.6	76	21	NNGG
SpCas9	4.1	4.8	5.5	76	20	NGG

Figure 1. Discovery and Optimization of MM101 for Enhanced Base Editing Efficiency. A, Mechanistic illustration of the EGFP-activation reporter. **B,** Evaluation of the editing efficiency of various MM proteins using an EGFP-activation reporter system. **C,** Comparative analysis of MM101, IscB, SpCas9, and other small Cas9 orthologs. **D,** Schematic representation of the protein engineering for MM101. **E,** Assessment of nickase activity for eMM101 and wild-type MM101 using the EGFP-activation reporter system. **F,** Diagram illustrating the design of eMM101ABE8e. **G,** Average editing efficiency of ABE8e derived from IscB, eMM101, SpCas9, SlugCas9, evoCjCas9, and eNme-C in HEK293T cells.

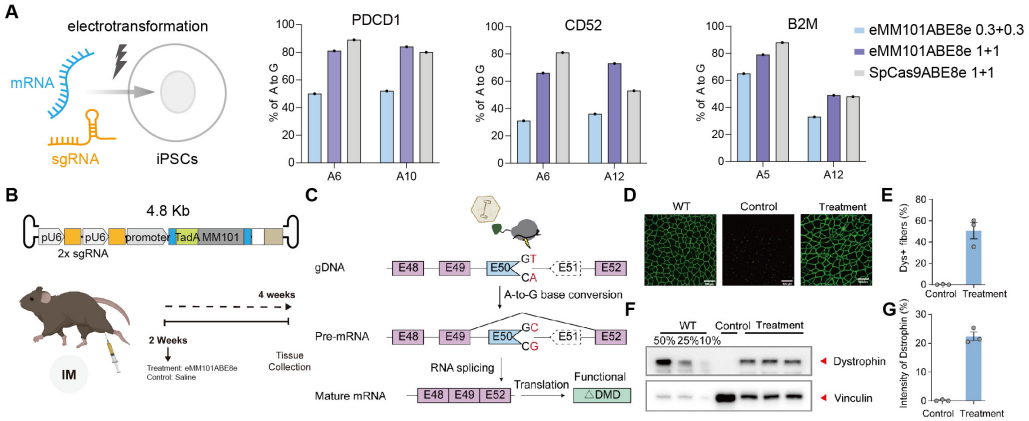


Figure 2. Therapeutic Potential of eMM101ABE8e in iPSCs and Mouse Models. A, Editing efficiency of splice-site adenine bases at PDCD1, CD52, and B2M in iPSCs transfected with eMM101ABE8e or SpCas9ABE8e mRNA along with their respective sgRNAs at varying doses (1 μg/2 × 10^5 cells, n = 1). **B and C,** Schematic representation of the intramuscular injection of a single AAV delivery system in a humanized DMD mouse model. **D,** Immunofluorescence staining for dystrophin in treated DMD mouse muscle tissue. **E,** Quantification of dystrophin-positive fibers in muscle tissue treated with eMM101ABE8e. Data are presented as mean ± standard deviation (n = 3). **F,** Western blot analysis showing dystrophin recovery in DMD mouse muscle after treatment with eMM101ABE8e. Vinculin (VCL) protein levels were used as an internal control. **G,** Percentage of dystrophin recovery quantified from western blot results. Data are presented as mean ± standard deviation (n = 3).

Liver Targeted Genetic Therapies

102 LNP-formulated Prime Editor enables *in vivo* therapeutic precise editing of the *ATP7B* p.H1069Q and p.R778L mutations causing Wilson disease

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Wilson disease (WD) is a monogenic autosomal recessive disease caused by loss-of-function mutations in the *ATP7B* gene, encoding a copper-transporting ATPase critical for copper homeostasis. Current therapies are supportive, life-long with side-effects and require intensive management. Clinical trials of AAV-mini-*ATP7B* are underway, and the safety, efficacy and durability are still under evaluation. Prime Editing offers the opportunity to correct *ATP7B* mutations back to their wildtype sequence thereby retaining normal gene regulation and full protein function. The *ATP7B* p.H1069Q and p.R778L mutations are highly prevalent and found in more than 40% of patients in the US & EU. Correcting these mutations by Prime Editing offers a potentially curative and one-time therapeutic strategy to address a significant unmet medical need.

We established an iterative high-throughput screening platform to rapidly and robustly optimize all components of Prime Editing *in vitro*, including pegRNA, ngRNA, mRNA and Prime Editor (PE) protein resulting in the discovery of several lead candidates for validation *in vivo*. To model WD *in vivo*, we utilized humanized-*ATP7B* mouse models that carry either p.H1069Q or p.R778L mutation and recapitulate WD patient phenotypes. Following further optimizations to all components of our liver-targeted LNP and RNA cargo, we demonstrated dose-dependent editing in p.H1069Q fully humanized mouse models, achieving >70% precise editing of hepatocytes leading to >75% reduction of copper in the liver. Gene correction and phenotypic rescue were durable 12 weeks after treatment. Further studies are underway to characterize copper homeostasis. Using Prime's suite of biochemical and targeted sequencing assays, no off-target editing was detected in human hepatocytes and WD patient-derived cells, underscoring the precision and accuracy of Prime Editing.

To achieve safe and precise gene correction to the liver, we developed a universal liver-targeted LNP that contains N-acetylgalactosamine or GalNAc conjugated to a PEG-lipid, positioning the GalNAc moiety on the surface of the LNPs. GalNAc facilitates selective uptake by the hepatocyte-abundant ASGPR receptor, enabling precise liver targeting via IV injection. To assess the impact of the universal LNP distribution to liver and testes, rats were administered LNP formulated +/- GalNAc lipid. Tissues were collected at multiple time points and mRNA cargo was assessed as a measure of biodistribution. Based on AUC, the relative liver-to-testes distribution was improved from 36X to 495X with LNPs containing the GalNAc lipid. Notably, mRNA distribution to testes was reduced 5 to 8-fold while mRNA levels in the liver were increased by 60% with addition of the GalNAc lipid.

To demonstrate the safety and effectiveness of our universal LNP platform in large animals, we developed surrogate PEs that target either the *ATP7B* p.H1069 or the *SLC37A4* gene. These surrogate PEs encapsulated as RNA cargo in LNPs, when delivered by IV injection, result in >50-80% precise editing in cynomolgus monkey hepatocytes. Additional NHP studies using these Prime Editors will be presented.

Based on these data, we have initiated IND enabling studies to develop the drug candidate PM577 for the treatment of Wilson disease. Thus, Prime Editing offers the potential of a one-time safe and effective curative approach for Wilson disease patients.

103 Engineering an Endoplasmic Reticulum (ER) Stress Responsive RNA Structural Switch for mRNA and Gene Therapies

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Protein misfolding and aggregation in the ER leads to ER stress and triggers the unfolded protein response (UPR) pathway, which in turn can activate the innate immune system. Upon activation, 'adaptive UPR' promotes a series of transcriptional and translational events to re-establish ER homeostasis. However, if ER stress persists, an alternate 'terminal UPR' program induces apoptosis. Overexpression of Factor VIII (FVIII), a blood clotting factor, and related derivatives following AAV liver gene delivery for treatment of severe hemophilia A has been linked to ER stress induction in preclinical studies and hepatotoxicity in clinical studies. Similarly, other endogenous or exogenous secreted proteins, such as monoclonal antibodies, can elicit ER stress upon over-expression exceeding critical thresholds, thus decreasing efficacy. Consequently, there is an unmet need to circumvent pro-apoptotic UPR, while maintaining sufficient long-term protein expression in mRNA and gene therapies. One strategy is to take advantage of UPR-linked cell survival promoting mechanisms to preserve an optimal rate of protein production from delivered genes.

Here, we exploit an endogenous feedback loop that involves RNase-mediated rearrangement of X-box binding protein 1 (XBP1) mRNA during adaptive UPR to engineer an ER stress responsive switch. To deploy unconventional XBP1 mRNA splicing in gene delivery, we first incorporated XBP1 mRNA fragments (XBP1F1-5) upstream of reporter genes to validate mRNA splicing and protein expression regulation under chemically induced ER stress. We designed the fusion mRNA in a way that XBP1F splicing introduces stop codons preceding the reporter gene sequence. Then, we replaced reporter genes with therapeutically relevant ER stress causing FVIII and Leronlimab (an anti-CCR5 monoclonal antibody being evaluated for HIV treatment). For both therapeutic proteins, we observed efficient splicing of XBP1F constructs and a significant decrease in ER stress markers in various cell lines when expression was modulated by XBP1F splicing. We also demonstrate a potential application for the XBP1F switches in mRNA therapy by evaluating XBP1F splicing efficiency and ER stress marker expression in cells transfected with in vitro transcribed XBP1F1-Leronlimab mRNA. Notably, key structural domains based on XBP1F stem loops were essential for optimal attenuation of ER stress levels. In order to assess this technology in a preclinical proof-of-concept study, we packaged Leronlimab and XBP1F-Leronlimab into AAV8 and administered vectors to mice intravenously. Including XBP1F in the cassette design reduced interindividual variability of therapeutic mRNA expression and ER stress marker levels in the liver.

To conclude, by leveraging an unconventional splicing mechanism during UPR, we developed an RNA-based gene switch to control expression from delivered nucleic acids (mRNA or DNA) encoding ER stress causing proteins. Further testing and refinement of XBP1F modified therapeutic constructs in animal models with the goal of developing mRNA and gene therapies with decreased immunotoxicity and improved safety profiles is underway.

104 A Hybrid AAV PiggyBac Transposon and mRNA-LNP Transposase System for Arginase Deficiency: Long-Term Survival and Arginine Control, with a 50-fold Dose Reduction Compared to AAV Alone

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Introduction:
Arginase Deficiency is a rare metabolic disorder of the urea cycle that results in neurodevelopmental abnormalities

including intellectual disability, progressive neurological decline, and a characteristic gait termed spastic diplegia. While patients have at times elevated ammonia, they characteristically have persistent hyperargininemia and the marked presence of guanidino compounds (e.g., GAA). The main therapy today is dietary which, at best, is only partially effective. Traditional AAV-based gene therapy in a mouse model prevents the disorder with moderate to high doses of a liver-specific AAV vector.

Methods:
We sought to develop an approach that allowed for substantial dose reduction with normalized biochemical characteristics in afflicted mice and potential for durable genomic integration in young mice during liver growth. We applied a hybrid approach in which an AAV9 transposon expressing an ARG1 cDNA is co-administered with a super piggyBac (SPB) transposase as an mRNA-lipid nanoparticle (LNP) to provide rapid, transient SPB transposase expression for genomic integration of the DNA cargo. Adult (8-week-old) conditional arginase 1 knockout mice were divided into 5 groups and followed for 9 months. To initiate arginase deficiency, all mice received Cre Recombinase-mRNA LNP at 1 mg/kg. Negative control mice received vehicle and positive control mice received traditional AAV8 expressing ARG1 under a liver-specific promoter at a dose of 5e13 gc/kg. Transposon/transposase experimental mice were divided into two groups: one receiving active SPB transposase and the other receiving catalytically dead (CD, i.e., inactive) transposase LNP RNA. Along with active or CD-SPB, mice received varying doses of transposon AAV encoding ARG1. All groups included male & female mice (n = 3 each).

Results:
Mice were followed for 9 months with daily weighing (surrogate for health) and monthly blood collection. Vehicle control mice demonstrated substantial weight loss and all perished by day 50 (6 of 6 dead) after administration, while all traditional AAV8.TBG.ARG1 mice survived (6 of 6 alive). While 1 male having received the highest dose of AAV transposon + CD-SPB survived for the length of study, all others perished early (8 of 9 dead) with substantial weight fluctuation. With catalytically active SPB, male mice of all 3 doses survived to the end of study (except one 1e12 dose mouse lost due to anesthesia overdose) (i.e., 8 of 9 alive) as did female mice of all 3 doses (i.e., 9 of 9 alive). While in vehicle control & CD-SPB mice GAA, ammonia, and arginine increased, these were low & stable in active SPB mice. Ammonia challenging (6 mmol/kg ammonium chloride) demonstrated marked improvement of ammonia control with the transposon-based approach compared to classical non-integrating AAV. In a follow-up ongoing study, additional mice were similarly administered an even lower dose of AAV.ARG1-transposon + SPB. Presently at 8 months, 5 of 6 mice are alive with stable weights; analysis of blood ammonia and arginine is underway.

Conclusions:
These studies in aggregate demonstrate promising results of a transposon/AAV carrying ARG1 and mRNA-LNP for hepatic transduction and long-term hepatic arginase expression producing ammonia, arginine, and GAA control at significantly lower AAV doses. Next steps are to examine the approach in a neonatal model with actively growing liver and dividing hepatocytes.

105 Identifying Novel Single Amino Acid Substitutions of Human Factor VIII with Enhanced Cofactor Function for Next Generation Hemophilia A Gene Therapy

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Introduction:
Hemophilia A (HA) is a monogenic bleeding disorder that causes prolonged or spontaneous bleeding due to insufficient activity of clotting factor VIII (FVIII). FVIII is integral in the clotting cascade as a cofactor to factor IX (FIX), binding to both activated FIX and factor X (FX) to increase the rate of FX cleavage. The use of AAV gene therapy to permanently treat HA has been impeded by insufficient levels of FVIII expression or the loss of FVIII

expression over time after treatment. While the underlying mechanism for this lack of durable expression has not been elucidated, we have hypothesized that HA gene therapy outcomes can be improved with the use of a FVIII transgene with enhanced activity, similar to the improvement seen in hemophilia B gene therapy with the use of the hyperactive FIX-R338L transgene. Here, we used domain-restricted evolution-directed gene engineering (DREDGE) technology to design and screen single amino acid substitutions of human FVIII to identify individual substitutions that significantly increase FVIII cofactor activity for use in a next generation gene therapy for HA.

Methods:
We have developed the DREDGE technology for protein engineering by focusing on highly conserved amino acids that are not known to cause any reduced function when mutated within a specific domain of a protein of interest. We selected over 50 amino acid positions in human FVIII protein and designed over 200 variants. Substitution variants were generated on a B domain deleted FVIII-SQ backbone, which were then individually transfected into Huh7 cells. The resulting conditioned media was tested for FVIII activity by one-stage activated partial thromboplastin time (aPTT) assay and for FVIII antigen by ELISA. The ratio of activity to antigen was then used to determine changes in the specific cofactor activity of the resulting FVIII protein.

Results:
Initial screening of the over 50 chosen positions found 3 positions at which the substitutions had a positive effect: K570, R571, and K707. Testing every amino acid substitution at each of these 3 positions via site saturation mutagenesis revealed that changes at positions K570 and R571 increased cofactor activity. Changes at position K707 increased FVIII concentration in the media without an increase in specific cofactor activity. Single amino acid substitutions at either position K570 or R571 enhance the specific cofactor activity of FVIII. At position K570, K570V is the most active substitution with an approximate 3-fold increase in cofactor activity. At position R571, R571S is the most active substitution with an approximate 5-fold increase in cofactor activity. One variant of note, R571A, that was identified in our in vitro screen with a ~3-fold increase in specific activity, has just been reported in a human subject showing ~600% specific activity, validating the effectiveness of DREDGE.

Conclusions:
We have efficiently identified and created hyperactive FVIII variants using DREDGE. The variants generated have increased FVIII activity to antigen ratios, allowing for providing high levels of activity at low expression levels. While work is ongoing to validate these variants in vivo and precisely define their specific activity and mechanisms of action, we believe these variants could provide more durable therapeutic benefit in next-generation HA gene therapy. Further, the DREDGE technique could be a useful tool to identify enhanced transgenes for other indications.

106 Development of a Novel Non-Viral Genetic Therapy for Hemophilia A Utilizing Durable, Redosable, and Titratable Approach of Ultrasound-Mediated Delivery of an Oversized Episomal hFVIII DNA Vector

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Introduction:
Hemophilia A (HemA), the absence or deficiency of Factor VIII (FVIII) protein is associated with episodes of uncontrolled bleeding resulting in marked increases in morbidity and mortality. Current treatment paradigms require serial injections of recombinant FVIII protein or bispecific antibodies. Recently FDA-approved, viral-mediated, gene therapies offer an alternative one-dose treatment option, albeit with uncertain durability. Once treated, patients may experience variable FVIII therapeutic expression levels and the ability to redose is not an option should the FVIII expression level become subtherapeutic. Transcutaneous ultrasound-mediated gene delivery (UMGD)

represents a novel, targeted, therapeutic option permitting serial redosing as required to sustain therapeutic FVIII levels. As designed, UMGD offers a safe, individualized, and durable treatment option for patients with HemA.

Methods:
SonoThera provides unique genetic engineering expertise in the development of next-generation DNA payload designs including optimized DNA constructs encoding an oversized FVIII transgene (FVIII-ST). Previously, transgenic FVIII expression measurement was performed in vitro using human and mouse cells and subsequently, in vivo in mouse plasma to compare the activity of FVIII-ST with the clinically validated FVIII-V3. The next-generation DNA constructs were coupled with SonoThera's UMGD platform technology for targeted and non-invasive delivery to the subject liver in combination with ultrasound contrast agents and externally applied ultrasound.

Results:
Based on in vivo testing of wild-type mice, Hemophilia A disease model mice, and non-human primates (NHP), SonoThera's FVIII-ST vector revealed superior expression resulting in enhanced efficacy and durability of human FVIII as compared to FVIII-V3. With ongoing optimization of UMGD parameters, FVIII-ST expression levels exceeding 175% of normal were achieved in mouse blood, persisting for over six months following a single treatment without significant elevation of safety endpoints. Further, using serial treatments, marked increases in transgenic FVIII were observed, providing potential dosing regimens to maintain clinical efficacy of FVIII levels. Recently, NHPs undergoing serial UMGD therapy demonstrated reproducible, elevations of human FVIII in blood upon ultrasound-mediated delivery of FVIII-ST to the liver.

Conclusion:
Based on in vitro and preclinical safety and efficacy data in wild type, Hemophilia A mice and NHPs, SonoThera offers a non-viral treatment paradigm as a potential genetic therapy for patients with Hemophilia A.

107 Liver Fibrosis Negatively Impacts *in Vivo* Gene Transfer to Hepatocytes

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Liver fibrosis is a pathological process occurring in several genetic and acquired diseases. The fibrotic damage impacts liver architecture and the metabolism of hepatocytes and may negatively affect gene therapy approaches. Adeno-associated viral vectors (AAV) demonstrated clinical efficacy and safety for many inherited diseases. Lentiviral vectors (LV) represent an alternative that allows stable long-term transgene expression in preclinical models of liver disorders. Recently, lipid nanoparticles (LNP) have emerged as promising non-viral vehicles to deliver RNA-based therapeutics or genome-editing agents to the liver. Here, we perform a detailed characterization of the impact of liver fibrosis on *in vivo* gene transfer to hepatocytes by AAV, LV, and LNP in four mouse models. We exploited two chemically induced mouse models that mimic common fibrosis types. Repetitive carbon tetrachloride (CCl_4) administrations for 3 or 6 weeks cause mild or moderate toxic fibrosis in the pericentral area, while diet supplementation with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 3 or 5 weeks leads to moderate or marked biliary fibrosis in the periportal area. We exploited CCl_4- and DDC to induce liver fibrosis in wild-type (WT) and floxed-tdTomato mice. We intravenously administered LV or AAV encoding for reporter genes (human factor IX or mCherry) to WT mice or LNP carrying the CRE-recombinase mRNA to floxed-tdTomato mice. Both pericentral and periportal fibrosis negatively affected LV and LNP-mediated hepatocyte gene transfer. On the contrary, the efficiency of AAV transduction was reduced just in case of periportal fibrosis. Moreover, we employed *Abcb11*$^{-/-}$ and *Agl*$^{-/-}$ mice, which recapitulate features of progressive familial intrahepatic cholestasis type 2 and glycogen storage disease type III, as representative models of genetic disorders characterized by liver fibrosis. We observed mild and minimal periportal fibrosis in the liver of 6-month-old *Abcb11*$^{-/-}$ mice and 9-month-old *Agl*$^{-/-}$ mice, respectively. We administered them with LV or AAV encoding for mCherry and observed slightly reduced

transduction in *Abcb11*$^{-/-}$ mice, while we did not find changes in *Agl*$^{-/-}$ mice. These data suggest that the type and extent of damage determine the impact on gene transfer efficiency. The different gene transfer outcomes may depend not only on the deposition of extracellular matrix but also on blood flow alteration, inflammatory cell infiltrate, or specific cell responses caused by the underlying pathogenic mechanisms. However, we did not detect any alteration in LV clearance and biodistribution in other organs or liver cell types in the presence of CCl_4 or DDC-induced fibrosis. Interestingly, we observed an overall change in the efficiency of gene transfer in different zones of the liver lobule (zonation) in the various models. In the presence of liver damage, LV periportal transduction bias was always reduced, while AAV pericentral transduction bias was modified just in *Abcb11*$^{-/-}$ and *Agl*$^{-/-}$ mice. LNP gene transfer zonation was altered with both CCl_4 and DDC-induced fibrosis. Overall, this work shows that hepatic fibrosis generally reduces hepatocyte gene transfer efficiency but never completely abolishes it. This study has relevant implications for developing liver-directed gene therapies and highlights the need to evaluate delivery vehicles, vector doses, time of administration, and patient selection depending on the underlying liver damage.

108 Steatosis increases human hepatocyte susceptibility to AAV vector transduction

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Background & Aims:
Adeno-associated virus (AAV) vector therapies that target the liver have emerged as transformative approaches to deliver systemic proteins for diseases such as hemophilia A (FVIII deficiency) and are under development for many liver conditions. However, for reasons that remain poorly understood responses vary widely between individuals. Metabolic dysfunction-associated steatotic liver disease (MASLD) affects approximately 30% of the global population including 44-57% of hemophilia patients. Given the widespread MASLD prevalence, understanding the impact of human hepatocyte steatosis on liver-targeting AAV transduction efficiency may help optimizing therapeutic outcomes.

Methods:
For in vivo studies, primary human hepatocyte (PHH) engrafted (huFNRG) mice were fed a hypercaloric Western diet (WD) and/or 10% sucrose water for one month to induce liver steatosis or maintained on control chow. Mice were then injected with AAV3B capsid-derived vectors expressing either mScarlet or codon-optimized (co)F8 from a chicken beta-actin (CBA) promoter, and liver tissues or isolated PHH were analyzed for vector uptake and transgene expression two weeks later. For in vitro studies, PHH isolated from huFNRG livers were cultured, treated with oleic acid (OA) to induce steatosis, transduced with AAV3v4 vectors expressing fluorophores from a CBA promotor or FVIII variants from a transthyretin (TTR) promoter, and fluorescence or FVIII activity in supernatants were quantified over weeks.

Results:
A greater proportion of human hepatocytes in steatotic huFNRG mice expressed mScarlet compared to those on control chow, ranging from 1.7-fold higher for mice on 10% sucrose water to 3-fold higher for those on a WD+10% sucrose diet. Higher transduction of steatotic hepatocytes was consistent across various AAV3B-derived capsids. Steatotic livers also demonstrated increased vector DNA uptake and mRNA expression compared to chow-fed mice, with a 3-fold increase for AAV3v4-CBA-mScarlet and a 5-fold increase for AAV3v4-CBA-coF8 vector DNA. OA-treated PHH cultures showed significantly higher fluorophore expression than controls as early as day 3 post-transduction with self-complementary AAV vectors. Similarly, steatosis led to higher FVIII activity levels in AAV3v4-TTR-coF8 transduced PHH cultures starting from day 9 and persistent through day 18, with this effect observed across several variants, including coF8-X5, which exhibited the highest activity.

Conclusion:
Steatotic primary human hepatocytes are more susceptible to productive AAV vector transduction with AAV3B-derived capsids. These results suggest that MASLD may affect AAV gene therapy transduction and highlight the need for optimizing therapeutic strategies for individuals with hepatic steatosis.

CAR T-Cells for Solid Tumors

109 Armored anti-macrophage CAR T cells remodel the tumor microenvironment and control metastatic ovarian and lung tumor growth.

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Background
Despite advances in CAR T cells (CAR-T), anti-tumoral efficacy remains elusive for solid tumors. This is primarily due to a lack of specific and persistent antigens on tumor cells and the highly immunosuppressive tumor microenvironment (TME) dominated by tumor-associated macrophages (TAM). To address this problem, we sought to develop CAR T cells that would specifically target and kill immunosuppressive TAMs and deliver immunostimulatory cytokines into the cold tumor.

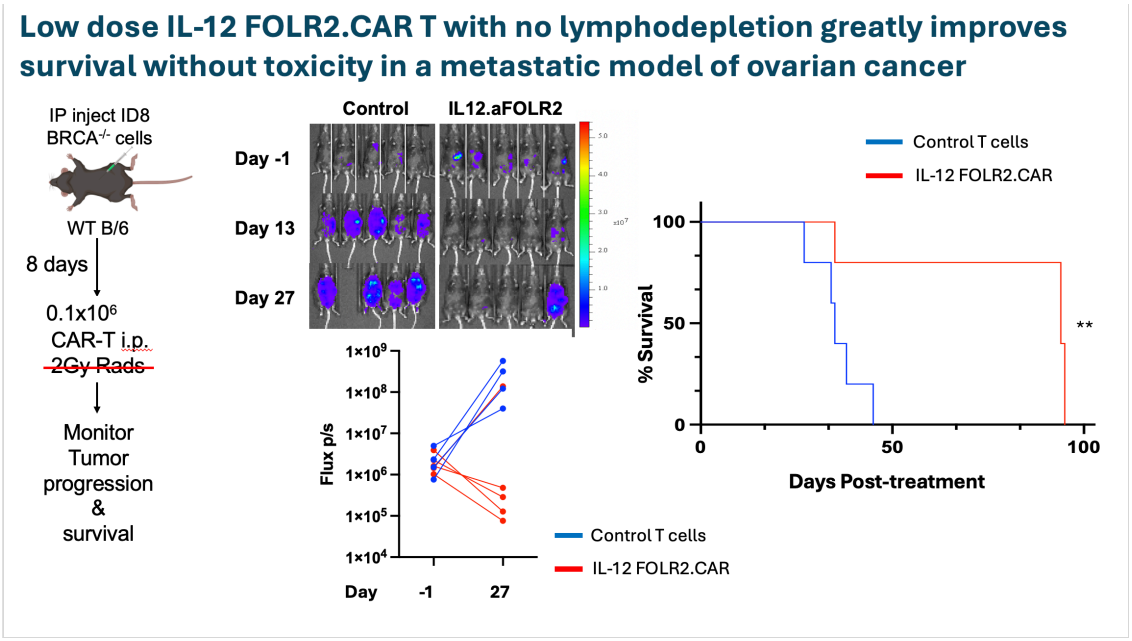
Methods
We generated chimeric antigen receptor (CAR) targeting FOLR2 and TREM2, two cell-surface molecules that are highly and specifically expressed on TAMs found within many human tumors and not expressed by non-macrophages or even most healthy tissue macrophages. We also engineered the vectors to express IL-12, a pro-inflammatory cytokine. IL-12 has been shown to benefit T cell therapies but has also been challenged by high toxicity, necessitating that its delivery is localized to tumors. We tested the IL-12 anti-FOLR2 CAR T cells and IL-12 anti-TREM2 CAR T cells in syngeneic, orthotopic mouse models of metastatic ovarian and lung cancer,

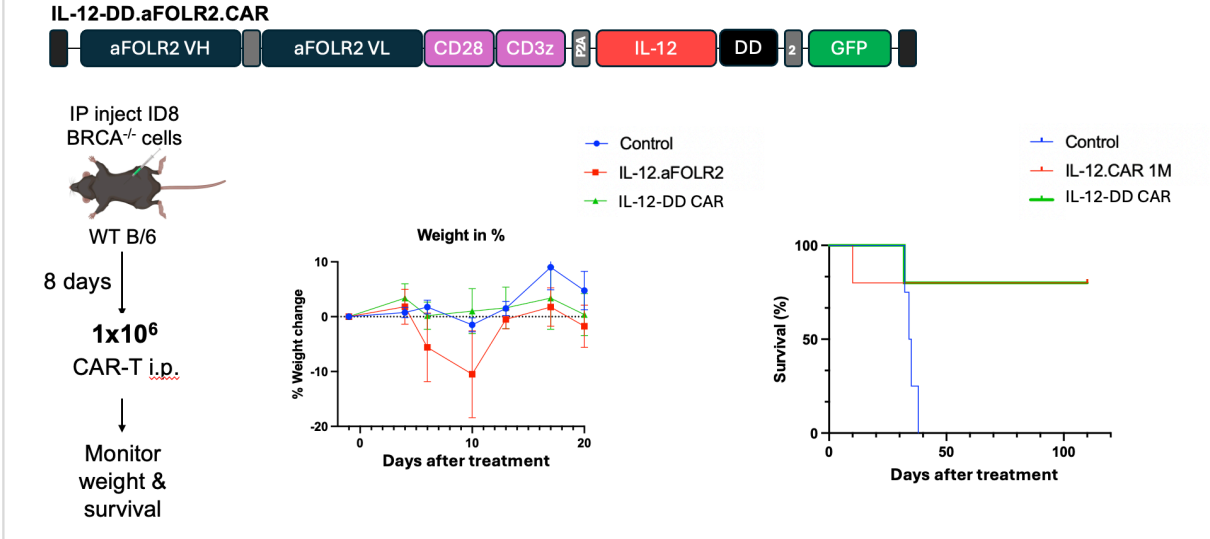
respectively. Mice were treated with control or CAR T cells after tumors had formed in the peritoneal cavity (ovarian) or lungs. We studied how the armored anti-TAM CAR T cells remodeled the TME and impacted animal survival.

Results
In mice with disseminated ovarian tumors, the transfer of control T cells had no impact on tumor growth, and animals died within 35 days of implantation. In striking contrast, when we injected IL-12 armored anti-FOLR2 CAR T cells, ovarian tumors shrunk within 3 weeks, and the mice survived >90 days. Importantly, this was achieved with only 105 CAR T cells, without any pre-conditioning, and little to no toxicity was observed. This result depended on the anti-macrophage CAR allowing the localization of the secreted IL-12, as transfer of IL-12 expressing T cells did not provide survival benefit. Similarly, survival was almost doubled when we treated immunocompetent mice with aggressive lung tumors with 1.5x105 IL-12 anti-TREM2 CAR T cells (no pre-conditioning) compared to the control T cell treated group. Examination of the tumors in both the ovarian and lung cancer models found that expression of IL-12 from the CAR-T led to a significative infiltration of activated endogenous T cells and a shift in macrophage to an immunostimulatory phenotype, indicating TAMs had been reprogrammed and an endogenous T cell response was promoted. Notably, IL-12 induced upregulation of FAS on tumor cells, facilitating FAS-dependent killing. Incorporation of a destabilization domain for IL-12 enabled conditional control of cytokine release while preserving anti-tumor efficacy.

Conclusions
These results demonstrate that targeting tumor-associated macrophages (TAMs) with IL-12 armored CAR T cells

enables significant and lasting anti-tumoral effects in preclinical models of two aggressive cancers. Anti-TAM CAR-mediated local delivery of IL-12 represents a promising approach for solid tumor therapy by resetting and reprogramming the TME, inducing FAS-mediated killing, and activating endogenous immunity with limited toxicity. The incorporation of a controllable IL-12 secretion system enhances safety and clinical translatability. With continued refinement, these therapies hold the potential to overcome the barriers of solid tumor treatment and provide durable outcomes for patients with aggressive cancers.





110 Peptide Drug-Secreting CAR T cells Targeting the VIP/VIPR Pathway Display a Novel Phenotype and Superior Antitumor Efficacy

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Chimeric antigen receptor (CAR) T cell therapy is a highly potent cellular immunotherapy that still faces significant obstacles that limit clinical success. Novel approaches to improve the CAR T cell phenotype, metabolic fitness, and the engagement of endogenous immunity are necessary to create effective products. Herein, we target the vasoactive intestinal peptide (VIP) pathway to enhance CAR T cell therapy efficacy. VIP, a neuropeptide that binds to VIP receptors, plays an immunosuppressive role by dampening adaptive immune responses, including CAR T cells. VIP is produced by activated T cells and by several hematological and solid tumors, as a mechanism to suppress T cell activity. To counteract this, we engineered CAR T cells that secrete a novel synthetic short peptide drug antagonist of VIPR (CAR/VIPRa). This design enables localized delivery of antagonistic peptide drug to enhance CAR T cell function.

CAR/VIPRa T cells exhibit reduced exhaustion, improved viability, and enrichment of stem/ central memory phenotypes after manufacturing. By RNA sequencing, antigen-stimulation induces cellular pathways in CAR/VIPRa T cells that support enhanced mitochondrial function, ribosome biogenesis, and oxidative phosphorylation compared to CAR T cells that are primarily engaged in lymphocyte proliferation and differentiation. CAR/VIPRa T cells are metabolically quiescent yet display plasticity, supporting robust energetic profiles needed to endure frequent antigen stimulation within the tumor microenvironment (TME). Furthermore, CAR/VIPRa T cells showing elevated levels of FOXP1 (associated with T cell quiescence) and KLRB1 (a marker of CD8 memory), among other key genes.

Functionally, CAR/VIPRa are proliferative after multiple rounds of antigen stimulation. In xenograft cancer mouse models, CAR/VIPRa T cells exhibit superior expansion while maintaining a less exhausted memory phenotype and greater tumor infiltration. Additionally, in immunocompetent mouse models, CAR/VIPRa T cells increased endogenous T cell infiltration of endogenous T cells into the tumor. These findings suggest that the secreted antagonistic peptide acts both in an autocrine manner to support CAR T cells and in a paracrine manner to modulate the TME, thereby enhancing overall anti-tumor activity.

Lastly, the translational impact of CAR/VIPRa T cells was confirmed in patient-derived T cells and tumors. The differentiation state of CAR/VIPRa T cells prior to antigen-stimulation is shifted from effector to central memory phenotypes compared to CAR T cells. In aggressive patient-derived tumor mouse models, CAR/VIPRa T cells control tumor burden more effectively than standard CAR T cells.

We designed and engineered a novel antagonistic peptide drug-secreting CAR T cell that targets an emerging immune checkpoint pathway. Targeting the VIP/VIPR pathway is a promising strategy to preserve critical CAR T cell survival phenotypes and augment the intrinsic metabolic plasticity of CAR T cells that drives improved *in vivo* expansion and tumor infiltration to reduce disease burden. Ultimately, CAR/VIPRa T cells serve as micro-pharmacies that can deliver a drug both autocrinally to enhance CAR T cell phenotype and metabolism and in a paracrine manner to boost endogenous T cell infiltration, thereby strengthening anti-tumor responses.

111 Engineered CXCR3-A Expression Promotes Intracerebroventricularly Delivered B7-H3-specific CAR T Cell Trafficking and Efficacy against Diffuse Intrinsic Pontine Glioma in Orthotopic Mouse Models

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Diffuse intrinsic pontine glioma (DIPG) is an aggressive brainstem tumor that is the leading cause of death in children with brain cancer. DIPG is incurable with most patients passing away in a year, so more effective treatments are desperately needed. A promising approach is the use of chimeric antigen receptor (CAR) T cells, which has shown encouraging clinical evidence of feasibility, tolerability, and some activity against DIPG in early phase studies, including Seattle Children's BrainChild-03 trial conducted by our group. However, clinical benefit was not universal, and insufficient CAR T cell trafficking to the tumor is a known obstacle against solid tumors such as DIPG. Therefore, to improve CAR T cell trafficking and therapeutic efficacy, we engineered our clinically tested anti-B7-H3-CAR T cells to overexpress the chemokine receptor CXCR3-A, which detects the concentration gradient of its ligands, such as CXCL10, and directs cell migration.

Our BrainChild clinical trials have shown evidence that some CAR T cells would encounter tumor and produce CXCL10 following antigen-stimulation. Here, we hypothesize that these CXCL10 will attract substantially more CXCR3-A-modified anti-B7-H3-CAR T cells to the DIPG tumor site when compared to the standard CAR T cells. More CXCR3-A-modified CAR T cells engaging the tumor would lead to more CXCL10 production, which can further amplify CAR T cell recruitment through positive feedback, thus improving the trafficking and efficacy of CAR T cell therapy against DIPG.

In this study, we demonstrate that compared to the standard anti-B7-H3-CAR T cells (CAR-only), the CAR T cells engineered to overexpress CXCR3-A (CAR-3A) migrate more efficiently towards the CXCR3 ligands, including CXCL9, CXCL10, and CXCL11, in chemotaxis assays (Figure A). These effects are statistically significant from three biological replicates using CAR T cells of different donors, with p values determined by ratio paired t tests. In vivo, intracerebroventricularly (ICV) delivered CAR-3A T cells show significantly improved efficacy in treating an orthotopic DIPG (SU-DIPG-13) xenograft mouse model (Figure B), based on both tumor bioluminescent imaging and mouse survival, with p value of the latter determined by Mantel-Cox log-rank test. Additionally, immunohistochemistry staining of human CD3, CD4, and CD8 confirms the increased presence of the CAR-3A T cells around the tumor sites 24-hour and 48-hour post ICV injections, compared to the CAR-only and non-transduced (NTD) T cells of the same donor. The enhanced therapeutic efficacy by the CAR-3A T cells via ICV delivery has been further validated in a second DIPG (PBT-27FH) mouse model with CAR T cells of another donor.

Overall, our data support the potential for engineering CXCR3-A expression in CAR T cells as a novel approach to enhancing CAR T cell trafficking and efficacy against DIPG. To our knowledge, we are the first to investigate the trafficking of chemokine receptor-modified CAR T cells via ICV delivery, which is clinically significant for CNS tumors.

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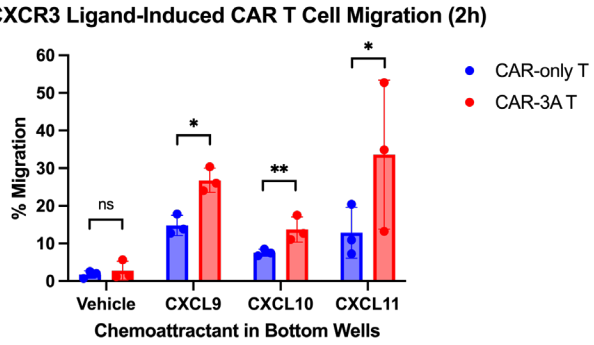
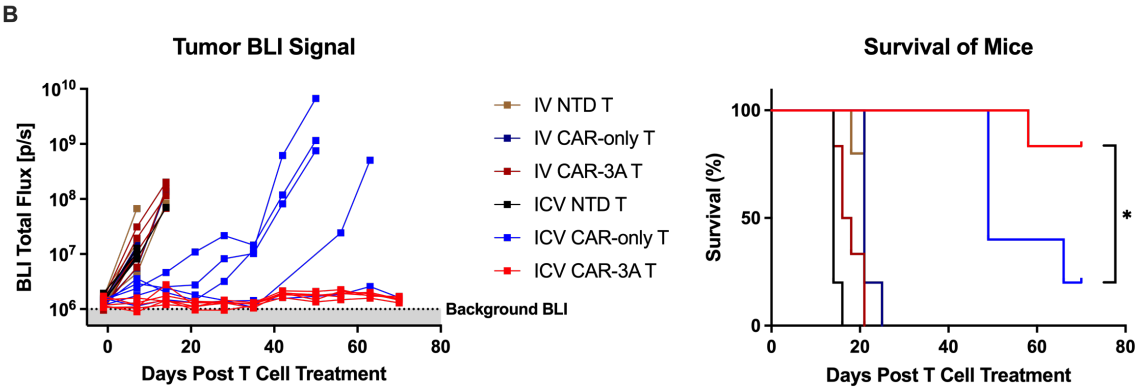


(A) Percentage of CAR T cells that migrated from insert wells to bottom wells with vehicle, 300 ng/mL CXCL9, 100 ng/mL CXCL10, or 300 ng/mL CXCL11 after 2 hours. p values were determined by ratio paired t tests using data from three different T cell donors. Results are presented as means ± SD. ns, not significant; *p < 0.05; **p < 0.01.



(B) Luciferase-labeled SU-DIPG-13 tumor cells were intracranially implanted into NSG mice on day -7. Bioluminescent imaging (BLI) was performed on day -1 to quantify the signals of the engrafted SU-DIPG-13 tumor cells. On day 0, the mice were treated with 2 x 10^6 unmodified or CXCR3-A-modifed CAR T cells or the equivalent number of NTD T cells via either IV or ICV injection routes. On day 1 and day 2, three mice from each treatment group were euthanized to collect their brains for IHC staining to evaluate the migration of T cells towards the tumor site. BLI was performed weekly for the rest of the mice with routine health checks and recording of mouse weight and survival. (Left) Bioluminescent total flux from SU-DIPG-13 tumor cells at the indicated time points. (Right) Kaplan-Meier plot for mouse survival. p value was determined by log-rank (Mantel–Cox) test. *p < 0.05.

112 Modeling and Use of Engineering to Overcome On-Target/Off-Tumor Toxicity of Claudin 18.2-Targeted CAR-T cells in Gastric Cancer

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Claudin 18.2 (CLDN18) targeted therapies, such as monoclonal antibody, zolbetuximab, and CAR-T cell, Satri-cel (CT041) are expanding access to immunotherapy in gastric cancer, however, high rates of nausea and vomiting were seen. Taking advantage of the high degree of homology of CLDN18.2 between mouse and human, we demonstrate this toxicity is likely driven by on-target/off-tumor (OT/OT) activity and inversely correlates with CAR binder affinity. We further develop novel, fully-human, nanobody-based CARs with superior therapeutic windows compared to CT041, decreasing OT/OT while preserving efficacy.

To evaluate the efficacy and OT/OT toxicity of targeting CLDN18.2, we engineered a CAR utilizing the scFv from CT041, with 4-1BB costimulatory domain and CD3ζ activation domain. NSG-DKO (MHC class I/II KO to avoid GvHD) mice were engrafted with human CLDN18.2-endogenously expressing esophageal adenocarcinoma cell line, OE19. Animals were treated with 1x10^6 CT041-scFv based CAR+ T cells and irrelevant BCMA-scFv CAR control. While tumor regressed rapidly in animals treated with the CT041-scFv based CAR-T, animals also rapidly lost weight, requiring euthanasia. Multiplex immunofluorescence from CT041-scFv based CAR-T treated animals show marked infiltrate of human CD3+ T cells into gastric tissue, coincident with CLDN18.2 expression (**Figure 1A-C**), and destruction of normal tissue architecture (**Figure 1D**). Control treated animals thrive and have no T cell infiltrate in the gastric epithelium. CT041-scFv based CAR-T mortality was even observed in tumor-free animals, suggesting tumor antigen driven expansion of CAR-T cell is not necessary to generate toxicity. To test the effect of dose on toxicity, OE19 tumor-bearing animals were treated with dose de-escalation of CT041-scFv CAR-T cells (1x10^6, 3x10^5, 1x10^5). Animals receiving lower doses had lower OT/OT but also less effective tumor control, making it difficult to identify an optimal therapeutic window. Following an antibody discovery campaign, novel fully-human heavy chain only antibodies (HCAb) specific to CLDN18.2; cross reactive to the human and mouse

protein, were cloned into the 4-1BB containing CAR. In vitro screening identified CARs with two highly active binders, "5795-VH" and "5797-VH". The binding kinetics of IgG reformatted versions of binders 5795, 5797 and CT041 were determined via SPR. CT041-IgG had highest affinity (K_D=3.64±0.2 nM) 5797-IgG had slightly lower affinity (K_D=4.5±3nM) while 5795-IgG had ~10-fold lower affinity (K_D=21.8±2nM; **Figure 1E**). Using the human xenograft in vivo model above, 5795-VH- and 5797-VH-based CAR-T were compared for tumor control and OT/OT toxicity with the CT041-scFv based CAR and irrelevant control at $1x10^6$ CAR T cell dose. Again, CT041-scFv based CAR-T treated animals all lost weight requiring euthanasia. 5797-VH CAR-T treated animals showed mixed results with some OT/OT toxicity requiring euthanasia and some survival with long term tumor control. No 5795-VH CAR-T treated animals lost weight from OT/OT tox, most animals demonstrating long term tumor control, until relapse at >100 days (**Figure 1F**).

A primary challenge to extending immunotherapies to solid tumors is selecting antigens with widespread tumor expression, but limited expression in normal tissues. We demonstrate that appropriate modeling of anticipated toxicity and careful examination of binding kinetics is critical to engineering a CAR with an optimal therapeutic window.

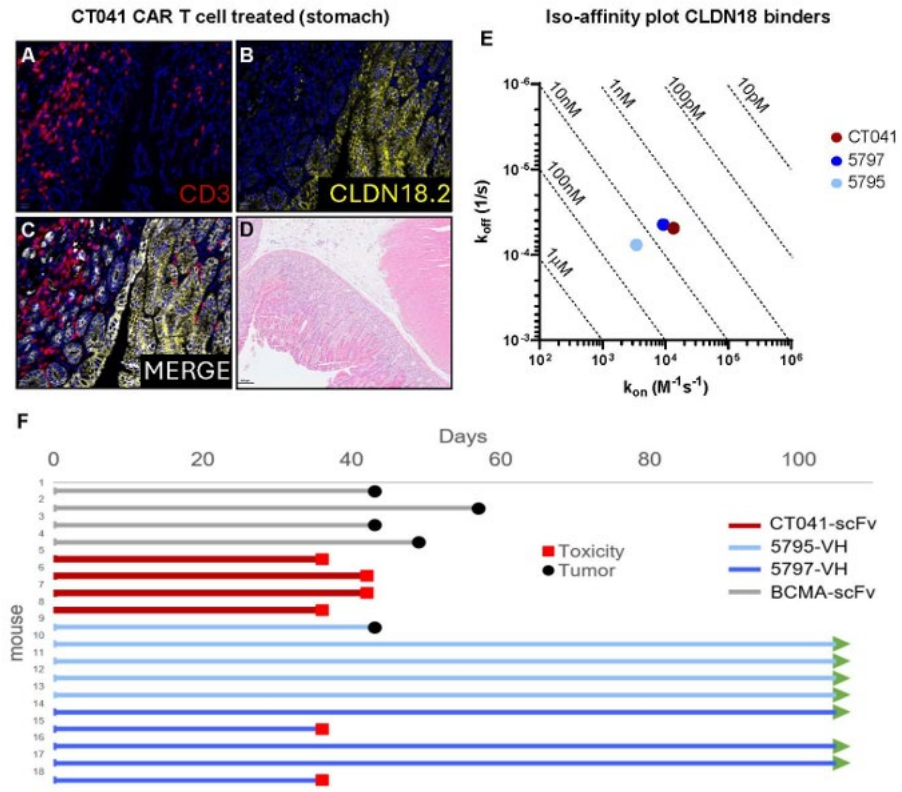

Figure 1: CLDN18-targeted CAR binder affinity determines on-target/off-tumor toxicity and therapeutic window. NSG-DKO mice bearing OE19 xenografts were treated on day 13 with $1x10^6$ CT041-scFv based CAR or BCMA-scFv based irrelevantly targeted CAR-T by tail vein injection. Multiplex immunofluorescence (mIF) on gastric tissue from a CT041-scFv based CAR T treated animal at the time of sacrifice due to toxicity, **(A)** stained for hCD3, **(B)** CLDN18.2 or **(C)** merged with PANCK (white) to highlight epithelial cells, DAPI (blue) highlighting nuclei. **(D)** H&E shows atrophy of normal architecture and inflammation (scale bar 100um). **(E)** Iso-affinity plot of binders as determined by surface plasmon resonance (SPR) of recombinant scFv-Fc or VH-Fc. **(F)** NSG MHC I/II double knock-out animals were injected with OE19, treated with aCLDN18.2 VH CAR-T, CT041-scFv CAR-T or BCMA-scFv CAR-T on day 16 and followed for tumor control, overall survival, and on target, off tumor toxicity. Swimmer plot extending to day 100.

113 Engineering CAR T cellular therapy around resistance mechanisms in TROP2+ solid tumors

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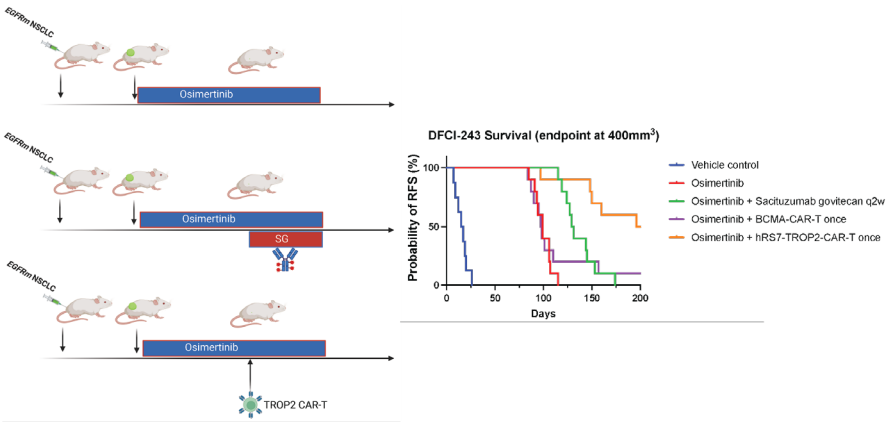
Introduction:
Solid tumors harboring actionable genomic alterations such as those frequently seen in non small cell lung cancer (NSCLC) have high response rates to tyrosine kinase inhibitors (TKI) and antibody drug conjugates (ADC) but most patients experience progression with few durable responses observed. Resistance to ADCs have been due to down-regulation or mutation of target antigen or resistance to the chemotherapy warhead target, and may explain the lack of durable responses. We utilized TROP2-targeted CAR T cells to treat TROP2+ solid tumors, including in the setting of resistance TKI targeted therapy (drug tolerant persister cells; DTPs), and ADC.

Methods/Results:
To study eradication of post-TKI resistant DTP cells, we treated a NSCLC PDX model with Osimertinib (Osi) for 6 weeks. In this model, bulky disease is eliminated but all tumors relapse. In the DTP setting we evaluated if repeat dosing of sacitizumab govitecan (SG), an anti-TROP2 ADC, could eradicate DTPs and prevent relapse, but saw only a small improvement in survival (mOS: Osi 94d vs Osi+SG 108d; n=10/arm). We then engineered a SG-scFv based TROP2-targeted CAR and evaluated how CAR T cells compared to the ADC in the post-Osi DTP model. We found that theTROP2 CAR induced significantly longer mOS compared to SG ADC (mOS: ADC 130d vs CAR 210d; n=10/arm, $p=0.005$, Fig. 1).

We also modeled known settings of resistance to TROP2 ADC due to low antigen expression, topoisomerase pathway mutations, and target epitope point mutations and demonstrated in NSCLC and Breast Cancer models that CAR T cells can be engineered to overcome them all. E.g., in an in vitro model of low TROP2 expression, cytotoxicity of ADC is decreased to 19%, while CAR T, at even low E:T, can eradicated 61% of tumor cells, $p<0.01$). To address emergence of point mutations we leveraged novel VH-only binders derived from a fully-human VH-only Tg mouse platform. This panel of VH-only binders was epitope mapped and engineered into novel VH-only TROP2 CARs that revealed their ability to target epitopes not accessible to clinically approved TROP2 ADCs. Leveraging this mapping, we rationally engineered structure-based biparatopic CARs with improved efficacy against point mutants. These biparatopic CARs showed significantly enhanced in vitro and in vivo efficacy compared to the benchmark SG-based CAR T in in vivo models of tumor heterogeneity (mOS: SG-based CAR 42d vs. biparatopic VH CAR not reached, $p<0.01$, Fig. 2).

Conclusion:
We demonstrate a high degree of in vivo activity of TROP2 CAR T against multiple models of TROP2+ solid tumors. We demonstrate that CARs have a unique advantage over ADCs, including in targeting TKI-resistant residual DTP cells, likely due to the senescence like state which is resistant to chemotherapy-based approaches, like ADCs, but sensitive to immune based therapies. We map the epitopes targeted by approved TROP2 ADCs as well as developed novel fully-human VH-only based TROP2 CARs that target novel epitopes. Biparatopic CARs demonstrated improved efficacy in vitro and in vivo, in models of emerging resistance, where single epitope targeted CAR failed. This work demonstrates that rational structure-based engineering of CAR can overcome mechanisms of resistance to ADC as well as improved targeting of post-TKI DTP cell reservoirs of relapse.



	Median survival (days)
Vehicle control	16
Osimertinib	99
Osimertinib + Sacituzumab govitecan q2w	130
Osimertinib + BCMA-CAR-T once	97.5
Osimertinib + hRS7-TROP2-CAR-T once	>200

ns, 0.2643
**, 0.0054
**, 0.0014

Figure 1. TROP2 CAR-T generate durable responses in EGFR-mutant PDX model of DTP state. **A.** Schematic of in vivo model with EGFR-mutant NSCLC PDX with osimertinib alone group, osimertinib and sacituzumab govitecan (SG) group and osimertinib and TROP2 CAR-T group. **B.**Relapse free survival of groups as well as median relapsed free survival. Log Rank Mantel Cox analysis was used for statistical analysis. **p<0.01.

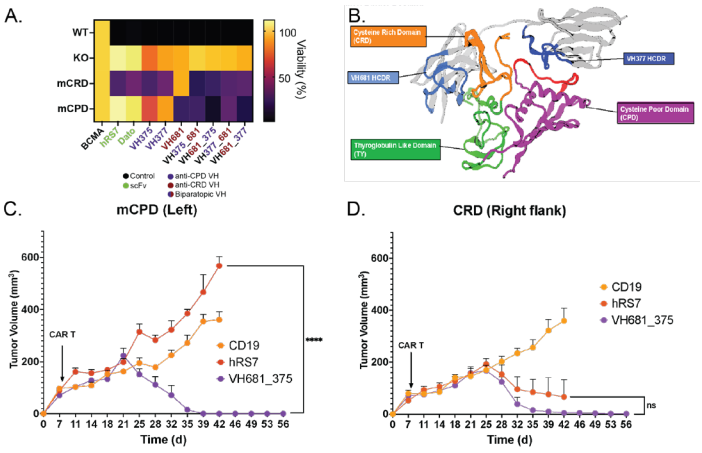


Figure 2. Rational epitope binding-based design of biparatopic CARs leveraging single domain VH-only binders overcomes models of resistance to single epitope-targeted approaches. A. In vitro cytotoxicity of TROP2 scFv, VH, and biparatopic VH CAR against PC9 NSCLC cell line with substitutions of TROP2 domains with murine domains which do not cross-react with TROP2 scFv or VH binders. VH binders target either CRD or CPD domain. Performed at 2:1 E:T and co-cultured for 24 h before viability was assessed via luciferase assay and normalized to irrelevant BCMA control **B.** In silico protein-protein docking prediction of biparatopic TROP2 VH681_375, modeled from FASTA Primary Sequence using antibody homology modeling (Molecular Operating Environment (MOE)), in complex with TROP2. **C.** PC9 harboring murine CPD or murine CRD tumors were injected subcutaneously into separate flanks of NSG mice and after tumors reached ~100 mm3, CARs were administered at a one-time dose of 2.5E6 CAR+ cells via tail vein, as indicated by arrow. Tumors measured by caliper measurement of mCPD tumor on left flank and statistics shown by 2-way ANOVA mixed model comparison, with error bars representing standard error of the mean (SEM) Data are representative of two different experiments with different donors **D.** Tumors measured by caliper measurement of mCRD tumor on right flank and statistics shown by 2-way ANOVA mixed model comparison, with error bars representing standard error of the mean (SEM) Data are representative of two different experiments with different donors. ns $p > 0.05$; **** $p \leq 0.0001$

114 Chimeric RNA-Binding Proteins Enhance CAR T Cell Function

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Genetically modified cell-based immunotherapy has been successful in multiple liquid tumors; however, the complex and immunosuppressive solid tumor microenvironment has limited the efficacy of cell-based therapies for solid tumors, which account for the majority of cancers. There is ongoing effort to achieve a more therapeutically effective T cell state by modulating the expression of endogenous genes to enhance chimeric antigen receptor (CAR) T cell activity against solid tumors and prevent T cell exhaustion. One promising strategy involves the use of RNA-binding proteins (RBPs), which regulate gene expression through mRNA splicing, export, stability, degradation, and translation. Previous research indicates that RBPs are crucial in T cell functions such as proliferation, cytokine production, inflammatory responses, and effector activities. However, genetic manipulation of endogenous RBPs have not been sufficient to produce improved effector phenotypes in CAR T cells for robust tumor clearance. To address this, we synthesized chimeric RBPs by combining effector and RNA binding domains from different natural RBPs involved in immune response pathways, each tagged with a unique DNA barcode. These chimeric RBPs were then integrated into a backbone with a CD19-28z CAR and a tNGFR selection marker. We edited primary human T cells using CRISPR-Cas12a to integrate a library of nearly 12,000 synthetic RBPs into the endogenous TRAC locus. In large scale pooled knockin screens, we repetitively co-cultured the library of edited CAR T cells with stress doses of Nalm6 target leukemia cells, and performed both acute and chronic antigen stimulation assays to assess CAR T cell survival and proliferation. Genomic DNA was collected from the T cell pool before and after the Nalm6 stimulations and was used for next-generation sequencing of the synthetic RBP library barcodes. The most effective chimeric RBPs were identified by comparing barcode readouts before vs after the acute or chronic stimulation assays, and linking the most abundant barcodes to enhanced CAR T cell proliferation. We identified multiple chimeric RBPs that outperformed both natural RBPs and CAR only cells. In particular, for the chronic stimulation assay, (N)HNRNPD–(RBP)PDCD4–(C)ELAVL1 emerged as a top hit with increased proliferation (fold change: 7.15). These enriched synthetic RBPs represent promising candidates for further study, potentially leading to improved CAR T cell therapies that can overcome the challenges posed by solid tumors. This approach represents a new method for the discovery and screening of new, synthetic genes for both CAR T cell therapies and in the future more broadly across cell types for gene and cell therapies.

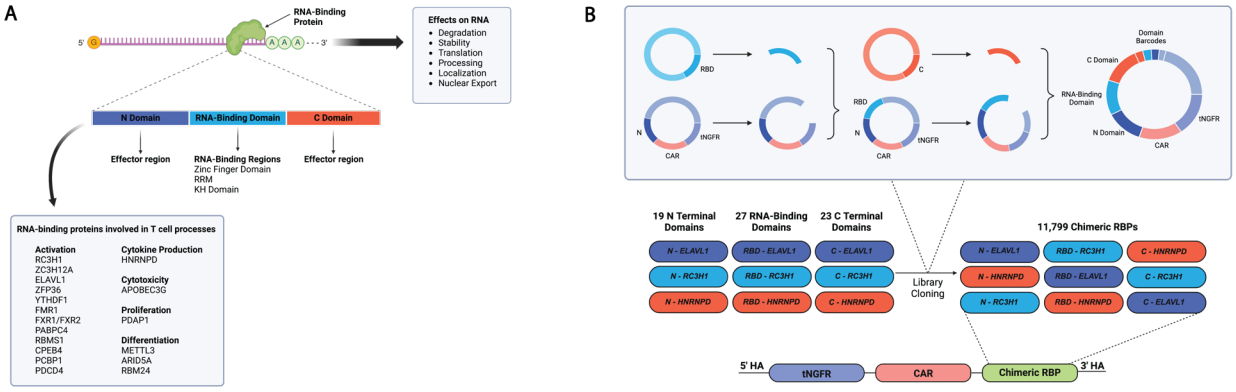

Figure 1. Design and cloning of 11,799 member chimeric RNA-binding protein library for CAR T cell engineering. (A) Three common types of RNA-binding regions were used to define the RNA-binding domain, with effector regions on either side to create interchangeable modules for combinatorial cloning. RBPs included in the screen impact cell fate of immune cells and/or are involved in many T cell response pathways. (B) Schematic of library construction using restriction enzyme digestion and ligation to iteratively add domains to a homology directed repair template backbone containing a tNGFR and a CAR (CD19-28Z).

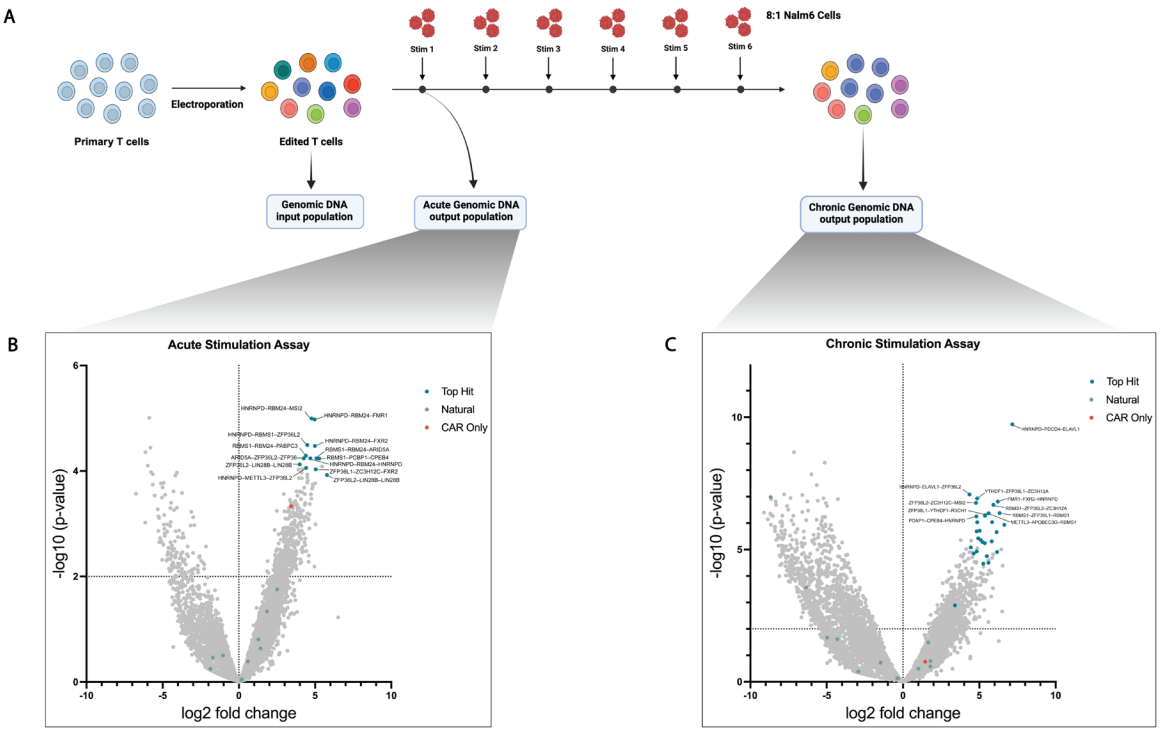

Figure 2. Chimeric RBPs can improve CAR T cell performance in a repetitive stimulation exhaustion assay. (A) Schematic of repetitive stimulation assay with target Nalm6 leukemia cells. (B/C) A comparison of barcodes from the edited CAR T cells pre- and post-stimulation found many top hits of highly proliferative chimeric RNA-binding proteins in both chronic and acute assays.

115 CRISPR-Based *In Vivo* Screen Identifies Key Gene Mediators of CAR-T Cell Dysfunction in Solid Tumors

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Introduction:
Clinical efficacy of CAR-T therapy in patients with solid tumors has generally been limited. Primary mechanisms underlying treatment failure include limited proliferation, lack of persistence and restricted functionality of CAR-T cells. Here, we have characterized dysfunction of CAR-T cells driven by chronic antigen stimulation in an *in vivo* model of solid tumors. Using this model, we screened potential gene mediators of CAR-T cell dysfunction. We hypothesized that CAR-T cell dysfunction may be prevented or delayed by targeting specific genes, resulting in an enhanced CAR-T therapy for the treatment of solid tumors.

Methods:
We optimized an *in vivo* model of CAR-T cell dysfunction using CD28-based anti-HER2 CAR-T cells against

ovarian xenograft tumors in NSG mice. Tumor-infiltrating T cells (TILs) were isolated during two phases: 1) initial CAR-T activation leading to tumor regression ("effective CAR-TILs") and 2) progressive CAR-T functional decline and tumor escape ("dysfunctional CAR-TILs"). Bulk RNA-seq and single-cell RNA-seq analysis were performed on dysfunctional CAR-TILs compared to effective CAR-TILs and preinfusion product. We identified 300 upregulated candidate genes in dysfunctional TILs and designed a custom sgRNA library to genetically modify CAR-T cells using CRISPR/Cas9 technology. Knockouts providing a functional advantage to CAR-T cells were identified through *in vivo* screening in our model of CAR-T dysfunction by Amplicon-seq. Top candidate genes after the screen were individually evaluated in an *in vitro* model of CAR-T cell dysfunction based on a restimulation assay, as well as in different *in vivo* models of xenograft solid tumors with distinct CAR constructs.

Results:
Dysfunctional CAR-TILs from our model displayed key features of exhausted T cells, including elevated expression of inhibitory makers, reduced proliferation and cytotoxicity and impaired ability to produce cytokines upon stimulation with tumor cells. Using bulk RNA-seq and Ingenuity Pathway Analysis on dysfunctional compared to preinfusion CAR-TILs, we observed enrichment of T cell exhaustion and NK cell-like signatures in our model. Using an *in vivo* CRISPR knockout screen, we found that sgRNAs targeting 14 genes were positively enriched. Knock-out of four out of six validated gene candidates enhanced the antitumor effect of CAR-T cells against ovarian xenograft tumors. Single-cell RNA-seq analysis confirmed that these new gene candidates are expressed in clusters of early and late exhaustion. Among them, the top hit was ZC3H12C (Regnase-3). Targeted ZC3H12C deletion in CD28-based CAR-T cells enhanced cytotoxicity and cytokine production after repeated stimulations with tumor cells *in vitro*, compared to AAVS1-KO CAR-T cells. *In vivo*, ZC3H12C-KO CAR-T cells mediated superior antitumor responses and enhanced T cell persistence compared to AAVS1- or LAG3-KO CAR-T cells. Similar results were observed using different *in vivo* models of lung and pancreatic tumors with Mesothelin-CD28z and CD19-BBz CARs.

Conclusions: By using an *in vivo* model of CAR-T cell exhaustion in solid tumors, we provide new insights into the mechanisms underlying CAR-T cell dysfunction. These results link ZC3H12C with CAR-T cell loss of functions after chronic antigen exposure and demonstrate enhanced potency of ZC3H12C-deficient CAR-T cells in mediating antitumor effects.

Gene Editing: New Tools and Technology Advances

116 Systemic Delivery of a Mitochondria-Targeting ARCUS Gene Editing Nuclease by AAV Eliminates Mutant Mitochondrial DNA, Demonstrating Therapeutically Meaningful Heteroplasmy Shifts In Vivo

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Pathogenic mutations in the multi-copy mitochondrial DNA (mtDNA) are commonly heteroplasmic, with both wild-type and mutant genomes co-existing in the same cell. Clinical symptoms manifest once the proportion of mutant mtDNA exceeds a particular disease threshold, resulting in multi-systemic disorders affecting high energy-demand tissues. The heteroplasmic m.3243A>G mutation is the most common pathogenic mtDNA point mutation and is responsible for approximately 1/3 of all mitochondrial diseases. Patients afflicted with m.3243-associated mitochondrial disease, for which there is currently no treatment, most commonly experience debilitating fatigue, muscle weakness, and exercise intolerance due to insufficient energy production within skeletal muscle. Taking advantage of the lack of double-strand DNA break repair within mitochondria and the unique ability of ARCUS nucleases to target DNA directly through protein contacts rather than guide RNAs, which cannot traffic across the mitochondrial membrane, we propose a therapeutic approach to correct m.3243-associated mitochondrial disease by utilizing an AAV-delivered mitochondrial-trafficked ARCUS nuclease (PBGENE-3243) to specifically cleave and eliminate mutant m.3423G mtDNA. This approach leaves the intact wild-type m.3243A mtDNA to repopulate the

cell, resulting in a shift in mtDNA heteroplasmy toward wild-type and restoration of normal energy production within skeletal muscle. While preclinical in vitro data for the PBGENE-3243 nuclease demonstrates a high degree of specificity and efficacy, there is no available animal model that contains m.3243A>G in which to evaluate PBGENE-3243 for in vivo efficacy. Therefore, we utilized the heteroplasmic m.5024C>T mouse to establish proof-of-concept for ARCUS-mediated heteroplasmy shift in skeletal muscle following systemic AAV delivery. We used a surrogate ARCUS nuclease targeting mutant m.5024T that demonstrates a similar level of potency to the PBGENE-3243 nuclease. Following delivery of 5.0 x 10^{13} vg/kg of surrogate AAV in adult mice, we observed transduction of key target tissues including the quadriceps, gastrocnemius, tibialis anterior, diaphragm, and heart, culminating in reductions in mutant m.5024T mtDNA ranging from 55% to 90% across the same organs at 12 weeks post-treatment. These reductions in mutant m.5024T mtDNA, along with concomitant increases in wild-type m.5024C mtDNA, were sustained at 24 weeks. While there is no phenotype associated with this mouse model, a reduction in mutant m.3243G mtDNA by 20-50% is cited to be sufficient to alleviate clinical symptoms of m.3243-associated mitochondrial disease in humans, and thus the efficacy observed with the surrogate in mice is more than sufficient. These data demonstrate the ability of ARCUS nucleases to shift heteroplasmy at levels beyond what is expected to be therapeutic and will further inform development of PBGENE-3243 in IND-enabling studies.

117 Programmable gene insertion in human cells using an evolved CRISPR-associated transposase

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The mutational diversity of many genetic diseases complicates their treatment with precision genome editing methods, which are typically allele-specific. Genomic integration of a healthy gene copy at a target locus could enable one-time, allele-agnostic treatments for loss-of-function genetic diseases, but current strategies for targeted genomic integration of large DNA sequences in mammalian cells are limited by low efficiency, limited programmability, or excess byproduct formation. Here we use protein evolution and engineering to develop CRISPR-associated transposases (CASTs) that enable efficient, RNA-guided integration of large DNA sequences into genomic sites in human cells. Using phage-assisted continuous evolution (PACE), we performed hundreds of generations of mutation, selection, and replication to identify transposase variants with ~200-fold averaged improved integration activity. Combining evolved transposase variants with rational engineering yielded an optimized, evolved CAST (evoCAST) system that supports ~10–30% integration efficiencies of kilobase-size DNA cargoes across 14 tested human genomic sites. We used evoCAST to install human factor IX cDNA into *ALB* intron 1; to insert a CD19-targeted chimeric antigen receptor into *TRAC*; and to integrate wild-type cDNAs of four genes implicated in loss-of-function genetic diseases into intron 1 of their respective endogenous loci. Collectively, our findings overcome key bottlenecks for CAST integration activity in human cells, establish a platform for the evolution of mammalian cell-active CASTs, and advance a broadly applicable system for the targeted genomic integration of large DNA cargoes.

118 Site-Specific DNA Insertion into the Human Genome with Engineered Recombinases

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**Corresponding Author*

Technologies for precisely inserting large DNA sequences into the genome are critical for diverse research and therapeutic applications. While large serine recombinases (LSRs) can mediate direct, site-specific genomic integration of multi-kilobase DNA sequences without a pre-installed landing pad, current approaches suffer from low insertion rates and high off-target activity. Here, we present a comprehensive engineering framework that

combines directed evolution, computational modeling, and DNA-binding protein fusions to dramatically enhance both the efficiency and specificity of LSR-mediated genomic integration.

Using the genome-targeting LSR Dn29 as a proof-of-concept, we performed deep scanning mutagenesis and substrate-linked directed evolution to identify mutations improving integration efficiency or specificity. Machine learning models trained on single mutation data accurately predicted higher-order combinatorial mutant activities (NDCG>0.97), enabling rapid optimization. Key variants included superDn29 with 10-fold improved efficiency, and goldDn29 with 5-fold enhanced specificity. Structural analysis of mutations using AlphaFold3 revealed distinct functional clusters - efficiency mutations in the coiled-coil hinge region likely modify tetramer stabilization, while specificity mutations near the DNA-binding interface modify target recognition.

To further enhance genomic targeting, we developed LSR-dCas9 fusions that facilitate recruitment to desired integration sites. Guide RNA optimization and multiplexing improved efficiency up to 6-fold, while incorporating guide binding sites on donor plasmids enhanced cargo recruitment. Additionally, we optimized the donor DNA sequence through comprehensive attachment site mutagenesis, identifying nucleotide preferences that improved integration rates.

Combining these orthogonal strategies generated highly efficient and specific recombinases. SuperDn29-dCas9 achieved 53% integration efficiency at an endogenous human locus, while hifiDn29-dCas9 demonstrated 97% genome-wide specificity. The optimized variants effectively integrated large DNA cargoes (5-12 kb tested) in challenging cell types - achieving 33% efficiency in non-dividing cells, 24% in human embryonic stem cells (hESCs), and 17% in primary human T cells. In hESCs, we demonstrated stable integration and expression of a 12 kb CRISPRi construct throughout differentiation into hematopoietic progenitor cells, enabling robust knockdown of endogenous genes.

Single cell analysis revealed that goldDn29-dCas9 produced predominantly single-copy, on-target integrations in hESCs. The system also demonstrates promise for cross-reactivity with various model organisms, including non-human primates and mice. This work provides a comprehensive blueprint for engineering site-specific recombinases and enables precise insertion of multi-kilobase DNA sequences into diverse cell types without exposed double-strand breaks. The developed tools have broad applications in gene therapy, particularly for replacing entire genes rather than correcting individual mutations, as well as in functional genomics studies requiring controlled transgene integration.

119 Monoallelic Knockout of Mutant *ELANE* in Congenital Neutropenia Patient HSPCs Rescues Neutrophil Development In Vivo

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Severe Congenital Neutropenia (SCN) and Cyclic Neutropenia (CycN) are primary hematopoietic lineage genetic disorders predominantly resulting from autosomal dominant mutations in *ELANE*, the gene encoding neutrophil elastase. Expression of the mutant allele leads to partial or near complete neutrophil lineage maturation arrest at the promyelocyte stage. This results in reduced neutrophil counts in the blood and frequent, recurrent infection. Therapeutic approaches that correct the *ELANE* mutation and restore neutrophil differentiation and function would provide significant benefit and/or a potential curative therapy. In the current study, we utilized a highly engineered, CRISPR-derived nuclease editing strategy to selectively knock-out the mutant allele in CycN and SCN $CD34^+$ HSPCs (PMID: 35795780). This approach eliminates only the dominant mutant allele thereby preserving neutrophil elastase function derived from the wild type allele. In this approach, the OMNI A1 V10 nuclease is co-delivered with two sgRNAs, one targeting a constant region creating a double stranded break in both alleles, and one targeting a heterozygous SNP on the mutant allele resulting in partial excision of the mutant allele leading to mRNA

destabilization and degradation after RNP delivery. Here, we describe our extensive pre-clinical studies of this approach for treatment of patient-derived HSPCs in a humanized mouse model. Patient cells were edited, and we were able to achieve input excision rates of 25.8% on average. For xenotransplantation studies, 1 million untreated patient (NT), edited patient, or healthy donor mPB $CD34^+$ cells were transplanted into immunodeficient NBSGW recipient mice and recipients were monitored for 16-22 weeks post-engraftment. At study endpoint, detailed immunophenotyping by flow cytometry was performed for bone marrow, spleen, and peripheral blood cells. The proportion of mature neutrophil precursors was defined as the percentage of $CD66b^+$ $CD15^+$ $CD11b^+$ $CD16^+$ within the engrafted human (h)$CD45^+$ hematopoietic population. Strikingly, gene editing resulted in a marked increase in the proportion of mature neutrophil precursors in the BM of mice that received edited cells in comparison to the untreated condition with 4.016% vs 0.372% in recipients of CycN HSPCs and 1.14% vs 0.017% in recipients of SCN HSPCs. The proportion of mature neutrophil precursors in the BM approached the level of the healthy donor in both settings. Excision rates in the BM averaged at 9.19% for CycN cells and 5.6% for SCN cells, indicating relatively low levels of editing are required to rescue the phenotype. In parallel, we performed ex vivo neutrophil differentiation using $CD34^+$ cells (or bulk BM cells) isolated from recipient mice at the time of sacrifice; demonstrating the retained the capacity to differentiate after editing and long-term engraftment. Additionally, HSPCs and myeloid cells were FACS-sorted from total BM of humanized mice for scRNAseq to analyze myeloid developmental trajectories and differential expression of neutrophil associated genes between the untreated, edited, and healthy donor groups; results showed a block in myeloid development in untreated mice which was rescued with editing. Taken together, these data demonstrate that monoallelic *ELANE* disruption rescues neutrophil development *in vivo* while also retaining the healthy *ELANE* allele and neutrophil elastase function; and support moving this approach forward for clinical application.

120 Highly efficient and seamless selection of long-range gene-edited HSPCs by targeting haploinsufficient genes

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Random integration of strong promoters by viral vectors resulted genotoxic in patients treated with eli-cel for X-linked adrenoleukodystrophy (X-ALD), who developed myelodysplasia and acute leukemia, and in previous murine models. Still, not all patients incurred in serious adverse events, indicating that strong promoters may be tolerated upon targeted gene integration.

Gene editing (GE) by homology-direct repair (HDR) allows for target integration of a therapeutic cassette in hematopoietic stem and progenitor cells (HSPCs), enabling characterization of genomic and transcriptional perturbations. However HDR GE is constrained by low efficiency, and often yields heterogeneous genetic outcomes, raising genotoxic concerns.

Rationale
We developed an innovative selection strategy that greatly enriches for HDR-edited HSPCs, while removing genotoxic byproducts, called CLEAN (Clear-cut integration of Large payloads into Essential Alleles with spontaneous counterselection of NHEJ outcomes). CLEAN is based on nuclease-based disruption of haploinsufficient loci in HSPCs, and reconstitution of the same loci by an HDR template that also include the gene of interest (GOI). As a result, all cells whose target locus is disrupted by the nuclease either have the GOI at the target location or are purged out along with their burden of large deletions and translocations (Fig 1).

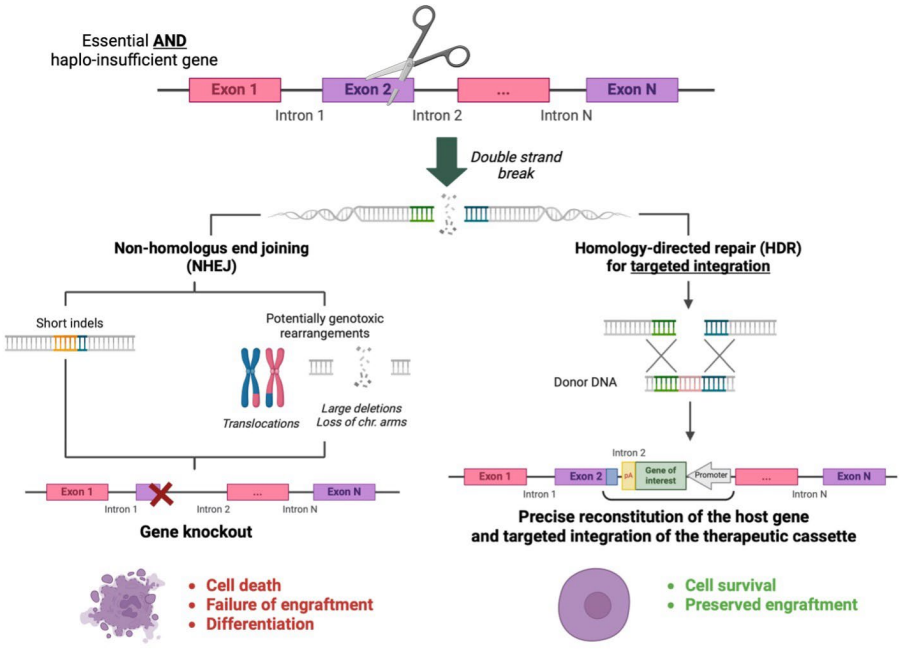
Results
After screening of putative haploinsufficient loci we identified 3 candidate genes (*UBA1, OGT, RPS19*) whose

knock-out impairs the clonogenic output of HSPCs in vitro. Conversely, rescue with a suitable HDR template, also including a GFP reporter, restored the clonogenic output to a significant degree, and 80-90% of the colonies were GFP+; precise bilateral HDR outcomes were then confirmed by long-read sequencing.

Using our optimized, GMP-ready GE protocol we achieved ≥60% HDR efficiency across tested loci. Upon xenotransplantation into immunodeficient mice, HSPCs bearing indels were spontaneously counter-selected over time. Bone marrow engraftment of human cells was lower (<10%) than the control group (40-60%), consisting of mice xenotransplanted with HSPCs bearing indels at the *AAVS1* site. HSPCs HDR-edited at all target genes resulted in engraftment of 40-60% and nearly 100% of human cells were GFP+. This compares favorably to cells HDR edited at the control *AAVS1*: at comparable levels of pre-transplant HDR efficiencies, only 40-50% of cells remained reporter positive at the experiment endpoint.

Depending on the target locus, GOI expression may be coupled to the target gene or placed under the transcriptional control of a strong promoter. Preliminary investigations into gene expression perturbations of the target and nearby genes upon integration of the model SFFV strong promoter did not raise concerns. Further comprehensive characterization of genomic and transcriptional perturbations is ongoing.

Conclusion
CLEAN tackles the limitations of HDR GE, i.e. efficiency and potentially genotoxic byproducts. It is a one-size-fits-all approach for HSPC gene therapy, well suited for targeted integration of corrective cassettes in a well characterized locus for the treatment of X-ALD, other inborn errors of metabolism requiring strong promoters and very high levels of correction, and gene therapies with a tight therapeutic interval. While selection constitutes an intrinsic bottleneck in clonal diversity, high GE efficiency reduces the magnitude of this effect.



121 Engineered Hematopoietic Stem Cells Give Rise to Therapeutic Antibody Secreting B Cells

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The therapeutic antibody market dominates modern medicine, with monoclonal antibodies representing half of the top ten selling drugs with global sales of >$200B. Despite their proven efficacy across a wide variety of diseases (i.e. cancer, autoimmunity, metabolism, etc.) they still require repeated administration to remain effective. Conversely, cell-based medicines have entirely different pharmacokinetics and pharmacodynamics than traditional medicines, including the potential to have lifetime durability. To this end, we have developed a genetically engineered stem cell-based therapy that enables continuous antibody production from a single dose. Using CRISPR/Cas9 homology directed repair mediated editing, we engineered hematopoietic stem and progenitor cells (HSPCs) such that when they differentiate into B cells they will secrete therapeutic levels of antibodies. We insert the antibody expression cassette in the *CCR5* safe-harbor locus under the control of a synthetic B cell promoter, enabling antibody expression across B cell subtypes while preserving normal hematopoiesis and endogenous B cell function. We validated this platform using two clinically approved antibodies: anti-TNFα for autoimmune disease and anti-PCSK9 for hypercholesterolemia. We achieved 35-40% targeted integration of the gene-targeted antibodies (GT-Ab) in human HSPCs and demonstrated robust engraftment (up to 85% chimerism) of these cells in sub-lethally irradiated NSG mice with maintenance of edited alleles (average 35%) in the bone marrow 16 weeks post-transplant. Because immunodeficient mice lack an immune system and do not reconstitute with mature IgG-secreting B cells, we used two other models to demonstrate antibody secretion using our system. First, we engineered GT-Ab human B cells in vitro and demonstrated efficient expression of anti-TNFα or anti-PCSK9 antibodies into the culture supernatant. We then modeled autologous hematopoietic stem cell transplant (HSCT) by transplanting engineered GT-Ab murine HSPCs into lethally irradiated C57BL/6 immunocompetent mice. We detected therapeutic antibodies in the serum of mice by week 4 post-transplant, with week 16 concentrations reaching 4-9 µg/mL for anti-TNFα and 125-300 µg/mL for anti-PCSK9 – meeting or exceeding therapeutic requirements in humans. Notably, these levels were achieved with less than 10% gene-modified cells in the peripheral blood, suggesting compatibility of the system with reduced-intensity, non-toxic conditioning regimens. In terms of safety, we observed normal B cell development in the mice, including formation of IgM^+IgD^+ mature B cells, with stable maintenance of the engineered GT-Ab alleles in B cells sorted from the spleen. The high levels of antibodies in the mouse studies prompted us to develop a system in which the levels could be modulated. By fusing the antibody to a destabilization domain, we demonstrated that levels of antibody secretion could be controlled through small molecule regulation. This ability to regulate antibody level may be essential to achieve both safety and efficacy of sustained cell-based delivery of monoclonal antibodies. Overall, this platform represents a modular approach for treating chronic diseases currently reliant on repeated administration of therapeutic antibodies, offering the potential for a durable production of antibodies from a single treatment.

122 RNA Switch-Based Inducible AAV-CRISPR System for *In Vivo* Genome Editing

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The adeno-associated virus (AAV) vector is so far the most efficient delivery platform for *in vivo* genome editing therapies targeting extrahepatic tissues, such as muscle and the central nervous system (CNS). However, a single administration of AAV can result in prolonged transgene expression lasting years to decades. This poses significant safety concerns, including (i) persistent off-target genome editing, (ii) immune clearance of nonself-editor-expressing (edited) cells, and (iii) double-strand break-induced integration of AAV DNA into host chromosomes. Synthetic RNA switches [refs 1-3], compact (~100 bp) RNA-based cis-regulatory elements that function independently of protein cofactors, hold great promise for addressing these challenges by enabling precise temporal control of genome editor expression. The key challenge is designing an RNA switch that provides both robust, inducible expression of genome editors and complete suppression of leaky expression in the dormant state.

Here, we first present our unpublished *in vitro* and *in vivo* data detailing the development of a multilayer-RNA-switch-regulated CRISPR-Cas12a system. This system is characterized by its leak-free behavior, efficient inducibility, and compact design suitable for packaging within a single AAV vector. In the optimized construct, Cas protein expression, crRNA expression, crRNA folding, and crRNA maturation are simultaneously suppressed by

two integrated cis-acting RNA elements. A single steric-blocking antisense oligonucleotide (ASO) targeting both cis-acting RNAs can deactivate all four regulatory layers, enabling precise temporal control. In mouse models, this multilayer-regulated system demonstrated negligible basal editing activity prior to induction and was effectively switched "ON" with a single dose of ASO. Furthermore, we will describe our latest efforts in developing a tightly regulated Cas9-ABE8e system toward leak-free and highly inducible *in vivo* base editing, leveraging an unpublished ultra-efficient RNA ON-switch systems (Z^{on}). These RNA switch-based inducible AAV-CRISPR system enables precise temporal control of *in vivo* genome editing, which may have broad potential for *in vivo* therapeutic editing applications.

Viral Vectors in Large Animal Models

123 Systemic Gene Therapy CAP-002 Demonstrates Potential for Disease-Modifying Treatment of Seizures and Motor and Cognitive Deficits of STXBP1-DEE Using an Engineered, CNS-Targeted AAV

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Syntaxin-binding protein 1 (STXBP1) developmental and epileptic encephalopathy is a rare and severe genetic epilepsy and neurodevelopmental disorder that impacts approximately 3.3 to 3.8/100,000 live births globally. De novo heterozygous mutations in the STXBP1 gene lead to impairments in synaptic vesicle release, neuronal communication, and network activity across the brain, resulting in numerous disease manifestations. STXBP1 encephalopathy is characterized by treatment-resistant epilepsy, profound intellectual disabilities, speech and motor impairments, behavioral issues, and increased risk of early mortality due to sudden unexpected death in epilepsy (SUDEP). To date, there are no approved disease-modifying therapies for this debilitating disorder.

Using an Stxbp1 haploinsufficient mouse model that recapitulates the major features of the human disease, including motor, cognitive and seizure phenotypes, we assessed the phenotypic efficacy of 3 doses of an intravenous (IV) administered adeno-associated virus (AAV) vector delivering the CAP-002 therapeutic cargo (human STXBP1 gene, or hSTXBP1). Study results demonstrate STXBP1 supplementation that achieves brain-wide biodistribution can reverse disease phenotypes across all three disease domains (motor, cognitive, and seizure) in a dose-dependent manner, with long-lasting correction out to a year post-intervention. We also utilized human iPSC-derived Stxbp1 knockout neurons to demonstrate that CAP-002 not only robustly transduces human neurons but also restores synaptic firing and network activity in vitro. These results demonstrate that AAV gene therapy, if able to achieve wide-spread biodistribution and supplementation of STXBP1 protein in neurons across the brain, has the potential to be disease-modifying and provide a long-lasting treatment for STXBP1 encephalopathy.

Using our high-throughput, non-human primate (NHP)-based AAV engineering platform, novel AAVs have been engineered to cross the blood-brain barrier and efficiently deliver therapeutic cargos at breakthrough levels throughout the brain after intravenous delivery. The CAP-002 candidate consists of one such engineered capsid, that achieves brain-wide distribution in up to 70% of neurons in the NHP, at low to moderate doses. We have identified a novel human receptor that binds to CAP-002; this receptor has complete homology between humans and macaques in the predicted CAP-002 binding pocket, increasing confidence in the translatability of CAP-002 performance from NHP to humans. When packaging hSTXBP1, CAP-002 achieves expression across the CNS at levels comparable to those that reversed disease phenotype in the mouse model. This breakthrough profile of brain transduction, coupled

with significant de-targeting of the liver compared to AAV9, allows CAP-002 to be delivered at lower doses than historically used with systemically delivered gene therapies, offering the potential for a greater therapeutic window. Based on these data and the well-tolerated efficacy profile of CAP-002, Capsida's STXBP1 program is targeting a 1H 2025 IND filing with the goal of achieving a disease-modifying treatment for patients suffering from STXBP1 encephalopathy.

124 Enhancer AAV Toolbox for Accessing and Perturbing Striatal Cell Types and Circuits

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The striatum, a key component of the basal ganglia, plays a critical role in motor control, habitual behaviors, and cognitive functions, and its dysfunction is central to a variety of movement disorders and substance use disorders. Effective targeting and manipulation of distinct cell types in the striatum is crucial for unraveling specific cellular contributions to functional circuits and for developing effective therapeutic interventions to treat basal ganglia disorders. Here we developed and validated an enhancer AAV toolbox for targeting several major striatal neuron populations, including pan-medium spiny neurons (MSNs), direct and indirect pathway MSNs, and Sst-Chodl, Pvalb-Pthlh, and cholinergic interneurons. Using enhancer elements derived from bulk and single-cell ATAC-seq data, we engineered AAV vectors that demonstrate high specificity in labeling these neuronal populations in the mouse brain. We evaluated these enhancer AAVs for their strength and specificity through various routes of administration and functional assays, including the use of Cre recombinase and opsins. Moreover, we investigated suitability of these enhancer AAV vectors for cross-species applications by comparing *in vivo* chromatin accessibility for select MSN enhancers across mouse, rat, and human, and conducting *in vivo* characterization of enhancer AAV activity in rat and macaque brain. The high conservation of enhancer activity across species underscores the immense potential of these tools for new directions in comparative cellular neuroscience and translational research. Finally, enhancer AAV screening and validation data from this collection can be viewed in the Allen Institute's Genetic Tools Atlas (GTA) at https://portal.brain-map.org/genetic-tools/genetic-tools-atlas, an open access community resource created with the support of the NIH BRAIN Armamentarium consortium.

125 Therapeutic efficacy of AAV-delivered HIV-1 bNAbs to prevent SHIV rebound in rhesus macaques

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Background:
In the past decades, antiretroviral therapy (ART) has become the gold standard for HIV-1 therapy for people living with HIV-1. However, ART does not eliminate the viral reservoir highlighting the need for alternative therapies. New approaches including the incorporation of broadly neutralizing antibodies (bNAbs) for HIV treatment have been explored. Our lab has been evaluating the use of adeno-associated virus (AAV) vectors to express HIV-1 bNAbs in rhesus macaques for prevention and therapy approaches against HIV. However, the host immune response routinely limits the achievable concentrations of AAV-expressed bNAbs. In an ongoing study, we have demonstrated that co-administration of PD-L1 with HIV-1 bNAbs limits the host immune response and results in sustained bNAb expression in rhesus macaques. In the present study, we aimed to demonstrate that when co-delivered with PD-L1, AAV-delivered bNAbs could maintain suppression of SHIV viremia in rhesus macaques after lifting ART.

Methods:
Two groups of eight rhesus macaques were infected with a dose of 10,000 IU/ml/animal of barcoded SHIV-AD8(EOM). All macaques were treated with ART after 17 days post-infection to suppress infection. Viral loads were monitored by plasma qPCR. Macaques in the treatment group received AAV9 vectors encoding 10-1074 and 3BNC117 co-delivered with AAV9 vectors encoding PD-L1 administered intramuscularly at 36 weeks post-infection. ART was at 46 weeks post-infection, viral loads were monitored by qPCR of the plasma, and bNAb concentrations were determined by ELISA. We will further quantify the unique barcodes of rebounding virus by deep sequencing and quantify the viral reservoir by IPDA. ELISA and ELIspot will determine anti-drug host responses.

Results:
All 16 rhesus macaques were successfully infected after one challenge with SHIV-AD8(EOM). Peak plasma viral loads between the treatment and control groups averaged from 2.0 to 2.7E+7viral RNA copies/ml, respectively. All macaques became suppressed by eight weeks after starting ART. On week 36, all macaques received AAV9 vectors to determine whether expressed bNAbs can maintain viral suppression without ART. Macaques receiving only AAV9.PD-L1 had viremia rebound within 2-4 weeks following the removal of ART. In contrast, six out of 8 macaques that received AAV9.PD-L1 with AAV9.101074 and AAV9.3BNC117 cocktails maintained viral suppression for at least 8 weeks after ART was discontinued. This observation was supported by measurable concentrations of both 3BNC117 and 10-1074 in the serum of the infected macaques.

Conclusions:
Our ongoing study reveals that the co-expression of PD-L1 is sufficient to achieve therapeutic concentrations of bNAb expression in rhesus macaques that can suppress a SHIV infection without ART. Additionally, these results suggest AAV-delivered bNAbs are a possible alternative to ART therapy in SHIV-infected macaques.

126 Focused Ultrasound Enhances Deep Brain Gene Delivery via Intra-CSF AAV Administration in Rodents and Non-Human Primates

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Gene delivery via recombinant adeno-associated viral (AAV) vectors offers the potential for durable therapeutic effects following a single treatment, yet effective distribution throughout the brain remains a critical challenge for addressing neurological disorders with widespread pathology. While intravenous AAV delivery provides broad systemic access, poor biodistribution in brain tissue necessitates high doses, raising safety concerns. Low-intensity focused ultrasound (FUS), in combination with intravenously injected gas-filled microbubbles, has been shown to safely and transiently modulate the permeability of the blood brain barrier (BBB) at targeted locations to enhance AAV brain delivery in non-human primates (NHPs), but only in the context of intravenous administration.

Infusion of AAV directly into the cerebrospinal fluid (CSF) is a clinically relevant route of administration that enables higher levels of transduction in superficial brain regions at relatively low doses. However, achieving widespread transduction of deep brain structures remains a major limitation. Here, we hypothesized that combining intra-CSF AAV administration with FUS-mediated BBB modulation could resolve this limitation by expanding AAV delivery into targeted deep brain regions.

In this study, we demonstrate that FUS-mediated BBB modulation combined with intra-cisterna magna AAV administration increases vector delivery to the rat striatum by 5-fold, with substantial enhancement in transduction of non-targeted brain regions, compared to intravenous AAV+FUS treated animals at equivalent doses. Building on

this success, we scaled this delivery strategy to NHPs. Following intra-cisterna magna infusion of AAV, FUS was applied unilaterally, targeting the caudate and putamen. BBB opening was confirmed throughout both targeted structures using T1-weighted scans with intravenous gadolinium contrast administration. Remarkably, three weeks post-administration, AAV vector genome delivery to the caudate and putamen was increased by up to 100-fold, and transgene expression was enhanced by up to 1000-fold, compared to the contralateral side. Robust transgene expression in the putamen, confirmed by *in situ* hybridization, was exclusive to the sonicated regions, with no detectable transduction in non-sonicated areas. Histopathological analysis of the anterior region of the sonicated structures revealed unresolved microscopic findings only in the caudate. These findings establish a compelling case for the use of FUS to extend the therapeutic reach of intra-CSF AAV delivery to deep brain structures with precision and scalability.

This approach represents a significant advancement in AAV-based gene delivery, offering a transformative platform for treating neurodegenerative diseases with widespread pathology. By enabling targeted and efficient transduction of deep brain regions through non-invasive FUS, these studies expand the transduction footprint of intra-CSF delivery, paving the way for the clinical translation of this technology to revolutionize the management of devastating neurological disorders.

127 Non-Human Primate (NHP) Safety Study of High-Dose EPI-321: A Novel AAV-Delivered Epigenetic Editing Gene Therapy for the Treatment of FSHD

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At Epicrispr Biotechnologies, we are advancing an innovative therapy for Facioscapulohumeral Muscular Dystrophy (FSHD) using our proprietary Gene Expression Modulation System (GEMS) platform. EPI-321, our investigational product, is a single-vector adeno-associated virus (AAV) serotype rh74 gene therapy designed to target the D4Z4 epigenome and permanently suppress DUX4 expression. EPI-321 incorporates an ultracompact, catalytically inactive, non-bacterially derived Cas protein fused to gene-suppressing modulators, under the control of the muscle-specific promoter, along with a guide RNA targeting the chromosome 4 D4Z4 locus.

Myotropic recombinant AAV vectors are widely used for systemic gene therapies for muscle disorders due to their unique ability to achieve clinically relevant levels of gene delivery to skeletal muscle. However, effective, global muscle delivery requires high-dose intravenous administration, which is associated with AAV class-effect toxicities. These toxicities commonly include systemic inflammatory responses characterized by complement activation, and coagulopathy. To address these challenges, we conducted a comprehensive safety study of high-dose EPI-321 in AAVrh74 seronegative cynomolgus macaques, both with and without immunosuppressive regimens aimed at mitigating AAV-related toxicities.

All animals survived until scheduled termination following high-dose - intravenous administration of EPI-321. Animals that received EPI-321 without immunosuppression (n=4) exhibited transient and stochastic complement activation, mild and transient thrombocytopenia, mild and transient elevation of hepato-biliary parameters, and mild and transient increases in prothrombin time (PT) and activated partial thromboplastin time (aPTT). These findings are hallmark features of AAV class-effect previously described in NHPs. Immunosuppressive regimens, including methylprednisolone sodium succinate (MPSS) alone (n=4) or a combination of MPSS, sirolimus, and rituximab (Triple IS; n=4), successfully mitigated these effects. Both regimens delayed and suppressed complement activation, ameliorated thrombocytopenia, and stabilized coagulation parameters, effectively reducing the severity of PT and aPTT changes.

These findings establish the safety profile of high-dose EPI-321 and highlight the benefits of immunosuppression, including methylprednisolone alone, in minimizing these AAV-related class effects in NHPs. We plan to initiate first-in-human trials in 2025, bringing us closer to delivering a novel therapeutic option for patients with FSHD.

128 Cardiovascular Investigation of SRP-9005 (AAVrh74.MHCK7.hSGCG) in Non-Human Primates: A Gene Therapy for Limb-Girdle Muscular Dystrophy 2C/R5

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Introduction: Limb-girdle muscular dystrophy 2C/R5 (LGMD2C/R5) is a rare and progressive neuromuscular disorder caused by mutations in the γ-sarcoglycan (SGCG) gene that leads to SGCG deficiency with consequent muscle weakness and atrophy. Additionally, LGMD2C/R5 is often associated with cardiac impairment or cardiomyopathy that may present in childhood. SRP-9005 (rAAVrh74.MHCK7.hSGCG), an investigational gene therapy, utilizes a self-complementary recombinant adeno-associated virus serotype rh74 (AAVrh74) vector to deliver a codon-optimized full-length human SGCG transgene to restore functional SGCG expression and reduce LGMD2C/R5 disease burden. The safety profile of SRP-9005 in large animals following systemic delivery has not been established. We therefore evaluated the safety of SRP-9005 in non-human primates with an emphasis on cardiac safety.

Methods: Cynomolgus monkeys (N=16; n=2 male and n=2 female per dosing group; age ~3 years) received a single intravenous (IV) bolus of SRP-9005 (7.00×10^{12}, 2.00×10^{13}, or 7.41×10^{13} vector genomes per kilogram [vg/kg]) or saline (control) and were observed for 12 weeks. Prednisolone 1 mg/kg was also administered orally to all animals on Days –1 to 60. Study endpoints included echocardiography (at baseline, Week 6, and Week 12), electrocardiography (at baseline and Day 81), quantification of SGCG protein expression in skeletal and cardiac muscle by western blot (at baseline [skeletal muscle only] and Week 12), and clinical pathology evaluation including hematology, coagulation, serum chemistry, and urinalysis (blood and urine samples were collected at Weeks –2, ~4, and 12). In addition, the effect of SRP-9005 on cardiac biomarkers (cardiac troponin I) was assessed. Organ weight assessment and macroscopic and histopathological examinations of the heart were performed at scheduled necropsy (Day 85).

Results: At all timepoints, no abnormalities in cardiac function (systolic/diastolic function, left ventricular dimensions, heart rate, rhythm, or waveform morphology) were detected following administration of SRP-9005 compared to the saline-treated animals. Histopathology analysis revealed no evidence of cardiac remodeling or degeneration with SRP-9005 at all doses tested. Of note, as expected from wild-type animals, no cardiac improvement was observed. Furthermore, no SRP-9005–related effects were observed on organ weight, gross pathology, or histopathologic findings. There were no SRP-9005–related effects on hematology, coagulation, clinical chemistry, urinalysis, or cardiac troponin I levels. There was no cellular immune response to the AAVrh74 capsid or human SGCG transgene. However, a humoral response to both AAVrh74 capsid and human SGCG transgene was seen by Week 12 in all dose groups. Molecular evaluation determined a dose-dependent increase in SRP-9005 vector biodistribution and SGCG protein expression in skeletal and cardiac muscle of non-human primates administered SRP-9005.

Conclusions: A single IV dose of SRP-9005 in non-human primates did not elicit any adverse effects or cardiotoxicity at $\leq 7.41 \times 10^{13}$ vg/kg, which may guide dose selection in clinical trials for LGMD2C/R5. In addition, these data indicate SRP-9005–induced hSGCG expression in heart and muscle following systemic administration in non-human primates.

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129 Evaluating a Bicistronic AAV9 Vector for Tay-Sachs and Sandhoff Disease: Impact of Promoter-Driven Genome Truncations on RNA Expression

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Tay-Sachs disease (TSD) and Sandhoff disease (SD) are neurodegenerative disorders that result from mutations in the genes that encode the α (HEXA) and β subunits (HEXB) of the hexosaminidase A heterodimeric enzyme. TSD and SD are clinically similar and manifest in infantile, juvenile, and late-onset forms. While patients with the infantile and juvenile forms face early mortality, late-onset patients can have a normal lifespan but become wheelchair-bound. A completed clinical trial for infantile TSD and SD, using two monocistronic AAVrh8 vectors encoding HEXA and HEXB, showed positive effects on disease progression and increases in cerebrospinal fluid HEXA. However, to circumvent the need for dual vectors and bi-thalamic injections, a bicistronic AAV9 vector encoding both *HEXA* and *HEXB* cDNA (AAV9Bicis_*HEXA*/*HEXB*), driven by a bidirectional CMV/chicken β-actin promoter (*BiCBA*) was developed. In a TSD sheep model, treated animals survived up to 5 years whereas untreated sheep typically die at ~nine months of age. The efficacy of AAV9Bicis_*HEXA*/*HEXB* was further supported via clinical examination and with correction of CNS histopathology 4-5 years after treatment. This data led to the submission of a pre-IND to the FDA outlining a toxicology study in rats, normal sheep, and non-human primates (NHPs).

To better understand the effectiveness of AAV9Bicis_*HEXA*/*HEXB*, we performed single molecule, real-time (SMRT) sequencing and AAV-genome population sequencing (AAV-GPseq) of packaged vectors, encoding for rat, sheep, and human cDNAs of the *HEXA* and *HEXB* transgenes. Unexpectedly and across all vector datasets, fully functional self-complementary *HEXA*- or *HEXB-only* genomes were also packaged, with these species being more prevalent than full-length genomes (Fig. 1a). The full-length species exhibited the lowest proportion of genomes in the sheep vector (11.5%), rat vector (22%), and human vector (20%) (Fig. 1b). Truncated genomes were also evident on both native and alkaline, agarose gel electrophoresis (Fig. 1c). These stark vector truncation patterns suggest that the *BiCBA* promoter is a hotspot for template-switching events during replication, likely as a result of the promoter being an inverted repeat and GC-rich.

To assess these effects on expression, wildtype NHPs, and sheep were randomly assigned to either an AAV9Bicis_*HEXA*/*HEXB* vector-treated (NHP: n=2; sheep: n=2) or a PBS control cohort (NHP: n=1; sheep: n=1). Vector or PBS was administered via combined bilateral intracerebroventricular, cisterna magna, and lumbar intrathecal injections, with treated animals receiving a total of 2.38E14 vg. At the endpoint, animals were terminated, and tissues were collected for vector genome biodistribution (Fig. 2), HexA enzymatic activity, and RT-PCR for HexA and HexB expression by qRT-PCR.

This study clarifies the efficacy of the AAV9Bicis_*HEXA*/*HEXB* vector utilizing the *BiCBA* promoter for TSD gene therapy. Truncation patterns demonstrate that *BiCBA* influences the packaging of AAV, leading to varying proportions of full-length genomes and *HEXA*- or *HEXB*-only self-complementary genomes. We hypothesize that the packaging of these unique vector constructs may have positively contributed to the efficacy of the AAV gene therapy. Forthcoming RNA expression profiles of AAV9Bicis_HexA/HexB transfected NHPs and sheep will underscore the impact of these truncation patterns on *HexA* and *HexB* RNA expression.

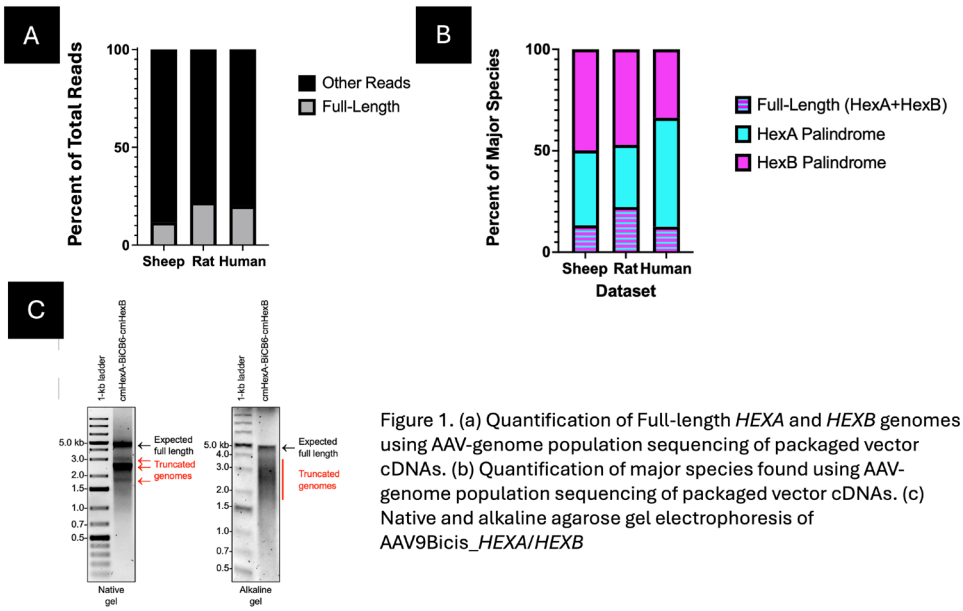

Figure 1. (a) Quantification of Full-length *HEXA* and *HEXB* genomes using AAV-genome population sequencing of packaged vector cDNAs. (b) Quantification of major species found using AAV-genome population sequencing of packaged vector cDNAs. (c) Native and alkaline agarose gel electrophoresis of AAV9Bicis_*HEXA*/*HEXB*

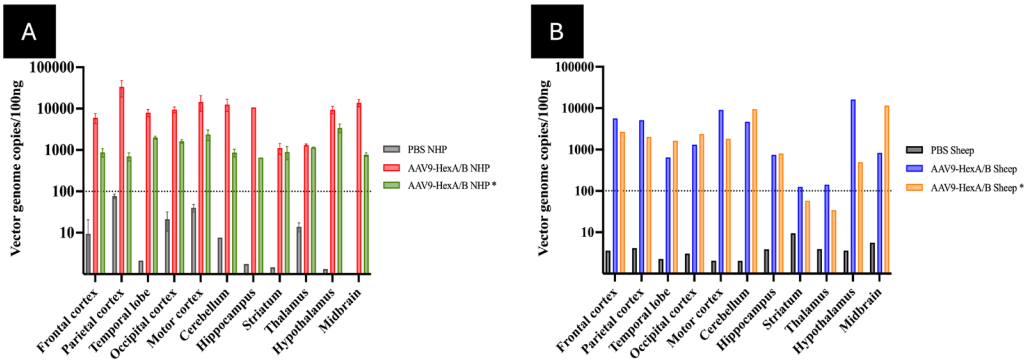

Figure 2. Vector biodistribution in brain tissue of NHPs (a) & sheep (b) assessed in control and experimental animals. this was evaluated using qPCR

Novel Neurological Disease Models and Therapeutic Approaches

130 Mouse Models of Jordan's Syndrome Provide New Insights Into Disease Pathophysiology and Therapeutic Development

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Mutations in Protein Phosphatase 2, Regulatory Subunit B' Delta (*PPP2R5D*, OMIM 601646) lead to a rare intellectual disability disorder known as PPP2 syndrome type R5D, or Jordan's syndrome (JS). Whole exome sequencing of JS patients has identified 16 unique pathogenic variants in *PPP2R5D*, with clusters of mutations found in exons 5, 7, and 12. The two most prevalent variants, p.E198K and p.E200K, represent approximately 50% of known cases and arise from G>A transition mutations. The underlying pathophysiology of JS remains unclear; however, recent clinical case studies offer some insights with reports of patients harboring E198K and E200K variants presenting with levodopa-responsive early-onset Parkinsonism. Disease modeling using human iPSC-derived midbrain neurons has also associated JS with altered dopamine signaling. Novel mouse models of JS containing human equivalent mutations in *Ppp2r5d* have been developed. Preliminary behavioral assessments in these mice have revealed hyperlocomotive phenotypes. In our study, we investigated the role of *Ppp2r5d* variants and their pathophysiological effects in mouse brain tissues. We also explored prime editing mediated gene correction as a novel therapeutic intervention for JS. Utilizing 12- to 16-week-old E198K and E200K variant mice, protein analysis showed that *Ppp2r5d* variants do not affect overall *Ppp2r5d* expression in the brain. Immunohistochemical analysis demonstrated no structural alterations to nigrostriatal dopaminergic circuits with no loss of tyrosine hydroxylase stained (TH^+) neurons in the substantia nigra or reduction in TH^+ neuronal projections innervating the striatum. Unbiased transcriptomic analysis of tissues from the prefrontal cortex, striatum, hippocampus, and cerebellum uncovered significantly upregulated genes across genotypes and tissues that affect protein folding, translation, and RNA processing; and upregulate key pathways associated with endoplasmic reticulum stress. Furthermore, we observe altered genes in striatal tissues with known or suspected roles in Parkinson's Disease pathogenesis. To evaluate site-specific gene correction of pathogenic *Ppp2r5d* mutations, we utilized Neuro2a cells and designed enhanced prime editing guide RNAs (epegRNAs) targeting *Ppp2r5d*. The initial epegRNAs yielded <0.1% editing. By optimizing epegRNAs and assessing alternative PBS and RTT sequences, we improved editing efficiencies by more than 100-fold and can achieve up to 18% editing *in vitro* with minimal insertions and deletions detected. In summary, this data provides new insights into potential cellular and molecular mechanisms underlying JS and, more importantly, generates novel targets for therapeutic development.

131 Comparison of Angelman Syndrome Candidate Therapeutics Using a Novel Humanized Ube3a-ATS Mouse Model Highlights Major Differences in Efficacy, Tolerability, and Biodistribution

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Angelman syndrome (AS) is a severe neurodevelopmental disorder caused by mutations of the maternally-inherited *UBE3A* allele (*matUBE3A*). In neurons, the paternally-inherited *UBE3A* allele (*patUBE3A*) lies dormant due to silencing by a long-noncoding RNA commonly referred to as *UBE3A-ATS*. Disruption of *UBE3A-ATS* can unsilence *patUBE3A*, including by small molecules, CRISPR/Cas9, or antisense oligonucleotides (ASOs) which are currently in clinical trials. Thus, targeting *UBE3A-ATS* is a major therapeutic strategy for the treatment of AS. While the function of *UBE3A-ATS* to silence *patUBE3A* is well conserved between rodents and primates, the sequence is highly divergent. Therefore, preclinical proof-of-concept studies targeting *UBE3A-ATS* must currently use a non-clinical surrogate rather than a test article which could be used in non-human primate or clinical studies.

To overcome this limitation, we generated and characterized a novel, humanized *Ube3a-ATS* mouse model harboring 170 kb of human *UBE3A-ATS* knocked into the syntenic mouse 7q locus by CRISPR/Cas9. Notably, the humanized region encompasses *SNORD115* and the target sequence for each ASO currently in clinical trials. We confirmed that the humanized *Ube3a-ATS allele* (*hsATS*) silences mouse *patUbe3a* as efficiently as the wild-type mouse *Ube3a-ATS* (*mmATS*). Accordingly, we also confirmed the full penetrance of several reliable AS mouse model behavioral phenotypes in *hsATS* mice bred on the AS mouse background: open field hypoactivity, reduced marble burying, poor rotarod performance, deficient nest building, increased hind-limb clasping, and markedly increased immobility in the forced swim assay. *hsATS*/AS mice further exhibited increased body weight and reduced brain weight, two morphological hallmarks of AS model mice.

Collectively, our studies validate this model for the testing of human sequence-specific strategies to downregulate *hsATS* and unsilence *patUbe3a*. gRNAs targeting Cas9 to the *hsATS* allele significantly upregulated *patUbe3a* in primary cortical neurons prepared from *hsATS* mice but not wild-type littermates. Similarly, ASOs in clinical trial including RO7248824 (Roche), 1273062 (Ionis), and 4.4.PS.L (Ultragenyx) significantly upregulated *patUbe3a* in primary cortical neurons prepared from *hsATS* mice but not wild-type littermates. Further, dose response experiments revealed differences in maximal efficacy and EC50 among these ASOs. Finally, we assessed in vivo *patUbe3a* unsilencing mediated by these ASOs following neonatal ICV injection, capturing differences in ASO efficacy that mirrored our in vitro findings, and identifying major differences in their tolerability and brain biodistribution. Overall, these studies highlight the power of the humanized *Ube3a-ATS* model for preclinical studies of potential Angelman syndrome therapeutics.

132 Investigating Blood-Brain Barrier Functionality in ACTA2 Multisystemic Smooth Muscle Dysfunction Syndrome: A Murine Model Study

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Objective:
Multisystemic Smooth Muscle Dysfunction Syndrome (MSMDS) is an ultrarare monogenic disorder caused by a missense mutation in the ACTA2 gene, where arginine at position 179 is replaced by histidine (R179H). This mutation disrupts actin polymerization, leading to shear stress on large vessels and microvascular dysfunction. The objective of this study is to characterize the effects of this vasculopathy on the blood-brain barrier (BBB) and determine whether CRISPR base editing of the ACTA2 mutation can restore BBB integrity and prevent neurodegeneration in MSMDS.

Background:
MSMDS is characterized by smooth muscle myopathy and major dysfunction in the vascular, respiratory, enteric, and genitourinary systems. In particular, cerebrovascular involvement results in strokes and white matter injury in childhood. Ischemic strokes are commonly triggered during episodes of hypotension or anesthesia, suggesting impaired cerebral autoregulation and neurovascular coupling. Previous studies in the MSMDS mouse model have shown evidence of BBB dysfunction, which contributes to the neurovascular abnormalities observed in the disorder. Additionally, CRISPR base editing has demonstrated success in restoring smooth muscle contractility and alleviating white matter disease in these same mice. This study aims to further explore the potential of CRISPR base editing to correct BBB dysfunction and mitigate neurodegeneration in MSMDS.

Design/Methods:
We developed a conditional knock-in ACTA2 R179H mouse model where the mutation is restricted to smooth muscle cells (SMCs) throughout the body. To study the effects on the blood-brain barrier (BBB), we isolated pericytes and primary endothelial cells to create an in vitro BBB model. We used dextrans of various sizes to assess the integrity of the barrier. Two-photon (2P) microscopy was employed to visualize BBB leakage using fluorescent dextran and to quantify blood flow velocity, baseline pulsatility, and functional hyperemia. Histological analysis was performed to assess BBB integrity, looking for signs of leakage, vessel damage, and neurovascular remodeling. To correlate our findings with human data, perfusion maps and MRIs were analyzed to identify vessel stenosis and straightening.

Results:
We observed accelerated neurodegeneration in the MSMDS model, displaying histopathological features similar to those seen in neuronal proteinopathies (Laur, 2021). Using CRISPR-Cas9 adenine base editing delivered by two adeno-associated virus (AAV) constructs, we corrected the ACTA2 R179H mutation in SMCs. This intervention successfully restored smooth muscle contractility and reduced BBB permeability. The in vitro BBB model showed differences in integrity, with consistent trans-endothelial electrical resistance (TEER) measurements indicating BBB

dysfunction in mutant cells. Two-photon microscopy confirmed BBB leakage in mutant mice, which correlated with accelerated neurodegeneration seen in histological analysis. Histological examination of the BBB in mutant mice revealed structural abnormalities in the blood vessels, confirming the presence of barrier dysfunction.

Conclusion:
Histological analysis revealed demyelination and neuronal loss in cortical and hippocampal regions, supporting the role of BBB dysfunction in neurodegeneration. Targeted correction of the ACTA2 mutation improved myelination and reduced pathological microglial and astrocytic activation. Future work will focus on optimizing AAV delivery.

133 AAV Delivery of Full-Length SYNGAP1 Rescues Epileptic and Behavioral Phenotypes in a Mouse Model of SYNGAP1-Related Disorders

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Syngap1-related disorders (SRDs) are rare but profound neurodevelopmental disorders that manifest in a spectrum of symptoms, ranging from epilepsy to motor deficits to autism spectrum disorder (ASD). Treatment options for patients with SRD are limited with many patients relying on a cocktail of medications to manage the diverse symptoms rather than addressing the underlying pathology. Around 80% of SRDs are caused by haploinsufficiency of the SYNGAP1 gene, which encodes a critical synaptic protein, SynGAP1. We developed a gene replacement strategy to deliver SYNGAP1 driven by a pan-neuronal promoter human synapsin1 (hSyn1) via adeno-associated virus (AAV). We were able to confirm packaging of full-length transgene and successfully produce full-length protein in vivo. Cultured neurons and brain sections from vector-treated mice further suggested appropriate trafficking and synaptic enrichment of SynGAP1, consistent with expected physiological localization. To assess the potential of our transgene as a therapeutic, we administered it to a Syngap1 heterozygous (Het) mouse model and measured the effects on epileptiform activity and behavioral phenotypes. Our results revealed a developmental and dosage-dependent effect. When delivered on postnatal day 2 (P2), we observed a partial reduction of interictal spikes (IIS) and a reduction in risk-taking behavior, but no rescue of the hyperlocomotor activity. However, when we delivered the transgene at P21, we observed rescue of IISs, reduced risk-taking behavior, and normalized locomotor activity. These results demonstrate the feasibility of rescuing multiple disease phenotypes by using a gene supplementation strategy. Importantly, efficacy was observed both early and later in development, showcasing the potential of an AAV-based gene therapy approach for SRDs.

134 Developing an AAV-based gene replacement therapy for leukodystrophy caused by mitochondrial alanyl-tRNA synthetase 2 (*AARS2*) deficiency

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The nuclear *AARS2* gene encodes mitochondrial alanyl-tRNA synthetase 2, an enzyme responsible for attaching alanine onto the cognate tRNA in mitochondria, a process known as tRNA charging. The resulting alanyl-tRNA plays a crucial role in mitochondrial translation, which produces essential proteins constituting the mitochondrial respiratory complexes. Mutations in *AARS2* have been implicated in two recessive diseases: infantile cardiomyopathy and late-onset leukodystrophy. Currently, no treatment is available for *AARS2* related diseases. To

develop a potential gene replacement therapy, we designed AAV9 vectors expressing human *AARS2* under a ubiquitous promoter (rAAV9.hAARS2). Systemic delivery to neonatal wildtype (WT) mice via facial vein injection resulted in rapid lethality due to liver toxicity, evidenced by apoptotic hepatocytes in TUNEL assays. Incorporating miR122 binding sites to enable liver-specific silencing (rAAV9.hAARS2. miR122BS) mitigated hepatotoxicity and prevented lethality. Developing animal models that accurately mimic human disease is critical for AAV gene therapy development. However, homozygous *Aars2* knockout mice are embryonically lethal. Therefore, we created a compound heterozygous mouse model that mimics the two mutations found in a patient. This mouse model harbors an exon 3 deletion and the M146T point mutation (*Aars2*$^{dEx3/M146T}$). The *Aars2*$^{dEx3/M146T}$ mice showed a 60–80% reduction in AARS2 protein levels across multiple tissues, and approximately a 50% reduction in mt-tRNA^{Ala} charging efficiency in the heart. Systemic treatment with rAAV9.hAARS2.miR122BS in adult *Aars2*$^{dEx3/M146T}$ mice partially corrected the mt-tRNA^{Ala} charging defect in these mice. In another approach to overcome embryonic lethality associated with constitutive *Aars2* knockout and to further test gene therapy, we generated a conditional knockout (cKO) mouse model by depleting AARS2 specifically in neurons. Compared to littermate controls, cKO mice exhibited significant body weight loss, severe motor deficits, and a maximum life span of 19 days. Intracerebroventricular (ICV) delivery of rAAV9.hAARS2.miR122BS to neonatal cKO pups extended their survival up to 150 days. These findings demonstrate the safety and therapeutic potential of *AARS2* gene replacement therapy. (AD and RX: Co-first authors, GG and DW: Co-corresponding authors)

135 Isoform-specific re-expression of human FMRP in mice rescues Fragile X syndrome-related translation and behavior phenotypes

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Fragile X syndrome (FXS) is the most common monogenic form of intellectual disability and frequently co-occurs with autism spectrum disorder. Patients with FXS exhibit symptoms of cognitive and social deficits, hyperactivity, sensory hypersensitivity, and sensory stimulation-induced seizure. Currently, there are no effective treatments for FXS. FXS is caused by CGG repeat expansion and hypermethylation within the 5' untranslated (UTR) region of the Fragile X messenger ribonucleoprotein 1 (FMR1) gene, resulting in transcriptional silencing and lack of FMRP protein. FMRP is an RNA-binding protein that regulates translation of a large number (~ 800) of mRNAs. Its absence often leads to excess protein synthesis, cortical neuronal hyperexcitability, and synaptic deficits. Because of the monogenic nature of FXS and the many mRNA targets of FMRP, gene therapy has been considered an attractive strategy for FXS treatment. However, it remains unclear which FMRP isoform should be re-expressed, as FMR1 undergoes alternative splicing to produce >20 unique isoforms that may have distinct functions. In this current study, we focus on two predominant FMR1 mRNA isoforms that either include or exclude exon 12 (FMR1+E12 or FMR1-E12). Sequence alignment has shown that exon 12 encodes the KH2 variable loop region, thought to be important for maintaining mRNA binding stability, on the KH2 RNA-binding domain. To examine relative abundance of FMR1+E12 or FMR1-E12 in total FMR1 during postnatal development, we performed RT-PCR with specific primers and RNA isolated from mouse and human cortex. Our results showed that the ratio of E12+ FMR1 to total FMR1 was highest at early postnatal stages and decreased with maturation in both mouse and human. By developing a novel FMRP+E12-recognizing antibody, we also found that the proportion of FMRP+E12 isoforms relative to total FMRP was highest during childhood (≤10 years) in human cortex. To determine which isoform best rescues FXS phenotypes, we then generated isoform-specific, inducible, and humanized FMR1+E12 and FMR1-E12 mice in which endogenous transcription of mouse Fmr1 mRNA is completely abolished but human FMR1+E12 and FMR1-E12 can be separately induced following breeding with the germline B-actin-Cre (ACTB-Cre). Isoform-specific FMRP was observed in the brain as early as embryonic day 15. Both FMRP+E12 and FMRP-E12 isoforms normalized excess protein synthesis relative to Cre$^-$ controls (Fmr1 KO equivalent) in BONCAT and puromycin assays, and also rescued hyperactivity in male Cre$^-$ control (Fmr1 KO equivalent) mice. However, only FMRP+E12 was able to fully correct learning deficits in fear-conditioned memory acquisition in Cre$^-$ controls (Fmr1 KO equivalent), indicating distinct roles for these isoforms during brain development and learning behaviors. We further designed an AAVPHP.eB-FMR1+E12 virus to selectively re-express FMRP+E12 in the Fmr1 KO mouse model through retro-orbital injection (at P1). FMRP+E12 re-expression was clearly observed in the Fmr1 KO mouse brain 2 weeks after injection. Consistently, AAVPHP.eB-mediated re-expression of FMRP+E12 also rescued fear-conditioned learning deficits. Together, our results suggest that FMRP+E12 is uniquely abundant in early postnatal

development and important for learning behaviors. Re-expression of FMRP+E12 therefore has the potential to become an innovative gene therapy strategy for treating FXS.

136 How Knocking Out Galectin-3 In Canavan Disease Model Mice Affects Disease Progression

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Canavan disease (CD) is a lethal neurodegenerative disease caused by mutations in the Aspartoacylase (ASPA) gene, leading to accumulation of N-acetylaspartate (NAA) in the brain. Due to the severity of neurodegeneration, most CD patients will only live to early childhood. Some common symptoms are inability to walk, lack of head control, macrocephaly, and failure to meet developmental milestones. Histopathologically, spongiform degeneration, astrogliosis and microglia activation are prominent findings. However, the underlying pathomechanism is unclear but crucial to develop and optimize gene therapy.

A hallmark of neurodegeneration is neuroinflammation, caused by microglial reactivity, which is widely studied as a therapeutic target. However, microglia are a diverse population. To understand the microglial role in CD, single cell sequencing was performed, comparing WT, KO, and AAV treated KO mice. A significant multi-fold increase in galectin-3 (gal-3) in KO mice compared to WT was observed. We hypothesized that gal-3 contributes to CD pathomechanism and can function as a biomarker to monitor response to gene therapy. Gal-3 is a protein released by activated microglia as a neuroinflammatory response and is associated with neurodegeneration in the central nervous system (CNS).

Given its association with neuroinflammatory response, gal-3 levels and colocalization with microglia was explored before and after treatment in Nur7 mice and WT. The Nur7 mouse model has a single point mutation in the ASPA gene but has near normal life expectancy while still showing neurologic phenotypes of other CD mouse models. Gal-3 positive microglia increased significantly in Nur7 mice, particularly in brain regions of pronounced brain pathology, and decreased after treatment. The gal-3 positive microglia were also shown to be ameboid shaped and returned to a resting state post-treatment. The ameboid shape is associated with microglia activation and release of gal-3. Interestingly, we found a similar pattern when mice were treated at different time points, suggesting that gal-3 might allow monitoring to therapeutic response as it can easily be detected in cerebrospinal fluid and blood.

To determine if gal-3 positive ameboid microglia have protective or damaging function, Gal-3/ASPA double knockout (DKO) mice were created. Using brain vacuolization as an initial readout, P21 DKO and Nur7 brains showed similar vacuolization in a brain region specific manner. A similar trend was seen at P70, however, DKO seem to show worsening vacuolization.

Overall, the findings in DKO mice suggest that gal-3 might have a protective effect in untreated CD. However, gal-3 also shows a strong response to gene therapy at different treatment time points and might therefore be a viable biomarker to monitor gene therapy.

Pharmacology/Toxicology Studies and Analytics/Assay Development Session I

137 A Comprehensive and Robust Analytical Platform for Clinical Grade CAR-T Cells

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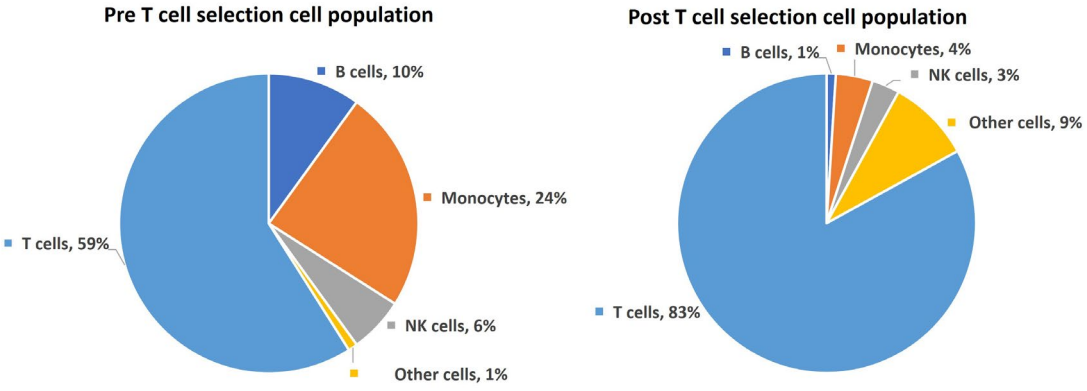
Background & Aim
A comprehensive and robust analytical platform has been developed using the Analytical Quality by Design (AQbD) approach to support clinical-grade Chimeric Antigen Receptor (CAR)-T cell therapy. This platform incorporates and optimizes both existing and new analytical methods to ensure robustness, reliability, and suitability for CAR-T cell manufacturing. By defining and monitoring Critical Quality Attributes (CQAs), the platform ensures consistent product quality, optimizes manufacturing processes, improves product yields, reduces variability, and identifies potential risks associated with cell therapy manufacturing.

Method
Studies were conducted to evaluate a panel of CQAs using various T cell development lots. T cells were isolated from leukopak using conventional and new cell isolation methods. A flow cytometry-based panel was developed to characterize different cell populations, including T, NK, B cells, and monocytes. Thereafter, Lentiviral Vector (LVV)-CAR was transduced to T cells, and analysis of transduction efficiency was optimized per product type by using anti-linker monoclonal antibodies-based flow cytometry. Potency was assessed through proliferation and cytokine release assays. Additionally, different characteristics of CAR-T cells were analyzed by various flow cytometry panels: A) memory, B) activation, C) cytokine, D) exhaustion, E) degranulation, and F) apoptotic panel.

Results
The analytical platform has successfully evaluated: 1) T cell purity and other cell populations (Figure 1: Flow cytometry-based analysis to evaluate the cell phenotype in pre and post T cell selection) when comparing various T cell isolation methods; 2) characterization of activation of T cells; 3) optimal transduction efficiency; 4) stemness of CAR-T cells; and 5) viability and presence of apoptotic CAR-T cells. Cytokine release analysis confirmed proper effector function and potency to kill specific target cells.

Conclusion
This integrative T cell therapy analytical platform accelerates the development of CAR-T cell therapies by reducing method development timelines for new CAR constructs. This platform is flexible and customizable to enhance the speed and quality of T cell therapy development. The platform systematically evaluates and mitigates risks associated with analytical methods and manufacturing processes, ensuring consistent product quality and advancing CAR-T cell manufacturing for clinical applications.



138 Development of Orthogonal Potency Assays for Multi-Characteristic Opsin Gene Therapy: Gene Expression and Light-Stimulated Activity

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Multi-characteristic Opsin (MCO-010) is an AAV2-based optogenetic gene therapy product, which, upon intravitreal delivery, photosensitizes retinal neurons in the photoreceptor-degenerated retina of patients. Quality assurance of such gene therapy products requires robust potency assays that mimic biological activity relevant to the mechanism of action. This requirement presents unique challenges for optogenetic therapies like MCO-010, which must demonstrate both successful gene expression and light-activated functioning. The development and validation of appropriate potency assays for such products must navigate multiple challenges due to the non-availability of standardized assays and the complexity of developing such assays while meeting ICH requirements. The development steps include standardization of cell-based systems, optimization of viral transduction parameters for reproducible measurement of transgene expression, selection of molecular probes, establishment of reference standard, and integration of light-stimulation device with device for activity measurement. We developed a potency assay matrix that comprehensively evaluates MCO-010's critical quality attributes.

The primary assay quantifies relative potency through flow cytometric measurement of MCO-010 expressing cells, utilizing an intrinsic fluorescence reporter. This assay employed a dose-response curve to evaluate relative potency with respect to a well-characterized reference standard. The method development and validation encompassed the study of various system suitability and assay criteria such as goodness of curve fit, precision (intra-assay, inter-assay), and specificity across multiple doses meeting ICH validation requirements for product release testing.

The secondary assay measures light-stimulated calcium activity, directly evaluating the functional aspect of the MCO-010 protein. This assay quantifies calcium influx following white light stimulation using calcium-fluorescence indicators, enabling evaluation of the biological activity of MCO-expressing cells in response to light as compared to control cells. The development of this functional assay includes the optimization of multiple parameters, including the selection of cell line, light stimulation protocol, calcium indicator (concentration, incubation period), and gating for cellular response measurement.

We will present data from both expression-based and functional potency assays for MCO-010. Our results include method development findings, robustness studies across critical parameters, and validation data demonstrating assay reliability and reproducibility. We will show how these assays effectively measure product quality through a comprehensive evaluation of viral transduction, transgene expression, and light-activated functioning. This potency matrix offered enhanced confidence in product quality while providing insights into different aspects of MCO-010’s biological activity, effectively balancing manufacturing requirements with biological relevance and regulatory compliance. The successful implementation of these potency assays supports product release and stability testing and establishes standards for optogenetic gene therapy products.

139 Preclinical safety evaluation of AAV5-hNR2E3 (OCU400), a mutation agnostic gene therapy candidate for retinitis pigmentosa and leber congenital amarousis

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Background:
Retinitis pigmentosa is a debilitating ocular disease that affects 1/4000 people worldwide. In the early stages, patients develop night blindness and lose their peripheral vision, which often leads to complete vision loss at advanced stages. Nuclear receptor subfamily 2 group E member 3 (NR2E3) is a nuclear hormone receptor that regulates multiple transcriptional networks that are known to modulate retinal cell homeostasis. In preclinical studies, subretinal delivery of mouse NR2E3 rescued the RP and LCA phenotype by resetting the molecular

pathways to restore the normal ocular structure and function in all the RP and LCA disease models. In this study we evaluated safety and biodistribution of OCU400 after its subretinal administration in Gottingen minipigs.

Methodology:
AAV5-NR2E3 was administered subretinally in Göttingen minipigs at dose levels of 1.0×10^{10}, 5.0×10^{10}, and 1.25×10^{11} vg/eye. The safety and toxicity of AAV5-h*NR2E3* were evaluated in the ocular and non-ocular tissues of the animals upon administration of a single dose. All positive tissues in which vector transgene was detected were analyzed for protein expression and sustenance. Additional parameters of the study included clinical observations, body weights, food evaluation, ophthalmology, tonometry (intraocular pressure; IOP), electroretinography (ERG), multimodal cSLO fundus imaging and spectral domain optical coherence tomography (SD-OCT). Clinical pathology was evaluated using hematology, coagulation, and clinical chemistry parameters. Anti-drug antibody (ADA) analyses were performed in serum and aqueous humor for viral capsid (AAV5) and transgene (NR2E3). Cellular immune response was evaluated by ELISpot in peripheral blood mononuclear cells (PBMC), spleen, and mandibular lymph nodes, organ weights, and macroscopic and microscopic examinations.

Results:
OCU400 was specifically expressed ocular tissues with transient, low expression in some non-ocular tissues. Expression of NR2E3 was sustained in retina through 6 months post-dosing, suggesting that OCU400 targets the appropriate tissue, and is present for longer duration. Ophthalmological findings were generally mild and related to the dosing procedure, as they were present in both treated and untreated animals and were resolved over time. OCU400 treatment resulted in the development of anti-viral capsid and anti-NR2E3 ADA responses. NR2E3 demonstrated a weak antibody response, while higher antibody titers, were detected against AAV5 capsid, particularly in aqueous humor, but there were no corresponding systemic clinical signs or ophthalmic changes. There was no immunogenic response in PBMCs or lymphocytes at any time during the study. Histology data showed changes in the bleb area of all the study animals related to surgical procedure, and no OCU400-related mortality, macroscopic changes, or organ weight changes were observed.

Conclusions:
We observed that OCU400 is specifically delivered to the target tissues and efficiently transduces retinal cells where it is translated to produce NR2E3 protein. Administration of OCU400 at all dose levels was well tolerated and resulted in ocular changes that were minor and mostly related to the dosing procedure with no significant signs of toxicity.

140 Development and Evaluation of AAV9.anti-IGF1R (Insulin-like Growth Factor-1 Receptor) Vector Potency Methods for KRIYA-586, a One-time Gene Therapy for Thyroid Eye Disease (TED).

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Introduction:
TED is a debilitating chronic autoimmune disease that causes inflammation and enlargement of muscle and fat tissues in the extraocular space, leading to symptoms including proptosis and diplopia which significantly impacts quality of life. A key pathological implication of TED is the overexpression of insulin-like growth factor-1 receptor (IGF1R) in several cell types. KRIYA-586 is a gene therapy designed to inhibit IGF1R activity using AAV9.anti-IGF1R to attenuate the downstream signaling pathways to mitigate disease pathology. Assessment of vector potency is critical prior to vector release in preclinical and clinical development.

Our vector potency strategy is based on three key aspects; 1. Assessment of vector cellular entry by capsid immunostaining for specific serotype, 2. Quantitation of expressed protein to evaluate the vector-delivered transgene expression, 3. Testing for biological activity reflecting the disease specific mechanism of action.

Methods:
Vector transduction was evaluated in HEK293 cells using different transduction enhancers-at several multiplicities

of infection (MOIs). Protein expression levels were evaluated to determine the most effective transduction method using an in-house developed Gyrolab™ sandwich-immunoassay. Quantification was based on a standard curve, established through the analysis of on-column fluorescence binding profiles using the Gyrolab™ Evaluator software. Following transduction, the cells were harvested at different time points, immunostained to identify the AAV9 capsid and analyzed via flow cytometry to determine the optimal cell harvest time to detect capsid cellular entry.

Biological activity was assessed using an in-house developed chemiluminescence assay based on an IGF1R overexpression cell line. Using the vector derived anti-IGF1R protein, inhibitor-dose response curves were generated to determine the IC50 value. Potency methods were also assessed using a biologically relevant cell line along with the HEK293 cells to determine the suitability of HEK293 cells to represent the vector potency.

Results:
Addition of a transduction enhancer during AAV9.Anti-IGF1R vector transduction demonstrated a significant increase in protein expression ≥3-folds at all tested MOI's (Figure 1). Harvesting the cells at 3.5 hours post transduction was selected as a feasible time point to assess vector cellular entry. The resulting anti-IGF1R protein was quantified using Gyrolab™ immunoassay with a dynamic range of 7.8 – 500 ng/ml of target protein (Figure 2). Comparison of potency methods between a more biologically relevant cell line and the HEK293 cell line resulted in comparable protein expression levels at 233 ng/mL and 493 ng/mL, and biological activity with similar IC50 values of 61ng/mL and 67 ng/mL, respectively.

Conclusions:
The KRIYA-586 potency assessment successfully demonstrated a robust evaluation of AAV vector transduction and therapeutic protein activity. The approach of using a simplified Quality-Control feasible cell line yielded comparable biological activity measurements to those obtained from a more biologically relevant cell line, validating a more efficient approach to vector potency testing. This analytical framework allows characterization of vector functionality prior to release, while maintaining feasibility for routine testing in a GMP environment.

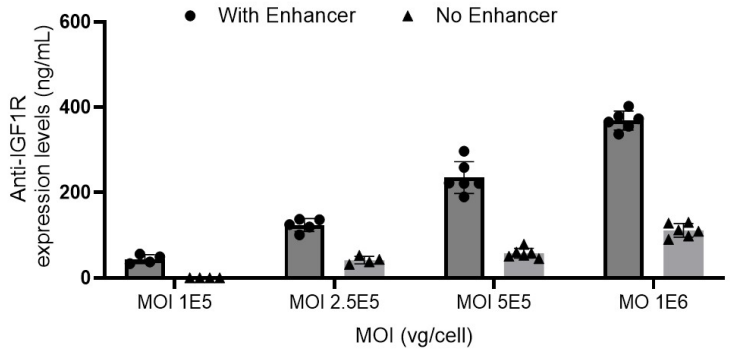

Figure 1. Comparison of the protein expression levels obtained post transduction of AAV9.anti-IGF1R vector with and without the transduction enhancer, tested on HEK293 cell transductions.

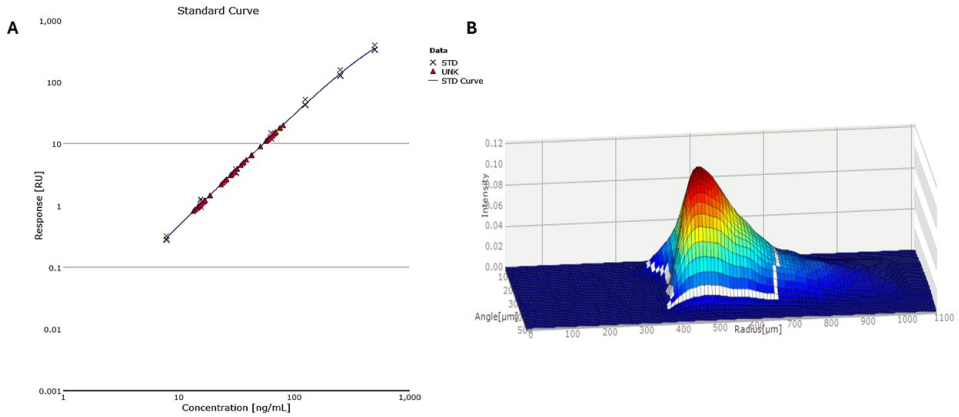

Figure 2. Gyrolab immunoassay development. A. Standard curve with a linear range of 7.8 - 500 ng/mL. STD – standard points, UNK – Unknown samples. **B.** On column fluorescent signal showing the binding profile of the detect indicating higher affinity of the interaction

141 Automated and Miniaturized Sonication Linker-Mediated PCR (SLiM-PCR) for High-Throughput Integration Site Analysis in Gene Therapy Applications

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Vector Integration Site (IS) analysis is crucial for assessing the safety and efficacy of preclinical and clinical gene therapy (GT) applications based on integrating vectors.We developedSonication Linker mediated-PCR (SLiM-PCR), a methodthat enables retrieval and quantification of IS through specific PCR amplification of vector/genome junctions from sheared genomic DNA, combined with next-generation sequencing (NGS) and bioinformatic analysisby dedicated software. SLiM-PCR allowed for accurate identification, quantification and long-term tracking of vector marked clones, in whole blood or bone marrow samples, and in several isolated hematopoietic lineages in clinical trials based on hematopoietic stem cell (HSC) GT. It is compatible with multiple vector systems, including lentiviral(LV), gammaretroviral, and Sleeping Beauty transposon-based vectors. Over the past 10years we analyzed >7000 samples from 156 patients enrolled in 7 clinicaltrialsand >8000 samples from several preclinical studies. Given the increasing number of samples, we fully automated our laboratory procedures with liquid handling robotscapable of processinghundreds of DNA samples per week in 96/384-well plates, and enabling barcoding for multiplexed NGS. To further optimize efficiency,we are miniaturizing reaction volumes by leveragingAcoustic Droplet Ejection (ADE) technology usingthe Beckman Labcyte Echo 525, which allows precise nanoliter-scale liquid transfers via acoustic waves.So far, working on LV transduced cell lines,we have successfully reducedreaction volumes 10-fold, down to5microliters, consequently achieving increased throughput, reduced reagent consumption, and enhanced reproducibility. Our results show that we can obtain comparable results to those obtained with standard volumes in terms of IS number retrieved fromthe same amount of genomic DNA, maintaining the precision in IS relative abundance estimation (down to 0.1%) and detection limit. To further refine SLiM-PCR, we are investigating PCR-induced artifacts and chimeric sequences, which can lead to false-positive IS detection and affect overall data accuracy. By applying SLiM-PCR to genomic DNA from monoclonal cell lines harboring IS at known genomic positions, we aim to systematically assess the frequency and nature of PCR artifacts. We are optimizing PCR conditions by testing different polymerases, reducing input DNA amounts, and minimizing the number of amplification cycles to strike the best balance between sensitivity and specificity. In parallel, we are developing bioinformatics pipelines incorporating clustering algorithms and artificial intelligence (AI)-driven

approaches to detect and filter putative chimeric sequences, enhancing the accuracy of IS analysis.These advancements in automation, miniaturization, and artifact detection significantly improve the scalability, precisionand reliability of SLiM-PCR, enabling high-throughputIS tracking and strengtheneningthe robustness of longitudinal safety assessments in vector-based gene therapy applications.

142 Evaluation and Modeling of TransAct Addition for synNotch--CAR Induction Assay in E-SYNC T Cells

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**Corresponding Author*

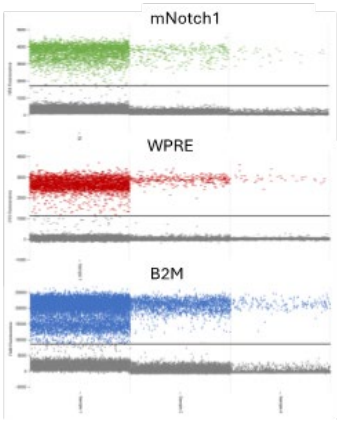
Background and aim:
Amplification and rearrangements of the epidermal growth factor receptor (EGFR) gene are frequently found in glioblastoma multiforme (GBM). The most common variant is EGFR variant III (EGFRvIII). At UCSF, we manufactured autologous E-SYNC T cell product to treat patients with EGFRvIII+ GBM. The percentage of induced Anti-EphA2/IL-13R alpha2 CAR is used to prepare the final dose formulation. During prior manufacturing runs, we noted that (1) the percentage of transduced cells measured by the CAR induction assay was lower than the expected percentage from the digital PCR (dPCR)-based genomic measurement of integrated copy numbers, and (2) the level of detectable CAR expression demonstrated a positive correlation with the activation state of cells. Here, we evaluated whether treating T cells with the Miltenyi TransAct, T cell activator, prior to measurement of the induced-CAR expression, would improve the sensitivity of this assay to more accurately determine the percentage of transduced cells.

Methodology:
To assess TransAct activation influence, three post-thawed cell samples from previous runs and one in-process cell sample were co-cultured with EGFRvIII-coupled beads, in the presence and absence of TransAct, to induce CAR expression from synNotch activation. Flow cytometry was utilized to assess CAR induction at the phenotypic level, while dPCR readouts were deployed as a complementary assay to evaluate transduction efficiency at the genomic level. Mathematical modeling based on the Poisson distribution principle was applied to correlate data from both genomic and phenotypic readouts.

Results:
Our data demonstrated that activating E-SYNC T cells with TransAct in CAR-induction assay improves assay sensitivity to determine transduction efficiency and calculations more accurately for vector copy number. This change improves the measurement of drug product identity, purity, and safety and ensures more accurate dose formulation. Comparison data supporting this change using in-process clinical manufacturing drug substance from patient PBMC and three additional healthy donor drug products demonstrate that the addition of TransAct more accurately aligns the flow cytometric measurements with genomic data as measured by dPCR.

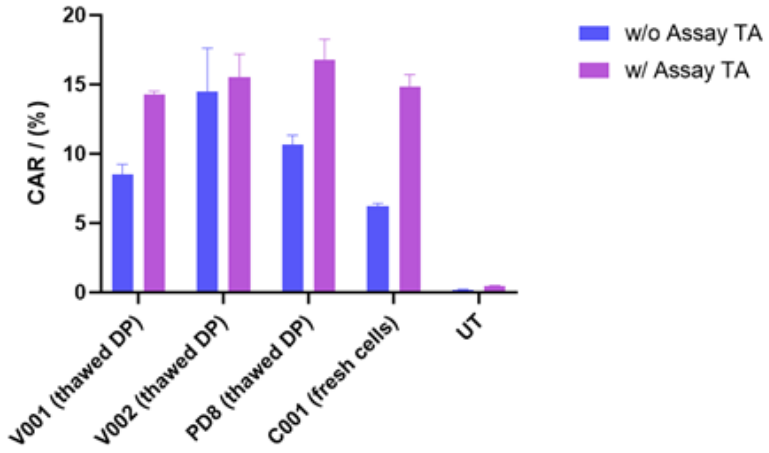
Conclusion:
The addition of TransAct improves the CAR induction assay based on more reasonable associations with dPCR-based VCN analyses. We have updated assay qualification to incorporate these changes and plan to use the modified assay for future E-SYNC T Cells product releases.

A



B

Sample	%synNotch-CAR by dPCR	Predicted VCN
V001	36.64	1.19
V002	40.89	1.22
PD8	28.26	1.15
C001	31.98	1.17



Molecular and Cellular Methodology

143 Highly Sensitive Detection and Characterization of Intended and Unintended Gene Editing Events by TES.

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Gene editing (GE) is a revolutionary technology that offers the potential to treat genetic disorders and improve human health. Techniques like CRISPR-Cas9 have made GE more accessible and efficient, opening promising avenues for medicine and biotechnology. However, one of the significant safety concern is off-target effects, where unintended alternations occur in the genome. These **off-target editing events** can potentially lead to harmful consequences, such as the disruption of essential genes or deregulation of cancer-associated genes. As GE technologies continue to evolve, ensuring accuracy and minimizing these unintended alterations is essential to mitigate risks and ensure the safety of these powerful tools.

Commonly used off-target GE detection and characterization methods in cells and tissues, such as GUIDE-seq, Bless and Discover-seq are mainly suitable for developmental phase of GE tools. Due to reliance on exogenous tags, requirement for live and dividing cells or cells fixed upon double strand break induction, real time active repair-based detection, low sensitivity and complex workflows these methods might be impractical for routine, scalable monitoring of patient samples after treatment.

Thus, it is essential to develop methods that not only assess off-target activity during development but also enable the quantification of **genomic integrity in the cell-based product**. Additionally, these methods should allow for the **monitoring of gene-edited patient cells and tissues**. Applying our **advanced target enrichment sequencing (TES)** method we developed a novel approach for GE off-target detection and characterization, which can be applied for non-clinical as well as clinical setting. The method uses a set of RNA baits designed for enrichment of known on-target site as well as **>1600 predicted CRISPR-Cas off-target cut sites** on DNA samples obtained from CRISPR-Cas9 gene edited cells.

We optimized our experimental workflow on a novel knock in HDR developed strategy to edit RPL5 locus to apply it to several pipelines of interest, achieving very high sensitivity of our method for **DNA input as low as 50ng**. In particular we were able to characterize all predicted off-target sites. Using low DNA input is highly beneficial when testing for off-target effects in GE therapy, allowing analysis of more precious samples, which is particularly important when working with patient-derived or limited DNA sources.

Moreover, we were capable to detect internal control DNA sequences with known ratio of **small insertion-deletion mutation with a high precision and accuracy**. High level of accuracy and precision was also confirmed for the detection of more infrequent genetic alterations, such as **chromosomal translocations**. The ability to identify mutations at rare frequency ensures that even subtle genomic alternations, which could have significant biological implications, are reliably detected and analyzed.

Our method can be adapted and qualified for **any type of GE approach** by applying sequence-specific probes. Hence it will be a valuable tool in determination and characterization of potential off-target sites for GE- based gene therapy sample analysis. While assessing the precision and efficiency of developing GE therapies as well as providing precise monitoring of genomic stability in patients, it will become an essential tool for safety assessment in GE development.

144 Bioinformatic analysis of the genetic basis of differential AAV production capability of 293 variants

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A major application of 293 cells is the production of adeno-associated virus (AAV) vectors. Stable integration of adenoviral genes E1a and E1b, which are required for AAV production, in the 293 cell genome, makes 293 cell lines the predominant choice for AAV production in mammalian systems. During the process of AAV vector development, 293 cell lines have been evolved in parallel for maximum vector yield. Despite the excellent performance of these new variants in AAV manufacturing, the molecular basis of these new variants has yet to be investigated. In this study, we sought to study the genomes of two high AAV production cell lines, 293A, an adherent variant derived from a clone commonly used in our laboratory, and 293S, derived from a commercially available suspension cells (VPC2, Thermo Fisher Scientific.). Genomic DNA from these two HEK293 derivatives was sequenced by Nanopore long-read sequencing technology. Sequencing yielded an average depth of coverage exceeding 10×. For comparison, we retrieved Illumina short-read sequencing data from the SRA database for the original HEK293 cell line and three derivatives-HEK293F, HEK293Freestyle, and HEK293H-under the accession numbers SRR10129632, SRR10129614, SRR10129620, and SRR10129613, respectively. The origins of these cell

lines are summarized in **Figure 1**. For simplicity, these cell lines are referred to as 293, 293F, fs293F, and 293H, respectively.

Our study revealed that the two high rAAV-yielding cell lines, 293A and 293S, exhibit greater genomic similarity compared to other 293 variants, despite their markedly different origins. While 293A is an adherent cell line maintained in our lab for over 20 years, 293S is a recently developed suspension cell line from Thermo Fisher Scientific. This unexpected finding suggests that both cell lines may share underlying genetic mechanisms that confer efficient rAAV production. We for the first time identify a genomic duplication of adenovirus integration regions (~328kb region). The high AAV yield variants, 293A and 293S, both showed a duplication pattern in this region that is distinct from that of other 293 cell variants (**Figure 2**). In addition, the viral E1A and E1B genes are brought into close proximity to strong regulatory elements, such as enhancers GH19J043180 and GH19J043200. These enhancers, which are associated with the (PSG) family, are enriched with H3K27Ac marks and transcription factor binding sites, indicative of high enhancer activity. Following adenoviral gene integration, GH19J043180 and GH19J043200 may predominantly interact with the integrated viral genes, effectively "hijacking" the local regulatory environment and redirecting their activity to drive robust expression of E1A and E1B. These findings showed that the molecular states of the adenovirus integration region may be one of the key factors leading to high yield production of AAV vectors.

A limitation of this study is that HEK293 derivatives were sequenced using two different sequencing technologies, with different sequencing depths and read lengths. This difference may introduce systematic bias when comparing genomic features between two sequencing technologies, especially in the absence of internal controls at critical genomic loci. Future studies incorporating unified sequencing platforms and orthogonal method validation will be important to further confirm these findings.

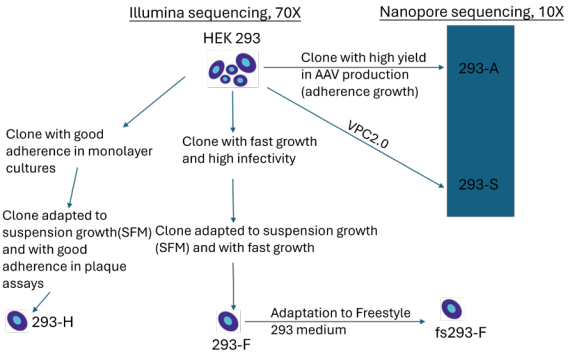
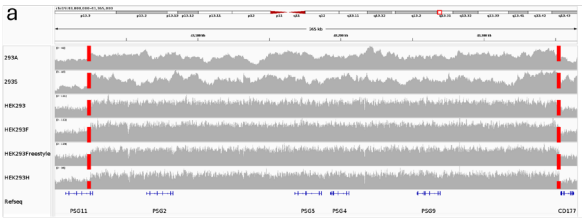


Figure 1. 293 genome sequencing and acquisition. The origins of these cell lines and sequencing methods utilized are summarized.



b

	# of reads supporting reference:alternative SV alleles		
Sample	Duplication of chr19: 43024964 - 43352663	Ad5:1 breakend	Ad5:4344 breakend
HEK293	94:43	10:33	20:100
HEK293F	113:51	7:31	17:82
HEK293H	90:33	6:12	14:73
HEK293 Freestyle	108:41	6:32	15:87
293A	16:9	0:21	0:22
293S	12:11	0:16	0:19



Figure 6. SV analysis reveals distinct integration and duplication patterns in HEK293 derivatives.

(a) IGV visualization of sequencing coverage confirms a duplication in the region marked in red, as identified by SV callers in all HEK293 derivatives. (b) SV analysis shows reads supporting a heterozygous duplication in all six HEK293 derivatives, a heterozygous insertion of Ad5 in HEK293, HEK293F, HEK293Freestyle, and HEK293H, and a homozygous insertion of Ad5 in HEK293A and HEK293S.

(c) An inferred model of Ad5 integration and duplication is presented, with the duplicated region highlighted in green, the Ad5 inserted sequence marked in yellow, and chromosome 19 depicted in blue.

145 ATCC® Cell Line Land: an OMICS Data Repository for ATCC® Cell Models that Drives Scientific Innovation and Improves Reproducibility

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Ensuring the molecular quality and authenticity of biomaterials is critical for the reproducibility and reliability of scientific research. ATCC® supports these efforts by maintaining a vast collection of highly authenticated and characterized biomaterials widely used across the biological sciences. However, as genetic and molecular data become increasingly complex, more rigorous quality assurance processes become essential.

To address these challenges, ATCC® is building a comprehensive reference omics database to warehouse genomic and transcriptomic sequencing data, detailed gene expression analyses and genetic status information for all ATCC® human and animal cell models. By integrating next-generation sequencing technologies, bioinformatics, and expertly curated reference data, we are creating a robust framework to ensure the validity and molecular integrity of ATCC® cell model collections. Access to the entire database is currently available through the ATCC® Cell Line Land from QIAGEN Digital Insights® and will be made available through the ATCC Genome Portal later in 2025.

Our work involves mapping transcriptomic landscapes, performing variant analyses, and standardizing data to deliver a reliable platform for researchers to verify the identity and quality of their biomaterials. We have completed whole-transcriptome and exome sequencing for over 600 human and mouse cell lines and expect to continue adding new data for at least 250 additional cell models per year. These datasets lay the foundation for improved quality control, the elimination of molecular ambiguity, the reduction of contamination risks, and enhanced reproducibility in biomedical research.

In this presentation, we will share our progress in sequencing cell models, discuss the challenges we've encountered, and outline long-term strategies to maintain these databases as indispensable global resources for the scientific community.

146 Comparison of Linker mediated PCR (LM-PCR) and Target enrichment mediated (TES) methods for genome wide retrieval of rare AAV integration events in preclinical models

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**Corresponding Author*

The retrieval of rare AAV integration events from preclinical and clinical specimens is important for understanding the safety and efficacy of gene therapies involving AAV and other DNA-based vectors. Given that AAV integration is considered a rare event and AAV genomes are frequently rearranged due to concatemerization in transduced tissues, it is vital to assess the sensitivity and quality of assays commonly used to detect AAV integration for the development of more standardized analytical assays supporting preclinical safety assessments. To address this, we generated a reference sample which consisted of mixing genomic DNA isolated from AAV transduced livers with synthetic dsDNA standards which were spiked in limiting dilutions. This reference sample set was used for evaluating and comparing two widely used methods—LM-PCR and TES—for their sensitivity, reproducibility, and efficiency in retrieving AAV integration sites with NGS platforms.

Results from mouse tissues injected with two doses of AAV vectors revealed integration frequencies ranging from 1 x 10^{-3} to 3 x 10^{-2} integration events per haploid genome depending on the method applied and dose analyzed. The synthetic standards were detected at a frequency of 1.5 x 10^{-5} using LM-PCR and 1.5 x 10^{-4} copy numbers per haploid genomes with TES, with LM-PCR showing higher sensitivity compared to TES. Generally, NGS-based relative clonal abundances were more easily resolvable with LM-PCR-based methods compared to TES. However, LM-PCR also yielded more PCR artifacts, as evidenced by the retrieval of false positive reads specific to the spiked in standards, suggesting that TES methods are less prone to such artifacts. A comprehensive genome wide distribution of AAV integration site patterns in the mouse genome and their association to cancer-related genes was evaluated based on over 20,000 integration sites detected in the liver of AAV dosed mice. A significant advantage of TES is its ability to capture integration events involving fusion of non-ITR regions to the host genome, which are challenging to detect with tiled primers in LM-PCR. This ability allowed for the generation of a map of fragile regions of the AAV genome involved in AAV integration and concatamerization events, indicating that the 3' end of the AAV vector may have a higher probability of being involved in integration and concatamerization events.

This comparative study highlights the strengths and limitations of the most commonly used NGS methods for detecting rare AAV integration events, providing the research community with valuable insights to guide the selection of appropriate methods for analyzing rare AAV/DNA integration events for preclinical safety studies.

147 Evaluation of Target Enrichment Sequencing to Assess AAV Integration Patterns for the Safety Assessment of Gene Therapies

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**Corresponding Author*

Recombinant adeno-associated viruses (rAAVs) are frequently used as vector for gene therapy due to their ability to transduce various cell types and their favorable safety profile. RAAV viral genomes primarily persist as episomes within the nucleus, but a small percentage of integration into the host genome has been reported in both nonclinical and clinical studies. In murine models, rAAV integration has been linked to the development of hepatocellular carcinoma, though this finding has not been observed in other species or clinical trials. To further investigate this risk across nonclinical and clinical samples, the development of a reliable pipeline to identify rAAV integration sites is paramount. While various methods have been successfully developed for lentiviruses, assessing the integration profile for rAAVs presents unique challenges due to their low integration frequency and the occurrence of viral genome rearrangements. Additionally, the absence of well-characterized controls impedes the assessment of different techniques.

In this study, we used stable cell clones as tools to evaluate Target Enrichment Sequencing (TES) for AAV integration site analysis. These clones were generated by leveraging the tendency of rAAVs to integrate in genomic double-stranded DNA (dsDNA) breaks. DsDNA breaks were induced in the *PTPN12* and *Meg8* genes via CRISPR/Cas9-mediated cleavage in the human MCF10A cell line, followed by transduction with a rAAV-GFP tool vector. Subsequently, single-cell cloning was performed. The clones were thoroughly characterized by Vector Copy Number (VCN) quantification via ddPCR, and long-range PCRs, which demonstrated homozygous and heterozygous integrations in the intended target site of varying sizes. Discrepancies between VCN and long-range PCR also suggested additional integrations at unknown sites. Further characterization using TES with PacBio long-read sequencing revealed that most integrations indeed occurred at the CRISPR/Cas9 target site, but approx. 40% of the clones exhibited integrations at random loci, including repetitive regions that were difficult to map. Integrated viral genomes were truncated, with sizes ranging from 70 bp to concatenated integrations of multiple AAV genome copies. Rearrangements of the vector sequence and integrations containing parts of the plasmid backbone used to generate the rAAV were also identified. Additionally, deletions were detected in the human genome around the AAV integration site. Whole genome sequencing confirmed the TES results. Finally, the limit of detection of TES was evaluated.

In conclusion, this study characterizes TES' performances for AAV integration site analysis, aiming to support the safe development of gene therapies. Additionally, our findings provide valuable insights into the patterns of CRISPR/Cas9-targeted AAV integration, demonstrating that integrations do not always occur at the intended target and can be accompanied by genomic deletions, vector rearrangements, and truncations.

148 Highly Sensitive Detection of Integration Sites by S-EPTS/LM-PCR and TES for Various Types of Therapeutic Vectors

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Safety is critical for development and application of every therapeutic vector in cell and gene therapy. Cases of insertional mutagenesis in previous clinical trials have demonstrated importance of detection, tracking and further characterization of **vector integration sites (IS)** from preclinical development to longitudinal monitoring of patients. Originally developed for integrating vectors, such as gamma-retroviral (gRV) and lentiviral vectors (LV), highly sensitive and specific detection methods have been further refined allowing detection of rare integration events of non- or low-integrating vectors, such as recombinant adeno-associated viral (rAAV) vectors or non-viral vectors.

We have developed **shearing extension primer tag selection ligation-mediated PCR (S-EPTS/LM-PCR)** to achieve high sensitivity, fast turnover and high specificity in IS detection. S-EPTS/LM-PCR was validated for LV based on common long terminal repeat (LTR) sequences. Via analysis of cell clones representing single, specific IS in polyclonal background, the assay's dynamic range was confirmed to cover accurate quantification for clinically relevant clonal contributions. S-EPTS/LM-PCR detects IS using a set of nested primers specific for the vector of interest. As other PCR based methods, S-EPTS/LM-PCR allows detection of vector IS containing the primer binding sites. S-EPTS/LM-PCR therefore is particularly well-suited for integrating vectors that are characterized by stable LTR regions present in a multitude of different vectors.

We have further developed an approach utilizing **target enrichment sequencing (TES)** to capture the entire vector sequence. Thereby, TES enables detection of vector fragments integrated into the host cell genome. This method is particularly advantageous for AAV vectors, which are known for rearrangements and partial vector integration (Batty et al., 2024). Both methods, S-EPTS/LM-PCR and TES, can be adapted and qualified for any type of vector by applying sequence-specific primers or probes.

Sequencing data generated from these experimental methods is analyzed using tailored, comprehensive bioinformatics framework, designed to facilitate IS analysis through the core tool, **GENE-IS** (Afzal et al., 2017). Beyond the representation and quantification of IS by GENE-IS, the framework extends to advanced downstream assessment, allowing, e.g., IS distribution profiling, identification of common IS and IS in proximity of cancer-associated genes, or assessment of sample diversity, enhancing insights into clonal dynamics. The key component of our framework is safety assessment and understanding the risk of insertional mutagenesis posed by vector integration into the host genome.

Complementing the two methods, we have developed a **whole-genome sequencing (WGS)** analysis framework. Combination of WGS with either S-EPTS/LM-PCR or TES delivers a comprehensive, whole genome perspective on IS as well as structural genetic variants, thus enabling thorough evaluation across preclinical and clinical studies and of particular interest in cases of serious adverse events in gene therapy.

To date, our methods for IS analysis have been applied in more than 60 clinical studies as well as many pre-clinical studies and generated data were part of safety assessment enabling approval of multiple gene therapies using gRV, LV and rAAV.

149 Redefining rAAV Vector Analysis and Quality Control with Orthogonal Long-read Sequencing Technologies.

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A major challenge in recombinant adeno-associated virus vectors (rAAV) production arises from the difficulty for traditional identity analytical methods such as PCR amplification and DNA sequencing of the encapsulated vector genomes to accurately assess the quality of the material produced. Massive parallel sequencing can be used to provide identity and quality information on rAAV drug products as well as the effect of upstream and downstream processes on the resulting rAAV. However, technical and bioinformatic processes greatly influence the quality and reliability of the produced information.

To overcome these challenges, we have developed an integrated platform for the analysis and quality control of rAAV based on high-throughput, long-read sequencing technologies and a standardized bioinformatic pipeline. Viral genomic DNA is extracted and processed to generate DNA library preparations using a set of high-quality quality standards to control potential extraction and sequencing biases. Viral DNA sequencing is implemented using both SMRT-seq (PacBio) and Oxford Nanopore (ONT) technologies.

Both platforms were compared in terms of their sequencing output, read quality, mappability on the vector sequences, percentage of partial and intact vector genomes, as well as the ability to detect contaminating host cell and plasmid DNA sequences. As an orthogonal method, duplex digital-PCR was used as a baseline for vector integrity quantification. We also dissected potential biases in the SMRT-seq and ONT library preparation processes, quantifying their impact on the downstream analysis. We quantified a bias towards long DNA fragments generated during SMRT-seq library preparation, which is not present in ONT libraries. SMRT-seq outcomes depend also on the second-strand generation strategy used, while ONT sequencing is instead able to sequence single-stranded DNA directly, though with less accuracy. We have developed and implemented internal process controls to correct for these technical challenges.

A novel bioinformatic pipeline quantifies ITR-to-ITR sequences as well as the potential presence of rep-cap, host cell and plasmid sequences. The pipeline performs first a raw quantification of the detected sequences, followed by a corrected estimation based on the inferred biases, allowing for a realistic assessment of the rAAV products.

This novel process relying on strong internal and process controls can be used to provide Critical Quality Attributes (CQA) of rAAV preparations for use in pre-clinical and non-clinical studies.

Innovations in in vivo Targeting of HSPCs and Immune Cells

150 *In Vivo* HSC Gene Editing for Correction of the Sickle Cell Mutation Using RNA Gene Writers

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Tessera Therapeutics is pioneering a new approach to genome engineering through the development of Gene Writing and delivery platforms. Our RNA Gene Writers leverage all-RNA components and harness the mechanism of target-primed reverse transcription, to edit genetic information without the need to introduce DNA double stranded breaks. RNA Gene Writers can introduce a broad range of genomic alterations, from writing in a transgene to introducing single nucleotide changes, providing a versatile toolkit for addressing monogenic diseases. Here, we applied RNA Gene Writers to correct the sickle cell disease (SCD) causing mutation HBB E6V, in hematopoietic stem cells (HSCs), with the aim of achieving therapeutically relevant levels of gene correction *in vivo*.

We first modeled the efficiency of HBB editing by introducing HBB Makassar variant (E6A) in WT CD34+ human HSCs. We observed an average of 74% editing *in vitro* after optimization of the RNA template and RNA Gene Writer expression. We measured high engraftment levels and maintenance of ~70% HBB editing in long-term repopulating HSCs and their multi-lineage progeny at 16 weeks post-transplant in mice. Comparable editing levels were also maintained after secondary transplant. We then demonstrated efficient editing of HSCs from multiple SCD patients, showing ~70% correction of sickle alleles to WT. Erythroid differentiation from edited cells showed 98% restoration of normal adult hemoglobin levels by HPLC and abrogation of cell sickling.

RNA Gene Writers are designed to be delivered as all-RNA compositions, facilitating *in vivo* delivery by lipid nanoparticles (LNPs) and eliminating the need for viral vectors and DNA template-based editing. We previously demonstrated that our HSC-targeting LNPs achieved ~95% delivery of GFP reporter cargo to HSCs in humanized mice and non-human primates (NHP) through intravenous administration.

We have now demonstrated 62% HBB Makassar install *in vivo* in LT-HSCs (Lin-CD34+CD38-CD90+ cells) in humanized NBSGW mice, after a single dose of RNA Gene Writer formulated in HSC-targeting LNP. Comparable editing levels were maintained in HSCs and multi-lineage progeny after transplanting *in vivo* edited CD34+ into secondary mice, demonstrating absence of functional impairment after *in vivo* editing and successful targeting of bona-fide LT-HSCs. We have also shown in repeat dosing studies that a second administration of LNPs enabled a near linear increase in the percentage of HBB editing in LT-HSCs.

Importantly, in cynomologous NHP we showed an average of 24% HBB Makassar install *in vivo* in LT-HSCs (day 14 post treatment). Editing levels were stable up to 4 months and edited cells were present in multiple hematopoietic lineages. Longitudinal analysis of edited cells in NHP provided insights on the kinetics of output after editing, showing stable myeloid output as early as 2 months after treatment.

Finally, we validated the robustness of our *in vivo* HSC editing system by using RNA Gene Writers targeting an independent genomic locus (B2M). We achieved high (>70%) and stable levels of *in vivo* editing of B2M in NHP LT-HSCs that was maintained for over 6 months post treatment, further demonstrating the applicability of our findings to other targets.

All together, we demonstrated that RNA Gene Writers can efficiently edit long-term HSCs *in vivo* in mouse and NHP, across multiple targets, and have the potential to provide therapeutic benefit to patients with SCD.

151 In Vivo Generation of TRAC-Targeted CAR T Cells via Site-Specific Integration Enables Cell-Specific Engineering and Potent Antitumor Activity

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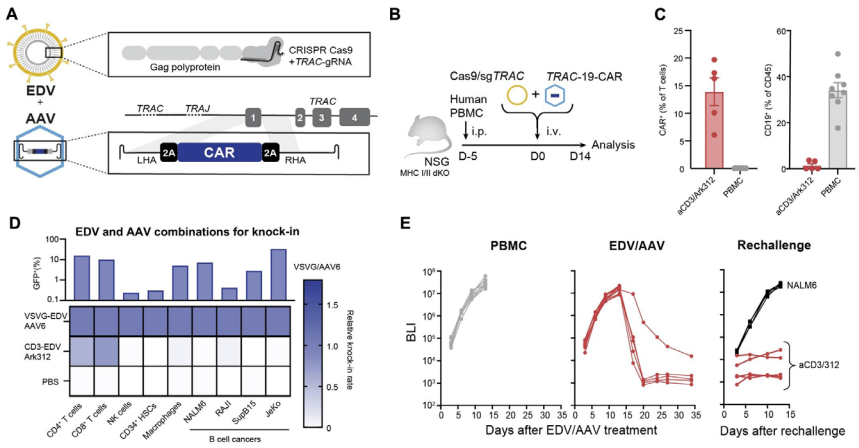
Engineered T cells, reprogrammed to express chimeric antigen receptors (CAR) or T cell receptors (TCR), have transformed cancer treatment and are being explored as therapeutics for autoimmune disorders and infectious

diseases. Enhancing T cell function through genome editing—either by disrupting endogenous genes or precisely inserting DNA payloads—has shown significant promise. However, ex vivo manufacturing of these therapies is costly, complex, and logistically challenging, limiting their scalability and accessibility. In addition, adoptive T cell therapies require lymphodepletion, which can lead to severe side effects such as cytopenias and subsequent infections.

In vivo generation of engineered T cells represents a promising advance in cancer immunotherapy because it bypasses ex vivo manufacturing and lymphodepletion preconditioning, thereby offering the potential for more accessible and effective treatments for cancer patients. However, current methods for in vivo delivery rely on transient or random integration of DNA payloads, which may limit their persistence and safety.

We hypothesized that integrating a promoter-less CAR transgene at the TRAC locus in vivo would combine T cell-specific and physiological CAR expression. To achieve this, we developed the first method for in vivo site-specific integration of large DNA payloads in human immune cells. We used a two-vector system in which an enveloped delivery vehicle (EDV) delivers a Cas9 ribonucleoprotein (RNP) and an adeno-associated virus (AAV) carrying a homology-directed repair (HDR) template ensures precise integration at the TRAC locus. We optimized both vectors for delivery specificity and gene targeting efficiency. First, we pseudotyped the EDV envelope with a mutated VSVG to prevent interaction with the LDL receptor and then targeted it to T cells using an anti-CD3 antibody, thereby ensuring both specificity and T cell activation. Second, we evolved a novel AAV capsid, named Ark312, which is resistant to neutralizing antibodies and exhibits T cell-biased tropism.

To assess our system, we performed a single injection of the two vectors encoding a TRAC-targeted CD19 CAR-2A-EGFRt into a humanized mouse model (MHC I/II DKO NSG engrafted with human PBMCs). Notably, our in vivo approach achieved TRAC-CAR engineering in up to 20% of T cells in the spleen—an efficiency unprecedented for in vivo genome editing techniques—and resulted in deep B cell aplasia. To demonstrate the exquisite specificity of our approach, we performed an ex vivo experiment in which we knocked in a GFP transgene at the clathrin A (CLTA) locus, a gene ubiquitously expressed in T cells, B cells, NK cells, macrophages, hematopoietic stem cells, and multiple malignant B cell lines. We showed that only T cells were engineered using our two-vector system, confirming that the combination of our delivery method and TRAC-restricted expression results in a highly T-cell–specific therapy. To validate the therapeutic potential of in vivo–generated TRAC-CAR T cells, we tested the vectors in an aggressive humanized B-cell leukemia tumor model (Nalm6) and observed complete tumor resolution. The edited T cells were functionally persistent, as demonstrated by their ability to control tumor growth upon rechallenge. Thus, in vivo–generated TRAC-CAR T cells combine cell-specific and physiological CAR expression while bypassing ex vivo manufacturing and patient preconditioning.



In vivo generation of TRAC CAR T cells A) Schematic representation of TRAC-targeting Cas9 EDV and the TRAC-CD19 CAR HDR template. **B)** Schematic of the humanized B cell aplasia mouse model. **C)** Flow analysis of CAR T cells and B cells in spleens. **D)** The vector combos delivering a GFP at the CLTA locus were tested ex vivo on the indicated cell types. heatmap of the relative KI **E)** Tumor burden measurement after Nalm6 + human PBMCs with or without the EDV/AAV combination. Age matched humanized mice were used for the rechallenge control (black)

152 Efficient and Liver-Detargeted *In Vivo* Multiplex Gene Editing of Human Hematopoietic Stem and Progenitor Cells

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In vivo genetic engineering of hematopoietic stem and progenitor cells (HSPCs) has the potential to transform the treatment of numerous diseases with significant unmet needs. An ideal delivery system for gene-modifying cargos should a) deliver transient species, such as ribonucleoproteins (RNPs), to reduce immunogenicity and off-target gene editing, b) efficiently target human HSPCs in their quiescent state, c) access HSPCs in the bone marrow niche, and d) minimize off-tissue delivery, particularly to the liver.

To this aim, we developed targeted virus-like particles (DLVR) carrying RNP gene-editing cargoes fused to a retroviral capsid and equipped with an envelope with targeting moieties designed to confer high tropism for human HSPCs. Our first design incorporated a Baboon endogenous retrovirus glycoprotein (BaEVTR) and an MMLV capsid fused to an RNP Adenine Base Editor (ABE) targeting the human Beta-2-Microglobulin (B2M) gene. These particles achieved high editing efficiency (up to 80%) *in vitro* in human HSPCs without cytokine stimulation. Systemic intravenous delivery in a short-term humanized NBSGW mouse model resulted in double-digit editing levels in BM-resident HSPCs (20%). Our BaEVTR DLVR also displayed a high tropism for B cells. To evaluate HSPC targeting under more challenging conditions, we dosed BaEVTR DLVR particles in long-term humanized mice with fully engrafted human BM (92%) and high proportions of human B cells in the peripheral blood (PB) and spleen (23% and 19%, respectively). In this scenario, BaEVTR DLVR achieved high editing levels in PB and spleen (26% and 35%, respectively) while maintaining robust BM HSPC editing (5%). Notably, transduction enhancers such as vectofusin-1 (VF1) are dispensable *in vivo*. Most importantly, and differently from other delivery systems aimed at HSPCs, our BaEVTR DLVR particles essentially avoid human hepatocytes *in vitro* and *in vivo* in the FRG mouse model (0.9% vs. 18% editing achieved with a VSV-G DLVR at equivalent dose).

To enable multiplex editing, we developed BaEVTR DLVR particles carrying ABE complexed with two gRNAs targeting B2M and BCL11A, achieving efficient editing at both loci *in vitro* in resting HSPCs (up to 85% and 64%, respectively) and *in vivo* in NBSGW mice (average of 22% and 12%, respectively). Further enhancing potency and specificity, we next designed a new class of engineered envelopes (BaEVX) swapping the cytoplasmic domain of BaEVTR with that of different retroviruses to improve compatibility with the DLVR platform. Two BaEVX constructs demonstrated equal multiplex editing capability, but superior editing potency compared to BaEVTR *in vitro* in absence of VF1 (up to 59% vs. up to 22%) and, when further engineered with a CD133-specific binder, achieved unprecedented levels of in vitro editing of resting HSPCs without transduction enhancers (up to 92%). *In vivo* dosing of BaEVX particles in humanized mice is ongoing. Importantly, BaEVX particles produce in high titers in suspension cell lines, and manufacturing scale-up is underway to enable studies in cynomolgus macaques. Overall, we demonstrated that our HSPC-tropic BaEVX particles efficiently achieve RNP-mediated editing in human HSPCs under resting conditions *in vitro* and *in vivo*, accessing HSPCs within their natural BM niche. BaEVX DLVR enables multiplex *in vivo* editing of HSPC while avoiding liver transduction, making it a promising candidate for clinical translation.

153 Targeted LNP Delivery of an RNA Gene Writer *In Vivo* Enables Generation of functional CAR-T Cells in a Humanized Mouse Model

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Autologous *ex vivo* Chimeric Antigen Receptor (CAR) T-cell therapies are a powerful treatment option for relapsed and refractory leukemias and lymphomas. However, significant challenges limit access to these therapies, including long vein-to-vein times for drug delivery, viral vector supply chain constraints, complex manufacturing processes, and high costs. While allogeneic CAR-T therapies offer an alternative, they have not yet matched the therapeutic efficacy of conventional autologous T-cell therapies, emphasizing the urgent need for same-day *in vivo* treatment solutions.

RNA Gene Writers are designed to edit cellular genomes using RNA templates by harnessing the mechanism of target-primed reverse transcription (TPRT). These Gene Writers can be engineered to perform various editing reactions, from introducing gene-length DNA sequences, to rewriting genomic regions by making single nucleotide substitutions, to small insertions or deletions. These edits can be achieved by delivering all-RNA compositions to primary cells *in vitro* or *in vivo*, eliminating the need for viral vectors and DNA template-based genome editing methods.

Initial testing of T cell specific lipid nanoparticles (LNPs) containing a Gene Writer with a B-cell maturation antigen (BCMA) CAR template *in vitro* led to an observed average of 16% BCMA CAR+ T cells, in primary human T cells that were pre-activated with TransACT. Moreover, these CAR+ T cells generated *ex vivo* could clear antigen specific tumors *in vivo* in human xenograft mice, mediating robust cytotoxic response and CAR-T cell expansion.

For *in vivo* applications, targeting and editing of resting T cells is a critical milestone to overcome. Utilizing a CD19 CAR template, we delivered T cell specific LNPs to freshly isolated human primary T cells *in vitro*, without pre-activation, generating an average of ~40% CD19 CAR^+ cells. These CAR+ cells were observed to be potent in their ability to drive cytotoxicity, cytokine production and CAR-T cell expansion in response to multiple tumor types. Additionally, we demonstrated Gene Writers delivered with T cell specific LNPs integrated CAR transgenes targeting CD20 into resting non-human primate (NHP) T cells *in vitro*, achieving approximately 60% CAR writing and enabling a path towards writing in NHPs *in vivo*.

For *in vivo* proof-of-concept studies, we tested two humanized xenograft mouse models for CAR specific activity. A single intravenous infusion in a tumor bearing xenograft mouse model of Gene Writer with a CD19 CAR template formulated in targeted LNPs generated an average of 24% CAR-T cells *in vivo* that were able to expand and eradicate tumor burden compared to control. Additionally, LNP delivery of Gene Writer with a CD20 CAR template in a CD34+ engrafted humanized mouse model resulted in an average of 30% CAR-T cells *in vivo* and consequent elimination of human B cells, highlighting its therapeutic potential in autoimmune diseases.

The ability to generate *in vivo* CAR-T cells using Gene Writers and our proprietary LNP delivery platform provides a unique opportunity to potentially eliminate the challenges associated with conventional *ex vivo* CAR-T cell therapies and viral based *in vivo* CAR-T gene therapies.

154 Targeted Multiplexed Virus-like Particles (MVPs) enable robust in vivo Hematopoietic Stem Cell (HSC) engineering

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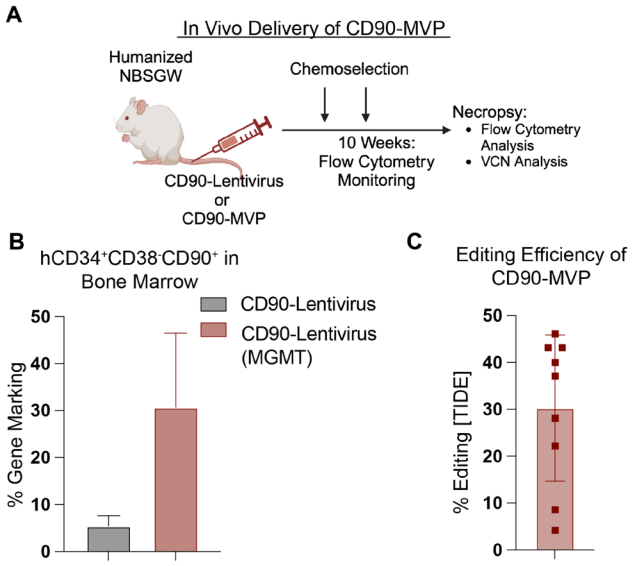
Background:
Ex vivo Hematopoietic Stem Cell (HSC) gene therapy has significantly changed the treatment landscape of many chronic disorders, including sickle cell disease (SCD). However, barriers persist regarding the accessibility of these treatments, as these platforms require significant medical infrastructure. The advent of in vivo delivery strategies in the clinic will substantially broaden the accessibility of gene therapy. However, current putative delivery platforms lack iterative capacity, requiring binary choices to be made on gene modification strategies and cell population

targets. Here, we report the development of HSC-targeted Multiplexed Viral-Like Particles (MVPs), enabling concomitant *in vivo* transduction and gene-editing in HSCs.

Approach/Results:
HSC targeting was enabled by studding MVPs with an anti-CD90 single-chain variable fragment (CD90-MVPs). Intravenous administration of these particles in humanized NBSGW mice without the use of mobilization regimens displayed high specificity, with an average of 83% of all gene marked cells in the bone marrow (BM) expressing CD90 (n=9). In primary injected mice, average gene marking in the HSC compartment ($CD34^+CD38^-CD90^+$) was 2.6%. Transplantation of CD34+ hematopoietic stem and progenitor cells (HSPCs) from injected mice into secondary recipient mice showed an increased average gene marking of 14.8% (n=5), suggesting CD90-MVPs preferentially transduced long-term self-renewing HSCs. Depending on disease pathology, some disorders require a minimal threshold of gene modification chimerism for pathophysiological relief. This includes a 20% gene modification threshold found in clinical trials for SCD gene therapy. To enable the enrichment of gene-modified cells, we concomitantly expressed the MGMT(P140K) transgene which protects modified cells from carmustine chemoselection. After two round of selection, gene marking reached up to ~65% in HSCs. Additionally, CD90-MVPs administration and chemoselection did not perturb differentiation capacity as gene marking was found in all hematopoietic lineages (i.e. B-cell, T-cells, Monocytes, etc.). We next sought to apply the CD90-MVP platform to the delivery of therapeutic payloads for SCD gene therapy. CD90-MVPs loaded with Cas9 protein and co-transfected with HBG-directed gRNA and MGMT(P140K) transgene were administered on $CD34^+$ HSPCs *ex vivo*. We observed an 8-fold higher gene transduction rate on $CD34^+CD90^+$ HSCs than 90^- HSPCs. Additionally, 66.33% of HBG promoter alleles in $CD34^+CD90^+$ HSCs contained indels. This was concomitant with a 50% increase in fetal hemoglobin expression in erythrocytes derived from edited $CD34^+CD90^+$ HSCs. *In vivo* administration of CD90-MVPs in humanized NBSGW mice confirmed successful gene modification of $CD34^+$ HSPCs with an average vector copy number (VCN) of 0.18 and HBG allele editing frequency of 30.3%.

Conclusions:
In summary, CD90-MVPs is a novel in vivo HSC gene delivery platform, allowing for the multiplexing of HSC engineering strategies and enabling the broad use of gene therapy. The multiplexing of both integration gene modification as well as gene editing strategies enables a more iterative platform, which could increase the efficiency of gene therapy broadly. Furthermore, the high specificity of CD90-MVPs without the use of mobilization, surpasses a high safety benchmark as gene therapy moves toward in vivo administration and enables a wider application of HSC gene therapy.



(A) Experimental design for in vivo transduction of human HSCs in xenograft NBSGW murine model. **(B)** Transduction efficiency of CD90-targeted lentiviral particles on HSC population with or without MGMT chemoselection . **(C)** Allele editing efficiency at the HBG -1113 locus of human $CD34^+$ (hCD34+) HSPCs isolated from xenograft NBSGW mice injected with CD90-MVP.

155 In Vivo Hematopoietic Stem Cell Gene Therapy for HIV by Base Editing of CCR5 Alone and in Combination with HIV Decoy Receptor Expression

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Our approach utilizes helper-dependent adenovirus (HDAd) vectors that target therapeutic gene delivery to HCSs in vivo. Here, we employed this approach for HIV therapy. We screened a panel of single guide RNAs paired with base editors in TZM-bl cells to achieve CCR5 knockout and found that a sgRNA targeting the CCR5 start codon (referred to as SILENCE2) combined with an ABE (ABE8e) and a sgRNA that creates a premature stop codon (referred to as STOP1) combined with a CBE (BE4max) or a laboratory-evolved CBE (TadCBE) exhibited the highest on-target editing rates as well as CCR5 down-regulation. We then produced all-in-one base editor HDAd vectors containing the sgSTOP1 and sgSILENCE2 alone or in combination with a Dual editor (TadDE). In addition to the editing machinery, the vectors contained an expression cassette for $mgmt^{P140K}$, which allows for enrichment of edited cells by selection with low doses of O^6BG/BCNU. Downregulation of CCR5 and inhibition of HIV infection was demonstrated in TZM-bl cells. CCR5 base editing by the HDAd-STOP1-TadCBE and HDAd-STOP1-SILENCE2-TadDE vectors conferred 98% inhibition of HIV-BaL infection. Next, we transduced human $CD34^+$ cells from the cord blood of three healthy donors and subjected them to O^6BG/BCNU selection and in vitro differentiation over 42 days resulting in $CD4^+CCR5^+$ T-cells. At the end of differentiation, cells were exposed to HIV. HIV genome titers in supernatants of T-cells derived from HDAd-STOP1-TadCBE and HDAd-STOP1-SILENCE2-TadDE-transduced $CD34^+$ cells were 21-fold and 4-fold lower, respectively, compared to T-cells derived from untransduced $CD34^+$ cells. Based on HIV-Gag flow cytometry of T-cells, inhibition of HIV replication was 84% and 70%, respectively. For in vivo studies, we have generated humanized mice by transplanting human $CD34^+$ cells that can differentiate into $CD4^+/CCR5^+$ T-cells, into NSG neonate mice. After the engraftment, mice were mobilized with G-CSF/AMD3100 and intravenously injected with HDAd-STOP1-TadCBE or HDAd.control followed by O^6BG/BCNU selection. Eight weeks after selection, mice were challenged by intraperitoneal injection of HIV. Viral load in plasma and tissues was measured 7 weeks later. CCR5 editing rates in bone marrow mononuclear cells were on average 50% at the end of the study without significant off-target editing at any of the top 20 sites identified. HDAd-STOP1-TadCBE treated animals had 8-fold lower HIV plasma titers than control animals. Significantly lower HIV loads were also found in the lower GI tract and the liver. A major problem in HIV therapy is the development of virus mutants that escape therapy, especially if the therapy targets only R5-tropic HIV strains. To reduce the chance of escape mutants, we combined the CCR5 knockout module with an eCD4-Ig decoy receptor expression cassette and compared it to single-module HDAd vectors in the inhibition of HIV infection of TZM cells. On average, HDAd-STOP1-TadCBE (CCR5KO), HDAd-eCD4Ig, and HDAd-combo inhibited HIV infection 93, 77, and 98%, respectively The combo vector exerted a significant additive inhibitory effect of the CCR5-KO and eCD4Ig expression modules and provided inhibition of both CCR5 and CXCR4 tropic HIV strains. We have started *in vivo* HSC transduction studies in non-human primates using an HDAd-combo vector and a chemotherapy-free approach to expand HDAd-combo transduced HSCs.

156 Analysis of safety, selectivity, and efficacy of in vivo hematopoietic stem cell transduction after intravenous administration of AVID adenovirus vector in Rhesus macaques

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Viral vectors represent a clinically validated platform for therapy of numerous genetic diseases with demonstrated potential of transforming patients' lives. Although several FDA-approved therapies are based on adeno-associated vectors (AAV), their relatively small therapeutic cargo capacity limits their utility for therapy of genetic diseases via gene editing approach. Viral vectors based on adenovirus (Ad), on the other hand, can deliver much larger

therapeutic genes compared to AAV and represent an attractive platform for therapeutic gene editing in vivo after intravenous vector administration. We recently engineered a novel AVID-388 Ad vector platform, which efficiently delivered reporter genes to human hematopoietic stem cells (HSCs) after intravenous administration without triggering cytokine storm, hepatotoxicity, nephrotoxicity, or thrombocytopenia. We envision that the AVID vector platform can deliver gene editing enzymes to HSC in vivo for correcting sickle cell disease (SCD) and other genetic diseases of the blood and immune system. In this study, we conducted the first-in-primate analysis of safety, selectivity, and efficacy of *in vivo* HSC transduction following intravenous administration of AVID-388-based vector, E7-GFP, in rhesus macaques. To better mimic the potentiated inflammatory state exhibited by SCD patients, we recruited rhesus macaques with a potentiated inflammatory state due to SIV infection. In our pilot experiment, rhesus macaques were conditioned with G-CSF, and bone marrow HSCs were mobilized into peripheral blood using plerixafor. The E7-GFP vector was administered intravenously at a single dose of 0.4×10^{12} v.p./kg, and bone marrow aspirates were collected 3- and 7-days after vector administration. $CD34^+$ cells were enriched to 80% purity using a primate-specific CD34 antibody and magnetic columns, then stained with an antibody panel to quantify GFP^+ cells in different bone marrow populations by flow cytometry. Our experiments showed that *in vivo* transduction of total $CD34^+$ cells was 0.3%. However, in the most primitive LT-HSC population (cells of $CD34^+CD45RA^-CD117^{high}CD90^+$ phenotype), the percent GFP^+ cells reached 15.1%, representing a ~50-fold increase in selectivity, consistent with the data from our study in humanized huCD34+ NSG mice. Importantly, the intravenous E7-GFP vector administration did not trigger thrombocytopenia, a hallmark of acute systemic inflammation and coagulopathy observed after intravenous administration of Ad vectors at high doses, thus demonstrating a significantly improved safety profile of the AVID platform compared to earlier generation Ad vectors. Taken together, the data from this first-in-primate study demonstrated improved safety, high selectivity, and near therapeutic level efficacy (~20% of HSC correction is required for ameliorating SCD) of in vivo LT-HSC transduction following i.v. administration of our novel engineered E7-GFP vector to NHPs. These data strongly support the rationale for developing clinically useful AVID platform-based vectors for in vivo HSC-targeted gene therapy of SCD and other genetic diseases of blood and immune system.

Conflict of interest statement: DSH is a co-founder, shareholder, and officer of AdCure Bio, LLC, which develops adenovirus technologies for therapeutic use. This work was supported by funds from the US NIH grants AI107960 and CA267694, Pediatric Research Alliance, as well as contracts from AdCure Bio, LLC.

Oligonucleotide Therapeutics I

157 Preclinical development and *in vivo* delivery of antisense oligonucleotides for targeted *NF1* exon 17 skipping.

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To give more specific and efficacious treatments, Neurofibromatosis Type 1 (NF1) patients need new targeted therapeutics that can be used alone or in combination with the currently approved MEK inhibitor, selumetinib. Antisense oligonucleotides (ASOs), chemistries of which are already clinically approved for other rare pediatric diseases due to their tolerability, could provide mutation-specific therapies. Certain NF1 pathogenic variants (PVs) can be rescued by modifying splicing using ASOs to induce skipping of exons containing PVs to restore expression of functional neurofibromin. Our research has published *in vitro* evidence highlighting the therapeutic potential of antisense oligonucleotides (ASOs) for numerous NF1 pathogenic variants through targeted exon skipping, including exon 17. To provide pre-clinical validation of ASOs *in vivo* and to facilitate translational development, various

mouse models have been developed. The first is a conditional tamoxifen-inducible systemic knockout *Nf1* mouse model with floxed NF1 alleles. Following tamoxifen inactivation of floxed *Nf1* alleles, adult CAGGCre-ER^{TM};*Nf*$^{Flox/Flox}$ mice lose expression/function of *Nf1* systemically; we refer to this as the "acute" model as mice die 12 days post tamoxifen induction. We have bred the acute model to the humanized exon 17 allele with patient-specific pathogenic variant G629R to obtain CAGGCre; NF$^{Flox/hG629R}$ mice. We have used these mice to evaluate two ASO delivery platforms: conjugation of ASOs with morpholino chemistry (PMO) to cell penetrating peptides (PPMO) and recombinant Adeno-Associated Viral (AAV) vectors carrying U7-SnRNA expression cassettes to give proof-of-concept of *in vivo* exon skipping efficacy of our optimized ASOs. We treated five CAGGCre; NF$^{Flox/hG629R}$ mice with a single ICV dose of 1.34 mg/kg exon 17 PMO conjugated to a CPP (PPMO) designed for cell membrane penetration and endosomal escape (5 mice with PBS injection were used as control). One week post PPMO/Ctrl injection tamoxifen induction was performed. We see PPMO-driven skipping of the humanized allele in RNA from brain and optic nerve (both NF1 relevant tissues). We also see restoration of neurofibromin protein expression and partial functionality (as measured by reduced pERK/ERK) in brain. PPMO has been limiting to date. We are working on improving PPMO solubility, purity, and production scale to allow multiple dosing schedules. We were also able to treat small cohorts of CAGGs-Cre$^{Flox/hG629R}$ mice with AAV-U7-SnRNA vectors delivered by ICV. We have been able to show evidence of *in vivo* exon skipping of the humanized allele in the brains and whole blood of AAV-U7-SnRNA treated mice using q-RT-PCR. This provides evidence of *in vivo* efficacy and gives rationale for dose and route that may be effective in future studies with improved vectors.

Conclusions: This preliminary data is the first to show *in vivo* splice modulation and restoration of neurofibromin protein expression in NF1-relevant tissues including the brain and optic nerve. Detection of skipping in blood provides an important clinical biomarker of on-target activity.

158 Silencing of *Inhbe* mRNA Using GalNAc-siRNAs Induces Durable Weight Loss in a Mouse Model of Diet-Induced Obesity

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We aimed to develop small interfering RNAs (siRNAs) to suppress expression of the liver gene inhibin βE (Inhbe). Human heterozygous INHBE loss-of-function (LoF) carriers have reduced abdominal fat, and reduced risk for type 2 diabetes (T2D) and coronary artery disease, suggesting that lowering INHBE expression by 50% may restore a healthy metabolic profile in obesity. We investigated the impact of reducing Inhbe expression, via N-acetylgalactosamine (GalNAc)-conjugated stereopure siRNA targeting Inhbe mRNA (Inhbe GalNAc-siRNA), on body weight and composition in a diet induced obesity (DIO) mouse model, alone or added to semaglutide, a GLP-1 receptor agonist (GLP-1RA). To develop siRNAs that suppress expression of Inhbe, we have optimized siRNA chemistry for desired pharmacological properties. In previously published work, we showed that incorporating phosphoryl guanidine backbone linkages in siRNAs, and controlling siRNA backbone stereochemistry, can improve the potency, tissue exposure, and durability of knockdown in mouse liver in vivo. These benefits corresponded with both an increase in thermal instability at the 5′-end of the antisense strand and improved Argonaute 2 (Ago2) loading. Since reporting this design, we have performed additional screening and optimization of our siRNA chemistry, further improving silencing durability. Using an updated design, siRNAs targeting mouse Transthyretin (Ttr) as a benchmark supported >50% knockdown of Ttr protein for ~50 days after a single 0.5 mg/kg dose, compared to a stereorandom reference siRNA based on a clinically proven format, which sustained >50% knockdown for only ~27-days post-dose. Using our advanced siRNA chemistry, we generated INHBE-00002 and INHBE-00003, stereopure GalNAc-siRNAs targeting mouse Inhbe. In DIO mice, INHBE-00002 supported >50% Inhbe mRNA knockdown compared to PBS, up to 28 days after a single 3 mg/kg subcutaneous dose, with significant weight loss compared to PBS-treated mice. The impact of the siRNA on weight loss was durable, as INHBE-00002-treated DIO mice remained significantly lighter than PBS-treated mice for the duration of the study (84 days). Using a different Inhbe siRNA (INHBE-00003), we explored the impact of adding Inhbe siRNA to

semaglutide treatment, as Inhbe regulates body weight through a peripheral mechanism distinct from GLP-1RAs. After 28 days of daily semaglutide, mice weighed ~9% less than PBS treated mice, which was similar to weight loss observed 28 days after a single dose of INHBE-00003. In INHBE-00003-treated mice, epididymal visceral fat mass was reduced by 40% relative to PBS, while quadricep muscle mass was not impacted. Adding a dose of siRNA at the start of the semaglutide dosing regimen (day 0) increased weight loss to 18% by day 28, compared to PBS-treated mice. By 28 days after last dose, mice treated with semaglutide had regained all lost weight whereas those treated with semaglutide and two doses of siRNA maintained their weight loss relative to PBS-treated controls. Taken together, these data show that we have developed an siRNA design that supports potent and durable knockdown. The impact of Inhbe siRNA on mouse body weight supports a strategy to achieve healthy weight loss in people with obesity by reducing INHBE expression. Our results indicate several potential uses for INHBE GalNAc-siRNA treatment as a therapeutic approach for people with obesity, including as a monotherapy, as an add-on to GLP-1RAs to potentiate weight los

159 Splice-Switching Antisense Oligonucleotide Drug Discovery for CLN3 Batten Disease

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Splice switching antisense oligonucleotides (SSOs) are short sequences of modified nucleic acids that can be designed to selectively base-pair to an RNA target and modulate pre-mRNA splicing. SSOs are a powerful drug platform with clinical success, including therapeutics designed specifically for individuals carrying unique pathogenic variants (n-of-1). CLN3 Batten disease is a fatal, pediatric lysosomal storage disease caused by mutations in the gene encoding CLN3, a lysosomal protein of unknown function. Symptoms, which begin in young children, include blindness, seizures, loss of motor function, and death in early adulthood. CLN3 Batten disease-causing variants can disrupt the open reading frame by creating a premature termination codon and produce a truncated, non-functional protein. We have devised a therapeutic strategy for treating CLN3 Batten disease caused by particular frame shift variants using SSOs to induce skipping of a targeted exon to re-frame the mutated transcript. Building off our previous work in successfully developing an SSO to reduce neurological disease burden in Cln3 Batten disease mouse models when delivered to the central nervous system, we next tested the SSOs in a pig model of the disease to target visual deficits associated with the disease. A single intravitreal treatment with an SSO induced robust exon skipping in the retina for up to 12 months and improved retinal function. We next leveraged our experience to target to a rare pathogenic variant, c.569dupG in exon 8 of CLN3. We devised an approach to induce skipping of the mutation-containing exon 8 by redirecting RNA splicing using SSOs. We designed and tested SSOs in patient-derived cell models of the disease and identified an SSO, Zebronkysen, that is potent and effective at inducing exon 8 skipping from CLN3 mRNA to correct the open reading frame. Zebronkysen has received FDA approval and is being used to treat twins with this rare pathogenic variant. To date, three doses have been delivered to the patients. Together our results suggest that SSO-mediated exon skipping is a promising therapeutic approach for multiple CLN3 Batten disease-causing variants.

160 Thiopurine-Based Oligonucleotide for Targeted Telomere Damage in Leukemia and Lymphoma Cell in Vivo

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Telomerase (TERT) is an enzyme activated in the majority of human cancers and critical for maintaining telomere length and cell survival. Telomere shortening is accelerated especially in hematologic malignancies with high

proliferative rate such as acute myeloid leukemia (AML) or diffuse large B-cell lymphoma (DLBCL). Previous attempts to target TERT^{+} cancers faced challenges, including the delayed clinical responses and on-target/off-tumor toxicities. Here, we present a cancer cell-targeted oligonucleotide designed to deliver a synthetic TERT substrate, 6-thio-2'-deoxy-guanosine (6tdG), damaging telomeres and triggering apoptosis. In vitro, 6tdG-oligonucleotides (6tdGOs) were selectively cytotoxic to human TERT^{+} AML and DLBCL cells without affecting activated T-cells or non-malignant TERT^{-} cells. Repeated intravenous administration of 6tdGO, but not 6tdG nucleoside, had significant and directly cytotoxic antitumor effects against xenotransplanted human AML and DLBCL models in immunodeficient NSG mice. 6tdGO showed enhanced antitumor activity when tested in syngeneic mouse inv(16) AML and in Em-myc/15A lymphoma models in mice. In immunocompetent mice, treatment with 6tdGO induced systemic, cancer cell-specific and CD8 T-cell-mediated antitumor immune responses. The abscopal effects of 6tdGO were abolished in mice lacking expression of *Sting1* or *Ifnar1* but not *Trl9*. These findings suggest that STING-mediated type-I IFN signaling was triggered in response to 6tdGO-induced cancer cell death, thereby promoting recruitment/activation of CD8 T-cells. Importantly, the repeated 6tdGO treatments were well-tolerated in humanized hCD34/NOG mice. Except for the reduced percentage of human B-cells, 6tdGO did not decrease the numbers of hematopoietic stem cells, myeloid cells, or T-cells. Overall, 6tdGO offers an effective and safer strategy against aggressive TERT^{+} hematologic malignancies with potential to activate T-cell based antitumor immunity.

161 Advancing HTT-lowering divalent siRNA therapy for enhanced safety and efficacy in the central nervous system

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Oligonucleotide therapeutics, including antisense oligonucleotides (ASOs) and small-interfering RNAs (siRNAs), are under clinical evaluation for neurodegenerative diseases such as Huntington's disease (HD). However, their efficacy and safety profiles have varied. Non-selective lowering of both wild-type and mutant huntingtin (HTT) mRNA and protein has demonstrated safety across species and shown the potential to reduce disease progression in HD mouse models and effective biodistribution through the brain of a large animal model. The specific thresholds of RNA and protein lowering required to alter disease progression remain undefined. The advent of divalent small-interfering RNA (di-siRNA) has provided a platform with enhanced efficacy and prolonged durability throughout the central nervous system (CNS) following a single administration. Here, we report an optimized HTT-lowering di-siRNA compound (di-siRNA^{HTT}) with superior efficacy and safety in small and large animal models. This advancement incorporates the novel extended nucleic acid (exNA) modification, which enhances stability by protecting the di-siRNA scaffold from endonuclease degradation. Additionally, the formulation includes artificial cerebrospinal fluid (aCSF) supplemented with calcium and magnesium to mitigate acute neurotoxicity during intracerebroventricular (ICV) delivery. This innovation enabled the safe evaluation of increased dosing regimens across species.

In YAC128 HD transgenic mice, a single ICV injection of the lead di-siRNA^{HTT} compound achieved significant silencing of both wild-type and mutant HTT protein throughout the brain. At a low dose, silencing was sustained for two months, and at a high dose, silencing was maintained for up to six months. Six months post-injection, we observed 85–95% HTT protein reduction in key brain regions, including the caudate, putamen, cortex, thalamus, and hippocampus. In lambs, the lowest dose achieved 74% HTT protein silencing in the caudate and 28% in the putamen, a 20% improvement compared to the previous di-siRNA architecture (no exNA). Using di-siRNA^{exNA}, dose-response experiments revealed maximum efficacy and safety at twice the initial minimum dose, silencing HTT protein >75% in the caudate and approximately 50% in the putamen. Higher doses, although more effective, were not well tolerated. Electroencephalogram (EEG) recordings conducted in lightly sedated lambs confirmed the

absence of seizures at lower doses, while higher doses showed focal seizures evolving to bilateral ictal activity, with initial spike-and-wave patterns in the left hemisphere progressing to generalized polyspikes followed by spike-and-wave discharges. Intrathecal delivery did not achieve comparable efficacy in brain regions relative to equivalent doses delivered ICV.

These findings highlight a novel di-siRNA^{HTT} therapeutic platform capable of robust, sustained HTT mRNA and protein silencing across species. The compound's safety profile and efficacy expand the opportunity to advance this modality into pre-IND enabling studies, offering a promising therapeutic avenue for Huntington's disease.

162 Computationally Designed Miniproteins Enable Tissue-Specific Oligonucleotide Delivery Through Cell Surface Receptor-Targeting

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Oligonucleotide-based therapeutics hold transformative potential for treating a wide range of genetic diseases, yet their full realization has been limited by the constraints of current delivery technologies. Untargeted oligonucleotides suffer from poor cellular uptake and rapid clearance, requiring high-doses which are often prohibitive due to dose-limiting, class-based toxicities. In muscular and hepatic diseases, receptor-mediated delivery of oligos (via TfR1 and ASGPR respectively) has enabled therapeutic dosing paradigms, but similar approaches have remained elusive in other compartments.

To overcome these challenges, we have developed a targeted delivery approach that uses computationally designed miniproteins to route oligonucleotide-based therapeutics to target tissues *in vivo*. By building upon recent breakthroughs in machine learning for *de novo* protein design, we can rapidly develop stable proteins with tunable affinity profiles for a broad array of target receptors, including those without known crystal structures.

Using TfR1 as a benchmark, we highlight the unique features of these miniproteins, including their robust stability, low immunogenicity, and high receptor affinity. Our data reveal that TfR1-targeted oligonucleotide payloads drive efficient uptake into muscle cells in mice, resulting in significant target gene modulation sustained for 7 to 11 weeks.

Building on this success, we expanded the platform to target novel receptors, unlocking delivery potential to kidney and CNS. Intravenous administration of miniprotein-oligonucleotide conjugates for kidney delivery and ICV injections for CNS applications resulted in robust knockdown of gene targets within the respective tissues, demonstrating enhanced efficacy compared to untargeted payloads. These findings affirm the potential of computationally designed miniproteins to precisely deliver therapeutics to tissues previously inaccessible to larger modalities like antibodies.

Collectively, our platform drives therapeutic delivery by efficiently directing oligonucleotide payloads to the intended tissue improving on-target activity and mitigating off-target effects. Our flexible and efficacious miniprotein platform represents a transformative solution to the longstanding challenges of precision delivery, enabling new frontiers in the treatment of diseases once deemed inaccessible to oligonucleotide therapeutics.

163 NEAT1 lncRNA Structure-Mediated Functional Modulation as a Novel Therapeutic Approach for MYC-Driven Cancers.

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MYC is a well-characterized oncogene implicated in the dysregulation of over 70% of human cancers. Despite its significance, MYC is widely regarded as an undruggable target due to its lack of structural pockets suitable for small molecule binding. Emerging evidence suggests that MYC physically interacts not only with DNAs but also with RNAs, raising the possibility that nucleic acid-based therapeutics could target MYC.

In this study, we identified MYC-interacting RNAs through MYC pull-down RNA sequencing and validated that NEAT1 lncRNA is a direct interactor of MYC. Intriguingly, suppression of NEAT1 using CRISPR interference (CRISPRi) or gapmer antisense oligonucleotides (ASOs) resulted in increased MYC protein levels, while MYC transcript levels remained unchanged. This finding indicates that NEAT1 regulates MYC protein stability.

To explore the potential of NEAT1 as an anti-cancer therapeutic target, we hypothesized that NEAT1 structure is critical for MYC stability regulation. We designed 88 tiled mixmer ASOs that do not degrade their target but instead bind to and modulate the secondary structure of the full transcript of NEAT1 and performed functional screens assessing both cell proliferation and MYC protein stability. Among these, ASO14 and ASO24 were identified as MYC destabilizers, whereas ASO43 and ASO85 were MYC stabilizers.

To elucidate the mechanism underlying NEAT1-mediated regulation of MYC protein stability, we conducted CRISPR-based screening using a proteasome-focused library (Bison library) in conjunction with ASO14 and ASO24 treatment. This revealed UBR5 as an E3 ubiquitin ligase important for NEAT1-mediated MYC degradation. Mechanistically, ASO14 and ASO24 enhanced the interaction between NEAT1, MYC and UBR5, thereby promoting ubiquitin-mediated proteasomal degradation of MYC.

Unlike traditional RNA-targeting strategies that focus on degrading the target RNA, our findings highlight a novel concept in which the structure of a target RNA can be modulated without degradation to either enhance or suppress a specific biologic activity. This study establishes the concept of RNA structure-mediated loss- and gain-of-function effects and proposes ASO14 and ASO24 as promising anti-cancer therapeutic agents targeting NEAT1 for MYC-dependent tumorigenesis.

HSC Transplantation and Gene Therapy

164 Attack and Defend Against HIV-1 Infection Using HSPC-based Gene Therapy With a Safety Kill Switch

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Antiretroviral therapy has improved health outcomes of people living with HIV but cannot sustain life-long remission. Although stem cell transplants with allogeneic CCR5Δ32/Δ32 donors have achieved HIV cure in a few cases, such HLA-matched donors are rare. Hematopoietic stem/progenitor cell (HSPC)-based anti-HIV-1 gene therapy modifies autologous HSPCs with a lentiviral vector and provides long-term anti-HIV-1 immune cells, potentially curing HIV with a single treatment. To ensure safety, incorporation of a kill switch allows elimination of genetically modified immune cells in case of adverse events.

Our multipronged lentiviral vector (LV) exerts anti-HIV-1 effects through different mechanisms comparable to antiretroviral therapy. The LV contains a CD4-based chimeric antigen receptor to attack HIV-1 infected cells, shRNA to knock down CCR5, and C46 fusion inhibitor to inhibit HIV-1 entry. We co-expressed a truncated human epidermal growth factor receptor (EGFRt) as a safety kill switch. Previously we successfully engrafted humanized BLT (huBLT) mice following transplant of gene-modified human fetal liver-derived CD34+ cells. The vector marking was stable at ~2 copies/cell. Viral load was reduced significantly by 1-log up to 8 weeks post-transplant. For safety, we investigated depletion of EGFRt+ cells via antibody-dependent cellular cytotoxicity using an FDA-approved anti-EGFR antibody, Cetuximab. Cetuximab-mediated negative selection significantly reduced EGFRt+ cells in peripheral blood, spleen and bone marrow. EGFRt+ population was reduced in CD34+/CD90+/CD38- HSPC population within the bone marrow as well.

Here, we present transplantation of anti-HIV-1 vector modified HSPC in a more clinically relevant pigtail macaque model. For optimal transgene expression and CCR5 downregulation, we modified our anti-HIV-1 genes to match the natural pigtail macaque sequences. CD4-based chimeric antigen receptor was codon optimized and a single nucleotide of CCR5shRNA was replaced. Our modified-vector showed improved transgene expression and CCR5 downregulation in pigtail macaque PBMCs in vitro. Pigtail macaque peripheral blood-derived (PB) CD34+ cells were transduced and transplanted back to the host. After transplantation of PB-CD34+ cells, vector marking was stable at 0.01 and 0.02 in peripheral blood cells in 2 animals.

Our anti-HIV-1 vector showed stable engraftment, robust HIV-1 inhibition, and safety in huBLT mice. The anti-HIV-1 transgene expression and vector marking persisted after transplant in pigtail macaques. As PB-CD34+ cells are the mainstay in clinical use, further research in improving transduction efficiency and in vivo positive selection methods is needed to enhance transgene expression.

165 Enhancing ADC-Based Non-Genotoxic Conditioning by Targeting Cycling Stem Cells and Lymphocyte Depletion for HSC-Directed Lentiviral Gene Therapy for Hemophilia A

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Hematopoietic stem cell (HSC)-directed lentiviral (LV) gene therapy has shown clinical promise in treating monogenic diseases, and we have recently shown proof of concept in clinical trials for hemophilia A (HA). HSCT therapies utilizing standard non-targeted conditioning with total body irradiation (TBI), or alkylating chemotherapy remain limited by potential genotoxicity-associated infertility and secondary malignancy. Since conditioning is required to create space in the bone marrow (BM) niche for HSC engraftment, non-genotoxic conditioning strategies are under development to advance HSC-targeted gene therapies. We and others have developed an antibody-drug-conjugate (ADC) using anti-CD117 and a ribosome inactivating immunotoxin saporin (sap) to selectively deplete CD117-expressing HSCs and progenitor cells (HSPCs). However, using the ADC alone did not provide sufficient HSC depletion to achieve robust engraftment. We, therefore, initiated studies to investigate the hypothesis CD117-sap is not effective as a single conditioning agent due to a lack of deep HSPC depletion in the BM. Therefore, we first optimized our ADC efficacy by testing various streptavidin linkers, biotinylating strategies, and dosing schedules. Although our optimized ADC achieved robust stem cell depletion, multipotent progenitor cells were not cleared. Interestingly, flow cytometric analysis of murine HSPCs revealed CD117-sap induces nearly complete

residual HSC exit from a quiescence G0 state and entry into a non-G0 proliferative state, in both wildtype and HA mice. Based on these results, we incorporated the timed administration of the antimetabolite 5-fluorouracil (5-FU, which is toxic to cycling cells) post-ADC treatment. Flow cytometry and colony-forming unit assays on BM from treated mice showed 5-FU administration resulted in deep progenitor depletion and decreased colony counts in a 5-FU dose-dependent manner. In addition, CD117-sap/5-FU conditioning prior to HSCT resulted in 80-95% peripheral blood chimerism of unmodified donor cells, but interestingly, the kinetics of this high engraftment, as well as survival, varied among mouse strains. Pre-conditioned wild-type C57Bl mice displayed slow engraftment (4 months to reach >80%) and complete survival. In contrast, HA mice had rapid engraftment in 1 month, but also showed >50% mortality, which could be overcome with factor VIII (FVIII) prophylaxis. We explored these transplant deaths and found our HA mouse strain has substantially lower baseline white blood cell (WBC) counts compared to wild-type mice, and the addition of splenic leukocytes into the regimen also resulted in 100% survival. Also, we have extensively backcrossed our HA strain to C57Bl, which has WBC counts like wild-type mice, and showed 100% survival without FVIII coverage or splenic leukocyte injections. We then tested the conditioning in HSC-directed LV gene therapy for HA using engineered HSPCs transduced with a high-expression FVIII transgene, which resulted in rapid myeloid engraftment at 2 weeks post-HSCT with moderate FVIII plasma activity levels (1-5%). By 4 months post-HSCT these plasma activity levels increased to mild levels (5-40%), and ongoing studies are exploring long-term transgene tolerance. Collectively, we show small molecules targeting stem cell cycling can synergize with ADCs in non-genotoxic conditioning platforms to clear proliferating residual HSPCs and augment LV-engineered HSC engraftment.

166 BCL11A-Deficient Human Erythropoiesis is Impaired in *In Vitro* Culture and Xenotransplanted Bone Marrow

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Genetic depletion of the transcriptional repressor BCL11A in red blood cell precursors alleviates b-hemoglobinopathies by inducing g-globin gene expression. An alternative strategy for γ-globin derepression is blocking BCL11A-binding motif in HBG1/2 promoter. These strategies demonstrate that BCL11A is a promising therapeutic target for fetal hemoglobin induction. However, the effects of knocking down BCL11A in primary erythroid cells derived from human mobilized peripheral blood $CD34^+$ cells remain insufficiently described.

Here, we examined the effects of BCL11A knock-down in erythroid cells by editing the BCL11A +58 erythroid enhancer in the healthy $CD34^+$ cells and compared it with editing of the CD33 coding region (editing control) and the HBG1/2 promoter BCL11A binding motif. These edits were assessed with and without a competitive environment both *in vitro* and in xenotransplanted bone marrow (BM). BCL11A knockdown significantly impaired overall expansion and increased apoptosis of erythroid progenitors. Using sequencing techniques, we found a substantial amount of differentially expressed genes and binding sites in the BCL11A enhancer-edited group. Mutant colony-forming unit erythroid (CFU-E) cells, proerythroblasts and basophilic erythroblasts exhibited dysregulation of 94 genes (> 2-fold change, FDR < 0.05), including 25 likely direct targets of BCL11A. Differentially expressed genes were associated with a range of biological pathways that impact cell expansion and survival.

Moreover, using a competitive repopulation assay with a 50:50 mixture of CD33- or HBG1/2 promoter- and BCL11A enhancer-edited $CD34^+$ cells revealed that the BCL11A enhancer-edited cells exhibited a competitive disadvantage specifically in erythroid cells compared to CD33 or HBG1/2 promoter-edited cells. These effects were more pronounced in erythroid cells from the BM of xenotransplanted immunodeficient NBSGW mice.

We further investigated sickle cell disease (SCD) patient-derived $CD34^+$ cells under similar competitive conditions. Consistent with healthy donor $CD34^+$ cells, we observed the BCL11A enhancer-edited cells demonstrated reduced erythroid expansion compared to CD33 or HBG1/2 promoter-edited cells. These findings show that BCL11A plays

a broader role in erythropoiesis beyond g-globin gene repression, with potential but yet unknown clinical implications.

167 Myeloid Cell Replacement Therapy Improves Function in Friedreich Ataxia Mice by Intercellular Mitochondrial Transfer

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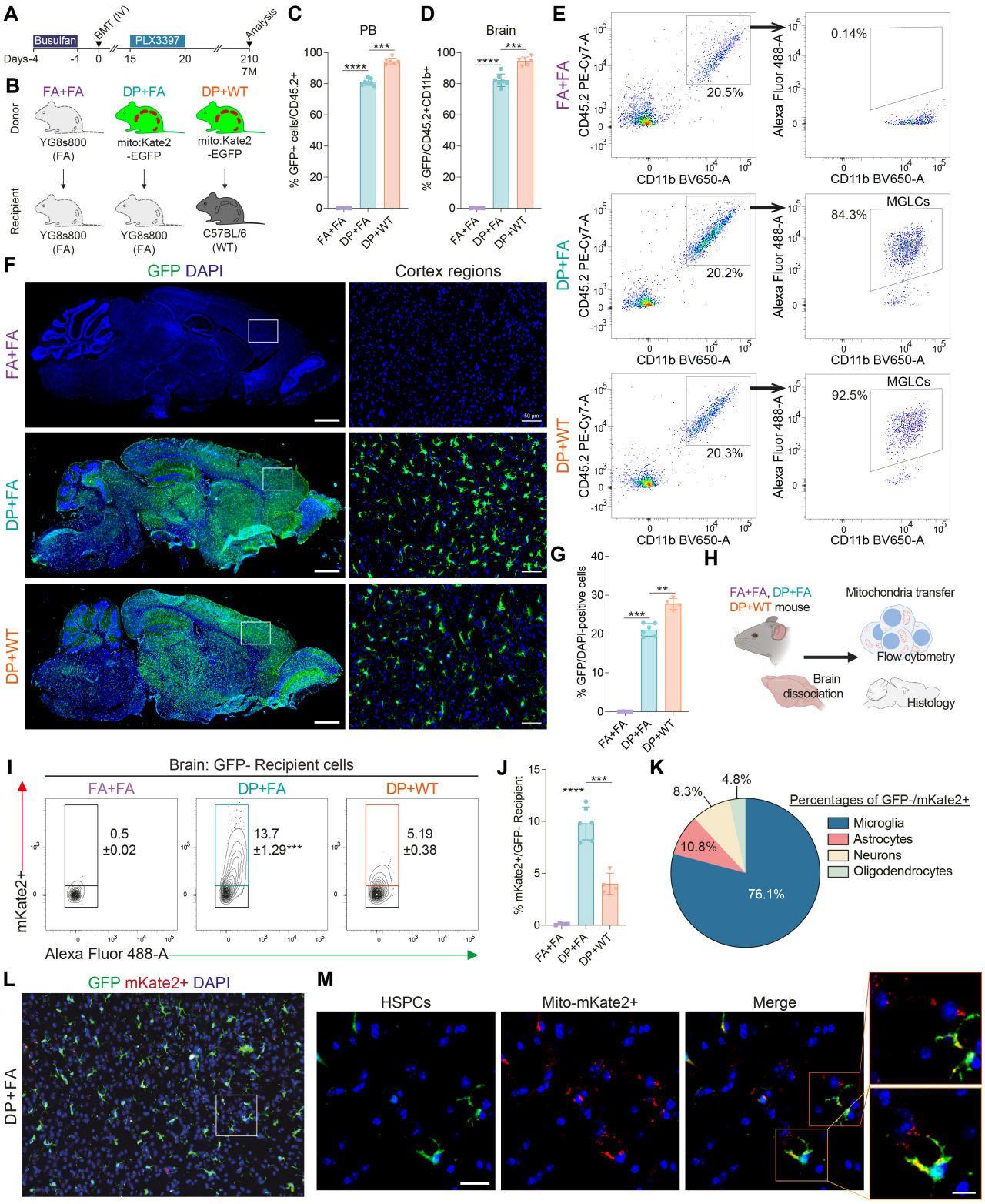
The primary objective of this study is to evaluate the therapeutic effects of bone marrow transplantation (BMT) or hematopoietic stem cell transplantation (HSCT) in a Friedreich's ataxia (FA) mouse model and to confirm the intercellular mitochondrial transfer from bone marrow-derived donor cells to FA cells. To achieve this, we performed BMT using the human FXN YAC transgenic mouse model (YG8s800) carrying over 800 GAA repeats, following a conditioning regimen with Busulfan and PLX3397 to enhance the engraftment of bone marrow-derived cells in CNS tissues.

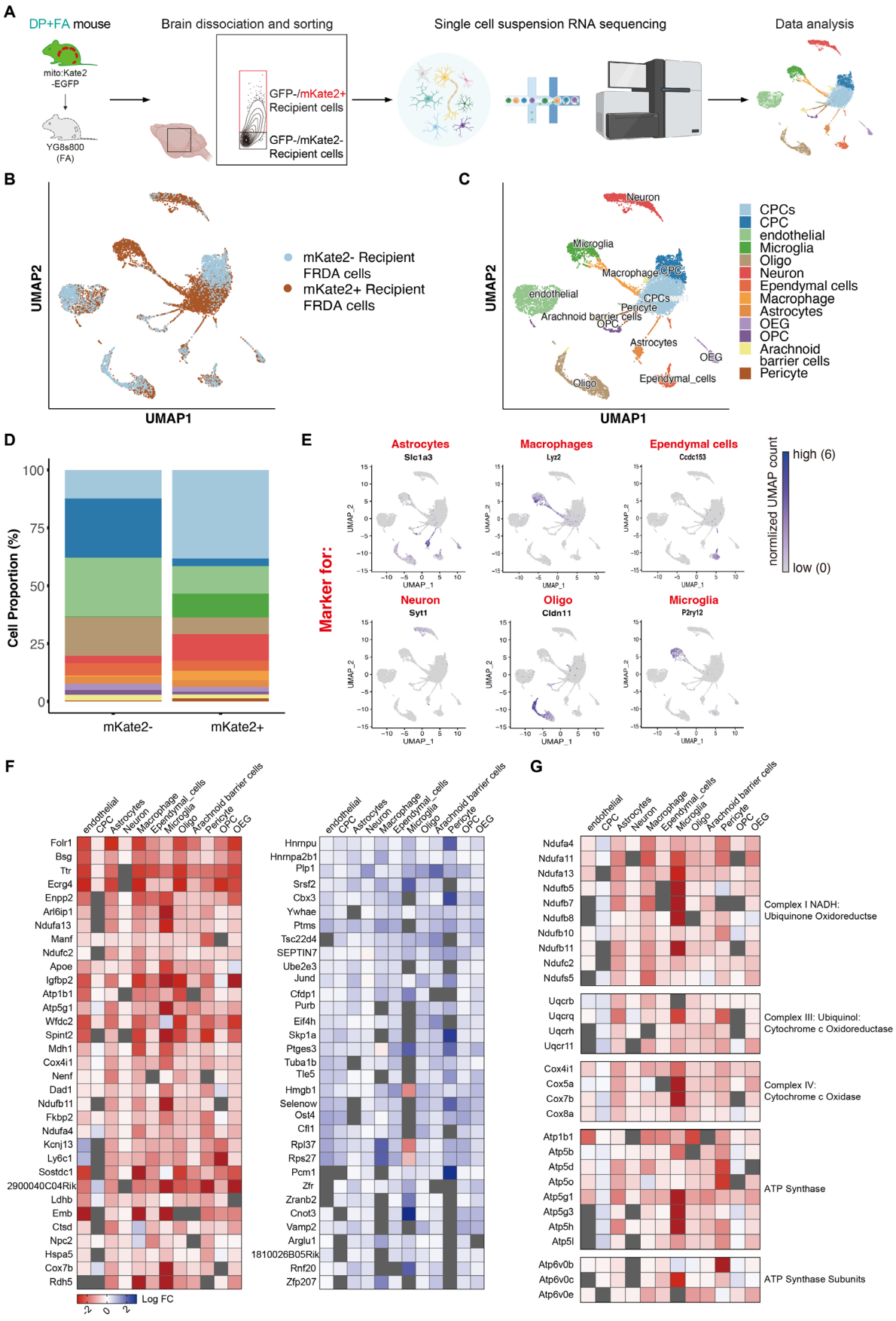
We confirmed mitochondrial transfer in multiple tissues, including the brain, following BMT and investigated how this process contributes to functional recovery in the FA model. Using single-cell RNA sequencing, we compared FA cells that acquired mitochondria with those that did not, identifying therapeutic mechanisms across key CNS cell types (neurons, astrocytes, microglia, and oligodendrocytes). This provided critical physiological insights into cell-type-specific therapeutic pathways.

BMT significantly improved neurological and cardiac function in YG8s800 mice, with robust engraftment and differentiation of transplanted cells in hematopoietic and CNS tissues. Additionally, mitochondrial function was restored, leading to enhanced motor coordination and cardiac function. Successful mitochondrial transfer from transplanted cells to FA cells resulted in reduced oxidative stress and the restoration of cellular functions.

To elucidate the mechanisms underlying mitochondrial transfer, we performed transcriptomic analysis of mitochondria-acquiring (mKate2+) and non-acquiring (mKate2−) cells using single-cell RNA sequencing. The results revealed an upregulation of genes associated with ATP synthesis and oxidative phosphorylation (Cox4i1, Ndufv1, Atp5o, Ndufa9) across major CNS cell types, with gene set enrichment analysis (GSEA) confirming significant activation of these pathways. In neurons and astrocytes, genes related to neuroprotection and protein homeostasis (Ttr, Ptgds, Igfbp2, Chchd10) were also upregulated. Overall, 33 enriched pathways were primarily associated with ATP metabolism and proton transport, while pathways related to RNA splicing and translation were downregulated. These findings suggest that mitochondrial transfer may partially restore energy production and regulate diverse cellular functions.

In conclusion, this study demonstrates the therapeutic potential of mitochondrial transfer through BMT in an FA mouse model. By identifying signaling pathways that facilitate mitochondrial uptake, our findings provide novel therapeutic targets for FA. This study serves as a foundational step toward developing treatments for FA and other mitochondrial dysfunction-related disorders.


A
Busulfan
BMT (IV)
PLX3397
Analysis
Days-4
-1
0
15
20
210
7M
B
FA+FA
DP+FA
DP+WT
Donor
Recipient
YG8s800 (FA)
mito:Kate2 -EGFP
C57BL/6 (WT)
C
PB
% GFP+ cells/CD45.2+
D
Brain
% GFP/CD45.2+CD11b+
E
CD45.2 PE-Cy7-A
CD11b BV650-A
Alexa Fluor 488-A
20.5%
0.14%
20.2%
84.3%
MGLCs
20.3%
92.5%
F
GFP DAPI
Cortex regions
G
% GFP/DAPI-positive cells
H
FA+FA, DP+FA
DP+WT mouse
Mitochondria transfer
Flow cytometry
Brain dissociation
Histology
I
Brain: GFP- Recipient cells
mKate2+
0.5 ±0.02
13.7 ±1.29***
5.19 ±0.38
J
% mKate2+/GFP- Recipient
K
Percentages of GFP-/mKate2+
Microglia
Astrocytes
Neurons
Oligodendrocytes
8.3%
4.8%
10.8%
76.1%
L
GFP mKate2+ DAPI
M
HSPCs
Mito-mKate2+
Merge


A
DP+FA mouse
mito:Kate2
-EGFP
YG8s800
(FA)
Brain dissociation and sorting
GFP-/mKate2+
Recipient cells
GFP-/mKate2-
Recipient cells
Single cell suspension RNA sequencing
Data analysis
B
UMAP2
UMAP1
mKate2- Recipient
FRDA cells
mKate2+ Recipient
FRDA cells
C
UMAP2
UMAP1
Neuron
Microglia
Macrophage
CPC
endothelial
CPCs
Pericyte
Arachnoid barrier cells
OPC
Astrocytes
OEG
Oligo
Ependymal_cells
CPCs
CPC
endothelial
Microglia
Oligo
Neuron
Ependymal cells
Macrophage
Astrocytes
OEG
OPC
Arachnoid
barrier cells
Pericyte
D
Cell Proportion (%)
100
75
50
25
0
mKate2-
mKate2+
E
Marker for:
Astrocytes
Slc1a3
Macrophages
Lyz2
Ependymal cells
Ccdc153
Neuron
Syt1
Oligo
Cldn11
Microglia
P2ry12
UMAP_2
UMAP_1
normlized UMAP count
high (6)
low (0)
F
endothelial
CPC
Astrocytes
Neuron
Macrophage
Ependymal_cells
Microglia
Oligo
Arachnoid barrier cells
Pericyte
OPC
OEG
Folr1
Bsg
Ttr
Ecrg4
Enpp2
Arl6ip1
Ndufa13
Manf
Ndufc2
Apoe
Igfbp2
Atp1b1
Atp5g1
Wfdc2
Spint2
Mdh1
Cox4i1
Nenf
Dad1
Ndufb11
Fkbp2
Ndufa4
Kcnj13
Ly6c1
Sostdc1
2900040C04Rik
Ldhb
Emb
Ctsd
Npc2
Hspa5
Cox7b
Rdh5
Log FC
Hnrnpu
Hnrnpa2b1
Plp1
Srsf2
Cbx3
Ywhae
Ptms
Tsc22d4
SEPTIN7
Ube2e3
Jund
Cfdp1
Purb
Eif4h
Skp1a
Ptges3
Tuba1b
Tle5
Hmgb1
Selenow
Ost4
Cfl1
Rpl37
Rps27
Pcm1
Zfr
Zranb2
Cnot3
Vamp2
Arglu1
1810026B05Rik
Rnf20
Zfp207
G
Ndufa4
Ndufa11
Ndufa13
Ndufb5
Ndufb7
Ndufb8
Ndufb10
Ndufb11
Ndufc2
Ndufs5
Complex I NADH:
Ubiquinone Oxidoreductse
Uqcrb
Uqcrq
Uqcrh
Uqcr11
Complex III: Ubiquinol:
Cytochrome c Oxidoreductase
Cox4i1
Cox5a
Cox7b
Cox8a
Complex IV:
Cytochrome c Oxidase
Atp1b1
Atp5b
Atp5d
Atp5o
Atp5g1
Atp5g3
Atp5h
Atp5l
ATP Synthase
Atp6v0b
Atp6v0c
Atp6v0e
ATP Synthase Subunits

168 CSF1R Inhibitor Treatment Facilitates Engraftment of Hematopoietic Stem Cell-Derived Microglia-Like Cells in Nonhuman Primates

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the current standard of care for neurotrophic lysosomal storage diseases (LSDs) such as Krabbe Disease and Metachromatic Leukodystrophy. The therapeutic mechanism of action is believed to be through the engraftment of enzyme sufficient, HSC-derived cells throughout the central nervous system (CNS) as microglia-like cells (MLCs). However, allogeneic transplantation is associated with significant morbidity and mortality. Recent work in murine models has demonstrated that MLC engraftment can be enhanced using gene-modified, autologous HSCT (auto-HSCT) paired with depletion of endogenous microglia by CSF1R inhibitors such as Pexidartinib (PLX). Here, we evaluated the engraftment of MLCs post GFP-labelled auto-HSCT in several nonhuman primates (NHPs) with and without PLX-mediated depletion of microglia.

To evaluate the impact of PLX in NHPs, we treated two animals with PLX either intrathecally (IT) or intravenously (IV). Depletion was characterized by FACS as well as immunofluorescent (IF) imaging. We then treated five animals with total body irradiation-conditioned, GFP-labelled auto-HSCT. A sixth animal was similarly transplanted, then treated with IT PLX two weeks post-transplant. We tracked hematologic recovery in all animals in the peripheral blood (PB) and bone marrow (BM) for at least six months post-transplant. At necropsy, engraftment was determined in the PB and BM by FACS and in the CNS by FACS and IF. We further characterized microglia and MLCs by morphological analysis as well as single cell RNA sequencing.

We found that both IT and IV PLX led to robust, region-dependent depletion of endogenous microglia. Maximum depletion was observed in superficial structures with a gradient of depletion to deeper brain structures. We did not appreciate any impact on hematopoietic lineages in the PB or BM due to systemic or local PLX administration. Given prior reports of adverse hematologic effects of PLX, we treated one animal with PLX IT following auto-HSCT. We did not observe any obvious deviations in hematopoietic recovery or behavior due to PLX treatment. At necropsy, we observed a range of engraftment in the PB and BM (10%-50%). However, in non-PLX treated animals we observed a two-log-fold lower engraftment to the CNS (0.1%-0.5%). IT PLX administration enhanced this engraftment four-fold, with the PLX-treated animal showing 20% engraftment in the PB/BM and 1% engraftment in the CNS. Characterization of MLCs demonstrated similarities to endogenous microglia, with up-regulation of homeostatic microglia markers and no significant differences in branching. However, MLCs remained distinct from endogenous microglia, with differential expression of several homeostatic microglial markers and activation-associated markers. This heterogeneity was also appreciated morphologically, with MLCs displaying longer maximum and average branch lengths.

This work stands as a landmark study exploring the application of CSF1R inhibitor-mediated MLC engraftment in a large animal model. Our data demonstrate the feasibility of these studies in NHPs and the potential to further study the safety and impact of MLC engraftment on health and behavior. Ways to further enhance MLC engraftment and functionalize MLCs with gene engineering strategies will pave the way for the application of these technologies for a range of neurotrophic disorders from LSDs to neurodegenerative diseases.

169 Investigating Novel Non-Genotoxic Conditioning Approaches for In Utero Hematopoietic Stem Cell Transplantation

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Hematopoietic stem cell (HSC) transplantation *in utero* (IUTx) has been performed safely for decades in humans and successfully cured X-SCID, with all the patient's T lymphocytes arising from the donor. However, for reasons that are not well understood, successful HSC engraftment after IUTx has only been achieved in X-SCID fetal recipients. We hypothesize that depletion of endogenous HSC and/or immune cells is necessary to achieve therapeutic levels of donor HSC engraftment following IUTx. Here, we investigated which antibody-drug conjugates (ADC) would be able to create niche availability, eliminate host HSC competition, and thwart nascent immunity, thereby enabling engraftment of adult donor HSC at therapeutic levels following IUTx. Using a fetal sheep model, we used immunohistochemistry to identify which immune cells and/or HSC populations are present in the fetal bone marrow (BM) at the time of IUTx. Flow cytometric analysis of sheep BM was then used to identify antibodies suitable for use as ADC to target HSC (CD45+GPI-80) and immune cells (CD45+IL2RG). Antibody internalization using a pH-dependent dye, and cell death assays using ADC and saporin as a toxin were also performed to confirm internalization and determine optimal concentration of the respective immunotoxins. Sheep fetuses at 55-57 gestational days were conditioned using GPI-80- or IL2RG-saporin ADC and were then transplanted 5-7 days later with human adult donor CD34+HSC, at a dose of 10^7 cells/kg. Engraftment levels of human donor cells were determined in peripheral (PB) and BM samples at 60 days post-IUTx and at various time points post-birth. In all conditioned IUTx recipients, levels of human HSC(CD34+), T(CD3+), (CD7), B(CD10+)(CD20+), and myeloid (CD123+) cells in PB and BM were determined by flow cytometry after exclusion of sheep CD45+ cells. At 60 days post-IUTX, levels of human cells in GPI-80-conditioned recipients were: CD34+(0.5±0.02%;5.8±0.5%), CD3+(5.72±0.4%;14.93±1.0%), CD7+(7.62±3.6%;13.4±0.73%), CD10(3.49±2.0%;9.49±4.9%), CD20+(3.88±0.9%;11.18±2.1%), and CD123+(6.48±0.9%; 6.2±2.1%), in PB and BM, respectively. Similar levels of human T, B, and myeloid cells were observed in IL2RG-conditioned fetuses: CD34+(0.68%;0.95), (CD3+(3.13%;14.2%), CD7+(2.78%;0.15%), CD10 (5.05%,0.59%), CD20+(8.63%,14.4%), and CD123+(6.12%; 3.2%), in PB and BM, respectively. Clonogenic assays in GPI-80- and IL2RG-conditioned IUTx recipients demonstrated the presence of human CFU-GEMM, BFU-E, CFU-GM, and CFU-E, therefore establishing the ability of the engrafted human HSC to proliferate and differentiate into multiple hematopoietic lineages. Total human donor cells in PB of both GPI-80- and IL2RG-conditioned animals at birth (4 months after IUTx) decreased considerably to around 2%. However, at 6 months after IUTx, the levels of human CD3+, CD7+, CD20+, and CD123+ cells in the PB of GPI-80-conditioned animals increased to 5.57%, 5.4%, 2.86%, and 1.12%, respectively, while engraftment in IL2RG-conditioned animals remained low. In conclusion, when compared with historical controls of HSC-transplanted fetuses without conditioning, the depletion of recipient HSC or immune cells prior to IUTx each appear to promote successful short-term donor HSC engraftment, while the selective depletion of recipient HSC seems to be critical to achieve long-term engraftment of donor HSC.

Disease Models and Pre-Clinical Applications for Lysosomal Storage Diseases

170 Persistent Expression of Liver-Directed AAV Gene Therapy Improves Long-term Outcomes in a Mouse Model of Lysosomal Acid Lipase Deficiency

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Lysosomal acid lipase deficiency (LAL-D) is a rare autosomal recessive disorder caused by mutations in the lipase A (LIPA) gene, which encodes the enzyme lysosomal acid lipase (LAL). This enzyme is responsible for converting cholesteryl esters and triglycerides in the lysosome to free cholesterol and fatty acids. There are 2 manifestations of this disease: a severe infantile-onset form (formerly Wolman disease) and a later-onset form (formerly cholesteryl ester storage disease). The current standard of care for LAL-D is once-weekly or once-biweekly enzyme

replacement therapy (ERT). Though this treatment is generally well tolerated, there is still a need to develop a more curative therapy, especially for infantile-onset patients that sometimes do not respond well to ERT. We have recently developed a liver-directed AAV gene therapy (rsc.AAVrh74.LP1.*LIPA*) that was shown to improve disease symptoms in a mouse model of LAL-D. Intravenous treatment of *Lipa*$^{-/-}$ mice at 8 weeks of age, when disease is already present, reduced hepatosplenomegaly, serum transaminase levels, organ triglyceride and cholesterol levels, and measures of liver fibrosis and inflammation when mice were analyzed at 24 weeks of age (16 weeks post-treatment). Almost all of these measures in treated *Lipa*$^{-/-}$ mice did not differ significantly from those found in wild-type mice. LAL enzyme activity was also restored at 16 weeks post-treatment (24 weeks of age) in all organs to or beyond levels found in wild type mice. Based on these promising results, we performed a life-span study. Untreated *Lipa*$^{-/-}$ mice show a median survival age of about 33 weeks. 8-week-old *Lipa*$^{-/-}$ mice treated intravenously injected with rsc.AAVrh74.LP1.*LIPA* at a dose of $1x10^{13}$ vg/kg (n=20) all survived past the longest survival age of untreated *Lipa*$^{-/-}$ mice. To date, 5 of the treated mice were euthanized due to moribundity, with an average age at death of 63 weeks, and this level of mortality matches that found in wild-type FVBn mice. When analyzed, none of these mice presented with hepatosplenomegaly or fatty liver disease. Tissue biodistribution of AAV vector genomes showed presence of AAV in key organs such as the liver and spleen at the same levels as mice analyzed at 24 weeks of age. *LIPA* transgene expression also persisted, leading to lasting reduction of cholesterol and triglycerides. LAL enzyme activity in all organs and tissues, including serum, remained at or above wild-type levels. These data demonstrate persistent expression and functional activity from single-treatment rAAVrh74.LP1.*LIPA* gene therapy, suggesting this therapy has the potential to be a curative treatment for LAL-D.

171 Hematopoietic stem cell gene therapy for Mucopolysaccharidosis type IIIC

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[1]UC San Diego, La Jolla, CA, [2]University of California, San Diego, La Jolla, CA, [3]University of California San Diego, San Diego, CA, [4]University of California, San Diego, San Diego, CA, [5]UCSD, San Diego, CA, [6]University of California, San Diego, La Jolla, California

Mucopolysaccharidosis type IIIC (MPS IIIC) or Sanfilippo Syndrome type C is a severe neurodegenerative lysosomal storage disorder (LSD) caused by loss-of-function of the lysosomal transmembrane protein acetyl-CoA:heparan-ℝ?-glucosamine N-acetyltransferase (HGSNAT). MPS IIIC is characterised by accumulation of the glycosaminoglycans (GAGs) Heparan Sulfate and currently has no treatment. This disease presents with developmental delay and neuronal cell loss leading to death from severe neurodegeneration. Our group has previously shown that transplantation of hematopoietic stem cells (HSPCs) rescue cystinosis, another LSD due to mutations in a transmembrane lysosomal protein. One of the mechanisms of rescue involved lysosomal cross-correction from HSPC-derived macrophages to the disease cells via tunnelling nanotubes. We believe that the same principles could be used to treat MPS IIIC.

We generated a new MPS IIIC mouse model and confirmed disease phenotypes such as GAG accumulation, splenomegaly, neurological defects, and presence of disease specific non-reducing end carbohydrates. To explore a new therapeutic strategy for this condition, we transplanted wild type (WT) hematopoietic stem and progenitor cells (HSPCs) into lethally irradiated 2-month-old *Hgsnat*$^{-/-}$ mice and analyzed the resulting impact 6 months later. Transplanted HSPCs differentiated into macrophages in tissues and microglia-like cells in the brain. This resulted in partial restoration of *Hgsnat* expression and enzymatic activity along with the significant reduction of the MPS IIIC-specific non-reducing end carbohydrate in the treated *Hgsnat*$^{-/-}$ mice compared to untreated *Hgsnat*$^{-/-}$ mice or *Hgsnat*$^{-/-}$ mice transplanted with *Hgsnat*$^{-/-}$ HPSCs. In addition, WT HSPC transplant resulted in improved neurological defects, reduction in splenomegaly and urine retention in the *Hgsnat*$^{-/-}$ mice. Furthermore, presence of glomerular hyaline bodies with focal fibrosis and sclerosis were observed in the kidney of the disease controls whereas these abnormalities were alleviated in the *Hgsnat*$^{-/-}$ mice treated with WT HSPCs.

For the clinical translation of this approach, we are developing an autologous transplantation of *ex vivo* gene modified HSPCs. We have generated self-inactivated (SIN)-lentivirus vector containing human *HGSNAT* cDNA (NM_152419) and performed *in vitro* characterization. Following transduction of murine and patient cells lines, we observed a recovery in *HGSNAT* expression and activity as well as HGSNAT lysosomal localization, decreased GAG storage, decreased lysosomal expansion, decreased autophagosome accumulation. We also transplanted gene-

modified HSPCs in MPS IIIC mice, and have confirmed successful engraftment with vector copy number (VCN) between 1.2 and 3.6. Our preliminary data showed improvement in locomotor phenotypes in MPS IIIC following gene modified HSPC transplantation. Additionally, RNA-seq analysis revealed increased expression of inflammatory genes as well as an increase in synapse pruning, microglial activation and upregulated lysosomal gene expression in the MPS IIIC mice compared to WT mice. Notably, this phenotype was ameliorated following transplantation of gene-modified HPSCs. These data support that HSPC transplantation presents a promising therapeutic avenue for MPS IIIC and represent the first step towards a clinical translation of HSPC-mediated therapy strategy for MPS IIIC.

172 Testing a Dual AAV Gene Therapy Vector Construct to Treat Sialidosis and Galactosialidosis Using Small and Large Animal Models

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Sialidosis and galactosialidosis (GS) are rare lysosomal storage disorders caused by mutations in the *NEU1* and *CTSA* gene, which encode for lysosomal enzyme neuraminidase-1 (Neu1), and it's chaperone protective protein cathepsin-A (PPCA), respectively. Neu1, PPCA and b-galactosidase (b-gal) form a lysosomal multi-enzyme complex (LMC) that together degrade various glycoconjugates within the lysosome. Deficiency in either of these enzymes causes toxic accumulation of glycoconjugates in cells throughout the body resulting in similar but distinct diseases; sialidosis, GS or G_{M1} gangliosidosis. Sialidosis manifests in two forms: type I, is characterized by late-onset symptoms including myoclonus, ataxia, and vision loss; type II, the severe early-onset form, is associated with multi-systemic involvement and childhood mortality. GS is divided into three forms, the early infantile form results in premature death, fetal hydrops, progressive skeletal abnormalities and neurologic disorder, and the late-infantile form being a later-onset, milder phenotype of the infantile disease with no neurological defects. In the juvenile/adult form patients have slowly progressive CNS symptoms, including motor disturbance, mental retardation, skeletal abnormalities, dysmorphism, macular cherry-red spots, and angiokeratoma. Although life expectancy varies by disease type, both GS and sialidosis are ultimately fatal due to lack of existing treatments. Given that Neu1 is dependent on PPCA for stability and enzymatic function and because GS patients have loss of PPCA and Neu1 primarily, our goal is to develop a dual gene therapy to treat both diseases combining *NEU1* and *CTSA* in the same vector design (AAV9-Ctsa-Neu1). Previously our group has shown efficacy in treating sialidosis mice via intracerebroventricular injection (ICV) with our AAV9-Ctsa-Neu1 compared to other single gene vectors (Fig. 1A-D). However, ICV injection was unable to significantly ameliorate kidney pathology and therefore we hypothesize combined ICV and intravenously (IV) delivery will treat both the CNS and peripheral disease of sialidosis and GS. Neu1 KO mice were given a combined ICV (1.6E11 vg) and IV (1.3E12 vg) injection of AAV9-Ctsa-Neu1 at 5 weeks of age and allowed to continue to humane endpoint. Preliminary data shows that combined IV and CSF treated Neu1 KO mice have significantly increased motor ability and decreased anxiety-like behavior in the open field test at 4 months old, compared to untreated Neu1 KO mice as well as overall increased body weight (Fig. 1E-G). Further assessments are still ongoing, as well as initial testing in GS mice using the same delivery method. To facilitate translation to the clinic, large animal models are necessary and due to the lack of neurological pathology in the Neu1 KO mouse model, developing a sialidosis large animal model will be beneficial. We have successfully generated the first two sheep models of sialidosis type I (via knock-in) (Fig. 2D), and type II (via frameshift indel (A320del)), using CRISPR-SpCas9 technology and AAV donor. Homozygous type II sheep recapitulate hallmark disease features, including increased sialic acid levels, reduced Neu1 activity, severe neurological deficits, hepatosplenomegaly, hydronephrosis, skeletal abnormalities and short lifespan (Fig. 2A-C). Collectively, our findings establish a foundation for advancing gene therapy using a dual-gene construct as a viable treatment for GS and sialidosis.

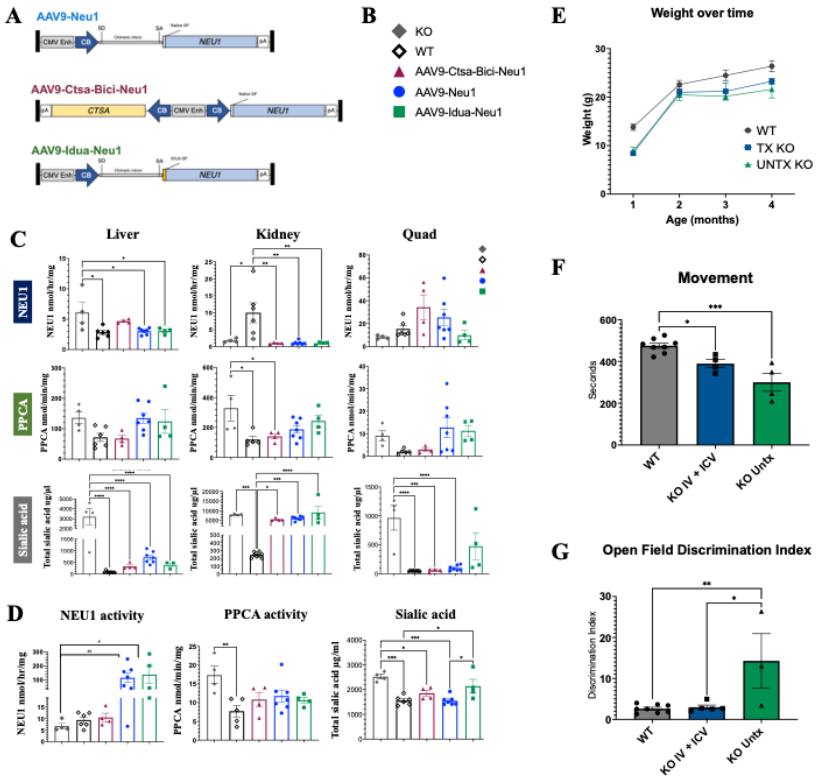

Figure 1: Summary of mouse experiments. *Panels A-D are from preliminary data testing neonatal ICV injections in Neu1 KO mice. Panels E-G are from current experiments using IV + ICV injections of dual gene vector (AAV9-Ctsa-Neu1).* A) Vector construct design: AAV9-Neu1, AAV9-Ctsa-Bici-Neu1 and AAV9-Idua-Neu1 (CMV Enh= cytomegalovirus enhancer, CB = Chicken ß-actin promoter). B) Figure C&D legend. C) Enzymatic activity for Neuy1 (1st row), PPCA (2nd row) and sialic acid levels (3rd row) in the liver, kidney, and quad. In summary, AAV9-Ctsa-Neu1 treated mice have overall increased Neu1 activity and decreased PPCA and sialic acid levels compared to other treatment groups, with the exception of the kidney (One-way ANOVA followed by Tukey's post-hoc analysis *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). D) Results of enzymatic activity of PPCA and Neu1 and sialic acid levels measured from brain homogenates. In summary AAV9-Ctsa-Neu1 treated mice had more appropriate levels of Neu1, decreased PPCA and significantly decreased levels of PPCA compared to other treated animals (One-way ANOVA followed by Tukey's post-hoc analysis *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). E) Graph showing weight over time across 4 months for WT (gray), Neu1 KO mice treated via IV + ICV with AAV9-Ctsa-Neu1 (blue) and untreated Neu1 KO mice (green). F&G) Results of Open Field test showing movement (how often the mouse is ambulatory within the arena) and the discrimination index (amount of time spent in periphery/time spent in center). Untreated KO mice move significantly less than WT animals, with treated KO mice having more movement than untreated KO although not significant. Untreated KO mice spend significantly more time in the perimeter of the arena compared to WT and treated KO mice.

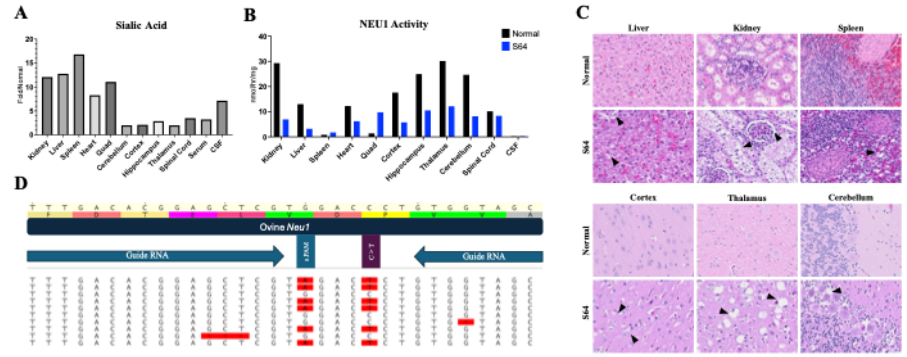

Figure 2 Successful generation of a type II sialidosis sheep model and preliminary results of generating a type I sheep model of sialidosis via knock-in. A) Sialic acid quantification of S64 (A320del homozygous animal) represented as fold over normal, in various tissues. All tissues analyzed showed increased sialic acid compared to a normal control lamb (1 month old). B) Neu1 enzymatic activity measured in various tissues for S64 compared to a normal control lamb. Neu1 activity is moderately increased in all tissues analyzed. C) Representative images of S64 and normal lamb (control) from the liver, kidney, spleen, cortex, thalamus and cerebellum. Black arrows indicate pathology, such as storage and vacuolization. D) Diagram showing representative sequences from nanopore sequencing from an early-stage sheep embryo that had successful insertion of an AAV delivered humanized DNA donor template containing P360S (C>T) mutation. Blue arrows indicate guide location, small blue box indicates silenced PAM (s.PAM), and purple box showing P360S C>T mutation reported in sialidosis patients.

173 Evaluating the Efficacy of iPSC-Derived Neural Stem Cell Transplantation in an MPS I Mouse Model

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Mucopolysaccharidosis type I (MPS I) is a severe metabolic disorder caused by deficient α-L-iduronidase (IDUA) enzyme activity, leading to impaired degradation of glycosaminoglycans (GAGs). This accumulation triggers progressive tissue damage and organ dysfunction, including neurodegeneration. Although FDA-approved treatments such as hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) are available, their efficacy in mitigating neurodegeneration is limited due to their inability to cross the blood-brain barrier (BBB).

To address this challenge, we successfully generated human neural stem cells (NSCs) by differentiating induced pluripotent stem cells (iPSCs) derived from reprogrammed human cord blood cells. These NSCs were purified via fluorescence-activated cell sorting and transplanted into neonatal immunocompromised MPS I (*Idua$^{-/-}$)* mice on a NOD/SCID/Il2rγ (NSG) background. Remarkably, eight-months post-transplantation, the NSCs demonstrated migration, partial restoration of IDUA enzyme activity, and a significant reduction in β-hexosaminidase levels in the brains of the MPS I mice, highlighting their potential for addressing neurodegeneration in MPS I.

Our comprehensive histopathological analyses elucidated the distribution of transplanted human NSCs in MPS I mouse brains with human-*specific antibody, STEM121. The staining highlighted* a widespread migration of *transplanted cells, a critical characteristic ensuring broad distribution of IDUA into the Idua$^{-/-}$ brain. Co-staining of STEM121 with GFAP and Olig2 antibodies demonstrated the differentiation of many transplanted human NSCs into* glial cell types such as *astrocytes and oligodendrocytes. Notably, the presence of engrafted cells was associated with a reduction in certain elevated biomarkers in MPS I mouse brains, including CD68 and LAMP1. These findings suggest a promising therapeutic impact of the transplanted cells in addressing the deficiencies associated with MPS I.*

Further examinations involving *IDUA*-overexpressing NSCs will be conducted to evaluate their efficacy *in vivo* within our platform, paving the way as an alternative option for potential patient-specific cellular therapy for MPS I.

174 Durability of FLT201: an Investigational Gene Therapy for Gaucher Disease Type 1 Encoding an Engineered Variant of the GCase Enzyme

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In Gaucher disease Type 1 (GD1), mutations in the *GBA1* gene attenuate or abrogate the activity of the lysosomal enzyme glucocerebrosidase (GCase-WT). FLT201, a clinical-stage investigational liver-directed gene therapy for GD1, consists of an engineered AAV capsid (AAVS3) containing an expression cassette encoding a novel engineered GCase variant, GCase85, with improved stability compared to GCase-WT. Studies in rhesus macaques have shown that FLT201 resulted in a rapid and robust increase in plasma GCase activity, produced from the liver, when treated with a single infusion of 2×10^{12} vg/kg. GCase activity was observed from day 8 and sustained for 83 days (n=1) and 170 days in six animals (n=3 female and n=3 male). Fluctuations in GCase levels were noted

between day 21 and 43; at day 170, GCase plasma levels ranged from 336–1308 nmol/h/mL, pre-treatment levels ranged from 7.4 to 9.3 nmol/hr/mL. From this study, one male and one female rhesus macaque was retained for ongoing observation up to 60 months, and we present interim 3.5-year data demonstrating durable plasma GCase levels of 388 nmol/hr/mL and 1344 nmol/hr/mL respectively.
FLT201 was well tolerated in rhesus macaques. At 85 days (n=1) and 177 days (n=4) and 1211 days (n=2) following FLT201 infusion, there were no deaths or treatment-related effects on clinical condition, post-dose observations, body weight, food consumption, hematology, blood chemistry, or immunophenotyping of peripheral leukocytes. In FLT201-treated animals terminated at 85 days (n=1) and 177 days (n=4), minimal or mild lymphohistiocytic or lymphoplasmacytic histiocytic infiltrates (slightly increased above known background levels) were observed in the dorsal root ganglia (DRG) (n=4/5) or the kidneys (n=2/5) respectively, were considered not to be adverse and unlikely to be related to the administration of FLT201. A reduction and recovery of plasma GCase activity may be attributed to transient detectable anti-GCase antibodies against the human protein, observed between 42 and 308 days in 3 out of 7 animals. The totality of the data demonstrates that FLT201 was well tolerated with no treatment-related adverse events and results in durable long-term expression.
The impact of a human monoclonal neutralizing antibody (NAb) to GCase on GCase85 uptake and activity in human macrophages and subsequent substrate clearance was determined in a conduritol-β-epoxide (CBE) induced in vitro model and was shown to be minimal. The clinical relevance of this data is discussed.

175 Optimized Liver Targeted GBA Expression via AAV3b for Type 1 Gaucher Disease Treatment

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Gaucher Disease (GD) is a lysosomal storage disorder caused by loss-of-function mutations in the *GBA* gene, which encodes the glucocerebrosidase (GCase) enzyme. Approximately 90% of GD patients have Type 1 GD, characterized by lipid-engorged macrophages (Gaucher cells) in multiple organs such as the spleen, liver, and bone marrow, with little to no involvement of the central nervous system (CNS). While enzyme replacement therapy (ERT) provides overall therapeutic benefits for Type 1 GD patients, it involves a burdensome regimen of intravenous infusions every other week and does not improve all clinical manifestations, particularly in the pulmonary and skeletal aspects. We propose a one-time, intravenously (IV) dosed AAV gene therapy approach for liver-targeted sustained expression of GCase. After optimizing capsids and promoters, we selected a liver-tropic capsid (AAV3b) and a liver-targeted promoter to enable sustained expression of a codon-optimized *GBA* transgene. Proof-of-concept studies demonstrate cross-correction across multiple organs, including the spleen, with no overt toxicity as seen in a 3-month GD mouse study and a 6-month non-human primate study. In GD mice 1e13 vg/kg injection achieved a 10x increase in GCase in the liver and normalized elevated glucosphingosine lipid levels to wildtype levels in both the liver and spleen. In NHPs, 3e13 vg/kg treatment resulted in a 100x increase in GCase and a 60x increase in the liver and spleen, respectively. These studies support the liver-targeted expression of GCase as a therapeutic strategy for treating Type 1 Gaucher Disease.

176 An Innovative Platform Approach for the Parallel Development of HSPC-GT for Rare/Ultra-Rare Lysosomal Storage Disorders with Severe Skeletal and Neurological Manifestations.

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Lysosomal storage disorders (LSDs) are caused by the deficiency of a specific lysosomal enzyme, causing the accumulation of undigested macromolecules, which results in severe multi-organ damage. Currently approved therapies provide an external source of therapeutic enzyme, but they show several limitations. Previous clinical data from our Institute proved the safety and efficacy of Hematopoietic Stem and Progenitor Cell-Gene Therapy (HSPC-GT) to treat neurological and skeletal manifestations in other forms of LSDs. Building on this experience and the common pathological mechanisms of LSDs, we are developing a HSPC-GT platform approach to treat a group of rare/ultra-rare LSDs with severe skeletal and neurological involvement (MPSIVA, GLB1-related disorders, and a-Mannosidosis), utilizing the lentiviral vector (LV) backbone previously employed in the Institutional clinical trials and a platform standardized transduction protocol. Based on these characteristics, we optimized the Chemistry, Manufacturing and Controls activities, Non-clinical and Clinical Development plans to generate a complete regulatory dataset for the leading disease (MPSIVA), complemented by disease specific data for the other indications with the aim of submitting a single Clinical Trial Application to initiate clinical evaluation. This approach moves towards a simultaneous and parallel developmental approach, making more sustainable the generation of novel therapeutic interventions for ultra-rare diseases. To this aim, we tested and selected the therapeutic LV that allows the supraphysiological release of each specific enzyme in human and murine HSPCs, without inducing in vitro and in vivo toxicity. Both human and murine transduced cells demonstrated clonogenic and proliferation capacities similar to those of control untransduced cells. Furthermore, they properly engrafted in the bone marrow of recipient mice, successfully reconstituting the hematopoietic systems at levels comparable to those of the control cells. We proved that the amount of enzyme released by the progeny of platform LV-transduced human HSPCs induced the metabolic cross-correction of patients' cells, including skeletal cells. To support clinical translation, we developed a transduction protocol using clinical-grade materials combined with transduction enhancers. This protocol achieved high transduction efficiency and showed no signs of toxicity, both in vitro and in vivo. A GLP platform study for parallel evaluation of enzyme overexpression toxicity is ongoing. Moreover, proof of concept studies have started to assess the safety and efficacy of HSPC-GT compared to standard HSPC transplantation in disease-specific murine models. Preliminary data showed that the enzyme activity was restored more effectively in metabolic tissues and bone samples from MPS IVA mice treated with GT than in those receiving donor HSPCs. Additionally, we observed a reduction in urinary GAGs and a downregulation of markers associated with systemic inflammation. Overall, our preliminary data support the platform development of HSPC-GT for LSDs and its clinical application.

Epigenetic Editing and RNA Editing

177 Programmable RNA Writing with Cleavage Enhanced Trans-splicing

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Trans-splicing-based RNA editing technologies, which introduce exogenous templates via competition with the endogenous pre-mRNA, have the potential to ntroduce edits, insertions, and deletions into nucleic acid sequence without permanent off-target effects but can be challenging to engineer. Here we describe Programmable RNA Editing & Cleavage for Insertion, Substitution, and Erasure (PRECISE), a versatile and efficient RNA editing method for generating changes of arbitrary length and sequence into existing pre-mRNAs via 5′, 3′, or internal trans-splicing. By cleaving pre-mRNAs with the RNA-targeting CRISPR-Cas effector Cas7-11, we bias towards editing outcomes and boost RNA trans-splicing rates by 10–100 fold to between 5–50% and 85% on endogenous and

reporter transcripts, respectively. We apply PRECISE editing in diverse cell types to 10 distinct endogenous transcripts across a range of expression levels and introduce more than 50 types of edits, including all 12 possible transversions and transitions, deletions, and insertions ranging from 1 to 1,863 nucleotides. PRECISE lacks detectable off targets by whole transcriptome sequencing. To demonstrate clinical relevance, we replace exon 4 of MECP2, addressing most mutations that drive the Rett Syndrome; edit the SHANK3 transcript, a gene involved in Autism; and replace exon 1 of HTT, removing the hallmark repeat expansions of Huntington's disease. To enable protein-free PRECISE, we combine payload engineering and ribozymes to remove the polyA tail of the donor transcript, and show this compact system can edit HTT exon 1 via single-AAV delivery in non-dividing neurons. PRECISE editing markedly broadens the scope of genetic manipulation by enabling both small and large edits without changes to the genome or detectable off-targets, and can be delivered using clinically relevant vectors without exogenous protein expression, creating a new platform for the treatment of genetic and other diseases.

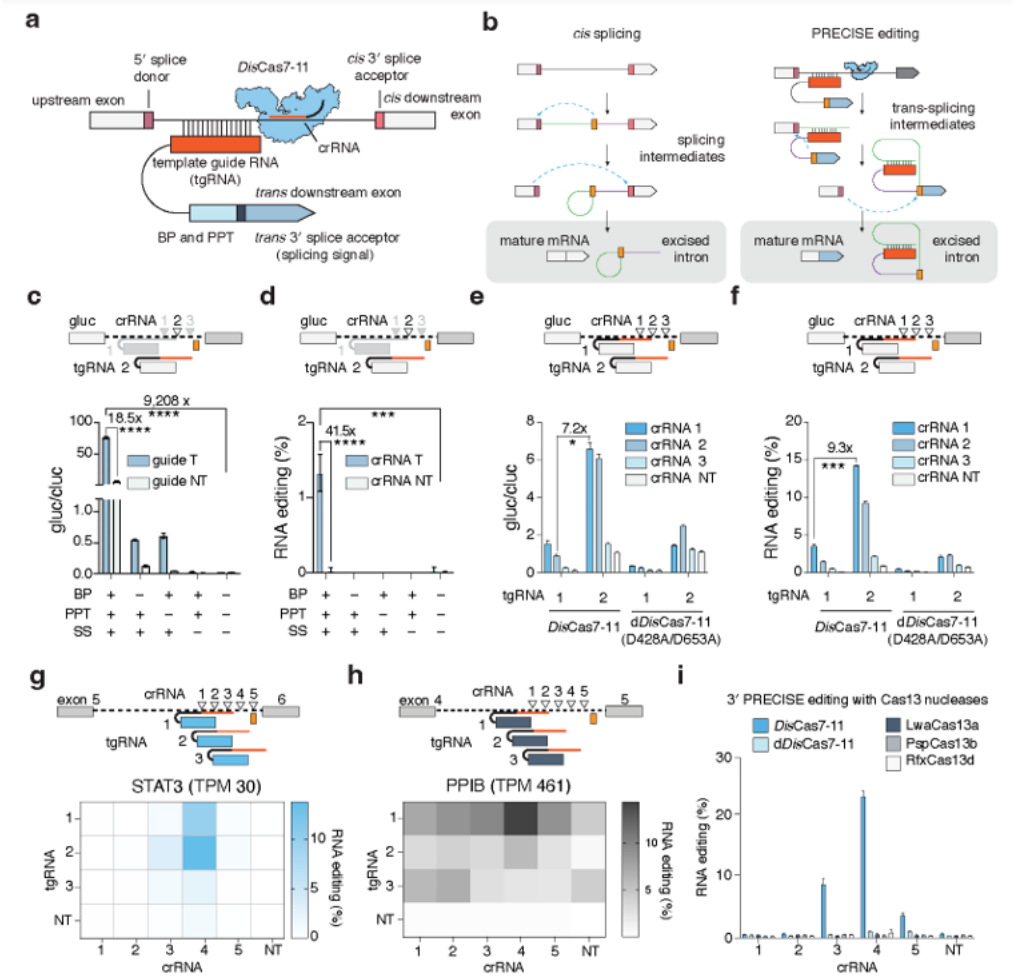

Figure 1

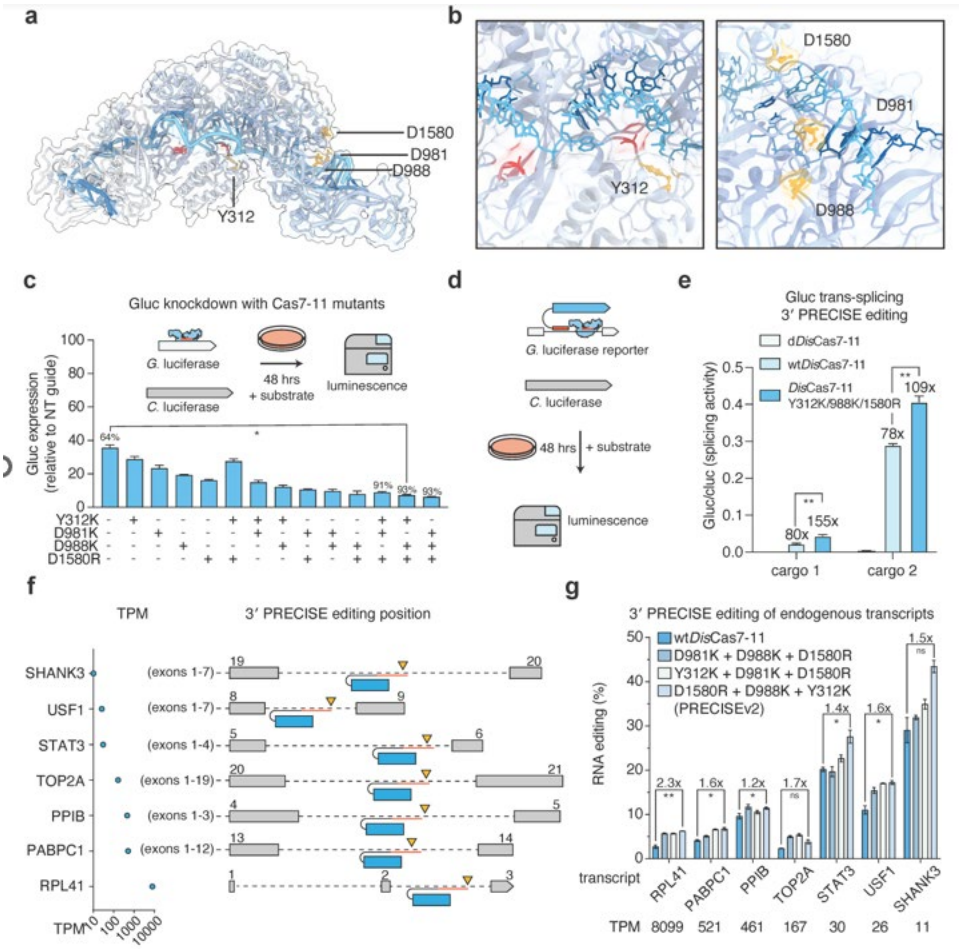

Figure 2

178 Neuronal-Type Specific *SNCA*-Targeted Epigenome Therapy for Precision Medicine in Synucleinopathies

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Elevated *SNCA* levels are causative in synucleinopathy pathogenesis, specifically, Parkinson's disease (PD) and dementia with Lewy bodies (DLB), while normal physiological SNCA levels are essential to maintain neuronal function. We developed precise neuronal-type specific *SNCA*-targeted epigenome therapies for synucleinopathies that mediates fine-tuning of *SNCA* levels based on CRISPR/deactivated(d)Cas technology. The system is *an all-in-one* lentiviral vector comprises of CRISPR/d*Sa*Cas9 and gRNA targeted at *SNCA*-intron1 fused with a synthetic repressor molecule. To achieve neuronal-type specificity for dopaminergic and cholinergic neurons, the vectors were driven by TH or ChAT promoters, respectively.

We utilized a patient hiPSC-derived dopaminergic and cholinergic neurons to carry out the *in vitro proof-of-concept* studies. The differentiated matured neurons showed an efficient and neuronal-type specific downregulation of *SNCA*-mRNA and protein, and rescued disease-related cellular-phenotypes including, Ser129-phophorylated α-

synuclein, neuronal viability, and mitochondrial dysfunction. Next, preclinical *in vivo* studies were performed by stereotactic injection into the substantia-nigra (SN) of our developed PD-mouse model induced by an AAV/A53T-human (h)SNCA vector that contains the mutated human-*SNCA* coding sequence driven by the human native promoter/intron1 regulatory region. These experiments demonstrated a significant reduction in hSNCA protein in the SN, and ameliorated neuropathological characteristics including, robust reduction in Ser129-phosphorylated and aggregated alpha-synuclein and greater retention of dopaminergic neurons in the SN and striatum. Furthermore, behavioral studies showed significant improvement in motor function. Next, we refined the therapeutics *all-in-one* vector to be compatible for AAV delivery. We employed our recently published AAV-epigenome-editing platform combined with an engineered small dopaminergic-specific regulatory element based on neuronal-specific ATAC-seq profiles. The novel AAV/short(s)THp-dCas-repressor vector recapitulated the significant reduction in total, Ser129-phosphorylated and aggregated hSNCA, and retention of dopaminergic neuron projections in the SN and striatum.

In this study, we demonstrated the therapeutic potential of our *SNCA*-targeted technology for PD, DLB and related disorders, providing the foundation to advance towards IND-enablement and clinical trials. The platform is applicable and can be tailored for the development of other gene-targeted therapies for a spectrum of CNS disorders. In conclusion, Our *all-in-one* AAV platform for gene repression represents the 'next generation' gene therapy approach for AD-PD spectrum diseases and other CNS disorders based on fine-tuning of gene expression via targeted epigenome editing.

179 Efficacy and Long-term Safety of CRISPR/Cas13 RNA-targeting Medicine in Mouse and Non-human Primate Models of Neovascular Age-related Macular Degeneration

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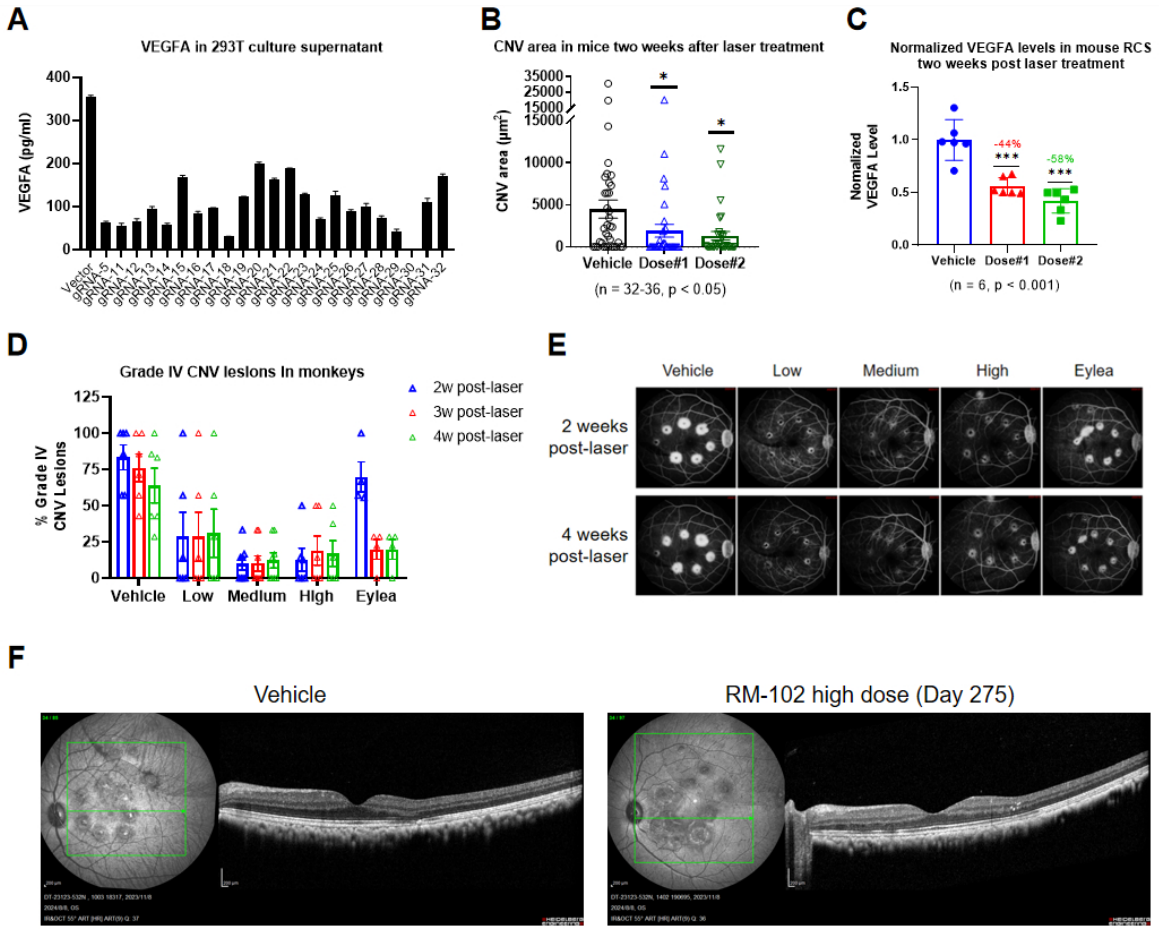
Purpose:
Regular intravitreal injections of anti-vascular endothelial growth factor (VEGF) drugs have proven effective in treating neovascular age-related macular degeneration (nAMD). However, frequent invasive procedures can lead to serious complications, and up to 40% of cases are non-responders, potentially due to immune responses against the injected drugs or anatomical barriers in the retina that hinder drug uptake. Here, we introduce RM-102, a novel AAV and CRISPR/Cas13 (CasRm)-based therapy designed for long-term effectiveness in nAMD.

Methods:
VEGFA-targeting CRISPR/CasRm plasmids, consisting of different sgRNAs, were transfected into 293T cells for sgRNA screening. The most effective sgRNA and CasRm were incorporated into an all-in-one plasmid, packaged into AAV vectors (RM-102), and injected subretinally into VEGFA-humanized mice and non-human primates (NHPs) at different doses. After weeks, choroidal neovascularization (CNV) spots were induced by laser photocoagulation. Fluorescein fundus angiography (FFA) and isolectin-B4 (IB4) staining of the retinal pigment epithelium-choroid-sclera (RCS) tissues were performed to evaluate the suppression of CNV by RM-102. Safety evaluations included ophthalmic examinations, ocular pathology studies, and a biodistribution study in monkeys.

Results:
VEGFA-targeting CRISPR/CasRm plasmids significantly reduced VEGFA levels in culture supernatant, with gRNA-30 being the most effective (Fig. A). In mice, two weeks after laser treatment, FFA showed that lesions indicating the greatest damage (grade 2B) were less frequent in the RM-102-treated groups (1.7% and 5.4%) compared to the vehicle group (14.1%). IB4-stained CNV areas in RCS were significantly reduced by 52% and 71% in the treatment groups (Fig. B), mainly attributed to the downregulation of VEGFA in RCS (Fig. C). In monkeys, the incidence of grade IV lesions in the RM-102-treated groups was significantly reduced, comparable to 2 mg of Eylea administered intravitreally one week post-laser treatment (Fig. D & E). No product-related abnormalities were

found in ophthalmic examinations during the 275-day observation period (Fig. F). Biodistribution results indicated that RM-102 was primarily distributed in the retina and RCS of the subretinal bleb.

Conclusions:
RM-102 effectively suppresses VEGFA expression and laser-induced CNV in NHPs, with excellent long-term safety. These results show that RM-102 is a promising treatment for nAMD and offer an option for non-responders to anti-VEGF therapies.



180 Applying AIMer-Based RNA Editing Technology to Correct a Nonsense Mutation in the Lung

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AIMers are short, chemically modified oligonucleotides that engage endogenous adenosine deaminase acting on RNA (ADAR) enzymes to induce highly efficient and specific A-to-I RNA base editing. Harnessing endogenous ADAR enzymes using oligonucleotides is an emerging approach to targeted therapeutic RNA editing for the correction of nonsense mutations. However, achieving highly efficient RNA editing *in vivo* is challenging for tissues beyond the liver. Recently, we described the optimization of AIMer design to increase the potency and target space

capabilities of RNA editing and demonstrated the impact of this design in mouse liver. Here, we report the development of optimized AIMers for application to the lung and demonstrate correction of a nonsense mutation and protein restoration of a therapeutically relevant target in human lung cells *in vitro*. We applied AIMer technology in the lung to edit the house-keeping transcripts *Ugp2* and *ACTB* in mouse and non-human primate (NHP), respectively. One week after a single dose of AIMer, we observed 50% editing of *Ugp2* in the lung of transgenic mice expressing human *ADAR1* and 10% editing of *ACTB* in NHPs. To assess AIMer activity against a clinically relevant target, we investigated whether AIMers could be applied to correct the W1282X nonsense mutation in the *CFTR* mRNA, which causes Cystic Fibrosis, a multi-organ disease that ultimately leads to respiratory failure. These AIMers are designed to convert the premature stop codon to tryptophan, promoting transcript stabilization and protein restoration. In human bronchial epithelial cells expressing $CFTR^{W1282X}$, we progressively optimized AIMer chemistry for the *CFTR* mRNA target to enhance editing efficiency. CFTR AIMers with our initial screening AIMer-D design supported 21% (EC50 = 376nM) *CFTR* mRNA editing. Notably, judicious selection of the sugar modification at the position across from the target adenine in the AIMer increased maximum editing from 56% (with 2'deoxy) to 75% (with 2'OMe) in partially optimized AIMers. Further optimization led to AIMers that support maximum editing up to 75% (EC50 =246nM). Treating human bronchial epithelial cells with our highest efficiency CFTR AIMers increased *CFTR* transcript levels by 3-fold and restored CFTR protein levels to 50% of wild-type. These preclinical investigations lay the foundation for the development of AIMers as potential RNA medicines to treat disease in the lung.

181 Molecular and behavioral rescue following dual AAV targeted CRISPR epigenome editing in CDKL5 deficiency disorder patient-derived organoids and transgenic mice

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CDKL5 (cyclin-dependent kinase like 5) deficiency disorder (CDD) is a rare developmental epileptic encephalopathy caused by pathogenic loss of function variants in the *CDKL5* gene. CDD is characterized by onset of medically refractory multifocal and myoclonic epilepsy in the first four to six weeks of life with an estimated incidence rate of one in 40,000 – 60,000 live births. In addition to the early infantile-onset epileptic spasms and tonic seizures, other common features include low muscle tone, intellectual disability, sleep difficulties and cortical visual impairment. In female somatic cells, one *CDKL5* allele is epigenetically silenced by X chromosome inactivation. One potential strategy to treat CDD in females is to reactivate the silenced, wildtype allele in cells expressing the mutant copy of the gene. In contrast to somatic cell gene editing, through which modification of the DNA sequence following genetic intervention is permanent, the advantage of an epigenome editing approach is that these edits are reversible. Following our previously published data demonstrating that targeted epigenetic reactivation of the *CDKL5* gene on the X chromosome is feasible, we have further expanded on the preclinical utility of our targeted gene reactivation approach. In order to deliver large epigenome editors to the central nervous system in the developing brain, we utilize intein-mediated *trans*-splicing of protein, demonstrating *in vitro* reconstitution of full-length dCas9 and *in vivo* distribution and reconstitution of split dCas9 following direct intracranial injection of AAV9 encoded vectors. To further optimize viral genome size, we show multiplexed gRNA expression from a single tRNA-gRNA cassette and utility of a hypercompact super core promoter and synthetic poly(A) vector design demonstrating equivalent *in vitro* editing efficiencies to oversized constructs. Following optimization of this epigenome editing approach, we demonstrate disease-modifying activity in human and mouse models of CDD. To this extent, we have generated cortical organoids from a patient-derived iPSC line carrying a heterozygous *CDKL5* c.1412delA mutation that clonally express either the wildtype or mutant allele. We demonstrate *in vitro* DNA methylation editing of *CDKL5* using split dCas9, as well as highly specific on-target gene regulation of *CDKL5* as measured by whole transcriptome sequencing following AAV transduction of cortical organoids. Reactivation of CDKL5 results in a significant increase in phosphorylation of the kinase substrate EB2-Ser222. Intracerebroventricular AAV delivery of the dual epigenome editors in the exon 6 deletion rodent model of CDD restores *Cdkl5* on the mRNA and protein levels across cortex and whole brain, respectively. *Cdkl5* epigenetic editing *in vivo* results in a significant EB2-Ser222 phosphorylation increase in treated heterozygous mice. Lastly,

treatment of adult heterozygous animals results in rescue of behavioral domains as measured by open field for gross motor activity, clasping, and the Y-maze assay for spontaneous alternations to measure cognition. Our results highlight the preclinical utility of *in vivo* epigenome editing for the treatment of previously undruggable genetic conditions that are, in part, caused by epigenetic silencing mechanisms.

182 Investigator-initiated Development Paths for Oligonucleotide and Epigenetic Therapies in Prion Disease

Fiona Serack[1], Juliana Gentile[2], Alissa Coffey[3], Edwin Neumann[4], Tessa Bertozzi[5], Taylor Corridon[2], Nikita Kamath[6], Meredith Pokatayev[3], Vanessa Laversenne[2], John Harvey[7], Pamela Brauer, Catherine Pirtle[7], Michael Howard[3], Kenia Guzman[3], Dimas Echeverria[8], Ken Yamada[9], Julia Alterman[9], Ken Chan[6], Benjamin Deverman[1], Anastasia Khvorova[9], Jonathan Weissman[10], Eric Minikel[3], **Sonia Vallabh[1]**
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Prion disease is a rapidly progressive neurodegenerative disease that is universally fatal and currently untreatable. Pathogenesis is driven by the misfolding of a single causal protein, the prion protein (PrP, encoded by PRNP), into a self-propagating conformer. Genetic proofs of concept have long shown that reduction of PrP gene dosage is protective against prion disease, with full knockout conferring complete protection. More recent studies have leveraged new PrP knockout animal models and PrP-lowering antisense oligonucleotides to show that this protection is dose-dependent and observable across species and prion strains. At this moment in biotechnological history, it is conceivable that either an oligonucleotide therapeutic or a virally vectored gene therapy could reduce PrP levels in the brain sufficient to treat, delay or prevent prion disease. Our lab is therefore pursuing both strategies. This talk will describe our efforts to advance to small academic-initiated first-in-human studies both a divalent siRNA therapy for prion disease, and a one-time epigenetic therapy called CHARM (Coupled Histone tail for Autoinhibition Release of Methyltransferase). Our di-siRNA molecule consists of two linked siRNA duplexes that target the Prnp RNA, lower total PrP in the mouse brain up to 70% at one month post- dose following naked delivery into CSF, and maintain significant target engagement up to six months post-dose. CHARM, by contrast, is an epigenetic editor that recruits the endogenous DNMT3A methyltransferase to the Prnp promoter, resulting in up to 80% PrP reduction across the mouse brain, stable up to at least six months, when delivered systemically via blood-brain barrier crossing AAV. Both di-siRNA and CHARM have been shown capable of dramatic disease modification in prion-infected mice. As both di-siRNA and CHARM are novel drug types first reported in 2019 [1] and 2024 [2] respectively, our ongoing efforts have the prospect to generate the first human data for both modalities. In the context of these two rapidly advancing programs, this talk will discuss the comparative advantages and disadvantages of oligo and gene therapies for prion disease. Topics will include comparison of single-cell drug activity across cell types and regions in the CNS, and how contrasting target engagement profiles may interact with disease biology; distinct timelines and challenges for manufacturing and IND-enabling studies; and regulatory feedback received to date regarding both programs. Finally, this talk will discuss the central clinical challenge in this rapidly progressive dementia – treating preventively. We will share our work, shaped by FDA input, across the domains of biomarker development, natural history and preclinical studies to enable preventive trials in healthy individuals with known high-penetrance PRNP variants. We will further describe how the mandate to extend healthy life, rather than just illness – one that is shared broadly across the field of neurodegeneration – is guiding our clinical plans. This talk will be delivered from a patient-scientist perspective as Sonia Vallabh is both a prion disease researcher and herself a carrier of a high-penetrance pathogenic PRNP variant causal for genetic prion disease.

[1] Alterman 2019, PMID: 31375812
[2] Neumann and Bertozzi 2024, PMID: 38935715

183 An epigenetic gene therapy delivered using lipid nanoparticles as a treatment for HPV-associated malignancies

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Human Papillomavirus (HPV) causes a range of epithelial malignancies associated with oropharyngeal, cervical, and anal carcinomas with ~47,000 invasive cases in the USA annually causing significant morbidity and mortality. Despite an HPV vaccine that reduces cancer risk; denialism, cost, and lack of awareness means the continued risk of HPV-related cancers. Integration of the circular, episomal HPV genome into the host's DNA is an essential event in oncogenesis. Out of the episome context, the HPV long control region (LCR) promoter overexpresses the viral E6 and E7 proteins, which inhibits the tumor suppressors, p53 and Rb1 respectively, driving transformation. Furthermore, the viral oncogenes are critical for tumor maintenance and in a state of 'oncogene addiction' making the oncogenes ideal targets for therapeutic intervention. The viral oncogenes are considered undruggable, but ideal for gene therapy interventions. However, a gene therapy faces significant challenges such as targeting multiple HPV oncogenes within sequence-diverse HPV subtypes, which may require a multi-component treatment, complicating clinical translation. Furthermore, the transient epigenetic suppression of the oncogenes would avoid unwanted genotoxicity associated with nuclease-based strategies. Finally, delivery of genetic medicines is a hurdle and a clinically viable mRNA format packaged within a scalable non-viral delivery platform would make a gene therapy for HPV-associated cancers a reality.

To this end, we have exploited a viral self-regulatory genetic circuit to develop a compact, single-component epigenetic repressor targeted to universally conserved sites in HPV's LCR, which can silence multiple viral oncogenes in virtually all HPV subtypes. This epigenetic modality, termed BPV-E2-KRAB, inhibited the proliferation of multiple HPV-associated cancer cell lines including oropharyngeal, cervical, and anal cancer, and in both high-risk HPV16 and HPV18-associted cervical cancers. Furthermore, BPV-E2-KRAB suppressed E6 and E7 expression resulting in the reactivation of the p53 tumor suppressor and caspase-mediated apoptosis. The BPV-E2-KRAB was formulated as mRNA into MC3 lipid nanoparticles (LNPs) and when delivered intratumorally reduced tumor volume *in vivo* in a xenograft HPV-associated oropharyngeal carcinoma model with improved clinical scores and survival in the treated mice compared to controls. Furthermore, safety profiling showed no effects on the proliferation of a non-HPV cell line, serum ALT/AST levels, or liver histology. Finally, global mRNA profiling reveal upregulation of interferon pathways by the BPV-E2-KRAB, which may additionally contribute to the anti-proliferative effects in HPV-associated cancers. Overall, as a transient, druggable, scalable LNP-mRNA formulation, the BPV-E2-KRAB represents a promising new epigenetic mRNA treatment towards a generalizable gene therapy for HPV-associated malignancies.

Novel Approaches to Gene Targeting and Gene Correction

184 Essential HDRescue: Enhancement of Genome Editing by Harnessing Cellular Vulnerabilities

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Homology-directed repair (HDR) is a DNA repair pathway required to install precise, user-defined alterations in the genome following a nuclease-induced DNA double-stranded break (DSB). Although HDR can install precise edits at a locus-of-interest (LOI), mutagenic non-homologous end-joining (NHEJ) occurs more frequently than error-free HDR in mammalian cells. This higher rate of NHEJ limits the ability to identify and select precisely edited cells. Existing genome editing selection strategies improve HDR efficiencies by introducing a selectable transgene or

marker, such as drug resistance, that is tied to a user-defined modification. These selection methods require the use of small molecules or specialized equipment to isolate cells containing the intended edit and may also require labor-intensive cloning techniques. Selective agents also risk unwanted phenotypic changes to targeted cells and are not ideal for pre-clinical applications in genome editing, particularly if the modified gene is not under the control of its endogenous regulatory elements. Therefore, there is a need for a broadly applicable and practical method to improve the frequency of user-defined editing events in a cell-intrinsic manner.

To address this problem, we developed a novel enrichment method, termed Essential HDRescue, to increase the frequency of precise editing events at a LOI by co-targeting an essential genomic locus. We hypothesized that using intrinsic positive and negative selection at a common essential gene would enable enrichment of precise editing events at a separate LOI. To select against cells that have undergone NHEJ, an essential gene is targeted using CRISPR to induce gene knockout via NHEJ repair, resulting in rapid cell death. To enrich cells that have undergone HDR, a donor template installs a silent mutation to rescue the essential gene function and prevent recutting and subsequent NHEJ repair. Using targeted next-generation sequencing, we demonstrate that co-targeting essential genes *SF3B1*, *RAN*, or *GAPDH* in cancer cell lines and iPSCs significantly increases HDR rates—up to 30% precise editing—without the need for an exogenous reporter or selective drug. Further analysis of single-cell-derived clones showed that up to 52% of clones contained at least one precisely edited allele, and up to 21% of iPSC clones contained a homozygous precise mutation, representing a nearly 6-fold and 4-fold increase in clones with the respective edit compared to single-targeted control cells. Off-target activities and translocations were observed in less than 1.0% of edited clones with our top Essential HDRescue single-guide RNAs. We demonstrate enrichment of HDR-mediated small inserts and large protein tags using both single-stranded and double-stranded DNA donors and across several LOI with Essential HDRescue. These results suggest that Essential HDRescue is an effective strategy to boost HDR rates, without bearing deleterious consequences at on- or off-target sites, particularly in cell types, such as iPSCs, which are advantageous for authentic *in vitro* model creation.

Essential HDRescue allows for the enrichment of endogenously edited loci and does not require the use of an exogenous reporter or selective drug. Instead, this technique harnesses intrinsic cellular vulnerabilities caused by DSB repair at essential loci, making Essential HDRescue a widely applicable approach to enhance genome-editing outcomes in mammalian cells.

185 Precise Removal of a Large Pathogenic Repeat Expansion *In Vitro* and *In Vivo* Using a Dual-guided TevCas9 (Dualase®) Genome Editor Encoded in a Single AAV

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Repeat expansion disorders (REDs) are challenging targets for current genome editing approaches since the pathogenic repeated nucleotides are often hundreds of bases to kilobases long. Furthermore, the repeat lengths can vary widely between patients and even between affected cells within a single patient. RNA-programmable genome editing using CRISPR-Cas nucleases creates double-strand breaks at a target site, which are predominantly resolved through error-prone non-homologous end-joining (NHEJ), a process that introduces random insertions or deletions (indels). Efforts to excise these repeats using two nucleases targeting non-repeat regions flanking repetitive sequences have been reported but can be limited by low efficiency and unintended indels around either cut site, which may affect the function of nearby regulatory or coding sequences. Additionally, the large coding size of many gene editors necessitates splitting the editor between two adeno-associated virus (AAV) vectors. This creates challenges for editor design optimization and *in vivo* delivery, as well as increasing the costs of production. A genome editing therapeutic capable of precise repeat sequence removal and in which the regulatory sequences, gene editor and guide RNA(s) could be packaged and delivered within a single AAV would have several important advantages over existing systems.

Here, we present proof-of-concept using an RNA-guided TevCas9 gene editor for the efficient and precise removal of repeat sequences in patient cells and in a humanized murine model. The previously reported TevCas9 gene editors are fusions of the GIY-YIG family I-TevI nuclease and linker domains to RNA-programmable nuclease

domains, which in this study is *Staphylococcus aureus* Cas9. I-TevI functions as a monomeric nuclease targeting a preferred 5'-CNNNG-3' motif that is appropriately spaced from the Cas9 binding site. I-TevI-mediated cleavage at the target site leaves a 2-nucleotide 3'-overhang. We use a version of TevCas9 in which the Cas9 domain is inactivated by mutation and use two guide RNAs that direct the Dualase to flank the repeat region and orient the active I-TevI domains into the repeat sequences. Cleavage by both I-TevI domains leaves complementary 2-nucelotide 3'-overhangs that, when joined, result in precise removal of the large, intervening repeat to leave a non-pathogenic number of repeats.

TevCas9 genome editors are approximately 3.7 kilobases of coding sequence and can be encoded in a single AAV together with two or more targeting guide RNAs in one construct ("all-in-one") and can be specifically directed to virtually any target. Using such an all-in-one AAV9-encoded TevCas9 editor programmed with two guide RNAs to target a pathogenic repeat expansion, we show genotypic and phenotypic correction in patient-derived differentiated cells in culture. In a humanized mouse model of the same repeat expansion disorder, we also demonstrate removal of the repeat sequence as well as correction of pathologic phenotypes. Additionally, we report optimizations of the all-in-one construct that improve the performance of AAV9 encoded TevCas9 to remove repeats in patient-derived cells. Our AAV-encoded, dual-guided TevCas9 genome editors are capable of precise, therapeutic removal of pathogenic genomic repeat expansion sequences for a broad range of REDs.

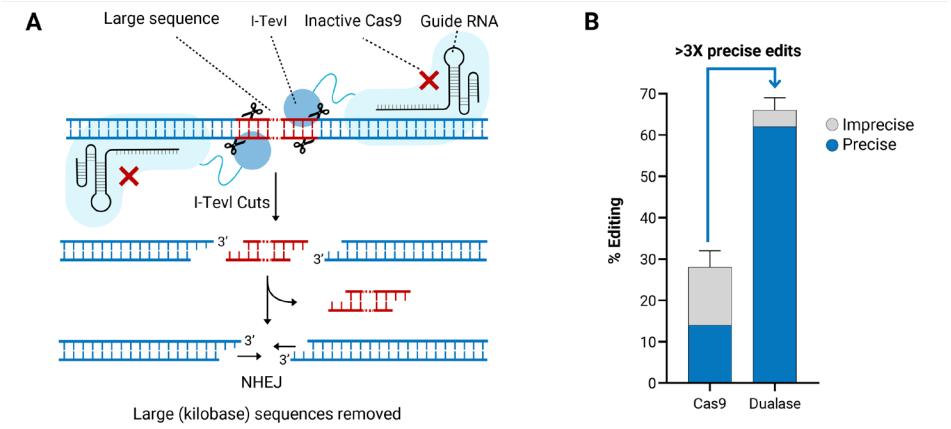

FIG 1. Dual-guided I-TevI for efficient & precise large sequence removal. **A.** Inactivating mutations are introduced into an RNA-guided Cas9 domain in TevCas9 such that active I-TevI domains are oriented into either side of a large repeat sequence with two guide RNAs. Upon cutting at both I-TevI sites, the intervening large repeat sequence is removed and the two complementary 2-nucleotide 3'-overhangs are joined to leave a non-pathogenic number of repeats. **B.** Editing outcomes in AAV-transduced a patient-derived cell model of a large repeat expansion show the precision of repeat removal based on clonal sequencing and efficiency based on percent of repeat vs non-repeat sequences. TevCas9 ("Dualase") results in 3-times more precise edits when compared to dual-guided saCas9 alone.

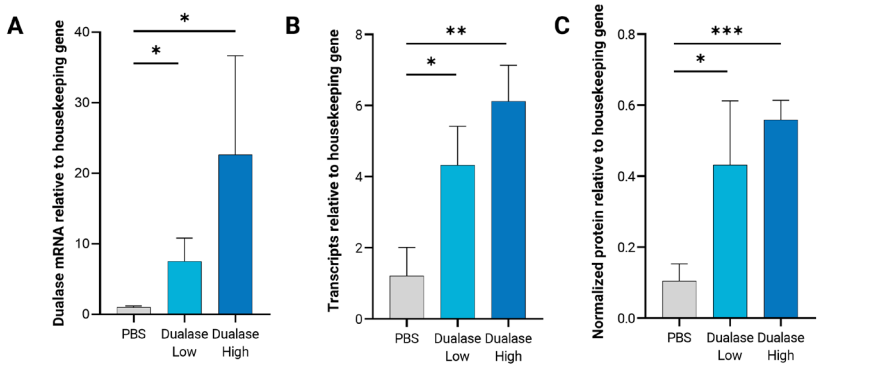

FIG 2. A. Humanized repeat expansion mice were intracerebroventricular (ICV) injected with AAV9-encoded dual-guided TevCas9 ("Dualase"). A dose-dependent increase in expression of TevCas9 relative to a housekeeping gene was confirmed by quantitative reverse transcription PCR (RT-qPCR) in bulk cerebellum tissue relative to mice injected with phosphate buffered saline (PBS) as a control. **B.** The mRNA transcripts of a downstream missing enzyme suppressed by the repeat expansion was measured by RT-qPCR relative to a housekeeping gene. A TevCas9 dose-dependent increase in the transcripts was observed, with a greater than 6-fold increase at the highest TevCas9 dose relative to PBS-treated mice. **C.** The expression of the downstream enzyme was also assessed by Western Blot relative to a housekeeping gene in cerebellum tissue. Similar to the recovery in transcription observed in panel B, an increase in the levels of the missing enzyme was observed, with up to a 5-fold recovery at the highest dose of TevCas9 relative to PBS-treated mice. N=3; * P<0.05, ** P<0.01, *** P<0.001.

186 In Cellulo DNA Assembly for Targeted Genomic Integration in Human Cells

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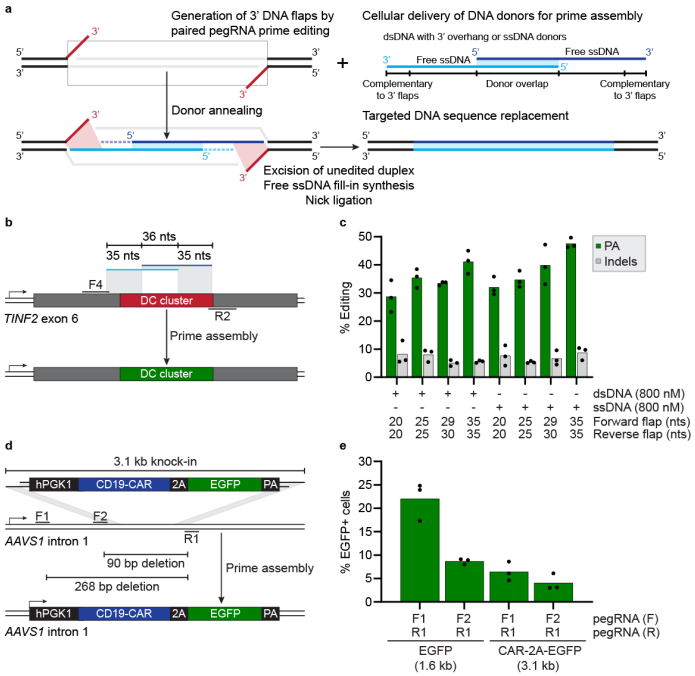
Therapeutic genome editing holds great potential to remedy diverse severe Mendelian disorders. Although progress has been made, multiple cell type-specific barriers constrain the installation of medium to large genomic modifications in therapeutically relevant hematopoietic cells. To expand the genome editing toolkit, we developed an approach that enables DNA sequence assembly and integration in human cells using CRISPR-targeted flap synthesis. Denoted as prime assembly (PA), our approach enables the integration of single- or double-stranded DNA (ssDNA and dsDNA) donors to specific genomic loci. As a proof of concept, we devised and optimized a therapeutic exon recoding strategy for *TINF2* dyskeratosis congenita (DC) to potentially correct almost all known gain-of-function mutations associated with this short telomere syndrome, achieving an average efficiency of 47.6% in K562 cells, as determined by amplicon sequencing. Using this approach, we established design rules and demonstrated the capacity of PA to accommodate different types of donors, including synthetic ssDNA donor libraries that can be directly integrated to the genome for functional genomics applications.

To highlight the potential of PA for targeted transgene integration, we developed strategies to integrate reporter transgenes at the *TRAC*, *AAVS1*, and *IL2RG* loci. At the *AAVS1* locus, we achieved average efficiencies of 22.0% and 6.4% $EGFP^+$ cells for the integration of 1.6 kb and 3.1 kb transgene cassettes, respectively. Using a CDK4/6 inhibitor and cell cycle analysis, we observed efficient PA in non-dividing cells arrested in G0/G1. Under selective pressure, we also demonstrate the assembly and integration of up to four ssDNA fragments at the *ATP1A1* locus. Finally, we successfully targeted a reporter transgene to the *IL2RG* locus using ssDNA donors in hematopoietic stem and progenitor cells from different healthy donors, albeit with low efficiency without optimization. Together, prime assembly enables site-specific DNA integration without requiring homology-directed repair, cell cycle progression, recombinases, or donor dsDNA, providing a new tool for functional genomics and therapeutic genome editing.



Prime assembly enables efficient site-specific DNA integration in human cells. (a) Schematics representation of prime assembly (PA). (**b**) Schematics of *TINF2* DC cluster recoding using PA. (**c**) PA and indel quantification at *TINF2* as determined by CRISPResso2 analysis from amplicon sequencing. K562 cells were electroporated with PA vectors and donors, and genomic DNA was harvested 3 days post-nucleofection. $n = 3$ independent biological replicates. (**d**) Schematics representation of targeted transgene integration at the *AAVS1* locus using PA. (**e**) Percentage of EGFP+ cells as determined by flow cytometry. K562 cells were electroporated with PA vectors and donors targeting *AAVS1*, and the percentage of EGFP expressing cells was quantified 21 days post-nucleofection. $n = 3$ independent biological replicates.

187 Development of Novel HDAd Vectors for Efficient Targeted Integration via Prime Editing and Site-Directed Recombination

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Targeted transgene integration into pre-selected genomic sites is critical for precise, safe, and predictable gene therapy outcomes. Unlike random integration, which risks insertional mutagenesis or variable expression, targeted strategies aim to insert transgenes at specific "safe harbor" loci (e.g. *AAVS1*, *ROSA26*) or disease-relevant sites. Conventional CRISPR-mediated HDR (homology-directed repair) is inefficient in non-dividing cells, such as primitive hematopoietic stem cells (HSCs), and suffers from genotoxicity associated with double-strand DNA breaks generated by CRISPR. Helper-dependent adenovirus (HDAd) vectors have a payload greater than 30 kb, are devoid of viral coding genes, and can efficiently transduce HSCs for *in vivo* applications. Here, we aim to develop novel helper-dependent adenovirus (HDAd) vector systems carrying a donor cassette, a prime editor (PE), and a highly efficient serine recombinase Bxb1 for targeted transgene integration. The prime editor is responsible for installing one or two Bxb1 attachment sites (attP) at the *AAVS1* locus; while the Bxb1 mediates insertion of the donor DNA linked with one or two attB sequences. We designed three strategies with different mechanisms for circularization of the donor DNA. Each included two vectors: one expressing the PE elements and the Bxb1 recombinase; the other containing the donor cassette joined to a Bxb1 attachment site. Specifically, strategy A featured a flippase to generate the donor minicircle. Strategy B installed two heterologous attachment sites *in cis* and capitalized on the recombinase-mediated cassette exchange (RMCE) for donor integration. Strategy C included one attachment site (attP-GA) and its cognate attB-GA for donor DNA circularization, while utilizing a heterologous attP-GT/attB-GT pair for integration. We demonstrated that, without the formation of donor minicircles, linear HDAd viral DNA linked with only one attachment site did not stably integrate. This is consistent with the mechanism of Bxb1-mediated recombination. By transfection in cell lines, we observed that all three strategies

resulted in similarly efficient integration of the donor template. Compared to the random integration mediated by a Sleeping Beauty transposase (SB100x) and a CRISPR-based HDR, the frequency of integration by the three strategies was approximately two and three-fold higher. We successfully produced HDAd vectors for strategy B, except the PE vector, which showed significant rearrangement, likely due to repetitive sequences of the four U6 promoter-epegRNA elements. We are attempting to switch to alternative type III RNA polymerase promoters to reduce sequence repetition in this vector. By HDAd transduction, we found that the PE vector generated near saturating levels of attP docking site installation. With regards to integration, strategy C outperformed strategy A, suggesting that the flippase in the latter strategy is likely to limit its efficiency. Targeted integration was confirmed by in-out PCR and inverse PCR. Additionally, single cell derived clones demonstrated that the overwhelming majority clones had at least one copy of transgene integration at the target site. Building on these strategies, ongoing studies are testing novel HDAd vectors expressing therapeutic transgenes for HIV-1 infection and hemoglobinopathies.

188 Engineering High-Efficiency, High-Specificity Recombinases for Therapeutic *In Vivo* Genome Engineering

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Genomic medicine promises to revolutionize human health by enabling off-the-shelf therapies that cure disease with a single treatment. However, current gene delivery approaches are hobbled by fundamental limitations in potency and safety and are failing to deliver on this promise.

Large serine recombinases (LSRs) offer a path forward. LSRs mediate high-fidelity, site-specific integration of large genetic payloads without exposing double-strand breaks and can be delivered by lipid nanoparticles. Naturally occurring LSRs lack the specificity and/or activity necessary for use as genomic medicines without cumbersome workarounds to insert native attachment sites. We hypothesized that engineered, site-specific, therapeutic-grade recombinases could be designed to integrate payloads at therapeutically relevant sites within the human genome.

Here we report a high-efficiency, high-specificity recombinase targeted to a safe harbor locus in the human genome with broad applicability for genomic medicine. It is the output of an iterative design-build-test-learn process that leverages a platform of structure-guided design enabled by insights from proprietary cryo-EM structures, bacterial directed evolution screens, and real-world AI/ML-based protein design and data analysis.

The modular LSR architecture facilitates parallel subdomain evolution and optimization. We identified efficiency-enhancing mutations throughout the protein and found that combining individually identified variants resulted in cumulative improvements. For example, insertional efficiency at the primary safe harbor site was increased sevenfold, reaching over 50% in human cells. Specificity-enhancing mutations were predominantly confined to DNA binding regions, where bacterial evolution selections were critical for engineering. We identified LSR variants for which primary site insertion efficiency exceeded that at all secondary sites by more than 10-fold as evaluated in an unbiased NGS cell-based readout and, specificity relative to the top three secondary sites improved by 90x, 391x, and >500x – all while retaining high insertional efficiency at the safe harbor site.

When electroporated into primary human T cells with a CAR payload, our engineered LSR resulted in over 40% CAR-positive cells with more than 25% integration by ddPCR. Additionally, payloads ranging from 3 kb to 13.5 kb were integrated with no loss in efficiency relative to size.

The design of therapeutic-grade recombinases for predetermined sites in the human genome represents a novel and powerful avenue for precise, non-viral genomic engineering. We are now applying this strategy to develop recombinases targeted to additional therapeutically relevant sites in the genome. This work lays the foundation for a genomic medicines platform capable of enabling off-the-shelf, in vivo LNP-based therapies that are both highly

effective and broadly accessible.

189 Alemtuzumab-Resistant Virus-Specific T (VST) Cells Developed for the Prevention of Viral Infections After Hematopoietic Stem Cell Transplantation Retain Antiviral Activity In-Vivo and In-Vitro

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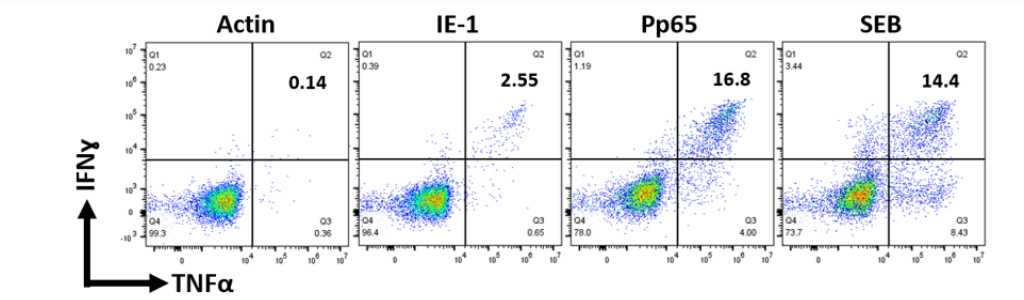
Hematopoietic stem cell transplantation (HSCT) is a curative treatment for many malignant and non-malignant hematologic conditions. Alemtuzumab is often used as conditioning pre-HSCT but is associated with delayed T cell reconstitution and an increased risk of infections. Virus-specific T cell (VST) immunotherapy enhances immune reconstitution post-HSCT but may be eliminated by the use of alemtuzumab. CD52, the target of alemtuzumab, has no known crucial role in the immune system and CD52 knockout (CD52-KO) may allow generation of alemtuzumab-resistant VSTs. We proposed to use CRISPR/Cas9 gene editing to develop a "next generation" VST product with broad applicability and efficacy. Furthermore, we anticipated CD52-KO VSTs to exhibit similar efficacy and persist in NOD SCID Gamma (NSG) mice after alemtuzumab treatment.

Design/Method:
Peripheral blood mononuclear cells were isolated from CMV positive donors and incubated with CMV-specific peptides pp65 and IE-1 to create CMV-specific VSTs. CD52-KO VSTs were generated by nucleofection of sgRNA (UUACCUGUACCAUAACCAGG) and Cas9 protein. VSTs were analyzed via flow cytometry to determine knock out efficiency, phenotype, and cytokine expression on a cellular level. Additionally, DNA was extracted to determine knock out efficacy on a genetic level by using Interference of CRISPR Edits (ICE) Analysis.

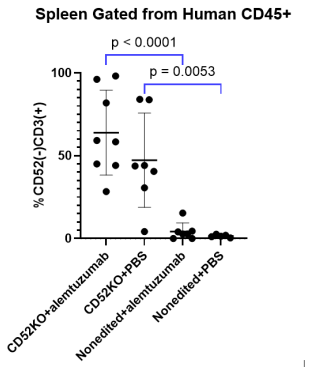
VSTs were tested with 5 groups of NSG mice. Groups 1 and 2 were injected intravenously with 10E6 CD52-KO VSTs, while groups 3 and 4 received 10E6 nonedited VSTs, and group 5 received phosphate-buffered saline (PBS) only. Following VST injection, groups 1 and 3 were treated with a single intraperitoneal injection of alemtuzumab at 5mg/kg while remaining groups were treated with PBS. Mice were sacrificed at 72 hours for the harvest of spleen, bone marrow, and PBMCs to determine knock out efficacy and replicability in-vivo using flow cytometric analysis.

Result/Discussion:
Across 3 runs, CD52-KO efficiency, determined by surface staining of CD52 and ICE analysis, ranged from 58-85% (mean = 71.5%) with no significant off-target edits predicted by Change-seq, CRISPRme, or COSMID. Additionally, intracellular cytokine staining for IFNg and TNFa demonstrated that CD52-KO VSTs retained antiviral activity comparable to non-edited VSTs in the presence of alemtuzumab in-vitro (Figure A). Furthermore, CD52-KO VSTs were detectable in vivo (Figure B). Mice who received CD52KO VSTs and no alemtuzumab treatment had significantly fewer CD52+ T cells (n=7; CD3+/CD52+ 15.73%) than those receiving non-edited VSTs (n=5, CD3+/CD52+ 70.78%, $p<0.0001$). After treatment with alemtuzumab, mice who received CD52KO VSTs maintained a significantly higher percentage of CD3+CD52- T cells (n=8, mean CD3+/CD52- 63.33%) compared to mice receiving nonedited VSTs (n=7, mean CD3+CD52- 3.74%, $p < 0.0001$).

Our study suggests that CD52-KO VSTs, in combination with alemtuzumab, could offer a potential approach to restore antiviral immunity while preventing graft versus host disease after HSCT. Long term persistence and antiviral function of cells in-vivo, as well as multiviral CD52-KO VSTs are being investigated with the eventual intent to create a readily available CD52-KO VST that can fight infection while resisting T cell depletion after HSCT.



B. Persistence of CD52-KO VSTs versus unedited CMV VSTs in NSG mice spleen



190 Nuclease-Free In Vivo Genome Editing of the CNS Following Systemic Administration of AAVHSC15 Vectors

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The development of genetic therapies for central nervous system (CNS) diseases and for neurologic manifestations of systemic diseases has generally proven challenging. While gene augmentation strategies work well for diseases where over- or under-expression is not toxic, the lack of native physiologic regulation can be pathogenic where highly regulated transgene expression is required. This is compounded by the incomplete identification of precise regulatory elements for many disease-associated genes, further complicating the design of therapeutic gene transfer vectors. In addition, the episomal nature of many gene transfer vectors creates uncertainty regarding the long-term stability of gene augmentation. In contrast, the ability to directly correct mutations at the level of the genome offers significant advantages for both the retention of all known and unknown regulatory signals as well as durability. Here, we evaluated the potential of systemic in vivo genome editing to insert promoterless sequences at specified genomic locations within the CNS and in peripheral tissues. We utilized the single component nuclease-free AAVHSC editing platform which edits via the high-fidelity BRCA2-dependent homologous recombination. AAVHSC editing is cell cycle independent and functions in both dividing and non-dividing cells. Genome editing was evaluated in the CNS and peripheral tissues at 2 different loci, the murine Mecp2 and Rosa26 loci following intravenous (IV) delivery. Genome editing was assessed by the insertion of a promoterless luciferase open reading frame downstream of Exon 4 of the murine Mecp2 gene or within Intron 1 of the Rosa26 gene such that expression would be driven by the native chromosomal promoters. Results revealed that IV injection of AAVHSC15 editing vectors resulted in specific in vivo luciferase expression from both Mecp2 and Rosa26 genes. Systemic genome editing was observed both within the CNS and in peripheral tissues. Sequence analyses revealed that editing was precise. No insertion / deletion mutations or ITR insertions were observed at the target site. Transcriptional analyses

revealed that the edited Mecp2 and Rosa26 transcripts were expressed in both the CNS as well as peripheral tissues including liver and heart. Importantly, widespread editing of neurons, astrocytes, and microglia was observed in different regions of the CNS following systemic administration, including the cerebellum, mid brain, medulla, pons, striatum and hippocampus. These results indicate that AAVHSC editing offers a promising platform for the treatment of neurologic diseases that require correction of neurons and glial cells. Based on our overall results, we conclude that in vivo systemic AAVHSC genome editing represents an attractive therapeutic platform for the universal systemic correction of disease-associated mutations.

Downstream Manufacturing for AAV Vectors

191 Integrated AAV Capture and Polishing via BioRMB™, a Continuous, Column-free Chromatography Platform

Thiago Millen[1], **Dalton Kinnard**, Oleg Shinkazh, Alex Schaffer[2], Sreeja Edara, Dwayne Kenney, Collin Britten[3]
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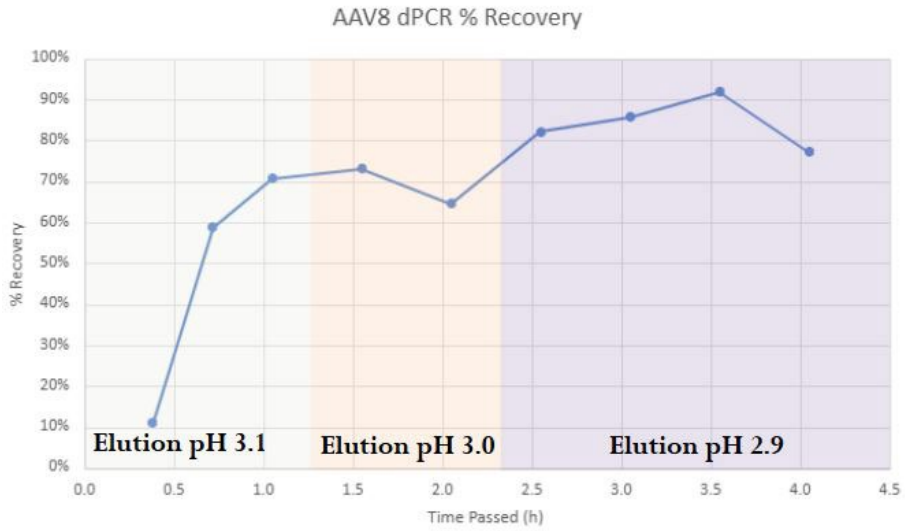
BioRMB™ is a column-free and steady-state purification platform that is specifically tailored for sensitive modalities such as gene therapies, vaccines, mRNA, and complex antibodies, which combines the principles of Real Moving Bed (RMB) and columnless chromatography, allowing different operations to be performed simultaneously on the resin slurry as it is pumped in a continuous loop like a conveyor belt. BioRMB™ overcomes many limitations of batch columns as well as circumventing the drawbacks of multi-column systems, all while providing 5-10x greater productivity with a fully disposable, single-use flow path. Additionally, BioRMB™ demonstrates >80% reduction in resin volume vs. batch columns, higher recoveries and lower HCP.

Previously, we have demonstrated integrated mAb capture and polishing using BioRMB™. In this work we will present capture data for two different serotypes of AAV, using titers obtained by dPCR, qPCR, BLI (Bio-layer interferometry), as well as impurity removal characterized by HCP ELISA, and Host cell DNA by dPCR. We will also be presenting preliminary findings on a proof-of-concept data set for a novel PAT technique for real-time monitoring of AAV whole capsid titter as well as a strong application for real-time AAV empty-full analysis on a continuous purification platform, by integrating BioRMB™ and Real-Time MALS. This will be compared with data acquired by both mass photometry and SEC-MALS.



192 Data-Efficient and Adaptive Machine Learning Framework for Accelerated AAV Downstream Processing.

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Optimizing downstream purification of adeno-associated viral (AAV) vectors remains a fundamental challenge in advancing gene therapy development and commercialization. We have developed a machine learning approach utilizing Gaussian process (GP) surrogate models coupled with quasi-Monte Carlo acquisition functions to predict and optimize AAV vector yield during downstream processing. Our method involved training GP models on critical chromatography parameters across seven clinically relevant serotypes (AAV10, AAV2, AAV5, AAV6, AAV9, AAV3, and AAV8). These parameters included fixed conditions such as a 306 cm/h system flowrate and equilibration conditions set to pH 7.0 with 2.5 conductivity. The optimization space explored three AAV serotypes (AAV2, AAV5, AAV6, and AAV9) with variable elution and wash pH ranging from 5.0 to 9.0 in 0.5 increments. Elution conductivity was varied continuously between 10.0 and 100.0 mS/cm, while wash conductivity was adjusted in 1.0 increments from 1.0 to 15.0 mS/cm. Sample volumes were tested at discrete intervals of 5 mL between 5 and 30 mL, and sample flowrates were continuously varied between 130 and 600 cm/h. All purifications were done using Toyopeal-NH2-750F resin conjugated with the peptide CVIDGSASTDDDRIC. The GP model, integrated with the acquisition function, guided an iterative Bayesian optimization process that efficiently sampled the search space of process parameters to find specific values of those that significantly optimized quality attributes of the purification process (Fig. 1). The model achieved remarkable accuracy for yield prediction predictive accuracy with a coefficient of determination (R^2) of 0.92 across all serotypes tested. The model converged on parameters to maximize yield and purity by more than 98% in only three experimental iterations, outperforming traditional process development methods that typically yield around 70% (Fig 2). This data-driven strategy significantly accelerates process optimization while enhancing critical quality attributes of AAV vectors. To add a layer of process understanding, we used SHAP (SHapley Additive exPlanations) to assess how each process parameter affects the model's predictions. This gave qualitative and quantitative insight into how each input parameter affected the process yield. Our analysis revealed that sample volume was the dominant process parameter affecting capsid yield, with higher volumes positively correlating with increased production. Other parameters, such as wash conductivity and elution flowrate, had moderate effects: higher wash conductivity reduces capsid yield, while lower conductivity increases it; and higher sample flowrate increases capsid yield, while lower flowrate decreases it. Wash pH demonstrated minimal impact on the process outcome. This analysis provides quantitative insight into each parameter's importance for process optimization. Beyond improving manufacturing efficiency and product quality for gene therapy programs, our methodology shows promise for broader applications in biomanufacturing process optimization. These advances directly address current manufacturing bottlenecks that impact the availability and cost of gene therapies for patients.

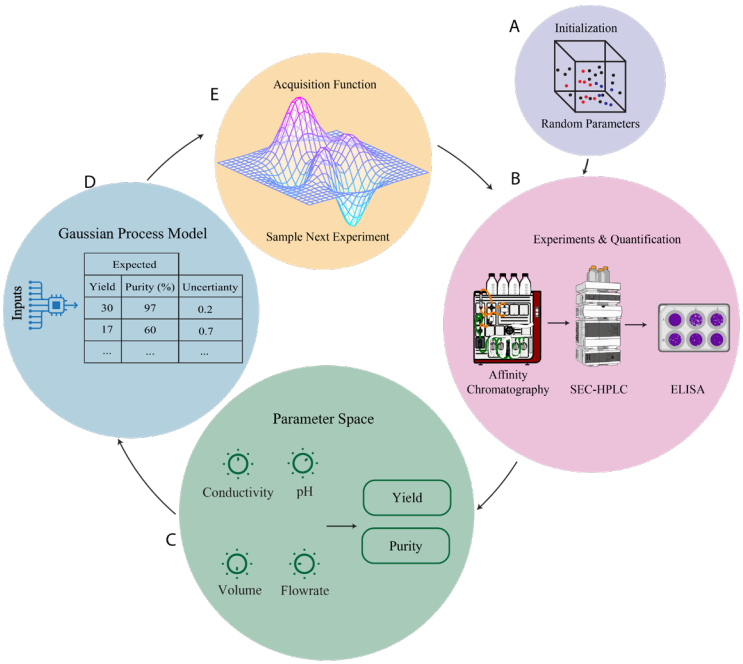

Fig. 1 . Closed-loop purification process (A) An initial set of random experiments is drawn from the experimental space. (B) Experiments are performed. (C) Experimental data (parameters) from step (B) collection and curation. (D) Gaussian process surrogate model building using curated data from step (C). (E) Design of the next experimental round. Proposed optimization cycle (B)-(C)-(D)-(E) continues until the objectives are satisfied.

	AAV2 Batch 1		AAV2 Batch 2		AAV2 Batch 3	
	Yield (%)	Purity(%)	Yield (%)	Purity(%)	Yield (%)	Purity(%)
1	45.35	0.973	92.41	0.976	85.52	0.971
2	4.45	0.899	75.03	0.972	84.79	0.978
3	55.94	0.991	91.55	0.959	92.23	0.959
4	60.78	0.941	87.06	0.953	92	0.961
5	24.05	0.999	82.12	0.959	93.99	0.958
6	53.96	0.859	5.92	0.937	99.95	0.971
7	56.58	0.926	5.99	0.981	97.38	0.943
8	58.72	0.973	75.98	0.938	97.69	0.95
9	22.86	0.893	75.8	0.906	99.02	0.947
10	44.9	0.965	75.58	0.897	96.61	0.945

Fig. 2. Yield and purity improvements for AAV2 in three optimization steps

193 Combining Steric Exclusion with Anion Exchange Chromatography – Development of an Innovative, Affinity-Independent, and Scalable AAV Downstream Process

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Adeno-associated viruses (AAV) are prominent vectors in the field of in vivo gene therapy, yet their purification remains a major challenge due to the need for multiple process steps, often leading to product loss and high costs. Traditional downstream processes are typically serotype-specific and heavily reliant on costly affinity resins (**Figure 1 A**). To overcome these challenges, we have developed a serotype-independent purification method utilizing steric exclusion chromatography (SXC), which can be seamlessly integrated with a subsequent anion-exchange polishing step (**Figure 1 B**). AAV elution from the SXC column can be realized with a buffer of choice. This in turn eliminates the need for intermediate concentration and buffer exchange steps, thereby reducing the overall number of process steps while achieving high-purity enrichment of filled AAV particles.

In the initial phase of process development, we optimized AAV capturing via SXC using a design of experiment (DoE) approach (**Figure 2 A**). The AAV particles captured via SXC were further purified using anion-exchange chromatography with a monolithic stationary phase. This step not only removed unspecific impurities but also enriched filled AAV particles. The process achieved a viral genome recovery rate of 51.7%, with impurity reductions of 99.9% for DNA and 99.8% for protein (**Figure 2 B**). The method was successfully adapted to various AAV serotypes and different genes of interest, demonstrating its robustness and versatility. Additionally, the scalability of SXC was confirmed, underscoring its potential for large-scale manufacturing.

This streamlined, universal, and scalable process offers a robust and efficient alternative to traditional AAV purification methods, addressing critical challenges in gene therapy production and paving the way for broader implementation in both research and manufacturing environments.

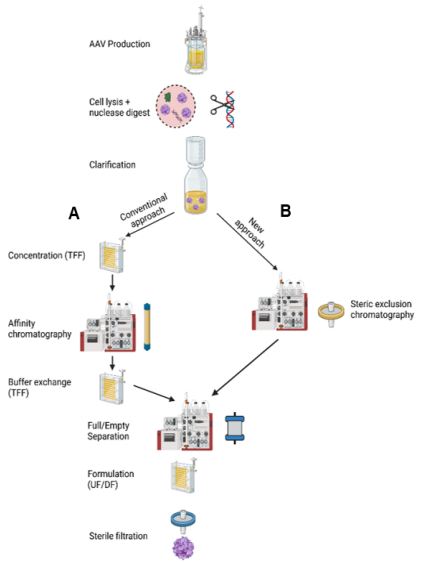

Figure 1: Schematic representation of a standard AAV downstream process. A) Two tangential-flow filtration (TFF) steps are mostly used to reduce the loading volume before the affinity column and to achieve a buffer exchange for the anion-exchange based empty/full separation. B) AAV capture using SXC allows an elution with a buffer of choice. The eluate can be directly applied to the anion-exchange column for empty/full separation.

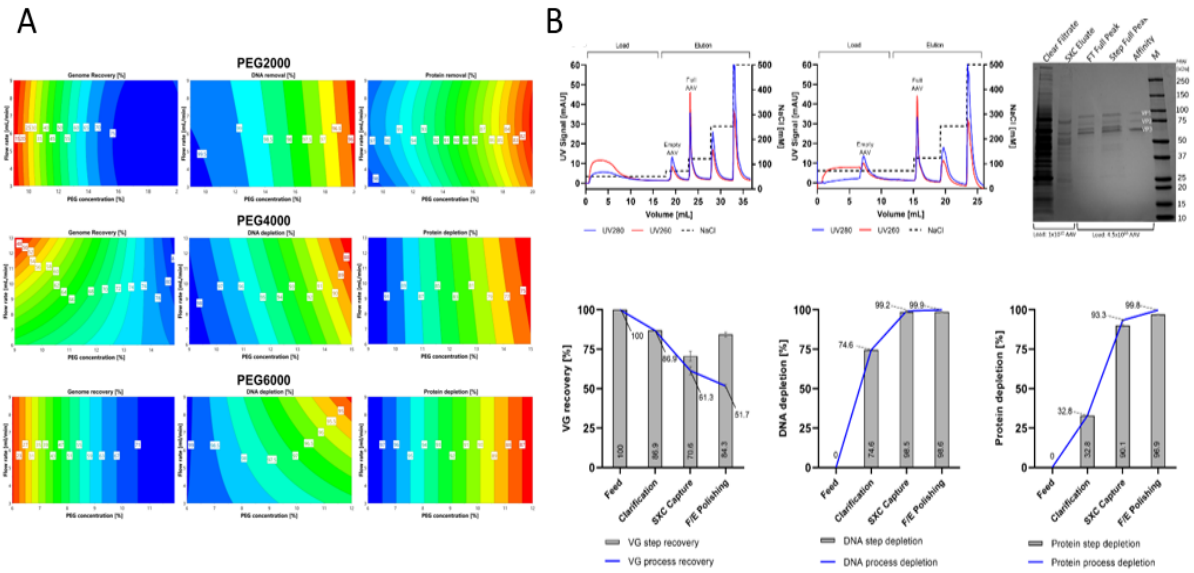

Figure 2: **A**) DoE results of three different PEG variants (PEG2000 (top), PEG4000 (middle) PEG6000 (bottom). Genome recovery, DNA removal and protein removal were used for multivariant statistical analysis and process optimization. **B**) Top left and middle shows two chromatograms of empty/full separation of SXC purified AAV samples. Top right shows an SDS-PAGE of different process steps. The histograms on the bottom show the stepwise VG recovery, DNA depletion and protein depletion.

194 Neutral pH Elution of AAV using Affinity Liquid Phase Separation (ALPS) in Centrifugation and Tangential Flow Filtration (TFF) Formats for Flexible Downstream Processing

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The purification of adeno-associated virus (AAV) using Affinity Liquid Phase Separation (ALPS) introduces a novel method that challenges traditional gene therapy production paradigms. The IsoTag™ AAV reagent combines an affinity ligand with a stimulus responsive, phase separating biopolymer to purify AAV based on size and affinity in a single process without chromatography. The ALPS process was implemented in a centrifugation format to screen binding and elution conditions for AAV9 in parallel, utilizing only 1mL of harvest per condition. This screen identified a neutral pH elution buffer that allows for >90% recovery of AAV9, comparable to results achieved with a standard pH 3.0 elution. This elution buffer was tested in a tangential flow filtration (TFF) setup utilizing hollow fiber filters, demonstrating centrifugation screening results were translatable to scalable ALPS-TFF process. The ALPS TFF process developed at the 1L scale was then scaled to 2L and 16L by maintaining a constant filter loading, shear rate and flux for viral genome capture and recovery >95% by ITR2 qPCR in less than 4 hours for all three scales. Finally, an ALPS-TFF process was developed using flat sheet cassette format with externally validated data, demonstrating flexibility of the IsoTag™ AAV ALPS technology for AAV affinity purification. The IsoTag™ AAV reagent allows for neutral pH elution of AAV in a high throughput centrifugation format as well as scalable TFF processes to address a wide range of AAV affinity purification applications.

195 Development of a Scalable and Cost-Effective Clarification Strategy for Adeno-Associated Virus Bioprocessing Using Single-Use Anion Exchange Fibrous Technology

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Adeno-associated virus (AAV) is widely used in gene therapy, but its bioprocessing is less mature than recombinant proteins. This is primarily attributed to the structural complexity of AAV particles and challenges in removing

impurities, particularly soluble host cell DNA (hcDNA) that associates with AAV particles. These challenges necessitate dedicated and systematic development of clarification strategies for an efficient AAV bioprocess platform.

First, this study systematically evaluated the effects of diverse system variables (e.g., ionic/non-ionic and zwitterionic lysis reagents, nucleases, clarification methods) and process variables (e.g., incubation time, conductivity) on host cell lysis in a model HEK293 expression system. Key metrics such as lysis efficiency, AAV recovery, hcDNA size and concentration, and particle size distribution were assessed. Additionally, the impact of lysis methods on AAV quantification by ELISA and PCR was investigated to develop a robust quantification workflow for various metrics. Then, we processed lysates (with varying AAV serotypes, hcDNA concentrations, and residual detergents) to screen for a scalable and platform-compatible clarification strategy suitable for diverse feed streams. A single-use anion exchange (AEX) technology with quaternary amine-grafted synthetic fibers, was identified as an attractive solution enabling single-stage, nuclease-free lysate clarification. Systematic operating parameter optimization (e.g., resident time, conductivity, etc.) achieved consistent performance (>90% AAV recovery, >95% turbidity reduction, and >2-log hcDNA reduction) across diverse lysate feed streams and scales. Notably, this charge-based clarification method, leveraging ionic interactions to capture hcDNA in a flow-through mode, eliminated the need for nuclease digestion, delivering comparable results to nuclease-treated workflows while reducing estimated costs by >35% relative to conventional depth filter-based processes requiring nuclease treatment.

After processing various clarified fluids with AAVX affinity chromatography, we evaluated the purified AAV quality, residual hcDNA/plasmid DNA concentration, and hcDNA size distribution across unit operations in the AAV bioprocess. The analytical results identified the key clarification step to address downstream purification challenges. Overall, our study developed a novel, versatile and single-stage nuclease-free AAV clarification approach that effectively processes feed streams with varying detergent types, conductivity, and impurity profiles. The approach integrates seamlessly with downstream capture chromatography, ensuring purified AAV yield and quality. With its demonstrated scalability from laboratory to manufacturing scales, this single-use AEX fibrous clarification offers an efficient and robust solution for AAV bioprocessing.

196 Purification of a broad range of adeno-associated virus serotypes from HEK293 cell lysate using peptide-based affinity *AAVW*$_{II}$ adsorbent and quantitative proteomic analysis of residual host cell protein via LC-MS/MS technology

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**Corresponding Author*

Cell and gene therapies (CGTs) are at the forefront of innovation to treat rare degenerative & dysregulation diseases, immunological disorders, and genetic defects diseases, such as Hemophilia. Adeno-associated viruses (AAVs) have been widely utilized as delivery vectors for in-vivo replacement of functional genes to target cells for gene therapy. Currently, commercialized affinity resins have been utilized for the capture step of downstream manufacture of AAV vectors, but the expensive price and extremely acidic elution would limit their application on the biomanufacturer of AAV-base gene therapy products. To support the development of CGT products, innovative peptide-based affinity ligands, specifically designed to capture a broad range of adeno-associated virus serotypes from human embryonic kidney 293 (HEK293) cell lysate with high binding affinity and selectivity, were developed through high-throughput screening technology via microfluidic device in the previous work (Chu, Shastry et al. 2023). Due to high AAV binding capacity of above 1×10^{13} vp/mL, a broad range of binding affinity (serotype 1, 2, 4, 5, 6, 8, 9, and rh10), comparable AAV recovery of above 40%, and excellent pertaining of transduction activity of above 60% of AAV particles at near physiological pH of 7.0, *AAVW*$_{II}$-Toyopearl resin was selected for the product purity optimization (Fig. 1). AAV elution with high-purity could be achieved using 10 CVs of stepwise elution of 100% buffer A (ddH_2O) and 5 CVs of stepwise elution of 5% buffer B (1 M $MgCl_2$ in 20 mM Bis-Tris HCl buffer at pH 6.0) at the resident time of 2 min. Furthermore, seven other AAVs were purified from crude or Charcoal-treated HEK293 cell lysate via *AAVW*$_{II}$-Toyopearl resin under optimized elution strategy. Regardless of viral serotypes in

Fig. 2(A), *AAVW*$_{II}$-Toyopearl shows high dynamic binding capacity (DBC) of AAV of above 1×10^{13} vp/mL with strong salt-tolerance, ranging from 10-30 mS/cm, and excellent purity of over 200-fold reduction of HEK293 HCPs, in accordance with normalized SEC-UPLC analysis in Fig. 2(B). Noteworthily, it provides 10-fold higher DBC of AAV8 (~1×10^{14} vp/mL) regardless of transgene payload size (2.5~4.7 kb). Except AAV5 and AAV9, it provides comparable recovery of over 50% and high retaining of transduction activity (above 90%) of AAV particles under physiological elution pH of 7.0 and conductivity of below 7 mS/cm. CE-SDS analysis in Fig. 2(C) was performed for the elution fraction of AAV8 (Luc) from *AAVW*$_{II}$-Toyopearl resin with a VP1:VP2:VP3 ratio of 1: 1.07: 9.87, closing to typical 1: 1: 10 for a high transduction activity. Finally, the feedstocks and elution fractions from *AAVW*$_{II}$-Toyopearl resin were analyzed via nanoLC-MS/MS using a Thermo Scientific VanquishNEO UHPLC interfaced with a Orbitrap Eclipse™ Tribrid™ Mass Spectrometer to quantify the total removal of the AAV product-related impurities, such as green fluorescent protein (GFP, transgene plasmid), Rep78 (AAV2S Rep gene), Early E2A DNA-binidng protein and Early 4 ORF6 protein (AAV helper plasmid), the individual host cell proteins of HEK293 cell line, such as histone-related proteins, Ubiquitin-related proteins, and phosphatase-related proteins, as well as the co-eluted problematic residuals, such as cathepsin, catalase and autotaxin. In sum, the peptide ligand of *AAVW*$_{II}$-enables an economical, fast, robust, and platformable purification technology for a broad range of AAV serotypes under physiological condition.

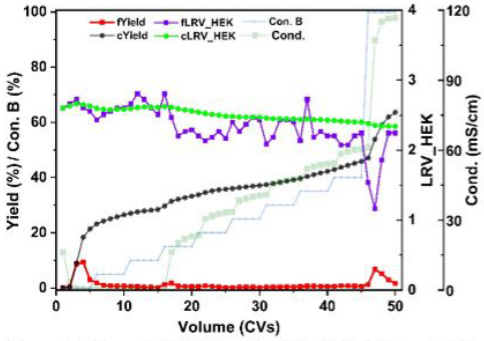

Figure 1. *Values of fYield (%), cYield (%), fLRV_HEK, and cLRV_HEK of AAV8 purified from a clarified HEK 293 cell lysate using AAVW$_{II}$ -Toyopearl resin with the increase of the stepwise elution volume, concentration of buffer B (Con. B, %), and sample conductivity (Cond., mS/cm). Note: stepwise elution with respective 5 CVs of 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 100% buffer B for final regeneration.*

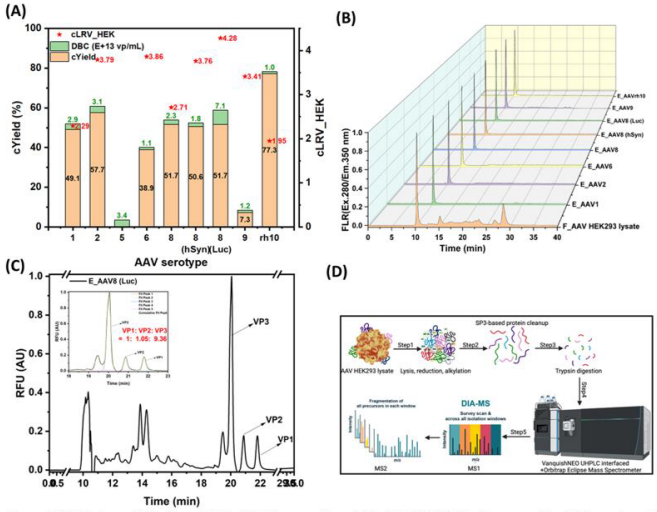

Figure 2. ***(A)*** *Values of cYield and DBC of AAVs, as well as cLRV of HEK293 HCPs for respective AAV serotype 1, 2, 5, 6, 8, 8 (hSyn), 8 (Luc), 9, and rh10 obtained via chromatographic purification from a clarified HEK 293 cell lysate (AAV titer: ~1×10^{12} vp/mL; HCP titer: ~0.2 mg/mL) using AAVW$_{II}$- Toyopearl resin;* ***(B)*** *normalized Size Exclusion Chromatography - Ultra Performance Liquid Chromatography (nSEC- UPLC) analysis of feedstock of AAV HEK293 cell lysate (F_AAV HEK293 lysate), and elution fractions of respective AAV1, AAV2, AAV6, AAV8, AAV (hSyn), AAV8 (Luc), AAV9, and AAVrh10 from AAVW$_{II}$- Toyopearl resin;* ***(C)*** *Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS) analysis of elution fraction of AAV8 (Luc) from AAVW$_{II}$- Toyopearl resin with a VP1:VP2:VP3 ratio of 1: 1.05: 9.36;* ***(D)*** *Proteomic analysis workflow for the characteristics and quantification of HEK293 HCPs from respective feedstocks and elution fractions of AAVW$_{II}$- Toyopearl resin.*

197 Novel anion exchange chromatography elution approach to improve the Adeno-associated virus process robustness

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Adeno-associated virus (AAV) based therapeutics are emerging as novel modalities for the treatment of genetic diseases. AAV manufacturing processes produce the desired full capsid containing the target vector genome along with various product-related impurities, e.g., empty capsids, which lack the vector genome required for function, and partial capsids, which are a heterogeneous mixture of capsids containing truncated vector genomes, residual host cell DNA, etc. It is imperative that the purification process for AAVs removes these impurities to a sufficient degree to ensure safety and efficacy.

A common purification technique for the enrichment of full capsids leverages anion-exchange chromatography to exploit charge differences among the capsid species. Typically, an increasing salt gradient is employed to separate full capsid populations from other product variants. Since achieving high resolution during the separation can be a challenge, the ability to effectively control product collection during elution is critical to ensure consistent full capsid enrichment and recovery from batch to batch.

In this work we present a novel approach for elution by leveraging a conductivity signal to trigger the start and end of product collection. Conductivity-based collection has the advantage over other signal-based methods, including UV, since the signal itself is independent of common chromatography variables such as column load density and starting material variability. We have demonstrated this method to be highly reproducible and effective across a wide range of process scales. Additionally, the effectiveness of this approach has been demonstrated for several AAV capsid serotypes and with different elution salt/buffer combinations.

Viral Vector and Transgene Biology

198 Engineering of Next-Generation Bocavirus Vectors by Surface Loop Modification and Peptide Insertion

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Adeno-associated virus (AAV) has emerged as the leading candidate for virus-mediated *in vivo* gene therapy as illustrated by hundreds of clinical trials and the rapidly increasing number of approved AAV-based therapeutics. Yet, its DNA packaging capacity of ~4.7 kb hampers its use for the delivery of larger genes including base editors and some clinically relevant cDNAs. Our group has previously demonstrated that bocavirus, another member of the Parvoviridae family, can encapsidate larger AAV vector genomes of up to ~6 kb and transduce various cells including human primary airway epithelia. Moreover, others reported potent *in vivo* gene delivery with recombinant Human Bocavirus 1 (HBoV1) encoding AAV genomes to the lungs of ferrets and non-human primates, underscoring the promise of bocaviruses as alternative gene therapy vectors. However, the current first generation of bocavirus vectors suffers from a low transduction efficiency, in turn necessitating the administration of high vector doses. In particular, *in vitro* transduction in cell lines is often inefficient, which hampers basic research on molecular virus-host interactions or high-throughput screenings of capsid variants. Thus, we aimed to fill in the gaps in our knowledge of bocavirus biology by identifying residues that govern the transduction. Interestingly, Gorilla Bocavirus 1 (GBoV1) exhibits significantly higher transduction efficiencies in multiple cell lines compared to HBoV1, despite the high amino acid identity of these variants (86%). Following our hypothesis that distinct residues on the capsid surface loops explain this difference, we replaced each variable region (VR) of GBoV1 with that of

HBoV1 and measured the transduction efficiency of the ensuing chimeras in Huh-7 cells. Unexpectedly, several variants yielded better transduction, including GBoV1 vectors carrying VRIII or VRVI from HBoV1 that outperformed genuine GBoV1 by up to four-fold. Remarkably, VRVI of HBoV1 and GBoV1 are almost identical except for one amino acid (Asp to Asn). Based on this, we concurrently replaced VRIII and VRVI with those of HBoV1 in the same GBoV1 capsid and found that the resulting double-swap chimera transduces the cells even better, as much as 15-fold compared to the GBoV1 wild-type. A similar trend was also observed in HEK293T cells. Furthermore, we hypothesized that insertion of peptides known to boost AAV transduction would also enhance bocavirus vectors considering the similarity in capsid structure. First, we assessed two 7-mers with an NXXRXXX motif (X = any residue) reported by our group to improve transduction of multiple AAV serotypes in Huh-7 cells. As hoped for, insertion of either peptide into GBoV1 VRIV enhanced transduction by roughly four-fold. This motivated us to insert a longer, melittin-derived peptide (>20 residues) reported to aid endosomal escape of AAV when displayed on its surface. Strikingly, this peptide not only boosted the GBoV1 wild-type by five-fold but it yielded an up to 30-fold improvement in transduction when inserted into the VRIII-VI GBoV1/HBoV1 chimera. To our knowledge, this is the first study reporting successful rational engineering of bocavirus capsid variants through domain swapping and peptide display. Granted their superior efficacy can be confirmed in primary cells and animal models, this work could pave the way for a new generation of parvoviral gene therapy vectors with significant potential to overcome some of the shortcomings of AAV.

199 Investigating the Influence of Viral and Cellular Insulators on Stable HSV-1 Vector-Mediated Transgene Expression in Various Tissues

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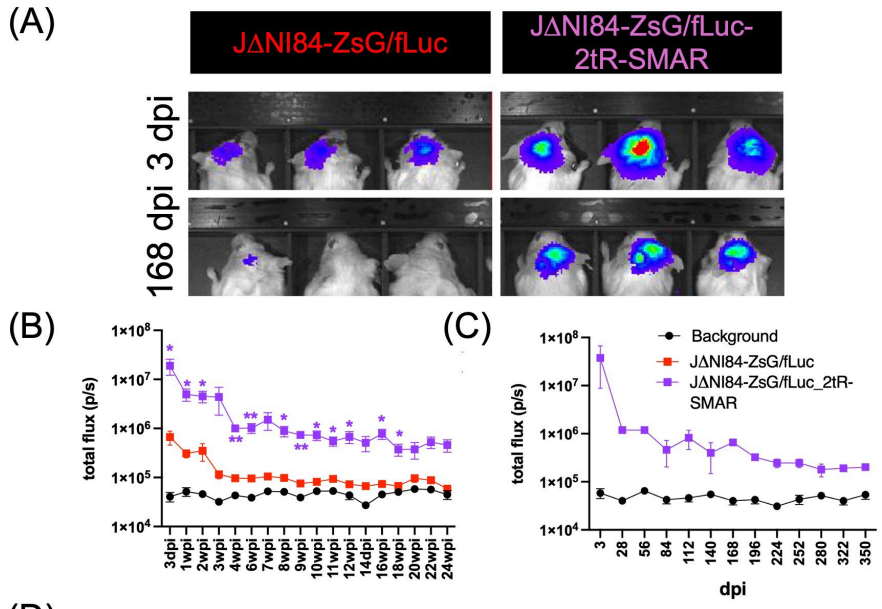
Controlling the epigenetic environment is crucial for the successful long-term expression of therapeutic gene cassettes in gene therapy platforms. Unlike other gene therapy platforms that are constrained by the inclusion of insulator sequence, replication defective (rd) HSV vectors offer the unique advantage of a large payload capacity, allowing direct intervention and manipulation of the transgene cassette epigenetic environment. To enhance the safety and efficacy of rdHSV vectors, we have developed a novel gene transfer platform by functionally deleting all immediate-early (IE) genes and the reiterated JOINT region flanking the unique long (U_L) and unique short (U_S) genomic components, resulting in a payload capacity of ~30-kbp. However, in the absence of ICP0 expression, transduced cells rapidly silence the viral genome, including the transgene cassettes. Our preliminary data indicate that sustained transgene expression requires strategic flanking of the transgene cassette with natural viral insulators, such as those near the IE and latency-associated transcript (LAT) loci. HSV viral insulators contain multiple recognition motifs for the CCCTC-binding factor (CTCF), a key regulator of chromatin structure. These modifications rendered the vectors devoid of viral gene expression while leveraging the insulator sequences to enable robust transgene expression in neuronal and non-neuronal cells *in vitro.* Furthermore, *in vivo* delivery to skeletal muscle demonstrated stable transgene expression from the LAT locus for at least one year, whereas persistent but declining expression from the ICP4 locus was observed in the mouse hippocampus over five months. While the role of HSV insulators in regulating wild-type HSV transcription during latency and reactivation has been studied, the binding of CTCF to viral insulators in rdHSV vectors and its impact on transgene expression remains unexplored. Here, we provide the first evidence of CTCF occupation of two CTCF binding clusters in rdHSV vectors. Moreover, we evaluated the influence of insulators on transgene activity across multiple cell types, including neurons, muscle, retinal pigment, and bronchial epithelial cells. Recognizing the importance of viral insulators, we further evaluated additional anti-silencing elements to sustain high-level expression. A novel insulator design incorporating scaffold/matrix attachment regions (SMARs) and tRNA sequences significantly enhanced and stabilized transgene expression, underscoring the potential of insulator selection in regulating transgene activity in rdHSV vectors. Notably, *in vivo* delivery of this optimized insulator design yielded substantially higher transgene expression in the brain compared to viral insulators alone, maintaining robust expression for at least one year. These findings highlight the critical role of epigenetic modifiers in advancing the reliability of gene therapy. By integrating optimized insulator sequences, this new cassette design ensures high, durable transgene activity across multiple tissue types, offering promising therapeutic potential for treating complex diseases affecting neurons and other tissues.


(A)
JΔNI84-ZsG/fLuc
JΔNI84-ZsG/fLuc-2tR-SMAR
3 dpi
168 dpi
(B)
total flux (p/s)
(C)
Background
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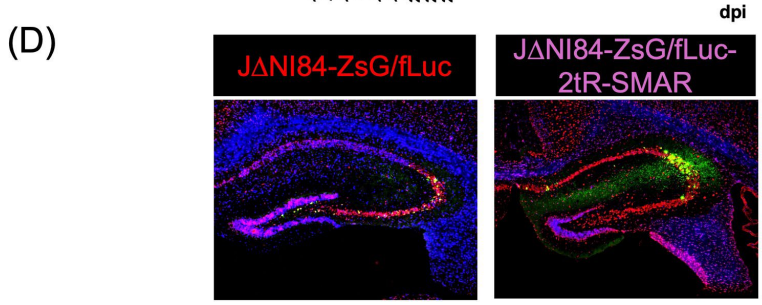
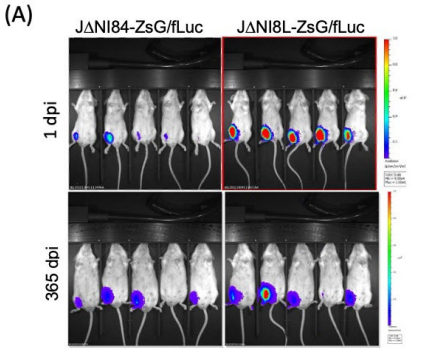
JΔNI84-ZsG/fLuc_2tR-SMAR
total flux (p/s)
dpi


(D)
JΔNI84-ZsG/fLuc
JΔNI84-ZsG/fLuc-2tR-SMAR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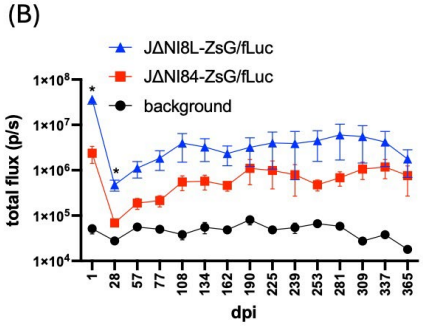
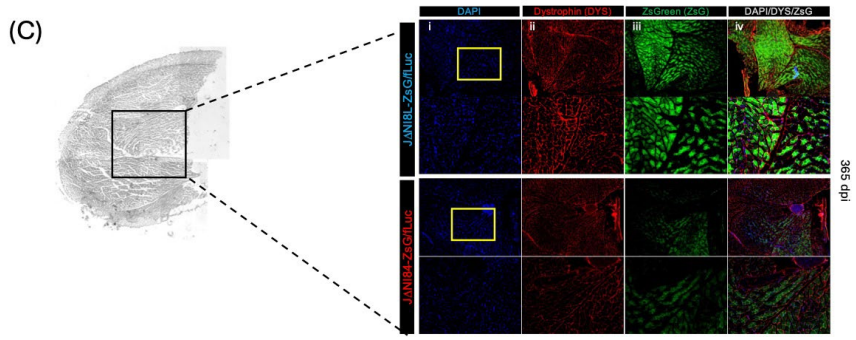
JΔNI8L-ZsG/fLuc
1 dpi
365 dpi


(B)
JΔNI8L-ZsG/fLuc
JΔNI84-ZsG/fLuc
background
total flux (p/s)
dpi


(C)
DAPI
Dystrophin (DYS)
ZsGreen (ZsG)
DAPI/DYS/ZsG
JΔNI8L-ZsG/fLuc
JΔNI84-ZsG/fLuc
365 dpi

200 Novel adenovirus vaccine vectors lacking binding to the thrombosis associated Platelet Factor 4 protein

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Corresponding Author

The adenoviral (Ad) vector based AstraZeneca and Janssen COVID vaccines have been linked to rare cases of vaccine-induced thrombotic thrombocytopenia (VITT), a condition that appears to be linked to Ad binding to the blood protein Platelet Factor 4 (PF4). Meanwhile, several adeno-associated virus vectors (AAVs) in clinical use for gene therapy have been associated with cases of thrombotic microangiopathy (TMA), although a potential link to PF4 binding remains yet to be identified.

Harnessing the broad diversity of natural and synthetic Ads and AAVs, we strived to identify vectors that do not interact with PF4, to locate the binding site of PF4 on commonly used Ad vectors and to establish vaccine vector platforms using these PF4-non-binding Ad vectors.

Using a range of techniques, including SPR and ELISA-qPCR, we assessed the PF4 binding ability of 12 AAV and 50 Ad vectors including genetically or chemically modified Ad5 hexon or fiber variants. Unlike most of the vectors tested, AAV1, AAV6 and AAV7, as well as Ad34 and Ad80 did not bind to PF4. Likewise, the deletion of the hexon hyper-variable region (HVR) 1 of Ad5 or its shielding by PEGylation ablated the vector's PF4 binding. This suggests that HVR1 is the primary binding site of PF4 on the Ad5 capsid, which was supported by Brownian Dynamics (BD) modeling of molecular interactions. These simulations indicated further that PF4 binding is primarily driven by HVR1 loop steric occupancy of the hexon surface rather than by its electrostatic potential, suggesting that genetic manipulations that modify HVR1 surface charge are unlikely to ablate Ad binding to PF4 unless the loop is also shortened.

We then constructed PF4 non-binding Ad vaccines expressing the SARS-CoV-2 spike S1 domain and tested their immunogenicity in mice. The candidate COVID vaccine vector derived from HVR1-deleted Ad5 induced robust S1-specific CD8 and CD4 T cell responses matching those elicited by the parental Ad5 vector. Interestingly HVR1-deleted Ad5 did not induce detectable vector-binding antibody titers, suggesting an improved potential for homologous prime-boost vaccination. The Ad34-based vaccine vector also induced anti-spike antibody responses, although at lower levels than those elicited by the parental Ad5 vector.

Taken together, we document important advantages of novel PF4-non-binding adenovirus vectors for vaccine delivery, raising expectations for safer vector platforms with decreased risk of VITT in prophylactic as well as therapeutic applications.

201 Understanding the Clinical Immunogenicity of Locally Injected HCAd Vector Provides Insight Into Optimizing Dosing Strategy

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Introduction: Gene therapy using viral vectors has potential to treat many diseases. However, vector immunogenicity could affect transduction, efficacy, safety, and durability, limiting successful gene therapy development. PCRX-201 (enekinragene inzadenovec), a novel high-capacity adenovirus serotype 5 (Ad5) vector carrying a transgene encoding for the inflammation-inducible expression of interleukin-1 receptor antagonist (IL-1Ra), is locally injected to the intra-articular (IA) joint space to treat osteoarthritis (OA) of the knee. In the open-label phase 1 trial (NCT04119687), PCRX-201 proved safe and beneficial in pain and function. Here we evaluate the role of clinical immunogenicity in safety, efficacy, and durability and its impact on PCRX-201 dosing and redosing strategy.

Methods: Patients aged 30-80 years (N=72) with painful OA of the index knee and Kellgren/Lawrence grade 2, 3, or 4 were eligible. The first cohort received IA injection of PCRX-201 (n=36) in the target knee at 1 of 3 doses (low: 1.4E10 genome copies [GC]; middle: 1.4E11 GC; high: 1.4E12 GC); the second cohort was pretreated with IA methylprednisolone 40 mg immediately before PCRX-201 administration (n=36) at the same doses. Western Ontario and McMaster Universities Osteoarthritis Index pain (WOMAC-A) and stiffness (WOMAC-B) scores were measured up to 104 weeks. Anti-Ad5 neutralizing antibody (NAb) titers in serum and synovial fluid (SF) and payload protein IL-1Ra levels in SF were measured up to 52 weeks.

Results: Preexisting serum or SF NAb did not affect PCRX-201 efficacy (Figure 1). There was no difference in pain or stiffness scores across NAb titers (serum: 0, 1-499, 500-5000, >5000; SF: ≤500, >500) (Figure 2). Serum NAb titers induced by PCRX-201 injection were highest at 4-12 weeks in both cohorts, and by 38-52 weeks, most serum NAb titers fell below the highest NAb titer observed in a PCRX-201 patient responder. Clinically observed NAb resolution rates to below maximal baseline thresholds highlight the possibility of redosing PCRX-201 at some defined point after initial IA injection. Pretreated patients receiving the middle and high doses showed faster reduction of serum induced NAb titer over time than those not pretreated, indicating that steroids lower the overall immune response to viral vectors. Additionally, a positive correlation between serum and SF NAb titers indicates that less invasively obtained serum can be used as a representative matrix for NAb measurement in the clinical setting. The most commonly reported adverse event (AE), treatment-related index knee effusion, occurred less frequently for participants in the pretreated (13/36; 36%) than the not pretreated cohort (22/36; 61%). Steroid pretreatment delayed effusion onset, indicating that effusion may be driven by immune activation. Because transgene expression is triggered by nuclear factor κB–inducible promoter, effusion-related immune activation may be quickly suppressed by IL-1Ra expression by PCRX-201. Effusion onset was earlier in the high- versus the lower dose groups, highlighting a positive correlation between dose and immune activation.

Conclusion: Preexisting NAb did not affect PCRX-201 efficacy or safety at all 3 doses, and steroids helped to reduce induced NAb titers and dose-related AE incidence. These results support the planned phase 2 study strategy of PCRX-201 with 10-fold lower dose (1.4E11 GC), steroid pretreatment, and potential for PCRX-201 redosing in future trials.

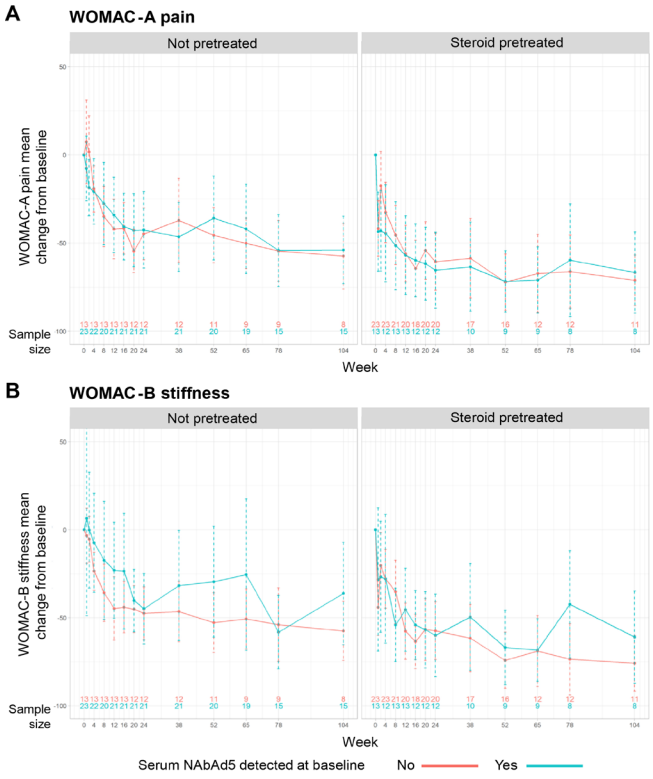

Figure 1. Impact of baseline serum NAb on change from baseline for **(A)** WOMAC-A pain and **(B)** WOMAC-B stiffness in the not pretreated and steroid pretreated cohorts through week 104. Error bars are the 95% confidence interval. NAbAd5, neutralizing antibody adenovirus serotype 5; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

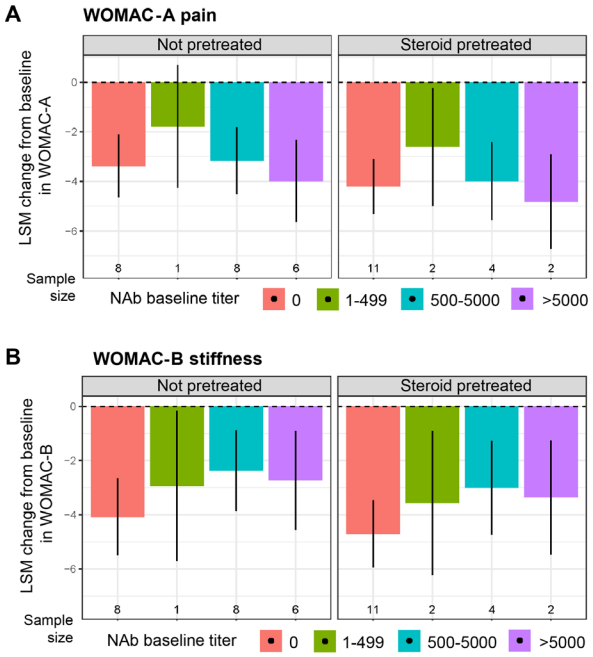

Figure 2. Impact of baseline serum NAb titer on LSM change from baseline for **(A)** WOMAC-A pain, and **(B)** WOMAC-B stiffness across 3 doses in the not pretreated and steroid pretreated cohorts through week 104. Error bars are the 95% confidence interval. LSM, least squares mean; NAb, neutralizing antibody; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

202 Suspension Cell Platform for the Production of Cocal-pseudotyped Lentiviral Vectors

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Cocal-pseudotyped lentiviral vectors offer significant advantages for ex vivo engineering of hematopoietic stem cells (HSCs) and T cells, as well as in vivo gene delivery. Compared to their VSV-G counterparts, Cocal-pseudotyped vectors demonstrate broader species and cell tropism, enhanced serum stability, and reduced serum neutralization in patient samples. Furthermore, their significance in in vivo gene therapy is emphasized by the studies conducted by Hans-Peter Kiem's group and Umoja Biopharma in dog and NHP models, respectively, using Cocal-pseudotyped lentiviral vectors. However, therapeutic potential of viral vectors is often hindered by low-yield manufacturing processes.

As a proof of concept, we developed a robust and scalable suspension-cell platform to produce third-generation Cocal-pseudotyped lentiviruses. Using commercially available producer cells that grow in chemically defined serum free media, we optimized production conditions to enhance viral yield. 0.5ug of total plasmid DNA per million cells was used for PEI-mediated transfections, leading to reduction by 80% when compared with the total DNA that was used for vector production using adherent cells. A single harvest at 56 hours post-transfection, followed by concentration, yielded viral titers that were twice as high as those obtained from adherent producer cells. The suspension cells produced vector until passage 60-65 enabling 20 cycles of production, after which the cell viability started declining.

Transduction experiments revealed no evident cellular toxicity. Functional and physical titrations indicated a higher infectivity index for Cocal-pseudotyped lentiviral vectors compared to VSV-G equivalents. Notably, scaling up production from 20 mL to 200 mL cultures maintained both viral titers and infectivity.

These findings highlight the potential of this scalable suspension-cell platform to enable high-yield production of Cocal-pseudotyped lentiviral vectors, offering a viable solution for therapeutic applications in gene therapy.

203 Widespread AAV chimerism and other sources of noise confound multiplexed enhancer screening in brain

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[1]Allen Institute for Brain Science, Seattle, Washington, [2]Allen Institute For Brain Science, Seattle, WA, [3]Department of Genome Sciences, University of Washington, Seattle, WA, [4]Allen Institute for Brain Science, Seattle, [5]Seattle Hub for Synthetic Biology, Seattle, WA
**Corresponding Author*

Cis-acting regulatory enhancer elements are valuable tools for gaining cell type-specific genetic access. Leveraging large chromatin accessibility atlases, putative enhancer sequences can be identified and deployed in adeno-associated virus (AAV) delivery platforms. However, a significant bottleneck in enhancer AAV discovery is charting their detailed expression patterns *in vivo*, a process that currently requires gold-standard one-by-one testing. Here we present a barcoded multiplex strategy for screening enhancer-AAVs at cell type resolution using single cell RNA sequencing and taxonomy mapping. We executed a proof-of-concept study using a small pool of validated enhancer AAVs expressing in a variety of neuronal and non-neuronal cell types across the mouse brain. Unexpectedly, we encountered substantial technical and biological noise including extensive chimeric packaging products (scrambled barcodes, from 37% to >60% of packaged molecules). We observed this AAV chimerism in

parallel lines of experimentation at two separate institutions. We found through dedicated barcode-swap experiments that this chimerism is sequence homology-dependent but capsid-independent. To circumvent these problems we developed novel techniques to accurately deconvolve enhancer-AAV expression patterns in brain. Overall these results underscore the need for carefully designed reporter architectures and improved noise mitigation strategies, and highlight the complexity of enhancer-AAV biology *in vivo*.

204 PACS1 Gene Integration and Clonal Expansion in HIV-1 Persistence: Insights from Longitudinal Analysis of HIV Patients

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**Corresponding Author*

Introduction: HIV persistence is a major challenge in current HIV treatment. Despite the effectiveness of antiretroviral therapy (ART) in suppressing viral replication, HIV continues to persist in latent reservoirs within the host genome. Recent studies have shown that specific HIV integration sites are linked to clonal expansion and persistence of infected cells. These integration sites may confer a survival advantage to CD4+ T cells, allowing them to resist current treatments and persist indefinitely.

Methods: To investigate the impact of HIV integration on cellular persistence and clonal expansion, we use LAM-PCR (S-EPTS/LM-PCR) to analyzed integration sites in over 100 HIV patients from Germany and China. We mapped viral integration sites within the host genome and identified common integration sites (CIS). Our analysis focused on the PACS1 gene, which has been identified as a significant CIS for HIV-1 integration. We correlated our findings with clinical data to assess the impact of PACS1 integration on clonal expansion and cellular fate.

Results: Our analysis revealed several key findings: 1. PACS1 Gene as a CIS Hotspot: The PACS1 gene was identified as a significant hotspot for HIV-1 integration, with a high frequency of integration events. 2. Clonal Expansion and Cellular Fate: Cells with HIV-1 integration in the PACS1 gene were more likely to form dominant clones, suggesting that integration within PACS1 confers a selective advantage, promoting clonal expansion and potentially influencing cellular fate. 3. Temporal Dynamics of Integration: We observed a competitive interaction between integration sites in the PACS1 gene during the early and late stages of infection, indicating that the integration landscape evolves over time. 4. Validation with External Datasets: Our findings were validated using independent external datasets, confirming that PACS1 integration is associated with clonal dominance and supporting the link between PACS1 integration sites and clonal expansion. In conclusion, our study highlights the role of the PACS1 gene in HIV-1 integration and clonal expansion. Integration within PACS1 may influence cellular proliferation and survival, providing infected cells with an evolutionary advantage. This insight is crucial for understanding HIV persistence and developing strategies to target and eliminate latent reservoirs.

Novel Genetic Approaches for Muscle and Skeletal Diseases

205 Non-human primate muscle gene editing via single systemic AAV delivery of ultra-compact CRISPR nuclease

Renan Sper[1], Christopher Schmitt[1], Jason Li[1], Ning Chai[2], Winston Chang[1], Ngan Nguyen[1], Aashish Lamichhane[1], Danni Frimannsson[1], Clarissa Rhines[1], Julia Nussbacher[1], Matan Retwitzer[1], Aaron DeLoughery[1], Sara Ansaloni[2], Emel Alpay[1], Bridget Mckay[1], Sahana Somaiah[1], Maggie Bobbin[3], Megan Masnaguetti[1], Keith Abe[2], Gabor Veres[4], Bohong Zhang[2], Lucas Harrington[2], Subhadra Jayaraman[1], Benjamin Rauch[1]
[1]Mammoth Biosciences, San Francisco, CA, [2]Mammoth Biosciences, Brisbane, CA, [3]Mammoth Biosciences, San Mateo, CA, [4]Mammoth Biosciences, Novato, CA

In vivo therapeutic gene editing of muscle tissues holds great promise for the development of genetic medicines for a variety of genetically inherited muscle degenerative diseases including Duchenne Muscular Dystrophy (DMD), Myotonic Dystrophy Type 1, and Facioscapulohumeral Dystrophy, among others. The use of adeno-associated virus (AAV) represents a valuable option for gRNA and nuclease co-delivery in a clinical setting, considering various gene therapy clinical trials have demonstrated improved muscle function following systemic AAV transgene administration. However, the size constraints of AAV has limited its application for CRISPR-based gene editing. Here we describe a novel ultra-compact nuclease named NanoCas^{TM} and test its ability to edit muscle cells via single AAV delivery. To explore the editing capacity of the AAV-NanoCas, we targeted the human *DMD* locus. We developed a single AAV construct co-expressing gRNA and NanoCas, and designed 3 gRNAs targeting the splice acceptor and/or predicted premature stop codon of 3 different exons of the human *DMD* locus, and compared with single AAV saCas9 control. AAV particles were evaluated in vitro via myotube transduction, in vivo in a DMD mouse model, and in NHP. Human myotube transduction resulted in *DMD* editing levels ranging from 20 to 40%, while mice in vivo dosing led to editing in the heart, gastrocnemius, and quadriceps, ranging from 10 to 40% indel. AAVs were administered intravenously to male cynomolgus macaques. Muscle biopsy analysis was performed at 4 and 8 weeks and at 12-week necropsy. NGS analysis of multiple muscle tissues resulted in a broad range of indel activity, reaching as high as 46%, while liver editing remained low. In summary, we described an ultra-compact nuclease for in vivo therapeutic proof-of-concept and demonstrated significant in vitro activity in human myotubes, in vivo activity in mice, and in NHP. To our knowledge, this is the highest skeletal muscle editing demonstrated in NHP following systemic AAV delivery of any CRISPR nuclease. Importantly, the NanoCas gene is extremely compact, leaving ample space within the AAV design for additional regulatory elements and fusion proteins for things like RT and Epigenetic editing. NanoCas represents a valuable therapeutic nuclease for in vivo muscle editing via single AAV delivery.

206 Rescue of Full-Length Dystrophin Protein and Motor Performance in a Mouse Model of Duchenne Muscular Dystrophy Using an AAV-tRNA Therapeutic

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Duchenne muscular dystrophy (DMD) is a fatal X-linked disorder caused by the absence of functional dystrophin, a large structural protein critical to the integrity of skeletal and cardiac muscle cells. DMD is characterized by progressive loss of muscle, respiratory insufficiency, and dilated cardiomyopathy. Existing therapeutic strategies are unable to restore full-length dystrophin due to the large size of the protein (427 kDa) and the diversity of unique mutations.

Nonsense mutations, in which a premature termination codon prevents translation of full-length dystrophin, are found in approximately 15% of patients with muscular dystrophy and are associated with more severe forms of the disorder. Suppressor tRNAs are tRNA molecules in which the anticodon has been altered to enable ribosomes to read through nonsense mutations and produce full-length, functional protein. Since there are only three premature termination codons (TGA, TAA and TAG), a limited number of suppressor tRNAs potentially allows for treatment of all DMD patients with nonsense mutations.

We have used the D2-mdx mouse model, which contains a nonsense mutation and recapitulates key aspects of DMD pathology in humans, to test whether a transgene encoding a suppressor tRNA delivered systemically using an Adeno-Associated Virus (AAV) vector can restore dystrophin protein and lost motor function. At 6 weeks post-dosing, we show that the muscles of treated animals express full-length dystrophin protein in a dose-dependent manner and that the rescued protein is organized in the stereotypical fashion of wild-type dystrophin protein. Additionally, we report that by 12 weeks post-dosing, there is significant restoration of motor function as demonstrated by an increase in latency time in the rotarod performance test and significantly increased forelimb and hindlimb grip strength. There is no evidence of adverse treatment effects as measured by behavioral or histologic changes in major organs or in blood chemistry.

To our knowledge, this study is the first demonstration of sustained restoration of full-length dystrophin accompanied by functional muscle improvement in an animal model of DMD using a practical therapeutic vector. In addition to being a potentially highly effective treatment for DMD patients with nonsense mutations, these results establish suppressor tRNAs as a viable therapeutic platform for a range of neuromuscular disorders and cardiomyopathies.

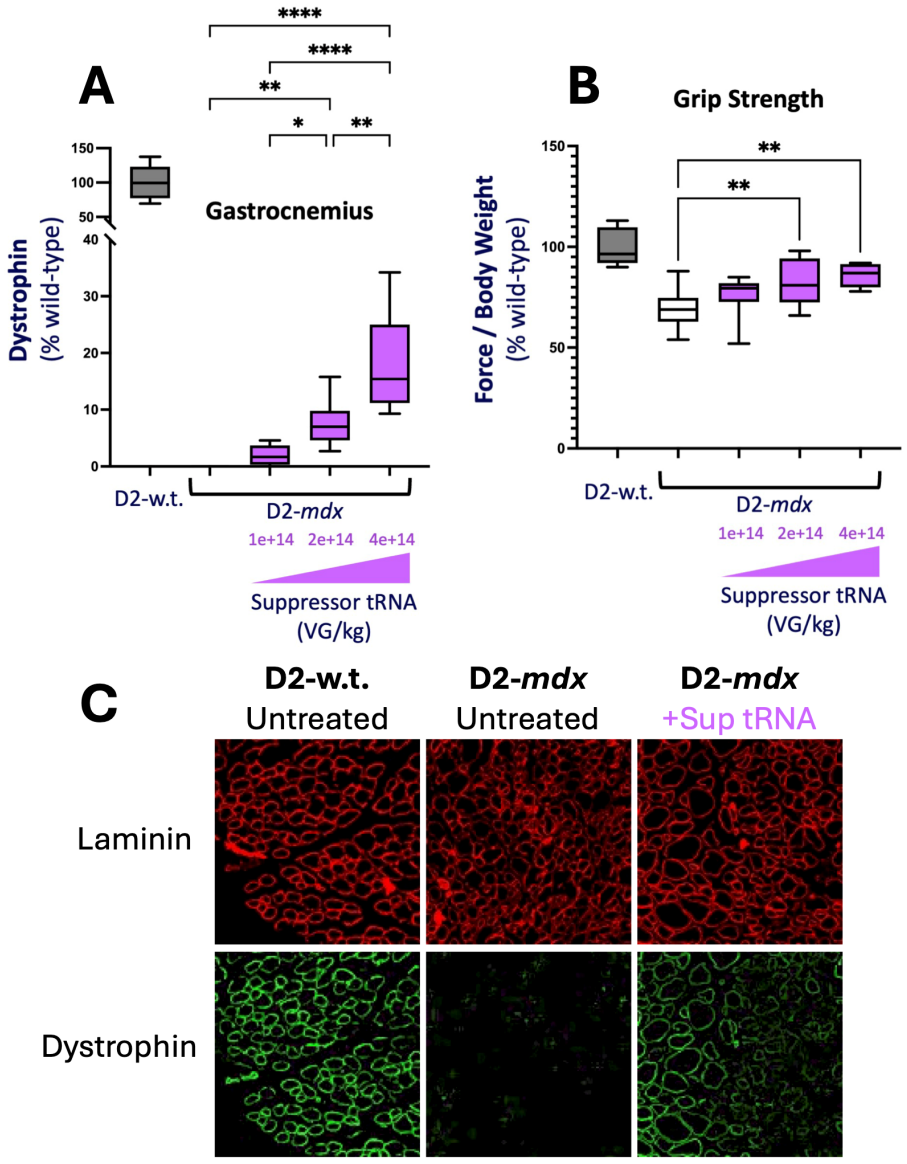

Figure 1. (A-B) Quantification of full-length Dystrophin as detected by western blot in gastrocnemius muscle **(A)** and grip strength **(B)** at 12 weeks post-injection (intravenous administration; indicated doses). **(C)** Immunohistochemistry for Laminin and Dystrophin in tibialis anterior muscle at 6 weeks post-injection (intramuscular administration; 1e+11 VG/muscle).

207 CRISPR-Cas9 Mediated Endogenous Utrophin Upregulation Improves Duchenne Muscular Dystrophy

Maëlle RALU[1], Simon GUIRAUD[2], Paola Galbiati[3], Sumitava Dastidar[4], Anne De Cian[5], Giuseppe Ronzitti[6], FrancescoSaverio Tedesco[4], Mario Amendola[7]*
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Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disorder caused by mutations in the *DMD* gene, leading to a loss of expression of the dystrophin protein. This induces inflammation, necrosis, fibrosis, and muscle wasting. There is currently no curative treatment for this devastating disease, which is lethal around the age of 20-30 years old. Several dystrophin-based therapies are currently under study such as exon-skipping, AAV-mediated delivery of mini/micro-dystrophin constructs or CRISPR-mediated editing strategies. However, these strategies propose to restore truncated forms of dystrophin, converting the phenotype to a Becker-like dystrophy, and might induce immune response to the newly expressed dystrophin. In addition, exon skipping and CRISPR correction are mutation-dependent, thus only subsets of patients could benefit from the treatment. An alternative therapeutic approach, potentially suitable to all DMD patients irrespective of their genetic defect, consists in upregulating utrophin (UTRN), a structural and functional paralogue of dystrophin, able to compensate for the dystrophin deficit. Several studies have shown that its overexpression ameliorates the dystrophic phenotype in mice and dogs with a lower risk of immune response. While a 1.5-fold increase of utrophin results in a therapeutic benefit, a 3-4-fold overexpression prevents the dystrophic pathology. Here, we developed a CRISPR-Cas9-mediated strategy to increase utrophin expression by relieving its repression. Previous work has shown that UTRN is post-transcriptionally downregulated by the Let-7c microRNA (miR) binding its 3' untranslated region (3'UTR). Using a Cas9/gRNA ribonucleoprotein complex we disrupted the binding site (BS) of this miR at the DNA level in human DMD and murine myoblasts to permanently upregulate utrophin. This induced a 3.5- and 3-fold increase of UTRN expression at the mRNA and protein levels, respectively. Results were confirmed in three-dimensional human DMD cultures, where Let-7c BS disruption resulted in over 2-fold UTRN upregulation and functional improvements of calcium intake and muscle contraction. In addition, we performed Guide-Seq analysis and didn't observe any major off-target editing for the selected gRNA. Finally, we evaluated this strategy in the mdx mouse model of Duchenne. Since some miRs are dysregulated in DMD, we first confirmed by qPCR that miR Let-7c expression is not affected in the tibialis anterior, diaphragm and heart of this model compared to the wild-type C57BL10J mice. We performed intravenous injection of two recombinant adeno-associated viruses type 9 (rAAV9s) encoding for Cas9 and gRNA. This resulted in levels of DNA editing in the tibialis anterior (13%), heart (21%) and diaphragm (5%) that were sufficient to upregulate UTRN expression by about 1.5-2-fold depending on the organs. Interestingly, this level of upregulation resulted in an amelioration of the muscle histopathological phenotype (centronucleation, necrosis, fibrosis). As genome editing efficiency remains limited, we are currently treating mdx mice with a more myotropic AAV, aiming to increase delivery and consequently, editing. Other gRNAs to increase UTRN expression - transcriptionally or post-transcriptionally- have also been designed and validated in human DMD myoblasts and are currently tested in 3D organoids. These findings provide the foundations for a universal gene editing therapeutic strategy for DMD.

208 Overexpressing Murine *Prg4* Isoforms with High-Capacity Adenoviral Vectors (HCAd) by Intra-Articular Injection Improves Articular Cartilage Preservation in a Murine Model of Post-Traumatic Osteoarthritis (PTOA)

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Proteoglycan 4 (*Prg4*) acts as a chondroprotective agent within joint tissues, and its expression is altered in osteoarthritis (OA). This reduction makes intra-articular injections of exogenous PRG4 a promising treatment. Loss-of-function mutations in PRG4 in humans cause camptodactyly-arthropathy-coxa vara-pericarditis syndrome (OMIM #208250), which includes early onset OA. *Prg4* knockout mice also develop OA, consistent with its chondroprotective role. Our group previously demonstrated that using High-Capacity Adenovirus (HCAd) over expressing either *Prg4* or *IL-1Ra* via intra-articular (IA) injection preserved cartilage and delayed OA progression in both small (for both) and large (for *IL-1Ra*) preclinical models. These studies have paved the way for a current phase I clinical trial of HCAd-*IL-1Ra* gene therapy in OA patients (NCT04119687) and its recent designation as a Regenerative Medicine Advanced Therapy (RMAT) by the FDA.

However, the physiological roles of PRG4 isoforms across different tissues and their regulation under pathological conditions remain unclear, and different orientations of *Prg4* isoforms in HCAd vectors may affect *Prg4* transcription levels. Based on this and the role of hemopexin in limiting oxidative stress, binding of TIMPS, we hypothesize that the *Prg4* isoform containing the hemopexin-like domain may contribute to joint preservation.

The murine isoforms packaged into HCAd include a truncated C-terminal murine PRG4, primarily containing the hemopexin-like domain (*EF1-mPrg4-999*), and a longer PRG4 isoform that also comprises the full Mucin and Somatomedin B (SMB) domains (*EF1-mPrg4-3276*), both in -1 or -2 orientations. The orientation "-1" is used when the 5' to 3' orientation of inserted transgene is toward the ITR of HCAd, and orientation "-2" is used when 5' to 3' orientation of inserted transgene is directed away from the ITR. Meanwhile, HCAd0 (a control virus without transgenes), PBS, and the NFkB-responsive murine IL-1Ra HCAd were utilized as experimental negative and positive controls. Bilateral destabilization of the medial meniscal tibial ligament (DMM) surgery was performed to induce murine post-traumatic osteoarthritis (PTOA). Two weeks postoperatively, the above HCAds and PBS were administered via intra-articular injection of the knee joint. Twelve weeks postoperatively, we harvested both knee joints for 2D histological analysis, 3D phase contrast micro-CT analysis, and immunohistochemical staining using F4/80 and CD206 to differentiate the total and M2 macrophage populations.

Employing 3D phase contrast micro-CT scanning, we observed that *in vivo* administration of HCAd-*EF1-mPrg4-999-2* significantly attenuated cartilage volume loss during OA progression compared to HCAd0 and PBS control groups. Additionally, HCAd-*EF1-mPrg4-3276-1* administration notably preserved cartilage-covered bone surface compared to PBS control, achieving results comparable to HCAd-*mIL-1Ra* injection. We are evaluating cellular changes and immune cell infiltration using Safranin-O staining, H&E staining, and immunohistochemical analysis of F4/80 and CD206 to understand the therapeutic effects of these PRG4 isoforms on chondroprotection and inflammation.

In these studies, we demonstrated a novel chondroprotective effect of the hemopexin-like carboxy-terminal domain of PRG4 in the context of PTOA. The data suggest that different components of the PRG4 protein may contribute to diverse activities in mediating chondroprotection.

209 A Prenatal Gene Editing Approach to Congenital Musculoskeletal Disease with Systemically Delivered Lipid Nanoparticles

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Introduction.
Congenital musculoskeletal disorders, including Duchenne muscular dystrophy (DMD), represent an opportunity for gene therapy. Lipid nanoparticles (LNP) represent a promising alternative to viral vectors for systemic administration, though achieving robust skeletal muscle delivery remains challenging. We aimed to evaluate both the muscle transduction potential of LNPs and the efficiency of CRISPR-Cas9 adenine base editing (ABE) at a therapeutic target for exon-skipping in the dystrophin gene, both in adult mice and *in utero.*

Methods.
CAG-Sun1/sfGFP (Sun1GFP) mice with floxed nuclear-localized green fluorescent protein (GFP) were injected with LNPs bearing different ionizable lipids (LNP1, LNP2) encapsulating Cre mRNA which drives nuclear GFP expression in successfully transduced cells. Mice received 1.5 mg/kg IV injections at gestational day 14 (E14) or 16 (E16) via the vitelline vein, or at 8 weeks of age via the retroorbital sinus. Quadriceps and diaphragm were harvested at 4 weeks of life (E14, E16) or 4 weeks post-injection (adults) and underwent nuclear extraction and fixation, followed by antibody staining for myonuclear (PCM1+) and muscle stem cell (PAX7+) markers. Fluorescence-activated nuclear sorting (FANS) quantified GFP+ fractions for each tissue and cell type. To assess *in vivo* base editing efficiency in skeletal muscle, adenine base editor mRNA and nucleoside-optimized guide RNA targeting the exon 45 splice acceptor site of the dystrophin gene were encapsulated within an LNP from prior Sun1GFP studies and injected into E14 and adult BALB/c mice at 2mg/kg or 3mg/kg and later harvested at similar time points. In addition to sequencing whole tissue DNA, muscle tissue underwent FANS protocol with nuclear sorting into myocyte nuclear fractions, followed by DNA recovery and sequencing to assess myocyte-specific base editing efficiency.

Results.
Efficient transduction of Sun1GFP myocytes and muscle stem cells were observed with *in utero* LNP injections, but not following adult injections. Up to 90% of quadricep myocytes and stem cell nuclei expressed GFP after E14 and E16 injections with both LNP formulations. In the diaphragm, myocyte transduction was similarly efficient; however, decreasing muscle stem cell transduction was seen from E14 to E16 (LNP1: 72% vs 56%; LNP2: 75% vs 43%). LNP2 was chosen for base editing studies. Next generation sequencing at the dystrophin exon 45 locus demonstrated efficient on-target adenine-to-guanine conversion in both E14 and adult livers. *In utero* LNP administration increased skeletal muscle editing compared to adult administration in the diaphragm and quadriceps. Importantly, significant on-target base editing was achieved specifically in myocyte nuclei in the diaphragm and quadricep after in utero LNP injections.

Conclusion.
In utero systemically administered LNPs can transduce skeletal myocytes and stem cells throughout the body, and base editing of the diaphragm and skeletal muscles via LNPs is more efficient when administered prenatally. Most importantly, however, as data suggest only a minority of dystrophin loci need to be corrected in skeletal muscle to be clinically therapeutic, we herein present data that suggest we can leverage systemically administered LNPs to deliver base editing constructs to skeletal and diaphragm myocytes in doses that may be sufficient to treat DMD, and potentially other congenital myopathies, prior to birth.

210 AAVrh74.tMCK.hBAG3 Gene Therapy Improves Phenotype in a Hereditary IBM Model, VCP-A232E Mouse

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Introduction:
Sporadic inclusion body myositis (sIBM) is the most common acquired myopathy in older adults. sIBM is characterized by chronic progressive weakness and its pathology involves an interplay between inflammatory and degenerative pathways. Histopathology indicates mitochondrial abnormalities and protein accumulation. Mutations in the valosin-containing protein (VCP) gene leads to a hereditary type of IBM (hIBM), in which sarcoplasmic and myonuclear inclusions with TDP-43 pathology are observed. Pathophysiological conditions in the cell cause the protein quality control system to depend on the autophagy-lysosome pathway (ALP) for degradation of accumulated misfolded proteins. BCL2-associated athanogene 3 (BAG3) protein enables aggresome formation and initiates the ALP.

Aim:
We aim to ameliorate disease process resulting from misfolded protein aggregation by upregulating the ALP. We

assessed our strategy of reducing the aggregate burden in the muscle by overexpressing the BAG3 in the VCP-A232E mouse, a model for hIBM.

Methods:
Seven male VCP mice received $3x10^{12}$ vg total dose of AAVrh74.tMCK.hBAG3 via tail vein injection at three months of age and eleven sex- and aged-matched VCP mice served as untreated (UT) controls. Mice were tested on rotarod and treadmill at the experimental endpoint, nine months post gene delivery. The other outcome measures conducted were quantitative muscle histopathology and morphometry assessing the amount of TDP-43 accumulation, as well as expression levels of autophagy markers.

Results:
BAG3 treatment resulted in functional improvements; 35% greater distance run on the treadmill (BAG3: 70.0 ± 4.4 vs UT: 51.7 ± 4.6 m, p=0.015) and 46% better performance in the rotarod (BAG3: 32.3 ± 2.5 s vs UT: 22.1 ± 2.1 s, p=0.007) at the endpoint compared to untreated mice. To assess the efficacy of AAV.hBAG3 gene therapy in treating abnormal mitochondrial content and distribution, we quantified abnormal muscle fibers in gastrocnemius (GAS) and tibialis anterior (TA) muscles, demonstrating that the BAG3 treatment lead to a significantly smaller number of abnormal fibers in both muscles (GAS, BAG3: 13.6 ± 2.6 vs UT: 29.4 ± 4.8 number/mm^2, p=0.034; TA, BAG3: 7.0 ± 2.1 vs UT: 20.0 ± 3.5 number/mm^2, p=0.010). Our quantifications in TA muscle samples also showed a significant decrease in the number of fibers with internal nuclei following BAG3 gene therapy. Muscle fiber analysis on SDH-stained samples revealed that BAG3 gene therapy in VCP mice caused a significant increase in the size of fast-twitch fibers in both muscles analyzed. We utilized immunofluorescence staining to assess the TDP-43 accumulation levels in VCP mice and we observed a significant decrease in the number of TDP-43-positive aggregates in the treated muscle samples (BAG3: 191.7 ± 20.6 vs UT: 261.0 ± 21.1 aggregate/mm^2, p=0.037). These observations were associated with significantly increased autophagy markers, p62 and LC3 on western blots of muscle samples from the BAG3 treated cohort, compared to UT counterparts.

Conclusion:
Systemic delivery of AAVrh74.tMCK.BAG3 at $3x10^{12}$ vg in VCP-A232E mouse model for hIBM resulted in significant improvements in functional, histopathological, and molecular analyses, compared to UT cohort. Our strategy of supporting ALP system by overexpressing BAG3, has potential therapeutic use for other myodegenerative conditions associated with abnormal protein aggregates.

211 Identifying Adeno-Associated Virus Serotypes that Transduce Fibro-Adipogenic Progenitors in Mouse Healthy and Fibrotic Skeletal Muscles *In Vivo*

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The skeletal muscles extracellular matrix (ECM) serves multiple critical functions to maintain skeletal muscles' health. In most myopathies and dystrophies, the ECM undergoes maladaptive fibrotic changes due to the excessive production of dysfunctional ECM. These changes become major contributors to the pathogenesis and progression of various disorders affecting muscle. In addition, some muscular dystrophies are caused directly by pathogenic variants in the genes encoding skeletal muscles ECM proteins. One of those proteins is collagen VI, and pathogenic variants in its coding genes cause collagen VI-related dystrophies (COL6-RDs).

Fibro-adipogenic progenitors (FAPs) are one of the major cell types that produce ECM proteins in skeletal muscles. To date, no delivery vector has been shown to transduce skeletal muscles FAPs via systemic injection *in vivo*. This has markedly contributed to the current absence of effective gene therapies for many of the myopathies caused by ECM pathogenic variants and dysregulated ECM production.

Here, we screened 9 clinically relevant naturally occurring adeno-associated virus (AAV) serotypes to identify AAVs capable of transducing mouse skeletal muscles FAPs efficiently and selectively *in vivo*.

We intravenously (IV) injected 7-9-week-old Cre/FAPs reporter male mice (Ai14;PDGFRα^{EGFP}) with each serotype at 1.5x10^{12} vector genomes (VGs)/mouse (n=3/serotype). Tissues were collected 4 weeks post-injection, and the percentage of transduced cells was quantified using fluorescence-activated cell sorting.

We assessed the 9 AAVs' efficiencies at transducing FAPs and non-FAPs in skeletal muscles and the liver. We identified a serotype that was significantly more efficient at transducing FAPs in both tissues than most serotypes, while its transduction of non-FAPs was no different from the majority of the other serotypes. These results suggest this serotype has a selectively greater transduction efficiency towards FAPs in different tissues compared to other serotypes. Besides the top-performing serotype, we identified 3 moderate FAP-transducing serotypes that were significantly more efficient at transducing muscle FAPs than the remaining 5 poor-performing serotypes.

We are currently quantifying the number of VGs per diploid genome, further characterizing the serotypes' biodistribution, and identifying the AAV capsid motifs specific to the top-performing serotypes to inform rationally designing potentially more efficient and selective FAPs-targeting capsids. Additionally, we are in the process of assessing the efficiency of the top-performing serotypes at transducing FAPs in male and female fibrotic skeletal muscles of a COL6-RDs mouse model. Once confirmed in the COL6-RDs mouse model, the top-performing serotypes will be further validated in human *in vitro* cellular models.

Lipid Nanoparticles I

212 Discovery of peptides for targeted delivery of mRNA lipid nanoparticles to cystic fibrosis lung epithelia

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Cystic fibrosis (CF) is a genetic disease affiliated with hyperconcentrated mucus that lines the epithelia of multiple organs. In the lungs, this mucosal abnormality is the main cause for morbidity and mortality for CF patients, leading researchers to focus their attention on genetic correction in the airways. Non-viral approaches to gene therapy have shown clinical promise; however, the thick mucus barrier found in CF afflicted lungs remains a significant challenge for cell uptake of the carrier and thus the therapeutic cargo. Given this obstacle, we were encouraged to develop a phage display selection method to identify a mucus and cell penetrating peptide that could overcome this delivery barrier and be used in the context of a non-viral delivery system (i.e., lipid nanoparticles) to deliver mRNA. In this work, we used primary human bronchial epithelial cells from CF patients cultured at air-liquid interface for our selection model. At the air-liquid interface, primary human bronchial epithelial cells produce mucus and differentiate into multiple cell types, making it clinically relevant. With this method, we discovered a lead candidate peptide and incorporated it into lipid nanoparticles to deliver mRNA to primary human bronchial epithelia *in vitro* and mouse lungs *in vivo*. Compared to lipid nanoparticles without our peptide, peptide-lipid nanoparticles demonstrated up to 7.8-fold and 3.4-fold higher reporter luciferase bioactivity *in vitro* and *in vivo*, respectively. Importantly, these peptides facilitated higher specific uptake of nanoparticles into lung epithelia relative to other nontarget cell types. Since nucleic acid delivery to primary human bronchial epithelia is a significant challenge, we are encouraged by these results and anticipate that our peptide could be used to successfully deliver cystic fibrosis gene therapies in future work.
Reference: Soto MR, Lewis MM, Leal J, Pan Y, Mohanty RP, Veyssi A, Maier EY, Heiser BJ, Ghosh D. Discovery

of peptides for ligand-mediated delivery of mRNA lipid nanoparticles to cystic fibrosis lung epithelia. Molecular Therapy Nucleic Acids. 2024 Dec 10;35(4).

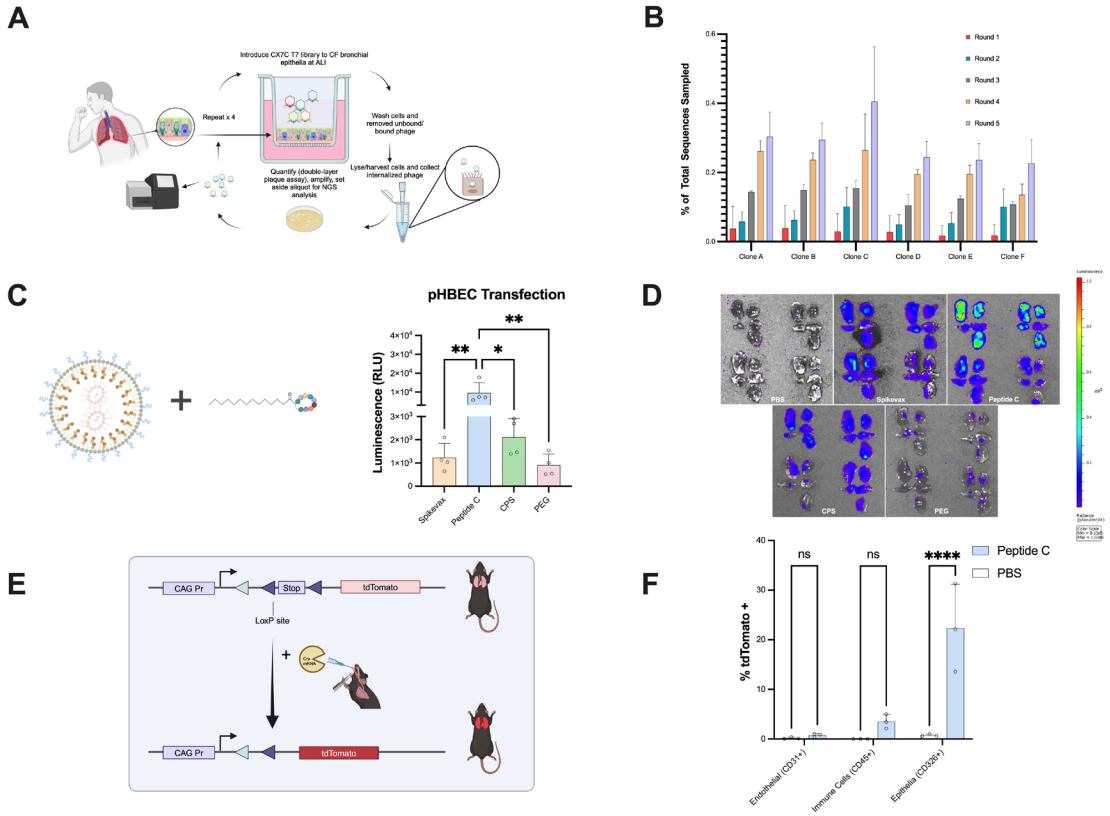

Figure 1. (A,B) Selection strategy (A) and enrichment of top performing clones (B) using a cysteine constrained random 7-amino acid peptide T7 phage display library (CX_7C) against differentiated primary human bronchial epithelial cells (pHBECs) for a total of five rounds. **(C)** Schematic of Peptide LNP components (Left) and comparison of pHBEC transfection between lead Peptide C LNP and controls after delivering NLuc mRNA (Right) (*n*=4; mean (SD)); One-way ANOVA (Tukey's multiple comparisons test); *$p < 0.05$, **$p < 0.01$ **(D)** To BALB/c mice, we delivered either PBS or Nluc LNPs, harvested lungs at 24 hours post administration, and immediately measured bioluminescence via IVIS imaging. Shown are representative images for each treatment group (*n*=4 to 8) **(E,F)** Schematic of Cre mRNA delivery to Ai9 mice and flow cytometry analysis of tdTomato positive cells in various cell types in the lungs following intratracheal delivery of Cre mRNA to Ai9 mice (*n*=3; mean (SD)); Multiple unpaired t-tests (Holm-Šídák multiple comparisons test); ****$p < 0.0001$. Illustrations made with BioRender.

213 Novel Non-Viral DNA-based Gene Therapy Vector for CAR T Engineering I*n Vivo*

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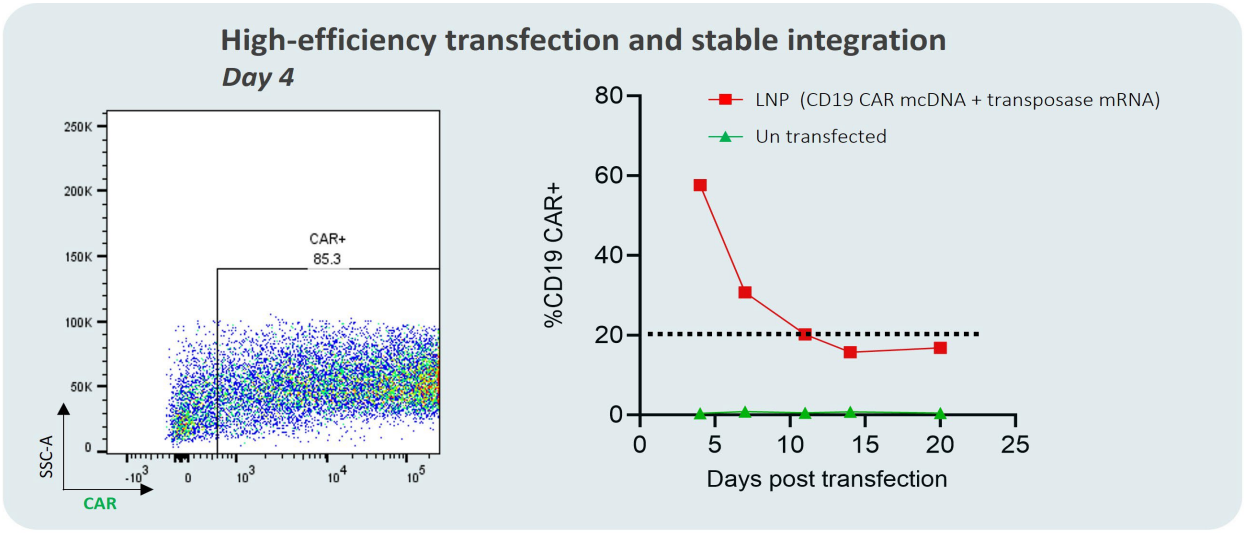
The engineering of immune cells to express Chimeric Antigen Receptors (CARs) has revolutionized cancer treatment, with ex vivo CAR-T therapies achieving significant clinical and commercial success. However, these therapies come with major limitations, including high costs, lengthy manufacturing processes, potential toxicity, and logistical challenges associated with patient-specific cell production. These constraints emphasize the need for next-generation approaches to improve accessibility and efficiency.

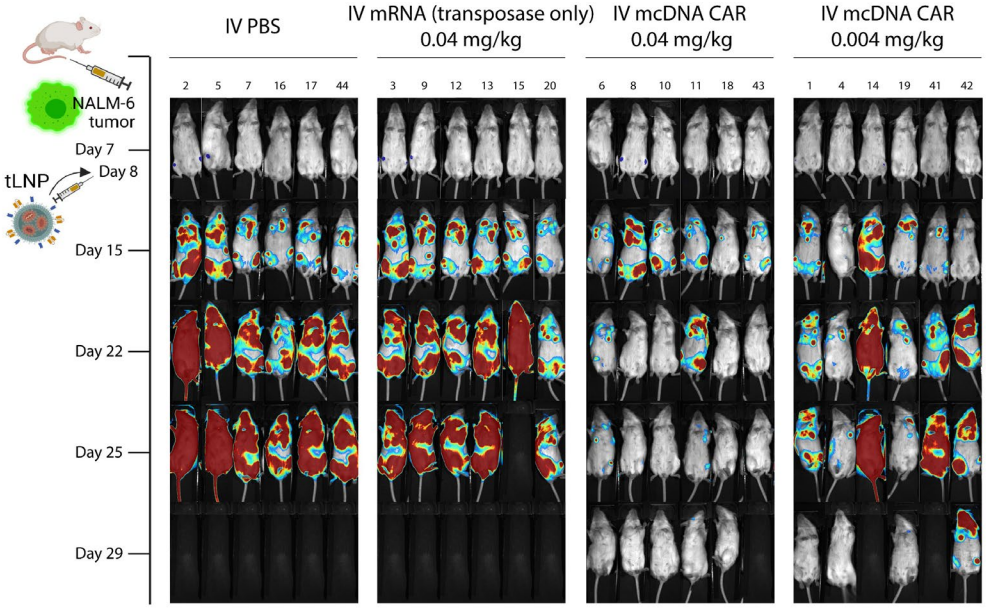
We present an innovative cell-targeted lipid nanoparticle (tLNP) platform designed to deliver both DNA and RNA directly to T cells, enabling in situ CAR-T cell generation (Fig. 1). This tLNP system consists of lipid nanoparticles carrying transposon DNA (encoding CAR), mRNA (encoding transposase), and targeting/activating protein

components. We demonstrate that this approach can activate resting primary T cells and facilitate stable CAR expression without requiring traditional ex vivo expansion or external activation steps.

Beyond in vitro modification, we show that this non-viral vector drives functional CAR-T cell generation in vivo. We would like to present and discuss our latest data demonstrating successful generation of CAR T in an NSG mouse model injected with a human B-cell leukemia cell line. We will discuss that a single dose of tLNP (carrying DNA/RNA) results in durable CAR-T cell generation, tumor control, and prolonged survival (Fig. 2).

By shifting CAR-T engineering from a labor-intensive, facility-dependent process to a streamlined, in vivo approach, this platform has the potential to overcome critical barriers in cell therapy, making CAR-T treatments more scalable, cost-effective, and widely accessible.





214 Lipid Nanoparticle-Mediated CRISPR-based Therapy Enables Mutation-Agnostic Gene Editing Solving for Autosomal Dominant Polycystic Kidney Disease

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Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a prevalent hereditary disorder caused by mutations in the *PKD1* (85%) or *PKD2* (13%) genes, which encode polycystin-1 and polycystin-2, respectively. This condition leads to the formation of fluid-filled renal cysts, often resulting in end-stage renal disease (ESRD). Restoring *PKD1/PKD2* expression in preclinical models has shown potential to attenuate and reverse cystogenesis. To address the broad mutation heterogeneity in *PKD1*, with over 800 pathogenic variants, we have developed a mutation-agnostic strategy for modulating certain regulatory elements that impact *PKD1* expression.

Helex's proprietary kidney-tropic lipid nanoparticle (LNP) platform addresses the delivery challenge for gene editing in kidney cells. Tail vein administration of LNPs in BALB/c mice demonstrated significant kidney tropism, confirmed via *in vivo* and *ex vivo* biodistribution studies. Using our proprietary ionizable lipid, the LNP achieved even biodistribution between the kidney and liver, reducing off-target liver accumulation by 100 times compared to commercial lipid-based formulations. Renal artery (RA) injection further enhanced kidney tropism, achieving ~50 times higher kidney accumulation than intravenous delivery and with minimal to nil off-target biodistribution in other organs.

In ADPKD patient-derived kidney cell lines, LNP-delivered CRISPR-Cas9 successfully edited the regulatory target, leading to a four-fold increase in *PKD1* mRNA levels. This was accompanied by increased polycystin-1 expression and modulation of downstream cystic pathways, including pCREB, PKA-C, and mTOR pathways confirmed by RT-PCR and western blotting. The proprietary LNP demonstrated a significantly improved safety profile compared to formulations using commercial lipids, supporting its suitability for therapeutic translation.

By integrating a kidney-tropic LNP platform with Helex's Epic-Cure™ drug design framework, this mutation-agnostic approach offers a transformative strategy for ADPKD. Beyond halting disease progression, it holds promise for reversing the disease phenotype, presenting a significant advancement over existing symptomatic treatments.

215 Rational Design and Preclinical Applications of Long Circulating Lipid Nanoparticles (lcLNPs) for Extra Hepatic Delivery of Nucleic Acids

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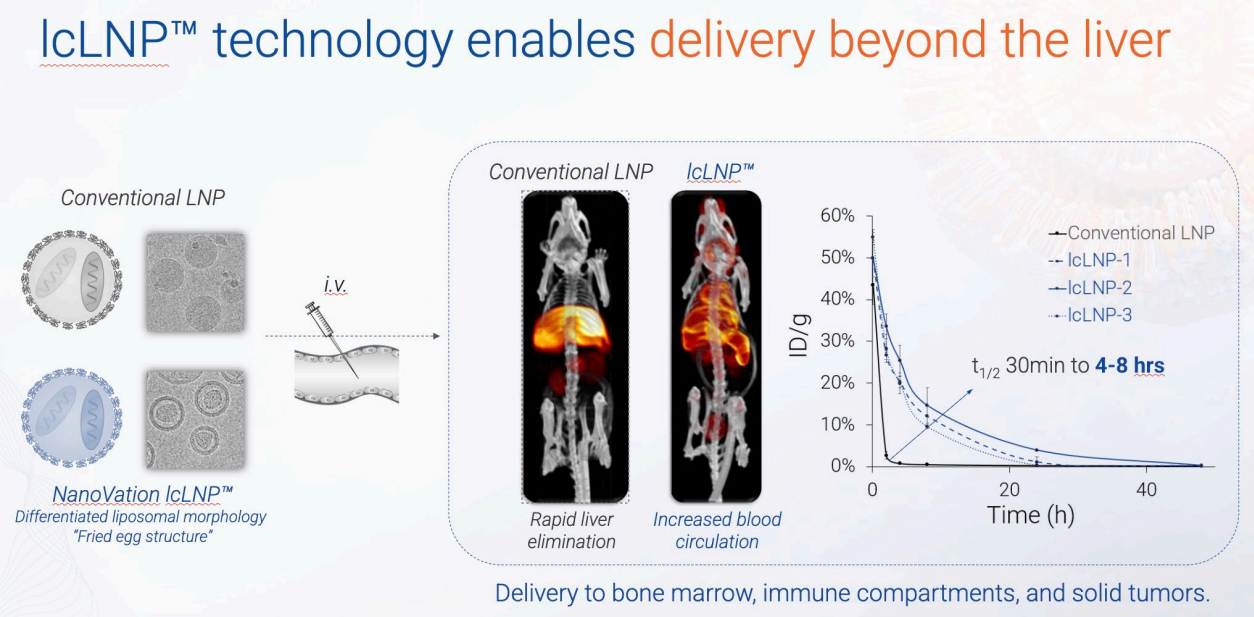
Historically, the potential for gene therapies has been limited due to the delivery. The most conventional approach includes the use of modified viruses that offer long lasting impact following single dose administration. However, they do suffer from safety issues, adaptive immune responses, lower packaging capacity and poor manufacturing scalability. Non-viral drug delivery systems, however, when designed appropriately can overcome these limitations and offer more access to gene therapies. Lipid nanoparticles have already enabled successful delivery and manipulation of targets in the liver as demonstrated by the first ever approved RNAi therapeutic, Onpattro. This composition comprising of ionizable lipids, phosphatidylcholine, cholesterol and PEG-lipid (50/10/38.5/1.5 mol%, respectively) was able to deliver the paylod (SiRNA) into the cytosol of hepatocytes. Onpattro was designed to accumulated rapidly in the liver following intravenous administration, which made the extrahepatic tissues inaccessible. Various solutions have been implemented to overcome rapid liver accumulation including PEGylation (persistent PEG-lipid), particle surface modification including ligands and charged lipids. However, they all face limitations in their clinical utility due to adaptive immune responses, acute immune stimulations and manufacturing complications.

Inspired by the development of long-circulating liposomes for small molecule therapeutics, we rationally designed a novel set of LNP formulations to deliver nucleic acids to extrahepatic tissues by extending the circulation half-life of the nanoparticles.Firstly, we demonstrated that formulations with elevated distearoylphosphatidylcholine (DSPC) displayed extended circulation profiles that was five-fold greater than benchmark LNPs, and they exhibited a substantially different morphology. Next, we were able to demonstrate that these unique and novel long circulating LNP systems (lcLNPs) can deliver multiple nucleic acid payloads including mRNA, SiRNA to target tissues of interest including abdominal skin. Furthermore, we demonstrated that optimizing the lcLNP compositions and ionizable lipids resulted in a 300-fold increase in mRNA expression in the abdominal skin when compared to conventional LNP compositions. We also saw a similar increase in mRNA expression in the long-term hematopoietic stem cells in the bone marrow, monocytes in circulation and splenic T-cells.

Our findings demonstrate that changing LNP compositions alone can modulate LNP tissue accumulation negating the need to add additional components to LNPs. This further supports the hypothesis that extrahepatic delivery can be achieved by extending circulation profiles of LNPs.



216 Enhancing Liver DNA Delivery with a Fully Non-Viral Multifunctional LNP Approach for In Vivo Transposon-Mediated Genomic Integration of Large DNA Cargo

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Integrating DNA into a patient's genome holds great promise for treating genetic diseases by restoring function through gene addition. The Super piggyBac (SPB) DNA integration system can achieve genome integration of large cargo up to 200 kb without inducing double-strand DNA (dsDNA) breaks. A non-viral delivery platform capable of delivering transposon DNA and the transposase enzyme could enable repeat dosing for titratable gene addition.

To enable cellular delivery of dsDNA, we developed an LNP format incorporating an ionizable lipid and a novel non-lipid intracellular trafficking agent (ICTA). ICTAs are amphipathic organic compounds that enhance encapsulation and release of complex genetic payloads like pDNA. First-generation ICTAs improved delivery but led to aggregation and poor storage stability. A second-generation ICTA series was synthesized and screened in mice to elucidate structure-activity relationships for liver DNA delivery. Several ICTAs enabled LNPs with a 60-

130 nm size distribution (depending on ICTA and loading ratio), facilitating cellular uptake and passage through liver sinusoids. ICTAs provided a 10-fold increase in luciferase reporter transgene expression and improved delivery of phenylalanine hydroxylase and hFVIII transgenes by 3-5-fold in adult and juvenile mice. ICTA LNPs achieved therapeutically relevant protein levels, with PAH protein in >10% of hepatocytes and hFVIII exceeding 100% normal plasma levels, as determined by ELISA 6 days post-administration.

Uptake of DNA-carrying LNPs by innate immune cells can trigger pro-inflammatory cytokine secretion, activating cell death pathways in adjacent cells. We explored a strategy to neutralize inflammation and promote cell survival using transient expression of chimeric cytokine transmembrane receptors, where an intracellular pro-survival RTK domain was paired with an extracellular pro-inflammatory cytokine receptor. Adult mice given 1 mg/kg LNPs co-encapsulating transgene donor DNA and mRNA for these receptors showed up to a 60% reduction in serum MCP-1 at 4 hours and a 50% reduction in liver transaminases at 24 hours, compared to control mRNA LNPs. Transgene expression remained robust 6 days post-administration.

The ability to fine-tune transgene expression would enable patient-tailored treatment, crucial for clotting disorders. To achieve this, we tested an inducible iCasp9 “modulator switch” in our transposon, allowing the reduction of hFVIII levels via an inducer molecule. This modulator, linked via T2A in our FVIII transposon, was co-encapsulated with SPB mRNA in an LNP. Adult wild-type mice received a single intravenous LNP dose, achieving ~75% normal hFVIII levels by day 6. Subsequent IV doses of rimiducid (0.001–1.0 mg/kg) reduced hFVIII to between 31% and ~1% of normal, dose-dependently, with no hepatotoxicity.

The non-viral platform presented here may enable a safer, tunable, and durable solution for genetic medicines with broad applicability for rare and non-rare diseases.

217 Pharmacodynamic Activity, Pharmacokinetics and Tolerability of Lipid Nanoparticle Formulations of mRNA Following Repeated Intravenous Dosing in Monkeys

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With the increasing adoption of lipid nanoparticle (LNP) technology in the development of nucleic acid-based therapeutics, particularly in the areas of gene editing and gene modulation, there is an increasing need for a detailed understanding of factors influencing LNP activity, pharmacokinetics (PK), and tolerability, to continue to rationally improve the therapeutic index of LNP systems and to support their development as therapeutics. To investigate these, we have evaluated the activity, plasma PK profiles and tolerability of three different LNP formulations in cynomolgus monkeys, using two different mRNA payloads. LNP were administered at 0.5 or 1.5 mg/kg by 1-hour intravenous (IV) infusions, once per month for three months. Pharmacodynamic activity was quantified by measuring levels of human IgG (ELISA) or alpha-galactosidase (LC-MS/MS) encoded by the encapsulated mRNAs; plasma PK profiles of ionizable lipid and PEG-lipid concentrations were measured by LC-MS/MS; and mRNA-LNP tolerability was assessed via standard clinical pathology endpoints, multiplex assays for cytokines (MSD), ELISA/EIA methods for complement and anti-PEG antibodies, and histopathology. Distinct pharmacodynamic activity profiles were observed in monkeys for the three different LNP compositions evaluated, based on differences in the ionizable lipids and payloads (mRNA), but the pattern of response did not change with repeat dosing. PK analysis of the three LNP revealed distinct plasma PK profiles and relatively large differences (~13-fold) in plasma exposure of the ionizable lipids; however, the PK profile and plasma exposure of the PEG-lipid was independent of the LNP and was the same for each LNP evaluated, consistent with rapid exchange out of the LNP in the circulation. PK profiles of the three LNP were unaffected by different payloads and repeated dosing. The substitution of a different mRNA into the same LNP composition resulted in overlapping PK profiles, and repeated monthly infusions did not alter plasma exposure. Changes in clinical pathology parameters, including increases in liver enzyme levels (ALT/AST), were observed to be dose-related and transient (peaking 48 hours after dosing). Differences in the magnitude of ALT changes were observed with different payloads, indicating a payload effect,

while peak ALT levels were consistent on each dosing occasion with repeated dosing of the same payload. Cytokine/chemokine levels were transient, generally peaking at 6 hours post-dose, and peak levels remained consistent with repeated dosing of the same payload. Despite the relatively large difference in plasma exposure between three LNP, the profile of histopathology findings and the target organs were the same: liver (vacuolation, single-cell necrosis and/or swelling of hepatocytes; mixed leukocyte infiltration), spleen (vacuolation and/or hypocellularity in red pulp; lymphoid depletion) and adrenal glands (lipid depletion in the cortex). Overall, these results provide a comprehensive characterization of the activity, PK and tolerability of different LNP in monkeys, following repeated monthly infusions. Each of the LNP were well tolerated, resulting in non-adverse, transient, predictable and reversible changes in clinical pathology (e.g., ALT/AST) parameters and histopathology findings, indicating that repeat dosing of mRNA-LNP is feasible and safe.

218 Modulation of Lipid Nanoparticle-Formulated Plasmid DNA Drives Innate Immune Activation Promoting Adaptive Immunity

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Nucleic acid vaccines have grown in importance over the past several years, with the development of new approaches remaining a focus. We describe a lipid nanoparticle-formulated DNA (DNA-LNP) formulation which induces robust innate and adaptive immunity with similar serological potency to mRNA-LNPs and adjuvanted protein. Using an influenza virus hemagglutinin-encoding construct (HA), we show that these DNA-LNPs drive potent inflammation dependent on the cGAS-STING-TBK1 pathway but independent of TLR9. Priming with HA DNA-LNP demonstrated strong upregulation and activation in migratory DC (mDC) subpopulations, and these changes were STING-dependent. Transcriptomics elucidated activation and upregulation of pro-migration factors among multiple innate immune populations after priming with DNA-LNP. Relative to mRNA-LNPs or adjuvanted protein, HA DNA-LNP induced superior antigen-specific CD8+ T cell responses with memory responses persisting beyond one year. In rabbits immunized with HA DNA-LNP, we observed immune responses comparable or superior to mRNA-LNPs at the same dose. Using an additional pathogen model, SARS-CoV-2 spike-encoding DNA-LNPs elicited protective efficacy comparable to spike mRNA-LNPs. Our study identifies a platform-specific priming mechanism for DNA-LNPs divergent from mRNA-LNPs or adjuvanted protein, suggesting avenues for this approach in prophylactic and therapeutic vaccine development.

Novel Therapeutic Gene Editing Applications

219 Efficient Cas9 LNP Mediated Repair of a Pathogenic *TCAP* Mutation in Skeletal Muscle

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Pathogenic insertion and deletion (InDel) mutations that result in loss of protein expression account for the vast majority of debilitating neuromuscular diseases, such as Duchenne Muscular Dystrophy (DMD) and Limb Girdle Muscular Dystrophy (LGMD), for which few therapeutic options exist. Gene editing-based therapeutics hold promise for a cure for monogenic neuromuscular diseases by repairing the disease-associated mutations. Nuclease-based gene editing is among the simplest and most effective approaches to modify the genome of a target cell population in vivo. Delivery of Cas9 nuclease and a single guide RNA creates a double-strand break (DSB) at its target site, which is precisely or imprecisely repaired by the endogenous DNA damage repair machinery. In proliferating cells, the InDel distribution resulting from imprecise repair is predictable based on the local sequence context and can be dominated by a single allele (e.g. Non Homologous End Joining (NHEJ)-mediated 1 bp insertion or Microhomology-mediated end joining (MMEJ) deletion). These dominant repair outcomes can be leveraged to restore a disrupted reading frame within a gene caused by a pathogenic mutation. In addition, MMEJ-based deletions can correct pathogenic insertion mutations consisting of a short stretch of duplicated sequence (microduplication), but the efficiency of MMEJ-based DSB repair in post-mitotic skeletal muscle has not been extensively characterized.

We examined a nuclease-based strategy for therapeutic correction of an 8bp microduplication mutation in the skeletal muscle gene, TCAP. Biallelic loss of function mutations in TCAP result in LGMDR7. We performed in vivo editing by delivering Cas9 mRNA or ribonucleoprotein (RNP) complexes intramuscularly in an LGMDR7 mouse model containing the humanized TCAP 8 bp microduplication. We have previously shown that this microduplication mutation can be efficiently corrected via MMEJ repair in proliferating cells.[1] We employed a Selective ORgan Targeting (SORT) LNP formulation that facilitates the local delivery of Cas9 mRNA or RNP to skeletal muscle.[2] We compared Cas9 editing outcomes at two target sites for Cas9 mRNA or RNP when employing end modified or heavily modified sgRNAs. Our best LNP-Cas9 formulation achieved local editing rates >30%. In addition, LNP size impacted the distribution of editing throughout different muscle groups in the hindlimb for Tibialis Anterior (TA) injection. Cas9 editing restored expression and proper sarcomere localization of Telethonin (encoded by TCAP) heterogeneously throughout the injected TA. Interestingly, indel distributions at the target site differ between LGMDR7 patient-derived induced Myoblasts in vitro and mouse skeletal muscle in vivo, which can be attributed to differences in the active DNA repair pathways in post-mitotic cells. We also evaluated the temporal cytokine, humoral and cellular immune responses associated with LNP Cas9-mRNA and RNP delivery to understand their potential impact on editing outcomes and persistence. These studies revealed differences in the host response between the two different Cas9 effector modalities. Our findings yield important insights into in vivo editing outcomes, LNP distribution and immune response in skeletal muscle that will be beneficial for developing effective gene editing technologies for a variety of muscular dystrophies.

1. Iyer S, *et al. Nature* 2019 568(7753):561-565.
2. Wei T, *et al. Nat Commun*. 2020 11(1):3232.

220 Brain-wide Genome Editing via STEP-RNPs for Treatment of Angelman Syndrome

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Brain-wide genome-editing remains a major hurdle for the treatment of neurogenetic disorders. Gene editing using CRISPR/Cas9 for neurogenetic disorders is challenged by difficulty in crossing blood-brain barrier, limited penetration, and narrow therapeutic windows. While modified adeno-associated virus (AAVs) overcome some of these barriers, their immunogenicity and higher risk of off-target effects due to the long-term presence of Cas9 protein, limited their translational value in human neurogenetic disorders. To address this, we developed an innovative We report a non-viral, non-nanoparticle, chemical modification-based method, called Stimuli-Responsive

Traceless Engineering Platform (STEP), which deliver conjugated Cas9 protein and sgRNA (RNP). STEP-RNP achieved effective delivery of CRISPR to neuronal cells in the brain, and the transient Cas9 protein substantially minimized the risk of off-target effect. We tested the STEP-RNP in vitro and in vivo for Angelman syndrome (AS), a neurodevelopmental disorder caused by the deficiency of neuron and maternal-specific *UBE3A* gene expression. The repressed expression of *UBE3A* in the paternal chromosome is mediated by the paternally expressed non-coding *UBE3A* antisense transcripts (*UBE3A-ATS*). Inactivation of *UBE3A-ATS* via antisense oligo (ASO) has shown positive clinical efficacy in ongoing phase 1/2 clinical trials in human AS. However, the transient effect of ASO that requires monthly repeating intrathecal delivery post a challenge as a standard clinical treatment. We designed STEP-RNP delivery system selectively inactivates *Ube3a-ATS* expression and potentially achieves permanent therapeutic effects via a single treatment. Using *Ube3a-YFP* reporter mice, we observed high gene editing efficiency (>75% targeting neurons) with widespread brain penetration. We administered STEP-RNP via intrathecal to newborn (P1-2) and adult AS (P21 or P42) *Ube3a*$^{m-/p+}$ mice. The treatment resulted in significant reduction of *Ube3a-ATS* and reactivation of *Ube3a* protein expression up to 40% of normal level across the cortex, hippocampus, and cerebellum. Accordingly, this treatment led to significant improvement in multiple behavioral domains, including locomotor function, anxiety-like behaviors, learning and memory, and also extended the latency for chemical induced myoclonic and tonic seizures in adult AS *Ube3a*$^{m-/p+}$ model. Importantly, we have not observed any acute toxicity and immunogenicity associated with STEP-RNP, neither detectable off-target events by comprehensive genomic profiling. Moreover, we have found the STEP-RNP effectively reactivate the *UBE3A* expression from the paternal chromosome in human 2D neurons and cortical organoids differentiated from AS patient iPSCs. In addition, we have shown the intravenous delivery also reach widespread penetration and efficient editing in brains. We are currently conduct FDA IND studies for Angelman syndrome using STEP-RNP supported by NIH Somatic Cell Genome Editing Consortium. Genome editing via STEP-RNP will have broad applications and the great potential to treat many neurogenetic disorders.

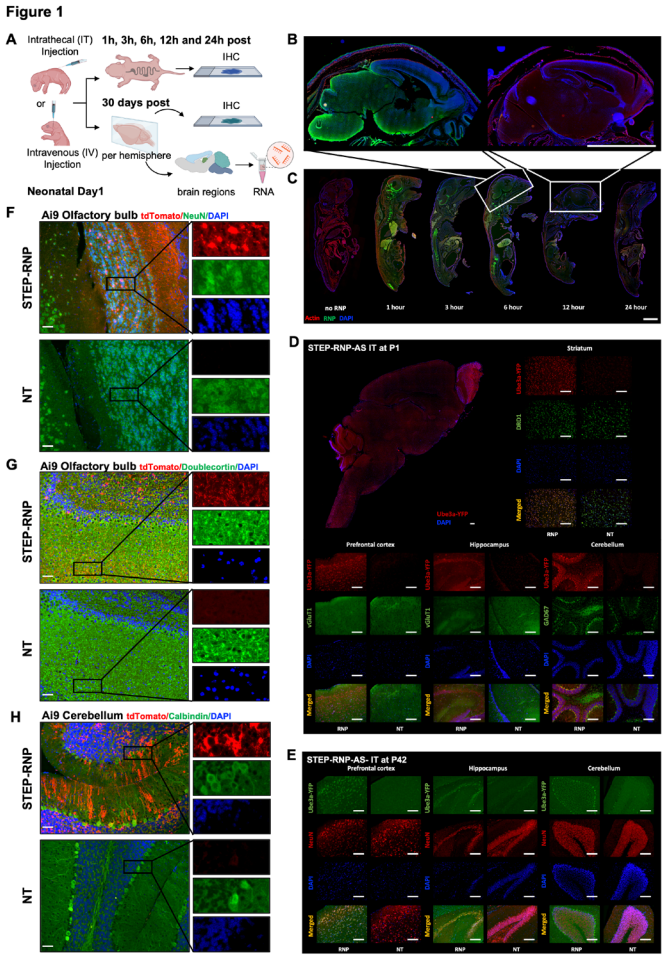

Fig 1. Biodistribution and cell type specificity of STEP-RNP
(A) Delivery of STEP-RNP via intrathecal (IT) and intravenous (IV) injection routes were assessed for whole body distribution at 1-, 3-, 6-, 12-, and 24-hours post-injection in pups (upper), and at 30 days post injection, brain of both neonates and adults were collected for further analysis.
(B-C) Representative images of STEP-RNP detection in the neonatal mouse brain (B) and whole body (C), over time following IT administration (10ug/ul, 10ul/mouse). STEP-RNP is detected by immunostaining on His-tag fused with Cas9. STEP-RNP is detectable in the brain 1 hour following administration, including Midbrain (single arrow), Hypothalamus (double arrow) and Pons Medulla (star) regions and is undetectable in the brain by 12 hours (n = 3 each time point, scale bar = 5 mm).
(D) Representative images of UBE3A-YFP expression in whole brain (upper left) and brain regions (insets) 30 days after IT injection (10ug/ul, 10ul/mouse) of STEP-RNP-AS (G5 + sgRNA33) into P1 neonatal *Ube3a*$^{m+/p-YFP}$ mice. UBE3A-YFP expression colocalizes with markers for multiple neuron types: dopaminergic neurons (DRD1), glutamatergic neurons (vGLUT1) and GABAergic neurons (GAD67), in the striatum, prefrontal cortex, hippocampus and cerebellum, respectively (n=5, scale bar = 150 μm).
(E) Representative images of UBE3A-YFP expression 30 days after IT administration (20ug/ul, 20ul/mouse) of STEP-RNP-AS (G221 + sgRNA33) into adult *Ube3a*$^{m+/p-YFP}$ (42 day old) mice. UBE3A-YFP expression colocalizes with NeuN+ neurons across the prefrontal cortex, hippocampus, and cerebellum (n=5, scale bar = 150 μm).
(F-H) Representative image of tdTomato expression colocalized with NeuN (F), Doublecortin (G) and Calbindin (H) in Ai9 mice 30 days after IT administration of STEP-RNP-sgRNA-AB (10ug/ul, 10ul/mouse) (n=5, scale bar = 50 μm).

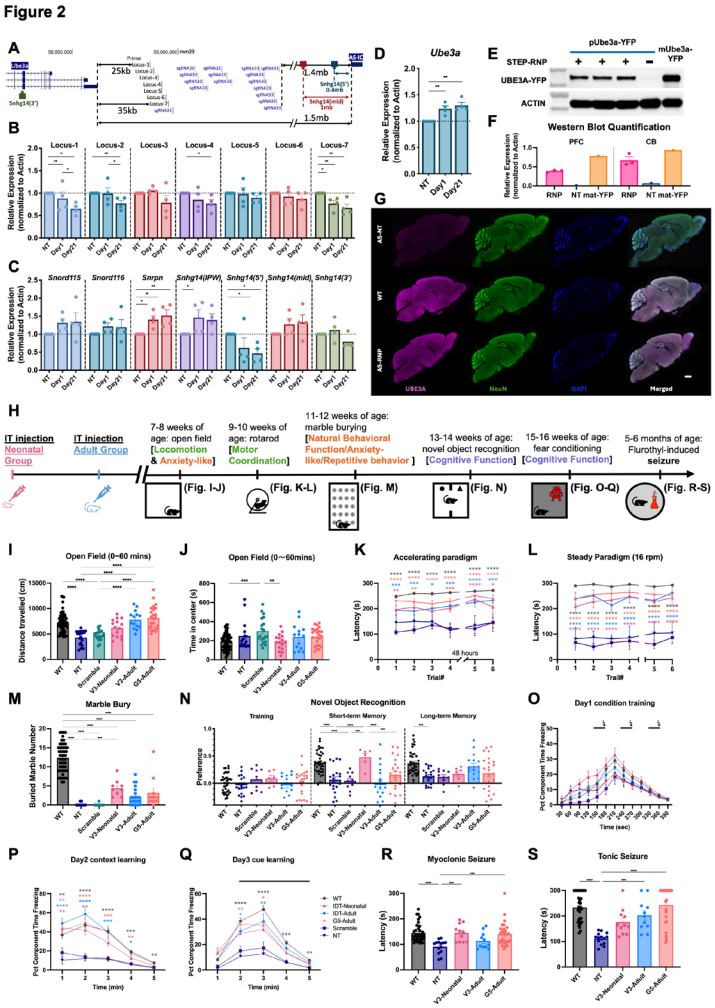

Fig 2. In vivo efficacy of STEP-RNP targeting Angelman syndrome (AS) mouse model

(A) Design of a series of primers targeting multiple loci of *Ube3a-ATS* (black), and multiple targeting loci of sgRNA33 (blue), and their location relative to *Ube3a* and Angelman Syndrome Imprinting Center (AS-IC).

(B) Relative expression of *Ube3a-ATS* in the mouse prefrontal cortex 30 days following STEP-RNP administration to postnatal day 1 and day 21 mice. qRT-PCR primers targeted multiple loci (locus 1-7) as shown in (A).

(C) Relative expression of genes surrounding *Ube3a-ATS* as assessed by qRT-PCR. Notably, there were no significant changes in the expression levels of *Snord115* and *Snord116* in the prefrontal cortex of mice treated with STEP-RNP when compared to untreated controls. A consistent decrease in the expression of *Snhg14* at the 5' end paralleled the observations in the qRT-PCR of *Ube3a-ATS* (n=4 for each group, *p<0.05; ** p<0.01).

(D) Relative reactivated expression of *Ube3a* assessed by qRT-PCR. The expression level of *Ube3a* in both postnatal day1 and day21 are significantly increased compared to untreated controls (n=4 for each group, *p<0.01; **p<0.001)

(E-F) Immunoblot analysis confirmed the reactivation of UBE3A protein 90 days after IT administration of STEP-RNP to P1 neonatal mice. Maternal UBE3A was used as a positive control. UBE3A from paternal chromosome is not detected without STEP-RNP. Paternal UBE3A was reactivated 49.53 ± 2.22% of maternal expression level in prefrontal cortex (PFC), and 71.83 ± 9.70% in cerebellum (CB) after STEP-RNP.

(G) Immunofluorescence showed that the effect of STEP-RNP gene editing was sustained for over 8 months after IT administration at P1-2. WT: Wild type; AS-NT: Angelman syndrome non-treated.

(H) Schematic diagram of behavioral analyses designed to evaluate the efficacy of STEP-RNP applied to AS (STEP-ANP-AS) mouse model (*Ube3a*$^{m-/p+}$). Neonatal (P1-2 day) (pink) and P21 and P42 days (adult) groups received a single injection of STEP-RNP before being subjected to all tests H – R. WT: Wild type; NT: no treatment.

(I) Open field test shows significant amelioration of locomotor function for AS mouse model groups treated with STEP-RNP-AS with either V3 Cas9 or G5 Cas9 compared to no-treatment (NT) and scrambled gRNA.

(J) Open field test shows significantly reduced time in center, a proxy for anxiety-like behavior, for neonates treated with STEP-RNP-AS compared to no-treatment (NT) and scrambled gRNA.

(K-L) Rotarod test shows significantly improved motor coordination performance under both accelerating (K) and steady speed (L) conditions in AS mouse model groups treated with STEP-RNP-AS compared to untreated and scrambled gRNA groups.

(M) Marble burying test shows partially increased marble recovery number in AS mouse model groups treated with STEP-RNP-AS compared to untreated and scrambled groups.

(N) Novel object recognition test shows significantly increased object preference in short term memory in AS mouse model groups treated with STEP-RNP-AS compared to untreated and scrambled groups, suggestive of improved short-term memory in these groups. Specifically, AS mice treated on day 1 have complete rescue for the recognition on the novel object in the short memory test. STEP-RNP treatment also improved the performance for long term memory in all groups.

(O-Q) Fear conditioning test shows significantly improvement of contextual learning and cue learning of AS mice treated with STEP-RNP-AS compared to untreated and scrambled groups at both ages.

(R-S) Flurothyl (bis-either) induced seizure test shows significantly increased latency and hence reduced susceptibility for both myoclonic seizure and tonic seizure in AS mouse model groups treated with STEP-RNP-AS compared to non-treated mice. In the myoclonic seizure test, both adult and neonatal AS mouse groups treated with STEP-RNP-AS show the full rescue of latency compared to the wild-type level. (*p<0.05, **p<0.01, ***p<0.001)

221 Development of *in Vivo* Genome Editing for the Treatment of Progressive Familial Intrahepatic Cholestasis Type 2

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Progressive familial intrahepatic cholestasis type 2 (PFIC2) is a monogenic inborn error of metabolism caused by the deficiency of the bile salt export pump (BSEP), transporter of bile acids (BA) across the canalicular membrane of hepatocytes, encoded by *ABCB11* gene. PFIC2 patients display jaundice, itching, and cholestasis and usually develop liver fibrosis and end-stage liver disease before adulthood. Current treatments include diet control and pharmacological or surgical interventions to decrease BA blood concentration. Most patients undergo liver transplantation as the only curative option. Early therapeutic intervention would be fundamental in diseases like PFIC2, presenting with early onset and rapid disease progression, to limit hepatic damage. Since BA levels and other stimuli tightly regulate *ABCB11* expression, we are developing a liver-directed CRISPR-Cas9 based-genome editing approach to integrate the corrective cDNA into the first intron of the *ABCB11* gene. This strategy may allow the stable correction of most disease-causing mutations, even in pediatric patients, while maintaining physiological gene regulation. We initially assessed lentiviral vectors (LV)-mediated liver gene addition in *Abcb11-/-* mice, a

PFIC2 model, to correlate therapeutic effects with the extent of liver transduction. We administered 2-week-old *Abcb11-/-* mice with LV encoding the murine BSEP transgene under the control of a hepatocyte-specific cassette. We observed that transgene mRNA levels of about 30% of the endogenous murine BSEP resulted in a significant decrease, up to normalization, of serum BA, bilirubin, and alkaline phosphatase (ALP) until 6 months of age in both males and females, indicating prevention of cholestatic damage. Towards genome editing, we selected the most efficient *Abcb11*-targeting guide RNA (gRNA) in hepatocyte cell lines. Moreover, we compared different mCherry-expressing donor DNAs designed to exploit homology-directed repair (HDR) or homology-independent targeted integration (HITI) strategies for site-specific integration. *In vitro*, we achieved the highest integration rate using the HDR-based donor DNA (up to 12%). *In vivo*, we tested the different mCherry-donor DNA configurations delivered via adeno-associated viral vectors (AAV) to 2-day or 2-week-old Cas9 transgenic mice. In the liver of mice of both ages, we observed a slightly higher integration rate (up to 16%), mCherry expression, and mCherry-positive liver area (up to 16%) exploiting HDR rather than HITI strategy. To move towards a translatable approach, we tested in 2-week-old wild-type (WT) mice the delivery of AAV-carrying HDR-based donor DNA followed by the administration of lipid nanoparticles (LNP) carrying Cas9 mRNA and gRNA at the same time, 48 hours later or 1 week later. We found that the coadministration of AAV and LNP results in the highest integration rate (up to 18%) and mCherry-positive liver area (up to 13%). The resulting mCherry expression was comparable to the BSEP expression obtained with the therapeutic LV gene transfer. We are currently testing the selected editing strategy in 2-week-old *Abcb11-/-* mice and evaluating therapeutic efficacy by measuring blood BA, bilirubin, and ALP. Overall, this study informs about the optimal strategy and timeframe of in vivo liver-directed genome editing and represents a first step towards site-specific durable gene correction of PFIC2.

222 *In vivo* site-specific T cell engineering allows complete remission of human leukemia in mice

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T cells engineered to express chimeric antigen receptors (CAR-T cells) show great promise in treating hematological malignancies. However, the *ex vivo* culturing and engineering of autologous T cells is cumbersome, time-consuming, and expensive. Here, we develop methods for the ***in vivo*** engineering of CAR-T cells, obviating pre-conditioning and reducing timelines and expenses without risking GVHD or graft rejection. We use adeno-associated viral vectors (AAV) coding for the CAR as well as for a nuclease targeting the CAR into the TCR alpha constant (TRAC) locus. Uniquely, our design allows co-encapsulation in a single AAV of the CAR gene, flanked by homology arms, together with a cassette coding for the "ARCUS" nuclease, an engineered derivative of the I-CreI homing endonuclease targeting the TRAC locus. Furthermore, to efficiently and specifically target cytotoxic T cells in vivo, we incorporate, in the capsid of our AAV, a designed ankyrin repeat protein (DARPin) targeting the human CD8 receptor. Importantly, we demonstrate both *ex vivo* and *in vivo* engineering of primary human T cells with our single AAV in mice xenotransplanted with human leukemia. In particular, subsequent to IP injection, *in vivo* engineered CAR T cells with the expected $CD8^+$, $CD3^-$, CAR^+ phenotype expand in the leukemia-bearing mice, reaching levels of up to ~12% of blood $CD8^+$ T cells. When applying IV injections, complete remission of the human leukemia was achieved in the xeno-transplanted mice. Site-specific *in vivo* engineering of CAR T cells may revolutionize the safety, efficacy, and scalability of cellular immunotherapies for hematological malignancies and beyond.

223 Preclinical Development of a CRISPR-Cas9-Based Therapeutic for the Treatment of Herpes Keratitis

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In this study, we explored a CRISPR-SaCas9-based therapeutic as a potential treatment for herpes keratitis, one of the leading global causes of blindness. Herpes keratitis is caused by infection with Herpes simplex virus 1 (HSV-1), one of the most common human viruses affecting approximately 3.7 billion individuals under the age of 50 (67%) worldwide. Upon infection, HSV-1 replicates at the site of entry before traveling via sensory neurons to establish lifelong latency in the trigeminal ganglia (TG) and other ganglia reservoirs. Reactivation from latency, often triggered by stress or immunosuppression, results in recurrent infections. These recurrences can manifest in many forms such as herpes keratitis, labialis (cold sores), encephalitis, or genital herpes. Despite the availability of therapies that alleviate symptoms and reduce recurrence, there is no approved cure or therapeutic vaccine to eliminate latent HSV-1 virus.

We employed CRISPR-SaCas9 to simultaneously target two essential HSV-1 genes, ICP0 and ICP27, leveraging a "dual cut" strategy to induce large deletions in the HSV-1 genome and prevent viral escape. Off-target analysis using GUIDE-seq and hybrid capture confirmed no off-target editing of the selected guide RNA pair, demonstrating the high specificity of the gRNAs. For in vivo proof-of-concept studies, we used a latent rabbit keratitis model, which closely mimics recurrent HSV-1 infection in humans. To evaluate promoter efficacy, we compared a minCMV promoter and a neuron-specific CaMKIIα0.4 promoter for driving SaCas9 expression. Intravenous administration of all-in-one AAV9 vectors containing either promoter achieved promising results. The AAV9-minCMV vector eliminated viral shedding in 83-100% of treated rabbit eyes and reduced HSV-1 DNA in the TG by 64-81%. The AAV9-CaMKIIα0.4 vector eliminated viral shedding in 90% of treated rabbit eyes and reduced HSV-1 DNA in the TG by 51%. Residual HSV-1 DNA in the TG showed insertions and deletions (Indel mutations) at the targeted HSV-1 sites and large genomic deletions of the HSV-1 genome, indicating effective disruption of the latent HSV-1 reservoir. Both promoters demonstrated robust SaCas9 expression in the TG at six weeks post-AAV9 administration, with the neuron-specific CaMKIIα0.4 promoter offering tissue-specific expression.

In summary, this preclinical rabbit study highlights the potential of a CRISPR-Cas9-based therapeutic to eradicate latent HSV-1 infection in patients with herpes keratitis. This gene-editing approach could also be extended to treat other HSV-1-related diseases. Furthermore, identification of conserved and robust gRNAs for both HSV-1 and HSV-2 may enable the development of a single therapeutic product for the treatment of all herpes-related diseases.

224 Enhancing HDR-Mediated *Pah* Transgene Insertions in a Murine Model of Phenylketonuria

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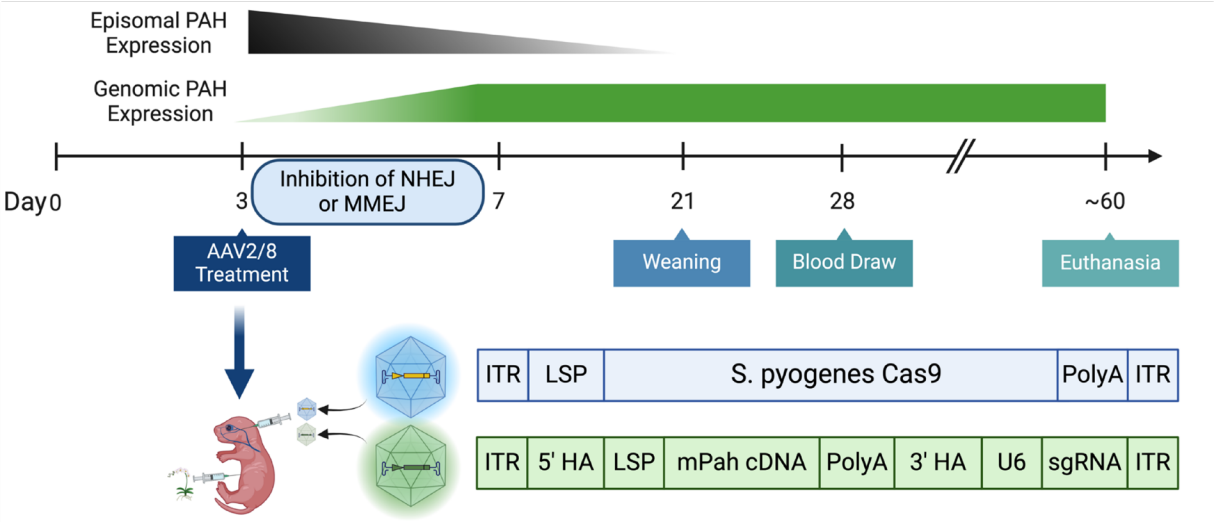
Introduction
A major challenge in resolving genetic diseases lies in the plethora of mutations that can each independently give rise to the same disorder. Over the past decade, significant progress has been made in developing gene correction and gene addition methods to treat these diseases. However, current gene addition techniques, which rely upon transient expression of viral episomes, offer only temporary relief in many diseases due to their limited stability in dividing cells. Alternatively, insertion a whole transgene using homology-directed repair (HDR) would provide permanent rescue of gene expression while being agnostic the specific genomic mutation. Previous work in our laboratory demonstrated significant reduction in serum phenylalanine concentrations in a murine model of phenylalanine hydroxylase (PAH) deficiency, a model of human phenylketonuria (PKU), following HDR-mediated gene correction. However, this response was only effective in neonatal mice that also received injections of vanillin,

an inhibitor of the non-homologous end joining pathway (NHEJ). We hypothesized that inhibition of DNA repair pathways that compete with homologous recombination (HR), such as NHEJ and microhomology-mediated end joining (MMEJ), would enhance the *in vivo* efficacy of large transgene insertions as well as gene corrections.

Results
PAH-deficient mice were treated with dual AAV2/8 vectors as neonates. One AAV vector expressed spCas9 and the other delivered a 2,379 bp *Pah* transgene repair template and expressed a guide RNA targeting exon 7 of the murine *Pah* gene (**Figure 1**). Simultaneous intraperitoneal administration of vanillin, an inhibitor of NHEJ, improved HDR insertion efficiency, reducing blood phenylalanine levels by 56.8%. Combining vanillin with novobiocin (NVB), an MMEJ inhibitor, in a treatment dubbed "Vanbiocin", further improved efficacy, with transgene insertions detected in 9.57% of hepatocyte alleles and blood Phenylalanine concentrations reduced on average by 70.6% to 631 µM. Vanbiocin treatment reversed hyperphenylalaninemia-associated hypopigmentation in affected mice and enabled dams to successfully rear their pups through weaning, overcoming the deficiencies of maternal PKU. Finally, oral administration of sapropterin dihydrochloride, a pharmacologic cofactor of PAH, reduced phenylalanine levels in Vanbiocin-treated mice to 396 µM, approaching 360 µM, the recommended treatment threshold for individuals with PKU.

Conclusion
Pharmacologic manipulation of DNA repair is a promising solution for enhancing HDR *in vivo*. Further refinement of this approach is required to provide a fully efficacious treatment for diseases such as PKU. However, the benefits of inserting a transgene into the genome would represent a generalizable treatment strategy that could be provided to a host of inherited errors of metabolism.



225 Editing Hematopoietic Stem and Progenitor Cells by Non-Viral Gene Knock-In Produces Anti-HIV Antibody *In Vivo*

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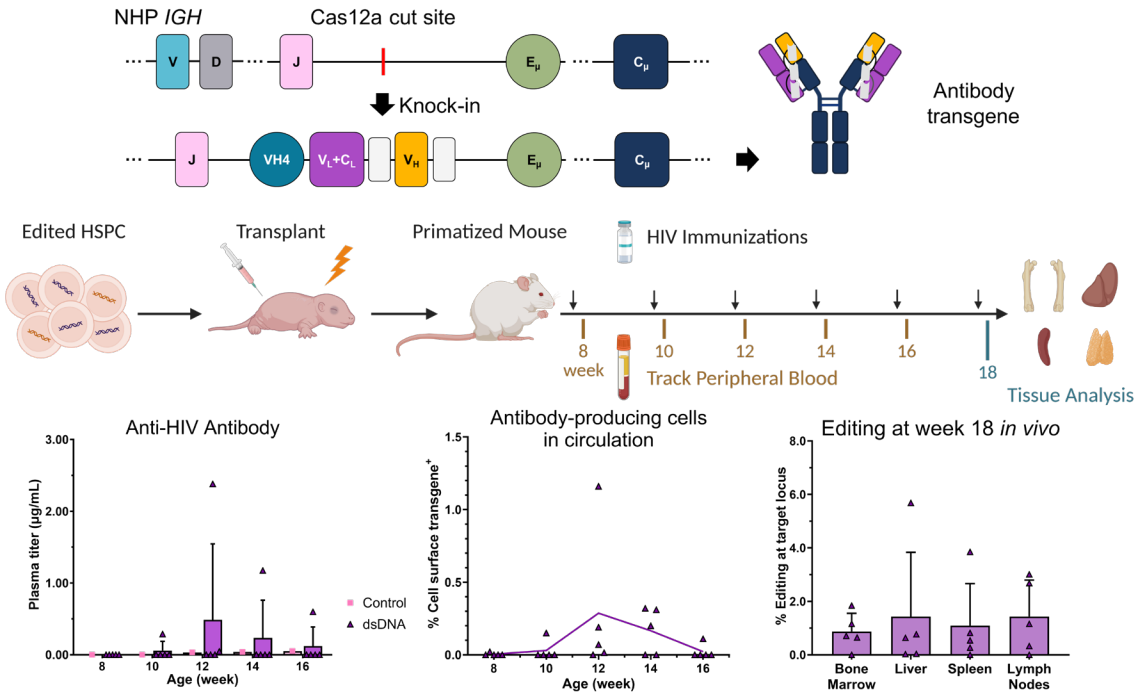
Non-viral gene editing offers a promising alternative to viral delivery for durable biologics production. Non-human primate (NHP) studies and human clinical trials have shown that adeno-associated virus encoding broadly neutralizing antibodies can protect against HIV, but result in limited, short-lived responses. The development of non-viral gene editing approaches in hematopoietic stem and progenitor cells (HSPC) holds promise for long-term antibody production. Here, we evaluate CRISPR/Cas9 and CRISPR/Cas12a for gene knock-in at the

immunoglobulin heavy chain locus in non-human primate HSPC as a preclinical model of HIV infection. Delivering the nuclease as a protein alongside a DNA template, we optimized editing with Cas12a demonstrating higher knock-in efficiency and fewer non-specific edits than Cas9 at this locus. We achieved up to 8.0% homology-directed repair events by electroporating Cas12a in the LCL 8664 NHP B cell line, with a larger fraction of productive insertions than Cas9 (85% vs 59%). We showed that delivery of large DNA templates (>2,250 base pairs) results in the durable expression of a reporter transgene from this locus in LCL 8664 cells. A double-stranded DNA template produced 2.6% transgene-positive cells, in comparison to 0.02% using a single-stranded oligodeoxynucleotide template. This approach was employed in primary CD34^{+} NHP HSPC, which were then transplanted into MISTRG mice. In this primatized mouse model, we observed engraftment, B cell differentiation, and reporter transgene expression, without any significant difference between electroporated and control conditions. Editing was detected *in vivo* at the target locus up to 16 weeks post-transplantation, with levels declining over time. To assess anti-HIV antibody expression, we developed a template encoding the broadly neutralizing antibody 10-1074 under the control a B cell-specific promoter, derived from a NHP variable heavy domain. After confirming recombinant antibody production *in vitro*, we transplanted MISTRG mice with NHP HSPC electroporated with Cas12a and this 10-1074 template. Beginning at 8 weeks of age, these mice were immunized with gp120 monomers. Anti-HIV antibodies were detected in peripheral blood circulation 4 weeks after immunization and declined thereafter. 20% of mice (n=1 of 5) developed titers of up to 2.4 µg/mL, similar to levels required to neutralize simian-HIV in NHP models. Surface expression of the antibody transgene was identified in all mice, although at low levels (≤1.2%). Mean editing frequency remained 1.5% in peripheral blood at week 16 post-transplantation; edited cells were also detected in the bone marrow, liver, spleen, and lymph nodes. NHP-CD19^{+} B cells were present in the lymph nodes and spleen, as well as NHP-CD34^{+} HSPC in the bone marrow. Our findings demonstrate that, following non-viral knock-in, HSPC remain capable of engraftment in an immunodeficient mouse model, where they can differentiate into immune cells producing biologically relevant levels of antibody from an endogenous locus. These data support proof of concept for this approach as a potential strategy for sustained biologics production in the treatment of chronic diseases such as HIV. Ongoing studies directly compare viral and non-viral editing using MISTRG6 mice, a strain with improved B cell differentiation and antibody production. Future work will assess efficacy in a NHP model of simian-HIV infection.



Manufacturing and Transduction for Viral Vectors

227 Suppressing APOBEC3 in Lentiviral Vector Producer Cells to Maximize Transgene Fidelity

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Introduction:
Within the fields of gene and cell therapy, HIV-derived lentiviral vectors (LV) are an established method of delivering therapeutic transgenes into target cells. Overall, LVs have a strong efficacy and safety profile, but one downside is the frequent incorporation of mutations into the transgene. Mutations may be synonymous, but typically protein function is disrupted and the efficacy of the therapy reduced. Oncogenic gain-of-function mutations are also possible for certain proteins. Transgene mutations have long been attributed to the relatively low fidelity of reverse transcriptase (RT), but in LV-treated ADA SCID patient samples analyzed by our lab, most observed mutations were C→T. That is characteristic of the antiviral protein family apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3), which evolved to defend against retroviruses by hypermutating their genome. Wild-type (WT) HIV encodes Vif to defend against APOBEC3s, but during the development of LVs, Vif was removed. Therefore, it was hypothesized that eliminating or inhibiting APOBEC3s within HEK293T (293T) LV-producer cells would reduce mutations in the integrated LV transgene.

Methods:
To test this hypothesis, CRISPR/Cas9 was utilized to create an APOBEC3A→G knockout (KO) 293T cell line. EFS-ADA (adenosine deaminase) LVs were packaged in the APOBEC3A→G KO line and separately in WT 293Ts transfected with a Vif expression plasmid. Control LVs were packaged in WT 293Ts. The resultant LVs were transduced into CD34s, and the integrated transgenes were library prepped, enriched, and duplex sequenced. The sequencing provided high coverage and accuracy, as dual unique molecular identifiers facilitated the exclusion of duplicate reads and errors incorporated during PCR or sequencing. Importantly, since APOBEC3 proteins preferentially target lagging strand ssDNA, the C→T mutations appear as G→A upon sequencing of the leading strand.

Results:
Sequencing identified 95 mutations (91 G—>A) in EFS-ADA transgenes packaged in WT 293Ts. That number was reduced to 9 total mutations (8 G—>A) in transgenes packaged in the APOBEC3A→G KO line, representing a 90.5% reduction in total and a 91.2% reduction in G—>A mutations. Separately expressing Vif in WT 293Ts led to a 50.5% reduction in both total and G—>A mutations. Additionally, 89%, 75%, and 84% of all G→A mutations were associated with an APOBEC3 substrate signature in transgenes packaged in WT, APOBEC3A→G KO, and Vif-expressing 293Ts, respectively.

Conclusions and Future Directions:
The data provides strong evidence to support the hypothesis that the APOBEC3 family is the predominant cause of mutations within the LV transgene, not RT. Future directions will concern identifying when/where the APOBEC3 proteins deaminate the transgene within the production and transduction pathways, via sequencing of 293T cDNA mid-packaging and the LV genome pre-transduction. Similarly, it will be determined whether a full knockout of APOBEC3 (via additional KO of A3H) in producer 293Ts will maximally eradicate C→T mutations, or if Vif needs to be incorporated in the LV capsid to protect against APOBEC3s produced in the target cell. Overall, this work provides evidence that simple modification(s) to the packaging process of LVs can dramatically increase their fidelity, and by extension, their efficacy and safety profile for all gene and cell therapy applications.

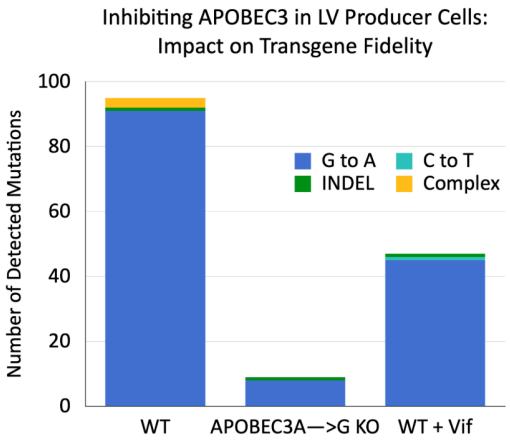

Figure 1: APOBEC3A→G KO or Inhibition Dramatically Reduces the Number of Mutations Within Integrated EFS-ADA Transgenes
EFS-ADA LVs were packaged under three different conditions: in WT 293Ts (WT), in APOBEC3A→G KO 293Ts, or in WT 293Ts expressing Vif (WT + Vif). The resultant LVs were transduced into CD34s and sequenced. The graph displays the number and type of all mutations identified in the EFS-ADA transgenes from each condition. APOBEC3s deaminate C→T, but the sequencing pipeline displays those mutations as G→A. INDEL = Insertion/Deletion.

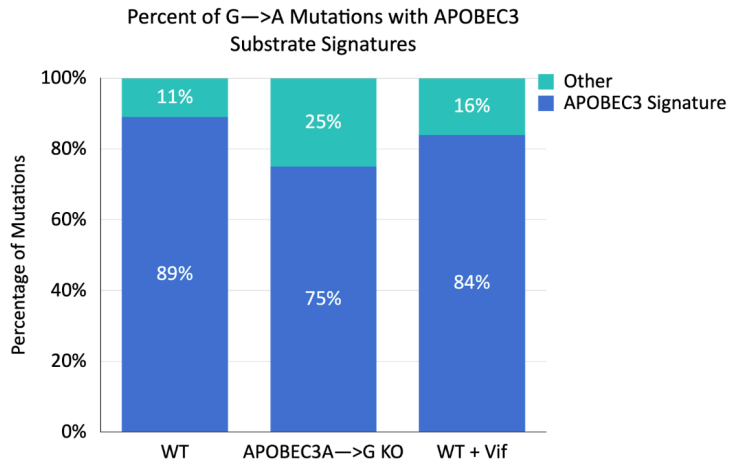

Figure 2: Majority of G→A Mutations Identified Within Integrated LV Transgenes are Associated with an APOBEC3 Substrate Signature
EFS-ADA LVs were packaged under three different conditions: in WT 293Ts (WT), in APOBEC3A→G KO 293Ts, or in WT 293Ts expressing Vif (WT + Vif). The resultant LVs were transduced into CD34s and sequenced. APOBEC3s deaminate C→T, but the sequencing pipeline displays those mutations as G→A. Thus, the graph displays the percentage of G→A mutations identified within each condition that are associated with an APOBEC3 substrate signature.

228 Engineering Lentiviruses for Enhanced Transduction of Primary Human T Cells

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In recent years, CAR T immunotherapies have proven curative for multiple cancers; however, high costs and manufacturing challenges have limited patient access to such treatments. To address these needs, Syenex is

developing enhanced viral gene delivery vectors to generate CAR T cells more efficiently, optimizing functional particle yield per production batch to significantly reduce per-patient dose costs. By screening libraries of envelope functionalization proteins and membrane display scaffolds, we developed SNX-T1, an additional plasmid component for lentiviral production systems that enhances transduction of activated primary human T cells by 10-fold over a standard VSV-G pseudotyped virus. SNX-T1 is compatible with a variety of lentiviral systems, including different producer cell types, different lentivirus generations, different fusogens, integration-deficient designs, and large transgene cargos. To further reduce manufacturing time and cost, we have expanded on this concept to create SNX-T2, a multi-plasmid mix that enables activation and transduction of resting T cells in mixed populations. This allows for the removal of external activation reagents and isolation steps in CAR T cell production, thereby shortening timelines for patient cell transduction. We have demonstrated efficient transduction of resting T cells in as little as one hour and greater than 99% T cell specificity in a PBMC population, an important step toward bedside CAR T engineering. Taken together, Syenex's suite of T cell engineering systems maximizes the output of viable therapeutic particles from each production run and/or shortens timeline processes, thereby lowering the overall cost per patient dose and improving accessibility of gene therapies. By screening libraries of designs, we have gained a deeper understanding of the rules of T cell-vesicle fusion, which are being applied to our in vivo delivery systems.

229 Purification and Chemical Shielding of Adenoviral Vectors for Gene Therapy, Vaccines, and Oncolytic Virotherapy

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In most *in vivo* applications, adenovirus (Ad) vectors are more potent than other viral or non-vector systems. However, Ads suffer from strong innate and adaptive immune responses that start by the binding of host proteins to the surfaces of their virions in a serotype-specific fashion. For example, Ad serotypes can be bound by natural antibodies or neutralizing antibodies that can trigger complement activation, innate immune responses, and their destruction by macrophages and Kupffer cells. This problem of host protein binding to Ad virions was made more apparently by observations of vaccine-induced thrombotic thrombocytopenia (VITT) in Ad COVID-19 vaccines. It has been proposed that different blood protein, platelet factor 4 (PF4), also binds Ad virions. This binding and the patterned display of PF4 on the virus surface is thought to induce antibodies against PF4 to induce VITT. One approach to reduce these bindings and to increase safety is the chemically "shield" the viruses with polymers like polyethylene glycol (PEG). PEGylation protects Ad from pre-existing and new neutralizing antibody and T cell responses. Relevant to VITT and other clotting side effects, we showed that 5 kDa linear NHS-PEGylation reduces binding to and activation of platelets and endothelial cells, blocked platelet loss, and reduced D-dimer levels after IV injections. While NHS-PEG is protective, it randomly covalently conjugates all of the lysines on the surface of Ad and reduces receptor binding and vaccine efficacy. To avoid this inactivation, we have inserted a single cysteine or 4 cysteines into the 7 the hexons of Ad to target PEGylation to one specific site on the virus using maleimide-PEG. This avoids inactivating the Ad's fiber receptor binding protein and maintained Ad potency. We will describe here the process for adenovirus vector purification and how NHS and maleimide PEGylation is applied during purification to better shield Ad for its various applications.

230 AnelloBricks: Development of a Scalable, Low-Cost, In-Vitro Assembled Anellovirus-Derived Platform for Gene Therapy Applications

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Anelloviruses, a family of small, non-enveloped, single-stranded DNA (ssDNA) viruses ubiquitously present in humans, represent a promising yet underexplored platform for gene therapy. Their unique biology, including their persistent yet asymptomatic and immune silent nature, positions them as ideal candidates for potentially safe and efficient gene delivery. Here we report the development of a cell-free, in-vitro assembled genetic medicines platform offering a modular approach with potential to treat a broad range of diseases across diverse therapeutic modalities.

This novel platform is composed principally of two components: recombinant capsid proteins and nucleic acid payloads (e.g. DNA, RNA), offering payload diversity while also simplifying production to conventional and readily scalable systems. In vitro assembly is initiated by the interaction of recombinant anelloviral capsid proteins with the nucleic acid payloads in a cell-free reaction system. The process is driven by the specific binding of the nucleic acids to the capsid proteins. Assembly proceeds through highly cooperative addition of payload bound to capsid proteins, guided by electrostatic and structural complementarity, to form a fully mature icosahedral Anellovector. This modular system allows for precise control over the stoichiometry and composition of the assembled vector, with the potential for reproducible manufacturing and efficient encapsidation of therapeutic nucleic acids.

We demonstrate successful packaging of ssDNA payload encoding GFP, efficient transduction in vitro, and in rodents. These findings highlight the potential of anellovirus-derived vectors as a novel gene therapy platform for a wide range of diseases, offering potential advantages in manufacturing scalability and cost compared to existing viral vector systems.

231 A Massively Parallel Reporter Assay for Evaluating Lentiviral Transfer Plasmid Design

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Lentiviral plasmids play a pivotal role in cell and gene therapies, but due to the intricacies of their genetic design, it is challenging to predict how different combinations of components influence their behavior. In principle, massively parallel reporter assays (MPRAs) can enable high-throughput evaluation of designs, however MPRAs have not yet been used, to our knowledge, to survey the impact of lentiviral transfer plasmid design on viral genome packaging and infectious titer. To address this, we developed an MPRA approach based on our commercially-available semi-stable lentivirus packaging system and used it to interrogate a library of nearly 5,000 lentiviral transfer plasmid designs. The assay evaluated the impact of design on lentiviral life cycle at two stages: RNA-seq was used to assess viral genome packaging and targeted genomic DNA-seq was used to assess infectious titer of a HEK293 cell line. We analyzed the resulting MPRA data with a novel two-stage hierarchical Bayesian regression model in order to simultaneously remove sequencing artifacts and quantify contributions from individual genetic parts. To do this, the model employed a zero-inflated negative binomial distribution to account for technical dropout and overdispersion. The resulting model encompasses both viral genome packaging and infectious titer while precisely identifying the impact of each plasmid design.

To validate our approach, we individually characterized 10 modeled designs that spanned a broad dynamic range of viral genome packaging and infectious titer. Viral genome packaging was assessed using RT-qPCR, and infectious titer was assessed using ddPCR. Spearman's rho between model activity estimates and observed MPRA activity for the single plasmid designs were 0.66 for viral genome packaging and 0.71 for infectious titer, supporting the validity of our screening approach and model. Across all designs, the hierarchical Bayesian regression model demonstrated high generalizability and highly accurate estimates for part contributions, capturing the vast majority of the variance (five-fold cross-validation mean Pearson's r of 0.96 and 0.94 between observed and posterior estimates of viral genome packaging and infectious titer, respectively, for a single representative gene of interest).

Our high-throughput evaluation of plasmid designs at multiple stages of the lentiviral life cycle provides new understanding of how specific design elements influence the overall system. For instance, we found that the commonly used woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and the SV40 origin of replication both had minimal impact on infectious titer. However, in the case of WPRE we discovered that the element actually decreased viral genome packaging while simultaneously enhancing viral delivery. As another example, our final model required no interaction terms. This indicates that the genetic parts we tested contributed independently to titer, and suggests a means for streamlined MPRA-based exploration of vast combinatorial design spaces. Overall, these results demonstrate that pooled assays can generate quantitative insights for lentiviral plasmid design and enable precise modeling of the impact of design on titer.

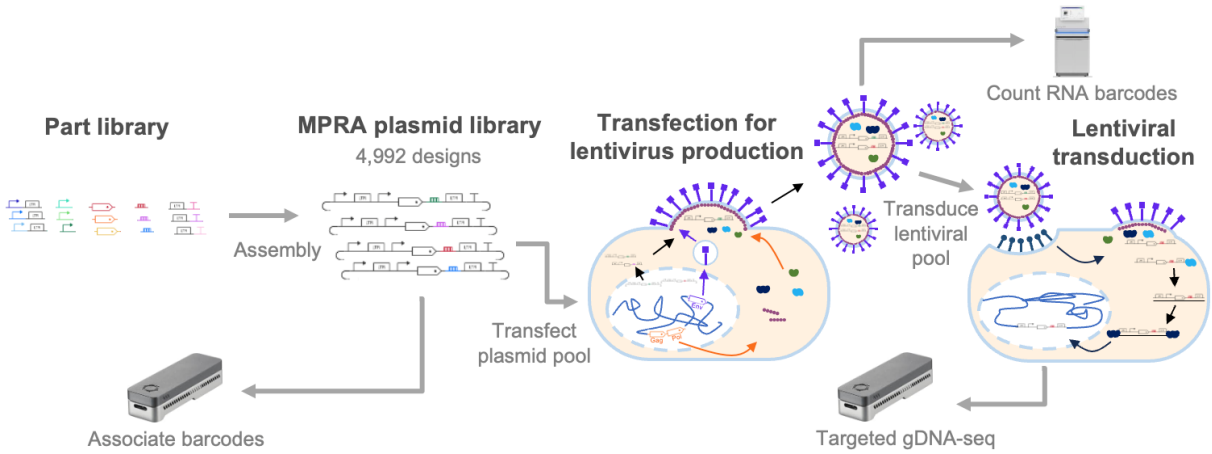

Figure 1: Overview of the lentiviral transfer plasmid MPRA workflow. A plasmid library containing 4,992 designs was introduced into a semi-stable packaging cell line to generate a lentiviral pool. This library was sequenced using RNA-seq to assess viral genome packaging and was also used to transduce a HEK293 cell line. Targeted gDNA-seq on the transduced cells was employed to assess infectious titer of the lentiviral pool.

232 Non-Integrative and Long-Lived Expression of Chimeric Antigen Receptor with Self- and Trans-Amplifying RNA

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Introduction:
Although CAR-T cell therapies have transformed the treatment of B cell lymphoma and multiple myeloma, *ex vivo* cell engineering is costly and time-consuming. *In vivo* gene therapies offer a resource-efficient alternative but can result in off-target genomic integration. Transient mRNA-based therapies mitigate this risk but may require frequent re-dosing due to the rapid turnover of mRNA. Recent studies show that synthetic circular RNA is a promising solution to attain long-term expression; however, translation of the transgene can be inefficient and thus require greater, though less frequent, dosing to achieve the same efficacy as that of linear mRNA.

This study aims to improve both transgene expression and RNA longevity by employing self- and trans-amplifying RNAs (saRNA and taRNA). The saRNA in this study replicates transgene RNA using the Venezuelan equine encephalitis virus (VEEV) RNA-dependent RNA polymerase, or replicase, which recognizes VEEV conserved secondary structures in the UTRs. The taRNA construct includes minimal 5' and 3' UTRs for RNA replication but lacks the ~8 kb VEEV replicase, leading to more efficient expression of transgene. As little as a single saRNA copy can propagate, leading to sustained CAR expression in both parent and daughter cells. saRNA is particularly well-suited to T and NK cell transfection, which exhibit low basal translational activity and would therefore benefit from exogenous amplification of transgene-coding RNA.

Methods:
An anti-human CD19 CAR construct was cloned into a canonical linear mRNA backbone with an α-globin 5′ UTR and AES-mtRNR1 3′ UTR. A bicistronic anti-hCD19 CAR-T2A-BFP cassette was incorporated into an saRNA backbone encoding the VEEV replicase. The taRNA construct was designed with an mNeonGreen reporter downstream of the anti-hCD19 CAR-T2A sequence and contains minimal VEEV-derived UTRs required for replication. CleanCap AU and a 100% 5-methylcytidine substitution were incorporated into the *in vitro*-transcribed RNA.

Functionality of ta- and saRNA were initially validated in HEK293T cells. Then, PBMC-derived human T cells were electroporated with linear mRNA, saRNA, taRNA, or saRNA + taRNA combinations encoding anti-hCD19 CAR.

Results:
In HEK293T cells, saRNA encoding anti-hCD19 CAR demonstrated improved protein expression, with 6.5% of cells expressing CAR 24 hours following transfection using wild-type replicase and 20% CAR-positive cells using hyper-replicase. Moreover, the combination of saRNA and taRNA maintained expression of CAR-positive cells and increased CAR levels 2-3-fold above that of taRNA alone, as measured by mean fluorescence intensity (Figure 1).

In primary human T cells, electroporation with combined saRNA and taRNA constructs demonstrated greater and more sustained expression of mNeonGreen reporter compared to that of conventional mRNA—expression was maintained for 96+ hours, as shown in Figure 2. In ongoing experiments, we are assaying the cytotoxicity of T and NK cells electroporated with CAR by saRNA-taRNA against CD19+ tumor cells.

Conclusion:
This study demonstrates the ability of saRNA and taRNA to sustain robust CAR expression in T cells. These findings highlight the potential for non-integrative, long-lived RNA-based therapies tailored to immune cells. Future efforts will focus on optimizing this platform for T and NK cells to enable *in vivo* delivery via lipid nanoparticles.

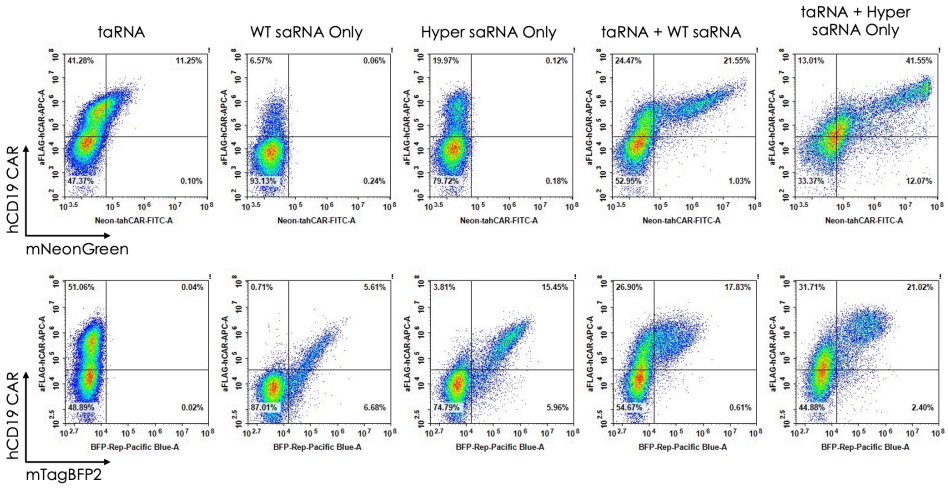

Figure 1. Flow cytometry 24 hours post-lipofection of HEK293T cells with taRNA alone, saRNA alone, or both ta- and saRNA. HEK293T cells were seeded at 30,000 cells/well in a 96-well plate one day before RNA transfection with Lipofectamine MessengerMax. The cells were transfected with 100 ng of taRNA, 100 ng of saRNA, or 100 ng of saRNA + 100 ng of taRNA. mNeonGreen and mTagBFP2 were the fluorescent reporters for taRNA and saRNA, respectively. The hyper-replicase demonstrated superior RNA replication to that of the wild-type replicase, resulting in higher levels of CAR and more cells expressing CAR in both the saRNA only and saRNA + taRNA conditions.

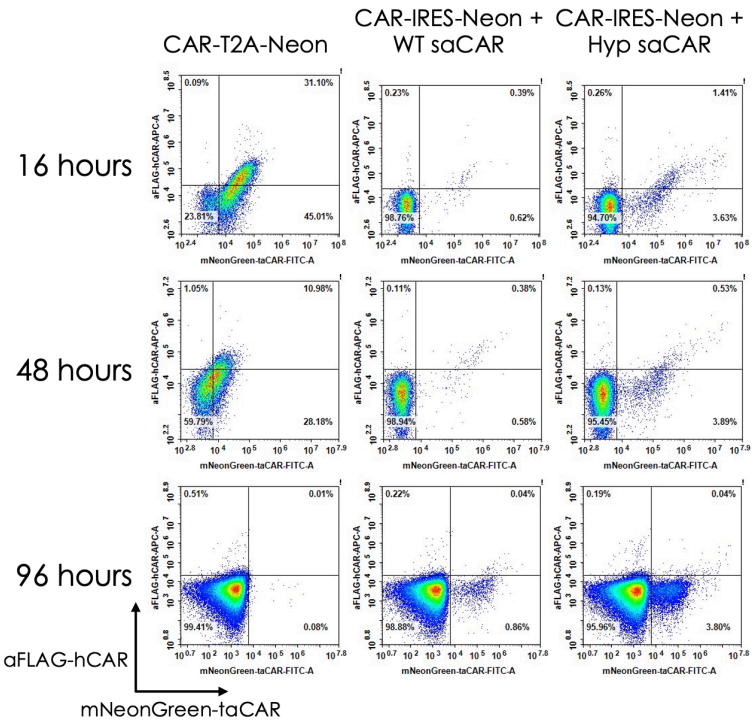

Figure 2. Flow cytometry analysis of RNA-electroporated primary human T cells. T cells, 7 days after removal of CD3/28 beads, were electroporated with 15 µg of linear mRNA encoding CAR-T2A-mNeonGreen, or with a combination of 5 µg taRNA (CAR-IRES-mNeonGreen) and 10 µg saRNA (WT or hyper saCAR). Results demonstrate that after 96 hours, the linear mRNA encoding CAR-T2A-mNeonGreen lost expression altogether, while both the conditions with sa- and taRNA maintained mNeonGreen expression. As T cells continued to expand at 48 and 96 hours, the mNeonGreen signal remained steady in cells transfected with the sa- and taRNA combination unlike that with cells transfected with linear CAR-T2A-Neon, indicating that RNA or protein was transferred to daughter cells and enabled longer-lasting expression.

AAV Vector Manufacturing: Experimental Design & Analytics

233 Quantification of Empty and Full Adeno-associated viral Capsids using Chip-Based Molecular Diagnostics

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*****These authors contributed equally

Introduction:
In the past decade, gene therapy has enabled a transformative shift in the treatment of genetic disorders. Of the options currently on the market, AAVs have gained popularity as the vehicle of choice for gene therapeutic delivery. The past decade has witnessed an accelerated approval pipeline of gene therapies by the FDA, as well as a rapid expansion of AAV therapy production. Despite these exciting developments, the biomanufacturing industry supporting process analytical technology (PAT) for AAV, specifically on differentiating full vs empty capsids, continues to lag. More importantly, ongoing research studies have revealed that administering a patient with an AAV dose that has partially filled or empty capsids has the risk of triggering immune responses and may compete for binding sites, resulting in reduced transduction.

Methods:
In this work, we developed a two-chip-based molecular diagnostic assay for at-line AAV quantification: the D4 (Dispense, Dissolve, Dissolve, and Detect) immunoassay for capsid titer and the CRISPR-AAV Evaluation (CRAAVE) assay for genome titer. The D4 assay utilizes fluorescently labeled detection antibodies (dAb) and capture antibodies (cAb) printed on a non-fouling polymer-coated glass slide. The dAb is printed onto soluble substrate so that it dissolves into the sample matrix upon sample addition and forms an immunocomplex at the cAb spots for fluorescent signal generation. The CRAAVE assay employs CRISPR-Cas12a for amplification-free genome detection. The CRAAVE assay utilizes the CRISPR/Cas12a system to recognize the AAV genome and subsequently cleave the F-Q reporters, where fluorescence from the cleaved reporters is correlated to genome titer. The assay composition is fully optimized in its lyophilized form on a miniature chip.

Results:
Through our two-chip assay, we achieved an empty-to-full capsid ratio within 30 minutes. Our D4 assay is multiplex friendly as the construction consists of multiple cAb zones specific for each serotype and AAX antibody fragment as our broadly nonspecific dAb. Additionally, the D4 assay has an impressive LOD of 6.5× 10^7 capsids/mL. The CRAAVE assay demonstrated high sensitivity, detecting AAV titers as low as 7 × 10^7 vg/mL with excellent precision (error <3%).

Conclusion:
We successfully developed a lab-on-a-chip solution that can provide the empty-to-full capsid ratio following a simplified, robust, and rapid protocol. Both the CRAAVE assay for genome titration and the D4 assay for capsid titer returned results comparable to ddPCR and ELISA but significantly reduced the user time and cost. The D4 assay and CRAAVE assay have been tested on AAV2, AAV5, and AAV6 serotypes and show great potential for adaption onto other AAV serotypes.

234 Leveraging Charge Detection Mass Spectrometry to Overcome Key Characterization Challenges in Recombinant Adeno-Associated Viruses

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Recombinant adeno-associated viruses (rAAVs) are widely known as promising therapeutic modalities to treat a wide variety of genetic diseases. As AAV-based gene therapy progresses, there is an increasing need for advanced analytical tools to better characterize these therapeutics and streamline their production. Utilizing rAAVs for gene therapy requires evaluating and characterizing their critical quality attributes, which can significantly impact the safety and efficacy of the final product. Charge detection mass spectrometry (CDMS), using an electrostatic linear ion trap (ELIT), is a cutting-edge technique for ultra-high mass analysis. It simultaneously measures the mass-to-charge ratio (m/z) and charge (z) of individual ions, enabling calculation of their mass. CDMS is highly effective in characterizing rAAVs and their product-related impurities, which often present significant challenges for current methodologies and conventional mass spectrometry.

AAV empty and full capsid standards of various serotypes (AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9) were buffer exchanged into 200mM ammonium acetate solution with 0.01 % Pluronic F-68 (Gibco) using Micro Bio-Spin columns (Bio-Rad). Ions were generated in positive ion mode using nanoelectrospray ionisation and mass analysis was performed using a prototype CDMS instrument. Signal processing and data visualization were performed using software developed in-house. Ions were trapped for 100 ms, and total acquisitions times were between 5 and 20 minutes. Detected time-domain signals were Fourier transformed; the measured frequency and the magnitude correspond to an individual ion's m/z and z respectively, enabling direct calculation of mass values. Data for individual ions were histogrammed to generate m/z, charge and mass spectra as well as 2-dimensional heat-maps.

An AAV serotype panel analysis was conducted, and the measured average masses were in good agreement with the theoretically expected mass values of the empty (3.8 MDa) and full capsids (4.5 – 5.5 MDa). This investigation also revealed distinct differences in the charge distributions of the various AAV serotypes, possibly due to the differences in viral protein content within the capsid structure. Additionally, for the full AAV capsid samples, empty particles were detected in AAV1, 2, 5, 8, and 9 with partial/intermediate particles detected in AAV1, 5, 6, and 9. These data highlight the ability of CDMS to readily distinguish between empty, full, and the partially full/intermediate capsids. A higher percentage of empty particles, around 12%, were detected in the full capsid samples of AAV5 and AAV8, which were manufactured in 2016. In contrast, the empty particle percentage in AAV1 and AAV9 samples, manufactured in 2020, was half of that or lower. For one of the samples, upon longer term storage at 4 °C, aggregates were detected at double the mass of the empty capsid (~ 8 MDa). This indicates that storage conditions of AAVs significantly impact their stability, which can ultimately affect the potency of the therapeutic product. The data presented here demonstrates that CDMS can effectively support the extended characterization of capsid population profiles, including capsid content determination based on AAV peak area ratios in the mass histogram. This advanced technique ensures accurate and comprehensive analysis, enhancing the overall quality control of gene therapy products.

235 Production of adeno-associated viral vectors by a novel human derived cell line HAT and comprehensive characterization of purified vectors

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[Introduction]
Currently, the production of AAV vectors is mainly carried out using the transfection method with HEK293 cells or the baculovirus expression system. One of the major challenges in production is to obtain the AAV drug substance with a high full particle percentage, and there are two approaches to achieving this. One is to improve the full particle percentage during upstream process, and the other is to remove particles other than full particles by improving the purification method in downstream process. Meanwhile, how differences in manufacturing methods affect quality attributes are unclear, and the relationship between quality characteristics and efficacy and safety is still not fully understood. In this presentation, we will report on the manufacturability of AAV vectors using the novel human derived suspension cell line HAT and the quality characteristics of the manufactured AAV vectors.

[Methods]
In this study, we tested productivity by HAT cells of AAV vectors with serotypes, AAV2, AAV5, AAV6, AAV8 and AAV9, with GFP as the GOI. After producing AAV vectors using HAT cells with the triple transfection method, the titer and full-to-empty particle ratio (F/E ratio, F/(E+F)) was measured after the cell lysis followed by affinity chromatography, and then AAV vectors were prepared with more than 90% EF ratio through our small-scale purification platform. AAV vectors were also produced using the triple transfection method with HEK293 cells, AAV vectors with more than 90% EF ratio were prepared. Twelve quality attributes of each AAV vector produced

using the two cell lines were comprehensively determined and compared. We further studied AAV2, AAV5, and AAV9, with GFP as the GOI for detailed analysis. The production efficiency of AAV using HAT cells in flask was compared with those using bioreactor.

[Results and Discussion]
For all five serotypes, HAT cells produced AAV vectors with higher F/E ratio than those of HEK293 cells. Surprisingly, F/E ratio of AAV9 vector in cell lysis followed by affinity purification was more than 70% in the case of HAT cells, demonstrating high potential of HAT cells for the production of AAV vectors. Highly purified AAV vectors produced in HAT cells and HEK293 cells showed equivalence in all quality attributes except VP ratio. Bioreactor-based culture showed similar manufacturability to flask culture.

[Conclusion]
Based on these results, HAT is a novel human suspension cell line suitable for the production of AAV vectors with quality attributes equivalent to those from HEK cells, and now that we have confirmed its manufacturability in bioreactor, we plan to verify its usefulness for clinical trials and commercial production as well as for actual use.

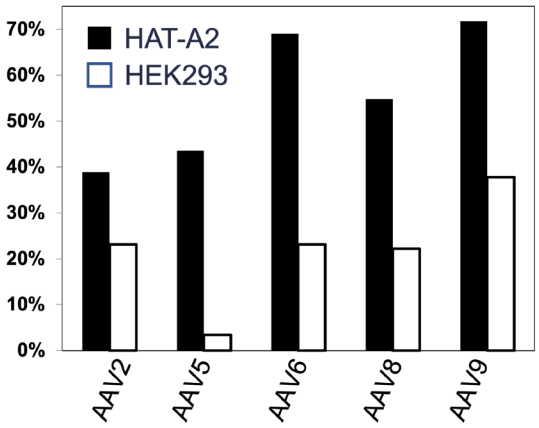

Figure 1. Full-to-Empty ratio (F/(F+E)) after cell lysis followed by affinity chromatography

236 Building efficient AAV testing platform: Prioritizing critical quality attributes with risk ranking-based scoring

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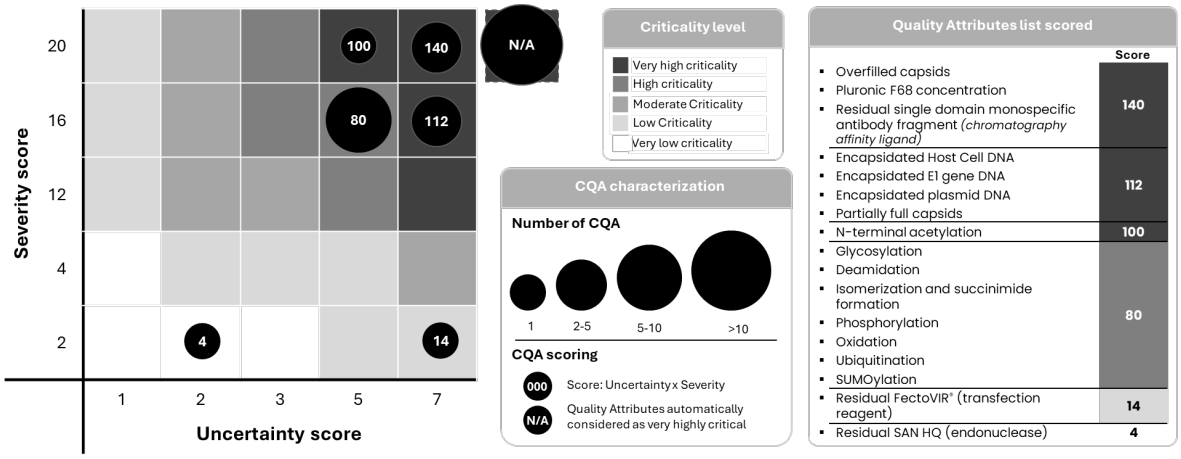
Developing adeno-associated virus (AAV) gene therapies requires a robust and scalable manufacturing process ensuring consistent quality, safety, and efficacy. A critical component is the establishment of a Chemistry, Manufacturing, and Controls (CMC) strategy that addresses the complexities of viral vector production. Using Quality by Design (QbD) methodology support early identification, and control of critical process parameters and quality attributes (CQAs) throughout the product lifecycle. By proactively integrating QbD principles, manufacturers can mitigate risks and meet regulatory requirements supporting the successful development, approval, and commercialization of AAV-based gene therapies.

We adopted three different methodologies based on data availability, CQA's nature and complexity. The first methodology, risk ranking and filtering, applies when access to clinical data is limited. The second one, preliminary hazard analysis, is used when (pre-)clinical data are available. The third assess process-related impurities based on the calculation of an impurity safety factor.

For the AAV ExoREADY™ platform at current developmental phase, the first tool was used to assess the criticality of the quality attributes. The criticality score is determined based on severity (efficacy, PK/PD, immunogenicity, safety) and uncertainty scores, leading to the ranking of 32 highly critical CQAs (see Figure 1). Based on compendial and regulatory expectations, 24 CQAs were identified as obligatory CQAs. Critical quality attributes for AAV gene therapy include capsid and genome identity, integrity, and purity, ensuring the correct structure and function of the viral vector. Additional factors such as empty capsids, vector aggregates, residual host cell components (proteins, DNA, E1 gene, plasmids), titers (capsid, vector genome, infectious particles), genetic insert expression, biological activity, and absence of contaminants (adventitious viruses, mycoplasma) are essential for safety and efficacy.

This methodology helped prioritize analytical development and confirm the manufacturing process's ability to produce high-quality AAV. A robust control strategy ensures investigational drugs are safe and effective for clinical applications. Additionally, the ExoREADY™ platform provides an optimized, pre-developed framework for efficient, cost-effective gene therapy production. With at least 134 AAV products in clinical trials in 2022 and 9 already FDA/EMA-approved as of 2022, staying aligned with evolving scientific understanding of AAV is crucial. Continuous CQA scoring guiding review, analytical portfolio expansion, and process improvements will ensure the platform adapts to new insights, clinical data, and industry advancements.



237 Enhanced AAV Capture Using Membrane Chromatography and Optimized Mid-Stream Processing

Anand Alembath[1], Andre Kiesewetter[2], Shivam Patel[1], Thibaut Deschamps[2], Yuanchun Zeng[3], Joseph Hersey[1], Jonathan Steen[1], Oliver Rammo[2]
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Enhanced AAV Capture Using Membrane Chromatography and Optimized Mid-Stream Processing
Membrane chromatography offers an innovative solution for the capture of adeno-associated viruses (AAV), addressing bottlenecks associated with traditional resin-based technologies, offering significantly improved productivity and reduced processing time by increased throughput and high dynamic binding capacity.

This work demonstrates the advantages of using strong cation exchange Natrix® CH membrane chromatography in an AAV2 and AAV9 non-affinity capture process following an optimized mid-stream approach for simple and robust processing. The process is shown to be a viable, cost-effective, and flexible alternative that meets the need for a serotype-independent capture solutions. By optimizing critical mid-stream steps, this new process enables fast processing at up to 8 MV/min while maintaining high dynamic binding capacities (>1E14 vp/mL) and low pressures during the capture phase. Importantly, these strategies ensure that both genome titer and AAV infectivity are retained throughout the purification process.

238 Stable maintenance of full-length ITRs in AAV transfer plasmids

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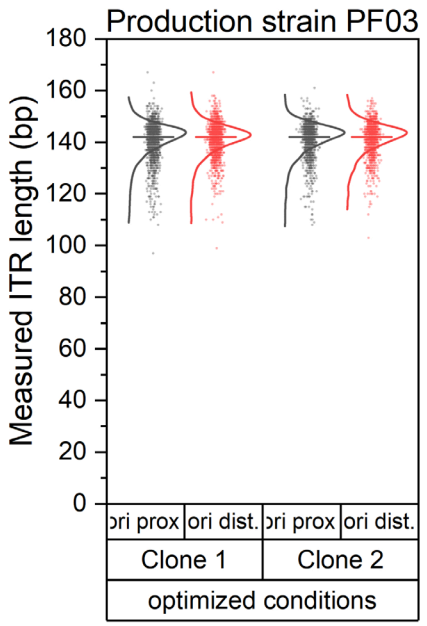
The inverted terminal repeats (ITRs) of adeno-associated virus (AAV) are a critical feature for the rescue of the AAV genome from the transfer plasmid containing the transgene and for efficient rAAV genome packaging into capsids. Full-length ITRs are effective in producing full capsids and homogeneous payloads. However, these largely palindromic sequences are prone to secondary structure formation and often tend to acquire deletions during bacterial propagation of transfer plasmids. Such plasmid populations containing truncated ITRs seriously undermine plasmid homogeneity and cause shortcomings in AAV yield and genomic purity.

In this study, we show that, by turning to optimized fermentation conditions such as cultivation temperature and by choosing the right bacterial strain containing mutations in genes responsible for genome organization, *E. coli* was denied the conditions that would potentially lead to ITR sequence deletions. These experiments were complemented by a robust analytical tool to measure ITR lengths in a plasmid population using next generation sequencing (NGS). Additionally, ITR length variants were characterized using capillary gel electrophoresis, which was found to be a complementary, rapid in-process control method. Furthermore, we replicated these results in a bioreactor cultivation and produced an AAV transfer plasmid containing full-length wild type ITRs. Taking this development a step further, we recently produced the first AAV transfer minicircle containing full-length ITRs and devoid of bacterial backbone sequences. The minicircle resulted in high yields of vectors in initial transfection experiments in HEK-293 cells.

We thus demonstrate our ability to obtain ITR sequences in their full length (wild type length), maintain them in that state during plasmid production and effectively track them by a robust analytical method. These findings open new possibilities for AAV manufacturers to increase their vector yield and quality.



239 Shedding Light on AAV Manufacturing: Application of Raman Spectroscopy for Real-Time Process Monitoring

Olaide Ibiyemi[1], Jennie Gullick[2], Samantha Higgins[2], Stefanie Frank[3], Stephen Goldrick[3]
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Introduction:
The success of adeno-associated virus (AAV) gene therapies has driven the demand for scalable, reliable, and reproducible manufacturing processes to meet clinical needs. However, AAV production by transient transfection is complex and presents significant challenges. The efficacy and consistency of the process is influenced by multivariate interactions between raw materials, process conditions and transfection parameters. Achieving reproducible processes with high product quality requires precise control of the dynamic biological system. Real-time monitoring of process variables can help improve process understanding to facilitate informed decisions for improved yields and batch-to-batch consistency.

Traditional analytical methods rely on offline sampling, which is time-consuming and lacks the ability for real-time measurements. Process Analytical Technologies (PAT) address this limitation by providing in-situ measurements for enhanced monitoring and control. Raman spectroscopy is a non-invasive analytical technique that can be used for this. It is based on the inelastic scattering of monochromatic light and enables measurements of a sample's composition from a single spectral measurement. Here, we apply Raman spectroscopy to the monitoring of key parameters including viable cell density (VCD), glucose, lactate and ammonia concentrations during AAV production.

Method:
Raman spectroscopy was applied to a 3L transiently transfected AAV2 production process in HEK293F cells (Figure 1). Spectra were recorded every 12.5 minutes using a 785 nm Raman analyser (Endress+Hauser), while offline samples were collected twice daily to measure VCD using the ViCell Blu (Beckman Coulter) and to determine glucose, lactate, and ammonia concentrations using the BioProfile FLEX (Nova Biomedical). Due to the large and highly dimensional datasets generated, multivariate data analytics tools are required for data interpretation. Partial Least Squares (PLS) regression was used to develop models correlating Raman spectra with offline measurements. The predictive performance of these models was evaluated using the coefficient of determination (R^2) which quantifies how closely the model predictions match the measured values. An R^2 value closer to 1 indicates strong predictive performance and a good fit between predicted and measured data.

Results and Conclusions:
The PLS models demonstrate strong predictive performance with R^2 values exceeding 0.7 on unseen data. The VCD model achieved an R^2 of 0.77 for cell densities ranging from 0.3 to $2.5x10^6$ cells/ml. As transfection is often based on VCD, this model could be used to optimize viral titre and productivity. Glucose and lactate models showed high accuracy, both with R^2 values of 0.99, strongly aligning with the offline data. The ammonia model achieved an R^2 of 0.93, demonstrating reliable predictions.

These results highlight the potential of Raman spectroscopy to enhance process understanding and control in AAV manufacturing while reducing the reliance on offline sampling. By providing real-time insights into culture dynamics, it can enable more effective monitoring, improved process control, and support the development of optimized feeding strategies. This approach can contribute to greater consistency and improved product quality in AAV production.

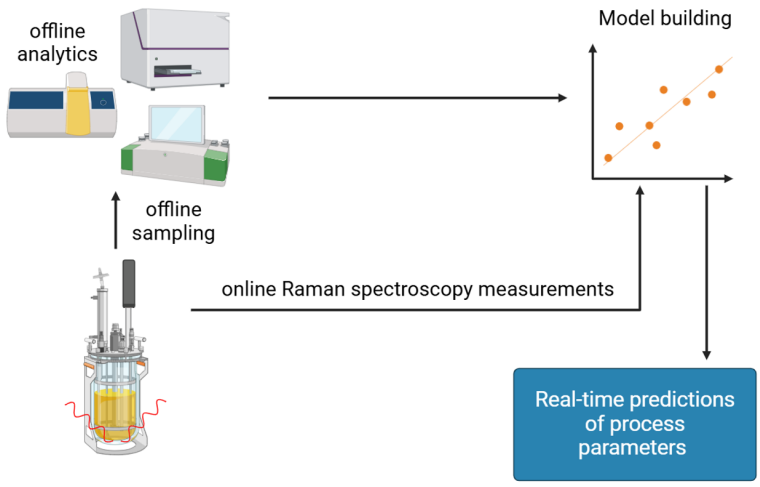

Figure 1. Methodology for online Raman spectroscopy analysis of process variables in AAV2 production in 3L bioreactors.

Vaccines and Immunotherapy for Cancer

240 Programmable Adeno-Associated Virally Delivered Lifelong In Vivo T Cell Engagement Provides a Novel Framework for Prevention and Treatment of Metastatic Cancer

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Cancer remains the most common cause of death due to disease in children and the second leading cause of death due to disease in adults, behind heart disease. Deaths in patients with solid tumors mostly result from the development of metastases, as exemplified by neuroblastoma in children and breast and gastrointestinal cancers in adults. Metastases are thought to arise from circulating tumor cells, in what could be thought of as the “leukemic compartment” of solid tumors. Furthermore, metastatic relapses that often occur years after treatment is finished result from reactivation of quiescent, disseminated cancer cells that escape prior therapies. Preventing the initial dissemination and the subsequent reawakening of these cells will thus require a treatment that is systemically and consistently active over years. We reasoned that circulating tumor cells, or those early in the metastatic process or in isolated deposits, exist outside of their immunosuppressive microenvironment and may thus be vulnerable to immunotherapy. We propose that long-term, persistent cytotoxic pressure directed at validated tumor associated antigens can be achieved using gene therapy and be effective in preventing the development of metastases. To test our hypothesis, we created two adeno-associated virus (AAV) constructs expressing a secreted bispecific T cell engager (termed "TransJoin") targeted to either the disialogangioside GD2, highly upregulated on neuroblastoma, or HER2, a well-known cancer driver upregulated on breast, gastric and other cancers. Using the purified bispecific proteins, we found dose-dependent cytotoxicity of cancer cell lines cocultured with human peripheral blood mononuclear cells (hPBMCs) that was proportional to cell surface target density, with no detectable killing in cells with low cell surface target density. In immunodeficient (NSGS) and immunocompetent (Balb/c and C57BL6) mice, we achieved consistent steady-state serum levels of the transgene product for >1 year following a single AAV administration, compared with the 2-hour half-life of the protein. To evaluate anti-metastasis efficacy, we tested

these vectors in models of luciferase-tagged GD2+ neuroblastoma or HER2+ breast and gastric cancers, enabling us to measure and track metastases by bioluminescent imaging over time. Following administration of a bolus of intravenous human cancer cells into immunodeficient mice peripherally humanized with intravenous blood mononuclear cells from normal human donors, systemic injection of the relevant AAV construct prevented >99% metastases in each model compared with animals given a control virus. The effect was seen even if we waited a week to allow establishment of metastases. Utilization of a murine host doubly knocked out for HLA class I and II (DKO) reduced the development of graft-vs-host disease seen in NSGS mice and enabled us to observe protection against metastasis >140 days after tumor cell injection. We are currently testing the HER2 construct in formal toxicology and IND-enabling antitumor efficacy studies. Overall, our data suggest that expression of protein-based immunotherapeutics via an AAV platform represents a novel adjuvant therapy for the treatment and prevention of cancer metastasis and recurrence.

241 Employing Pre-Existing Immunity Against Pathogens for Enhanced Cancer Immunotherapy via mRNA Vaccines

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Introduction
We explored a novel cancer immunotherapy strategy that suppresses tumor growth and extends the lifespan of tumor-bearing mice. This strategy introduces pathogen antigens into tumors via mRNA-lipid nanoparticles, leveraging pre-existing immunity to enhance therapeutic efficacy.

Objectives
Our goal was to assess whether mRNA-based vaccines could tag tumor cells with pathogen antigens and mobilize pre-existing immune responses to eliminate cancer cells. Specifically, we tested if prior immunization with the BNT162b2 COVID-19 vaccine would enhance the anti-tumor response against tumors tagged with the same protein.

Methods
The study used multiple tumor models in mice. Mice previously immunized with the BNT162b2 encoding the SARS-CoV-2 spike protein received intratumoral injections of the same vaccine. We measured tumor growth suppression, lifespan extension, mobilization of memory immunity, reprogramming of the tumor microenvironment, and tumor antigen-specific T-cell responses. Statistical analyses determined the significance of the results.

Results
Intratumoral injections of BNT162b2 in previously immunized mice tagged tumor cells with the mRNA-expressed spike protein. This rapidly mobilized pre-existing memory immunity against SARS-CoV-2, leading to the elimination of cancer cells displaying the spike protein and reprogramming of the TME by attracting immune cells. Partial elimination of tumor cells in a normalized TME triggered extensive tumor antigen-specific T-cell responses through antigen spreading, resulting in potent systemic tumor-targeting immune responses. Combining BNT162b2 treatment with anti-PD-L1 therapy yielded a substantial therapeutic impact, even in "cold tumors" typically less responsive to treatment.

Conclusion
Leveraging pre-existing immunity against pathogen antigens via mRNA-based vaccines, such as BNT162b2, significantly enhances cancer immunotherapy efficacy. Given widespread memory immunity against various pathogens from prior infections or vaccinations, this approach could rapidly transition into clinical use. mRNA vaccines against other pathogens, such as HBV, Common Human Coronaviruses, and influenza virus, hold great potential for treating different cancers and overcoming drug resistance, providing a promising alternative for cancer patients.

242 mRNA-LNP Vaccine Providing Antigen and Co-Stimulation in the Tumor Microenvironment (CART-Vac) Enhances CAR-T Cell Function

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Introduction
The immunosuppressive tumor microenvironment (TME) in solid tumors induces T cell exhaustion, compromising the expansion and functionality of CAR-T cells. Preserving the stemness of CAR-T cells within the TME is crucial for sustaining long-term efficacy. Previously, we established a CAR-T cell manufacturing process that preserves stemness by co-culturing CAR-T cells with antigen-presenting PBMCs overexpressing target antigens (TA) and co-stimulatory molecules (CSMs). This approach highlighted the importance of physiological T cell stimulation through antigen presentation and co-stimulation in maintaining CAR-T cell memory function. We hypothesized that inducing TA and CSM overexpression within the TME could similarly enhance CAR-T cell expansion and improve anti-tumor activity in an immunosuppressive environment. To validate this, we examined CAR-T cell functionality in the context of genetically modified sarcoma cells engineered to overexpress TA and CSMs. Furthermore, we assessed the synergistic effects of intratumoral administration of an mRNA lipid nanoparticle (LNP) vaccine encoding TA and CSMs (CART-Vac) to investigate its potential in boosting CAR-T cell efficacy against solid tumors.

Methods
To generate genetically modified tumor cells, the EPHB4-positive sarcoma cell line Rh30 was transduced with truncated EPHB4, CD80, and CD137L, creating Rh30-TACS cells. EPHB4 CAR-T cells were developed using *piggyBac* transposon-mediated gene modification, as previously described. CART-Vac was produced by synthesizing mRNA encoding truncated EPHB4, CD80, and CD137L, which was then encapsulated in LNPs with specific tropism for Rh30 cells. We evaluated the *in vitro* and *in vivo* anti-tumor efficacy of these CAR-T cells, focusing on their expansion and activity within the TME against Rh30-TACS cells. Additionally, we assessed the synergistic anti-tumor effects of combining CART-Vac with EPHB4 CAR-T cell therapy via intratumoral administration.

Results
Flow cytometry confirmed that Rh30 cells expressed EPHB4 but lacked CD80 and CD137L, whereas Rh30-TACS cells overexpressed all three. EPHB4 CAR-T cells exhibited enhanced cytotoxicity, robust proliferation, and increased secretion of pro-inflammatory cytokines against Rh30-TACS compared to Rh30 cells *in vitro*. *In vivo*, EPHB4 CAR-T cells achieved superior tumor control in Rh30-TACS-bearing mice, with increased T cell proliferation and infiltration into tumor tissues, compared to Rh30-bearing mice. CART-Vac successfully transfected Rh30 cells, inducing overexpression of EPHB4, CD80, and CD137L, with peak expression observed 24 hours post-treatment. Pretreatment of Rh30 cells with CART-Vac enhanced the in vitro anti-tumor functionality of EPHB4 CAR-T cells, promoting robust CAR-T cell expansion. In Rh30-bearing mice, CART-Vac pretreatment efficiently induced tumor-localized expression of EPHB4, CD80, and CD137L. Furthermore, the combination of intratumoral CART-Vac and systemic EPHB4 CAR-T cell therapy significantly enhanced tumor growth suppression and CAR-T cell expansion *in vivo*.

Conclusion
LNP-based CART-Vac effectively induces tumor-localized expression of TA and CSMs, significantly enhancing CAR-T cell efficacy. This combinatorial strategy holds promise for overcoming TME-associated challenges and improving CAR-T therapy outcomes in solid tumors.

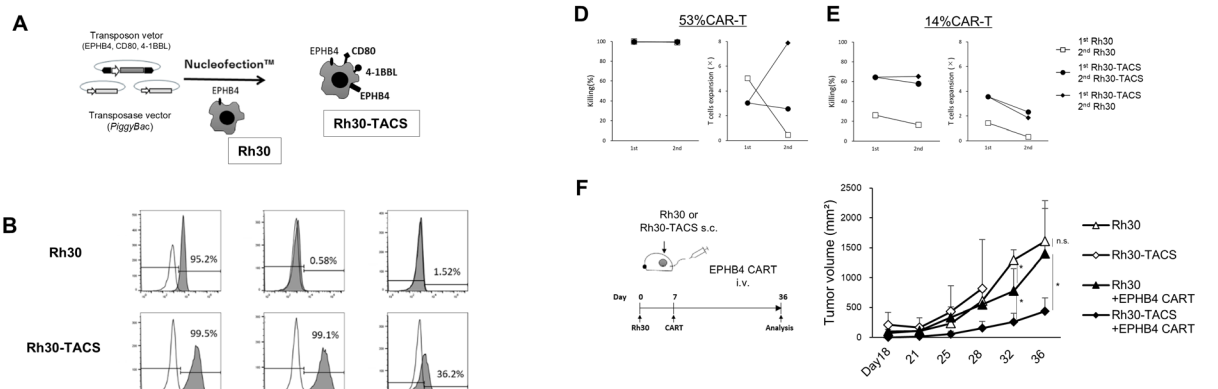

Figure 1. Overexpression of TAA and co-stimulatory molecules enhanced the efficacy of EPHB4 CARTs

(A) Schema of genetically modified Rh30 cells with stably expressing TA and CSMs (Rh30-TACS).
(B) EPHB4, CD80, and 4-1BBL expression in Rh30 or Rh30-TACS cells by flowcytometry.
(C) EPHB4, CD80, and 4-1BBL expressions in Rh30 or Rh30-TACS tumors by immunohistochemistry.
(D) Overexpression of TA and CSMs significantly enhanced the anti-tumor effects of EPHB4 CART cells *in vitro*. EPHB4 CAR-T cells with CAR expression of 53% were used.
(E) Similar serial co-culture experiment results as (D) but using CARTs with CAR expression of 14% were used.
(F) Efficacy of combinatorial treatment of CART-Vac and EPHB4 CART cells *in vivo*.

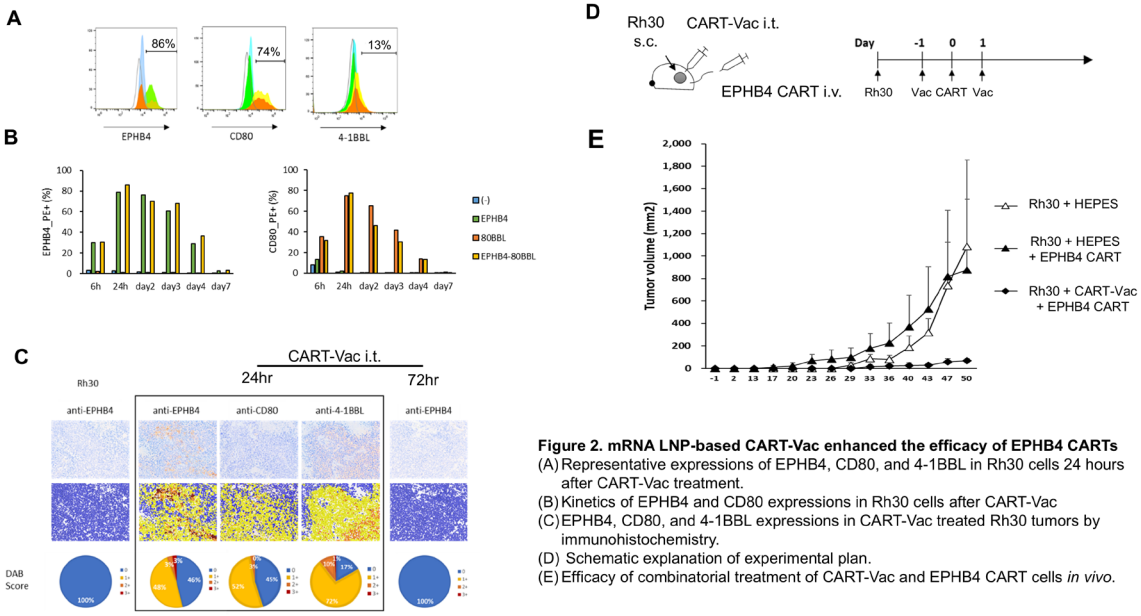

Figure 2. mRNA LNP-based CART-Vac enhanced the efficacy of EPHB4 CARTs

(A) Representative expressions of EPHB4, CD80, and 4-1BBL in Rh30 cells 24 hours after CART-Vac treatment.
(B) Kinetics of EPHB4 and CD80 expressions in Rh30 cells after CART-Vac
(C) EPHB4, CD80, and 4-1BBL expressions in CART-Vac treated Rh30 tumors by immunohistochemistry.
(D) Schematic explanation of experimental plan.
(E) Efficacy of combinatorial treatment of CART-Vac and EPHB4 CART cells *in vivo*.

243 Cancer Immunotherapy Using AIRE Conditioning of the Tumour Epitopeome

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T cell immune tolerance is established in part through the activity of the Auto-immune Regulator (AIRE) transcription factor in the medullary Thymic Epithelial Cells (mTEC) of the thymus by expressing a broad profile of SELF peripheral tissue-specific antigens for presentation to naïve T cells to promote activation/deletion of potentially autoreactive T cells. Here, we hypothesized that AIRE expression in tumors may mimic its role in mTEC - but with the opposite purpose of setting a steady-state profile of SELF epitopes against which no functional T cell reactivities exist thus cloaking the tumor from T cell attack. We show that changes in AIRE expression in tumor cells altered their profile of SELFNESS by changing the balance of AIRE-regulated epitopes occupying MHC Class I molecules. AIRE-induced altered SELFNESS significantly enhanced adoptive T cell therapy and stimulated heteroclitic $CD8^+$ T cell-mediated rejection of tumors through dendritic cell-mediated vaccination or *in vivo* delivery of AIRE-expressing vectors. Anti-tumor therapy was supported by AIRE-mediated generation of helper $CD4^+$ T cell epitopes and enhanced by immune checkpoint blockade. Our data are significant in that they demonstrate that changes in AIRE steady state levels re-sets the SELFNESS of cancer cells and converts a highly tolerized T cell compartment into a heteroclitic tumor-reactive T cell population. Exploiting AIRE as a master switch to manipulate tumor SELFNESS removes the need to identify specific TAA as targets for generation of *de novo* $CD8^+$ and helper $CD4^+$ T cell responses which can clear tumors and can be supported by additional immunotherapy interventions.

244 Unlocking Durable Anti-Tumor Immunity: Oncolytic Virotherapy as a Solution to Tumor Vaccine Shortcomings

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Tumor vaccines have shown clinical promise in certain cancers, yet their efficacy is limited by two major challenges: (1) the immune-suppressive tumor microenvironment (TME), and (2) limitation of only targeting antigen-positive tumor cells, which can result in the overgrowth of antigen-negative cells and eventual relapse.

To address these barriers, we explored a combination approach using mRNA tumor vaccines and oncolytic virotherapy in HPV and HER2 mouse tumor models. Our results indicate that while mRNA vaccines effectively eliminate antigen-positive tumors and inhibit their initial growth, tumors rapidly develop resistance, allowing them to escape the vaccine's effects.

However, introducing oncolytic virotherapy after mRNA vaccination—whether the virus expressed HPV or HER2 antigens or not—significantly extended the durability of anti-tumor responses in both models. The oncolytic virus, through its lytic infection, facilitated broad and dynamic tumor antigen presentation, lysing tumor cells and releasing a wide variety of tumor-associated antigens. This process not only increased the antigenic diversity within the tumor but also reprogrammed the TME, converting it from an immune-suppressive to an immune-supportive environment.

The combination of mRNA vaccination with oncolytic virotherapy generated a robust local immune response, driven by enhanced antigen presentation from viral infection. Additionally, the reprogrammed TME allowed the systemically established anti-tumor immunity from the mRNA vaccine to infiltrate and act within the tumor, overcoming previous immune barriers. We also observed the development of systemic antigen-specific T cells, which provided resistance to subsequent tumor challenges, indicating strong immune memory.

These findings underscore the unique role of oncolytic virotherapy as an intratumoral “boost” agent, capable of broadening tumor antigen exposure through tumor cell lysis while reconditioning the TME to amplify the effects of the systemic tumor vaccine. When combined in a prime-boost regimen, mRNA vaccines and oncolytic virotherapy synergize to create a robust, durable anti-tumor response that acts both locally and systemically to protect against recurrence.

In conclusion, our study demonstrates that combining mRNA tumor vaccines with oncolytic virotherapy is a powerful strategy to enhance tumor antigen presentation, reshape the TME, and overcome immune evasion, offering a promising approach for achieving durable anti-tumor immunity.

245 A Dual-Stimulatory Receptor Provides T-Cell Activation-Dependent Costimulation to Augment Native and Transgenic TCR-Based T-Cell Therapies

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TCR-based cell therapies produce highly sensitive tumor targeting. Unlike CAR-based receptors, a TCR complex lacks inherent ancillary costimulatory (Signal 2) and elicits reduced cytokine (Signal 3) signals required for functional T-cell persistence in vivo. Physiologically, these signals are provided in sync with Signal 1 and are contingent on TCR stimulation. In contrast, most current strategies to provide such signals to T cells include incorporating an additional antigen receptor (e.g. a costimulatory CAR) or integrating constitutive signals (e.g. cytokine modification) which function independently of the main antigen receptor. Therefore, we developed a synthetic Dual-Stimulatory Receptor (DSR) which provides both Signals 2 and 3 in response to the TCR activation (Signal 1).

An optimized DSR consists of a 4-1BB ligand (4-1BBL) extracellular domain linked to the cMPL (thrombopoietin receptor) intracellular domain. The 4-1BBL in the DSR interacts with the endogenous 4-1BB which is transiently upregulated upon TCR stimulation, thus inducing native 4-1BB co-stimulation (Signal 2) and downstream NF-kB signaling. Additionally, this engagement initiates cMPL signaling from the DSR leading to STAT3 and STAT5 phosphorylation in activated T cells, providing a cytokine-like Signal 3. In the absence of TCR stimulation, 4-1BB is downregulated and the DSR signaling is switched off, preventing constitutive activation. Indeed, T cells engineered with DSR (n=7) persisted over 8 rounds of repeated CD3 stimulation *in vitro* while unmodified T cells fail to expand beyond 3 rounds. Control T cells modified with *wild-type* 4-1BBL or truncated DSR lacking the cMPL endodomain also ceased expanding after 4 rounds of stimulation. The DSR+ T-cell fraction was enriched over time during repeated antigen stimulation demonstrating the phenotypic advantage that promotes T-cell expansion and persistence. Of note, DSR+ T cells did not expand upon cessation of CD3 stimulation demonstrating signal 1-dependent DSR-mediated T cell expansion.

We assessed the benefit of DSR in preclinical models of hematological malignancies: Acute Myeloid Leukemia (AML) targeted by CD123 T-cell engagers (TCEs)-secreting T cells and B-cell leukemia targeted by survivin/HLA-A2-specific transgenic TCR. The anti-tumor activity and cell persistence of TCE-secreting T cells (TCE-T) against an aggressive CD123+ Molm-13 AML cell line was dramatically enhanced by DSR modification in a short-term *in vitro* assay (>155-fold higher T-cell expansion than unmodified TCE-T on Day 9 of coculture) and long-term *in vivo* (Median Survival, TCE-T: 24 days vs DSR+TCE-T: >90 days). DSR-modified T cells persisted in mice long-term and mounted a secondary anti-tumor response upon re-challenge on day 35 after the initial challenge. Similarly, DSR armed CD8 T cells expressing a survivin-specific TCR expand and persist in vivo improving tumor clearance in an ongoing BV173 B-cell leukemia xenograft model (53-fold less tumor burden and 18-fold higher T-cell expansion over control on Day 21 post T-cell infusion as measured by in vivo bioluminescence signal). Overall, these results support using the DSR to boost TCR-based T cell therapies of cancer.

246 APC-Targeted LNP Enables Systemic Delivery of Neoantigen mRNA Vaccines and Enhanced Antigen-Specific T Cell Responses

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mRNA cancer vaccines have demonstrated promising clinical efficacy in patients with solid tumors. Currently, the primary clinical delivery methods for mRNA cancer vaccines are intramuscular (i.m.) injection using classic lipid nanoparticle (LNP) formulations and intravenous (i.v.) administration of lipoplex (LPX) that target antigen-presenting cells (APCs). However, even with i.m. delivery, classic LNP formulations can cause significant liver expression, raising concerns about liver toxicity, especially with repeated dosing. APC-targeted delivery of tumor antigen mRNA can enhance antigen-specific T cell responses while minimizing off-target toxicity. The permanently charged cationic lipids in LPX is associated with heightened toxicity, thereby limiting their tolerable dosage and clinical efficacy. In this study, we identified and developed a novel APC-targeted LNP (APC-LNP) formulation through the Dakini™ platform. Through an extensive screening of diverse ionizable lipid structures, lipid compositions and formulations, evaluated on human peripheral blood mononuclear cells (hPBMCs) and mouse models, we optimized a formulation that efficiently targets APCs across multiple organs with minimal off-target transfection in liver and lung parenchymal cells after i.v. injection, potentially enabling safe systemic administration. We compared the APC-LNP's ability to induce antigen-specific T cell responses with clinically used LPX and classic LNP formulation using the classic OVA-OT-I model. The effective dose for inducing OT-I-specific T cells with OVA-APC-LNP was ten times lower than with OVA-LPX via i.v. injection. Moreover, i.v. injection of OVA-APC-LNP resulted in significantly higher OT-I T cell induction compared to i.m. administration with classic LNP. The therapeutic efficacy of neoantigen vaccine was evaluated in a lung metastases model with CT26 tumor cells being intravenously inoculated. Twenty neoantigens, predicted using our AI-based neoantigen discovery platform NEOvigator™, were delivered via APC-LNP, achieving complete tumor suppression. In the aggressive and poorly immunogenic B16-F10 tumor model, the neoantigen vaccine showed significant tumor suppression. ELISpot analysis of neoantigen-specific responses in the spleen revealed a 52.6% response rate for predicted neoantigens. These results led to the development of our neoantigen cancer vaccine pipeline, GT601. The manufacture process for GT601 aligns with the standard for mRNA-LNP vaccine production. Preclinical toxicology studies across multiple species confirmed its favorable tolerability. GT601 is currently undergoing clinical evaluation in an investigator-initiated trial in China.

Organoids and iPSC Disease Modeling for Drug Discovery

247 Engineering Functionally Mature iPSC-derived Hepatocytes through Mapping Transcription Factor Regulatory Mechanisms

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The growing interest in differentiating induced pluripotent stem cells (iPSCs) into hepatocytes (iHeps) is driven by the shortage of liver donors for transplantations and the scarcity of primary human hepatocytes (PHHs) for toxicology studies. Thus far, iHeps have recapitulated many functions of primary hepatocytes enabling their usage in humanized transgene-free Il2rg-/-/Rag2-/-/Fah-/- (TIRF) mouse models and in vitro modelling assays. However, current protocols yield iHeps with immature gene expression and epigenetic signatures. In fact, we observed that primary hepatocytes have >75-fold and >7-fold higher expression of mature hepatocyte markers ALB and SERPINA1, respectively, compared with iHeps generated by conventional protocols. Additionally, our multi-omic single-cell ATAC-seq and RNA-seq analysis of iHeps demonstrates that only a subset of the cell population gains expression of mature hepatic markers. Deficiencies in maturity lead to limitations in the usage of iHeps as a cell

therapy and in disease modelling. Transcription factors (TFs) have pioneering roles in coordinating gene expression networks during liver development, and the inability of current protocols to recapitulate correct TF expression throughout the differentiation protocol likely contributes to the functional deficiencies of iHeps. To address these limitations, we employed TFome-wide open reading frame (ORF) overexpression and CRISPRko screens to identify factors that promote iHep maturity. Two iPSC lines were lentivirally transduced with libraries of either ORFs or guide RNAs and differentiated to iHeps. Subsequently, cells were immunostained for albumin and subjected to fluorescence-activated cell sorting (FACS) to isolate cells based on albumin levels. This process generated a list of 45 TFs putatively implicated in driving mature liver phenotypes when overexpressed, including TFs with known roles in liver development such as HNF4A, FOXA2, and NR5A2. Interestingly, several TFs had no previously established role in liver development or hepatocyte differentiation, suggesting the advantage of an unbiased screen in discovering novel regulators of in vitro cell fate. Validation experiments by RNA-seq corroborated the screen and confirmed the ability of most hits to upregulate the expression of maturation markers, including upregulation of ALB and SERPINA1 over 30- and 100-fold compared with negative controls, respectively. Further, scRNAseq demonstrated that the TF perturbations push the cells towards a more homogenously mature population. Functional experiments show that hit TFs produced iHeps with increased secretion of the liver-specific proteins albumin, angiotensin, fibrinogen, apolipoprotein A2, alpha-1-acid glycoprotein 1 and serpina1, and a morphology more closely resembling PHHs. To identify TFs that have both synergistic and complementary maturation effects, top validated hits were studied in combination. Three TFs (PPARG, NR5A2, and KLF7 (PNK)) uniquely led to augmented hepatic transcriptomic profiles when overexpressed together relative to each TF alone. PNK-iHeps enable long-term, functionally mature phenotypes in vitro. Ongoing work includes studying the gene regulatory networks through ATAC-seq and RNA-seq that enable the PNK factors to have a superior role in driving the maturation of iHeps and the potential for PNK-iHeps to be useful therapeutically in a humanized TIRF mouse model.

248 Phenotypic Characterization and AAV-Mediated Therapeutic Delivery in *C9ORF72* ALS iPSC-Derived Motor Neurons

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive paralysis and premature death. Hexanucleotide repeat expansions in the C9ORF72 gene are the leading genetic cause of ALS accounting for approximately 40% of familial cases. At the cellular level, 97% of ALS cases exhibit nuclear loss and cytoplasmic aggregation of the RNA-binding protein TDP-43, which is known to interact with ATXN2 in stress granules. Previously, we demonstrated that microRNA-mediated knockdown of Atxn2 (miAtxn2) improves mean survival by 54% in a Tdp-43 overexpressing mouse model of ALS (Amado et al. 2024). However, efficacy data in human cells is needed to support translation to clinic. Here, we identify disease-relevant phenotypes in C9ORF72 ALS patient iPSC-derived motor neurons (iPSC-MNs) and test the ability of our novel MN-targeting AAV, PM-AAV9, to transduce them and effectively deliver miATXN2. Doxycycline-inducible iPSC lower motor neurons were generated from an ALS patient with a hexanucleotide repeat expansion in the C9ORF72 gene and a healthy sibling control. C9ORF72 LMNs exhibited increased stress granule burden, elevated cell death, and reduced neurite length at DIV45. Delivery of eGFP by PM-AAV9 resulted in 90% transduction of LMNs at MOIs four logs lower than the published literature. PM-AAV9-miATXN2 reduced ATXN2 mRNA in a dose dependent manner up to 83% at an MOI of 5x105. Importantly total TDP-43 mRNA levels were not altered. A larger cohort of C9orf72-ALS patient cell lines and their isogenic control lines are being used in further validation studies wherein the expanded repeat in C9ORF72 (Meijboom et al. 2022) is deleted via CRISPR/Cas9 . This platform will enable further testing of miATXN2 and other ALS therapeutics in a human-relevant model.

249 Understanding Schizophrenia-Associated Loci Using iPSC-Derived Neurons and CRISPR Screening

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Schizophrenia (SCZ) is a chronic neurological disorder with a lifetime risk of 1% and is associated with increased morbidity and mortality rates. Given that genetic differences play a large role in SCZ risk, further understanding the genetic underpinnings of SCZ holds potential for advancing effective therapeutics for SCZ patients. A recent GWAS identified >22,000 variants in 287 distinct loci where common variation is associated with SCZ risk. However, SCZ GWA is not able to directly identify target genes nor demonstrate causality for common non-coding variants. High-throughput CRISPR screens have enabled researchers to dissect disease-associated loci, shedding light on the cis-regulatory elements (cCREs) and non-coding variants that modulate gene expression in a locus. Identifying active CREs and linking variants that map within these CREs to their target genes using CRISPR screens will refine our understanding of the non-coding regions of the genome that underly SCZ risk.

We utilize a scalable fluorescent-based readout of gene expression, HCR-FlowFISH, which enables us to epigenetically silence hundreds of cCREs with CRISPRi and measure the downstream effects on gene expression all within a single experiment. We have focused on expression of SCZ genes that have been identified by case/control whole exome sequencing (WES) and map within GWA regions. We have conducted CRISPRi + HCR-FlowFISH screens in iPSCs and iPSC-derived neurons (iNs) to identify active CREs and target genes in three WES-prioritized SCZ-associated loci. We have identified 78 genetic elements across three SCZ GWA loci that significantly alter expression of WES-identified SCZ genes when perturbed in iPSCs and/or iNs. These CREs include non-coding enhancers/silencers as well as promoters of genes and lncRNAs. Future prime editing screens of these loci will further aid in the prioritization of disease-relevant genetic variants that underly SCZ risk through modulation of SCZ genes. Findings from this work will improve our understanding of the genetics of SCZ and will lay the foundations for improved disease risk prediction and therapeutics for SCZ patients.

250 From Gene to Therapy: Unraveling WWOX and Its Role in Neurological Diseases

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Gene products of common fragile sites (CFSs) have been well-documented in cancer. In recent years, emerging evidence links these products, such as WW domain-containing oxidoreductase (WWOX), with neurological diseases, including epilepsy, multiple sclerosis (MS), and Alzheimer's disease. Patients harboring pathogenic germline bi-allelic *WWOX* variants have been described with rare devastating autosomal recessive spinocerebellar ataxia 12 (SCAR12) and WWOX-related epileptic encephalopathy (WOREE syndrome). Individuals with these syndromes present with a highly heterogeneous clinical spectrum, the most common being severe epileptic encephalopathy, profound global developmental delay, and premature lethality. Using mouse genetics, we modeled WWOX loss of function and revealed that specific neuronal *Wwox* ablation leads to brain hyperexcitability, intractable epilepsy, ataxia, profound hypomyelination, and postnatal lethality. Moreover, we recently established brain organoids from CRISPR-engineered human embryonic stem cells (hESCs) and patient-derived induced pluripotent stem cells (iPSCs). We discovered dramatic cellular and molecular CNS abnormalities using these human cell systems, including neural population changes, cortical differentiation malfunctions, and Wnt-signaling and DNA damage response impairments. Prompted by these results, we next designed and provided a proof-of-concept that ectopic WWOX expression, using an adeno-associated viral vector (AAV9) harboring human *WWOX* cDNA and driven by the human neuronal Synapsin I promoter (*AAV9-SynI-WWOX*) could rescue WOREE syndrome phenotypes. Our findings underscore the utility of modeling childhood epileptic encephalopathies using genetically engineered mouse models and brain organoids and their use as a unique platform to test possible therapeutic intervention strategies.

251 A fully human pluripotent stem cell-derived blood-brain barrier model validates the therapeutic potential of neurotropic adeno-associated viruses

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The blood-brain barrier (BBB) is a highly functionalized vascular interface which regulates the exchange of substances between the neural parenchyma and its periphery. BBB leakage, leading to its uncontrolled permeability, is increasingly recognized to facilitate the onset of neuropathologies and aggravate their clinical progression. In vitro models of the BBB have rapidly evolved into elaborated structures that mimic its spatial architecture and multicellular nature. However, their cellular components are currently highly heterogeneous in origin and maturation state. Here, we have developed novel procedures to establish reproducible and scalable sources of endothelial, mural and astroglial cells generating a fully human pluripotent stem cell (hPSC)-derived BBB model, termed thBBBA. hPSC-derived BBB cell types are readily assembled into thBBBAs that develop mature functional properties with high barrier impermeability. Mature thBBBAs can also be generated by frozen hPSC-derived cell samples, providing a simple and scalable off-the-shelf system for general use. thBBBAs are an invaluable system for evaluating the clinical potential of novel engineered AAV neurotropic capsids, previously selected in animal models or in vitro systems. In fact, we were able to rank a group of novel engineered AAV capsids for their ability to cross the thBBBA. Moreover, we established a thBBBA with the stable expression of the murine Ly6a, which encodes for the PHP.eB receptor in BBB endothelial cells. This Ly6a-thBBBA is the first and most convenient system for the direct comparison of BBB crossing capabilities between NHP-specific AAVs and the benchmark mouse-selective PHP.eB capsid. Additionally, thBBBAs were instrumental in identifying the critical pathological role of an IL-6 autocrine source in disrupting thBBBA integrity, increasing its permeability to NMDAR antibodies from autoimmune encephalitis patients, and revealing the therapeutic effects of tocilizumab in this disease.

252 Advancing Cardiovascular Drug discovery with iPSC-Derived 3D Cardiac Microtissues in High-Throughput Screening

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Human induced pluripotent stem cell (hiPSC)-derived models are revolutionizing drug discovery by providing human-relevant cell types for more accurate preclinical evaluations, especially for cardiovascular diseases and drug-induced cardiotoxicity. 3D cardiac microtissues (cMTs), which replicate the architecture and function of the human heart, offer improved drug screening for both efficacy and safety compared to traditional models. These models, developed from patient-derived iPSCs, increase the relevance and predictability of cardiovascular drug testing, allowing for more reliable preclinical data.

The study used Ncardia's iPSC-derived Ncyte® vCardiomyocytes, along with other patient specific iPSC-derived cardiomyocytes. Cardiomyocytes were co-cultured with Ncyte® and other iPSC-derived cardiac fibroblasts, and endothelial cells to form 3D cMTs. These cMTs were integrated into high-throughput screening (HTS) platforms, enabling large-scale drug screening. Automated formation of the microtissues was achieved with the TECAN Fluent robot, ensuring consistency in size and morphology. Calcium transient recordings were used to assess drug effects, while statistical analyses like one-way ANOVA evaluated the impact of various compounds on cardiac function and biomarkers such as NT-proBNP release.

As hiPSC-CMs in cMTs exhibit improved structure and metabolism compared to when in monocultures, we investigated whether cMTs could also enhance the predictivity of hiPSC-CM responses to therapeutics. In cMTs, the positive inotropes compounds increased both the beat rate and Ca2+ peak amplitude, while in 2D multi-cell type cultures, these compounds only induced positive chronotropic responses without affecting the amplitude or kinetics Automating the procedure revealed highly reproducible drug responsiveness and predictive accuracy in a reference compound screening highlighting their potential for high-throughput cardiac safety assessments.

In modelling catecholaminergic polymorphic ventricular tachycardia (CPVT), arrhythmias were observed that could be rescued by flecainide, validating their use in drug testing. Screening using the Specs Repurposing library resulted in an ~4.6% hit rate. The study also demonstrated the cMTs sensitivity to changes in cardiac function, including a concentration-dependent increase in NT-proBNP release and varying responses in ischemia-reperfusion studies.

This study highlights the effectiveness of hiPSC-derived 3D cMTs in advancing cardiovascular drug discovery. Their low variability, high predictability, and ability to model disease conditions, like CPVT, make them powerful tools for drug screening and disease modelling. The results highlight their potential to bridge the gap between in vitro and in vivo systems, improving the accuracy of preclinical evaluations and accelerating the development of novel cardiovascular therapies.

253 *In Vitro* Assays to Study Neuroprotection and Axon Regeneration in Human Neurons Differentiated from Neurogenin-2 Engineered Induced Pluripotent Stem Cells

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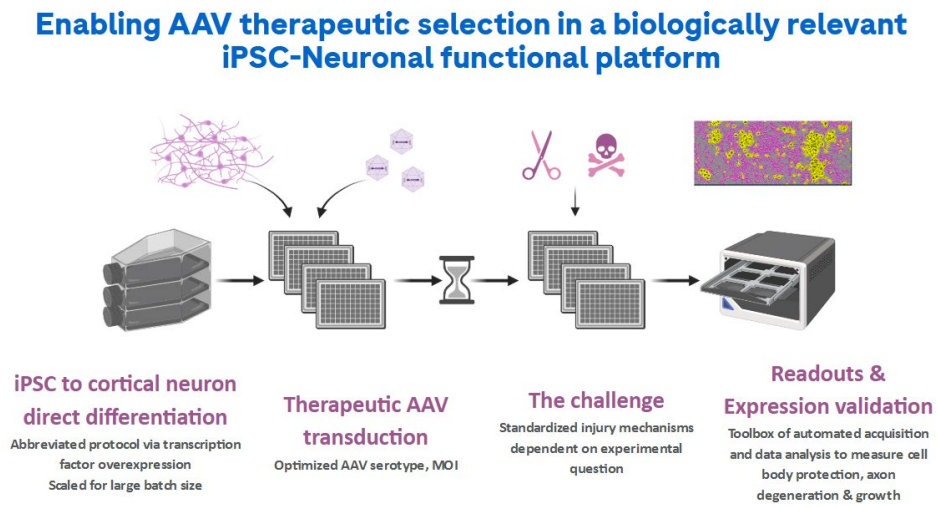
Drug discovery in neurodegenerative diseases is one of the most challenging therapeutic areas due to the lack of suitable models. Developing physiologically relevant in vitro neuroprotection and axon regeneration models is crucial for elucidating the mechanisms underlying neurodegeneration and designing effective therapeutic interventions. Neuroprotection focuses on maintaining the health of surviving neurons after injury, while regeneration seeks to repair or replace damaged neurons and synapses. Relevant *in vitro* experimental models of neuronal damage are necessary to evaluate any treatment's effectiveness. The discovery of induced pluripotent stem cells (iPSCs) has revolutionized the modeling of human diseases, significantly boosting the confidence of *in vitro* neurological disease models. We have developed in vitro assays using some common insults to model and study neuroprotection/axon regeneration, including chemical toxicity and mechanical damage in Neurogenin-2 engineered iPSC-derived human neurons (iNeurons). Integrating automated imaging and artificial intelligence (AI) analysis methods to measure axon degeneration/regeneration showed robust neuroprotection and regeneration *in vitro,* with little hands-on time. These models could help us accelerate therapeutic candidate validation and selection for neurodegenerative diseases.



CAR T Innovations in Autoimmune and Infectious Disease and Allergy

254 Selection for an HIV-Resistant Immune System by Multiplex Base-Edited CD45 CAR-T Cell Therapy

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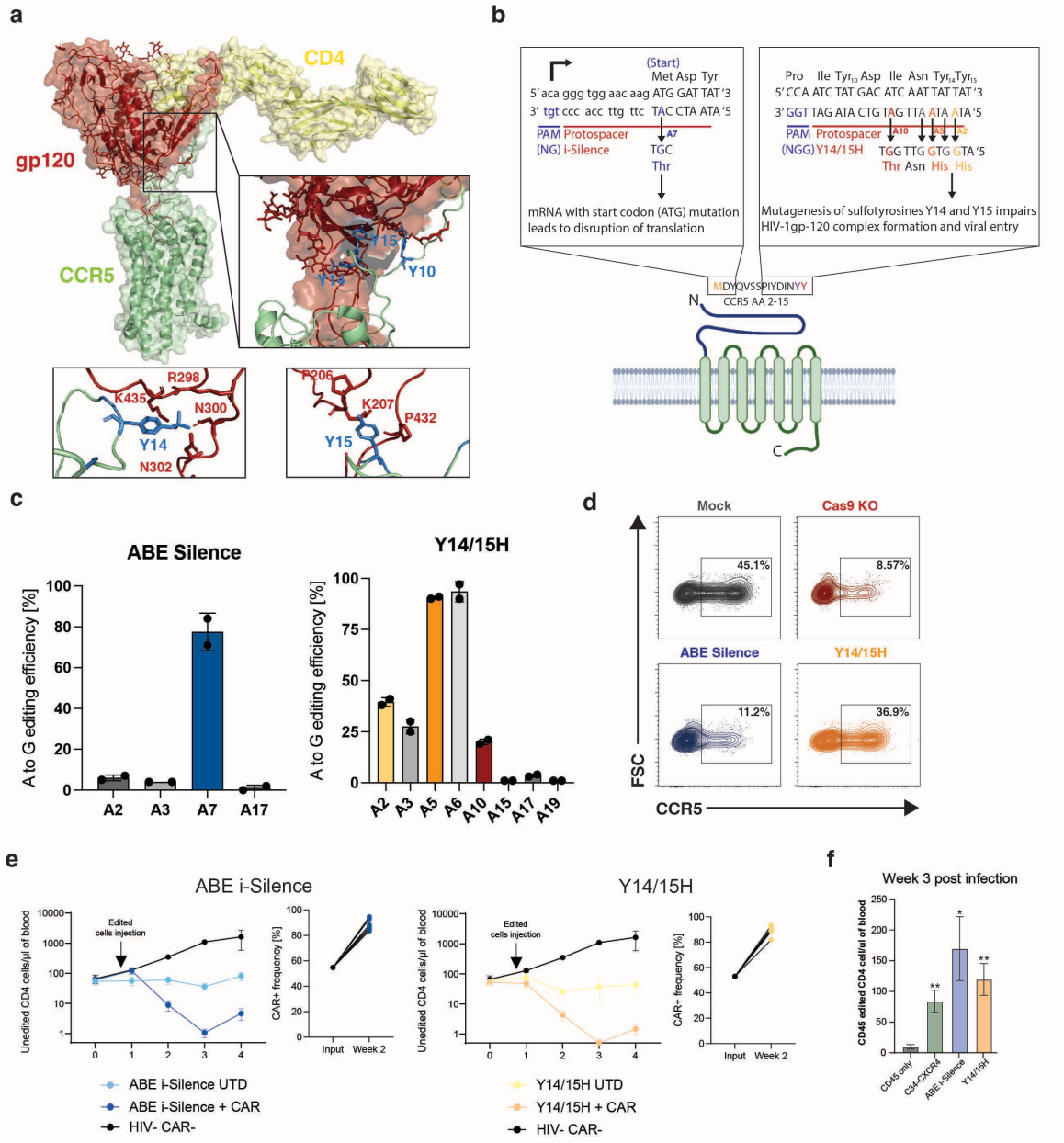
Introduction
Eradicating cellular reservoirs of human immunodeficiency virus (HIV) in patients on antiretroviral therapy (ART) is critical to curing HIV, since integrated provirus remains in CD4+ cells even in patients without detectable viral load on anti-retroviral therapy (ART). There are rare but compelling reports of HIV cures in patients who underwent allogeneic hematopoietic stem cell transplantation (alloHSCT) from CCR5Δ32/Δ32 donors, wherein myeloablative chemotherapy and donor alloreactive T cells completely eradicated the recipient's immunohematopoietic system and allowed it to be replaced by donor-derived hematopoiesis lacking the HIV co-receptor CCR5. While applying this strategy systematically as a treatment option is not feasible due to the paucity of HLA-matched CCR5Δ32/Δ32 donors and the high-risk nature of alloHSCT, these reports highlight that immunologic depletion of HIV reservoirs in combination with an HIV-resistant HSCT can achieve HIV eradication. To exert potent yet broad immune pressure against the cellular components of the viral reservoir we harnessed an HIV- and fratricide-resistant CAR-T cell therapy against the pan-leukocyte antigen CD45.

Results
Fratricide resistance of the anti-CD45 immunotherapy is achieved by epitope editing CD45 using an adenine base editing (ABE) as we recently described [PMID: 37651540]. At the same time, HIV-resistance is accomplished by either base editing the CCR5 start codon to silence CCR5 expression or mutating tyrosine residues (Y14 and Y15) in the N-terminal domain of CCR5 that are known to bind HIV gp120. Base editing CCR5 in primary human CD4 T cells is highly efficient with ~84% A to G editing at the CCR5 start codon, successfully silencing CCR5 protein expression. Similarly, a single gRNA achieved ~90% editing for A5 (Y14H) and ~38% for A2 (Y15H), accomplishing mutagenesis of the sulfotyrosine binding residues for HIV gp120 docking while preserving CCR5 expression. To test whether CCR5 base-edited cells (CCR5BE) are resistant to HIV infection, we infected CCR5BE or unedited CD4 T cells with the CCR5-tropic HIV stain BaL. Both groups of CCR5BE CD4 T cells showed reduced susceptibility to HIV BaL infection both in vitro and in vivo, whereas unedited control cells were readily infected. Resistance to HIV infection translated to a ~4-fold higher preservation of peripheral blood T cell numbers and a ~10-fold lower vRNA copy number in mice that received CCR5BE T cells compared to unedited T cells. Furthermore, we observed a strong enrichment of CCR5 edited cells from ~60% to >99% upon HIV infection, suggesting that CCR5BE T cells are protected from infection-induced cell death. To generate CD45 CAR-T cells that are resistant to fratricide and HIV infection, on-target base editing must co-occur biallelically at both the CCR5 loci and the CD45 epitope with high efficiency. Using single-cell DNA sequencing, we show that biallelic, multiplex base editing is efficient in primary human T cells with co-occurence of biallelic editing of ~75% (CCR5 start codon + CD45 and ~85% (CCR5 Y14/15 + CD45) respectively. Lastly, we demonstrate that multiplex base editing CD45 with CCR5 generates fratricide-and HIV-resistant CART45 cells that can eliminate HIV-infected CD4 T cells in mice while preventing the spreading of HIV particles from infected CD4 T cells to the CD4 positive CAR-T cells, thus facilitating their persistence compared to CD45-only edited CAR-T cells.



(a)Overall structure of the CD4-gp120-CCR5 complex (PDB:6MET); adapted from Shaik et. al43. (b) Adenine base editing strategies that are hypothesized to confer HIV-resistance to R5-tropic viruses. Left panel: gRNA that results in deletion of CCR5 expression by mutating CCR5 start codon using an NG-ABE8e (ABE Silence). Right panel: gRNA that mutates sulfotyrosine residues Y14 and Y15 which have been shown to impair viral entry when mutated. (c) Editing efficiency for ABE-mediated gene silencing and Y14/15H N-terminal mutagenesis in human T cells by targeted amplicon sequencing followed by analysis with EditR. Graphs depict all adenosine nucleotides within the 20 nucleotide protospacer regions with position one being the most PAM-distal nucleotide of the protospacer (n=2 biological replicates). Data are represented as mean ± SD (d) CCR5 protein expression measured by flow cytometry demonstrates that ABE-mediated CCR5 silencing can achieve comparable loss of protein expression compared to Cas9 nuclease mediated knockout. In contrast, Y14/15H mutation does not result in loss of protein expression despite high editing efficiencies. (e) Left panel: Number of unedited human CD4 T cells (infected target cells) in peripheral blood after the injection with CAR-transduced or untransduced epitope edited cells in comparison with mice that received neither HIV nor CAR injection. HIV- CAR- group was intentionally duplicated in each graph to facilitate visual comparisons at scale. Data are represented as mean ± SEM. Right panel: Percentage of CAR+ cell in human T cells before injection and 2 weeks post CAR T cell injection. (f) Number of CD45 edited human CD4 T cells (effector cells) in peripheral blood 3 weeks after HIV infection. (n= 6 mice per group)

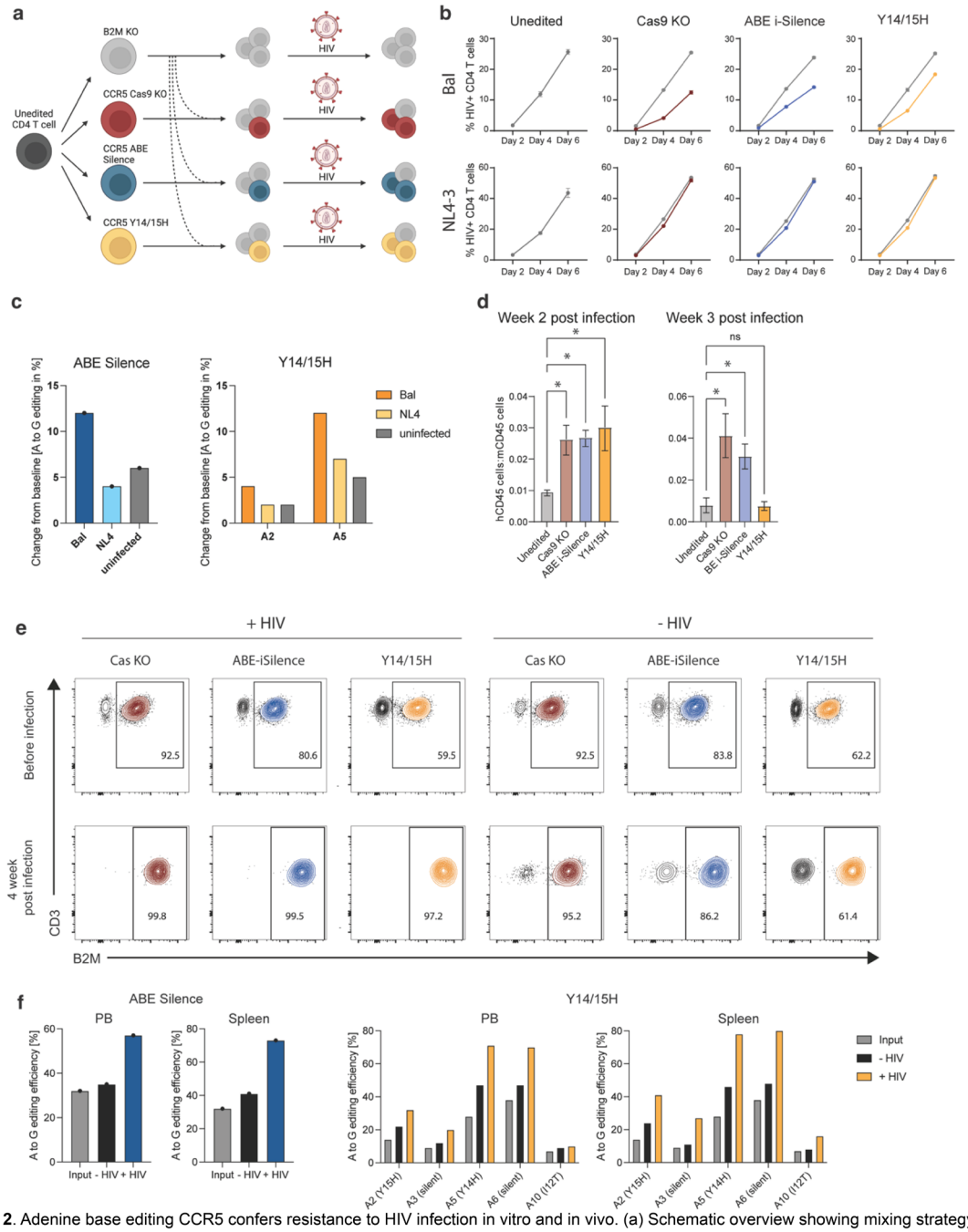


Figure 2. Adenine base editing CCR5 confers resistance to HIV infection in vitro and in vivo. (a) Schematic overview showing mixing strategy in both in vitro and in vivo experiments using beta-2-microglobuling negative cells as a surrogate for CCR5 unedited cells. CD4 T cells were edited at the targeted CCR5 locus and mixed with B2M KO CD4 T cells at 1:1 ratio and infected with HIV Bal (CCR5 tropic) or HIV NL4-3(CXCR4 tropic). (b) HIV infection was measured through intracellular FACs staining of HIV Gag core antigen every other day (n=3 technical replicates). Data are represented as mean ± SEM. (c) Quantification of on-target editing efficiencies by targeted amplicon sequencing 8 days after cells have been infected with either HIV BaL or HIV NL4-3. Left panel shows editing at A6 (on-target for CCR5 ABE silence) and right panel shows editing at A2 (Y15H) and A5 (A14H). (d) Two- and three-weeks post HIV infection, the ratio between human CD45+ cells and mouse CD45+ cells in mouse peripheral blood were analyzed. (n= 5-7 mice per group, *p<0.05, unpaired t-test). Data are represented as mean ± SEM. (e) The frequency of CCR5 edited cells (B2M+) in peripheral blood was measured through FACs staining of B2M before HIV infection and 4 weeks after HIV infection. (f) After 4 weeks, mice from each group (n=5-7 mice per group) were pooled and human CD45+ cells were sorted from spleen and peripheral blood (MACS) followed by targeted amplicon sequencing of human CCR5.

255 Multivalent CAR T cell therapy targeting membrane distal epitopes restricts HIV escape kinetics and enhances control of HIV replication in humanized mice

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Human Immunodeficiency virus 1 (HIV-1) primarily infects $CD4^+$ T cells establishing a chronic infection and leading to severe immunodeficiency. Although antiretroviral therapy can suppress HIV replication, it is not curative and requires lifelong treatment. Chimeric antigen receptor (CAR) T cell therapy offers a promising method to enhance the natural T cell response against HIV infection. Using broadly neutralizing antibodies (bNAbs) that target relatively conserved regions of the HIV envelope (Env) as the CAR ectodomain may further improve immunotherapy efficacy against a broad range of HIV isolates.

We generated a panel of 19 bNAb-based CAR T cells targeting six well-characterized regions of the HIV Env: V1/V2 apex, V3 glycan, CD4 binding site, gp120-gp41 interface, silent face, and MPER. CAR functionality was assessed *in vitro* through killing and suppression assays. Notably, CARs targeting distal epitopes (V1/V2 apex, V3 glycan, CD4 binding site) exhibited higher killing potency and cytolytic potential, suggesting that the accessibility of the epitope plays a key role in determining CAR effectiveness. Antigen sensitivity was also evaluated using time-course intracellular cytokine staining (ICS) after stimulation with low- and high-Env expressing cells. Notably, the V3 glycan-specific CARs demonstrated the highest antigen sensitivity. Furthermore, different Env trimer-based binding assays indicated that the V3 glycan-targeting CARs showed the highest avidity.

To evaluate the *in vivo* efficacy, we treated HIV-infected humanized mice with CAR monotherapy. Deep sequencing of the HIV *env* gene revealed the emergence of escape mutations at defined bNAb contact sites, highlighting the need for targeting multiple epitopes to limit viral escape. Mapping of escape mutations helped identify potent CAR product combinations with orthogonal escape patterns. Combination CAR therapy - consisting of 3 monospecific CARs - restricted HIV escape kinetics and resulted in a significant and durable reduction in plasma viremia in humanized mice. We next developed tri-valent CAR products, simultaneously expressing the 3 most potent bNAb-CARs. These multivalent CAR products showed an *in vivo* expansion advantage over mono- or double-specific products, suggesting greater antigen sensitivity. Notably, some mice in the cohort exhibited complete control of plasma viremia, while even non-controllers had significant protection from memory $CD4^+$ T cell depletion.

In conclusion, our findings demonstrate the potential of bNAb-based CAR T cells for HIV immunotherapy. We identified bNAb CAR classes with superior potency and elucidated the mechanisms driving their effectiveness. Targeting multiple conserved epitopes on HIV Env can limit viral escape and lead to durable control of viremia. The development of multivalent CAR products, simultaneously expressing the most potent bNAb CARs, represents a promising strategy for advancing HIV cure efforts.

256 CAR19 Engineered Regulatory T Cells, a Novel Approach for Immune Reset in B and T Cell Mediated Autoimmune Disorders

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Conventional CD19 CAR T therapy has demonstrated promising efficacy in SLE by resetting the immune system via robust B cell depletion. However, long-term remission in these patient populations may require changes to additional pathogenic immune cell activities, including the suppression of autoreactive T cells. Besides safety issues associated with cytokine release syndrome (CRS), high manufacturing costs of autologous cell therapy limit the accessibility of conventional CAR T therapy. To address these limitations, here we report on allogeneic CD19 CAR engineered regulatory T cells (CAR19 EngTregs) that combine the best features of both CAR Ts and Tregs with potential for improved immune reset, improved safety, and reduced manufacturing cost. We show that CAR19 EngTregs maintain the clinically validated mechanism of B cell depletion like CAR Ts while also directly suppressing the T cell arm of the immune system, distinct from CAR Ts. In addition, compared to CAR Ts, CAR19 EngTregs display significantly reduced production of inflammatory cytokines associated with cytokine release syndrome and ICANS.

We first engineered CAR19 EngTregs using a technology platform that addresses key challenges facing Treg cell therapeutic success including phenotypic instability, environmental IL-2 scarcity, and lack of tissue specificity through modifications designed to enable (1) stable FOXP3 expression, (2) CAR19 for B-cell targeting and (3) a chemically inducible signaling complex (CISC) that provides a rapamycin driven IL-2 like signal to promote engraftment, activity, and stability in the IL-2 scarce environment of autoimmune disease, while also enabling enrichment with rapamycin to high purity during cell production.

CAR19 EngTregs produced with the above platform demonstrated a stable Treg phenotype (FoxP3, CD25, CTLA-4 and ICOS) and upon CAR stimulation, upregulation of classic activation markers (CD69) and Treg specific markers (TNFR2, LAP and GARP). In vitro proinflammatory cytokine secretion (INFg, TNFa, IL6) was low, especially compared to CAR T cells. Interestingly, CAR19 EngTregs mediated robust cytotoxicity against both the Nalm-6 B cell line and primary B cells while expressing lower cytolytic enzymes (Perforin, Granzyme A, and Granzyme B) compared to CAR T. CAR19 EngTregs prevented B cell proliferation and differentiation into plasmablasts and plasma cells while abrogating immunoglobulin production. Importantly, CAR19 EngTregs also mediated CD19-driven T cell suppression demonstrating modulation of both B cell and T cell compartments.

Allogeneic CAR19 EngTregs dosed in a humanized pristane model in adult NSG mice, depleted both B cells and T cells in vivo, while eliciting lower serum inflammatory cytokines and lower immunoglobulin titers compared to CAR T or the anti-CD19 monoclonal antibody, ineblizumab. Histopathology demonstrated CAR19 EngTregs reduced inflammatory lesions in tissue more deeply vs comparator treatments. These data support CAR19 EngTregs as a uniquely differentiated therapy for B cell driven autoimmune diseases that target both B and T cell arms of the immune system demonstrating a superior efficacy and safety profile compared to CAR T.

257 Chimeric Antigen Receptor T Cells Targeting the IgE B Cell Receptor Specifically Eliminate Human IgE Producing B cells

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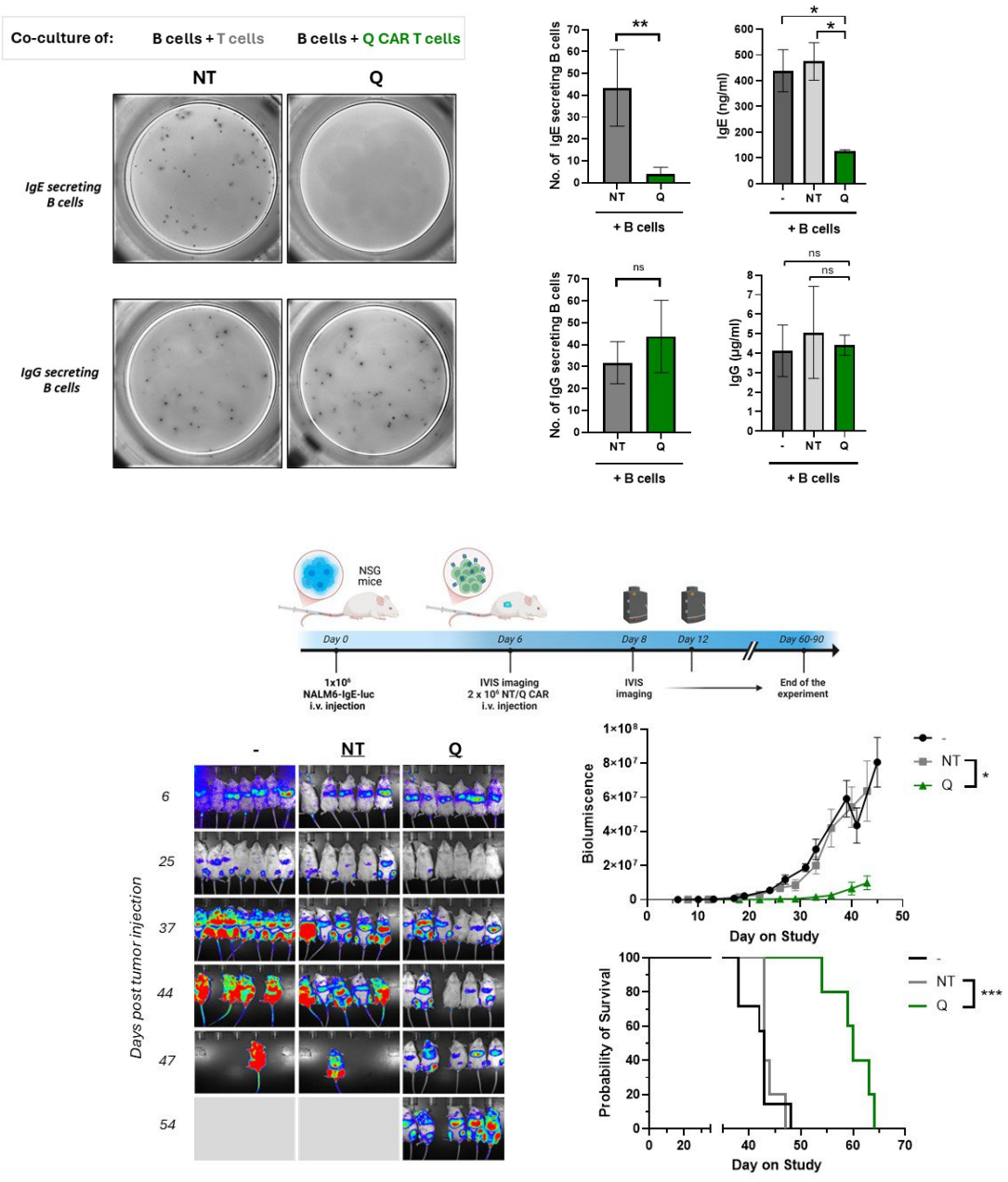
The prevalence of IgE-mediated allergic disorders has risen markedly in industrialized societies, posing significant clinical and socioeconomic challenges. These disorders are characterized by hypersensitivity reactions that range from acute, life-threatening anaphylaxis to chronic conditions such as allergic asthma and autoimmune chronic urticaria. Despite advances in therapeutic strategies, existing treatments—including pharmacological agents, monoclonal antibodies, and immunotherapy—remain limited in their capacity to provide definitive cures. Here, we

present a novel chimeric antigen receptor (CAR)-T cell therapy targeting M1', a unique extracellular domain of the IgE B-cell receptor (BCR). This domain, absent from free IgE, offers an unparalleled opportunity for selective targeting of IgE-producing cells. Our "Q CAR" construct, derived from the monoclonal antibody Quilizumab, demonstrated robust cytotoxicity against primary human IgE-secreting cells and significantly reduced IgE production in vitro. In an in vivo NSG mouse model, Q CAR-T cells inhibited the growth of IgE-expressing tumors and extended survival. By circumventing the limitations of prior CAR designs that target free IgE as well—this strategy provides a novel framework for the development of cellular therapies for allergic diseases. Our findings pave the way for further investigation in transgenic allergy models, with the ultimate goal of clinical translation to offer curative solutions for IgE-mediated disorders.

Q-CAR T cells, targeting IgE-BCR, specifically eliminate IgE-secreting B cells



258 A Two-Infusion Regimen with a Novel In Vivo Non-Viral Chimeric Antigen Receptor (CAR) Achieves up to 90% CD8+ T Cell Engineering and Tissue Depletion of Target Cells in Non-Human Primates (NHPs)

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Introduction: A major objective in the field of autoimmunity is to advance novel therapies capable of achieving durable drug-free responses by resetting the immune system, without prolonged or residual on- or off-target toxicities. This requires memory and autoreactive B cell depletion in tissues, followed by repopulation with newly formed B cells comprising a naïve phenotype. To that aim, we developed a novel platform technology (CellSeekerTM) comprising proprietary ionizable lipid-based LNPs, functionalized to effectively target CD8+ T cells in vivo to deliver encapsulated mRNA payloads to transiently generate CAR T cells (Fig. 1). This platform does not require lymphodepletion conditioning and is tunable in dose level and frequency to achieve the necessary exposure. Previously, we demonstrated that an intensive dosing regimen (3 infusions, Q72hr) with >0.3 mg/kg of anti-CD20 CAR mRNA in CD8-LNPs, resulted in global B cell depletion in blood and lymphoid organs of NHPs (Fig 2). To facilitate the clinical translation of this technology, we evaluated the potency and mechanism of action of a streamlined 2-infusion cycle aiming to achieve B cell depletion sufficient for immune reset, with minimum drug exposure. Methods: We generated two similar tLNP formulations comprising a highly biodegradable ionizable lipid, that were decorated with humanized anti-CD8 monoclonal antibody: i) CPTX2309 containing mRNA encoding for an all human anti-human CD19 CAR and ii) CPTX2309(S) containing mRNA encoding for a murine anti-human CD20 CAR. These formulations showed comparable human T cell engineering rate and pharmacological effect against human primary B cells in vitro; however, only CPTX2309(S) was cross reactive against NHP B cells. Cynomolgus monkeys were intravenously administered one (n=2/group) or two (Q72hr) doses (n=3/group) of CPTX2309(S) (1-2 mg/kg) without pre-medication, or with PBS as a control. Both single-dose animals were euthanized on day 3, while one animal per 2-dose group was euthanized on day 10 and the other two on day 43. A comprehensive PK/PD analysis in blood and tissues by flow cytometry for CAR expression, immune cell phenotypes, activation markers (ICOS and PD-1), and cell enumeration were performed. Immunohistochemistry for B cell and microenvironment characterization, and MSD analysis for cytokines and other markers in blood, were also performed. Results: Two infusions of CPTX2309(S), 3 days apart, resulted in rapid and deep B cell depletion in in blood evident within 24 hrs post 1st dose, paralleled by global depletion of B cells in tissues including bone marrow, spleen and lymph nodes measured on day 10 (Fig 3). A single dose of 2mg/kg appeared less effective than 2 lower-dose infusions (1mg/kg, or 1.5mg/kg, or step up dosing with 1mg/kg followed by 2mg/kg) in achieving global B cell depletion. In the effective 2-dose regimen, B cells began to recover within 3 weeks, predominantly with naïve B cells, in 5/6 animals, maintaining this profile skewed toward naïve B cells and not reaching the pre-treatment levels of total or memory B cells during the 6-week observation period (Fig 3B). Flow cytometric analysis demonstrated rapid engineering of CD8+ T cells with increased CAR expression upon 2nd infusion, reaching up to 90% of CD8+ T cells (Fig 4A). There was no detectable engineering of B cells and minimal CAR expression by CD4+ T cells. CAR was transiently expressed by CD8+ NK cells and myeloid cells. CAR expressi

Figure 1. Design and target product profile of an *in vivo* CAR product based on CellSeeker™ tLNP technology

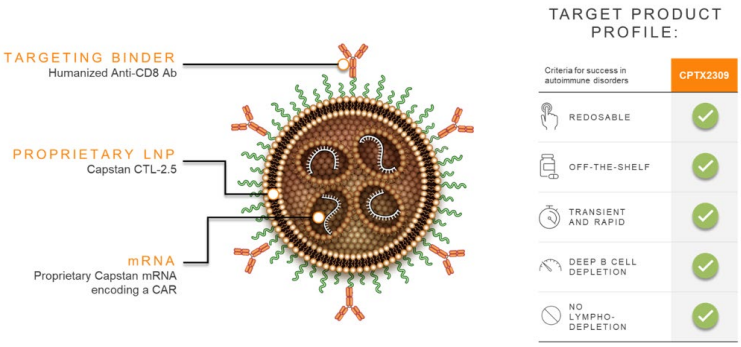


Figure 2. Dose-dependent B cell depletion in NHPs achieved with an *in vivo* anti-CD20 CAR in mRNA format, utilizing CellSeeker™ tLNP technology (Dosing: 3x Q72hrs)

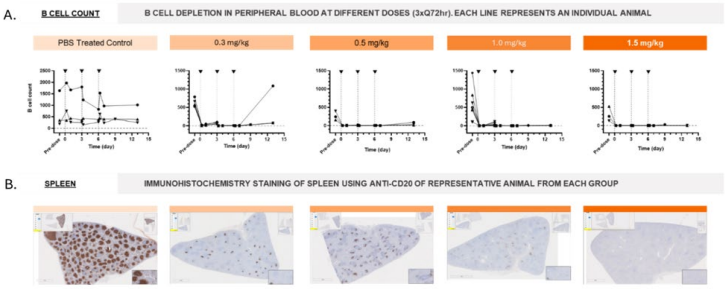


Figure 3. Profound B cell depletion in tissues, followed by recovery with predominantly naïve B cells characterized in blood, achieved with an in vivo anti-CD20 CAR in mRNA format, utilizing CellSeekerTM tLNP technology (Dosing 2x Q72 hrs)

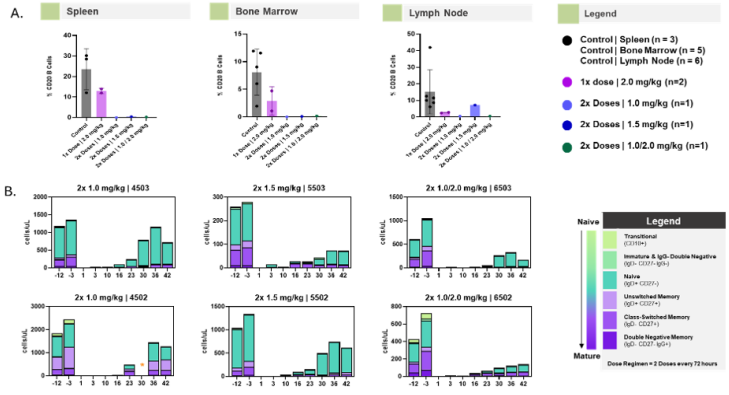
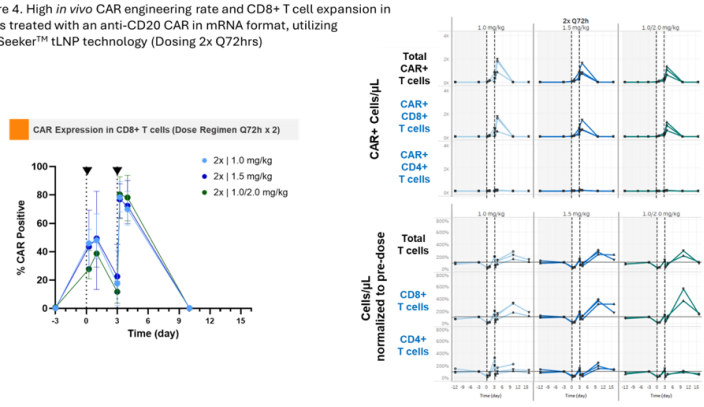


Figure 4. High *in vivo* CAR engineering rate and CD8+ T cell expansion in NHPs treated with an anti-CD20 CAR in mRNA format, utilizing CellSeeker™ tLNP technology (Dosing 2x Q72hrs)



259 Depleting the Latent HIV-1 Reservoir with Anti-CD4 CAR T-cells

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Background:
HIV-1 and related viruses (SIV, SHIV) infect CD4+ cells in humans and nonhuman primates (NHPs), respectively. Although antiretroviral therapy (ART) suppresses virus replication, cessation of ART leads to viral rebound. Our recent data suggest that the persisting HIV-1 reservoir may be immunologically silent, necessitating anti-HIV immunotherapies that do not require viral antigen expression. Here we developed an anti-CD4 chimeric antigen receptor (CAR) approach to transiently ablate the CD4 compartment, including latently-infected cells. Our novel anti-CD4 CARs were evaluated ex vivo for relative expression, specificity for autologous CD4+ target cells, and potency/duration of cytotoxicity. The most effective CAR was selected for an ongoing in vivo study (n=3) in SHIV-infected, ART-suppressed NHPs. Our in vivo study will inform the safety and feasibility of anti-CD4 CAR T-cells as a curative therapy for people living with HIV.

Methods:
Six anti-CD4 CAR transgenes, featuring two scFv orientations (VH-VL or VL-VH) and three spacer domains (short, medium, or long), were cloned and packaged into lentiviral vectors for transduction into rhesus macaque CD8+ T-cells. CAR T-cell proliferation was assessed with and without co-cultured autologous CD4+ T-cells in single-round antigen stimulation and long-term repeat stimulation assays (RSA). After identifying the most potent and specific CAR ex vivo, CAR T-cells were infused into SHIV-infected, ART-suppressed rhesus macaques via the intravenous route. The kinetics and magnitude of CAR expression and CD4 depletion were quantified in blood and tissues using flow cytometry, immunohistochemistry, and PCR-based assays. CAR vectors co-expressed truncated EGFR (EGFRt) to label CAR+ cells. In our NHP study, we used the anti-EGFR antibody Cetuximab to assess EGFRt as a "CAR-OFF" safety switch.

Results:
All 6 anti-CD4 CARs were functional ex vivo; CARs with VH-VL scFvs and short/medium spacer lengths had the highest CAR expression, with up to 90% expression in CD8+ T-cells after antigen stimulation. CAR expression increased 1.3-3-fold after stimulation with autologous CD4+ cells. Each anti-CD4 CAR molecule rapidly eliminated CD4+ targets (Figure 1) and proliferated specifically in response to antigen ex vivo. Early NHP data in peripheral blood indicate robust expansion of CAR T-cells and complete CD4 depletion. Cetuximab effectively depleted CAR T-cells to <2% in peripheral blood (Figure 2).

Conclusion:
NHPs are an ideal in vivo model to validate both the safety and efficacy of novel CAR T-cell therapies. We found that NHP anti-CD4 CAR T-cells exhibited potent function ex vivo and in vivo. Our CARs did not display significant fratricide, likely due to the use of double-purified CD8+ T-cells and exclusion of CD4+ T-cells during manufacturing. Cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) were observed in the first CAR-treated animal. To our knowledge, we are the first to validate Cetuximab as a CAR safety switch in NHPs, opening new doors to further optimize regulated CAR function in the NHP model. Currently, we are quantifying the impact of anti-CD4 CAR T-cells in our model of HIV latency. We predict that efficient CD4 depletion may be associated with curative endpoints.

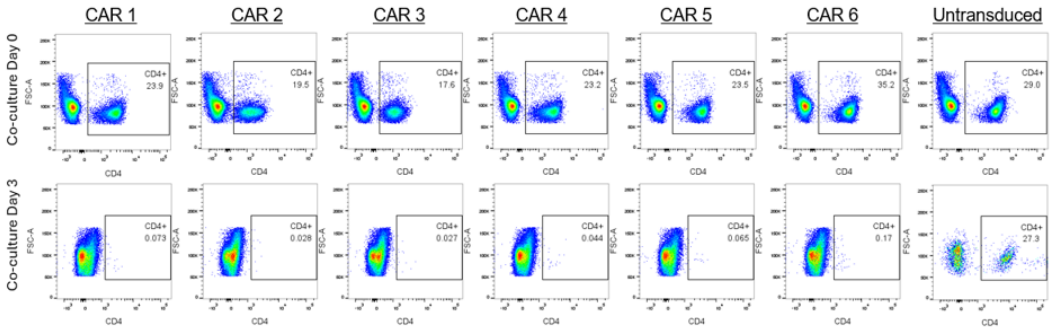

Figure 1. Anti-CD4 CAR T-Cells Kill CD4+ Targets ex vivo. Candidate anti-CD4 CARs 1-6 were expressed in primary rhesus macaque CD8+ T-cells, followed by co-culture with autologous CD4+ cells at a 5:1 effector-to-target ratio. The number of CD4+ targets was quantified immediately after mixing (Day 0, top row) and following 3 days of co-culture (Day 3, bottom row). Mock: negative control in which CD8+ T-cells were not modified with an anti-CD4 CAR vector.

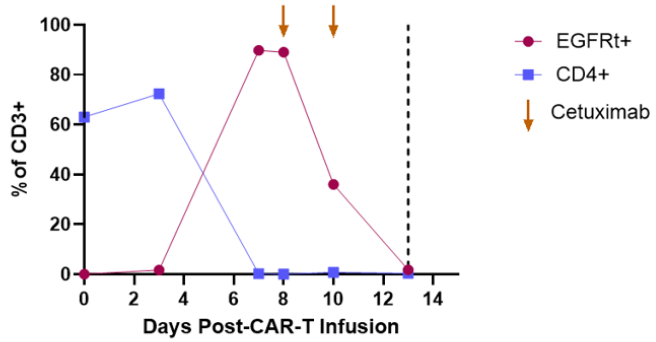

Figure 2. CAR expansion and CD4 depletion in an NHP model. Quantification by flow cytometry of CD4+ T cells and CAR+ cells via the truncated EGFR tag in peripheral blood after CAR infusion. Cetuximab was administered on days 8 and 10. End of study is indicated by the dashed vertical line.

260 CD19 CAR T Cells Generated In Vivo by T Cell Targeted Lipid Nanoparticles Demonstrate Robust and Durable B Cell Depletion in Non-Human Primates

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T cells transduced ex vivo with lenti- or gamma-retro viral chimeric antigen receptor (CAR) targeting the CD19 and BCMA antigens have shown significant clinical benefit to patients with hematologic malignancies, resulting in six FDA-approved products to date. Despite this clinical success, these treatments require a complex manufacturing process that results in barriers to patient access. To obviate the complexities of ex vivo manufacturing, we are developing T cell targeted lipid nanoparticle (LNP)-based therapeutics that enable *in vivo* cell-specific reprogramming, providing off-the-shelf gene therapies to patients while eliminating the need for pre-conditioning treatments. Specifically, LNPs were engineered with a CD8-targeting NANOBODY® molecule on their surface which enables the delivery of anti-CD19-based CAR mRNA cargo to T cells in vivo. Rhesus macaques were infused with single or multiple doses of CD8-targeted LNPs containing CD19 CAR mRNA with no infusion-related toxicities observed. We demonstrate CD19 CAR expression on the surface of CD8+ T cells as early as 1 hour

following infusion of targeted LNP peaking at 24 hours. CD19 CAR expression is associated with a robust decrease in B cells (identified via CD20 expression) resulting in >95% B cell depletion in peripheral blood 24 hours after treatment relative to the baseline B cell frequencies before dosing. Maximal B cell depletion in the peripheral blood is observed at 24 hours post targeted LNP infusion, followed by a return to the pre-infusion baseline at 50 days post-infusion. Importantly, we also find that CD8+ T cells and NK cells in the bone marrow express CD19 CAR 24 hours after infusion of targeted LNPs and we observe a concomitant decrease of bone marrow resident CD20+ B cells >90% relative to control animals. Similar to peripheral blood, maximal B cell depletion in the bone marrow is observed at 24 hours post targeted LNP infusion and returns to baseline at 29 days post-infusion. We observe gross changes in B cell receptor CDR and isotype distribution from B cells harvested from the bone marrow at 57 days post initial infusion relative to B cells harvested from the bone marrow pre-infusion. In summary, the robust B cell depletion in peripheral blood and bone marrow following infusion with CD8 targeted LNPs containing CD19 CAR mRNA supports further development of T cell targeted LNPs towards clinical applications in oncology and autoimmunity.

Immune Cell Therapies

261 Armored HIV-specific CAR NK and Activated NK Cells Are Safe and Led to Viremic Control in a Subset of HIV-Infected Humanized DRAGA Mice

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Over 39.9 million people are living with HIV-1 globally. Although small-molecule antiretroviral therapy (ART) has dramatically improved survival rates and quality of life, these drugs are expensive, have severe side effects, require lifelong adherence, and are not a cure. Reservoirs of HIV persist within B cell follicles of lymphatic tissues even during ART treatment. This persistence is partly due to a paucity of HIV-specific cytotoxic cells accumulating in these locations, as they largely do not express the follicular homing receptor CXCR5. Our group has pioneered the development of HIV-specific CAR T cell therapies, which also express CXCR5, allowing these cells to have heightened HIV-killing potential and specifically migrate to sites of viral replication within B-cell follicles. CAR T cell therapies are typically autologous, as treatment with allogeneic T cells can cause GVHD. This poses a challenge for treating HIV, as T cells are compromised and engineering HIV-infected cells may pose additional risks. We are now exploring CAR/CXCR5 NK cell therapies as an alternative as they can be used in allogeneic settings and have preexisting antigen-independent activation modalities for immunosurveillance of virally infected cells. We engineered bispecific, HIV-envelope-targeting CAR/CXCR5 NK cells armored with soluble IL-15 and knocked out PD-1 using base editor technology to prevent exhaustion. We evaluated these cells in vitro to determine their specific killing capacity and ability to migrate to CXCL13, the chemokine ligand of CXCR5. We then assessed the efficacy and safety of these therapies in vivo by treating HIV-infected humanized DRAGA mice (NRG background with HLA I and II transgenes for enhanced engraftment of human stem cells) with activated, armored CAR/CXCR5 NK cells (1-1.5X105 cells/g), treated 3 days post ART interruption). They were compared to mice treated with activated but unedited control NK cells or saline treatment. We monitored HIV viral loads, CD4 count, CAR NK levels, and adverse symptom onset for 2 months post-cell infusion. We found that CAR/CXCR5 NK cells had increased specific killing activity and migratory function to CXCL13 compared to unengineered NK cells in vitro. Our in vivo studies found that 62.5% of CAR/CXCR5 NK treated, 50% of activated NK treated, and 0% of saline-treated animals had viral loads below the detection limit at 56 days post-treatment. Despite this impact, all groups showed a continued decline of CD4+ T cell counts post-infusion. In CAR/CXCR5 NK-treated animals at 6 days post-treatment, abundant vRNA+ cells were detected within the follicle-like regions of the spleen. However, few CAR NK cells were detected and none were found in proximity to HIV+ cells. Importantly, we saw no evidence of

adverse health outcomes related to treatment. Our data indicate that armored CAR/CXCR5 NK cells and activated NK cells are safe and effective at reducing viral loads in a subset of treated animals. The low levels of CAR/CXCR5 NK cells in lymphatic tissues suggest that they may be inefficient at migrating into lymphoid tissues and may explain why they did not outperform unedited NK cells. This study demonstrates the promise of activated NK and engineered CAR NK therapeutics for clearing HIV infections and our future studies will focus on improving the lymphoid-homing potential of our therapies. This work supports further study of novel NK cell therapies for the durable remission of HIV.

262 Broadly-neutralizing antibodies and *CCR5*-edited hematopoietic stem cell transplantation synergistically delay virus recrudescence in a nonhuman primate model of HIV

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Introduction
Autologous, gene-edited hematopoietic stem and progenitor cell (HSPC) gene therapies have the potential to cure HIV, but require near-complete replacement of the host immune system in blood and tissues. In our vetted nonhuman primate (NHP) model, we are optimizing transplantation regimens to maximize engraftment of CRISPR-edited HSPCs, redirect cytoreductive approaches to reduce the number of infected cells *in vivo*, and merge orthogonal antiviral approaches. Here, we tested the hypothesis that infection-resistant D*CCR5* HSPCs would synergize with a humoral immunotherapy, intravenous delivery of a virus-specific broadly neutralizing antibody (bNAb), to eliminate latently-infected cells.

Methods
Rhesus macaques were infected with an HIV-like virus (simian/human immunodeficiency virus, SHIV.C.CH505.v2) and placed on antiretroviral therapy (ART) to model suppressed HIV infection. Bulk $CD34^+$ HSPCs were collected after G-CSF/Plerixafor mobilization, edited with NHP *CCR5*-specific CRISPR ribonucleoprotein (RNP) complexes, cryopreserved, and transplanted after conditioning with either myeloablative busulfan or an anti-CD45 antibody-drug conjugate (ADC). At week 26-30 post-transplant, ART was withdrawn and plasma viral load was monitored for signs of recrudescent virus replication. At ART withdrawal, a subset of animals received a single infusion of VRC07.523.LS, a safe and effective bNAb with potency against HIV and SHIV.C.CH505.v2, and is currently in clinical trials in people living with HIV (PWH).

Results
Myeloablative busulfan and CD45-ADC elicited mild lymphopenia while maintaining potent suppression of plasma viremia, with a similar range of *CCR5* editing efficiencies in peripheral blood. Shortly after transplantation, we observed depletion of $CD45^+$ cells in lymph nodes and spleen after ADC conditioning. Observed levels of *CCR5* editing in HSPC-derived PBMCs (~8-26%, Figure 1) did not impact the kinetics of post-RT viral rebound with either conditioning regimen compared to non-transplanted controls. While bNAb alone delayed rebound, the combination of transplant + bNAb further delayed recrudesence, by an average of 38 days compared to untreated controls (Figure 2).

Conclusions
Our NHP model of suppressed HIV infection is well- suited to identify conditioning regimens that replace or augment hematopoietic subsets with minimal impact on host immunity and their ability to synergize with other long-term treatment options for viral control. Our results advance our clinically relevant model, highlight long-term persistence of CCR5-edited HSPC and progeny *in vivo,* and confirm that antibody-based therapies augment HSPC-based approaches. Our ongoing efforts are focused on quantitative comparisons between busulfan- and CD45-ADC conditioning regimens, the latter of which may also directly deplete latently-infected cells.

Figure 1:*CCR5* Editing in PBMCs

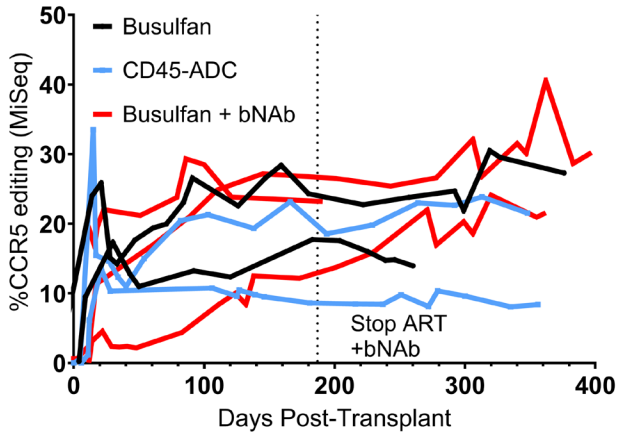
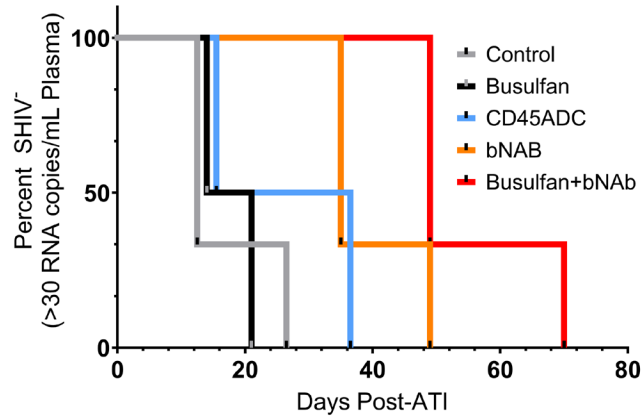


Figure 2: Days to SHIV Rebound Post-ATI



263 Epigenetic Reprogramming of Tumor-Infiltrating Lymphocytes for Improved Anti-Tumor Killing

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Tumor-infiltrating lymphocytes (TILs) have emerged as a promising autologous cell therapy to treat solid tumors by expanding and reinfusing tumor-reactive T cells. While TIL therapy has had some success in specific cancers, including recent FDA approval for advanced melanoma, its efficacy has been limited by the heterogeneity of expanded TIL products and the prevalence of dysfunctional exhausted CD8+ T cells (T_{EX}). A subset of CD8+ TILs that are double-positive (DP) for the CD103 and CD39 cell surface proteins is enriched for tumor-reactive TILs across multiple cancer types, but also expresses other proteins associated with the T_{EX} state. To identify factors that may improve T_{EX} phenotype by reprogramming gene networks, we used a lentiviral Open Reading Frame (ORF) library encoding all human transcription factors (TFs) to screen for master regulators of proliferation in tumor-reactive human TILs from non-small cell lung cancer patients. Two proliferation screens were conducted in parallel by initially isolating either human CD8+ or DP TILs, activating with anti-CD3/CD28, transducing the lentiviral library the following day, and sorting highly proliferative or lowly proliferative cells after 16 days of culture via CellTrace staining. TFs associated with cell proliferation (e.g. MYC, MYC-L) emerged as top hits for the CD8+ TIL

screen, but only RELB and RELA emerged as top hits driving proliferation in DP TILs. Individual validation of RELB overexpression confirmed a drastic improvement in TIL proliferation, with up to a 10-fold change across four different human TIL donors and a skew towards CD8+ TILs after in vitro expansion. Transient delivery of RELB mRNA to TILs also conferred an improvement in proliferation and a skew towards CD8+ TILs that persisted for up to 8 days post-electroporation for one donor. Transcriptomic changes were measured via bulk RNA-seq on RELB-overexpressing TIL subsets (CD4+, CD8+, and DP+), revealing a more memory-like phenotype with upregulation of T cell memory genes (BATF3, SELL, CCR7) and downregulation of naïve (TCF7) and exhaustion markers (PD-1). T cell receptor diversity of the same TIL subsets overexpressing RELB was assessed via bulk T cell receptor sequencing (TCR-seq) before and after *in vitro* expansion, which demonstrated a maintenance in TCR repertoire diversity and a marginal improvement in diversity to the DP TILs across four distinct donors. To assess functional capacity of engineered TILs, TILs overexpressing RELB or RELA were armed with a chimeric antigen receptor (CAR) targeting a HER2-expressing breast cancer cell line. RELB and RELA conferred improved persistence after multiple rechallenges with the cancer cells, and a final surface marker staining of the remaining TILs revealed that RELB-overexpressing TILs were mostly CD8+, while RELA and CAR-only conditions had no remaining CD8+ TILs. Using the same CAR-TIL model targeting HER2, mice were xenografted with a HER2+ tumor line and CAR-TILs overexpressing RELB significantly improved tumor control over control CAR-TILs. Current work is focused on identifying tumor-reactive TCRs and tracking these TCRs after *in vitro* expansion in engineered TIL products, functional validation of RELB killing *in vivo* with matched patient-derived tumor organoid xenograft models in different cancer contexts, and using transient RELB delivery methods for clinical translation of enhanced engineered TIL products.

264 Producing Genetically Engineered, Cytokine-Secreting Allogeneic Monocytes from CD34+ Cord Blood Cells as Cell Therapy for Solid Tumors

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Introduction:
Solid tumors are challenging targets for existing cell therapies due to poor infiltration of the tumor mass by immune cells and immunosuppression in the tumor microenvironment (TME). Current approaches to overcoming these challenges have had limited success, with only two FDA-approved cell therapies (both autologous T cell-based) for solid tumors. In addition, autologous cell therapies are expensive, patient-dependent, and can require weeks to produce limited cell numbers. To address these issues, we developed an *ex vivo* process that first expands CD34+ hematopoietic stem and progenitor cells (HSPCs) from pooled healthy donor cord blood units and then differentiates the expanded HSPCs into therapeutically relevant numbers of cryopreservable monocytes, immune cells that traffic to and persist in solid tumors and play a large role in coordinating anti-tumor T cell and NK cell responses. Furthermore, we have engineered our monocytes to secrete cytokines to resist immunosuppression in the TME and to recruit immune effector cells, providing an allogeneic off-the-shelf cell therapy option with the potential to revolutionize the treatment of solid tumors.

Methods & Results:
Using RNA-seq analysis, we demonstrate that monocytes generated with our proprietary *ex vivo* culture process exhibit similar expression levels of differentiation, function, and immune-regulatory genes compared to peripheral blood-derived monocytes. We have also incorporated lentiviral delivery methods to transduce pooled cord blood-derived CD34+ HSPCs that are then expanded and differentiated to genetically engineered monocytes. Using the eGFP transgene, we demonstrate that lentiviral transduction during CD34+ HSPC expansion generates 75% eGFP+ myeloid progenitors and that these levels are maintained following *in vitro* differentiation to monocytes and macrophages. Though increased transgene size results in reduced transduction efficiency, we still achieve 51% CAR-EGFRt+ monocytes that can differentiate *in vitro* to 44% CAR-EGFR+ macrophages. We also demonstrate lentiviral delivery to HSPCs of four unique transgenes encoding immune activating cytokines followed by the mCherry reporter, resulting in stable transgene expression in differentiated myeloid cells (33-51% of monocytes, 42-59% of macrophages) and cytokine secretion by both monocytes and macrophages. With removal of the mCherry reporter from the transgene construct, we expect to achieve levels of cytokine transgene expression comparable to the high, stable levels seen with the eGFP transgene.

Conclusions:
We have validated that our proprietary *ex vivo* culture platform generates CD34+ HSPC-derived monocytes expressing a gene profile characteristic of peripheral blood-derived monocytes. Further, we have expanded the capacity of our platform, demonstrating production of genetically engineered monocytes and macrophages. The addition of lentiviral transduction to our platform allows for long-term, stable expression of transgenes resulting in engineered monocytes and macrophages that are capable of secreting cytokines. We are currently assessing the ability of these engineered monocytes and macrophages to drive anti-tumor immune responses while allowing the transduced cells to resist the immunosuppressive TME.

265 TITLE: TIGIT Deletion Rescues the Antitumor Activity of Low Avidity T cells by Increasing TCR Signal Strength

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Pancreatic ductal adenocarcinoma (PDAC) is a major unmet medical need, with a high mortality rate mainly related to the rapid development of the disease. This aggressive progression lowers the 5-year survival rate to 12%. While conventional treatment often fail, adoptive cell therapy (ACT) is a promising therapeutic option. A key barrier to the clinical success of ACT is the difficulty in isolating high-avidity tumor specific TCRs, and to generate T cells able to resist the signals from the immunosuppressive microenvironment. To select valuable target antigens, we first investigated published datasets and identified 19 relevant tumor-associated antigens. By clonal tracking of the TCR repertoire of T cells stimulated with autologous APCs loaded with immunogenic peptides derived from the selected antigens, we isolated 5 different HLA-A*0201 restricted TCRs specificities. Importantly, all these antigens are expressed in our internal cohort of primary and metastatic PDAC patients. We exploited a cytosine base editor (CBE) to generate TCR-edited T cells and enhanced their potency by knocking-out TIGIT, a key inhibitory molecule expressed on exhausted T cells infiltrating PDAC. We confirmed the specificity of each TCR-edited T cell product, which killed PDAC cell lines expressing the antigen and the proper HLA, while sparing negative controls. To boost the efficacy of anti-tumor T cells, we coupled TCR editing with TIGIT knockout (KO). By challenging $TIGIT_{KO}$ and $TIGIT_{COMP}$ T cells in orthotopic models of PDAC primary tumors and colorectal cancer liver metastases, established from patient-derived organoids, we noticed that TIGIT KO induced different effects according to the TCR avidity. Indeed, low-avidity TCR-edited T cells redirected toward MSLN controlled tumor growth only in the absence of TIGIT, which rescued their antitumor capacity against PDAC. On the other hand, high-avidity T cells controlled tumor progression independently from TIGIT expression. Mechanistically, TIGIT disruption enhanced the TCR signal strength of low-avidity T cells by increasing the phosphorylation of ZAP70, enforcing then cytoskeletal rearrangements that enhanced T cell avidity and stabilized the immunological synapse with cancer cells. These events ultimately improved the degranulation capacity of $TIGIT_{KO}$ low-avidity T cells, reaching the level of high-avidity lymphocytes. We then evaluated the safety profile of our manufacturing system, by targeted sequencing of sgRNA-dependent off-target events and ultra-deep whole exome sequencing for sgRNA-independent off-target events. By a direct comparison of CBE and CRISPR/Cas9, we observed that both editing procedures proved highly efficient in simultaneously disrupting *TRAC*, *TRBC1*, *TRBC2* and *TIGIT*. While T cell phenotype was unaffected, CRISPR/Cas9 treatment caused translocations between our four target loci and aberrant T cell expansion. We then confirmed that CBE produces high on-target editing with minimal off-target nucleotides conversion and observed that it did not introduce biologically relevant spurious DNA off-target mutations. Collectively, our findings suggest that our T cell manipulation procedure is safe and specific, and that the genetic disruption of TIGIT can rescue the anti-tumor capacity of low-avidity T cells, highlighting TIGIT KO as a potential

strategy to broaden the repertoire of exploitable TCRs for the treatment of PDAC and other solid tumors with adoptive T cell therapy protocols.

266 CRISPR-Engineered CAR T Cells for Targeting Senescence-Associated Urokinase-Type Plasminogen Activator Receptor (uPAR)

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Cellular senescence is a critical biological process that limits cell proliferation and prevents the propagation of damaged cells. However, the accumulation of senescent cells contributes to aging and disease, necessitating innovative therapeutic strategies for their selective elimination. Here, we present a novel approach leveraging nonviral CRISPR genome editing to generate potent chimeric antigen receptor (CAR) T cells to precisely target the human urokinase plasminogen activator receptor (uPAR), a senescence-associated surface protein highly expressed on pathological senescent cells.

By precisely integrating a uPAR-targeting CAR transgene into the T cell receptor, alpha chain (*TRAC*) locus, we produced CAR T cells that exhibited robust, selective cytotoxicity against both induced and patient-derived senescent cells while minimizing off-target effects on healthy proliferating cells. Additionally, uPAR-*TRAC*-CAR T cells underwent spontaneous fratricide-driven refinement, leading to the emergence of a highly pure (>99%) CAR+/TCR- population displaying a CD45RA+/CD62L+ stem cell memory-like phenotype within days (**Figure A-B**). This refinement signature is maintained post-cryopreservation, enhancing therapeutic potential and streamlining manufacturing efficiency. Furthermore, we demonstrated that this strategy can be broadly applied to enhance the manufacturing of potent CAR T cells across other targeting systems (e.g., GD2-CART, **Figure C**).

Our integration of a uPAR-CAR through precise genome engineering not only establishes a scalable and effective strategy for senescent cell clearance but also introduces a novel manufacturing strategy to autonomously generate a highly enriched population of CAR T cells. With future development, uPAR targeting CAR T cells could accelerate the development of therapeutics targeting age-related diseases, fibrosis, and cancer.

[1] Martínez-Cué C, *Front. Cell. Neurosci*. 2020. [2] Bussian, T.J., *Nature* 2018. [3] Amor, C. *Nature* 2020. [4] Amor, C. Nature Aging 2024.

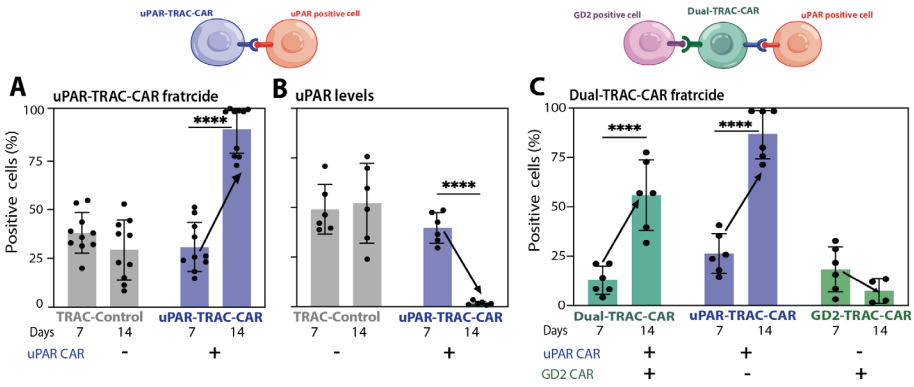

Figure. uPAR-*TRAC*-CAR T Cells Target Senescent uPAR+ T Cells in Culture and can be adapted to enhance other CAR targeting systems. (A) CAR, and **(B)** uPAR expression in T cell cultures before and after a seven-day refinement period: day 7 and 14 post-nucleofection in *ex vivo* T cell culture. uPAR-*TRAC*-CAR (blue, n=10), *TRAC*-Control (grey n=10). **(C)** CAR expression T cell cultures before and after a seven-day refinement period: day 7 and 14 post-nucleofection in *ex vivo* T cell culture. Dual-*TRAC*-CAR (teal n= 6), uPAR-TRAC-CAR (blue, n=6; i.e., *without* GD2-CAR), GD2-*TRAC*-CAR (green n=6; i.e., *without* uPAR-CAR).

267 Engineered T Cells Combining Stackable Reprogramming Technologies Enable Durable Anti-tumor Activity in Xenograft Solid Tumors

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Background
CAR T-cell therapies have achieved extraordinary outcomes in hematological malignancies, but translating this success to solid tumors requires strategies to enhance T-cell functionality and durability in the challenging solid tumor microenvironment. The number of approved cell therapies for solid tumors remains limited, with tumor-infiltrating lymphocytes (TIL) expanded from patient tumors recently receiving approval in melanoma. However, these TIL products have a highly exhausted phenotype and require systemic high-dose IL-2 for their survival, leading to toxicity in patients. Additionally, failure of T cells to expand and persist long-term and a paucity of costimulatory signals in the tumor microenvironment further limit efficacy. Lyell has developed multiple genetic reprogramming technologies to address these challenges: 1) overexpression of the activator protein 1 (AP-1) family transcription factor, c-Jun, to delay exhaustion and increase sensitivity to IL-2/15 cytokine stimulation (Lynn RC et al. 2019); 2) a chimeric costimulatory receptor (CCR) incorporating an NKG2D extracellular domain coupled with intracellular costimulatory domains to enhance activation in the context of TCR signaling; and 3) membrane-bound IL-15 (mbIL-15) driving signal transducer and activator of transcription 5 (STAT5) signaling to promote T-cell proliferation and persistence. Together, these technologies may enable the generation of potent and durable T-cell therapies, demonstrating significant efficacy and persistence *in vitro* and *in vivo* in exhausted TIL.

Methods
T-cell products were manufactured using protocols designed to maintain stemness, activated with standard reagents, and transduced with a vector encoding the following technologies alone or in combination: c-Jun, NKG2D CCR, and mbIL-15. Technologies were tested in healthy donor T cells and exhausted donor TIL. Cytotoxicity, cytokine production, and phenotype were evaluated *in vitro* after repeated antigen stimulation assays. Engineered TIL were evaluated for antitumor efficacy and engraftment *in vivo* in NSG MHC I/II double knock out mice implanted with an A375 tumor cell line engineered to express a low-affinity αCD3 molecule to activate T cells and the NKG2D ligand MICA to engage the CCR.

Results
TIL engineered with all three technologies demonstrated superior expansion and cytotoxicity over six rounds of repeated antigen stimulation *in vitro*, proliferating and killing tumor cells for 1-3 additional rounds compared to TIL engineered with two technologies, and 4-5 additional rounds compared to non-engineered TIL. During this period, TIL engineered with all three technologies enriched 40-60% and secreted at least 2-fold higher IFNγ and 6-fold higher IL-2 compared to non-engineered TIL. *In vivo*, survival of non-engineered TIL is typically transient in the absence of cytokine support, preventing durable anti-tumor efficacy (Zhao Y et al. 2022). In contrast, TIL engineered with all three technologies successfully engrafted *in vivo* without exogenous IL-2 and exhibited robust antitumor efficacy and significantly improved survival compared to TIL lacking one or more technologies (p-value=0.02).

Conclusions
Engineered T cells combining c-Jun overexpression, costimulation, and mbIL-15-driven STAT5 signaling demonstrate potent activation, robust T-cell expansion without exogenous IL-2 support, and effective, durable antitumor activity in xenograft solid tumors.

Emerging Delivery Platforms for In Vivo Gene Editing

268 Engineered Extracellular Vesicles Enable Efficient Gene Editing in the Mouse Brain

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Introduction
Gene editing holds immense promise for revolutionizing the treatment of human diseases. However, achieving safe and efficient non-viral delivery of gene editing tools in vivo remains a significant challenge, particularly to the brain. Extracellular vesicles (EVs) have emerged as promising vehicles for delivering Cas9 ribonucleoproteins (Cas9-RNPs), but their delivery efficiency is hindered by two key obstacles: suboptimal encapsulation of Cas9-RNPs and insufficient binding between single-guide RNA (sgRNA) and Cas9. Addressing these limitations is critical to unlock the full potential of EV-based gene editing.

Methods
Building upon advanced EV engineering strategies that enable highly efficient intracellular protein delivery (manuscript in revision in Nat Comm (X. Liang et. al, 2024), preprint available: https://doi.org/10.21203/rs.3.rs-3329019/v1), we further optimized the system for Cas9-RNP delivery. As reported in the preprint, our strategy for delivering Cas9-RNPs via engineered EVs utilized an intein-mediated self-cleavage. Cas9 was tethered to an EV-sorting protein via an intein linker, enabling active loading of Cas9-RNPs into EVs upon co-transfection with sgRNA. Intein's self-cleavage mechanism facilitated the functional release of Cas9 RNP from the EV-sorting domain. For further improvement of cargo release, novel temperature-sensitive intein variants were incorporated into engineered EVs. To address the second obstacle, the sgRNA binding to Cas9, we modified the sgRNA scaffold and validated its improved binding affinity using microscale thermophoresis (MST).

We tested the editing efficiency of these EV engineering strategies in vitro in reporter cells and on endogenous targets, either by flow cytometry or Sanger sequencing, respectively. Furthermore, using an osmotic minipump, we evaluated in vivo gene editing by intracerebroventricular (ICV) infusion of optimized engineered EVs into adult mice. Mice were sacrificed 2 weeks post-infusion, and the brain regions were dissected for bulk analysis with Sanger and next-generation sequencing.

Results
The use of an optimized modified sgRNA increased Cas9-RNP delivery efficiency by over 10-fold compared to our previous system in reporter cells. Further enhancement, exceeding 10-fold, was achieved by introducing a novel temperature-sensitive intein variant, which improved Cas9-RNP packaging into EVs. Combining the modified sgRNA and intein variant produced synergistic effects, resulting in a remarkable 100-fold improvement in editing efficiency for both, reporter cells and endogenous targets. To evaluate the in vivo potential of the engineered EVs, we infused them intracerebroventricularly (ICV) over 24h via an osmotic minipump to deliver Cas9-RNPs targeting the transthyretin (TTR) locus in mice. Selected brain regions were divided into infused vs. non-infused hemispheres and analyzed as a bulk sample with Sanger and next-generation sequencing (NGS), both of which methods confirmed over 30% TTR editing in the cortex and hippocampus and more than 25% in the cerebellum, irrespective of the infusion site.

Conclusion
Our work demonstrates innovative strategies to enhance EV-based Cas9-RNP delivery through sgRNA modification and improved packaging. The engineered EVs achieved remarkable gene editing throughout different brain regions

of adult mice, showing significant promise for treating central nervous system (CNS)-related diseases with non-viral delivery vector.

269 The First Coacervate-based Delivery System for Advanced Gene Therapy

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Gene therapy marks a significant breakthrough in the pharmaceutical field, necessitating the development of advanced delivery technologies to facilitate its clinical application. Here, we report the identification of a mammalian endogenous protein capable of forming coacervates with various nucleic acids. These coacervates exhibit an mRNA encapsulation capacity 1000-fold greater than lipid nanoparticles (LNPs), demonstrating efficient cellular entry, robust cytoplasmic release of nucleic acids, and broad compatibility with diverse cell types, including primary human and mouse immune cells. Based on these properties, we developed the first coacervate-based system for gene delivery, named EASY, utilizing human and mouse endogenous proteins (PCT/CN2024/124967). EASY supports a wide range of genetic cargoes for gene overexpression, knockdown, and knockout.

With EASY, we achieved over 90% transfection efficiency for mRNA encoding green fluorescent protein (GFP) in primary human T cells, while maintaining 90% cell viability. GFP expression persisted in 64% of cells up to 12 days post-transfection, highlighting its high payload capacity. EASY also achieved high transfection efficiency across various types of primary cells, including NK cells, B cells, and hematopoietic stem cells (HSCs). Notably, EASY facilitated efficient gene knockout by co-delivering Cas9 mRNA and sgRNA. For example, EASY achieved >80% efficiency in targeting the TRAC, B2M, and PDCD1 genes in human primary T cells, improving the safety and efficacy of T cell therapies. Indeed, EASY demonstrated a robust capacity for delivering large genetic cargoes, enabling simultaneous editing of up to ten genes by co-delivering Cas9 mRNA and the corresponding sgRNAs in human primary T cells, with consistent editing efficiency across all targets. To further explore its therapeutic potential, we targeted BCL11A in HSCs, a transcription factor that represses γ-globin and fetal hemoglobin expression in erythroid cells. The knockout of BCL11A increases fetal hemoglobin expression, offering a potential therapeutic strategy for hemoglobinopathies. Our results showed that approximately 80% of alleles at this locus were successfully modified, with no evidence of off-target editing in HSCs using EASY, highlighting its potential for advancing cell therapy.

In addition to gene knockout, EASY supports targeted genomic integration through homology-directed repair (HDR). By inducing double-strand breaks and delivering single-stranded DNA (ssDNA) templates, EASY achieved >30% efficiency in inserting an anti-CD19 chimeric antigen receptor (CAR) sequence into the PDCD1 gene locus. This approach generated CAR-T cells with sustained CAR expression and disrupted PD1 expression. These enhanced CAR-T cells exhibited potent anti-tumor activity in NSG mice xenografted with $CD19^+$ Raji cells.

In conclusion, EASY is a versatile, non-viral, LNP- and electroporation-free gene delivery platform that combines high efficiency, low toxicity, and user-friendliness. By eliminating risks such as insertional mutagenesis and dependence on specialized equipment, EASY represents a transformative tool for gene editing and cell therapy manufacturing, significantly expanding the therapeutic potential of CAR-T, HSC other cellular therapies.

270 Engineered Protein Delivery Vehicles Enable Single Intrastromal CRISPR-Cas9 Therapy for TGFBI Corneal Dystrophies with High Efficacy and Safety: A Comprehensive Preclinical Assessment in Non-Human Primates

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Introduction
TGFBI corneal dystrophies (CDs), driven by mutations in the TGFBI gene (e.g., R124H, R555W), are a group of inherited disorders characterized by progressive corneal opacity and vision impairment. Current treatment regimes such as keratoplasty and laser therapy are invasive, associated with recurrence risks, and fail to address the underlying root genetic pathology. CRISPR-Cas9-mediated gene editing offers a curative approach by directly disrupting mutant TGFBI to halt pathogenic protein accumulation. However, the lack of efficient and transient in vivo delivery systems for corneal keratocytes remains a major translational barrier. To address this, we developed an investigational CRISPR-Cas9 ribonucleoprotein (RNP)-based therapy delivered via engineered protein delivery vehicles (PDVs), as an investigational single intrastromal treatment for CD. PDVs combine the selectivity of viral vectors with the transient nature of TGFBI-targeting RNPs, avoiding genomic integration and prolonged exposure risks.

Methods
Preclinical evaluation encompassed *ex vivo* human corneal organoids, biodistribution studies in Ai9 reporter mice, and efficacy/toxicity assessments in non-human primates (NHPs) and rodents. NHPs received intrastromal injections at low, medium, or high doses. Ocular safety was monitored via McDonald Shadduck scoring, intraocular pressure (IOP) tracking, and histopathology over 28 days. Pharmacokinetics, immunogenicity, and safety were assessed, including serum Cas9 and vehicle structural protein (p30) detection, anti-drug antibody (ADA) assays, and non-cornea tissues editing analysis. Acute hepatotoxicity and nephrotoxicity were evaluated in rodents.

Results
In human organoids, the PDV achieved 24.2% on-target editing efficiency. In NHPs, it demonstrated dose-dependent efficacy with significant stromal diffusion and local confinement. The medium dose group exhibited a 60.4% peak efficiency. No detectable Cas9 and p30 (structural component of PDV) proteins were noted in serum. Minimal editing (<0.05%) was observed in extraocular tissues. The ocular adverse reactions were transient and manageable: the high-dose NHP group developed reversible corneal neovascularization or opacity. Transient IOP reduction (≤6.0 mmHg) occurred in high-dose NHPs but self-resolved within 7 days. Systemic ADA response was limited to the two high-dose NHPs with pre-existing anti-Cas9 titers, whereas low and medium doses showed minimal systemic ADA responses. Rodent studies confirmed unremarkable hepatotoxicity and nephrotoxicity of the PDV.

Conclusion
In this preclinical study in NHPs, a single intrastromal dose of the PDV led to dose-dependent targeted knockout of the TGFBI gene and no severe adverse events were observed. Balancing high editing efficiency with controlled local inflammation, the PDV here is a promising therapeutic candidate for TGFBI CDs and a first-in-human trial is justified.

271 CRISPR/Cas9 delivery using acerola-derived nanoparticles for targeted gene editing in the central nervous system

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We have developed drug delivery systems using extracellular vesicles including exosomes as carriers, and in particular, exosomes with cancer tissue-specific directivity by genetic modification were expected to have clinical applications. However, in consideration of drug development, (1) methods for mass purification of exosomes from cells, (2) low efficiency of nucleic acid loading into exosomes, and (3) immunogenicity issues associated with HLA expression have made practical application difficult. Therefore, to overcome the above issues, we focused on exosomes derived from edible plants instead of exosomes derived from animal cells. Previous studies have shown

that plant-derived exosomes themselves are not susceptible to degradation by digestive enzymes such as bile acids and pepsin, and from this it was inferred that they would be useful as DDS carriers that protect against degradation from digestive enzymes such as gastric acid and enable oral administration of protein and nucleic acid preparations, etc. Then we have attempted to isolate exosomes from various edible plants, and found that exosome-like nanoparticles (AELNs) derived from acerola have the most advantages in clinical applications. First, AELNs can be extracted from acerola fruit at low cost and in large quantities. Furthermore, the AELN-nucleic acid complexes are stable against digestive enzymes, indicating that AELNs can be used as DDS carriers for oral administration of nucleic acid drugs. In addition, previous studies have confirmed that fluorescently labelled AELNs administered orally to wild-type mice can reach the brain after systemic delivery. Therefore, the possibility that AELNs could be applied to brain treatment was considered. In this study, we developed a drug delivery system using acerola-derived exosome-like nanoparticles (AELNs) as carriers for oral and intranasal administration of CRISPR/Cas9 to edit genes within target organs, specifically the central nervous system (CNS). We chose AELNs due to their stability against digestive enzymes, which allows for the oral administration of sensitive protein and nucleic acid therapies. We extracted AELNs from acerola juice, achieving high recovery rates of particles sized between 200-400 nm. When these particles were combined with Cas9 protein and guide RNA (gRNA) to form ribonucleoprotein complexes (RNPs), they successfully edited the GLA gene in HEK293 and HUVEC cell lines. Notably, these complexes were as effective as RNAiMAX, a commercial gene delivery reagent, in editing genes. Furthermore, we explored using AELNs to deliver the CRISPR/Cas9 system designed to target the GGGGCC repeat expansion in the C9orf72 gene, which is implicated in ALS. By attaching GLP2 peptide to the AELNs/RNP complexes, we enhanced their uptake in cells expressing the GLP2 receptor, suggesting that GLP2 can direct these complexes to specific tissues. We demonstrated in a mouse model that oral and intranasal administration of these peptide-tagged AELNs/RNP complexes led to successful genomic editing within the brain, highlighting the potential of this system for treating neurodegenerative disorders. This study presents a novel, potentially transformative approach for in vivo genome editing using a targeted, non-invasive delivery system. Further research into the mechanisms of complex formation and the broader applicability of ligand-directed delivery systems is necessary to realize the full potential of this technology in gene thera

272 Enhancing Nanoparticle Delivery of Therapeutic Neurofibromatosis Type 1 Epigenetic Regulatory Protein Payloads *In Vivo*

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***Introduction & Methods*:**
Safe, effective delivery to the central nervous system is an ongoing challenge for the gene therapy field. One approach to solving this problem is by using an iterative design-build-test-learn (DBTL) polymer nanoparticle (PNP) discovery platform to produce and screen novel PNP designs *in vitro* and *in vivo*. For this study, top performing PNPs that had been previously produced via reversible addition-fragmentation chain transfer polymerization were intrathecally administered and evaluated for biodistribution in C57BL/6 wild type (WT) mice using proprietary nucleic acid barcoding technology. After characterizing the PNPs for size, polydispersity index (PDI), loading efficiency, transfection efficiency, and cell viability *in vitro*, three lead candidate PNP designs identified in the biodistribution study were loaded with a therapeutic payload, dosed intrathecally in WT mice, and evaluated against an FDA-approved drug, selumetinib, in a functional performance study for knockdown of multiple targets known to be associated with Neurofibromatosis Type 1 (NF1).

***Results*:**
Seven out of fifty-one PNP designs evaluated in the biodistribution study were found in either the brain or sciatic nerve tissue in WT mice. Two PNPs were found in both tissues. Altogether, these PNP designs reduced particle size and PDI, while maintaining loading efficiency and cell viability levels plus increasing transfection efficiency in Schwann cells. Four of the seven PNP designs were produced in a larger quantity using a proprietary “scale-out” approach to evaluate reproducibility and ensure sufficient material quantities were available for functional performance studies *in vivo*. Three of these four PNP designs were selected for *in vivo* testing and were safely administered intrathecally without any adverse effects in WT mice. Functional performance results revealed one

PNP design that significantly knocked down one of the gene targets in the brain. In addition, the selumetinib control caused significant knockdown of one gene in the liver, but none of the PNPs tested caused any significant knockdown of gene targets in the liver. Sciatic nerve test results are pending.

***Conclusions*:**
This set of studies evaluated biodistribution, scale-out production, and functional performance of multiple PNPs loaded with a therapeutic epigenetic regulatory protein payload. PNPs capable of delivering to sciatic nerve and/or brain tissue were identified, reproduced at larger scale, and down-selected for gene knockdown testing *in vivo*. The top 3 PNPs were safely administered intrathecally to WT mice and did not cause off-target gene knockdown effects as observed with the FDA-approved drug selumetinib. One PNP also showed significant knockdown of one gene target in the brain. Overall, these results demonstrate promising initial preclinical safety and efficacy outcomes for PNP delivery vehicles loaded with a therapeutic epigenetic regulatory protein payload intended for nervous system use.

273 *In vivo* Hematopoietic Stem Cells transduction to Deliver Neuroprotective Extracellular Vesicles for Parkinson's Disease Treatment

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The therapeutic potential of delivering Glial Cell Line-Derived Neurotrophic Factor (GDNF) to neurons using macrophage/microglial cells is promising, as GDNF supports dopaminergic neurons, protects them from degeneration, and promotes their survival—key aspects in treating Parkinson's Disease (PD). Intranasal injection of ex vivo generated exosomes loaded with GDNF effectively alleviated PD symptoms in mice. However, the ex vivo production of engineered extracellular vesicles is costly cellular, requires labor-intensive purification from conditioned cell culture medium, and treatment necessitates repeated administration. Our goal is to engineer hematopoietic stem and progenitor cells (HSPCs) in vivo to produce GDNF-containing extracellular vesicles (EVs) from HSPC-derived, brain-localized macrophage/microglial cells and deliver GDNF to damaged neurons, resulting in the cross-correction of the PD phenotype. This strategy is based on an in vivo HSC gene therapy approach that we successfully employed in the past to treat hemoglobinopathies, hemophilia A, and cancer in moue models. This approach involves the mobilization of HSPCs from the bone marrow and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd) vector. We demonstrated that HSPCs, transduced in the periphery, returned to the bone marrow and, after in vivo selection, effectively contributed to gene-modified circulating myeloid cells and brain-localized microglia cells. Towards our goal, we first engineered HDAd vectors for efficient production of extracellular vesicles that deliver reporter genes. The reporter genes (GFP, Nano luciferase, and Cre recombinase) are fused with a CD63-exosome sorting domain, which is released within EVs by mini-intein self-cleavage. We characterized EVs derived from transduced HEK293 cells for size and specificity by Dynamic Light Scattering and Western blot. We demonstrated the presence of GFP loaded into EVs using flow cytometry. We further showed successful cargo delivery by adding EVs from the supernatant of engineered HEK293 cells to recipient K562 cells, resulting in up to 90% GFP delivery. Confocal microscopy confirmed cell-to-cell transfer of EVs in producer cells. To further validate this process, we created a reporter K562 cell line using a lentiviral system to express CFP upon Cre delivery. We produced HDAd vectors encoding CD63-Cre, transduced them into K562 cells, and created a stable line that continually produces EVs containing Cre recombinase. In co-culture experiments, we observed efficient transfer of EVs, with up to 5% CFP expression in reporter cells at a 1:3 ratio of reporter to producer cells, further confirming EVs-mediated cell-to-cell transfer. For in vivo validation, we generated a double homozygous human CD46/Ai9 transgenic mouse model. (Human CD46 is the receptor for our HDAd vectors.) After injecting mobilized mice with HDAd vectors encoding Cre recombinase, with and without the CD63 sorting domain, we analyzed in vivo reporter gene (tdTomato) expression in tissues, including the brain. For PD therapeutic applications, we constructed HDAd-shuttle plasmids expressing CD63-GDNF from macrophage- and microglial-specific promoters, and transfected them into HEK293, THP-1, and BV-2 cells. GDNF loading into EVs, isolated from these cells, was demonstrated by ELISA. We are now preparing HDAd vectors for in vivo HSC transduction in a chemically induced PD mouse model.

274 Enabling Extracellular Vesicles for Targeted Gene Delivery Using Deep Learning Models-Designed Ligands

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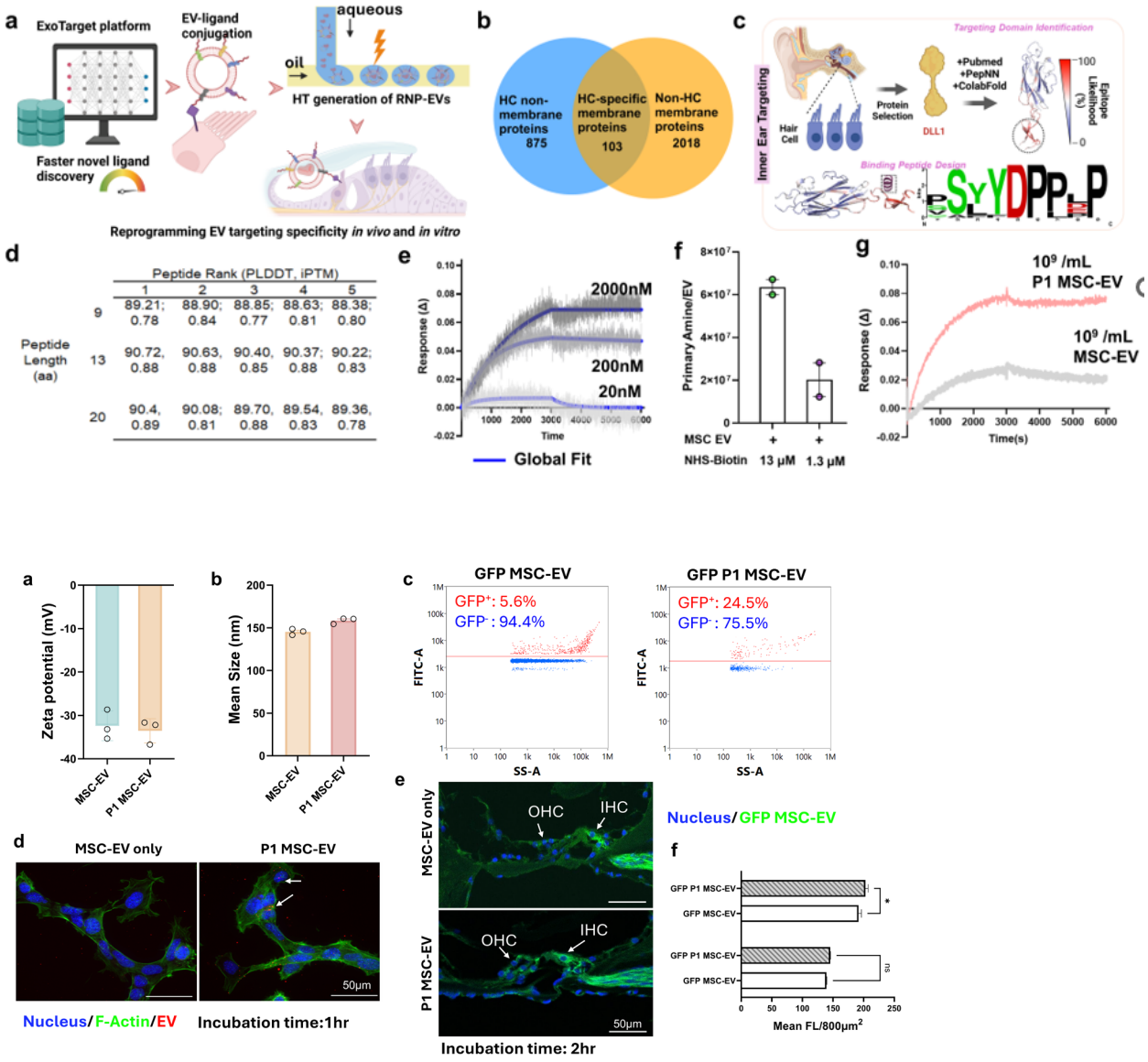
Effective drug delivery to the inner ear is essential for addressing hearing and vestibular impairments, improving health outcomes and quality of life. The inner ear, though small, is composed of highly specialized cells that perform distinct functions. Delivering the right cargo to the appropriate cell types in the inner ear is essential for developing safe and effective therapeutics for treating inner ear diseases. However, effectively generating ligands with high specificity and affinity for different cell types remains a significant challenge. Recent advancements in transcriptomic databases facilitated by single-cell sorting and sequencing technologies from gEAR portal provide valuable resources for ligand discovery for the inner ear-specific delivery.
We hypothesized that novel, cell-specific targeting ligands could be rapidly developed for the inner ear using inner ear transcriptomic database and Rosettafold-based deep learning models. The resulting ligands can then be feasibly conjugated to extracellular vesicles (EV) and screened for their ability to enhance EV-based gene delivery to the inner ear (Fig. 1a). Through database mining and comparative analysis (Fig. 1b), we identified 15 single-pass transmembrane proteins with relatively high expression in hair cells from a pool of 103 hair cell membrane proteins. Among these, Delta-like ligand 1 (Dll1) was selected as the most promising candidate for downstream ligand discovery, backed by comprehensive experimental studies. Using the PepNN model, the DSL domain of Dll1 was selected, and over 1000 peptides were generated with RFDiffusion and ProteinMPNN. AlphaFold were employed to rank and confirm peptide-protein interaction. (Fig. 1c-d) We then utilized biolayer interferometry (BLI) assays confirmed strong and consistent binding of Peptide 1 (one of the top 5 peptides) to Dll1 (Fig.1e). The N-hydroxysuccinimide ester functionalized biotinylation agent (NHS-Biotin) identified that around 10^7 primary amines per MSC EVs for the downstream peptide-EV conjugations via carboxyl-to-amine crosslinking chemistry. (Fig. 1f) The resulting Peptide1 MSC-EV (P1 MSC-EV) can effectively enhance the binding of MSC-EVs to the Dll1 protein in cell-free condition shown by BLI assay in Fig. 1g. The nanoparticle tracking analysis of P1 MSC-EV conjugates showed that there is no significant difference in size and zeta potential compared to natural MSC-EVs. The resulting P1 MSC-EVs were then electro-transfected with GFP proteins and free GFP was removed using immobilized metal affinity chromatography (IMAC) with nickel beads. The NanoFCM showed that 24.5% of GFP P1 MSC-EV has detectable GFP signals in the P1 MSC-EVs while MSC-EV has around 6%. *In vitro* cell uptake assays with $Dll1^+$ HEI-OC1 cells demonstrated effective binding within 1hr incubation period between P1 MSC-EV conjugates labelled with BioTracker 555 and target cells. The *in vivo* biodistribution study revealed differential targeting of inner hair cell (IHC) and outer hair cells (OHC) in the inner ear by P1 MSC-EV and MSC-EV. (Fig. 2e-f) These preliminary results demonstrate the feasibility of using deep learning models to design ligands for enabling EVs for targeted delivery in the inner ear.



AAV Gene Transfer (B): Ocular, Neurological & Immune Cell Systems

275 Introduction of a Lysine Acetylation Motif into the AAV2 VP1 to Enhance Macular Tropism and Intravitreal Administration

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Adeno-associated virus (AAV) vectors are the main gene delivery method for gene therapies, while capsid engineering is promising to improve tissue tropism and vector delivery efficiency. Lysine acetylation is a major posttranslational modification (PTM) involved in a broad array of physiological functions including rAAV-host cell interaction. In this study, rAAV constructs containing various peptide sequences were inserted into the VP1 loop III or IV region of AAV2 serotype. The peptide inserts contained a lysine acetylation motif within a consensus sequence of "xxxKP in which the x residues stand for those hydrophilic and uncharged amino acid residues (Q, S, T). A novel capsid AAV.N54, containing a lysine acetylation motif, xxxK_{595}P, having improved tropism to deep regions of retinas was identified through multi-species screening in mice, pigs, rabbits, and African green monkeys. Across the four species screened, AAV.N54 showed improved tropism over AAV2 and AAV.7m8, as evidenced by efficient delivery to the macular retina and detargeting from retinal ganglion cells. A 6-month study in Cynomolgus monkey eyes, injected with AAV.N54-GFP intravitreally, demonstrated a high and durable expression of GFP in macular and optic nerve head (ONH) regions. Highly purified AAV.N54-VEGF-Trap, manufactured using Sf9 and HEK293 cell culture systems was further characterized for lysine acetylation at K_{595} and was confirmed using LC-MS. The K_{595} lysine acetylation determines the selectivity of AAV.N54 capsid to target cells in retina tissue. In a laser-induced choroidal neovascularization (LCNV) mouse model, fluorescein angiography (FA) and flatmount analysis (FMA) showed that AAV.N54-VEGF-Trap prophylactically reduced retina lesions. In a DL-AAA rabbit disease model, AAV.N54-VEGF-Trap demonstrated remarkable efficacy in reducing vascular leakage and inflammation. In addition, a GLP toxicity study in NHPs demonstrated that AAV.N54-VEGF-Trap is well-tolerated. Overall, it was demonstrated that an engineered capsid, containing a K_{595} acetylation motif, has improved delivery to the macular retina and cross-species translatability was identified. A novel gene therapy using the capsid was developed for wet age-related macular degeneration (wAMD) including polypoidal choroidal vasculopathy (PCV).

276 Systemically delivered AAV5-based capsid variants enable up to 88% targeted RNA editing in primate brain

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AAV-delivered gene therapies hold enormous potential to treat neurological disease, but remain constrained by the challenges of crossing the blood-brain barrier (BBB) and AAV's inherent liver tropism. For example, our RNAfix® technology achieves over 90% targeted RNA editing in non-human primate (NHP) brain, but initial experiments using wild type AAV required invasive cranial surgery, and efficient editing was limited to injection-proximal regions. Encouragingly, novel engineered AAVs are emerging that can efficiently cross the primate BBB following systemic injection, but these capsids are almost all variants of AAV9. Given the high prevalence of anti-AAV9 antibodies in humans, these capsids may be limited in their addressable patient population. To overcome this challenge, we developed an AAV capsid engineering pipeline based on AAV5, which shows markedly lower population seropositivity than AAV9. We first disrupted AAV5's natural liver tropism by removing its known sialic acid-binding surface region, and replaced it with a massive diversity of novel potentially tissue-targeting sequences. Through progression from a billion-plex primary screen to a 5,000-plex secondary screen, we identified 30 capsids that demonstrated strong BBB penetration in NHPs. Singleplex analysis of three candidates confirmed robust BBB-crossing, with delivery of AAV DNA to >50% of brain cell nuclei while achieving >30-fold liver detargeting relative to AAV9. However, despite enabling higher RNA expression in brain relative to wild-type AAV9, absolute RNA expression remained insufficient for broad therapeutic application. To enhance RNA expression from our CNS-targeting capsids, we performed a deep mutational scan in NHP across all 30 initial candidates, identifying lead capsids with superior RNA expression in the NHP CNS. Notably, we observed that AAV5-based capsids tend to exhibit a reduced AAV RNA-to-DNA ratio relative to AAV9-based capsids, indicating impaired functional transduction. We hypothesized that the VP1/2 unique region of AAV5, which is critical for intracellular trafficking and uncoating and is divergent from almost all other serotypes, might underlie some of this deficiency. Through systematic screening of mutations and domain shuffles, we identified novel VP1/2u sequences that significantly boosted in vitro transduction across multiple cell lines for both wild-type AAV5 and its

variants. We then engineered a high performing VP1/2u sequence into our top CNS-targeting capsids and packaged a construct expressing an ADAR-based RNA editing guide RNA (gRNA) and a marker gene mRNA. We intravenously delivered to cynomolgus macaques at a dose of 1E13 vg/kg and harvested tissues after 4 weeks in life. Two highly promising capsids demonstrated robust payload expression (gRNA and mRNA detected in >40% of NeuN+ neurons across the brain) and achieved widespread targeted RNA editing (median 25-35% across brain regions). We observed particularly high transduction from these capsids in the substantia nigra (SN), a deep brain region of critical importance in Parkinson's disease (PD), with ~80% transduction of NeuN+ neurons and 69-88% editing of target RNA in this region. Together, our results represent three significant advances: the first demonstration of systemically delivered RNA editing in a primate brain; the highest primate neuronal targeting by a BBB-crossing AAV5 variant; and the highest targeting of the primate SN seen with any novel brain.

277 Rational Design to Develop Novel AAV Variants for Improved Gene Delivery to Retinal Microglia

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Recombinant adeno-associated virus (AAV) vectors have been widely used in gene therapy applications for central nervous system (CNS) diseases and have yielded favorable outcomes. These vectors can successfully transduce post-mitotic cells, such as neurons and astrocytes. Emerging evidence indicates that the microglia, the resident immune cells in the CNS and retina, play a central role in the pathobiology of neurodegenerative diseases. Consequently, these cells have become the focus of new treatment approaches. However, the targeted and effective manipulation of microglia is hampered by the lack of efficient viral and non-viral targeting methods. In addition, most studies on microglia have so far focused on the more accessible CNS population, while other populations, such as retinal microglia, have been less explored due to lower cell numbers and the lack of efficient primary culture methods.

To overcome these limitations, we have established easy and reproducible culture conditions that enable isolation, expansion and long-term culture of postnatal mouse retinal microglia cells. The method yields a high number of viable and pure cells (>85% Cd11b+Cd45+ cells) with intrinsic microglia features such as phagocytosis. The method can also be adapted to human retinal donor tissue samples from which we obtained a pure retinal microglia population (75% CD11b+CD45+ cells) displaying a characteristic immune-related marker and proteomic profile. We have used this unique *in vitro* model of retinal microglia as a screening platform to test novel AAV variants based on AAV1 and AAV6 capsids carrying genomes with a CMV-eGFP reporter expression cassette. The capsid variants carried single-point mutations of surface-exposed tyrosine, lysine, threonine, serine residues, and/or arginine residues, or the GL or NN peptide insertions from previously described AAV2.GL and AAV2.NN capsids. Capsid variants of AAV1 with the GL insertion showed transduction efficiencies that were more than 10-fold increase over the AAV1 wild-type and around 5-fold compared to the triple mutant AAV6 TM6 at 5 dpi. The increased transduction efficiency has been confirmed in resting and chemically activated microglia and allowed us to achieve dCas9-VPR (epi)genome editing to transactivate endogenous genes as well as gene knock-out without causing pro-inflammatory activation of the targeted microglia cells. Mechanistic studies suggest a more efficient nuclear entry and a higher affinity to cell-surface AAV receptors of the AAV1.GL variants. Finally, we started to validate our variants in mouse models of retinal disorders. In the Rd1 mouse model, which shows a strong induction and activation of microglia, we reached around 20% of Cd11b+Cd45+ cells after intravitreal and subretinal injection of AAV1.GL variants.

In summary, our newly developed culture method facilitates functional and molecular studies of retinal microglia cells, which have been poorly investigated to date. This innovative retinal microglia culture platform has helped to identify new, more efficient AAV capsids that could facilitate the development of future gene therapy and genome editing approaches.

278 A novel dual affinity selection (DAS) capsid screening platform for engineering AAV capsids with enhanced photoreceptor transduction via intravitreal delivery

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Inherited retinal diseases (IRDs) are a prime target for recombinant AAV (rAAV)-based gene therapy, exemplified by the FDA-approved Luxturna. Current therapies rely on invasive subretinal injections to target photoreceptors, the primary cells affected in IRDs, but this method suffers from localized efficacy and procedural risks. To address these limitations, novel AAV capsids are being engineered to enable efficient photoreceptor targeting via less invasive intravitreal (IVT) injections.

Prior studies using animal models to screen capsids for improved retinal transduction achieved only modest IVT-mediated photoreceptor targeting compared to existing capsids like 7m8. While reducing heparan sulfate (HS) binding enhanced retinal penetration, this strategy alone inconsistently improved transduction. We found HS localized to the inner limiting membrane (ILM) and inner retinal cells but absent in photoreceptors across mice, pigs, and NHPs. Conversely, sialic acids, a potential receptor class, were expressed on photoreceptor surfaces in these species.

We hypothesize that capsids with reduced HS binding and enhanced sialic acid affinity will achieve superior photoreceptor transduction via IVT injection. As proof of concept, we developed a dual affinity selection (DAS) capsid screening platform: (1) an HS-mediated transwell system to select capsid variants with reduced HS binding, followed by (2) a sialic acid-based magnetic bead pull-down assay to enrich variants with increased sialic acid affinity. A 12-mer peptide library was inserted at position N587 of the AAV2 capsid, generating a diverse library for first HS-based screening. Next-generation sequencing (NGS) identified 1,638 uniquely enriched capsid variants from the first screen with potentially reduced HS-binding ability. Among these, a leading candidate, termed IvtAAV.12mer1, was selected for in vivo validation. We produced single-stranded (ss) IvtAAV.12mer1.CB6.GFP and compared its transduction efficiency in mice via IVT injection ($2x10^9$ vg/eye) to ssAAV2.CB6.GFP and ss7m8.CB6.GFP. Our preliminary data revealed that IvtAAV.12mer1 achieved significantly more robust panretinal transduction, demonstrating a 14-fold increase compared to AAV2 and a 4.5-fold increase compared to 7m8. Additionally, IvtAAV.12mer1 showed enhanced GFP expression across all inner retinal cell types. Most notably, it exhibited a dramatic 174-fold improvement in photoreceptor transduction compared to AAV2 and a 3.5-fold improvement over 7m8, without inducing significant inflammatory cytokine expression compared to other two vectors, including TNFα, IL-6, IL-1β, and IFNγ.

To assess its performance in large animal models, we administered ssIvtAAV.12mer1.CB6.GFP and ss7m8.CB6.GFP ($1x10^{11}$ vg/eye) via IVT injection in pig eyes. Retinal whole mounts revealed similar transduction patterns for both vectors, primarily localized to the optic nerve head with gradual radial spread and diminished GFP intensity. Immunostaining confirmed transduction across all retinal cell types, including photoreceptors, but homogeneity was limited, likely due to inadequate photoreceptor-specific receptor binding. While reduced HS binding improved retinal penetration, robust photoreceptor transduction required a second screening step targeting sialic acid. We are currently conducting the second screening phase to identify capsids with optimized sialic acid binding for uniform photoreceptor transduction.

279 Targeted DART-AAVs as *In Vivo* Gene Delivery Platform for the Selective Transduction of TME Cell Subsets

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In vivo gene delivery has emerged as powerful tool for novel therapeutic concepts. A major challenge is the development of vectors capable of mediating highly selective gene transfer specifically into therapy-relevant cells. For this purpose, we have developed the concept of rational-based receptor-targeting of AAV vectors harnessing designed ankyrin repeat proteins (DARPins) in order to specifically deliver therapeutic genes into particular cell types of the tumor microenvironment (TME) such as CD8+ T cells and tumor cells (DARPin Targeting AAV: DART-AAV).

In our recent work [1], we engineered AAV2- and AAV6-based capsids displaying high-affinity DARPins specific for murine or human CD8 to specifically manipulate CD8+ cytotoxic T cells. In contrast to conventional AAV vectors, the modified vectors exhibit high selectivity for both murine and human CD8+ T lymphocytes. Upon systemic injection, the targeted vectors achieved an impressive *in vivo* gene transfer rate of up to 80%, which represents a breakthrough in the *in vivo* modification of T cells with AAV vectors. Most remarkably, near absolute selectivity for CD8+ T cells was observed in immunocompetent mouse models. The CD8-AAVs were detargeted from liver, where unmodified vectors caused a strong signal. Building on this platform, we engineered AAV9, which is known to be able to pass the blood-brain-barrier (BBB), for receptor-targeted delivery into tumor cells. Specifically, we developed HER2-AAV9 vectors displaying DARPins against the HER2/neu tumor antigen, resulting in significantly enhanced transduction of HER2+ tumor cells *in vitro*. When equipped with an anti-PD-1 or IL2 expression cassette, these vectors conferred robust checkpoint-inhibitor or IL2 secretion. In mice bearing subcutaneous HER2+ SKOV-3 xenografts, HER2-AAV9 mediated tumor-targeted delivery of a luciferase reporter with minimal hepatic transduction. To capitalize on the ability of AAV9 to penetrate BBB, we next evaluated the performance of DART-AAV9 in a glioblastoma model. The vector demonstrated a clear preference for HER2+ GL261 cells within the brain, with minimal transduction observed in healthy neural tissue after local or notably also systemic AAV administration. Moreover, when we inserted peptides described to enhance BBB penetration into the AAV9 capsid in combination with DARPin insertion, BBB penetration was further enhanced without increasing the liver transduction.

Collectively, these receptor-targeted AAV vectors exhibit exceptional efficiency and specificity, opening new avenues for directly modifying therapy-relevant cell populations *in vivo*. This platform holds promise for both, basic research and the development of next-generation gene therapy strategies, particularly in the context of tumor-specific immunomodulation.

Demircan MB, Zinser LJ, Michels A, Guaza-Lasheras M, John F, Gorol JM, Theuerkauf SA, Günther DM, Grimm D, Greten FR, Chlanda P. T-cell specific in vivo gene delivery with DART-AAVs targeted to CD8. Molecular Therapy. 2024 Oct 2;32(10):3470-84.

280 Selective Improvement in Retinal Bipolar Cell Targeting with Intravitreal Injection of a Novel AAV Capsid in Mouse and NHP

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Intravitreally (IVT) delivered optogenetic therapies that selectively target secondary neurons like retinal bipolar cells, such as in the RESTORE and STARLIGHT clinical trials, are demonstrating efficacy for patients with advanced vision loss. These trials rely on delivery by the natural AAV2 serotype with a bipolar cell-specific promoter, despite AAV2's limited transduction of bipolar cells when injected via IVT. To address this challenge, we developed and validated a novel AAV capsid that when injected via IVT has selectively improved transduction in bipolar cells across multiple animal models, outperforming all tested externally engineered capsids. This capsid's

cell tropism underscores its transformative potential in advancing optogenetic therapies for restoring vision in patients with severe retinal degeneration.

We engineered this novel capsid through in vivo screening and validation in adult cynomolgus macaque eyes. Analysis of a multiplexed capsid library delivered by IVT injection, revealed that this capsid achieved over a ~40-fold improvement in broad retinal transduction compared to AAV2, and outperformed all externally engineered capsids tested. To further validate its transduction efficiency, we conducted a head-to-head comparison by co-injecting four cynomolgus macaque eyes intravitreally with the novel capsid and an externally engineered capsid (1e10-1e11vg doses per capsid). Histological analysis revealed that the novel capsid showed expanded transduction from the peripheral retinal into the central retina, demonstrating broad and robust targeting. Notably, transduction efficiency across the retina increased by 3-4-fold compared to the externally engineered capsid, with the most significant improvement observed in retinal cells within the inner nuclear layer (INL), where transduction was increased by 4-5-fold. Co-staining with retinal markers confirmed that this transduction was primarily in PKC-α+ bipolar cells within the INL. Transduction levels in other retinal layers were not as improved, indicating that the enhanced efficiency was specific to bipolar cells. Similar evaluation in mouse retinas further supported these findings, also showcasing improved targeting to PKC-α+ bipolar cells after IVT injection. Specifically, the novel capsid showed a ~1.5-fold increase in mouse INL transduction compared to the externally engineered capsid, with over 40% of the INL layer transduced. The conserved transduction efficiency across both primates and mice illustrates the utility of this capsid for preclinical model testing of gene therapies and increases confidence in the translatability of its properties to other species, including humans.

These findings highlight the promise of this novel capsid as a powerful tool for advancing targeted gene therapies for retinal degeneration.

281 Generative AI Discovers GMU037, a Dual-Fitness Capsid with Simultaneous Superior NHP Ophthalmic Transduction and High Production Yield

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AAV gene therapy has made great progress in addressing previously intractable diseases. Despite this, many of these therapies have faced significant challenges, chief among them is the high cost to payers which limits uptake and presents a barrier to patient access, and toxicity associated with large clinical doses. Improving biodistribution and producibility of capsids could address both issues. Current molecular biology-based capsid engineering approaches have focused on improving only one property at a time, often to the detriment of the other. The capsid sequence search space (20^{735} combinations) is too large to simultaneously probe biodistribution and yield in a single capsid. Due to its ability to digitally simulate at whole-genome scale, the rapidly evolving field of generative artificial intelligence is ideal for this purpose. We have developed a pipeline that comprises a proximal exploration-based GenAI framework processed through a dual-fitness filter, to develop novel capsids with simultaneously improved tropism and manufacturability. This pipeline allowed us to investigate the unexplored sequence space around a current ophthalmic clinical capsid, interrogating >100-fold more capsids than the maximum possible by unbiased biological high-throughput screening. Using this pipeline, we uncovered a novel capsid, GMU037. Intravitreal administration of GMU037 in cynomolgus macaques resulted in 10-fold greater transduction of non-human primate peripheral retina compared to the current ophthalmic clinical capsid. At the same time, production of GMU037, either by research-grade triple transfection method or by a CMC-compatible process, yielded 10-fold higher vector genomes/cell than the current ophthalmic clinical capsid. Intriguingly, both properties are associated with a novel mutation, suggesting that our GenAI dual-fitness engine could accelerate discovery of complex first-principles of AAV biology.

Novel Models and Advances for Heart, Lung, and Kidney Gene Therapy

282 AAV-Mediated Gene Replacement Preserves Exercise Tolerance and Prevents Cardiac Remodeling in a Murine Knockout Model of SLC25A4 Deficiency

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Solute carrier family 25, member 4 (SLC25A4) is a gated pore that exchanges cytosolic ADP for mitochondrial ATP across the inner mitochondrial membrane of heart and skeletal muscle tissues. This exchange is necessary to supply substrate to complex V of the respiratory chain; functional SLC25A4 is thus a critical member of mitochondrial oxidative phosphorylation. Biallelic loss-of-function variants in *SLC25A4* result in skeletal myopathy, hypertrophic cardiomyopathy, exercise intolerance, and lactic acidosis marked by proliferation of hypofunctional mitochondria in myocytes. An ancestral frameshift variant of *SLC25A4* (c.523delC; p.Q175RfxX38) is relatively common among North American Mennonites and individuals homozygous for this variant exhibit cardiac dysfunction and progressive myocardial thickening that can culminate in end-stage heart failure necessitating transplantation.

We hypothesized that replacement of SLC25A4 activity through recombinant adeno-associated viral (rAAV) delivery could restore myocyte bioenergetics, prevent cardiac remodeling, and improve exercise capacity while normalizing tissue biomarkers and mitochondrial copy number. We generated a cross-species evolved, liver de-targeted AAV capsid (AAV.cc84) packaging the *SLC25A4* transgene under the control of a cardiac/musculoskeletal promoter. When delivered to human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) and mouse myoblasts *in vitro*, rAAV-SLC25A4 vectors enabled robust expression of SLC25A4 protein, which localized appropriately to the inner mitochondrial membrane.

We next generated and characterized an *Slc25a4* knockout mouse model (*Slc25a4 -/-*). By 14 weeks of age, *Slc25a4 -/-* mice have plasma alanine concentrations nearly two-fold higher than those of age-matched control mice ($p=0.022$). *Slc25a4 -/-* mice develop a concentric dilated cardiomyopathy characterized by increased cardiac mass, ventricular dilation, reduced contractile performance ($p=0.018$), and fibrosis. Exercise tolerance is also significantly reduced by 4 months of age ($p<0.0001$). Treatment of *Slc25a4 -/-* mice with a single systemic dose of rAAV-SLC25A4 resulted in robust SLC25A4 protein expression in cardiac and skeletal muscle target tissues, and reduced biodistribution and expression in off-target organs, including the liver. Neonatal rAAV-SLC25A4 treatment improved survival (log-rank $p=0.0482$), preserved exercise capacity ($p=0.0020$), and prevented cardiac remodeling in *Slc25a4 -/-* mice (0.0032). Functional outcomes from ongoing studies over longer time intervals will also be presented. In summary, AAV-mediated gene replacement is a promising therapeutic strategy for the treatment of SLC25A4 deficiency.

283 misiRNA: A Dual-Action mRNA Therapeutic to Treat Atherosclerosis

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Atherosclerotic cardiovascular disease (ASCVD) remains the leading cause of mortality and morbidity worldwide, placing a severe economic burden on healthcare systems. Current therapies, including lipid-lowering and antihypertensive drugs, primarily manage risk factors rather than directly targeting the diseased cells within atherosclerotic plaques. While percutaneous coronary intervention (PCI) with stenting is a common treatment for those experiencing acute coronary syndromes, it comes with significant risks, including in-stent thrombosis and neo-atherosclerosis, caused by delayed endothelial healing. One of the biggest limitations in the field is the lack of cell-

selective therapies that can specifically target diseased segments of the vasculature without harming the protective endothelium. Recently, the groundbreaking CANTOS trial demonstrated that inhibiting interleukin-1β (IL-1β) reduces recurrent cardiovascular events, highlighting inflammation as a critical driver of disease progression and demonstrating the need for targeted anti-inflammatory strategies.

To address this gap, we developed misiRNA, a pioneering, dual-action mRNA therapeutic that integrates miRNA-switch and siRNA technologies into a single mRNA construct to simultaneously inhibit plaque progression and suppress inflammation, while preserving endothelial integrity. This innovative therapeutic encodes the cyclin-dependent kinase inhibitor $p27^{kip1}$ to halt vascular smooth muscle cell (VSMC) proliferation and includes an endothelial-specific miRNA-responsive element to restrict translation in ECs, and an IL-1β siRNA within its poly(A) tail to knockdown inflammation. This unique design allows for precise therapeutic action, targeting pathogenic cells while safeguarding vascular homeostasis.

To ensure precise delivery, we used a self-assembling nanoparticle system using cell-penetrating peptides that selectively target to inflamed and damaged vascular regions, avoiding healthy tissues. *In vitro*, misiRNA effectively suppressed VSMC proliferation, reduced IL-1β expression, and preserved endothelial integrity. *In vivo*, systemic administration of misiRNA nanoparticles to $ApoE^{-/-}$ mice fed a high-fat diet led to preferential accumulation in atherosclerotic plaques and resulted in a significant reduction in plaque burden.

This novel, cell-selective mRNA-based nanotherapy has the potential to reduce the reliance on stents and could transform ASCVD treatment by selectively modulating plaque-forming cells while sparing protective endothelial cells. Furthermore, this platform technology could be adapted for other vascular diseases, representing a major breakthrough in precision medicine.

284 Corrective Editing to Treat Pseudoxanthoma Elasticum

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Pseudoxanthoma elasticum (PXE) is a rare, autosomal recessive connective tissue disorder caused by pathogenic variants in ABCC6, resulting in ectopic calcification of elastic fibers in the arteries, skin, retina, and gastrointestinal tract. Arterial calcification can lead to coronary artery disease, myocardial infarction, stroke, and intermittent claudication of peripheral arteries. The multisystemic manifestations of PXE contribute to substantial morbidity and mortality, and there are currently no targeted therapies.

Among the >300 ABCC6 variants reported in ClinVar, c.3490C>T (p.R1164X) and c.3421C>T (p.R1141X) are the two most frequent single-nucleotide variants and are potentially correctable with adenine base editing. The ABCC6 gene encodes an ATP-binding cassette protein, which functions as an efflux transporter localized to the plasma membrane mainly in the liver. While its specific substrate remains unknown, ABCC6 is a major regulator of pyrophosphate (PPi), and hepatic ABCC6 is a primary contributor of PPi to the circulation.

Motivated by these findings, we aim to develop adenine base editing therapeutics for PXE. Using prime editing or lentiviral transduction, we generated HuH-7 human hepatoma cell lines carrying the R1164X or R1141X variant. Screening various adenine base editors (ABEs) and guide RNAs (gRNAs), we identified ABE8.8 with PXE1 gRNA (targeting R1164X adenine at position 4 of its spacer sequence) and SpG-ABE8.8 with PXE2 gRNA (targeting R1141X adenine at position 5) as the most efficient at corrective editing.

To assess off-target editing with PXE1, we performed OligoNucleotide Enrichment and sequencing (ONE-seq) and targeted amplicon sequencing, verifying several off-target sites. While none pose oncogenic risks, to minimize off-target editing we explored a strategy using hybrid gRNAs in which certain positions of the spacer sequence are substituted with DNA nucleotides. We designed a series of 21 hybrid gRNAs (PXE1_hyb1 through PXE1_hyb21) with single, double, or triple DNA nucleotide substitutions and screened them in R1164X HuH-7 cells. In particular,

PXE1_hyb18, with substitutions at positions 5, 6, and 7 of the spacer sequence, reduced off-target and bystander editing while increasing on-target efficiency.

For in vivo evaluation, we developed a humanized mouse model in which a portion of mouse Abcc6 was replaced with the orthologous human sequence with the R1164X variant. Homozygous R1164X mice displayed reduced blood PPi levels and substantial muzzle calcification, mirroring PXE patients. We formulated lipid nanoparticles (LNPs) with ABE8.8 mRNA and either PXE1 standard or PXE1_hyb18 gRNA and administered them to R1164X mice. We observed that, compared to the PXE1 standard gRNA, the PXE1_hyb18 gRNA significantly increased the corrective on-target editing while reducing both off-target and bystander editing in vivo. LNP treatment of homozygous R1164X mice rescued the deficiency of PPi in the blood and, remarkably, eliminated muzzle calcification, suggesting that variant correction in the liver alone is sufficient to prevent ectopic elastic fiber calcification in distant sites and potentially address all multisystemic manifestations of PXE.

These findings support the feasibility of in vivo adenine base editing to permanently correct the two most frequent single-nucleotide pathogenic ABCC6 variants and, as a result, PXE phenotypes. This approach holds promise as one-time, long-term therapy for PXE patien

285 Delivery Route and Species-Specific Transduction Mechanisms Enable Robust Proximal Tubule Transduction of AAV-KP1 and AAV9 Irrespective of Neutralizing Antibodies in Non-Human Primates

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Kidney gene therapy using adeno-associated virus (AAV) holds promise for treating monogenic kidney diseases; however it is hindered by the organ's complex anatomical and functional structure. Additionally, presence of neutralizing antibodies (NAbs) further complicates effective transduction, limiting the eligible patient population and the potential for vector re-administration. It has been reported that even a low titer of pre-existing anti-AAV NAbs abolishes in vivo AAV transduction in non-human primates (NHPs) when administered systemically. We hypothesized that a local injection in renal pelvis can evade preexisting NAbs against AAV due to thousand times less immunoglobulin concentration in the urine than that in the blood. Here, we demonstrate that both AAV-KP1 and AAV9 can effectively and selectively transduce proximal tubules (PTs) following a real-time image-guided retrograde transureteral renal pelvis (RP) injection in both NAb-positive and negative NHPs. In addition, we reveal distinct renal transduction mechanisms via RP in mice and NHPs, where RP overcomes NAbs only in NHPs but not in mice, and enables AAV9 to effectively transduce PTs in NHPs despite its inability to transduce PTs in mice. This study involved a cohort of seven female rhesus macaques. In the initial experiments, AAV-KP1 and AAV9 were administered via RP in NAb negative NHPs and in the second experiments, AAV-KP1 and AAV9 were administered to NHPs with pre-existing NAbs. Blood samples were collected pre-injection and at multiple time points post-infusion for pharmacokinetic analysis, while tissues were harvested 3-weeks post-injection to assess AAV vector transduction and biodistribution. Similar to NAb-negative NHPs, robust transduction was observed by fluorescence microscopy in the cortex of the kidney of all three NAb-positive NHPs. Transduction pattern was also segmental corresponding to the injection path and the transduction was predominantly observed in the PTs, with no detectable transduction in glomerular cells. Notably, off-target liver transduction was almost completely suppressed in AAV-KP1 and AAV9-injected NHPs with pre-existing NAbs. In these animals, AAV was cleared from the bloodstream more rapidly compared to NHPs without NAbs. This accelerated clearance is likely attributable to the rapid removal of neutralized viral particles from the bloodstream by host immune mechanisms, implying beneficial effect of pre-existing NAbs in minimizing transduction in off-target organs including the liver. In contrast, mice injected with AAV-KP1 via RP were unable to overcome pre-existing anti-AAV-KP1 humoral immunity resulting in no significant kidney transduction in the presence of NAbs. Based on these observations, we have proposed a mechanistic model for RP-mediated PT transduction. In the model, PT transduction via RP is predominantly facilitated by basolateral entry in mice. In contrast, the primary route of PT transduction in NHPs occurs through

direct vector entry from the urinary (apical) side. This apical pathway minimizes interactions between AAV and blood or interstitial fluid containing anti-AAV NAbs, thereby enabling the vector to evade neutralization in NHPs. Thus, this study demonstrates for the first time that effective AAV-mediated gene delivery to PTs can be achieved using both AAV9 and AAV-KP1 in NHPs, even in the presence of anti-AAV NAbs. Therefore, local administration of AAV vectors via RP holds promise for clinical applications.

286 Podocyte Gene Therapy Enables Kidney Complement Modulation for IgA Nephropathy (IgAN) Treatment

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Adeno associated vector (AAV) gene therapy has the potential to treat a wide range of kidney diseases via targeted delivery of genes to podocytes, a kidney cell type instrumental in renal function. We have developed a novel AAV gene therapy platform able to deliver transgenes effectively, specifically and safely to podocytes. This approach was previously exemplified through pre-clinical development of PS-001, an AAV gene therapy that delivers the podocin gene, *NPHS2*, to the podocyte.

Here, we present a novel AAV gene therapy candidate, PS-002, that delivers the gene for complement factor I, *CFI*, to podocytes, enabling them to become bio-factories for the secretion of the complement regular CFI to specifically modulate complement activation in the glomerulus. Complement activation has been identified to play an important role in the pathogenesis of many kidney disorders including glomerularnephritis (GN). Immunoglobulin A nephropathy (IgAN) is one of the most frequent forms of GN world-wide, accounting for 25 – 50% of patients with primary GN.

PS-002 consists of a capsid that is highly effective at transducing human podocytes, and a promoter that drives gene expression of CFI specifically in the podocyte. Functional gene expression of CFI was demonstrated in *in vitro* studies in human podocytes, and PS-002 efficacy, safety, and potential for clinical translation was demonstrated in a rodent disease model and in large animal studies in Gottingen Minipigs.

Murine *in vivo* proof of concept studies were conducted in grouped ddY mice, a highly translationally relevant mouse model for IgAN. Using a murine analogue of PS-002, CFI expression was measured in kidney glomeruli and significant efficacy demonstrated via rescue of kidney function. Complement activation was inhibited in treated mice as demonstrated by a reduction in C3 deposits, and amelioration of disease phenotype by preservation of podocytes, reduction in histopathology, and decreased proteinuria.

Administration of PS-002 in pigs resulted in transgene mRNA expression across all kidney glomeruli at 28 days (100% glomeruli, n=3 pigs) and 56 days (99% glomeruli, n=3 pigs) post dosing as assessed by RNAscope, with a dose-response observed by RNAscope, qPCR and RT-qPCR. Using qPCR, RT-PCR, it was shown that, following local administration, CFI was expressed almost exclusively in the kidney glomerulus where podocytes are located, with gene expression undetectable in off-target organs such as the spleen and pancreas, and only low levels of gene expression detected in the liver. AAV delivered human CFI was detected at levels <1000 times physiological levels in the serum, demonstrating that systemic pig CFI activity would not be perturbed. Toxicological assessments of renal and other organ function and post-study histopathological assessments revealed no treatment-related safety concerns. These data demonstrate safe, consistent, and sustained transduction of all kidney glomeruli using PS-002.

Collectively, we present data that demonstrate (i) the feasibility of podocyte targeted gene therapy for glomerular specific modulation of complement, (ii) substantial non-clinical data to enable translation of PS-002 to clinical development for the treatment of IgAN, and (iii) the potential to use podocyte targeting AAV candidates to regulate complement activation in diseases beyond IgAN.

287 Developing Hotspot Prime Editors to enable therapeutic correction of multiple *CFTR* mutations in Cystic Fibrosis

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Cystic fibrosis (CF) is a life-threatening, monogenic autosomal recessive disease caused by mutations in the *CFTR* (Cystic Fibrosis Transmembrane Conductance Regulator) gene. The presence of these genetic mutations impairs the normal transport of chloride ions across epithelial cell membranes, consequently leading to the abnormal accumulation of mucus within multiple organ systems, most notably the lungs and pancreas. The mucus in the lung promotes chronic bacterial infections, inflammation and progressive lung damage, which are the primary causes of mortality in CF patients. Despite significant advancements in therapies such as CFTR modulators, patients that are responsive to the modulators require live-long treatment and more than 15% of CF patients with non-responsive mutations are left without treatment. Genetic therapies for CF have been under development utilizing various delivery methods, yet with limited success thus far. There remains a substantial unmet medical need for a single, disease-modifying treatment that can address the underlying causes of this disease for these patients. Prime Medicine is developing Prime Editors to durably and precisely correct multiple mutational hotspots with high unmet need and aiming to achieve permanent restoration of CFTR expression under endogenous control and normal CFTR function. These Hotspot Prime Editors taken together could address >93% of all CF patients.

Comprehensive high-throughput screening for Prime Editors led to the identification of potent editors capable of precisely correcting prevalent and nonsense *CFTR* mutations across multiple mutational hotspots, including the common G542X mutation. These Prime Editors demonstrated over 60% precise correction in patient-derived primary human bronchial epithelial (HBE) cells and were able to restore CFTR expression in HBE cells differentiated air-liquid interface culture. Correction of *CFTR* mutations also normalized chloride current in an electrophysiology assay, indicating a functional rescue of CFTR. To further evaluate the *CFTR* Prime Editors *in vivo* in a humanized mouse model with *CFTR* p.G542X mutation (*Cftr* $^{CFTR\ \text{p.G542X}/Cftr}$), we developed a lipid nanoparticle (LNP) system to deliver the Prime Editor components in the form of RNA (LNP-PE). We also delivered Prime Editors using a dual-AAV (adeno-associated virus) system in development. Following single doses of our LNP-PE or Dual-AAV PE, we observed encouraging editing activity in lung epithelium, including epithelial progenitor cells, prior to further in vivo optimizations, suggesting this approach can be disease-modifying. Additional data will be presented at the upcoming conference.

These findings demonstrate that Prime Editors delivered via LNPs or AAVs have the capability to accurately and effectively correct the high unmet-need pathogenic mutations causing CF. Prime Editing holds promise as a safe and efficacious curative approach for individuals affected by CF.

288 Optimized Non-Viral Fusogenix Proteolipid Vehicles for Precision Gene Therapy in the Lungs

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Despite the promise of gene therapy in treating various lung disorders, numerous clinical trials have failed to produce meaningful improvements in lung function primarily due to the limitations of current delivery platforms. Specifically, targeting the lungs after systemic injection is particularly challenging for non-viral platforms like lipid nanoparticles (LNPs) because of poor extrahepatic delivery and significant dose-limiting toxicity. The Fusogenix proteolipid vehicle (PLV) addresses these concerns by enabling efficient extrahepatic delivery while maintaining

tolerability. PLVs achieve robust intracellular nucleic acid delivery by incorporating a proprietary fusion-associated small transmembrane protein into a well-tolerated lipid formulation where it catalyzes fusion of the PLV bilayer with the membrane of target cells.

To optimize PLVs for lung-selective delivery, a panel of 20 novel PLV formulations were developed by manipulating manufacturing parameters and lipid composition. These formulations were screened for systemic DNA-GFP delivery, and we identified one formulation, called PLV-Lung (PLV-Lu), that successfully de-targeted the liver and achieved greater than 95% gene delivery to the lungs, as evaluated by GFP ELISA. Histology confirmed GFP expression in type I and II alveolar cells, as well as club cells of the conducting airways. Lung-tropism was maintained with other nucleic acid cargos, as systemic delivery of mRNA-GFP or mRNA-FLuc achieved significant expression of both reporters in the lungs with minimal expression in liver, kidneys, and spleen. Subsequently, we evaluated the ability of PLV-Lu to deliver gene editing technologies. In the Ai9 mouse model, PLV-Lu was used to deliver mRNA-Cre, leading to lung-selective tdTomato expression. Cell profile analysis using immunofluorescence and flow cytometry confirmed extensive tdTomato expression in club, ciliated, and basal stem cells of the conducting airways, as well as type I and II alveolar cells. We then optimized the PLV-Lu formulation for CRISPR-mediated gene editing and successfully co-delivered mRNA-Cas9 and sgRNA targeting the Ai9 'STOP' cassette. *In vivo*, this resulted in significant gene editing in type I and II alveolar cells, as well as club and basal cells in the conducting airways. To enhance delivery to the conducting airways, we tested whether PLV-Lu retained activity following aerosolization. Using a mesh nebulizer, we delivered PLV-Lu encapsulating mRNA-Cre and mRNA-Cas9/sgRNA to Ai9 mice and observed notable tdTomato expression in basal, club, and ciliated cells of the trachea and bronchioles.

To ensure broader applicability, we evaluated the platform's compatibility with siRNA by delivering siRNA targeting a housekeeping gene with PLV-Lu. Systemic delivery achieved 80% knockdown in the lungs and no measurable knockdown in the liver, kidneys, or spleen. Building on this, we assessed the efficacy of PLV-Lu in delivering siRNA targeting the clinically relevant endothelin 1 (EDN1) gene, implicated in pulmonary hypertension. We observed significant EDN1 knockdown in the lungs, with no knockdown detected in the liver or kidneys. This was associated with a decrease in circulating and lung EDN1 levels, as well as a decrease in endothelial-specific EDN1 expression. Overall, these results demonstrate the feasibility of using this unique PLV to selectively deliver nucleic acids to the lungs and modulate gene expression using gene editing technologies.

Modulation of Humoral Immune Responses in AAV Gene Transfer

289 Development of an Adeno-Associated Virus Expressing a Secreted T Cell Engager for Long-Term B Cell Ablation with High Transgene Expression to Minimize Vector Dose

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We previously reported an adeno-associated virus (AAV) expressing a secreted CD19-targeted bispecific T cell engager (PMID 35857488). A single intravenous dose resulted in persistent serum levels of the transgene product, GP101, and long-term elimination of B cells in marrow-humanized mice. Like CAR-T, AAV gene therapy is a single infusion with long-lasting effects, but potential advantages of AAV over CAR-T include it is off-the-shelf,

provides a gentle onset of action, doesn't require lymphodepletion chemotherapy, engages all T cells including those continuously produced from the bone marrow, and signals through the native T cell receptor. We have now made several modifications to the original construct to create a next generation vector. To reduce transgene-mediated immunogenicity, we used alternative codons to eliminate CpGs (toll-like receptor agonists), which maintained robust expression in cultured primary human hepatocytes. Based on our FDA INTERACT meeting, we also modified the downstream RNA stabilizing element derived from woodchuck hepatitis virus (WPRE) to prevent expression of "protein X," due to its theoretical potential for oncogenicity. We found that the construct using a ubiquitous promoter (CAG) exhibited higher transgene-encoded protein expression than a liver-selective promoter (LP1) in both primary cultured human liver cells transfected with plasmids and mice given intravenous AAV. The CAG promoter construct was also superior to a self-complimentary version, possibly in part due to the size constraints that necessitated using a shorter promoter (EFS) and eliminating the WPRE. We thus selected the CAG-driven vector utilizing an AAV capsid with high liver tropism, termed VNX-101, for further development. A single intravenous dose of VNX-101 in peripheral blood humanized NSGS mice was effective in eliminating leukemic cells in widely disseminated human xenograft mouse models. Treatment also eliminated bone marrow disease at the endpoint. GP101 was also still effective a year after the AAV dose as low as 1E10 vg/kg in animals initially given vector alone then challenged with tumor at day 340 and given huPBMCs at day 346. In a dose-range finding study, we found that a 3-fold increase in AAV dose resulted in ~10-fold increase in serum levels. Serum levels of GP101 were consistently above the lower threshold of the human therapeutic target range (300 pg/mL, based on clinical experience with blinatumomab) at doses of 3.0E10 vg/kg (females) and 1.0E10 vg/kg (males). In a 12-week GLP toxicity study, we observed a dose-dependent increase in serum GP101, with the lowest tested dose of 1.0E11 vg/kg resulting in GP101 concentration well above the target threshold. The No Observed Adverse Event Level was >2.7E13 vg/kg. VNX-101 treatment at 2.7E12 vg/kg was also well tolerated in hamadryas baboon monkeys with no significant changes in body weight or clinical signs reported. Collectively, these preclinical data support the efficacy and safety of VNX-101 as an off-the-shelf solution to achieve single-dose, long-term CD3 T cell – CD19+ cell engagement. We have now opened a Phase 1/2 clinical trial of VNX-101 starting at 1E11 vg/kg, called SENTRY-ALL, in patients with relapsed or refractory B cell precursor ALL, which is the first FDA-approved AAV immune-gene therapy for cancer clinical trial. Based on the mechanism of action, VNX-101 may also be applicable to B cell lymphoma, chronic lymphocytic leukemia, and autoimmune diseases.

290 Successful AAV Vector Re-administration via Two Distinct B Cell Immunomodulation Strategies in Non-Human Primates

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Introduction:
Adeno-associated viral vector (AAV) gene therapies have shown exciting promise for treatment of genetic disorders. However, a current limitation is the inability to re-dose due to development of high-titer AAV neutralizing antibodies (NAbs) that block transduction. In preclinical studies, immunomodulation has shown promise as a strategy to attenuate AAV NAb responses and enable re-dosing. However, in the clinic, common approaches such as B cell depletion with rituximab have so far yielded only modest NAb suppression. Here, in non-human primates, we tested two distinct strategies that we hypothesized would more effectively block development of AAV NAbs. First, we tested whether a more potent B cell depleter, a bispecific antibody targeting CD20 and CD3 (CD20xCD3), could enable AAV re-dosing by eliciting deeper B cell depletion in lymphoid tissues. In parallel, we tested if transient blockade of CD40, a receptor critical for B cell activation, affinity maturation, and formation of immunological memory, could also enable AAV re-dosing.

Methods:
Twenty-two AAV8-seronegative cynomolgus macaques received either no immunomodulation (Groups 1 & 2), CD40 antagonist mAb (Group 3, 50mg/kg weekly for 12 weeks), or CD20xCD3 bispecific antibody (Group 4, 0.1-0.5mg/kg weekly for 5 weeks). After three initial doses of CD20xCD3 or CD40 antibody, animals in Group 2-4 were administered an AAV8 CAG eGFP vector (1st AAV) i.v. at 1e13 vector genomes per kilogram (vg/kg). AAV NAb titers were longitudinally evaluated for 10 weeks. Subsequently, all animals (including previously AAV-naive

Group 1) received a second AAV8 vector encoding a secreted human transgene driven by a liver-specific promoter (2nd AAV) i.v. at 1e13 vg/kg. Four weeks later, the study was terminated, and transduction was assessed.

Results:
As expected, macaques receiving no immunomodulation (n=4) mounted a robust AAV8 NAb response after the 1st AAV exposure, peaking 10 weeks post AAV in most animals (NAb titer >1:1000 in 3 of 4 animals). Strikingly, however, CD20xCD3-treated animals (n=6) showed no evidence of a NAb response over the entire 10-week period. CD40 mAb-treated animals (n=6) exhibited a transient NAb response; however, titers peaked earlier and lower (2 weeks post AAV; peak titers ≤1:160) before declining to undetectable or near-undetectable levels by week 10 post AAV (4 of 6 animals ≤1:5). Animals were subsequently dosed with a 2nd AAV8 vector, and liver transduction was assessed by dPCR. As expected, animals treated with both AAVs, but without immunomodulation, showed few detectable vector genomes per diploid genome (vg/dg) in liver (Median: 0.28 vg/dg). By contrast, CD20xCD3-treated and anti-CD40-treated animals achieved much higher levels of transduction (Median: 59.4 and 40.2 vg/dg, respectively) approaching that of previously-naïve positive control animals (Median: 86.0 vg/dg). Similarly, transgene mRNA (in liver) and protein were detectable in CD20xCD3-treated, CD40 mAb-treated animals, and previously-naïve positive control animals, but not in AAV re-dosed control animals that were not treated with immunomodulation.

Conclusions:
Our findings suggest systemic AAV vector re-administration is achievable via both B cell depleting (CD20xCD3) and non-depleting approaches (CD40 blockade). Considerations of re-dosing timelines and magnitude of immunosuppression should be investigated further to understand the utility and use-case for each approach.

291 Innovative Strategies to Enhance AAV Gene Transfer in the Muscle in the Presence of Pre-existing Immunity

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Adeno-associated virus (AAV) vectors are widely used for gene therapy due to their ability to achieve stable transgene expression in a variety of tissues. However, their clinical application is often limited by host immune responses, including the development of anti-capsid and anti-transgene antibodies, which can reduce initial efficacy and prevent vector re-administration. Strategies to mitigate these immune responses are essential to extending the therapeutic potential of AAV gene therapies, particularly for chronic or progressive diseases that require repeated dosing. In this study, we focus on the muscle as a target organ for AAV-mediated gene delivery. Muscle is not only directly implicated in various genetic disorders but also serves as an efficient organ for the systemic secretion of therapeutic proteins, such as monoclonal antibodies (mAbs). Our goal is to identify vector modifications and combinatorial immunomodulatory strategies that enhance initial gene transfer and suppress anti-capsid and anti-transgene immune responses to enable safe and effective AAV re-administration.

We first evaluated whether co-administering known immunomodulatory agents could mitigate anti-capsid responses, allowing for efficient vector re-administration. Groups of C57BL/6 mice were IM administered $1x10^{11}$vg of AAV6.2FF expressing a human mAb mixed with doxorubicin(200uM), sirolimus(0.08mg/kg), SVP[Rapa](200ug), or SVP[empty](200ug). Mice were boosted in the same manner 3- and 6-months following the initial administration. Serum analysis revealed significantly lower anti-capsid antibody levels in the SVP[Rapa] treated mice compared to AAV alone, both pre and post boost; no significant increase in transgene expression was observed. As there was reduction but not ablation of anti-capsid antibodies, we then investigated whether the inclusion of a miR-142 binding site in the AAV-expressed mRNA (AAV-mAb-4x.mir142) would help suppress transgene expression in hematopoietic cells. Inclusion of the 4x.mir142 sequence in combination with SVP[Rapa] completely ablated both anti-transgene and anti-capsid responses demonstrating that genome modifications can synergize with SVP[Rapa] to further reduce antibody responses to AAV. T cell analysis was conducted on C57BL/6 mice that were administered AAV-mAb-4x.mir142 alone or in combination with SVP[Rapa] or SVP[empty], either as a single dose or two doses 3 months apart. Analysis of spleen and muscle tissue 5 days post second dose showed

an increase in exhausted $CD4^+$ and $CD8^+$ T cells (defined by $TIM3^+$, $LAG3^+$, $PD1^+$) in the muscle of the AAV-mAb-4x.mir142 + SVP[Rapa] treated mice but no impact on Treg quantity.

We then moved to using AAV expressing a model antigen, GFP-SEAP, to monitor transgene expression over time as well as facilitate more advanced immunological analyses. A total of 8 different GFP-SEAP genomes including all combinations of 4x.mir142, io2 (TLR9 antagonist sequence) and MAVI1(microprotein that suppresses type I IFN signaling) were designed. Groups of C57BL/6 mice were IM administered 3 doses of $1x10^{11}$vg of each vector 1 month apart to evaluate impacts on re-administration. Serum SEAP levels of mice treated with AAV-GFP-SEAP encoding 4x.mir142 or 4x.mir142-io2 were significantly higher than that of the control, there was no impact on the anti-capsid response. Ongoing work aims to further characterize the T cell response to both candidates with and without co-administration of SVP[Rapa] particles

292 Early Time Post-vector Activation of Complement by AAV Vectors in Nonhuman Primates

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In clinical trials with intravenously administered high dose adeno-associated vector (AAV) gene therapy, there are reports of complement activation-related serious adverse events (SAEs), including thrombotic microangiopathy and atypical hemolytic uremic syndrome. Although AAVs have been well tolerated in preclinical animal models, SAEs observed in clinical trials involving high dose AAVs suggest a need for improved preclinical models to understand AAV-induced complement-related immune responses. As a model for AAV-related complement-mediated adverse events, the present study assessed immediate (first hour post-vector administration) and late (1-10 days post-vector administration) complement activation in African Green non-human primates (NHP) with pre-existing immunity to AAV vectors. A NHP (7 kg) previously exposed to AAVrh.10 (serum AAVrh.10 neutralizing antibody titer, $2.7x10^2$) was administered AAVrh.10mCherry ($1.2x10^{13}$ gc/kg; intravenous), with repeat administration of AAVrh.10mCherry ($1.2x10^{13}$ gc/kg; intravenous) 2 wk later. Blood was collected before and at 10, 30, and 60 min, and then at 1, 3, and 10 days post each administration and serum assessed for terminal complement complex, the end-product of all complement pathways (Terminal Complement Component (TCC) ELISA, Svar). No immunosuppression was administered. No abnormal clinical signs, or abnormalities in hematology or serum chemistry parameters were observed. There were increases in serum total and neutralizing anti-AAVrh.10 antibodies resulting from the AAVrh.10 administration. No complement activity was present prior to 1st AAVrh.10 challenge but increased TCC levels (2,950 ng/ml at 10 min, peaking at 3,869 ng/ml at 60 min) were observed during the 1st hr post-AAVrh.10 challenge. By 24 hr timepoint post-1st challenge, TCC levels were reduced to 113 ng/ml and were undetectable at 10 days. A similar pattern was observed after the 2nd AAVrh.10 challenge, with TCC levels at 5,381 ng/ml at 10 min, peaking to 6,680 ng/ml at 30 min. Higher complement activation following the 2nd AAV administration suggests the effects were mediated by antibody binding to AAV capsids. The TCC levels were barely detectable >24 hr timepoint post-2nd challenge, with 195 ng/ml at 24 hr, 163 ng/ml at 72 hr and 180 ng/ml at 10 days. These data demonstrate repeatable AAV-mediated serum complement activation in an NHP with TCC activation limited to within the 1st hr post-AAV challenge and disappearing by 24 hrs. Importantly, this AAV-mediated early time point activation might be missed in clinical trials unless assessments of complement activation are made within the 1st hr post vector administration.

293 Potential for AAV-SLB101-mediated gene transfer treatment in the context of natural seropositivity and after an AAVrh74 treatment

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Adeno-associated virus (AAV)-mediated gene therapy is dependent on effective viral transduction and transgene expression in the target organs. Several studies have shown that pre-existing antibodies to AAV acquired either from natural infection or a previous AAV gene therapy treatment leads to inhibition of AAV transduction. Pre-existing titers are measured using a transduction inhibition assay for anti-AAV neutralizing antibodies (NAb) or an ELISA for anti-AAV total IgG antibodies (TAb). Often, the assay cut-off determined by the sensitivity of the assay is the titer threshold used for determining eligibility of patients for gene therapy treatment, however, this assay cut-off does not take into consideration the impact of the pre-existing antibodies on transduction. To evaluate the relationship between pre-existing titers and their effect on transduction, passive transfer studies were performed in mice to identify the TAb IgG titer values that reduced AAV transduction in target organs.

Immunocompromised Rag2 KO mice were infused with human serum at various anti-AAV-SLB101 TAb IgG titers 1 day before administration of 2.5E13 or 1E14 vg/kg AAV-SLB101-Luciferase. AAV-SLB101 biodistribution and expression were measured by whole body IVIS imaging and in target tissues to determine the impact of pre-existing titers. Loss of luciferase expression was dependent on pre-existing TAb IgG titer, AAV-SLB101-Luciferase dose, and the target organ. Luciferase expression at the 1E14 dose was observed in the quadriceps at titers up to 1:320 while expression in heart and diaphragm tissues was equivalent to control and not impacted at any of the TAb titers tested up to >1:640. These data highlight the capacity for AAV-SLB101 transduction in the presence of low anti-AAV titers. Findings warrant further investigation in larger models to enhance characterization of the relationship between assay cutoff and impact on transduction for clinical trial inclusion.

Several studies have shown that pre-existing NAb/TAb titers associated with natural AAV infections are orders of magnitude lower than the anti-AAV antibody titers post gene therapy treatment. To investigate the potential for effective AAV-SLB101 transduction after an AAVrh74 treatment, anti-AAVrh74 and anti-AAV-SLB101 NAb titers were measured in serum from mice, NHPs, and Duchenne muscular dystrophy (DMD) patients previously dosed with an AAVrh74 gene therapy. While AAVrh74 NAb titers were extremely high, the cross-reactive AAV-SLB101 NAb titers were substantially lower in magnitude, in a range closer to natural seropositivity. These low cross-reactive titers may provide the intriguing possibility of a therapeutic window for dosing patients with an AAV-SLB101 treatment after an AAVrh74 gene therapy.

294 KJ103: An igg-Degrading Enzyme That Reduces Neutralizing Antibodies Against AAV2 and AAV8 to 10% with a Single Dose, with Repeat Dosing Capability Allowing Further Reductions and Potential for AAV Re-dosingkj103: An igg-Degrading Enzyme That Reduces Neutralizing Antibodies Against AAV2 and AAV8 to 10% with a Single Dose, with Repeat Dosing Capability Allowing Further Reductions and Potential for AAV Re-Dosing

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The presence of pre-existing AAV-neutralizing antibodies (NAbs) in humans significantly challenges AAV-based gene therapy. We developed KJ103, a novel recombinant human IgG-degrading enzyme from a non-human, non-pathogenic subspecies, characterized by rapid action, high efficiency, and low immunogenicity. It is anticipated as a pretreatment strategy to reduce NAbs, broadening patient eligibility and enhancing gene therapy safety and efficacy.

In two randomized, placebo-controlled, single-ascending dose Phase 1 trials (0.01, 0.04, 0.12, 0.25, and 0.40 mg/kg) with healthy volunteers in New Zealand and China, we evaluated KJ103's safety, tolerability, pharmacokinetics (PK), pharmacodynamics (PD), and IgG cleavage efficacy. The data confirmed KJ103's safety and tolerability, with no serious adverse events (SAEs) or infections. IgG levels rapidly decreased within 45 minutes, staying minimal for a week before gradually returning to baseline, with results from both trials showing high concordance.

Notably, we assessed AAV2 and AAV8 NAb titers in clinical samples to validate KJ103's potential in enhancing gene therapy outcomes. A single dose of KJ103 exceeding 0.25 mg/kg was generally effective in increasing the NAb titer threshold for AAV-based gene therapy by approximately tenfold, regardless of the initial titer. For subjects with

high titers, an additional dose of KJ103 may be necessary to further reduce NAbs. We correlated IgG, F(ab')2 levels, and NAb titers to define therapeutic time windows for various clinical applications.

Overall, KJ103 can elevate the enrollment threshold for AAV NAb titers, enabling broader patient inclusion. This positions KJ103 as a promising candidate for reducing toxicity through lower dosing while also decreasing treatment costs. Furthermore, by effectively lowering NAbs and permitting its own repeat dosing, KJ103 may unlock viable re-dosing strategies for AAV-based therapies.

295 AAV-vectored PD-L1 Co-Expression as a Strategy to Enhance AAV-Delivered bNAb Efficacy

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Background: Immune responses often lead to the loss or subtherapeutic expression of rAAV-vectored transgenes. Such immune responses are a major barrier to the efficacy of AAV-vectored anti-HIV-1 broadly neutralizing antibodies (bNAbs) for the treatment and prevention of HIV-1 infection. The immune checkpoint molecule programmed cell death protein ligand 1 (PD-L1) interacts with programmed cell death protein 1 (PD-1) on T cells to suppress immune activation and promote tolerance. We previously reported that co-delivery of AAV9-vectored PD-L1 and 10-1074, (a bNAb), could suppress host anti-10-1074 immune responses, enabling persistent 10-1074 expression, which conferred protection against SHIV-AD8 challenge in nonhuman primates. Here, we extend these findings to 3BNC117, a more somatically hypermutated and thus more immunogenic bNAb, demonstrating applicability of this approach to other AAV-delivered therapeutics.

Methods: Five groups of six rhesus macaques each received the following vectors: 1) AAV9.PD-L1; 2) AAV9.10-1074; 3) AAV9.PD-L1 and AAV9.10-1074; 4) AAV9.3BNC117; and 5) AAV9.PD-L1 and AAV9.3BNC117. Serum antibody concentrations were determined by gp120 ELISA and B and T cell reactivity were assessed by anti-drug antibody ELISA and by ELISpot assay, respectively. Beginning at 20- or 30-weeks post AAV9 administration, all animals were challenged biweekly via low-dose, intrarectal challenge using SHIV-AD8. Protection from SHIV challenges was determined by monitoring plasma viremia by qPCR. Additionally, to assess the safety of our approach, we have ongoing studies to determine the immunomodulatory effect of AAV9.PD-L1 in PBMCs by RNA-seq and off-target AAV transduction in various organs by droplet digital PCR post necropsy.

Results: All six macaques that received AAV9.10-1074 and AAV9.PD-L1 had sustained concentrations of 10-1074 throughout the study compared to four of six macaques that only received AAV9.10-1074. Five of six macaques that received AAV9.3BNC117 and AAV9.PD-L1 had sustained concentrations of 3BNC117, compared to three of six macaques that only received AAV9.3BNC117 over the 52 weeks of the study. Macaques that had sustained bNAb expression for the duration of the study had lower anti-10-1074 or anti-3BNC117 antibodies and lower T cell reactivity to bNAbs compared to macaques that had declining bNAb expression. Lastly, all macaques that achieved sustained expression of 10-1074 resulted in significant protection from SHIV-AD8 challenges. Macaques that received AAV9.3BNC117 and AAV9.PDL1, but not AAV9.3BNC117 alone were significantly protected from SHIV-AD8 challenges, underscoring the utility of AAV9.PD-L1 in this study.

Conclusions: These studies suggest that co-expression of PD-L1 at the site of administration reduces the host immune response to an AAV-expressed transgene in rhesus macaques. We propose that our vectored PD-L1 co-expression strategy can facilitate the sustained expression of other viral-vectored transgenes.

Lipid Nanoparticles II

296 Novel Cyclohexane Based Ionizable Lipids (CHILs) for Non-viral Liver Delivery of Complex Nucleic Acid Genetic Medicine Payloads

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Gene editing technologies have the potential to transform medicine, especially if one can optimally deliver such technologies into patients as an in vivo genetic medicine. Key advancements include the development of powerful but complex technologies requiring co-delivery of multiple nucleic acid payloads, such as high-fidelity nucleases and transposon systems. These technologies offer the possibility for functional cures of genetic diseases. However, nucleic acid delivery to preferred cell types remains a challenge; in particular, mixed nucleic acid or DNA-containing payloads must be delivered in a safe and efficient manner.

Compared to viral systems, lipid nanoparticle (LNP) systems offer potential immunogenicity, nucleic acid payload versatility, repeat dosing, and manufacturing advantages. LNPs are typically comprised of a mixture of ionizable (IL), naturally occurring, and pegylated lipids. Most used ILs were designed to deliver single RNA(siRNA or mRNA) payloads and not mixed nucleic acid payloads like two guide RNAs (gRNAs) and a mRNA (as required for Cas-CLOVER™) or mRNA and a plasmid DNA (pDNA) (as required for the Super piggyBac transposon system (SPB)). We have recently identified a novel ILs containing cyclohexane hydrophobic moieties(aka CHILs). Cyclohexane was selected based on its ability to adopt different confirmations to form stronger hydrophobic associations than linear hydrophobic moieties. Cyclohexanes are also more resistant to oxidation than linear moieties containing double bonds such as those found in common ILs, like Dlin-MC3-DMA (MC3), and may have stability advantages.

A panel of CHILs was prepared to optimize cyclohexane placement. An initial screen in adult mice was performed using LNPs comprised of a base lipid composition and either mRNA or pDNA encoding luciferase. LNPs containing promising CHILs were further optimized utilizing a design-of-experiment (DoE) approach to optimize liver bioluminescence and minimize cytokine release and transaminase levels in adult mice. This yielded an evaluation of cyclohexane-based hydrophobic tails and identification of a preferred monoamine core,which was key for maximizing DNA delivery efficacy and tolerability. These findings provide insights into the structure-function relationships of CHILs and offer a promising platform for developing liver-directed nucleic acid delivery systems.

The lead CHIL, HBC365, has been deployed in PoseidaTherapeutics' hereditary angioedema (P-KLKB1-101) and hemophilia A (P-FVIII-101) programs. For P-KLKB1-101, LNPs containing HBC365 and the requisite Cas-CLOVER™ mRNA and guide RNAs have been optimized to promote robust gene editing, a sustained reduction in plasma kallikrein, and a desirable safety profile in adult mice and non-human primates. For P-FVIII-101, the durable expression of clinically relevant levels of clotting Factor VIII was achieved in adult mice when DNA cargo-optimized HBC365 LNPs were used containing an mRNA encoding the SPB transposase and a pDNA containing the Factor VIII transposon. Data demonstrating the evolution of this novel class of CHILs from conception through the identification and integration of HBC365 into P-KLKB1-101 and P-FVIII-101 programs will be covered in this presentation.

297 A CNS-Tropic Lipid Nanoparticle Platform Inspired by Natural Brain Chemistry

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Neurological conditions are the leading cause of illness and disability worldwide, but for many of these conditions, there are no disease modifying therapies available. While symptomatic treatments have improved quality of life for some patients, there remains a clear clinical need for targeted medicine development in this field. In recent years, genetic medicine—including gene replacement (mRNA), gene silencing (RNAi), and gene editing (such as CRISPR-Cas9) approaches—has emerged as a promising alternative to conventional small molecule drugs. Furthermore, human genetic studies have identified several genes that modifying onset and or progression of neurological diseases such as Huntington's disease, Parkinson's disease and many others. Although such genetically validated targets are attractive candidates for modulation by nucleic acid therapeutics, translation to the clinic remains challenging primarily due to difficulties in efficient and safe delivery of nucleic acids to central nervous system (CNS).

To overcome this barrier, Script Biosciences is using a proprietary CNS-specific lipid nanoparticle (LNP) platform—originally developed in the Whitehead Lab at CMU—capable of delivering nucleic acid cargo directly to the mammalian CNS, with minimal distribution to peripheral organs such as liver or spleen (Figure 1A). These novel LNPs incorporate lipids inspired by those found naturally in the brain but have similar physiochemical characteristics to previous FDA-approved LNPs used clinically.

Here we demonstrate that Script's CNS-LNP can not only efficiently encapsulate mRNA, siRNA and CRISPR-Cas complexes, but also effectively deliver them to cells both in vitro and in vivo. We show LNP-mediated transfection of ~95% of cells in vitro resulting in robust reporter mRNA expression (Figure 1D). Intrathecal injection of LNP-encapsulated mRNA results in widespread biodistribution throughout the brain and spinal cord of transgenic rodents as judged by TdTomato reporter gene activation in ~80% of neurons (Figure 1B, C). We also demonstrate efficient siRNA encapsulation and delivery: using the MALAT1 gene as a model target, our LNP-encapsulated MALAT-1 specific siRNAs achieved ~85% knockdown of MALAT-1 in vitro (Figure 1E). Finally, encapsulation and delivery of Script's proprietary gene editing system—CasPlus—can be also achieved using the CNS-LNP. In initial in vitro tests using TRAC as a model gene editing target, indel formation was seen in ~95% of sequences (Figure 1F), indicating high efficiency of cargo encapsulation, delivery, and functionality.

In conclusion, this CNS-LNP platform shows significant distribution and delivery to the rodent brain and can efficiently deliver various therapeutic RNA types. Our proprietary LNP platform paves the way towards addressing a wide range of neurological diseases by RNAi-mediated knockdown, CRISPR knockout, or gene augmentation approaches.

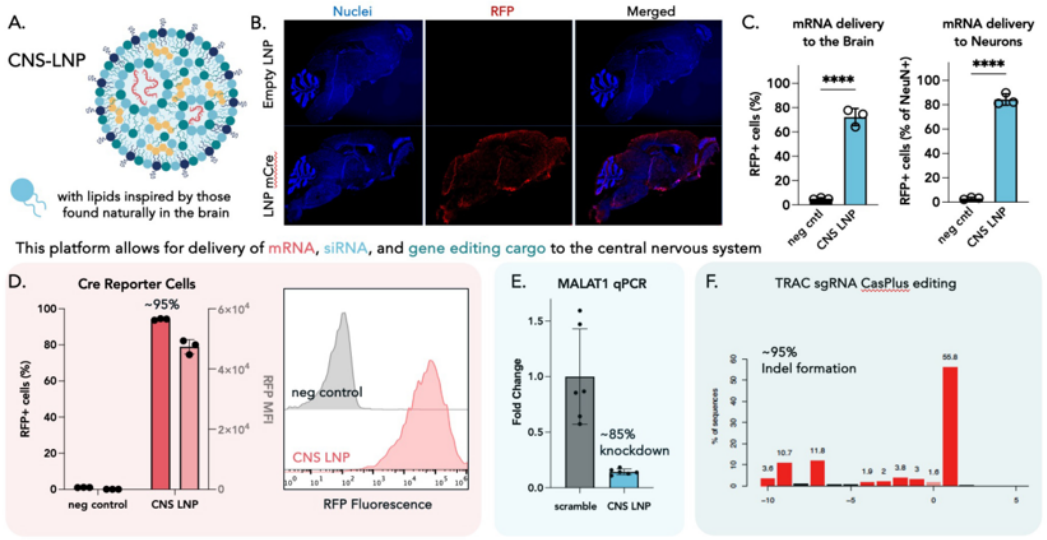

Figure 1. (A) A CNS lipid nanoparticle (LNP) platform for the delivery of multiple nucleic acid cargoes. (B) In Cre-reporter rodents, mRNA delivery and functionality can be seen throughout the brain. Visualized using immunofluorescence imaging. (C) The CNS-LNP delivers to ~70% of the brain and ~85% of neurons, measured using flow cytometry. (D) Cre mRNA can be delivered to ~95% of cells in vitro using the CNS-LNP. (E) MALAT1-targeting siRNA can be delivered to cells in vitro using the CNS-LNP, resulting in ~85% knockdown, measured by qPCR. (F) The CasPlus editing system—including sgRNA targeting TRAC—can be delivered via the CNS-LNP, resulting in indel formulation in ~95% of sequences, using Sanger sequencing and TIDE analysis

298 Ocular Gene Therapy Using the Proteolipid Vehicle Nucleic Acid Delivery Platform

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Purpose:
Age-related macular degeneration, Stargardt disease, and glaucoma are prevalent eye conditions that lead to vision loss. Gene therapy holds potential to combat and prevent vision impairment; however, conventional viral and non-viral gene delivery platforms face limitations due to challenges with nucleic acid delivery, immunogenicity, and tolerability. To address these limitations, we developed the Fusogenix™ proteolipid vehicle (PLV™) nucleic acid delivery platform. The PLV platform utilizes a proprietary fusion-associated small transmembrane (FAST) protein and well-tolerated lipid formulations to achieve efficient nucleic acid delivery to tissues throughout the body. FAST protein catalyzes the fusion of the PLV bilayer with the plasma membrane of the target cells, enabling delivery of encapsulated nucleic acid cargo directly into the cytoplasm, resulting in efficient and highly tolerable nucleic acid delivery to various tissues throughout the body. Here, we assess ocular gene delivery using the FAST-PLV platform.

Methods:
The FAST-PLV platform was used to deliver mRNA or DNA in vivo in mice, rats, and non-human primates. Routes of administration in vivo included subretinal injection, intravitreal injection, and subconjunctival administration. Evaluated outcomes included gene expression, protein expression and localization, tolerability (including pro-inflammatory markers, optical coherence tomography, and immunohistochemistry), and gene function.

Results:
The FAST-PLV platform enabled robust delivery and expression of mRNA and pDNA cargo in primary mouse and human retinal cell cultures without cytotoxicity. Intravitreal (IVT) injection of FAST-PLVs encapsulating pDNA or mRNA encoding firefly luciferase or eGFP resulted in localized and prominent expression in the eyes of both mice and rats. Immunofluorescence analysis confirmed stable expression in retinal ganglion and photoreceptor cells. To evaluate gene editing with PLVs, we assessed the co-delivery of mRNA-Cas9 and sgRNA targeting the Ai9 mouse

'STOP' cassette. IVT injection in Ai9 mice induced high tdTomato expression in photoreceptor cells, indicating effective gene editing. OCT imaging confirmed that PLVs were well tolerated. Next, we assessed the ability of PLVs to deliver clinically relevant cargo. We designed a pDNA vector encoding an anti-VEGF antibody as a potential treatment for wet AMD. IVT injection of PLVs encapsulating this construct in rats and mice resulted in high antibody expression detected in the vitreous for 60 days post-injection. The antibody was functionally active, and markedly reduced angiogenesis in a model of corneal neovascularization induced by alkali burn injury, 21 days post-PLV administration. We then designed a pDNA vector encoding the ABCA4 gene, which is impaired in Stargardt disease, encapsulated it within PLVs, and delivered it in rats by IVT injection. Expression of ABCA4 was observed 7 days post-injection in the retina, and co-localized with rhodopsin-positive photoreceptors. Building on these findings, we evaluated PLV activity in non-human primates. Subretinal injection of PLVs encapsulating pDNA led to widespread, robust expression in photoreceptor cells and the retinal pigment epithelium. Furthermore, slit-lamp examinations and OCT confirmed high tolerability following these injections.

Conclusions:
These findings highlight the promising potential of the FAST-PLV gene therapy platform for the treatment of multiple ocular diseases

299 Applying RNA-lipid nanoparticles to modulate protein expression in swine

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Background:
Genetic manipulation of rodents is fundamental to research in biology and medicine, however it is costly and challenging in large animal models. In vivo delivery of RNA is promising for the study of gene expression and the treatment of genetic diseases. Lipid nanoparticles (LNP) efficiently deliver small interfering RNA (siRNA) and messenger RNA (mRNA) to either knock down or overexpress specific proteins, representing a potential system for creating genetically modified large animals. LNP naturally accumulate in the liver, a major synthesis site for many blood circulating proteins. Swine are a preferred animal model for studying the cardiovascular and immune systems, but there are limited strategies for genetic manipulation, as they are sensitive to LNP-induced complement activation-related pseudoallergy (CARPA).

Aims:
Develop siRNA- and mRNA- LNP agents that can knock down or overexpress proteins in swine and subsequently determine the biodistribution of mRNA-LNP protein expression in a large animal model.

Methods:
We identified siRNA sequences targeting two coagulation proteins in swine: fibrinogen (siFga) and plasminogen (siPlg). We tested these sequences in silico and developed an mRNA sequence to express a reporter protein, NanoLuciferase (mNLuc). siFga, siPlg, and mNLuc were encapsulated in a clinically relevant LNP formulation and injected intravenously at a medicinal dose in swine. Knockdown and expression were analyzed based on antigen and mRNA expression levels.

Results:
siFga and siPlg efficiently modulated systemic blood coagulation and knocked down fibrinogen or plasminogen plasma protein, respectively, to below 5% of baseline values. When swine were injected with mNLuc, protein expression was detected in the liver, spleen, lung, heart, uterus, colon, stomach, kidney, small intestine, and sometimes brain. Furthermore, protein expression was found in the bone marrow, including megakaryocytes, hematopoietic stem cells (HSC), granulocytes, and in circulating blood cells such as white blood cells and platelets but not red blood cells. Swine treated with these LNP agents showed no clinical signs of CARPA.

Conclusion:
These findings advance RNA therapies in swine, and create a platform for generating large animal models for protein deficiencies and other genetic defects.

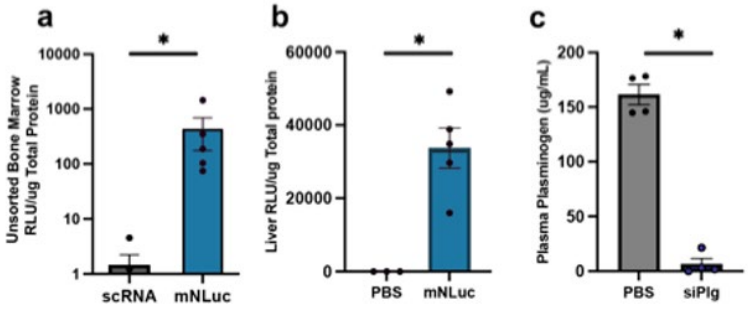

Figure 1: Proteins can be overexpressed or knocked down by intravenously injecting RNA therapies in swine. a) Bone marrow and b) hepatic NanoLuciferase protein signals measured *ex vivo* 24 h post-injection. c) Plasma levels of circulating plasminogen following siPlg or PBS. N = 4-5, **P<0.05*; Error bars represent mean ± SEM.

300 Leveraging Novel PEG-free Lipid Nanoparticles for Tissue and Cell Targeting While Reducing Immunogenicity and Toxicity

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Leveraging Novel PEG-free Lipid Nanoparticles for Tissue and Cell Targeting While Reducing Immunogenicity and Toxicity

Purpose:
Lipid nanoparticles (LNPs) are promising non-viral vehicles for delivering nucleic acids *in vivo* for gene and cell therapies. However, conventional LNPs primarily accumulate in the liver when systemically delivered, limiting their ability to target extrahepatic tissues and cells while also contributing to liver toxicity. Furthermore, these LNPs struggle to maintain integrity during nebulization for aerosol formation, making it difficult to deliver them to the lungs via inhalation. Additionally, conventional LNPs typically contain polyethylene glycol (PEG)-lipid, which can provoke PEG-mediated immunogenic responses, potentially leading to anaphylaxis, pseudo-allergic reactions, accelerated blood clearance, and challenges in repeated dosing. In this study, we present a groundbreaking LNP system, Cytofinity™ LNP, which is entirely PEG-free and can target T cells in the blood and spleen, as well as hematopoietic stem cells (HSCs) in the bone marrow through systemic delivery. The Cytofinity™ LNP can also effectively deliver nucleic acids to the lungs via nebulization and inhalation.

Approach:
We aimed to replace the PEG-lipid in conventional LNP formulations with gangliosides, which are naturally occurring in humans and animals, making them unlikely to be toxic or immunogenic. Gangliosides consist of a hydrophilic head with carbohydrates and sialic acids, and a lipid tail. Each ganglioside can bind to Siglec receptors on various cell types, allowing Cytofinity™ LNPs to target specific cells. By varying the type and amount of ganglioside, we can create LNPs with different tissue and cell targeting capabilities and performance characteristics.

Results:
We developed Cytofinity™, a PEG-free LNP that exhibits several unique features: 1) The exclusion of PEG eliminates PEG-mediated immunogenicity and allows for repeat dosing. 2) It enhances the delivery of RNAs to bone

marrow cells, increasing transfection efficiency by 3.5 times compared to conventional LNPs. 3) It boosts the delivery of RNAs to T cells by threefold. 4) It reduces liver expression of the delivered payload by ninefold. 5) The Cytofinity LNP retains its integrity during the nebulization process, whereas conventional LNPs experience increased particle sizes and reduced encapsulation efficiency. We further demonstrated that nebulized luciferase mRNA-loaded Cytofinity™ LNPs can be delivered to mice through inhalation, leading to successful expression of bioluminescence in the lungs. Immunohistochemistry studies of lung tissue collected from mice that inhaled the luciferase mRNA-loaded Cytofinity™ LNPs showed that various lung cells, including endothelial, epithelial, and ciliated cells, were all successfully transfected by the inhaled Cytofinity™ LNPs. 6) The Cytofinity LNP incorporating a Cas9 and a LoxP sgRNA demonstrated effective gene editing in CD34+ cells in the bone marrow in Ai14 mice.

Conclusions:
We have successfully developed Cytofinity™, a PEG-free LNP, by substituting the PEG-lipid with a natural ganglioside. These LNPs not only avoid PEG-related issues but also deliver nucleic acid payloads to various extrahepatic tissues and cells, including the bone marrow, lungs, T cells, and HSCs.

301 Retargeting Lipid Nanoparticles for Optimized mRNA Delivery in Non-Human Primates

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Messenger RNA-lipid nanoparticles (mRNA-LNPs) have been widely investigated and FDA-approved as intramuscular (IM) vaccines. Notably, mRNA-LNPs are under investigation in numerous therapeutic settings beyond infectious disease prophylaxis. We are interested in developing mRNA-LNPs as boosting agents to augment antigen-specific lymphocytes and engineered effectors such as chimeric antigen receptor (CAR) T cells. We and others have described antigen boosting strategies for solid tumors as well as infectious diseases such as human immunodeficiency virus (HIV). Whereas the IM dose route predominantly delivers antigen to draining lymph nodes to initiate potent adaptive immune responses, alternate dose routes and lipid composition may enhance the potency and efficiency with which mRNA-LNPs boost adaptive immune cells, particularly in secondary lymphoid tissues. We present a novel non-human primate (NHP) model to identify and evaluate innovative LNP formulations and dose routes. Our model provides unique and unprecedented opportunities to extend and preclinically qualify disease-specific applications for mRNA-LNPs. We established latent infection with an HIV-like virus (simian/human immunodeficiency virus, SHIV.C.CH505.v2) in rhesus macaques (RMs, n = 9). Each animal was injected intravenously (IV) or intranodally (IN) with one of two LNP formulations encapsulating eGFP reporter mRNA. Twenty-four hours later, animals were sacrificed in order to systemically quantify mRNA-LNP trafficking, cellular uptake, and expression of eGFP by flow cytometry and immunohistochemistry. We assayed toxicity via serum chemistry, histology, and cytokine assays, focusing specifically on the liver. LNP formulations and routes were well-tolerated, with no adverse reactions or liver toxicity. LNP-A contained typical 4-lipid components approved for use in humans, while LNP-B was supplemented with an anionic phospholipid. When injected IV, both LNP-A and LNP-B efficiently delivered eGFP mRNA to PBMC subsets including classical monocytes (CM, 33% eGFP+), nonclassical monocytes (80% eGFP+), and myeloid dendritic cells (mDCs, 62% eGFP+), but not plasmacytoid dendritic cells (pDCs, 8% eGFP+). In contrast, LNP-B displayed enhanced splenic delivery to CM (24% for LNP-A vs. 54% for LNP-B) and DCs (mDCs: 30% vs. 60%; pDCs: 16% vs. 38%). Relative to IV dosing, IN-delivery resulted in significantly greater eGFP expression in deep lymph nodes (CM: 22% for IV vs. 58% for IN; pDCs: 13% IV vs 32% IN), with high levels of protein expression observed within the subcapsular and medullary sinus by immunohistochemistry. Additionally, altering mRNA-LNP delivery route enabled targeting of genetic cargo to SHIV-infected cells, as detected by in situ hybridization. Dose route and LNP composition drastically altered biodistribution and the number of phagocytic and antigen presenting cells responsible for uptake and expression of mRNA-LNPs within distinct tissues, while detargeting the periphery. Our findings highlight the power and flexibility of mRNA-LNPs for customized targeting to specific tissue sites. Future studies will test delivery of mRNA encoding i) rationally designed HIV/SIV Envelope immunogens to stimulate virus-specific lymphocytes in

sites of HIV/SIV persistence, ii) broadly neutralizing antibodies (bNAbs) to reduce viral reservoirs and overcome barriers of inefficient tissue trafficking, and iii) CAR T cell cargoes to directly clear the latent viral reservoir.

302 Anionic double-layered nanocomplexes with receptor-targeting motifs penetrate Cystic Fibrosis mucus better than cationic LNPs and retains effective transfection efficiency *in vivo*.

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Background: Lipid-nanoparticles (LNPs) have emerged as promising vectors to deliver therapeutic nucleic acids such as siRNA and mRNA to the airway to treat respiratory diseases such as cystic fibrosis (CF). To achieve this, LNPs must first overcome the mucociliary barrier which consists of a network of anionic mucins. First generation LNPs used in clinical trials for CF were cationic which can become entrapped in the mucin network. Anionic LNPs may offer enhanced mucus diffusion rates but typically lower transfection efficiency. The aim of this study was to develop anionic, peptide-targeted LNPs that offer enhanced diffusion rates in mucus while retaining effective transfection properties *in vivo*.

Methods: We formulated cationic and anionic receptor-targeted nanocomplexes (RTNs) consisting of cationic, anionic, neutral and PEGylated lipids, and receptor-targeting peptides with a sixteen-lysine tail, and RNA molecules in different proportions and orders of mixing. Formulations were then characterised for formation of stable nanocomplexes by size and zeta potential measurement. Stable anionic formulations containing fluorescently-labelled RNAs were then analysed for their diffusion rates in CF mucus using a static mucus barrier model, which comprises CF mucus on polyester membrane inserts with 3.0 µm pores in 24-well plates. Transfections were also performed in *vitro* in CF basal cells and *in vivo* in murine lung.

Results: We developed a double-layered mixing method, in which a cationic liposome, receptor targeting peptide, and nucleic acid were mixed, and then mixed with a PEGylated anionic liposome. A stable anionic formulation termed LPRL3 containing mRNA was identified with a size of approximately 175.5±6.26 nm, PDI 0.13±0.01 and the strongly anionic zeta potential of -33.2±3.09 mV. The encapsulation efficiency was 98.1±0.31%. The diffusion rate of LPRL3 with mRNA in CF mucus (Dm) was $6.2x10^{-9}$±$0.09x10^{-9}$ cm^2s^{-1}, compared with cationic RTN ($2.01x10^{-10}$± $0.97x\ 10^{-10}$ cm^2s^{-1}), it was faster. The impedance (Dw/Dm) between the diffusion rate in water (Dw) and in CF mucus (Dm) was 4.9±0.8, which is approximately 62-fold less than that of the cationic RTN. The impedance of ionisable LNP formulation (D-LinDMA-MC3) was similar to LPRL3. On the other hand, the size and zeta potential of LPRL3 containing siRNA were 188.2±16.3 nm, PDI 0.30± 0.06 and -39.3±2.27 mV. The encapsulation efficiency was 98.2± 0.34%. The diffusion rate of LPRL3 containing siRNA in CF mucus was $3.23x10^{-8}$±$7.63x10^{-9}$ cm^2s^{-1} and the impedance (Dw/Dm) was 1.14±0.23, which was approximately 10-fold faster than that of a cationic RTN. The in vitro transfection efficiency of LPRL3 containing luciferase mRNA ($2.84x10^8$ RLU/mg protein) was approximately 20-fold lower than that of a cationic peptide targeted LNP in Cystic Fibrosis epithelial cells at submerged culture. which was not unexpected due to the high anionic charge although this was not observed in vivo. Anti-ENaC siRNA transfections in wild-type mouse lungs were performed with the double-layered formulation, LPRL3, which achieved approximately 30% ENaC mRNA knockdown ($p<0.001$), which was similar to a cationic formulation.

Conclusion: LPRL3 formulation is a novel anionic, receptor targeted lipid-nanoparticle that can penetrate CF mucus better than cationic LNPs while the transfection efficiency *in vivo* was at a similar level to cationic ones. Hence, it is promising in gene delivery for CF therapy via airway route.

AAV Preclinical and Proof-of-Concept Studies for Neurological Diseases

303 AAV9-Mediated Gene Targeting of a Natural Antisense Transcript as a Novel Treatment for Dravet Syndrome

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Dravet syndrome (DS) is a severe childhood genetic epilepsy which leads to life-long disability. Symptoms usually manifest in the first year of life and include prolonged tonic-clonic seizures, afebrile or febrile seizures, developmental delay and severe intellectual disability. DS patients have an increased mortality rate, due to sudden unexpected death in epilepsy (SUDEP). Approximately 90% of patients carry a heterozygous loss-of-function mutation in the *SCN1A* gene, which encodes a voltage-gated sodium ion channel, $Na_V1.1$. The $Na_V1.1$ channel is expressed in the brain and at a lower level, in the heart. A long non-coding RNA (lncRNA) from the natural antisense transcript (NAT) of *SCN1A* has been identified to specifically downregulate *SCN1A* expression. Previous studies have developed AntagoNATs, small synthetic oligonucleotides to inhibit *SCN1A* NATs and demonstrated a reduction in seizure phenotype after repeated intrathecal AntagoNAT injections to adult DS knock-in mice.

Here, we have developed novel AntagoNATs and incorporated these into a clinically relevant adeno-associated virus serotype 9 (AAV9) gene therapy vectors, to provide a one-off treatment to a heterozygous DS mouse model (*Scn1a*$^{+/-}$).

Eighteen AntagoNATs were tested *in vitro* in Neuro2a cells; of which thirteen significantly increased endogenous *Scn1a* expression compared to control. From the best performing candidates, we selected two AntagoNAT sequences (H and K) for *in vivo* assessment, as they had the highest homology (90%) to the human *SCN1A* NAT. The vectors were administered to newborn *Scn1a*$^{+/-}$ mice via intracerebroventricular (ICV) injection to target the brain and a combination of ICV and intravenous (IV) injections to target the brain and heart. AAV9-AntagoNAT-H & K demonstrated a higher survival when delivered via ICV & IV administration (total dose = $3.5x10^{10}$vg/pup), compared to ICV alone ($1x10^{11}$vg/pup). ICV & IV AAV9-AntagoNAT-H significantly increased survival to 84%, while ICV AntagoNAT-K resulted in 58% compared to 50% of PBS-treated controls over 100 days. ICV & IV AAV9-AntagoNAT-H treated *Scn1a*$^{+/-}$ mice demonstrated significant reduction in febrile seizures and reduced spontaneous seizure frequency compared to the PBS control group. Furthermore, we observed a significant increase in endogenous *Scn1a* expression in the brain and an increase of *Scn1a* expression in the heart. We also observed an increase in $Na_V1.1$ protein expression in the brain of ICV & IV treated AAV9-AntagoNAT-H group. We also administered AAV9-AntagoNAT-H to P14 *Scn1a*$^{+/-}$ mice *via* ICV & IV injection (total dose = $1x10^{10}$vg/pup), we observed an increase in survival, significant increased *Scn1a* and increased $Na_V1.1$ expression in the brain compared to PBS controls.

In this proof-of-concept study, we have demonstrated that the delivery of AntagoNATs via an AAV9 vector can be effective and thus offering the possibility of a one-time treatment for DS patients.

304 Development of a Secretable Frataxin for Enhanced Efficacy in Treating Friedreich's Ataxia

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Friedreich's Ataxia is a debilitating genetic disorder caused by loss of frataxin protein, resulting in progressive ataxia due to nervous system dysfunction and a shortened lifespan due to cardiac complications. The dual involvement of neuronal and cardiac tissues in the disease's pathophysiology has posed significant challenges for developing therapies that can treat all disease manifestations. Achieving frataxin repletion with a one-and-done therapeutic approach using a minimally invasive administration route has been a long-standing goal.

We have developed a first-of-its-kind, innovative gene therapy to address both cardiomyopathy and ataxia phenotypes using a single intra-CSF delivery of an AAV encoding an engineered frataxin variant. This approach integrates multiple innovations to enhance therapeutic efficacy. First, we engineered frataxin to enable secretion from transduced cells and uptake by non-transduced cells, facilitating cross-correction. This innovation expanded the therapeutic footprint beyond the AAV transduction area. Cross-correction of engineered human frataxin in mouse models rescued both neuronal and cardiac phenotypes, demonstrating unprecedented therapeutic potential. Second, we developed a novel capsid capable of transducing critical target tissues, including the cerebellum, dorsal root ganglia, and cardiomyocytes, with a single intra-CSF administration. This capsid demonstrated robust transduction and therapeutic efficacy in both mouse and non-human primate models. Third, we designed a tissue-enriched promoter to optimize frataxin expression. This promoter enhanced expression in cardiomyocytes, maintained therapeutic levels in the nervous system, and minimized expression in peripheral organs like the liver, ensuring safety and efficacy.

Pharmacology studies in non-human primates further validated our approach, showing widespread frataxin repletion in both the CNS and heart. Notably, therapeutic levels of frataxin were achieved in the heart following intra-CSF administration, with expression levels remaining within tolerable safety parameters. These findings, for the first time, demonstrate the therapeutic potential of secretable frataxin to simultaneously address neuronal and cardiac pathology associated with Friedreich's Ataxia. This groundbreaking approach offers a promising one-and-done gene therapy solution for this debilitating disease.

305 FDA-Aligned Strategy for AAV-Based TSC1 Gene Therapy: Advancing Preclinical Development Without Non-Human Primates

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Introduction:
Apertura Gene Therapy has developed AGTX-1111, an AAV-based gene therapy leveraging a novel transferrin receptor-targeted capsid, TfR1 CapX, for the treatment of epilepsy associated with Tuberous Sclerosis Complex (TSC1). AGTX-1111 addresses critical challenges in delivering therapeutic genes across the blood-brain barrier (BBB) to treat refractory epilepsy in TSC patients. A key focus of this program is the use of innovative preclinical models that exclude non-human primates (NHPs), supported by a robust regulatory strategy to streamline development and ensure scientific rigor.

Regulatory Framework:
Our approach was guided by published FDA guidances and insights from our FDA INTERACT meeting, which confirmed key aspects of our development strategy, including the classification of TSC1-associated epilepsy as a neurodegenerative disorder. This designation enabled the application of neurodegenerative disease-specific FDA guidelines. The FDA also concurred with the suitability of human transferrin receptor knock-in (B-hTfR1) mice as a definitive model for biodistribution and toxicology studies, given Apertura's position that NHPs are not a

pharmacologically relevant species for TfR1 CapX, which does not bind NHP transferrin receptor orthologs. This strategy significantly reduces costs and timelines, eliminating the need for large-scale NHP studies.

Preclinical Studies:
Key preclinical studies used a stepwise approach: (1) validating CNS-targeting efficacy of TfR1 CapX in B-hTfR1 mice, (2) using PHP.B as a surrogate capsid to establish proof-of-concept (POC), and (3) demonstrating superior CNS biodistribution and therapeutic benefit in TSC1 disease models. Studies confirmed that intravenously administered TfR1 CapX achieves broad CNS delivery via receptor-mediated transcytosis, providing a critical therapeutic advantage for addressing refractory epilepsy in TSC patients. Notably, biodistribution studies in NHPs corroborated the absence of CNS transduction, reinforcing the pharmacological irrelevance of NHPs for TfR1 CapX.

Potency Assay Development:
A cell-based ELISA assay using a TSC1 knockout cell line was developed to measure total and phosphorylated p70S6 kinase (P70S6K), a downstream target of mTOR. Loss of TSC1 leads to mTOR hyperactivation and increased phosphorylation of P70S6K, making it a key functional readout for TSC1 restoration. In vitro studies confirm that the P70S6K assay effectively measures mTOR pathway activity and is responsive to AAV-mediated TSC1 expression. This assay forms the basis for potency assessment in Phase 1/2 trials. FDA feedback further supported its use as a release assay while providing recommendations for future assay validation to support licensure.

Impact of Excluding NHPs:
This strategy aligns with the 3Rs of ethical animal use and achieves significant reductions in material requirements, costs, and timelines. Additionally, this approach emphasizes the translational relevance of alternative models, such as the B-hTfR1 mouse, for CNS-targeting gene therapies.

Conclusion:
AGTX-1111 exemplifies a streamlined regulatory and preclinical development pathway. This case study highlights the potential for innovative gene therapy approaches to address unmet needs in rare neurodegenerative disorders while reducing reliance on traditional animal models. These principles pave the way for efficient clinical translation and future applications of receptor-targeted AAV gene therapies.

306 Benefit and Tolerability of AAV Gene Replacement in ECHS1D Mice

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ECHS1 Deficiency (ECHS1D) is a rare Leigh syndrome-like disorder that lacks treatment. Loss of function mutations in ECHS1 impair the activity of short-chain enoyl-CoA hydratase, which oxidizes short-chain fatty acids and branched-chain amino acids (valine, leucine, isoleucine) within mitochondria. Patients present with severe developmental delays/regressions, hypotonia, dystonia, and infantile onset epilepsy. As a monogenic, loss-of-function disorder, ECHS1D is a viable candidate for gene replacement therapy using recombinant adeno-associated virus (rAAV) delivery, but this approach has yet to be explored. Towards this, we developed two vectors containing a ubiquitous promoter to drive expression of a codon-optimized form of human ECHS1, with or without additional CpG depletion (V1 and V2, respectively). Prior to packaging into AAV9, both plasmid constructs were validated to express similar levels of functional ECHS1 upon treatment in N2A cells. Interestingly, treatment with the viruses in Lec2 cells revealed that V1 resulted in much greater protein expression than V2, suggesting CpG depletion may enhance AAV-mediated transgene expression within this cell line. To determine if gene replacement can offer therapeutic benefit, the ECHS1D mouse model was used. This model has been previously shown to have significantly reduced ECHS1 expression with increased seizure susceptibility in adolescence and exercise intolerance throughout aging. For efficacy assessments, ECHS1D mice received vehicle treatment or 5e11vg of V1 or V2 via an intrathecal lumbar puncture (IT) at post-natal day 10 (P10). A short-term cohort was subjected to seizure induction at 3 months post-injection, while a long-term cohort underwent repeat endurance testing at 6, 9 and

12 months. After behavior, tissues were collected for biochemical analysis. While 80% of vehicle treated ECHS1D mice died following seizure induction, survival was increased to 50-60% in the virus treated groups. Wild-type (WT) mice retain 100% survival in this paradigm, so these results demonstrate a partial rescue of the epileptic phenotype. Further, virus treatment prevented the early exercise fatigue observed in untreated ECHS1D mice and restored performance to WT levels. While both viruses demonstrated similar behavioral efficacy, V1 treatment significantly increased ECHS1 expression compared to V2 treated mice and naive WT mice, in agreement with prior cell culture findings. To determine if high levels of ECHS1 are well-tolerated, WT mice were treated with a high (6e11vg) and low (1.5e11vg) dose of V1 at P10 via IT delivery. Weight was unaffected by treatment, but there was a mild reduction in survival in a dose-dependent manner. Unexpectedly, 80% of high dose treated mice developed progressive hindlimb clasping that did not resolve up to 1-year post-injection. This was not associated with any abnormalities noted by clinical blood chemistry or histopathology, however clasping appeared to correlate with degree of ECHS1 overexpression. Taken together, these findings support that gene replacement may be a viable therapeutic approach for ECHS1D patients; however, caution must be taken to avoid toxic levels of ECHS1 overexpression. Future work will develop vectors to allow cellular control of expression as a means to provide a safe and effective treatment for patients with this devastating disorder.

307 Proof-of-Concept AAV Gene Therapy for Christianson Syndrome (CS) in CRISPR-targeted Rat Model

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Christianson syndrome (CS) is a profound neurological disorder caused by loss-of-function mutations in the gene encoding the endosomal Na+/H+ exchanger 6 (NHE6) protein. CS is among the most common forms of X-linked intellectual disability. CS is characterized by intellectual disability, epilepsy, postnatal microcephaly, ataxia, and poor weight gain. Currently, there is no treatment for CS. In patient-derived induced pluripotent stem cells (iPSCs), we have shown that gene replacement leads to a cell-autonomous rescue of neuronal functions *in vitro* (Lizarraga et al., *Science Trans Medicine*, 2021). We are now investigating the efficacy of AAV9-NHE6 gene therapy in our CS rat model in vivo. We generated the CS rat model by CRISPR/Cas9 gene-targeting, and the CS rat strongly recapitulates several core CS symptoms such as ataxia and low body weight (Lee et al., *Brain*, 2022). We have now developed AAV vectors expressing the human *NHE6* gene. We then examined the efficacy of our therapeutic NHE6 vectors using our CS rat model. CS and WT male rats were assigned to 3 treatment groups: hNHE6 vector (AAV9-hNHE6), control vector (AAV9-eGFP), or sham surgery. Bilateral ICV injections or sham surgeries were performed at 7 to 8 weeks-old. This age was selected as we have previously shown CS rats exhibit motor dysfunction and neuropathology as early as 1-month-old. Using ubiquitous promoters, we detected NHE6 distribution throughout the brain using confocal microscopy. Outcome measures include body weight and functional motor tests. We also use computer vision and machine learning (ML) methods to assess naturally behaving rats in their home cage settings. Measurements were collected at baseline (i.e. 1 week prior to injection) and monthly through 6 months post-injection (PI). CS rats treated with the hNHE6 AAV9 vector (CS+NHE6) displayed significant and prolonged improvement in body weight compared to control-vector (CS+AAV-GFP) and sham (CS+sham) CS rats. Further, NHE6-AAV9 treated CS rat body weight was consistent with WT rats (all conditions). CS+NHE6 rats exhibited significantly greater motor activity, largely indistinguishable from control, in the open field task as compared to both CS+AAV-GFP and CS+sham rats through 6 months. Motor coordination, as measured by the rotorod assay, was significantly improved in CS+NHE6 rats compared to CS+AAV-GFP and CS+sham rats, although mutant rats demonstrated varying degrees of impaired coordination relative to WT rats. Gait abnormalities and ataxia were measured using DigiGait analysis. While both untreated CS rat groups (AAV-GFP and sham) displayed a wider gait, CS+NHE6 rats had a shorter gait similar to WT rats. Interestingly, our computer vision and ML methods are able to distinguish treated from untreated animals in a number of behavioral features in the natural setting, and these methods cluster the treated animals with the control animals. In conclusion, we show, that *NHE6* gene replacement therapy using AAV9 technology successfully ameliorates core symptoms in a CS rat model, such as low body weight and motor impairment *in vivo*. Importantly, these improvements in CS features observed in a CS rat model were long-lasting, through 6 months post-injection of our therapeutic NHE6 AAV treatment. Taken together, our *in vivo* results support proof-of-concept efficacy of *NHE6* gene therapy, with prominent improvements in motor behavior.

308 Development of a Self-Regulating AAV Gene Therapy for the Safe and Effective Treatment of Rett Syndrome

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Background:
Rett Syndrome (RTT) is a debilitating X-linked neurodevelopmental disorder that affects 1:10,000 girls. RTT is characterized by a period of normal development until 6-18 months, followed by rapid loss of previously acquired skills, resulting in severe neurological and physical disability. RTT is caused by mutations in *MECP2* which encodes methyl-CpG binding protein 2 (MeCP2), an epigenetic regulator that is highly abundant in neurons and critical for neurodevelopment and function. Affected females are heterozygous and due to random X-chromosome inactivation are mosaics of healthy and mutant cells. *MECP2* is a dosage sensitive gene, therefore effective gene therapies need to regulate *MECP2* expression to rescue mutant cells but avoid overexpression in healthy cells, which could be toxic as suggested by MeCP2 duplication syndrome where phenotypic presentation is similar to Rett syndrome.

Methods:
Our lab developed self-regulating, AAV-*MECP2* vectors for gene therapy of RTT. MeCP2 expression homeostasis is controlled by the upstream positive and negative regulators, Brain-Derived Neurotrophic Factor (BDNF) and microRNA-132 (miR-132), respectively. Our vector encodes the MeCP2 transgene with miR-132 recognition elements to exploit this negative feedback loop. We compared the therapeutic efficacy of these vectors delivered by intracerebroventricular (ICV) injection in neonatal (P0-P1) *Mecp2*$^{y/-}$ mice. *Mecp2*$^{y/-}$ males have neurological abnormalities and a survival expectancy of 8-12 weeks. We monitored mice for long-term survival and performed short-term (8-10 weeks) behavior testing and molecular analysis.

Results:
We performed a long-term survival study to screen five different AAV9 vectors encoding human MeCP2 under the mouse 229-bp promoter and various modifications of non-coding sequences. We identified a top AAV-*MECP2* vector that significantly extended lifespan in Mecp2$^{-/y}$ mice from median survival of 11 to 27 weeks with a range from 16.9 to 52 weeks. We assessed several behavioral parameters at 8-10 weeks of age and found significant improvements in motor coordination in Mecp2$^{-/y}$ mice treated with the lead candidate AAV9-MeCP2 vector compared to PBS controls. There was no significant improvement in treated Mecp2$^{-/y}$mice in locomotion, anxiety, or nesting behavior relative to controls. Western blot analysis showed that treatment restored MeCP2 protein expression in the brain and spinal cord of Mecp2$^{-/y}$ mice. Immunostaining of brain sections for MeCP2 expression revealed robust widespread distribution of MeCP2-positive cells in cerebral cortex but more sparse transduction in deep brain structures and cerebellum. We also performed bulk RNAseq of brain and liver from AAV9- and PBS-treated Mecp2$^{-/y}$ mice at 8-10 weeks of age to determine the impact of gene therapy in the transcriptomic profile of these mice.

Conclusion:
The AAV-*MECP2* treatment significantly extends lifespan of Mecp2-/y mice, rescues some behavioral phenotypes, and restores MeCP2 expression to the CNS. Future plans include exploring vector engagement with the regulatory loop, as this may be important for translation into our target heterozygous female population who have a mixture of healthy and mutant cells.

309 EXG202: A Next Generation AAV-based Gene Therapy for Neovascular AMD Delivered by Intravitreal Injection with Superior Retinal Transduction in NHPs and Remarkable Inhibitory Effect on Retinal Detachment in a Severe Neovascular Mouse Model

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The success of AAV-based gene therapy for most ocular diseases including wet AMD and DME depends on efficient delivery of the vector to the photoreceptors (PR), and/or retinal pigment Epithelium (RPE). While subretinal administration provides the most direct access to PR and RPE, it is more invasive, with a risk of damage to the retina. Intravitreal (IVT) injection is a safe, non-surgical procedure and has been widely used for delivery of protein therapeutics to treat common retinal diseases.

Here, we report the efficacy and preliminary safety of EXG202, a next generation rAAV-based gene therapy that expresses a fusion protein that binds all subtypes of VEGF as well as ANG2 and is packaged in a novel engineered AAV capsid designed for intravitreal injection.

The test systems are nonhuman primates (NHPs) and the Tet/opsin/VEGF double transgenic mouse model. In NHPs, 6 animals received EXG202 intravitreally in one eye and AV7m8 (a benchmark IVT capsid carrying the same transgene expression cassette as that of EXG202) in the contralateral eye (1×10^{11}vg/eye). Post injection animals were followed for 8 weeks. Levels of the transgene product were measured in aqueous and vitreous humors collected at week 4 & 8, as well as ocular tissues (iris-ciliary body, retina, RPE/choroid) collected at week 8. The levels of the transgene product in eyes that received EXG202 were consistently higher (up to 3-fold) than those in eyes that received AV7m8. Intravitreal injection of 1×10^{11} vg/eye of EXG202 was well-tolerated with no test article-related changes except for minimal vitreous cells in one of six eyes. There were mild vitreous cells in three of six eyes dosed with AAV7m8.

Efficacy of EXG202 delivered by intravitreal injection was tested in a dose-ranging study in Tet/opsin/VEGF double transgenic mice, with inducible expression of $VEGF_{165}$ that develop severe vascular leakage and exudative retinal detachment (RD) when treated with doxycycline Adult Tet/opsin/VEGF mice (n =7-9/group) were given an intravitreal injection of EXG202 at doses of 1×10^7, 3×10^7, 1×10^8, 3×10^8, or 1×10^9 vg/eye in one eye, and the negative control vector AVT-Luc in the other eye. Four weeks post injection, the mice were given doxycycline and after 4 days, fundus photographs were obtained. The results were categorized as no, partial, or total exudative RD. Compared with control vector-injected fellow eyes, total exudative RD was reduced by 61%, 71%, 86%, or 100% in mice treated with 3×10^7, 1×10^8, 3×10^8, 1×10^9 of EXG202. Compared to the first-generation gene therapy vector EXG102, which carries the same payload as EXG202 but is packaged in a wild-type AAV2 capsid for subretinal gene therapy, EXG202 delivered via intravitreal injection was ~10-fold more potent in prevention of RD in Tet/opsin/VEGF mice.

In conclusion, EXG202 demonstrated superior retinal transduction efficiency compared to the benchmark control AV7m8 in NHPs and strongly suppressed VEGF-induced vascular leakage and exudative retinal detachment. These data strongly support the clinical development of EXG202 for the treatment of nAMD or DME.

AAV Vector Manufacturing: Plasmids & Cell Line Development

310 Enhancing Upstream Processes for High-Yield, High-Quality AAV Vector Production Using a Novel In-House Cell Line

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High productivity and product quality have been critical in developing novel AAV-based drug candidates, not only to fuel clinical trials but also to reduce the high manufacturing cost per patient dose. The pursuit of high AAV productivity has led to an increased focus both on upstream process optimization, and the development of clonal cell lines that are highly transfectable and able to produce high titers across multiple serotypes.

A SV40 large T antigen knock-out HEK293 cell line was generated using Mad7-based genome editing of HEK293T cells. Clones were screened for vector genome productivity at shake flask scale and confirmation of removal of the SV40 large T loci via targeted nanopore sequencing. The top clone candidate (HEX2s52) was confirmed for full SV40 large T antigen and associated NeoR cassette removal via PacBio whole genome sequencing and lack of expression of large T antigen and NeoR with mass spectrometry.

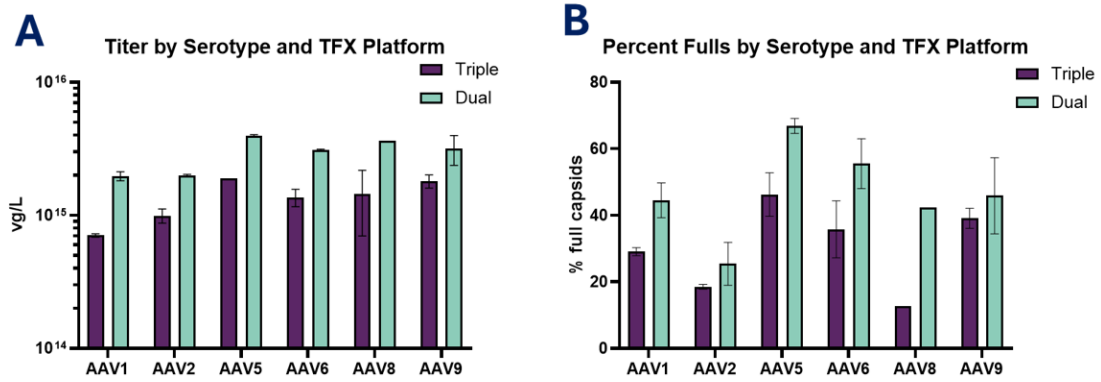
Performance of top clone HEX2s52 was confirmed at 2L bioreactor scale using OXB's dual plasmid inAAVate™ platform with AAV9 and was able to achieve a bioreactor titer of 5.7e14 vg/L and 35.7% full capsids. To improve HEX2s52 performance in the hopes of surpassing 1e15vg/L and 40% full capsids, further process development was performed focusing on factors such as transfection parameters, alternative transfection reagents, and incorporation of post-transfection additives.

With the implementation of this newly optimized process and the OXB in-house cell line at 2L bioreactor scale, AAV9 bioreactor titer increased >5-fold to 3.2e15vg/L, and percent full capsids increased to 45.9%. 2L bioreactor evaluation (N=2) was also performed in AAV1, AAV2, AAV5, AAV6, and AAV8 serotypes using this new process with HEX2s52 and ≥2e15vg/L was achieved for all serotypes using the dual transfection system, with AAV5 being the highest at 4.0e15vg/L and 66.9% full capsids.

2L bioreactor material was purified through to drug substance for all six tested serotypes and confirmed for good product quality and equal, if not better, final vg yield. Relative potency improved in OXB cell-line with optimized process material compared to historical platform Process C data for AAV1, AAV8, and AAV9 serotypes, with AAV1 showing >2.5x increase in potency.

Improvements from this optimized upstream process are not limited to OXB's in-house cell line. The commercial cell line also saw a >3-fold improvement in vector genome titer at 2L bioreactor scale when using the optimized upstream process for dual plasmid transfection in AAV9.

In summary, OXB's new in-house cell line alongside intensified upstream process delivers high quality, highly potent AAV vector across multiple capsid serotypes with bioreactor titers as high as 4e15vg/L. The benefits of this optimized process extend beyond OXB's in-house cell line, demonstrating a minimum three-fold increase in titer when applied to another commercially available cell line.



311 Establishment of a Novel Human Cell Line, HAT, for High-Yield and High-Quality AAV Manufacturing

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Introduction:
The field of AAV-based gene therapy has witnessed a rapid progression, with an accelerating number of product approvals and clinical trials over the last decade. However, manufacturing capacity cannot keep up with the growing demand using currently employed platforms, resulting in prohibitively high costs and limited accessibility of these treatments. In addition, though infrequent, the reported severe adverse effects in high-dose AAV administration further highlight the urgent need to improve AAV vector production. Despite considerable efforts to subclone and develop the commonly used HEK293 host cells, challenges remain in producing high-yield and high-quality AAV vectors. To address this, our study introduces a newly established Human Amniotic epithelial cell line for gene and cell Therapy (HAT cells).

Methods:
Placental tissues as raw materials for HAT cells were obtained from healthy expectant mothers after scheduled cesarean section. Written informed consent was obtained from all subjects before deliveries to comply with ethical and legal requirements. HAT cells were generated by transforming primary cells isolated from placental amnion, with DNA fragments encoding adenovirus 5 E1 genes. Adherent HAT cells selected from among a large pool of candidate lines were then adapted to serum-free suspension culture in chemically defined medium and single cell cloned with fully traceable history for widespread industrial adoption. The identity and quality of HAT cells were characterized to avoid the inherent risk of contamination in products manufactured in them. AAV vectors were produced using a triple plasmid transfection method. Genomic and capsid titer were measured by qPCR, ddPCR, and ELISA. The percentage of full capsids was determined by qPCR/ELISA ratio. Full capsid content was also estimated by anion-exchange chromatography (AEX). The scalability of HAT cells was assessed using a benchtop bioreactor.

Results:
The cell line identification analysis indicated that HAT is an authentic human-origin cell line. HAT cells exhibited high levels of proliferation and transfection efficiency. AAV productivity of adherent HAT cells and infectivity of HAT-produced AAV vector particles were equal or superior to those of HEK293 cells. HAT cells tested negative for adventitious pathogens and mycoplasma. These features indicate that HAT cells are suitable as a host cell platform. High-yield AAV vector production, routinely exceeding 6E13 vg/L, was achieved across multiple serotypes in clonal, suspension-adapted HAT cells. AAV2 and AAV8 vectors produced from HAT cells contained substantially higher full capsid content, greater than 1.5-fold compared to those produced from suspension HEK293 cells. A significantly higher peak in the AEX chromatogram presumed to be enriched for full particles was also observed in HAT-produced AAV vectors. Finally, we demonstrated a successful scale-up production of AAV8 vectors to 2-liter scale. The yield and capsid content were comparable and reproducible from shake flask to bioreactor.

Conclusion:
Here, we report the establishment of novel HAT cells and the potential of HAT cells to serve as a host for AAV vector production. We believe with further cell line development, process optimization, and comprehensive characterization of HAT-produced vectors, HAT cells will provide a promising manufacturing option that can enhance therapeutic efficacy and safety at lower AAV doses.

312 Improved Adeno-Associated Vectors for High-Yielding AAV Manufacturing

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Introduction
Adeno-associated viral (AAV) have emerged as a promising gene delivery tool for in vivo gene therapy, frequently requiring high doses to achieve the desired therapeutic effect. This underscores the critical need for enhancing AAV production titers while ensuring optimal AAV characteristics. However, achieving the higher production titers remains a significant challenge, driving the need for improved AAV vector design, along with production processes. A key element influencing AAV production is the membrane-associated accessory protein (MAAP), which is encoded from a frameshifted open reading frame in the viral protein 1 (VP1) capsid gene. Discovered as an egress factor, MAAP plays a role in AAV replication and infection. We have designed over 15 types of novel vectors per serotype by making substitution mutations to the MAAP protein (termed RepCapExcel vectors). Our current Catalent® Off-the-shelf (OTS) plasmids result in superior performance for titer and product quality and were an improvement over competitor, both in the Catalent HEK-293 cell-line, and commercial cell-lines. Work here investigates novel vectors conceived as additional possibilities for future improved versions of the RepCap plasmid. RepCapExcel vectors were evaluated using Catalent's® baseline AAV platform process and compared to Catalent® Off-the-shelf (OTS) plasmids. Vectors were evaluated for their potential to improve AAV product titers, without impacting the viral proteins expression, or the AAV product characteristics.

Methods
In this work, we generated a matrix of unique single-codon substitutions, amino acid substitutions, deletions, and combinations thereof in the membrane accessory protein across several AAV serotypes, including AAV5, AAV6, AAV8, and AAVrH10. We evaluated over 75 RepCapExcels vectors across various serotypes using the Catalent baseline UpTempo^{SM} platform process in shake-flasks, and compared their performance with the Catalent OTS RepCap vectors. AAV titer and quality attributes were compared with the Catalent version #1 OTS RepCaps, using the Catalent clonal HEK-293 Cell-line. Specific changes such as stop codon modifications, and amino acid substitutions had unique effects on the AAV product titer fold-increase, when tested using our model pGalacorin^{TM} as a gene-of-interest. In all cases changes were made to alter MAAP protein alone, while ensuring the Viral protein expression itself remained unchanged.

Results and Conclusion
Using these novel RepCapExcel vectors several key findings were observed: (1) AAV titers improved by 3-7 fold compared to RepCap plasmid controls (for example, an AAV5 RepCap variant achieved titers of up to 8 x 10^{11} vg/mL in shake-flasks, representing over a 3-fold increase over control RepCap, without a significant rise in residual packaged host cell DNA (hcDNA) or a decrease in percent full capsids; (2) The percent full capsid, and purity by viral proteins VP1, VP2, VP3 expression and protein ratios remained consistent, as confirmed by LC-MS and PTM analyses using samples from a single step affinity purification; (3) RepCapExcel variant #5 played a critical role in in recombinant AAV production across all serotypes; (4) A RepCapExcel vector variant #2 that exhibited the highest vector genome titer increase, as well as the highest AAV particle titer increase was identified.

313 Unveiling small non-coding RNA dynamics during rAAV production

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Recombinant adeno-associated viruses (rAAVs) are a cornerstone of modern gene therapy, offering precise delivery and stable transgene expression with minimal immunogenicity. Despite clinical advancements, large-scale rAAV production faces significant challenges such as low yield, high costs, and variability. Addressing these hurdles is critical to meet rising demand.

In this study, we explored the expression profiles of microRNAs (miRNAs) and small nucleolar RNAs (snoRNAs), which are increasingly recognized as key regulators in viral replication, during rAAV plasmid transfection and production in HEK293F cells. Therefore, HEK293F cells were either mock- or rAAV transfected and cultivated over 72 h. During cultivation and production, samples were taken to analyse cellular parameters and rAAV titers. Ultimately, RNA specimens of each sampling time point were isolated and analysed via microarray expression analysis.

Using a detailed and multi-faceted comparative approach, we identified a substantial number of miRNAs that were differentially expressed compared to both the non-transfected mock control and to the basal expression pre-transfection. The most significant differences in expression were observed during the main phase of rAAV production, particularly between 24- and 72-hours post-transfection (hpT). Here, 142 miRNAs were differentially expressed in all comparisons and of these, 128 miRNAs were associated with the GO term “viral process” based on enrichment analyses of their validated target genes.

In contrast, snoRNA analysis showed overall fewer differential expression events, with a higher number being upregulated relative to mock transfection and downregulated compared to basal expression. During the main rAAV production phase, five snoRNAs were identified as significantly differentially expressed across multiple time points, pointing towards their potential involvement in viral replication and production processes.

These findings provide valuable insights into cellular and viral responses underpinning rAAV production. By elucidating the regulatory roles of miRNAs and snoRNAs, this study lays the groundwork for future advancements in gene therapy manufacturing through host cell line engineering.

314 Enhancing Gene Therapy Access: Standardizing AAV Production With Cell Line Development

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As gene therapies approach widespread adoption, advanced adeno-associated virus (AAV) manufacturing platforms are essential to accelerate clinical and market entry. With the growing global demand, driven by the trend for higher recombinant AAV (rAAV) doses—62% of systemically administered rAAVs are now dosed between 10^{14} and 10^{16} vg—achieving high scalability, reducing production costs, and minimizing batch-to-batch variability are crucial. Ensuring the safety and efficacy of rAAV therapies, particularly at high doses, is becoming increasingly important. The industry must innovate to produce these viral vectors more robustly while reducing specific impurities.

Stable production methods have paved the way for monoclonal antibody production. However, rAAV poses unique challenges in cell line development and process-related impurities. While industrial production methods have advanced, most GMP rAAV processes still rely on transient transfection and plasmids. Contaminant removal traditionally focuses on impurities in solvents or vector surfaces, but contaminants encapsidated within rAAVs can't be removed with classical downstream purification technologies without disrupting the vector.

Leveraging high-throughput sequencing technologies alongside synthetic biology and gene editing tools, we have developed a highly efficient, helper-virus-free, stable rAAV producer cell line (PCL) that overcomes these challenges, enabling cost-effective, large-scale production of high-quality rAAV. Our proprietary, genetically modified HEK293 PCL integrates all necessary genetic components for vector production into the producer cell genome, eliminating the need for transient transfection or helper viruses. Additionally, our proof-of-concept cell lines use genetic modifications to inhibit specific cellular pathways, reducing host cell DNA encapsidation (including potential oncogenes) by up to 95% without affecting vector titers.

By embracing next-generation cell line development, the industry can tackle major challenges, ultimately enhancing global access to gene therapies.

315 Optimising AAV Vectors Quality and Characterisation Using Synthetic Linear DNA

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Recombinant Adeno-associated virus (rAAV) remains a popular vector for gene therapy, with over 250 trials recorded in the past ten years using rAAV as a delivery system. However, rAAV manufacturing still faces several challenges to keep pace with the demand for large scale, high quality batches. Indeed, the gold standard plasmid triple-transfection as a favoured mode of production presents several issues. One, availability of GMP grade plasmid and costs. Two, sequence fidelity of the Inverted Terminal Repeats (ITR). And three, FDA safety concerns over non-specific encapsidations of sequences from the plasmids used during manufacturing that may be a trigger for innate immunity.

As new forms of template DNA become available for rAAV manufacturing, assessing the quality of the produced vectors is of paramount importance. 4basebio has developed a proprietary, scalable enzymatic synthesis process for the production of linear, closed-end DNA constructs via its Trueprime™ amplification technology. The hpDNA™ produced is devoid of any bacterial backbone and circumvents cumbersome fermentation processes required for plasmid DNA. It also allows unprecedented conservation of the notoriously unstable Inverted Terminal Repeat (ITR) sequences. The process can significantly reduce the turnaround time for GMP grade DNA production.

Here, we compared the production of rAAV using synthetic linear hpDNA™ encoding for the typical Adenovirus helper functions, *rep* and *cap* genes, and an expression cassette consisting of AAV2 ITRs and a reporter gene driven by an ubiquitous promoter, with conventional plasmid triple-transfection as a control. We emphasised our focus on validating methods that take in account the fundamental difference of this new template with plasmid DNA to ensure the accuracy of our data, including physical titers and examination of packaged DNA.

We first looked at the Sanger and nanopore sequencing of various batches of hpDNA™, which revealed that 4basebio amplification process maintains ITR integrity across multiple constructs, even with wild-type sequences. We then optimised the transfection of our synthetic DNA through DOE and reached equivalency with their plasmid counterpart, in both an adherent and a suspension-adapted HEK293 lines. We also showed that various DNAseI were not as efficient as removing free floating DNA on synthetic DNA as they are on plasmid DNA. Following this important finding, we validated a new assay using a new high salt DNAse. Afterward, we looked into the detection of template backbone in rAAV productions made from either plasmid or synthetic DNA, and showed that while batches made from plasmid had up to 10% of beta-lactamase copies, these were below the qPCR limit of detection for batches made from hpDNA™. The examination of physical titers highlighted differences when testing different genome constructs, but overall similar titers between synthetic DNA vs plasmid DNA. Finally, transduction assays showed that rAAV particles made from plasmid or synthetic DNA were equally infectious.

While achieving similar viral genome titres and infectivity between the two production methods, we also demonstrated AAV vectors made from hpDNA™ were generally of better quality, which could greatly accelerate therapeutic development of gene therapy programmes.

316 Mechanistic Insights Enhance Multi-Serotype Production in the TruStable™ AAV Producer Cell Line

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Adeno-associated virus (AAV) vectors are increasingly important for gene therapy applications, yet traditional production methods face significant limitations in scalability and consistency. We previously developed TruStable™, a fully stable AAV producer cell line, demonstrating robust and reproducible vector production of AAV5. Here, we report the expansion of this platform to additional AAV serotypes through mechanistic understanding of capsid mRNA production requirements. Systematic investigation identified a key viral helper factor essential for efficient capsid mRNA production across several AAV serotypes. Integration of this helper factor enabled high-titer production of multiple AAV serotypes beyond AAV5, significantly expanding the versatility of theTruStable™ platform. The robust process characteristic to TruStable™ enables detailed mechanistic interrogation of AAV biology to further enhance vector production and quality.

Challenges in Immunological Responses to Therapeutic Interventions

317 Potential for Potent Intrahepatic CD8$^+$ T Cell Responses Despite Lack of Peripheral Responses Following Systemic Muscle-directed Gene Delivery with AAV

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Capsid-engineered adeno-associated virus (AAV) vectors are promising candidates for muscle-directed systemic gene delivery to treat neuromuscular disorders. However, therapeutic efficacy often necessitates high vector doses, which increase the risk of immunotoxicity. We recently found that intramuscular (IM) administration of $8x10^{12}$vg/kg of an AAV1 vector expressing chicken ovalbumin (OVA) from the CMV promoter induced a potent and destructive CD8$^+$ T cell response against OVA in C57BL/6 mice (with peak frequencies of 10-20% of CD8$^+$ T cells in peripheral blood at week 2) that was not be prevented by CpG depletion or other methods of disrupting innate immune pathways unless the vector dose was reduced or multiple pathways were targeted (Viruses 16:1507, 2024). Here, we packaged the identical expression cassette into AAVMyo, an AAV9-derived myotropic capsid with improved systemic muscle transduction (Nat Commun 11:5432, 2020). C57BL/6 mice (n=5/group) were intravenously injected with up to $1x10^{14}$vg/kg of AAVMyo-CMV-OVA. Surprisingly, circulating OVA-specific CD8$^+$ T cells were found only at ~10-fold lower peak frequencies and were the lowest (0.8%) at the highest vector dose. Frequencies further declined for the duration of the experiment (6 weeks). Transgene expression was detected by immunohistochemistry in the heart, diaphragm and limb muscle, accompanied by only very mild CD8$^+$ T cell infiltration (at 2 weeks, later time points pending), initially prompting us to conclude that systemic delivery elicited minimal CD8$^+$ T cell responses. However, a robust CD8$^+$ T cell response was unexpectedly found in the liver, with ~60% of intrahepatic CD8$^+$ T cells being OVA-specific and an overall increase in CD8$^+$ T cells in the liver at week 2. Tissue sections revealed substantial and wide-spread CD8$^+$ T cell infiltration of the liver parenchyma, despite undetectable OVA expression by immmunostaining. The liver also contained the highest vector copy numbers of all tissues examined, and significant elevation in serum ALT (3.2-fold) and AST (2.4-fold) levels were detected. Intrahepatic OVA-specific CD8$^+$ T cells expressed PD-1, 2B4, TIM3, and LAG3, exhibiting a phenotype of exhausted T cells, which may have limited liver damage. In our earlier IM administration study, we found that a vector containing a CpG-depleted transgene combined with a muscle-specific promoter (CK8e) and TLR9 inhibitory sequences (io2) elicited substantially lower responses against OVA (Viruses 16:1507, 2024). When AAVMyo-CK8e-OVA-io2-CpG$^-$ vector was given systemically, the intrahepatic CD8$^+$ T cell response was substantially reduced to on average ~9%, and only marginal CD8$^+$ T cell infiltration was observed. In conclusion, systemic delivery of AAV vectors has the potential to induce strong cellular immune responses in the liver that may not be detected in peripheral blood. Although the hepatic environment tends to limit T cell effector functions, this potential

for delayed liver damage by adaptive responses nonetheless constitutes a safety concern, as supported by liver toxicities seen several weeks after systemic AAV delivery in some patients. Our findings suggest that capsid modifications alone may not be sufficient to prevent liver-targeted immune responses, underscoring the importance of integrating vector genome engineering and other strategies to minimize off-target immune activation.

318 Intra-Ocular Delivery of the Bacterial Protease IdeS in Non-Human Primates: A Potential Strategy to Circumvent Pre-existing Immunity to Enable Successful AAV Gene Transfer to the Eye

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Adeno-associated viral vector (AAV)-mediated ocular gene therapy has the potential to provide a long-term treatment for many genetic or acquired ocular diseases; however, neutralizing antibodies (nAb) to AAV vectors are highly prevalent in the human population and can be a major challenge to successful gene therapy in the clinic. Preclinical studies in non-human primates (NHPs) have shown that nAbs can negatively impact AAV transduction following intravitreal injection (IVT) of an AAV gene therapy vector, leading to reduced transgene expression. Furthermore, nAbs that are induced from a primary intravitreal AAV vector administration will likely pose a challenge for future re-administration, if ever needed. While the impact of nAb levels and AAV vector dose in the human eye are currently being evaluated in multiple clinical trials, there remains a need for improved protocols for AAV-mediated ocular gene therapy in AAV seropositive patients. Here, we explore the use of IdeS, a protease that specifically degrades IgG and is currently approved for use in kidney transplantation, as a strategy to enable successful ocular gene transfer in the presence of pre-existing neutralizing antibodies to AAV.

We evaluated the efficacy of IdeS in the eyes of non-human primates with pre-existing neutralizing antibodies to AAV.SAN024, a capsid with high tropism for the eye. NHPs seronegative or seropositive for AAV.SAN024 nAbs received a bilateral IVT dose of either PBS or IdeS three days prior to IVT administration of an AAV.SAN024-sFLT02 vector (2E10 vector genomes/eye). Vector genome and transcript levels in the retina and nAb titers in the serum, aqueous humor (AH), and vitreous humor (VH) were measured five weeks after vector administration to assess the impact of intravitreally delivered IdeS on ocular transduction and immune response.

AAV.SAN024-sFT02 vector genome and transcript levels in the retinas of PBS-treated seropositive NHPs were an order of magnitude lower compared to PBS-treated seronegative NHPs, demonstrating an impact of pre-existing nAbs on AAV transduction and subsequent transcript expression in the eye. In contrast, seropositive NHPs that received IVT IdeS treatment prior to AAV.SAN024-sFLT02 administration achieved levels of vector transduction and transcript expression comparable to the seronegative NHPs.

In seronegative NHPs, the majority of NHPs exhibited minimal increase in serum nAb titer and no increase in ocular fluid nAb titers five weeks after IVT AAV.SAN024-sFLT02 administration. In contrast, seropositive NHPs that received IVT PBS elicited a strong AAV nAb response to AAV.SAN024-sFLT02, which was detectable in serum, VH, and AH. However, seropositive NHPs that received IVT IdeS had lower levels of AAV.SAN024 nAbs in serum, AH, and VH compared to seropositive NHPs that did not receive IdeS, with the majority of IVT IdeS-treated eyes having undetectable AAV.SAN024 nAbs in the AH or VH at the end of the study. Overall, serum AAV.SAN024 nAb titers were higher than intraocular nAb titers within an individual animal.

These data demonstrate that intravitreal delivery of IdeS can improve the efficiency of AAV gene transfer in the presence of pre-existing immunity and suggest a potential safe and effective strategy for addressing this challenge in the clinic.

319 Immune Modulation Sustains Alpha Cell Reprogramming and Mitigates Immune Responses to AAV in a Diabetic Non-Human Primate Model

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Introduction:
Diabetes mellitus is characterized by insufficient beta cell mass and inadequate endogenous insulin production. Gene therapy using recombinant adeno-associated virus (rAAV) has shown promise for curative therapy in diabetes mellitus in pre-clinical models. Retrograde intraductal infusion of rAAV6 to deliver Pdx1 and MafA, converting alpha cells into beta-like cells, reverses diabetes in mice. Direct infusion of AAV into the pancreatic duct for gene therapy has not previously been attempted in non-human primates (NHPs) and thus the immune response in this species is unknown. In clinical trials, anti-viral immunity to AAV can be a barrier to successful gene therapy. We evaluated the immune response to intraductal rAAV in NHPs with streptozotocin-induced diabetes.

Methods:
Diabetes was induced with streptozotocin (STZ) in cynomolgus macaques. NHPs received retrograde intraductal infusion of rAAV via laparotomy and duodenotomy for precise access to the pancreatic duct. rAAV capsids were chosen based on tropism for NHP pancreatic endocrine cells, and pre-existing neutralizing antibody titers (NAbs) were negative. Blood work including serum C peptide and IV glucose tolerance tests were serially obtained to monitor gene therapy efficacy. Immune response monitoring was performed for up to 4 months post-infusion and included serial NAbs, ELISpot assays, and immunophenotyping. Pancreatic tissues were analyzed using IHC and RNA-scope for beta cell markers and virus-encoded genes, as well as single-cell RNA transcriptomics.

Results:
One-month post-infusion, NHPs showed improved glucose tolerance and reduced insulin requirements. The AAV6 capsid with endocrine-specific promoters driving Pdx1 and MafA showed durable effects. ELISpot-positive cytotoxic T cells and neutralizing antibodies developed when steroids were absent. With steroid-sparing regimens, pancreatic B and T lymphocyte populations were noted on scRNA sequencing. Temporary immunosuppression (IS), using a combination of rituximab, rapamycin, and steroids, for a 3-month course is effective at preventing anti-viral immunity. Despite complete IS discontinuation at 3 months post-infusion, meaningful anti-viral immune response was not mounted up to one month after IS withdrawal, as evidenced by low NAb titers and negative ELISpot analyses. Additionally, rAAV gene therapy in these animals remained effective and glucose tolerance continued improving in the absence of immunosuppression.

Conclusion:
We developed a novel rAAV gene therapy approach and demonstrated that infusion of rAAV directly into the pancreatic duct of NHPs induces an anti-viral immune response. The anti-viral immune response in NHPs can be prevented by administration of a multi-agent IS and can allow for sustained therapeutic effects.

320 Co-Stimulatory Blockade Regimen Prevents anti-Transgene and anti-Vector Immune Responses for an Effective and Re-Dosable in vivo Gene Therapy for Hemophilia A.

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Lentiviral vector (LV)-based in vivo gene therapy is attractive for its potential applicability to permanently correct monogenic diseases, even in childhood, with negligible limitations derived from pre-existing immunity towards the

parental virus. Although a single systemic LV administration has achieved stable transgene expression by hepatocytes in mice, dogs, and non-human primates, immunity towards transgene and vector may limit the therapeutic outcome and LV redosing.

In a mouse model of Hemophilia A (HA), LV-mediated factor VIII (FVIII) expression by hepatocytes revealed that multiple factors may contribute to the induction of T cell-dependent immune responses to FVIII, such as the protein structure, the proportion of transduced hepatocytes, the translation rate, and the protein half-life. A high dose of LV encoding for human (hu)FVIII (~$5X10^{10}$ TU/kg) in adult HA mice established stable expression of huFVIII (50% of normal) in absence of anti-FVIII immune responses in 80% of treated mice, by a combination of fasting and type-I interferon (IFN) signaling blockade (anti-IFNa receptor, aIFNAR) which allowed maximizing LV transduction efficiency and dampening innate immunity. Conversely, the administration of LV alone did not result in detectable huFVIII and induced anti-FVIII immunity. Comparable doses of LV encoding for half-life extended huFVIII.XTEN resulted in minimal and only transient FVIII expression in vivo, again abrogated by anti-FVIII immunity. To improve FVIII output in vivo at lower LV doses, the transgene sequence was codon-optimized (coFVIII), enabling 10-fold increased expression compared to huFVIII. This strategy allowed to obtain high levels of circulating FVIII produced by a limited proportion of hepatocytes but resulted in the induction of a strong anti-FVIII humoral and cellular immune response leading to clearance of LV-corrected hepatocytes. Therefore, we tested T cell-targeted immunotherapies to control anti-transgene immunity. We found that the administration of anti-IFNAR immediately before coFVIII-LV or coFVIII-XTEN-LV administration combined with the co-stimulation blockade regimen allowed stable huFVIII expression at 110% and 500% of normal, respectively, in HA adult mice by lower LV doses (~$2X10^{10}$ TU/kg). This immunotherapeutic regimen abrogated the induction of anti-FVIII antibodies and CD8+ T cells, allowing the persistence of LV-modified hepatocytes releasing FVIII in the absence of antibody-mediated neutralization, largely beyond the time of its administration.

Furthermore, we did not detect any neutralizing anti-VSV.G humoral response, typically induced by in vivo administration of VSV.G-pseudotyped LV, thus enabling an effective re-dosing of the same vector in case of a sub-therapeutic first dose. Interestingly, this immunotherapy was similarly effective also for re-administration of adeno-associated viral vectors. Overall, these data indicate that immunogenicity to therapeutic transgene can be controlled by i) increasing the proportion of transduced hepatocytes and lowering the amount of huFVIII produced per cell and ii) applying a co-stimulation blockade regimen to achieve stable FVIII expression at therapeutic levels, even increasing the rate of transgene translation in a reduced fraction of hepatocytes. Moreover, the latter strategy gives the opportunity to adjust transgene output by re-dosing in vivo gene therapy, thanks to the absence of immunity to transgene and vector particles.

321 Stimulation with Self Peptides Led to Interleukin-17 Secretion by Peripheral Blood Mononuclear Cells from an AAV-Treated Patient with Duchenne Muscular Dystrophy and Unexplained Fatality

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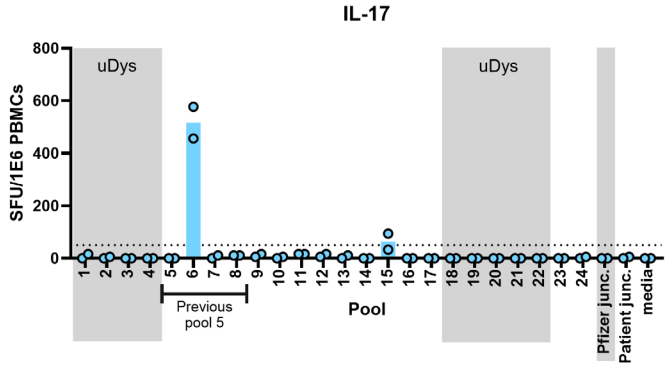
A 4-year-old boy treated with fordadistrogene movaparvovec had recurrent cycles of severe muscle pain, myoglobinuria and acute critical hyperCKaemia until his unexplained fatality 14 months following gene therapy. While another abstract at ASGCT (Lorentzos et al) focuses on his unusual clinical course, this abstract intends to present the findings from IFNγ/IL10/IL17 three-color FluoroSpots. The patient had a predicted in-frame deletion of *DMD* exons 45-48 accompanied by approximately 25-50% endogenous dystrophin expression by western blot and low-level immunofluorescent staining of endogenous dystrophin at the membrane on tissue sections. Despite this, he presented with a phenotype consistent with Duchenne muscular dystrophy (DMD). Peripheral blood mononuclear

cells (PBMCs) were isolated from whole blood at two time points during the course of the cyclical rhabdomyolysis events. The first blood collection occurred between crisis events, while the second occurred during an event. PBMCs were then used in a three-color FluoroSpot where cells were simulated with overlapping 15mer peptides spanning the patient's endogenous dystrophin (including the unique junction) and the transgenic microdystrophin (including unique junctions). Neither time point showed positive IFNγ or IL17 responses to peptides covering the microdystrophin. PBMCs from the first blood draw had a suggestive positive IL17 response to a peptide pool consisting of peptides contained only in the patient's endogenous dystrophin. This response was confirmed and the reactive peptides narrowed down with PBMCs from the second blood draw (see Figure). An additional pool of peptides covering only the patient's endogenous dystrophin was also identified as being weakly IL17 positive with PBMCs from the second blood draw (see Figure). While previous studies have shown patients with DMD to have IFNγ positive ELISpots upon stimulation of PBMCs with dystrophin peptides, we believe this is the first instance of IL17 responses being interrogated and documented in the same. The work described here was funded by Pfizer.



322 The Endosomal Recognition of AAV Genome is Critical for the Development of AAV Capsid-Specific Adaptive Immunity

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Adaptive immunity against the AAV capsid severely hampers vector transduction and prevents redosing, but the molecular mechanisms involved its development are not completely understood. In this study, we found that UNC93B1, a chaperone protein of endosomal toll-like receptors (TLRs)-3, -7/8, and -9, is critically involved in the development of both antibody responses and T cell responses against the AAV capsid. To demonstrate this, wild-type (WT) C57 mice or UNC93B1-knockout (KO) mice were administered 10^{14} vg/kg AAV vectors. Compared to WT mice, UNC93B1-KO mice presented markedly dampened antibody responses, characterized by delayed appearance and lower serum concentration of capsid-specific IgG. In contrast, the cytosolic DNA sensing STING-pathway did not affect the production of anti-capsid IgG. Similarly, UNC93B1-KO mice demonstrated significantly lower levels of capsid-specific T cell responses than WT mice, evidenced by fewer IFN-γ-releasing T cells following stimulation by AAV capsid peptide pool. The involvement of UNC93B1 was conserved across AAV8 and AAV9 and was independent of the encoded protein of the transgene. However, the presence of the AAV genome was required, since using empty capsids could partially, but not completely, reproduce the reduction of adaptive immune responses observed in UNC93B1-KO mice. Overall, we found that the endosomal recognition of AAV genome strongly promoted the development of adaptive immunity against the AAV capsid. As single TLR-7 or TLR-9 KOs did not show strong effects in reducing anti-capsid adaptive immune responses in previous studies, these results suggest that more than one endosomal TLRs, or additional UNC93B1-interacting partners, are involved in AAV genome detection. Furthermore, inhibiting UNC93B1 might be a possible route to reduce capsid-specific adaptive immunity.

323 Efficient Trafficking of AAV Encoded Protein from Hepatocytes to Hepatic Antigen Presenting Cells

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The ability of adeno-associated viral (AAV) vectors to transduce antigen-presenting cells (APCs) remains controversial. To address this, we utilized the LoxP-Stop-LoxP-tdTomato cassette in Ai14 mice in conjunction with a hepatotropic AAV vector expressing Cre recombinase. This approach enabled mapping of AAV transduction patterns in various liver APCs. In AAV-transduced cells, Cre excises the LoxP-flanked stop codon, enabling sustained expression of the fluorescent reporter protein tdTomato (tdT). Using this approach, we previously demonstrated widespread tdT expression in various liver APCs (abstract #1588, ASGCT 2024). Notably, variable levels of tdT fluorescence intensity (tdTmid and tdThi) were observed across APC populations

We hypothesized that these distinct intensities might result from different mechanisms. Here, we intravenously injected Ai14 mice with 2×10^{11} vg/mouse of an AAV8-CMV-Cre vector and evaluated the AAV transduction profile by tracking tdT expression kinetics in liver cells at 2, 4, and 6 weeks post-administration (n=4/timepoint). Consistent with prior findings, two distinct tdT-positive populations emerged: tdTmid and tdThi. The tdTmid population constituted 10–30% of type-2 conventional dendritic cells (cDC2), plasmacytoid dendritic cells (pDCs), and $CD11b^+$ cells (including Kupffer cells/KCs, monocytes and neutrophils), with only 5% of tdTmid cells being type-1 conventional dendritic cells (cDC1). In contrast, the tdThi population predominantly consisted of cDC1 (40%), with 6–10% comprised of cDC2, pDCs, and $CD11b^+$ cells. Additionally, we flow-sorted tdTmid and tdThi cells to analyze recombination events and vector genome copy numbers. Molecular analysis revealed Cre-mediated recombination in tdThi cells, while no recombination was detected in tdTmid cells. Vector genome quantification indicated higher AAV vector load in tdThi cells compared to tdTmid cells.

AAV8 has strong tropism for murine hepatocytes. To further elucidate the source of tdTmid and tdThi signals in APCs, we modified the AAV-CMV-Cre vector by incorporating four copies of liver-specific miR-122 target sequences (miR-122T) into its 3′ untranslated region (UTR). MiR-122, a liver-enriched microRNA, binds to complementary sequences (miR-122T) in the 3′ UTR, leading to mRNA degradation in hepatocytes. Intravenous administration of the modified vector reduced tdT expression in hepatocytes from over 95% to less than 5% at all time points in Ai14 mice. This intervention completely eliminated the tdTmid APC population, while significantly reducing the proportion of tdThi APCs. However, the percentage of $CD11b^+$ cells with tdThi expression remained largely unchanged. Together with molecular evidence showing that tdTmid APCs lacked Cre recombination events, while tdThi APCs showed recombination, these findings suggest that tdTmid signals predominantly result from the trafficking of hepatocyte-derived tdT, whereas tdThi arise from hepatocyte-released Cre protein or mRNA. However, AAV may directly transduce a subset of $CD11b^+$ APCs, which we identified as KCs.

Altogether, our results demonstrate efficient trafficking of functional transgene product from hepatocytes to diverse APCs in the liver, resulting in a spectrum of antigen expression among hepatic APCs, most of which does not require direct transduction but likely plays a central role in the orchestration of antigen-dose dependent T cell priming and tolerance induction within the liver microenvironment.

CMC for AAV Vectors

324 Diving Deeper: Using a SYBR Gold Capsid Ejection Assay as an Orthogonal Method of Measuring Potency and VP1 Deamidation in AAV Drug Product Samples

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A recent paradigm shift in the gene therapy industry has placed significant importance on the accurate determination of viral vector potency as a key measure of both product quality and efficiency. The idea that potency should be a top priority during production is gaining traction; however, there are several challenges that come with developing a robust potency assay. The field currently faces a critical gap in reliable orthogonal methods to assess potency in a fast, high-throughput manner, leaving scientists to rely on costly, time-consuming, traditional cell-based assay methods, often with wide variability. Though an orthogonal approach may not replace conventional potency methods, it may compliment them by providing quick, preliminary results to aid in go or no-go decisions. This is especially useful when making important decisions surrounding process development and drug product formulation screening.

In our work, we have discovered a direct correlation between initial fluorescence readings- using a SYBR gold assay to detect DNA leakage- and significant potency loss in AAV drug product samples stored at 25°C and 40°C over time. This correlation has been validated across multiple independent studies, demonstrating robustness and consistency. Through our analyses, we also found that initial fluorescence directly correlates with VP1 deamidation, which we hypothesize to be an additional indicator of potency. These small changes in genome integrity may not be detected using traditional viral genome titer assays but are easily identified thanks to the sensitivity of the SYBR gold capsid ejection method. Further, we have demonstrated a linkage between increasing hydrophobicity using hydrophobic interaction chromatography with increased SYBR gold fluorescence. This powerful analytical method provides us with even more insight as to what is occurring at the capsid level as it undergoes thermal stress.

By deriving an equation linking initial fluorescence to relative potency and VP1 deamidation, we introduce a rapid and practical method for assessing drug product potency and relevant post-translational modifications without significant cost or lengthy turnaround times.

325 The Need for an Unbiased Assay to Detect and Quantify Replication Competent AAV in Clinical Vector Products

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Replication-competent AAVs (rcAAV) are gene therapy product-related impurities that could have associated safety risks for patients. rcAAVs are conventionally detected in cell-based assays consisting of successive rounds of amplification in the presence of an adenovirus with a qPCR readout on viral gene sequences. The absence of rcAAV detection in 1E+08 vg has generally been used as an acceptance criterion.

Our data show that this cell-based method can generate significantly different results between testing facilities using different assay protocols. Moreover, we show that positive or negative results are highly dependent on the capacity of serotypes to infect the permissive cell line in vitro, which could be quite different from what is observed in vivo.

An analytical method that allows the unbiased detection and quantification of rcAAVs is thus needed. We have developed a method based on a multiplex digital PCR (dPCR) that targets 3 junctions specific to rcAAV genomes: ITR-Rep, ITR-Cap and Rep-Cap. It accounts for the presence of potential exogenous sequences and it takes advantage of the partitioning of the dPCR to isolate particles containing triple positive genomes. Several recombinant AAV vector batches of various serotypes, positive or negative for rcAAV in the cell-based assay showed similarly extremely low levels of triple positive genomes (less than 0.00008% of genomes quantified by qPCR) in comparison to wild type (wt)AAV. Interestingly the latter showed that only 3% to 7% of their genomes were triple positive. Results were independent of the HEK293/transfection manufacturing protocol. However, no

triple positive entities were found in baculovirus-generated preparations likely due to the modifications in constructs of the vector system.

Our data suggest that there are likely no HEK293/transfection-derived vector AAV preparations devoid of rcAAV genomes. In this context our efforts constitute the first step toward the goal of changing the way rcAAVs are detected and quantified. More results from other sponsors using AAV vectors are needed to solidify our preliminary data set and to engage in a field-wide discussion, including regulators, on how to generate specifications and acceptance criteria for this novel analytical method. This will help to better reflect on and provide mitigation strategies for potential safety risks associated with rcAAVs until means to eliminate them from vector batches have been identified.

326 pCO_2 Modulated Control of Recombinant AAV Production and Capsid Quality via Regulation of Pathways Linked to Rep and Cap Protein Expression

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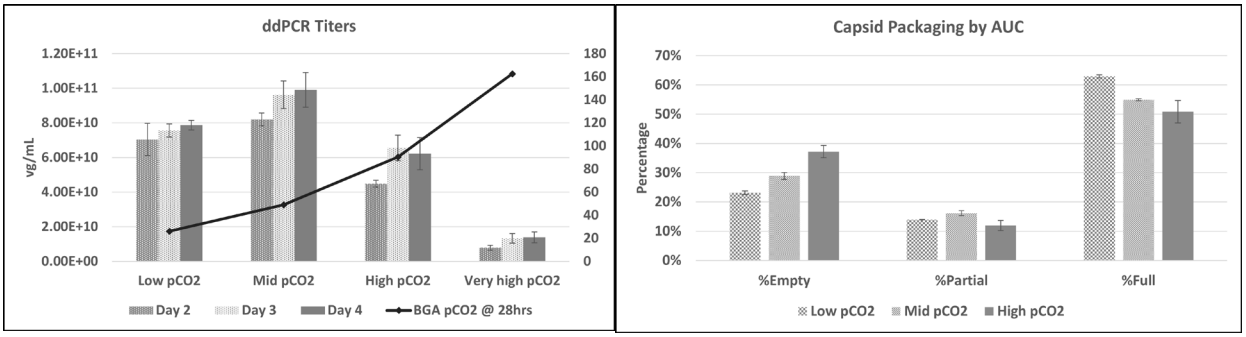
Recombinant adeno-associated virus (rAAV)-based gene therapies have shown promise as a one-time treatment to cure genetic diseases by delivering the encapsidated vector genome as therapeutic agents. To produce cost-effective, safe, and potent therapeutics, it is essential to optimize cell culture processes for rAAV production, with a focus on controlling capsid quality (full, empty, and partial capsids). This requires a comprehensive understanding of process parameters and their impact on rAAV productivity and quality. In this study, we identified the partial pressure of carbon dioxide (pCO2) as a regulator in upstream processes that significantly affects AAV titer as well as capsid quality in producer cell lines (PCLs). To investigate this further, we maintained different levels of pCO2 in separate bioreactors for the first 48 hours of AAV production. Analysis of harvest samples revealed an optimal range near the lower end of the investigated pCO2 range, as determined by droplet digital PCR (ddPCR). Purified harvest samples from these experiments were analyzed for capsid quality by Analytical Ultra-Centrifugation (AUC), which indicated a correlation between an increase in pCO2 level and a decrease in % full capsid. Western blot analysis was also performed on the purified samples to analyze Rep and Cap protein expression, which are linked directly to AAV productivity. The results showed the highest expression of Rep and Cap proteins at the lowest pCO2 levels, which suggests an underlying mechanism linking pCO2 levels with Rep and Cap protein expression and subsequent modulation of AAV productivity. In conclusion, our work across different cell lines and AAV serotypes demonstrated that PCLs benefit from a lowpCO2 range, with the timing and levels contributing to high titers and good capsid quality. Application of these insights to AAV manufacturing process development can significantly reduce the cost of goods manufactured (COGM) and ensure high % full capsid to reduce the risk of high levels of empty and partial capsids being delivered to the patients.



327 Stability of AAV2 Vector During Dose Preparation and Suprachoroidal Injection Using the Everads Injector

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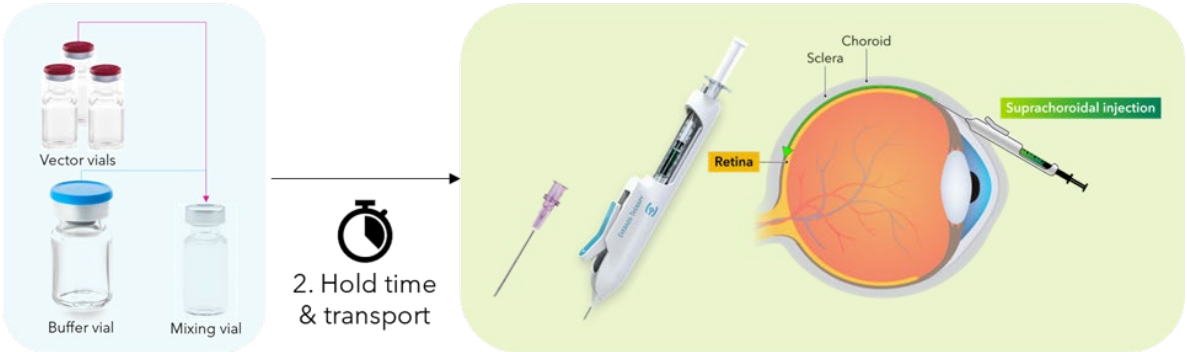
Kriya is pursuing a one-time AAV2-based gene therapy designed to block the complement pathway and reduce retinal lesion growth characteristic of geographic atrophy secondary to age-related macular degeneration. This therapy is to be delivered by suprachoroidal injection using a specialized device to better target the affected retinal tissues. As there is limited capacity for injection with any ophthalmic route of administration, drug products often must be diluted to prepare target doses. Ideally, dose preparations would occur as close to injection as possible. In practice, there is often a lag time between dose preparation in pharmacy and administration in clinic. Further, lubricants used in common syringes may challenge the strict subvisible particles safety limits for ophthalmic injections. It is thus critical to monitor product quality at all dose preparation and administration ranges proposed to ensure the doses patients receive are accurate, safe, and efficacious. A thorough in-use stability study was performed to establish stability of dose preparations and compatibility of our vectors with components to be used for preparation and administration.

A bracketed study design evaluated concentrations ranging from $1x10^{11}$ to $1x10^{13}$ vg/mL. The first part of the study assessed the accuracy and stability of dilutions prepared with lubricant-free syringes at refrigerated and room temperatures for up to 20 hours. The second part of the study evaluated loading and priming the Everads Injector, a novel suprachoroidal delivery device, with dose preparations stressed similarly to the first part of the study and then holding those doses for an additional 4 hours at room temperature before completing mock injections. Stability and compatibility were determined by comparing select product quality attributes (appearance, subvisible particles, vector genome titer, capsid titer, protein expression) to relevant controls or unstressed samples.

Concentrations of the high and low dose preparations were within expected variability of targets demonstrating target doses can be prepared accurately using syringe graduations. Mock doses achieved subvisible particle concentrations below USP <789> limits when 5µm filtered needles were used to extract prepared doses from compounding vials. No other notable product quality impact was observed from the use of filtered needles against unfiltered needles establishing their safety for use in clinical administration. No notable product quality impacts against respective controls were observed from the highest and lowest mock doses at the most strenuous conditions and durations evaluated. These results establish the stability of prepared dilutions for up to 20 hours at room temperature and stability of primed doses in delivery devices for up to 4 hours at room temperature allowing for flexibility between pharmacy and clinic. They also demonstrate that AAV2 vector is compatible with the components proposed (lubricant-free syringes and Everads Injector) to prepare and administer doses by suprachoroidal injection.



328 Development of a Flexible High Yielding, High Performing Process for Manufacturing of AFTX-201, a Novel Investigational AAV Gene Therapy for Treatment of BAG3 Dilated Cardiomyopathy

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Dilated cardiomyopathy (DCM) is defined as dilation and systolic impairment of the left ventricle and is the most common cause of heart failure (HF) in the young and most frequent indication for heart transplantation. More than 50% of patients with DCM have a genetic predisposition to the disease. B-cell lymphoma 2 (Bcl-2) associated anthanogene-3 (BAG3), a cochaperone that interacts with members of the heat shock protein (HSP) family, has been identified as a DCM-causing gene. BAG3-DCM represents a significant unmet medical need in this patient population with rapidly progressive cardiac dysfunction, and for whom no treatments targeting the underlying mechanism of disease exist. We are developing AFTX-201, an AAV-mediated gene replacement therapy to treat BAG3-associated DCM using a novel cardiotropic capsid with an improved safety profile.

The development of AFTX-201 required a flexible and scalable manufacturing process capable of handling diverse capsid-cargo combinations. To achieve this, we utilized Design of Experiment (DoE) studies to optimize the upstream process, resulting in high yields of >3e15 vg/L with >25% full particles at a 50L scale. To accommodate these yields, we developed a downstream process incorporating single-use chromatographic clarification, which increased throughput, reduced contaminants, and simplified setup. Affinity purification was optimized by focusing on capsid loading parameters rather than traditional viral genome loading, ensuring proper column sizing, reducing costs, and improving purity. A cesium chloride (CsCl) ultracentrifugation step was employed to achieve >90% enrichment of full particles. The process was successfully transferred to our CDMO partner, Forge Biologics, for toxicology material production, yielding high-quality vector with minimal host cell DNA, host cell proteins, and other common contaminants. The product has shown stability for over 6 months, with ongoing monitoring for extended time points. Additionally, the high yields enabled a reduction in Toxicology and Clinical lot production scale from 250L to 50L, resulting in significant cost savings without compromising quality.

329 Simplifying AAV Supply Chains: Demonstrating Equivalence of Long-Term Stability at -20 °C and -80 °C

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Most AAV products use the ultra-cold chain (≤ -60 °C) for long-term storage and distribution. However, the ultra-cold chain is expensive, burdensome, and requires specialized equipment and dry ice for shipments. While this has been accepted for ultra-rare diseases where AAV has been successfully utilized so far, Kriya is pursuing AAV-based gene therapies for prevalent diseases with larger patient populations. Simplifying the supply chain and reducing the cost of goods is necessary to bring gene therapies to a broader population. However, storing AAV products cold (-15 °C to -25 °C) raises concerns about product instability. These temperatures may be above the systems' glass transition temperatures where products may not be completely frozen which could lead to degradation not observed at lower temperatures. We sought to investigate this by evaluating comparative long-term stability of our investigational products at cold and ultra-cold frozen conditions.

Engineering lots of multiple drug products comprised of various AAV serotypes were placed on stability at multiple frozen conditions (≤ -65 °C, -20 °C) as well as multiple stress conditions (5 °C, room temperature) with appropriately spaced durations. Select product quality attributes including appearance, subvisible particles, genome titer, capsid titer, aggregation, capsid purity, genome integrity, and protein expression were evaluated at each duration using stability-indicating analytical methods. Product quality differences were assessed across conditions to

determine if the temperature of frozen storage had a significant effect on product stability. Durations of stability studies ranged from 3 months to over 18 months at the time of this writing, and all studies are ongoing.

Although product quality was impacted from stress conditions as expected (i.e. aggregation, protein modifications, losses of potency), no significant product quality changes have been observed at frozen conditions across all products. Further, no significant differences in any product quality attribute have been observed when comparing cold and ultra-cold stability results. These results suggest that -20 °C may indeed be a viable long-term storage condition for AAV products which could greatly simplify distribution and reduce the cost of next-generation gene therapies.

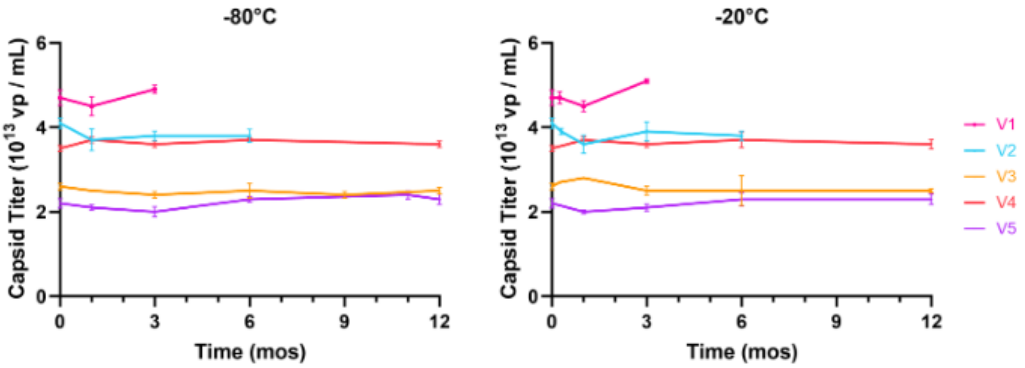

Figure 1: Capsid titer of different investigational AAV products stored at -80 °C and -20 °C over time.

330 Identifying AAV9 Degradation Pathways Caused by Stresses Encountered During the Product Lifecycle

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Kriya Therapeutics Inc. is a fully integrated biopharmaceutical company developing gene therapies to address common diseases. AAV gene therapies are comprised of capsid (protein) and vector genome (DNA), both of which have multifaceted degradation pathways from stresses encountered during the product lifecycle that would be expected to impact product quality. To increase our understanding of these degradation pathways and resulting impact on potency, we completed a forced degradation study of AAV9.

AAV9 vector was subjected to physical and chemical stresses experienced during the product lifecycle, including acidic (pH 2, 4), alkaline (pH 8.5, 10), thermal (40°C), photooxidative (~2000 lux), chemical oxidative (1 – 3% H_2O_2), shear (up to 1.8 Pa), and room temperature control, for various durations. Vector quality attributes evaluated included appearance, protein expression (cell-based assay), vector genome titer (ddPCR), capsid titer (Gyros), aggregation (SEC, DLS, light obscuration), capsid charge distribution (AEX), capsid purity (CE-SDS), and genome integrity (CE), which we have previously shown to be impacted by stress.

Multiple distinct degradation pathways were observed from different stresses. Increased 60 – 2000 nm aggregate populations were observed after exposure to pH 2.0 and shear stress, while increased subvisible particle concentrations (≥ 2 µm) were observed after exposure to pH 10 and 3% hydrogen peroxide. Decreased capsid and genome titers were observed with increased vector aggregation. Chemical modifications of the capsid increased with exposure to alkaline (pH 10 and 8.5), chemical oxidation (1000 ppm and 3% H2O2) and thermal stresses, while capsid degradation resulted from exposure to pH 2, pH 10, and 3% H_2O_2. Stress conditions also impacted vector genome stability, with vector genome degradation resulting from exposure to pH 2, 1000 ppm and 3% H_2O_2 and

progressive genome ejection from capsids occurring from shear stress. Reductions of potency (protein expression) were also observed to different extents when these various degradation pathways occurred.

This forced degradation study demonstrated that stresses experienced during the product lifecycle can cause distinct product degradation pathways for both vector genome and capsid proteins. These AAV9 degradation pathways also reduced potency. Investigating product degradation early in development can better inform exposure limits and product control strategies to maximize product quality.

Oligonucleotide Therapeutics II

331 Investigating the role of inflammatory cells in improving PMO delivery and exon-skipping efficiency in Duchenne muscular dystrophy

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**Corresponding Author*

Duchenne muscular dystrophy (DMD) is a genetic disorder that causes muscle degeneration due to the absence of dystrophin protein. This leads to a continuous cycle of muscle degeneration and regeneration, eventually resulting in muscle weakness and atrophy. For many years, there was no effective cure for DMD. However, exon-skipping therapy, which uses antisense oligonucleotide (ASO) drugs to restore dystrophin expression, has recently gained significant attention. To date, the U.S. Food and Drug Administration (FDA) has approved four exon-skipping ASO drugs (eteplirsen, gorodilsen, biltrasen, and casimelsen) for the treatment of DMD. In addition, new exon-skipping drugs are still being developed. ASOs based on phosphorodiamidate morpholino oligomers (PMOs) are neutrally charged and stable, minimizing immune activation and toxicity, making them ideal for targeted exon-skipping therapy. However, significant challenges remain, including the short retention time of PMOs in the bloodstream and their limited delivery to the heart and diaphragm muscles. To overcome these challenges, PMOs were modified with lipids or peptides to enhance their blood retention and tissue permeability. Despite these advancements, the fundamental mechanism by which PMOs are delivered to skeletal muscle tissue remains unclear.

In this experiment, we aimed to investigate how PMOs are taken up in skeletal muscle. To achieve this, we first generated transgenic mice capable of monitoring exon skipping *in vivo*. Using these mice, we studied the relationship between exercise and PMO uptake. Specifically, PMO was administered to mice subjected to low-intensity eccentric contraction (ECC) training, significantly increasing exon-skipping efficiency. To understand the underlying mechanisms, we performed RNA sequencing (RNA-seq) using the muscles that had undergone low-intensity ECC training. The results identified several genes associated with inflammatory cytokines, indicating that inflammatory cells might contribute to the uptake of PMOs in skeletal muscles. To support this hypothesis, PMOs were systemically administered to DMD model mice in which the number of inflammatory cells had been reduced through TNFα inhibition. As a result, the efficiency of exon skipping was significantly reduced. Additionally, when PMOs were introduced into cultured monocytes and subsequently administered to DMD model mice, exon skipping was successfully induced in the muscles of these mice.

These findings suggest that some of the administered PMOs may be delivered to skeletal muscles through inflammatory cells. We will further investigate the regulatory mechanism by which PMOs are transported to skeletal muscle.

332 Alpha Synuclein Lowering by miRNA-Based AAV Gene Therapy for Synucleinopathies: Proof of Concept and Biodistribution Studies in Rodent Disease Models and Non-Human Primates

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Synucleinopathies are clinically and pathologically heterogenous neurodegenerative disorders characterized by aggregation of alpha-synuclein protein (α-syn), encoded by the SNCA (Synuclein Alpha) gene. Pathological α-syn aggregates can be found in neurons and glia. Synucleinopathies are divided into two main groups: Lewy body (LB) diseases - Parkinson's Disease (PD) and Lewy Body Dementia - and Multiple System Atrophy (MSA). In PD, LB pathology originates in the brainstem and extends to midbrain and cortical regions with disease progression, in parallel with neurodegeneration of nigro-striatal dopaminergic circuits and other neurotransmitter systems. In contrast, MSA is characterized mainly by glial cytoplasmic inclusions (GCIs) in oligodendrocytes, with more extensive neuropathology including the olivopontocerebellar system. Only symptomatic therapies exist for PD. For MSA, symptomatic therapies are hardly effective. In both cases, there is a need for the development of disease-modifying strategies. Our hypothesis for a disease-modifying therapy is that lowering α-syn protein levels in relevant brain regions, i.e. putamen and midbrain, may reduce aggregation and degeneration, ultimately delaying disease progression.

We are developing an adeno-associated virus (AAV) gene therapy to deliver SNCA-targeting microRNAs (miSNCAs) engineered to decrease α-syn mRNA and protein levels. Here, we evaluate the potential phenotypic improvement by AAV5-miSNCA in two proof-of-concept studies in rodent PD and MSA models. Moreover, biodistribution and preliminary safety were evaluated in non-human primates.

In the PD study, adult rats received AAV1/2 overexpressing human A53T-α-synuclein ipsilaterally in the substantia nigra, at a dose known to cause neurodegeneration and PD-like motor phenotypes. Two weeks after AAV1/2-hA53T-α-synuclein administration, three different AAV5-miSNCA candidates were injected in the substantia nigra. All tested AAV5-miSNCA candidates showed correct microRNA processing and target engagement at both mRNA and protein levels. Moreover, dopamine metabolite deficiencies and motor deficits in hA53T-α-syn rats were corrected by AAV5-miSNCAs. Additionally, dopaminergic cell loss was rescued in the miSNCA-treated groups as compared to controls.

In the MSA study, a well characterized transgenic mouse model (MBP29) overexpressing wildtype human SNCA under the MBP promoter was used. Three different doses of our lead AAV5-miSNCA were administered at 2 months of age. At 9 months, dose-dependent target engagement at both mRNA and protein levels was observed. Moreover, myelin deficits were corrected, along with increased survival rate, in a dose-dependent manner.

Finally, we show safe and efficient delivery of AAV-miSNCA in non-human primates after MRI-guided intra-putamen bilateral injections. Furthermore, dose-dependent target engagement in putamen and midbrain were shown at both mRNA and protein levels. These results support the potential therapeutic value of α-syn RNAi-based gene therapy for disease modification in both PD and MSA.

333 ADAR-Mediated RNA Editing of *SLC10A1* (NTCP) as a Therapeutic Approach to Reduce Liver Bile Acid Re-Uptake in Cholestatic Diseases

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Bile acid homeostasis is crucial for liver function, and the Sodium Taurocholate Co-Transporting Polypeptide (NTCP, *SLC10A1* gene) is integral in the hepatic re-uptake of bile acids. Dysregulation of this process, leading to toxic bile acid build up, is implicated in liver cholestatic diseases such as Primary Sclerosing Cholangitis (PSC) and Biliary Atresia (BA). These conditions currently lack effective therapies, leaving a significant unmet medical need. Innovative therapeutic strategies, grounded in human genetics, could selectively lower NTCP's function in bile acid re-uptake without affecting its other cellular roles. Sufficient NTCP modulation leading to a 2-fold bile acid increase in plasma, is expected to have a therapeutic effect. Axiomer, an RNA editing platform, uses chemically modified editing oligonucleotides (EONs) complementary to the *SLC10A1* mRNA to recruit and guide endogenous Adenosine Deaminase Acting on RNA (ADAR) to perform targeted A-to-I editing on the double stranded structure. This results in a single amino acid change in the protein sequence (NTCP Q68R), enabling alteration of the bile acid re-uptake function in NTCP. Here we present preclinical proof of concept data of the approach and the efficiency of Axiomer EONs in *in vitro* and *in vivo* studies.

In-silico 3D structural modelling of the NTCP Q68R variant demonstrated disruption of hydrogen bonds and contacts for Na+ binding which are essential for bile acid transport. In *in vitro* assessment, a plasmid expressed NTCP Q68R variant demonstrated a 7-fold reduction in bile acid uptake vs. wild-type with no impact on protein expression nor localization. EONs targeting *SLC10A1* RNA demonstrated a 20% RNA editing efficiency *in vitro*, and a dose-dependent bile acids uptake inhibition (40% decrease with the 50nM dose vs. control to a near complete inhibition with the 100nM dose vs. control).

In follow-up *in vivo* studies, ADAR mediated editing of NTCP resulted in a mean editing efficiency of 13.3% in humanized liver mice and 18.3% in NHP respectively (reaching up to 24% in NHPs). These EONs led to a mean change of 4.4-fold in plasma bile acids, above the 2-fold change therapeutic threshold. Correlation between plasma bile acids and EONs editing efficiency (linear regression $R_2 = 0.51$) was also reported. The observed change in plasma bile acids levels was associated with a change in bile acids profile, from an unconjugated (73%) / conjugated (27%) ratio to an unconjugated (13%) / conjugated (87%) ratio post treatment in NHPs. In safety analysis, EONs confirmed class safety, with no hepatotoxicity or immunostimulatory score.

ADAR-mediated editing of NTCP is a promising approach to address unmet medical needs in cholestatic diseases, such as PSC and BA. By decreasing bile acid re-uptake in the liver and increasing plasma bile acids leading to compensatory mechanism limiting bile acid production, the technology can potentially alleviate the bile acid toxicity in the liver. A clinical candidate, AX-0810, using GalNAc-mediated delivery and with enhanced potency (reaching up to 57% editing, representing a 5.5-fold increase over early generation) and stability profile, is being investigated in longer term *in vivo* studies and will be further investigated in clinical trials starting this year.

334 A First-in-Human Double-Blind, Placebo-Controlled Single and Multiple Ascending Dose Study (SAD, MAD) in Healthy Volunteers to Evaluate the Safety and Tolerability of an Investigational Antisense Oligonucleotide Therapy (CMP-CPS-001) for the Treatment of Urea Cycle Diseases (UCDs): Interim Safety Readout

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Introduction
UCDs are a group of 8 rare genetic diseases, each caused by a distinct autosomal recessive or X-linked defect in one of the 6 enzymes or 2 transporters in the urea cycle. The urea cycle is responsible for elimination of the noxious metabolite ammonia. The most common clinical characteristic of UCDs is episodes of hyperammonemia, triggered by increased protein intake or protein catabolism (e.g., during infection or overexertion). Symptoms include irritability, headache, vomiting, and ataxia in milder cases. Seizures, encephalopathy, coma, and death can occur in cases with ammonia levels >200 μmol/L (adult levels). Symptomatic treatment is comprised of reducing nitrogen

production (via a strict low-protein diet) and augmentation of nitrogen elimination (nitrogen scavengers) on a chronic basis. Liver transplantation is a treatment option, however it is usually limited to early-onset cases and comes with its own morbidity and mortality risks. Several approaches to correct or compensate for the underlying causes are in development, including gene and mRNA therapies for OTC-deficiency. CMP-CPS-001 is an antisense oligonucleotide that targets a regulatory RNA of the gene encoding carbamoyl phosphate synthetase 1 (CPS1), the initial and rate-limiting enzyme responsible for catalyzing ammonia to carbamoyl phosphate and coordination of the cycle's regulatory function across downstream enzymes. Thus, CMP-CPS-001 is a new and differentiated therapeutic strategy to restore urea cycle function for OTC deficiency and potentially other UCDs.

Methods
Phase 1 study CPS-101 evaluates the effect of CMP-CPS-001 administered subcutaneously in healthy participants in 2 parts (NCT06247670). Part A is a SAD with 4 sequential cohorts of 12 participants (3:1 ratio of active drug:placebo). After dosing, each participant was followed for 6 weeks. Part B is a MAD with 4 cohorts of 12 participants (same 3:1 ratio as Part A). Participants in Part B will receive 3 monthly doses of study drug with a 56-day follow-up post last dose. Part A and Part B include measures of ureagenesis via isotope-labeling as a pharmacodynamic marker to be reported in the future. Part A has been completed and blinded interim results are presented here. Part B is ongoing.

Results
Part A (SAD) enrolled 48 individuals (36 active:12 placebo) across 4 dose cohorts of 0.2, 0.6, 1.8, or 4.0 mg/kg . Twenty males and 28 females, with a mean age of 32.6 years (range 18-55) participated. A total of 40 treatment emergent adverse events (TEAEs) were observed, with 22 participants (46%) reporting at least one TEAE. All TEAEs were Grade 1 (mild) or Grade 2 (moderate). No treatment-emergent serious adverse events (SAEs) were observed. No participant discontinued the study due to an adverse event. The top 2 most common TEAEs across SAD cohorts were headache (N=6) and nausea (N=4). Four participants experienced TEAEs related to injection/administration at the site of injection (2 pain, 1 injection site reaction, 1 injection site erythema), all of which recovered/resolved. No clinically significant liver function or thrombocytopenia abnormalities were observed. CMP-CPS-001 was well tolerated and there is no indication of a maximum tolerated dose at the dose levels tested.

Conclusions
Overall single doses of CMP-CPS-001 from 0.2 mg/kg to 4.0 mg/kg were well tolerated. No SAEs were observed. The majority of TEAEs were Grades 1 and 2.

335 Targeting Regulatory RNAs with Antisense Oligonucleotides for the Potential Treatment of SYNGAP1-Related Disorders

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Regulatory RNAs (regRNAs) are long, short-lived, noncoding RNAs expressed from enhancers and promoters that act locally to regulate gene expression. This regulation is accomplished through the ability of regRNAs to bind transcription factors and increase their local concentration near a gene's promoter and enhancer elements. Our RAP Platformä enables the identification of regRNAs and antisense oligonucleotides (ASOs) targeting these regRNAs to modulate target gene expression. Here, we describe the development ASOs that target regRNAs of *SYNGAP1* to potentially restore its expression in patients with mutations that cause SYNGAP1 haploinsufficiency.

Truncation and loss of function mutations in the *SYNGAP1* gene cause SYNGAP1-related disorders, a group of devastating neurodevelopmental conditions characterized by a constellation of burdensome phenotypes, including intellectual disability, severe behavioral problems, seizures, motor abnormalities, and impaired communication. Mutations result in loss of expression of the affected allele, resulting in haploinsufficiency. Insufficient levels of SYNGAP1 in postsynaptic terminals results in increased insertion of AMPA receptor at the post-synaptic membrane

and increases neuronal activity. Hyperexcitability throughout development and life results in the aforementioned phenotypes.

Increasing SYNGAP1 protein levels in amounts sufficient to yield a therapeutic benefit is a potential therapeutic strategy for SYNGAP1-related disorders. We employed our RAP Platform to identify ASOs that target a *SYNGAP1* regRNA to increase *SYNGAP1* transcription and protein levels in multiple cell types, including patient-derived neurons. The impact of increased SYNGAP1 protein levels on SYNGAP1 haploinsufficiency is explored. A lead ASO was administered to a humanized SYNGAP1^{hu} knock-in/knock-out mouse through intracerebroventricular injection and shown to increase SYNGAP1 protein levels by up to ~2-fold. Critically, intrathecal administration of this lead ASO to cynomolgus monkeys resulted in a significant ~1.5-fold increase in SYNGAP1 protein.

These data demonstrate that targeting a *SYNGAP1* regRNA with an ASO increases *SYNGAP1* transcription *in vitro* and *in vivo*. Importantly, administration of the ASO by a clinical route of administration in these animal studies demonstrated a marked increase SYNGAP1 protein level, supporting the continued advancement of this program as a potential treatment for patients with SYNGAP1 haploinsufficiency.

336 Antisense oligonucleotides targeting toxic CGG-repeat expansion in primary ovarian insufficiency due to Fragile X premutation

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A common cause of primary ovarian insufficiency (POI) is Fragile X premutation (FXPOI), a genetic condition found in 1 in 148-291 women. This condition is caused by an expansion of the CGG trinucleotide repeat in the 5' untranslated region of the fragile X messenger ribonucleoprotein 1 (*FMR1*) gene, leading to toxic CGG-RNA and aberrant poly-glycine (polyG) protein buildup. FXPOI not only causes early menopause, but also infertility, increased risk of osteoporosis, cardiovascular disease, dementia, and adult-onset debilitating neurological disorders. Currently, there is no targeted treatment for individuals with FXPOI.

To address this unmet need, we designed three antisense oligonucleotides (ASOs) with varying chemistries and lengths. Together with previously published ASOs, these ASOs were tested in human granulosa cells (KGN line) that overexpressed 99 CGG repeats tagged with green fluorescent protein (GFP). Treatment significantly reduced CGG-GFP RNA and protein levels, as shown by qPCR, immunofluorescence, and flow cytometry. We also established primary cultures from human granulose cells isolated from the follicular fluid of patients undergoing assisted reproductive technology. After treating the cells with ASOs, we observed reduced CGG-protein levels. Notably, ASO treatment decreased oxidative stress (measured by MitoSox) without altering estradiol hormone production. To assess off-target effects, we performed bulk RNA-seq on treated versus untreated and control primary human granulosa cells. Differential gene expression analysis is ongoing.

For *in vivo* studies, we have a knock-in mouse that harbors 117 CGG repeats in the endogenous *FMR1* 5'UTR region. Preliminary data show that Cy3-labeled ASOs persist in ovarian tissue for several days after intraovarian injections and ongoing studies will determine the efficacy of ASO treatment in ovarian function including ovulation.

In conclusion, we have developed a novel RNA-based intraovarian therapy that directly targets the genetic cause of FXPOI. Our ultimate goal is to translate these findings into clinical trials, offering a much-needed gene-based therapeutic strategy for women with Fragile X premutation, paving the way for similar gene therapies in reproductive medicine.

337 Targeting Regulatory RNAs with Antisense Oligonucleotides for the Potential Treatment of Urea Cycle Disorders

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Regulatory RNAs (regRNAs) are long, short-lived, noncoding RNAs expressed from enhancers and promoters that act locally to regulate gene expression. This regulation is accomplished through the ability of regRNAs to bind transcription factors and increase their local concentration near a gene's promoter and enhancer elements. Our RAP Platform™ enables the identification of regRNAs and antisense oligonucleotides (ASOs) targeting these regRNAs to modulate target gene expression. Using this platform, we successfully identified CMP-CPS-001, a clinical candidate targeting a regRNA of the gene encoding carbamoyl-phosphate synthase 1 (CPS1), the rate-limiting enzyme in the urea cycle.

The urea cycle is responsible for the elimination of ammonia from the body by its conversion to urea and subsequent excretion in urine. Mutations in enzymes of the urea cycle cause urea cycle disorders (UCDs), a group of rare disorders characterized by the toxic accumulation of ammonia that results in neurological symptoms and even death. At present, UCDs are inadequately treated, and a significant unmet need remains.

CPS1 is the first enzyme in the urea cycle that directly acts upon ammonia. We posited that increasing CPS1 activity could reduce ammonia levels by increasing metabolic flux through the urea cycle. We identified ASOs that target a human *CPS1* regRNA, as well as surrogate ASOs that target a mouse *Cps1* regRNA, and increase the expression of these genes. Increasing *Cps1* expression using a mouse surrogate ASO reduced plasma ammonia levels and increased urea production following an acute ammonia challenge in a disease-relevant mouse model carrying a mutation in ornithine transcarbamylase gene (*Otc*), a urea cycle gene mutated in ~60% of patients with UCDs. The clinical candidate – CMP-CPS-001 – increased *CPS1* gene expression *in vitro* in both wild-type and patient-derived primary hepatocytes, and reduced plasma ammonia levels in a humanized-liver mouse model. Finally, administration of CMP-CPS-001 resulted in increased ureagenesis in wild-type cynomolgus monkeys.

These preclinical data demonstrate that targeting a *CPS1* regRNA and increasing *CPS1* expression may directly ameliorate hyperammonemia, which is the key driver of symptomology in UCD patients. Moreover, the mouse model data show that increasing *Cps1* expression can lower ammonia and increase ureagenesis in the context of a mutation in *Otc*. CMP-CPS-001 is currently being evaluated in a Phase I clinical trial in adult healthy volunteers and is in development for the potential treatment of multiple urea cycle disorders.

Cell Therapy Product Engineering II

338 Feasibility of Manufacturing CARv3-TEAM-E T Cells for Intraventricular Injection in Recurrent Glioblastoma

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Background:
Promising Phase 1 clinical data in glioblastoma has been demonstrated following infusion of T cells transduced with a second-generation chimeric antigen receptor (CAR) targeting epidermal growth factor receptor vIII (EGFRvIII)

and secreting a T-cell–engaging antibody molecule (TEAM) against wild-type EGFR (Choi, NEJM 2024: INCIPIENT, NCT05660369). Feasibility of manufacturing CARv3-TEAM-E cells from glioblastoma patients for intraventricular delivery was a secondary study objective.

Methods:
The CliniMACs Prodigy® TCT program was used to generate a target of 6 doses of $10x10^6$ CAR T cells from autologous apheresis material over 7 days. Starting cell numbers and feeding schedule were optimized, with IL-2 used in expansion. Extensive validations were required to identify optimal cell concentrations and diluent solution to facilitate intraventricular delivery via Ommaya catheter and minimize amounts of cryopreservative and cellular debris.

Results:
CAR T manufacturing was successful for all 12 patients infused to date. Fresh starting material consisted of 12Ls apheresed (median 2.2 blood volumes, range, 1.9-3.5). Number of frozen doses for infusion achieved was median 8 per run (range, 4-11). CD3+ T cells comprised median 98.4% of the final product. Median CAR transduction efficiency was 22.4% (range, 15.6-28.1%), as assessed by flow cytometry detection of a CD19 epitope encoded on the CAR-containing viral vector. Median CD3+ T-cell fold-expansion over the course of culture was 20.8% (range, 13.2-25.1%). All sterility and PCR-based safety criteria were met (see Table). Median percent of CAR+ cells with secreted and then bound TEAM was 78.0% (range, 67.0-93.7%) compared to CAR negative cells with bound TEAM of median 3.0%. Notably, manufacturing characteristics of products derived from recurrent glioblastoma patients were similar to what we had observed in validation runs using cells from healthy donors. A feasible high concentration of total nucleated cells/mL was initially identified in normal donor validation studies to minimize amount of infused cryopreservative. For clinical runs this yielded frozen drug product volumes of median 0.45mL (range, 0.36-0.65mL). Thawing conditions and the diluent solution were optimized in normal donor validation studies for cell recovery and viability in the final 10mLs of diluted product to be administered intraventricularly.

Conclusions:
All intended autologous CARv3-TEAM-E T-cell products were successfully manufactured, yielding multiple doses. The manufacturing process of CAR transduction and expansion was consistent and reliable as reflected by low (two-fold) variability in T-cell expansion and transduction levels. Intraventricular delivery requires validation not just of manufacturing a CAR product but also of optimal cell concentration, freezing media and controlled rate freezing curves, and thawing conditions and diluent to ensure safe and effective delivery.

Table: CARv3-TEAM-E Drug Product Release Criteria

Release Criteria	Acceptable Values	Median	Range
Purity (%CD3+T cells of viable CD45+ cells)	≥ 90%	98.4%	96.4-99.2%
Transduction Efficiency (TE) (% CD19+ of viable CD3+CD45+ T cells)	≥ 10%	22.4%	15.6-28.1%
# Full Doses Frozen	Target 6 (Minimum 1)	8	4-11
Viability (Trypan Blue)	> 70%	95%	87-99%
Endotoxin	≤ 5 EU/kg/hr	<0.0013	<0.0009-0.0028
Mycoplasma (qPCR)	Absent	All Absent	N/A
Sterility (BacT)	NGTD at time of release (> 5 days in culture)	NGTD All negative at 14 days	N/A
VCN copies per transduced cell	0.2-10	2.0	1.5-2.4
RCL by qPCR	Decreasing and </= 100 copies/0.2ug DNA	Pass	N/A
EGFR-CD3 TEAM bound to EGFRv3 CAR+ Cells	≥ 5.0% CAR+ T cells with TEAM bound	78.0%	67.0-93.7%
EGFR-CD3 TEAM bound to EGFRv3 CAR- Cells	For Information Only	3.0%	1.9-6.6%

339 dnTGFβRII-Armored DLL3-Targeted CAR-T Cells Maintain TGFβ Resistance with Early Signals of T-Cell Exhaustion Modulation After Expansion in SCLC

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Introduction:
TGFβ promotes tumor growth through direct and indirect mechanisms by modifying the tumor microenvironment and inducing T-cell exhaustion. Lower TGFβ levels are linked with enhanced CAR-T efficacy. A dominant-negative TGFβ receptor (dnTGFβRII) was integrated into the DLL3-targeted CAR-T product LB2102, currently under phase 1 investigation (NCT05680922).

Methods*:*
Preclinical experiments utilized primary human T-cells from two independent donors, transduced with either the dnTGFβRII-armored DLL3-targeted CAR-T construct (LB2102) or the non-armored control (LCAR-T20). For clinical validation, LB2102 cells and endogenous lymphocytes were collected during CAR-T expansion from a phase 1 study participant who achieved a partial response.

Results*:*
LB2102 CAR VHHs demonstrated high specificity and no cross-reactivity to other DLL family members or unrelated proteins. LB2102 cells did not exhibit off-target killing in a panel of human primary cells (n=13). To assess whether dnTGFβRII expression confers resistance to TGFβ, LB2102 cells and unarmored LCAR-T20 cells were incubated with TGFβ1, and SMAD2 phosphorylation was assessed as a readout for TGFβ1-mediated signal transduction. SMAD2 phosphorylation was significantly inhibited in the armored LB2102 cells (Fig 1). Additionally, TGFβ1-induced expression of exhaustion markers was inhibited in the armored LB2102 cells, in contrast to that in the unarmored control cells.

In the patient-recovered LB2102 cells collected on Day 15 post-infusion, TGFβRII expression was observed in more cells and at a higher expression level in CAR+ T-cells (79.2%) compared to endogenous T-cells (5.61%). While baseline pSmad2 signaling remained low in both populations, ex vivo incubation with TGFβ1 induced SMAD signaling in the patient's endogenous lymphocytes but minimally in dnTGFβRII-armored LB2102 cells. Thus, the TGFβRII armoring effectively blocked TGFβ signaling upon exposure to TGFβ1 ex vivo (Fig 2). Notably, armored CAR+ T-cells also exhibited lower expression of exhaustion markers, specifically TIGIT expression was 22.9% in CAR-T cells compared to 50.8% in endogenous T-cells collected on Day 22 (Fig 3).

Conclusion and Discussion:
Recovered LB2102 CAR-T cells retained dnTGFβRII armor functionality, validating preclinical findings in a limited patient setting. This suggests that the phenotypic and functional stability of LB2102 CAR-T cells may be maintained after infusion and expansion in a patient with SCLC. Additionally, patient-recovered LB2102 cells exhibited reduced expression of exhaustion markers compared to the patient's wild-type lymphocytes. These findings support the hypothesis that dnTGFβRII armored CAR-T (LB2102) may counteract TGFβ-induced immunosuppression in this tumor, in part by reducing T-cell exhaustion. Despite limited data, the observed clinical response in this patient suggests that the armored CAR-T approach has the potential to enhance clinical efficacy.

Figure 1. Preclinical Inhibition of SMAD2 Phosphorylation by dnTGFβRII

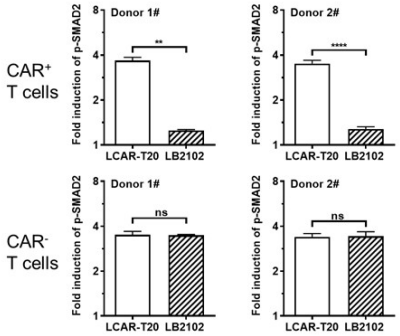


Figure 2. Patient-recovered cells: Inhibition of SMAD2 Phosphorylation in CAR-T cells or endogenous lymphocytes

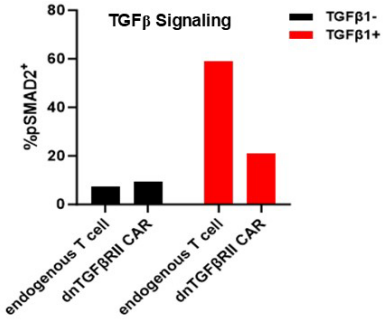
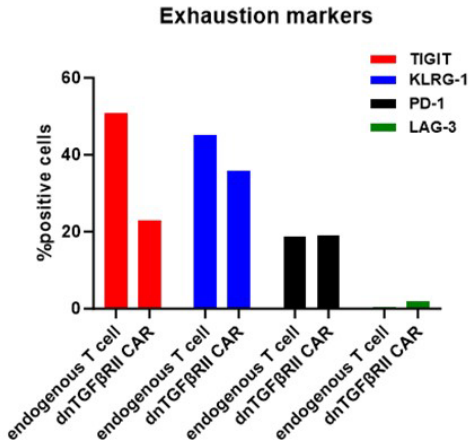


Figure 3. Patient-recovered: Expression of Exhaustion Markers in CAR-T cells or endogenous lymphocytes



340 A Novel Hybrid Lentiviral Envelope Pseudotype for the Efficient Transduction of Hematopoietic Stem and Progenitor Cells with Minimal Manipulation

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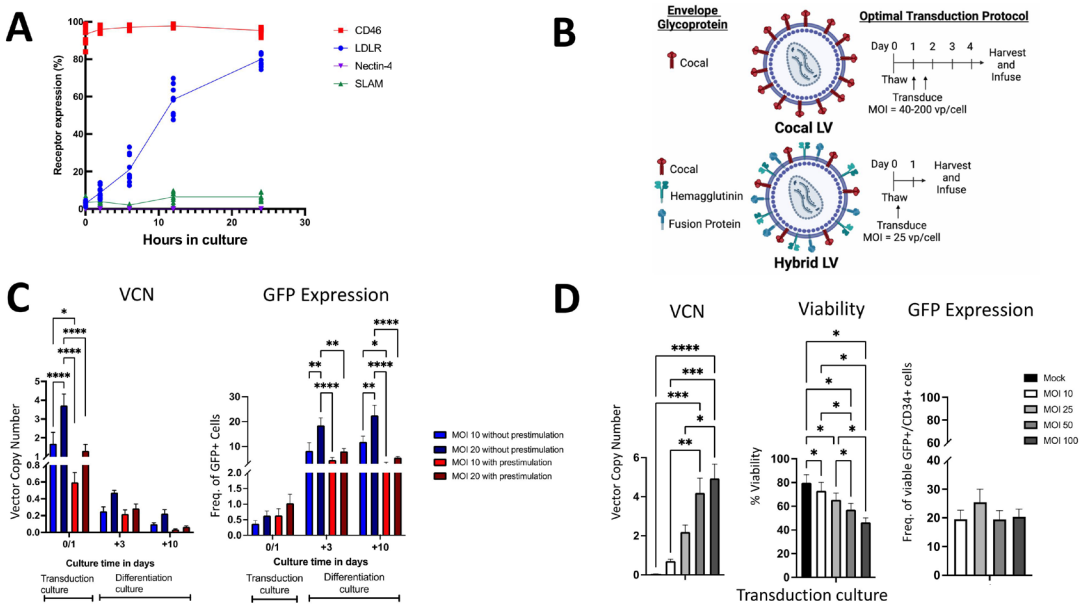
Hematopoietic stem and progenitor cells (CD34+) are promising targets for cell and gene therapies but their quiescent state limits transduction efficiency. A vector capable of transducing quiescent CD34+ cells could overcome these limitations. Vesicular stomatitis virus glycoprotein (VSVG)-enveloped lentiviral vectors (LV) are the current standard for CD34+ cell transduction but rely on the low-density lipoprotein receptor (LDLR), which is absent on resting CD34+ cells. Despite also displaying tropism for LDLR, we previously showed that cocal pseudotyped LV outperforms VSVG for transduction of CD34+ cells [Bayigga, L., ASGCT 2023]. Further, it is reported that LV pseudotyped with modified measles virus envelope glycoproteins, hemagglutinin (H) and fusion protein (F), utilize alternative receptors such as CD46 and SLAM to facilitate superior transduction in resting CD34+ cells [PMID: 29296856]. We assessed CD46, LDLR, SLAM and Nectin-4 expression in cryopreserved, G-CSF mobilized and purified primary human CD34+ cells (n=10 unique donors) over 24 hours in supportive culture for transduction (StemSpan SFEM II media + recombinant human cytokines). CD46 was consistently highly

expressed across all time points, whereas LDLR expression increased over time, reaching 58 ± 2% after 12 hours in culture (mean ± SEM). SLAM expression was minimal, while Nectin-4 expression was absent (Figure A).
We hypothesized that LVs incorporating both cocal and measles glycoproteins (hybrid LV) could enhance transduction of cryopreserved CD34+ cells at lower multiplicity of infection (MOI) to maximize vector batch yields (Figure B). Standard fourth generation LV preparation in 293T cells resulted in several formulations (F1, F4, F9, F11) with infectious titers ranging from 3.79E09 to 1.31E10 vector particles (vp)/mL. Of these, F4 demonstrated the highest transduction efficiency. Primary human CD34+ cells (n=4 donors) were transduced with cocal LV or F4 LV at MOI of 10 vp/cell as single hit transductions 24 hours following thaw with or without magnetically assisted transduction (MAT). MAT modestly increased transduction efficiency but was not statistically significant ($p > 0.05$). We next evaluated transduction timing by adding F4 LV at MOI of 10 or 20 vp/cell with or without 24-hour pre-stimulation culture (Figure C). Transduction was higher when vector was added immediately post-thaw (n=4 donors), suggesting preference for CD46 over LDLR. We further tested increasing MOI (10–100 vp/cell), with optimal transduction at MOI of 25 vp/cell (VCN: mean=2.2±0.3 copies/cell; %GFP+: 25.4 ± 4.5%), as higher MOI's were associated with reduced viability (Figure D). Clinical grade F4 LV (GFP- LV-158; titer = 1.4E09 vp/mL) was prepared for preclinical testing in a humanized NSG mouse model. CD34+ cells from four human donors were transduced immediately post-thaw (MOI of 25 vp/cell), cultured overnight and transplanted into mice following myeloablative total body irradiation at 1E06 viable cells/mouse (n= 8 mice/donor). The standard clinical transduction (24-hour prestimulation, 2- hit cocal
LV at MOI of 25 vp/cell) served as a positive control. Engraftment kinetics and transduction efficiency in peripheral blood are ongoing and will be presented. Our findings demonstrate proof-of-concept for a novel hybrid-envelope LV enabling low MOI, single-hit transduction of CD34+ cells immediately post-thaw, with no additional transduction enhancement required.



341 Biomaterials Manufacture and Deliver CAR T Cells In Vivo for Potent and Affordable Glioblastoma Treatment

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Background

Glioblastoma (GBM) is the most common brain malignancy in adults with poor prognosis and no effective treatment. Chimeric Antigen Receptor (CAR) T cell therapy is a promising anti-cancer immunotherapy but suffers from expensive (~$500,000) and labor intensive (>2 weeks) manufacturing per dose. The lengthy ex vivo expansion

also deteriorates T cell quality as it encourages T cell differentiation. Previously, our lab has fabricated implantable dry, macroporous, alginate scaffolds (drydux) that efficiently mediate viral transduction and in vivo delivery of T cells. Drydux transduction is dependent on the hygroscopic nature of the biomaterial that allows drydux to absorb T cells and virus, causing these particles to collide for gene transfer. Drydux reduces CAR T cell manufacturing time to three days and improves the quality and potency of CAR T cells due to in vivo CAR T cell manufacturing **(Fig 1A)**.

Methods and Results

We performed in vitro cytotoxicity studies and an in vivo GBM resection model to determine antitumor efficacy of drydux-produced CAR T cells directed at the tumor-associated antigen B7H3. B7H3 is a type I transmembrane protein highly expressed in 60-70% of clinical GBM samples but limitedly expressed on healthy brain tissue. We have shown that drydux are capable of transducing T cells with B7H3-encoding retrovirus at transductions efficiencies above 80%, comparable to the conventional CAR T transduction method of spinoculating activated cells and retrovirus on RetroNectin-coated plates **(Fig 1B)**. To asses cytotoxic function in vitro, B7H3-specific CAR T cells made using drydux or conventional methods and U87 GBM cells were seeded together at different effector to target (E:T) ratios. After 5 days of coculture, cytotoxicity was analyzed using flow cytometry to measure the frequency of residual tumor cells. Inflammatory cytokine expression was analyzed 48 h after the start of coculture. At all E:T ratios, drydux-produced CAR T cells performed as well as conventionally-produced CAR T cells. All tumor cells were eradicated at higher E:T ratios. At 1:5, 1:10, and 1:20 ratios, about 90%, 70%, and 35% of tumor cells were killed, respectively **(Fig 2A)**. Drydux-produced CAR T cells produced similar or increased inflammatory cytokines compared to conventional CAR T cells. Non-transduced T cells did not produce any cytotoxicity. To evaluate antitumor efficacy in vivo, U87-GFP-FFLUC cells were injected intracranially into NSG mice. After tumor growth, ~96% of the tumor was resected. Drydux loaded with activated T cells and B7H3-specific CAR-encoding retrovirus were implanted into the resection cavity and compared to B7H3-specific CAR T cells created conventionally and directly injected into the resection cavity. Drydux loaded with activated T cells, but no retrovirus, were used as a negative control. 71% of mice in the drydux group had noticeably reduced tumor burden compared to only 50% in the conventional group and 0% in the negative control group, indicating the drydux may have enhanced antitumor efficacy **(Fig 2B)**.

Conclusions

These results demonstrate drydux-produced CAR T cells work as well as conventionally-produced CAR T cells at treating GBM, but with a significantly shorter manufacturing time. Biomaterials may provide the boost CAR T cell therapy needs to successfully treat GBM.

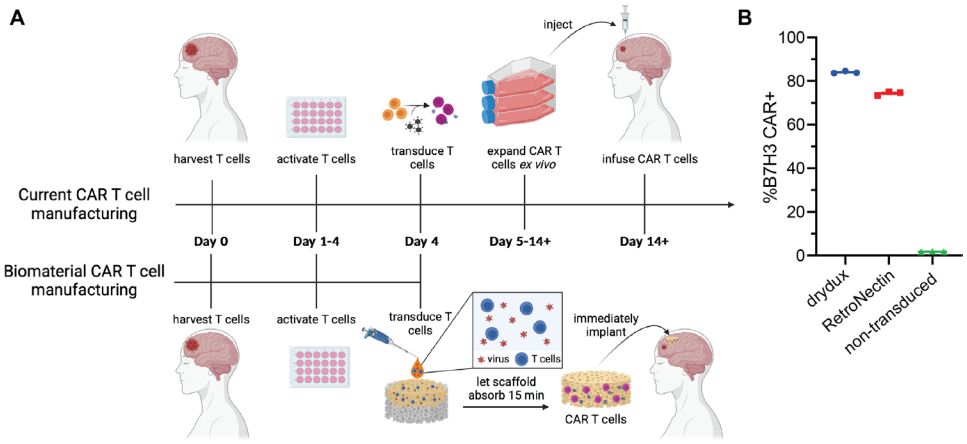

Figure 1. (A) Biomaterials (drydux) reduce the manufacturing time of CAR T cells to three days and can be implanted for *in vivo* manufacture and delivery. **(B)** Drydux transduces activated T cells with over 80% efficiencies, similar to spinoculation of RetroNectin-coated plates.

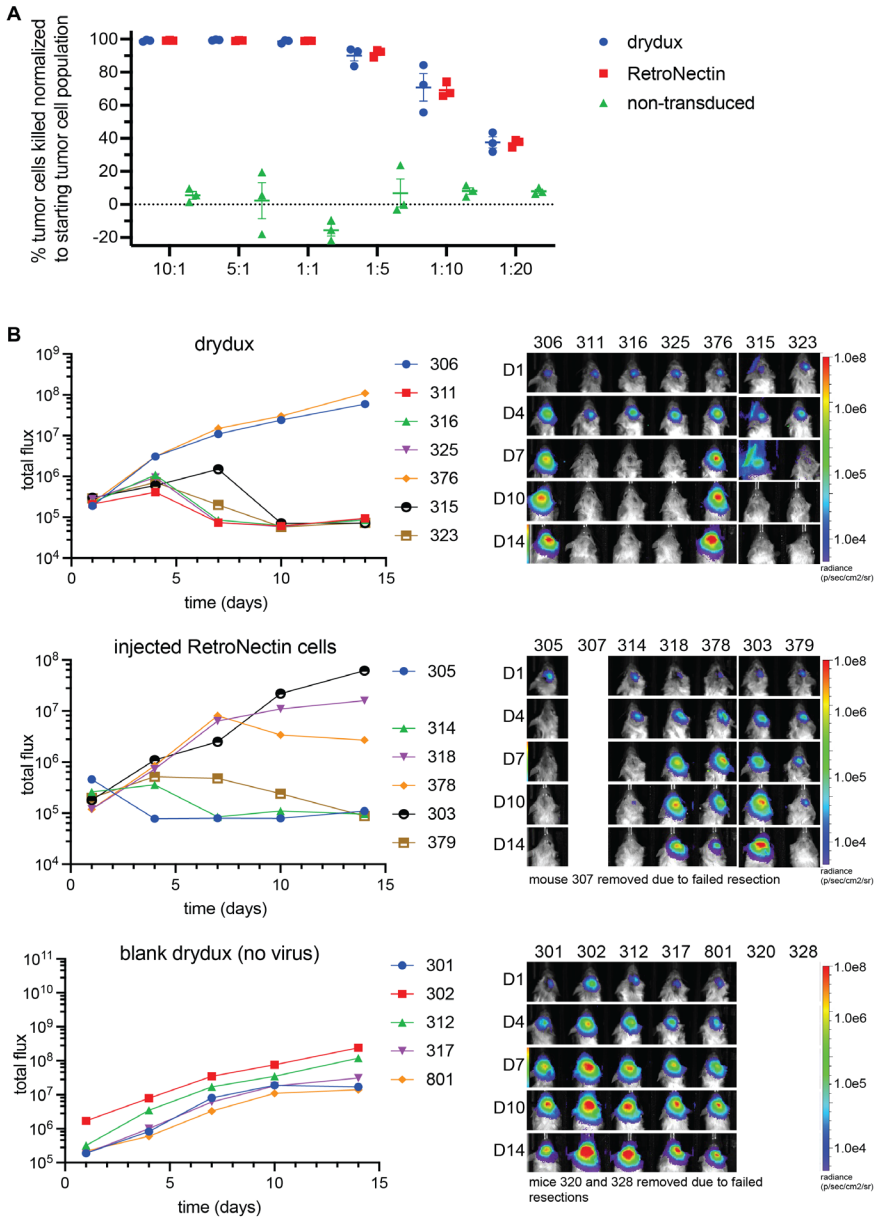

Figure 2. (A) B7H3-specific CAR T cells made using drydux or conventional methods and U87 GBM cells were seeded together at different effector to target (E:T) ratios. After 5 days of coculture, cytotoxicity was analyzed using flow cytometry. All tumor cells were eradicated at higher E:T ratios. At 1:5, 1:10, and 1:20 ratios, about 90%, 70%, and 35% of tumor cells were killed, respectively, by drydux and RetroNectin groups. No cytotoxicity was seen with non-transduced cells. **(B)** U87-GFP-FFLUC cells were injected intracranially into NSG mice and resected after growth. Drydux loaded with activated T cells and B7H3-specific CAR-encoding retrovirus were implanted into the resection cavity and compared to B7H3-specific CAR T cells created conventionally and directly injected into the resection cavity. Drydux loaded with activated T cells, but no retrovirus, were used as a negative control. 71% of mice in the drydux group had noticeably reduced tumor burden compared to only 50% in the conventional group and 0% in the negative control group.

342 Development of a manufacturing process for a Regenerative Macrophage Therapy to treat end-stage liver disease

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Macrophage-based regenerative therapies hold considerable potential for treating a variety of inflammatory and fibrotic diseases. Macrophages, as key regulators of tissue repair, not only mediate inflammation but also possess remarkable regenerative capacities, including tissue repair, resolution of inflammatory responses and immune modulation. We have advanced the manufacturing of a regenerative macrophage therapy (RMT), focusing on the optimisation of cellular engineering, scalability, and quality control in preparation for clinical application.

Clinical trials conducted previously by the University of Edinburgh demonstrated safety and efficacy of autologous RMT in liver cirrhosis patients (Moroni et al., 2019; Brennen et al, 2025]. We have further developed this approach through engineering cells to enhance anti-inflammatory and anti-fibrotic potency. Following manufacturing changes and optimisation, our lead asset, RTX001 is now entering Phase 1/2 clinical testing for treatment of end-stage liver disease in the UK and Spain.

We have further developed an *ex vivo* manufacturing platform to deliver multiple doses of a pro-regenerative macrophage therapy, ensuring functional consistency and potency across production batches. Process development has established a scaled GMP-compliant, robust protocol for the generation of autologous macrophages, integrating closed processing and optimised culture conditions to achieve high yields of macrophages with enhanced regenerative properties. We have developed a method of gene delivery to express IL-10 and MMP9 to boost the anti-fibrotic and anti-inflammatory activity of macrophages. A robust supply chain and the ability to provide multiple doses has been enabled through the development of cryopreservation and implementation of GMP-compliant storage and logistics systems.

Our process includes rigorous monitoring and quality control measures at every stage, from receiving input leukapheresis material through to final product release. Development of a comprehensive analytical package allows testing for safety, identity, quantity, purity, and potency to ensure that our final product meets regulatory standards. We have also built and incorporated a matrix of extended characterisation techniques which will enhance product understanding as we progress through clinical evaluation.

The application of process and quality controls minimises the risk of contamination and ensures the consistency of product quality.

In conclusion, our proprietary process for manufacturing a regenerative macrophage therapy has enabled us to initiate clinical testing against end-stage liver disease. By combining advanced cell engineering, scalable manufacturing, and rigorous quality control, we are creating a robust therapeutic platform with the potential to deliver transformative outcomes for patients. As we move into clinical trials and investigate the application of RMT in other fibrotic and inflammatory disease areas including, but not limited to, pulmonary fibrosis and graft-versus-host disease, we will continue to refine our processes for successful delivery of this next-generation regenerative therapy.

Moroni *et al. Nat Med* **25**, 1560–1565 (2019). https://doi.org/10.1038/s41591-019-0599-8
Brennan *et al. Nat Med* (2025). https://doi.org/10.1038/s41591-024-03406-8

343 Continued Clinical Scale Process Development to Enable Safe and Effective Homology Directed Repair Gene Edited Hematopoietic Stem and Progenitor Cells for Sickle Cell Disease

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Nulabeglogene autogedtemcel (nula-cel) is a phase I/II investigational drug product (NCT04819841), consisting of ex-vivo gene corrected, plerixafor-mobilized CD34+ HSPCs to cure Sickle Cell Disease (SCD). High-fidelity Cas9 is precomplexed with chemically modified gRNAs to induce innate Homology Directed Repair (HDR) mechanisms

via a recombinant AAV6 DNA donor template containing the wild-type *HBB* gene. This cell therapy directly corrects the disease-causing variant, simultaneously reducing sickle hemoglobin (HgbS) production and restoring physiologic adult hemoglobin (HgbA) levels.

Previously, a single patient (P1) was treated with cells manufactured using a first-generation process (V1.0). While P1 has done clinically well and met pre-defined safety and efficacy endpoints, including <10% HgbS at T24 months, the engraftment of red blood cells and platelets was delayed, requiring transfusions up to D+263 and eltrombopag to temporarily boost HSC growth. In addition, in the first phase of engraftment at T6 months, the level of gene corrected HDR alleles decreased from 33% in the drug product to 5-6% in the myeloid output from the bone marrow.

We address the delayed engraftment and low HDR correction with significant manufacturing process improvements. Here we describe the qualification of a V2.0 manufacturing process to improve nula-cel and address P1 observations. We switched to the Maxcyte GTx electroporator and initially increased %HDR from 39% to 47% and further tested an additional process change from culture bags to the G-Rex® bioreactor. The average from independent runs (n=5) showed: 1) a further increase in %HDR alleles to 59%±1.3% (mean±SEM); 2) an increase in cell yields from 112%±18% to 173%±26% despite a 1-day reduction in culture time. To better preserve HSCs, we shortened manufacturing to 3 days. We reduced the p53-mediated stress response by lowering the AAV6 MOI coupled with addition of a 53BP1 inhibitor HDR Enhancer peptide to favor HDR. We achieved a 4x AAV6 MOI reduction from 2500 to 625, while consistently achieving >50%HDR with no detectable increase in genotoxicity. A process comparability study (n=3) demonstrated that the above changes resulted in improved critical quality attributes including: increased HDR (36.6%±4.0% to 51.5%±6.7%); increased monoallelic/biallelic HDR events (39.7%±6.8% to 74.7%±7.4%); increased the LT-HSC marker profile by 49%±14%; improved viability via Annexin-V/7-AAD staining (76.3%±1.7% to 88.5%±0.7%); improved CFUs relative to an RNP only control without AAV6 (82.0%±14% to 110%±10%); while maintaining >100% cell yields despite a 2-day shorter manufacturing process.

The V2.0 process was successfully replicated at a CDMO in three independent clinical scale engineering runs. Comparability study results were maintained with 125%±8.3% cell recovery, 94.3%±0.7% cell viability, 97.0%±0.6% CD34+ purity, 53.7%±2.5% HDR correction and 101%±15% CFUs relative to an untreated control without editing. We also performed an *in vivo* long-term engraftment study in NSG mice and noted an average of 4-5 fold improvement in hCD45 engraftment in the bone marrow relative to prior studies using the V1.0 process. In conclusion, we demonstrated the clinical readiness of an improved V2.0 nula-cel product, with continuing FDA clearance for phase I/II clinical trial implementation.

344 Scalable Production of Hematopoietic Stem Cells and Microglia from iPSCs Using Stirred Tank Bioreactors for Consistent Cell Therapy Manufacturing

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Hematopoietic stem cells (HSCs) and microglia are critical for the development of blood, immune, and central nervous system (CNS) cells, offering immense potential for cell therapy and the treatment of various hematological and neurodegenerative conditions. The manufacturing of HSCs and microglia from human induced pluripotent stem cells (hiPSCs) with consistent quality and at scale is essential for their integration into therapeutic applications. However, achieving this goal is challenging due to the complexity of the differentiation process. Ncardia, with extensive experience in iPSC innovation, has developed robust protocols for HSC and microglia differentiation, ensuring large batch consistency and scalability.

We adapted HSC and microglia differentiation processes originally developed in small-scale 2D adherent culture into 250mL stirred tank bioreactors, with the goal of scaling up to 1L and 3L bioreactors. A Design of Experiments (DoE) approach was employed to systematically evaluate critical process parameters (CPPs) such as dissolved oxygen, agitation speed, and inoculation density. Parallel testing of multiple differentiation protocols was conducted

to establish proof of concept. In-process monitoring, combined with comprehensive data science analysis, enabled a robust understanding of critical quality attributes (CQAs). This methodology ensured quick optimization and high consistency of the differentiation process.

These protocols successfully yielded high-quality HSCs and microglia characterized by their ability to self-renew and differentiate into various functional cell types. The hiPSC-derived HSCs exhibited typical morphology, expressed key markers (>90% CD34, CD45, and CD43), and demonstrated potential for differentiation into multiple immune cell lineages, including natural killer (NK) cells, macrophages and microglia, highlighting their applicability in cell therapy and immunotherapy. Similarly, hiPSC-derived microglia, known as Ncyte® Microglia, exhibited the expression of typical markers (>80% IBA1, TMEM119, and CX3CR1) and functional properties such as phagocytosis and cytokine secretion in response to inflammatory stimuli. The ability to co-culture microglia with neurons and other CNS cell types further enhances their potential applications in drug discovery and toxicity testing.

Ncardia's innovative use of stirred tank bioreactors for HSC and Microglia differentiation from hiPSCs addresses the critical need for large batch consistency and scalability in cell therapy and research applications. By combining expertise in stem cell biology with advanced bioreactor technology, Ncardia ensures high-quality, reproducible production of HSCs and microglia. This capability not only accelerates the development of cell therapies but also enhances the potential for generating diverse immune and neural cell types for therapeutic use. Ncardia's comprehensive approach positions them as a leading partner in the advancement of iPSC-derived HSCs and microglia for clinical, research, and regenerative medicine applications, facilitating new opportunities in immunotherapy and neurodegenerative disease treatment.

Across Platforms for Hematopoietic Disorders

345 Long-term Inflammatory manifestations post lentiviral hematopoietic stem/progenitor cell gene Therapy for Wiskott Aldrich syndrome

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Introduction:
Wiskott-Aldrich syndrome (WAS) is a complex X-linked disorder caused by loss-of-function mutations of the WAS gene encoding to the WAS protein (WASp). WASp is a crucial regulator of the actin cytoskeleton in hematopoietic cells. WASp deficiency typically results in characteristic microthrombocytemia and lymphoid-myeloid dysfunction, with severity often correlating with residual WASp expression and function levels. However, emerging evidence highlights that WASp also plays a role in modulating autophagy and inflammasome activity.

Context:
In our open-label, non-randomized ex vivo gene therapy (GT) with LV-w1.6 WASp self-inactivating lentiviral vector clinical trial, which involved eight patients treated at Necker-Enfants Malades Hospital (Paris, France), and Great Ormond Street Hospital (London, UK), inflammatory manifestations emerged as a notable post-treatment issue. This study focuses on the inflammatory manifestations observed in the French cohort (NCT02333760).

Results:
All patients improved their inflammatory phenotype for four years, with normalization in inflammatory cytokine expression profiles. Despite overall improvement after GT treatment, three out of four patients from the French cohort developed inflammatory clinical manifestations ranging from grade 1 (N=2) to grade 2 (N=1) despite successful engraftment of gene-corrected Hematopoietic Stem and Progenitor Cells (HSPCs) expressing WASp and effective T-cell immune reconstitution. Interestingly, gene-corrected cells' engraftment depended on selective lineage and was particularly low in CD14+ and CD15+ cells (range 0.04; 0.43 copies/cells). One patient experienced a worsening of the lower extremity vasculitis that had been improving since the GT, requiring grade II analgesics for pain management ten years after the transplant. Two patients also developed late-onset oligoarthritis (one of whom had minimal vasculitis involvement) without systemic inflammation or specific auto-antibodies. All biological tests indicated a dysregulation of the innate immune system and impaired inflammasome activation, with elevated levels of IL-1 β, TNF-α, and IL-18 secretion. These findings were closely linked to the gene-corrected myeloid engraftment. All inflammatory symptoms responded well but only transiently, to immunoglobulin therapy. Two cases, including the most severe one, were successfully treated with a human interleukin-1 receptor antagonist, indicating that targeting the IL-1β pathway may be a viable therapeutic approach for managing inflammation after and potentially before gene therapy for Wiskott-Aldrich syndrome (WAS).

Conclusions:
This data highlights the importance of myeloid-corrected cells and emphasizes the need to closely monitor inflammation biomarkers and inflammasome activity to develop targeted immunotherapy for patients with WAS.

346 Lentiviral Vectors for Hematopoietic Stem Cell Gene Therapy for Alpha Thalassemia

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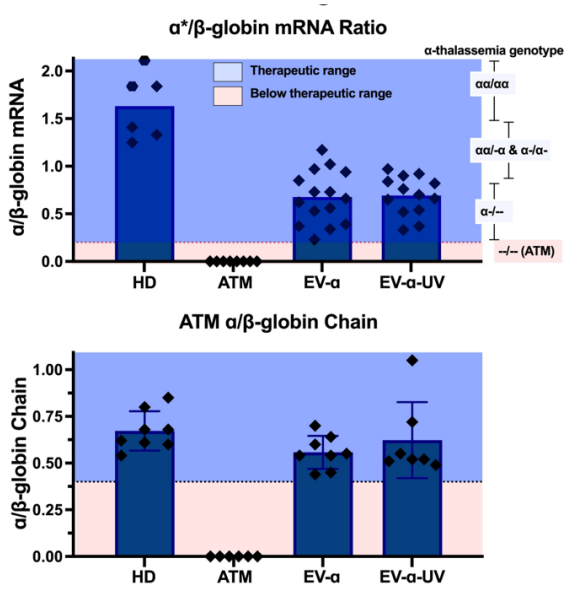
Alpha thalassemia major (ATM) is an inherited blood disorder caused by the absence of all four α-globin genes (*HBA2/1*), resulting in severe anemia from deficiency in hemoglobin production with lifelong dependence on blood transfusions and chelation therapy. While allogeneic hematopoietic stem cell transplant (HSCT) offers a potential cure, donor availability remains a significant challenge. Here, we present a novel gene therapy approach for autologous HSCT, utilizing lentiviral vectors (LVs) to deliver the *HBA2* gene under the regulation of optimized β-globin locus control region (LCR) enhancers, effectively restoring α-globin chain production in red blood cells (RBCs). The best performing LVs, EV-α and EV-α-UV, demonstrated 90-100% transduction efficiency in human hematopoietic stem and progenitor cells (HSPCs) transplanted into immunodeficient mice, achieving optimal vector copy numbers (<5) and a safe integration profile. Bone marrow-derived HSPCs from an ATM patient, treated with EV-α or EV-α-UV LVs, exhibited normalized α/β-globin mRNA and chain ratios (~1.0), recapitulating healthy RBC profiles. Furthermore, treated HSPCs derived from multiple cell sources across three ATM donors restored hemoglobin levels in RBCs by 50 to 100%. These findings establish the safety, efficacy, and translational potential of EV-α and EV-α-UV LVs, offering a promising autologous stem cell gene therapy for the treatment of ATM.



347 Lentivector Gene Therapy Mediates Reduction of Induced Inflammation in a Mouse Model of Deficiency of Interleukin-1 Receptor Antagonist

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Deficiency of Interleukin-1 Receptor Antagonist (DIRA) is a severe inherited autoinflammatory disease. IL-1Ra regulates inflammation by competing with IL-1α and β for binding to IL-1 receptor. Daily injections of Anakinra, recombinant hIL-1Ra, are used to treat DIRA. Significant injection site reactions, development of focal anakinra amyloidosis in some patients, breakthrough symptoms upon discontinuation, and the high cost of life-long treatment provide rationale to explore development of a single-treatment lentivector-based gene therapy for DIRA. We designed a SIN lentivector containing MND promoter, human wild-type IL-1Ra cDNA, modified WPRE and 650bp 5′-HS4 chicken β-globin insulator. Preliminary studies demonstrated secretion of functional hIL-1Ra after transduction. Here, we report promising results using this gene therapy to reduce inflammation in a DIRA mouse (B6.129S-Il1rntm1Dih/J) gastrointestinal inflammation challenge model.

DIRA mice lack spontaneous autoinflammation. We used a 7-day 2% Dextran Sulfate Sodium (DSS) water challenge to cause colitis in DIRA versus C57BL/6J mice that is associated with a marked rise in Serum Amyloid A (SAA) inflammation marker. SAA was similarly low at baseline (pre-DSS challenge) in B6 versus DIRA mice (~18-26 ug/ml), but increased to 199 versus 832 ug/ml, respectively (4-fold greater inflammation in DIRA versus B6 mice) post-DSS challenge. We established an Anakinra versus saline subcutaneous pump treatment regimen, demonstrating that continuous treatment of DIRA mice with 4 mg/kg/day Anakinra (continuous blood level of 195 ng/ml) during the 7-day DSS water challenge reduced the rise in SAA to less than observed in B6 mice saline pump controls (DIRA saline 1310 ug/ml, DIRA Anakinra 262 ug/ml, B6 saline 341 ug/ml).

DIRA mouse c-kit-positive HSCs transduced with hIL-Ra lentivector (120 MOI with LentiBOOST®), achieved VCN 6.1 with 96% of HSC colonies transgene positive and flow cytometry demonstrating expression of hIL-1Ra in ~36% of cultured cells. Five recipient DIRA mice conditioned with 900 cGy were transplanted with transduced DIRA HSCs. At 9 wks post-gene therapy serum hIL-1Ra averaged 253 ng/ml, about 30% higher than achieved with the Anakinra pump. When these gene therapy treated DIRA mice were subjected to the 7-day DSS water challenge, SAA levels rose to only 100 ug/ml, half of the SAA level seen with DSS challenged B6 mice and 8-fold less than

observed with DSS challenged naïve DIRA mice, demonstrating that this gene therapy treatment provided profound protection from DSS challenge gastrointestinal inflammation.

At 12 weeks DIRA mice were sacrificed to assess gene marking. In bone marrow, spleen and peripheral blood, VCN averaged 2, 1 and 4, respectively. Flow cytometry demonstrated hIL-1Ra expression in 15-35% of B cells, T cells and myeloid cells of bone marrow, spleen and blood. Our study demonstrates a novel lentiviral vector-based gene therapy for treating the DIRA mouse model. This gene therapy approach was well tolerated and demonstrated both superior IL-1Ra expression and superior reduction of inflammation from DSS gastrointestinal inflammation challenge, even compared to Anakinra pump, supporting further exploration of its potential as a long-term treatment for DIRA patients.

348 Functional Restoration of Immune Defects in STAT1 Gain-of-Function Immunodeficiency Following Gene Editing

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Hypothesis:
Gain-of-function (GOF) mutations in the *STAT1* gene result in a rare, combined immunodeficiency with a severe clinical phenotype. The only curative treatment option is allogeneic haematopoietic stem cell (HSC) transplantation (allo-HSCT) which carries high risks of morbidity and mortality for STAT1 GOF patients. Autologous gene therapy may be curative without the immunological complications associated with allo-HSCT. We hypothesise that gene editing of STAT1 GOF mutations in patient T cells and HSCs will restore normal STAT1 function.

Methods:
Guide RNAs specifically targeting the p.T385M mutation were electroporated into T cells and HSCs derived from peripheral blood (PB) in conjunction with mRNA encoding an adenine base editor. The degree of editing was determined by Sanger sequencing of target site amplicons. Functional analyses were performed in edited cells using flow cytometry to assess total STAT1, phosphorylated STAT1 (pSTAT1) and IL-17 production before and after stimulation. Real time quantitative PCR (RT-PCR) was used to measure transcript levels of key interferon stimulated genes (ISGs). Multiplexed digital droplet PCR was used to characterise on-target, unintended outcomes.

Results:
Base editing of p.T385M STAT1 GOF patient T cells resulted in 100% $\pm$ 0% (n=4) reversion of the patient mutation to the wild-type sequence, with an associated statistically significant ($p<0.01$) restoration of total STAT1 and pSTAT1 to healthy control levels, and a concurrent rescue in ISG expression. Up to 95% allelic correction was achieved following base editing of PB-HSCs from p.T385M STAT1 GOF patients. Levels of editing were maintained in HSC-derived monocytic progeny and corresponded to functional correction of total STAT1 and pSTAT1 in these cells. Importantly, initial experiments indicate that base editing does not induce any unintended on-target DNA aberrations, and that base edited PB-HSCs retain engraftment potential as measured by colony forming unit and phenotyping assays.

Conclusions & Future Directions:
Here, we show functional restoration of STAT1 gain-of-function following gene editing in patient T cells and HSCs. To our knowledge, this is the first case of base editing being used to successfully correct gain-of-function mutations causing inherited immunodeficiency. Future work will include off-target genotoxicity studies, as well as experiments utilising artificial thymic organoids to assess functional rescue in edited HSC-derived T lymphocytes. *In-vivo* chimerism experiments in a validated STAT1 GOF murine model are also underway.

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349 Site-Specific Gene Integration Strategies for IL7Rα-SCID Hematopoietic Stem Cell Gene Therapy

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Interleukin 7 Receptor subunit alpha Severe Combined Immunodeficiency (IL7Rα-SCID) is a rare inherited monogenic disorder that results in improper immune function because the patient cannot produce T cells, a group of immune cells that play a critical role in our adaptive immune response. The only approved treatment is an allogeneic stem cell transplant where the patient receives functional cells from an HLA-matched healthy donor. With limited donor availability, autologous hematopoietic stem cell gene therapy (HSCGT) using CRISPR/Cas9 is being developed by our group as a potential long-term treatment for IL7Rα-SCID. Previous work in IL7RA$^{-/-}$ mice that were treated with gammaretrovirus containing a constitutive promoter resulted in splenomegaly and neutrophilia, while an attempt to use alpharetrovirus with a lymphoid-restricted promoter in IL7RA$^{-/-}$ mice did not develop mature T cells *in vivo*. These results highlight the importance of maintaining the endogenous IL7Rα gene regulation for appropriate T cell development. In our strategy, we performed targeted gene editing in human CD34^{+} hematopoietic stem and progenitor cells (HSPCs) to insert the therapeutic IL7Rα transgene in the IL7RA locus through Homology-Directed Repair (HDR) and restore its function. Here, we evaluated three potential IL7Rα donor delivery methods: double-strand DNA (dsDNA), adeno-associated virus serotype 6 (AAV6), and integrase-deficient lentivirus (IDLV). After editing the HPB-ALL (IL7Rα-KO) cell line, all donor delivery methods restored IL7Rα expression using flow cytometry with mean fluorescence intensity similar to wild-type levels. A major challenge in HSPC gene editing is achieving high levels of HDR while minimizing toxicity observed from these donor delivery methods. We tackled this issue by adding AZD7648, a potent DNA-PK inhibitor, to influence DNA repair further towards the HDR pathway while adding lower amounts of the donor template. The addition of AZD7648 treatment increased HDR rates by 2-fold, and we achieved as high as ~50% with IDLV, ~71% with AAV6, but still ~1% with dsDNA *in vitro*. We further investigated the impact of gene editing combined with IDLV or AAV6 using downstream assays. Upon Colony-Forming Unit assay, we observed a higher clonogenic potential with IDLV (72% of Mock) than AAV6 (58% of Mock) even after using a lower dose of AAV6. Interestingly, we observed a slightly better outcome for both delivery methods with AZD7648 treatment. We also transplanted edited-HSPCs into immunodeficient NSG mice to investigate engraftment potential. After 16 weeks, we analyzed cells from the bone marrow and achieved higher total engraftment on HSPCs edited with IDLV (43% and 62%,+/-AZD7648) compared to AAV6 (38% and 23%,+/-AZD7648). More importantly, HDR% decreased by 3 and 2-fold in AAV6 (+/-AZD7648, respectively) treated cells, while HDR% increased by 1.5-fold in both IDLV conditions. Our results highlight the development of using site-specific gene integration with CRISPR/Cas9 for IL7Rα-SCID HSCGT; and suggest potential long-term advantages of using IDLV over AAV6. We are currently confirming lineage-specific expression and investigating the ability of HDR-edited HSPCs to become mature T cells using various *in vitro* differentiation protocols towards multiple lineages of the immune system. Crucially, we are also looking into sequence fidelity of the integrated transgenes due to reports of IL7Rα gain-of-function mutations in Leukemia patients.

350 Restore in vivo functions of mouse Fancc$^{-/-}$ hematopoietic stem cells using the CRISPR/Cas9 system.

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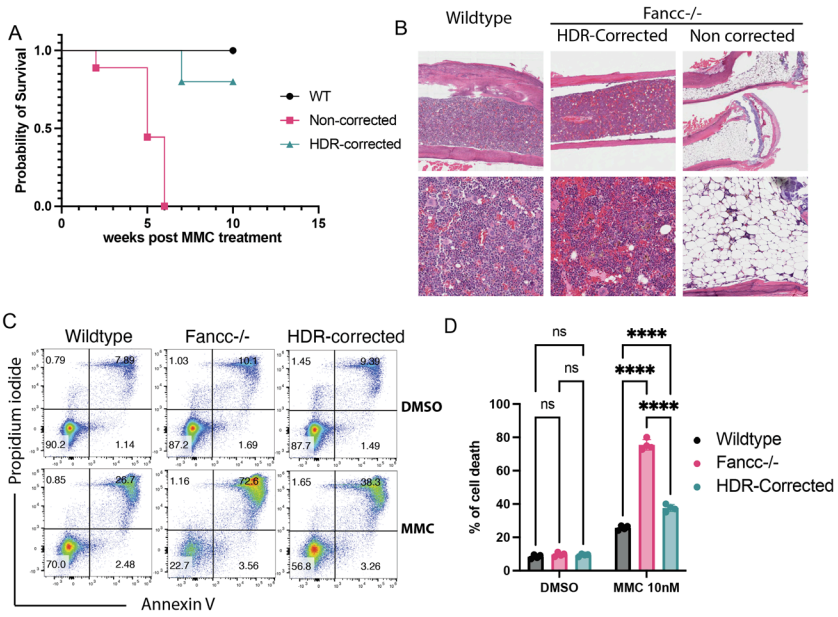
Fanconi Anemia (FA) is an incurable recessive disease caused by mutations in any members of the FA pathway. FA Complementation group C patients (FANCC), one of the most common subtypes, exhibit typical, but more severe symptoms than other subtypes. Gene therapy to repair mutated genes in autologous FA patient-derived hematopoietic stem/progenitor cells (FA-HSPCs) can permanently rescue FA-mediated bone marrow failure (BMF) and overcome the limitations of allogeneic bone marrow transplantation. FA-deficient cells are known to defect in the homologous recombination (HDR) pathway. However, we demonstrated an efficient HDR in mouse HSPCs using a reporter system, proving that the HDR pathway is active in Fancc-/- cells. Thus, CRISPR/Cas9- HDR is feasible for restoring functions of Fancc-/- HSPCs in vivo. We established a CRISPR/Cas9/AAV6-HDR system to integrate Fancc cDNA into the endogenous Fancc promoter. Of note, along with the reestablished expression of Fancc, corrected HSPCs restored the colony-forming ability and the resistance to mitomycin C (MMC) treatment. We assessed the functions of corrected HSPCs in vivo by transplanting these cells into irradiated Fancc-/- mice. 3 months later, we induced the BMF by MMC treatment. We found that mice transplanted with non-corrected HSPCs developed BMF and died after 5 doses of MMC injection, supported by the hypocellular structure of bone sectioning. However, mice transplanted with corrected HSPCs survived and did not develop BMF. Bone sectioning of survivors showed normal cellular structure. Collectively, we demonstrate that CRISPR/Cas9-HDR rescued functions of Fancc-/- HSPCs both in vitro and in vivo. Although, we can rescue the BMF in Fancc-/- mice, we observed significant cell death after the gene editing, as Fancc-/- cells are susceptible to secondary DNA damage caused by the CRISPR/Cas9. To improve the functions of corrected HSPCs, we are exploiting our established Spacer-Nick approach in Hypoxia that barely introduces double-stranded breaks and DNA damage. Spacer-Nick demonstrated improved functions of corrected HSPCs compared to the conventional CRISPR/Cas9 system. We are currently validating the functions of Spacer-Nick-corrected HSPCs in vivo.



351 *In vivo* HSC Transduction in Humanized Mice Mediated by Novel Capsid-modified HDAd Vectors

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We have developed an *in vivo* HSC gene therapy approach consisting of HSC mobilization and intravenous injection of HDAd vectors. While we have demonstrated safety and efficacy of the approach in CD46-transgenic mice and rhesus macaques, studies in mice with a humanized hematopoietic system could facilitate its potential clinical translation for the treatment of hemoglobinopathies and HIV. Here, we generated an Ad6-based vector containing

fibers from Ad3 (HDAd6/3) with enhanced affinity to DSG2. *In vitro* studies showed greater transduction of primitive HSCs ($CD34^+CD38^-CD90^+$) cells and colony forming cells by HDAd6/3-GFP compared to HDAd5/35-GFP. This was also confirmed *in vivo* in humanized NSGW41 mice. NSGW41 mice engraft cryo-preserved human $CD34^+$ cells in the bone marrow (BM) without preconditioning. However, human chimerism in peripheral blood (PB) is usually low, and mobilization of human cells by G-CSF/AMD3100 is inefficient. We showed that low-dose total body irradiation (100 Rad) or busulfan treatment (12.5 mg/kg) increases the percentage of human $CD45^+$ cells in PB two- and five-fold, respectively. In the low-dose busulfan treated mice, G-CSF/AMD3100 mobilization resulted in >20,000 human $CD34^+$ cells in the periphery, 40 min after AMD3100 injection (15% of human $CD45^+$ cells were $CD34^+$). At the peak of mobilization, humanized mice were injected with HDAd5/35-GFP or HDAd6/3-GFP vectors, followed by O^6BG/BCNU-*in vivo* selection to expand transduced HSPCs. At end of the study, HDAd6/3-treated mice showed significantly higher percentages of human $CD45^+GFP^+$ cells compared to HDAd5/35-treated mice (41.12±10.62% vs 22.38±8.17%, p=0.0338, respectively). This was also reflected by the percentage of GFP-expressing $CD45^+$ cells in the bone marrow (BM) and spleen. A similar pattern of increased GFP expression was found in the human $CD45^+$ cells in the BM of the secondary recipients of cells isolated from the BM of HDAd6/3-treated primary mice. Moreover, we tested G-CSF-free mobilization regimens, the CXCR2-inhibitor tGroβ together with AMD3100, or AMD3100+tGrob together with the VLA4 inhibitor WU-106, as G-CSF/AMD3100 mobilization has some limitations. The percentages of human $CD45^+CD34^+$ and $CD45^+CD34^+CD90^+$ cells in PB were measured at 15 min, 1, 2, 3, 6 hours, and 3 days after injection. The peak of G-CSF/AMD3100 mobilization was at ~1 hour, while the peaks for tGrob+AMD and tGrob+WU106+AMD3100 were at 15min post injection. Importantly, mobilization of $CD34^+CD90^+$ with tGrob+AMD3100 was significantly more efficient than G-CSF/AMD3100 at their corresponding peaks. HDAd6/3-transduction of HSCs mobilized with the different mobilization schemes followed by O^6BG/BCNU selection resulted in stable increase of GFP in peripheral blood with comparable GFP expression pattern in human $CD45^+$ cells within the BM of the different groups. Finally, we also tried to improve the HDAd5/35 vector platform by targeting the virus to a second HSC receptor, a_6b_1/CD49f (HDAd5/35_lam). *In vivo* transduction of human HSCs in humanized NSGW41 mice showed >20% transduction of $hCD34^+CD38^-CD90^+$ three days after the virus injection. In summary, our data in humanized mice show that human HSCs can be efficiently mobilized with G-CSF/AMD3100 or G-CSF-free regimens and *in vivo* transduced with HDAd vectors resulting in high GFP marking rates, which, theoretically, should be curative for hemoglobinopathies and HIV.

Gene Therapy Trials - In-Vivo Gene Therapy Modification

352 Initial safety data from ELIMINATE-B, the first clinical trial of a gene editing treatment for chronic hepatitis B

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Chronic hepatitis B virus (cHBV) infection affects approximately 300 million patients world-wide and is responsible for 1.1 million deaths annually. Premature death in patients is predominantly due to complications of cirrhosis, portal hypertension, and hepatocellular carcinoma even with current standard of care therapies. Currently there is no curative therapy for cHBV infection. ELIMINATE-B is an ongoing, global trial to evaluate PBGENE-HBV, a first-in-class gene editing therapy in patients with cHBV infection with the intent to cure. PBGENE-HBV consists of an ARCUS nuclease-encoding mRNA that is formulated in a lipid nanoparticle and optimized for safety and potency. PBGENE-HBV is designed to eliminate covalently closed circular DNA (cccDNA), the source of replicating virus, and inactivate integrated HBV DNA. Part 1 of ELIMINATE-B evaluates multiple ascending doses in HBeAg-negative patients controlled on nucleos(t)ide analogs. Initial data from the first cohort comprising 3 participants receiving their first dose administration of 0.2 mg/kg of PBGENE-HBV demonstrated the treatment was well-tolerated with no serious adverse events, including no clinically significant laboratory abnormalities. More

specifically, AST/ALT and platelet count have remained within normal limits for all dosed participants. The initial safety data supports continued evaluation of a finite course of dose administrations across multiple dose levels of PBGENE-HBV. Comprehensive datasets of the tolerability and efficacy of multiple administrations are being collected and will be analyzed on full dosing of cohorts.

Preclinical efficacy models across multiple species including nonhuman primates (NHPs) demonstrated that PBGENE-HBV eliminates cccDNA and inactivates integrated HBV DNA, resulting in durable reductions of HBV DNA and HBsAg. PBGENE-HBV demonstrated cumulative editing at three dose levels tested in an NHP HBV model, achieving 99% viral inactivation after two doses. Preclinical toxicology data indicated that PBGENE-HBV was well-tolerated after multiple dose administrations in NHPs at all dose levels evaluated. Consistent with other nonclinical studies utilizing systemic administration of LNPs, clinical chemistry findings in NHP included non-adverse, transient elevations in liver transaminases fully normalizing between 7 and 14 days. No adverse effects were observed in any parameter, including hematology, coagulation, immune function, or macroscopic/microscopic tissue evaluations after multiple dose administrations in NHPs. PBGENE-HBV distributed primarily to the NHP liver and showed no evidence of biodistribution to germ cells. In culmination, the initial clinical safety data discussed above demonstrates the translatability of the NHP model and supports the continued evaluation of multiple administrations and dose levels of PBGENE-HBV with the goal of cure.

353 Direct image-guided convective perfusion of the bilateral thalami offers a consistent approach to CNS dosing: first-in-human experience with gene therapy for frontotemporal dementia.

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Background:
Nervous system gene therapy delivery methods, including cerebrospinal fluid (CSF) or systemic administration, are limited by lack of target specificity, immune response/exposure and/or the blood-brain barrier. Convection-enhanced delivery (CED) overcomes these obstacles and permits for the direct, targeted, homogeneous, reliable and safe delivery of high concentration of viral vectors for transgene expression. Moreover, the use of real-time magnetic resonance (MR)-imaging allows for the precise monitoring of vector distribution during convective target perfusion. The current study exploits the properties of CED to deliver AVB-101 (recombinant adeno-associated virus serotype 9 (AAV9) encoding the human granulin (*GRN*) gene) to treat frontotemporal dementia (FTD) associated with progranulin deficiency. While large animal studies have demonstrated the safe and effective administration of AVB-101 into the thalamus, it has not been explored in humans previously.

Objective:
To define the properties of MR-guided intrathalamic convective gene therapy delivery, we analyzed findings in patients with FTD associated with a *GRN* mutation (FTD-*GRN*) that underwent administration of AAV9 expressing *GRN* as part of the ongoing ASPIRE-FTD Phase 1/2 study (NCT06064890).

Methods:
FTD-*GRN* patients underwent real-time MR-guided convective co-infusion of the bilateral thalami with AVB-101 and gadolinium (1 mM). A protocol-defined volume was divided equally between anterior/posterior thalamus, delivered as two pairs of simultaneous bilateral infusions.

Results:
Three consecutive patients (mean age 53.0 ± 18.2 years) underwent a total of 12 thalamic infusions with mean follow-up of 3 to 9 months to date. Real-time MR-imaging demonstrated increasing tissue volume of distribution (V_d) with increasing volume of infusion (V_i) that was consistent across patients. Infusion shaping by varying infusion rate and cannula position permitted in target distribution with mean 87.2% of perfused area within thalamus. Consistent with the expected variability in segmented thalamic size (5.4 to 8.4 cm^3), mean thalamic

coverage was 39.1 (range, 28.7 to 47.6%). There was no significant perivascular loss or leakback along the cannulae. There was no evidence of complication on imaging or clinical safety assessments. To date there have been no serious adverse events nor any adverse events related to the product or procedure reported in the follow-up period.

Conclusions:
First-in-human experience with real-time MR-guided convective delivery provides new insights into the consistency of thalamic gene therapy perfusion properties. The isotropic tissue composition, low propensity for perivascular leakage and its broad interconnectedness makes the thalamus an ideal gene therapy target for a wide variety of neurologic disorders.

354 RGX-202, an Investigational Gene Therapy for the Treatment of Duchenne Muscular Dystrophy: Interim Clinical Data

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Introduction:
Duchenne is a severe, progressive, degenerative muscle disease caused by mutations in the *DMD* gene which encodes for dystrophin, without which muscles degenerate and become weak, eventually leading to loss of movement and independence, required support for breathing, cardiomyopathy, and premature death. RGX-202 is an investigational, one-time AAV gene therapy designed to deliver an optimized microdystrophin gene that encodes for a novel microdystrophin protein that includes the C-Terminal (CT) domain found in naturally occurring dystrophin. In preclinical studies, the CT domain has been shown to protect muscles from contraction-induced stress and to improve muscle repair.

Methods:
AFFINITY DUCHENNE®, a phase I/II/III multi-center, open-label trial of a one-time intravenous administration of RGX-202 at two dose-levels (DL1, $1x10^{14}$ or DL2, $2x10^{14}$ genome copies/kg body weight) is enrolling ambulatory boys aged one and above with Duchenne to evaluate safety, tolerability, and clinical efficacy.

Results:
As of November 8, 2024, RGX-202 has been well tolerated in the Phase I/II portion (n=11) with no serious adverse events in boys aged 1-11 years old. Participants demonstrated robust RGX-202 microdystrophin expression three months following RGX-202 administration at DL1 (10.4 – 83.4%; n = 3) and DL2 (20.8-77.2%; n = 6). Functionally, RGX-202 participants demonstrated mean improved function on the North Star Ambulatory Assessment (NSAA) and time function tests at DL1 (12 months follow-up; n = 3) and DL2 (9 months follow-up; n = 2). These participants also exceeded available external natural history controls matched for age and baseline function.

Conclusion:
In the Phase I/II patients, RGX-202 has been well tolerated and demonstrated both robust RGX-202 microdystrophin expression and functional improvements.

355 Long-Term Functional Outcomes and Safety Following Delandistrogene Moxeparvovec Treatment in DMD: EMBARK 2-Year Results

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Introduction:
Delandistrogene moxeparvovec, an rAAVrh74 vector-based gene therapy approved in the US and other select countries, delivers a transgene encoding delandistrogene moxeparvovec micro-dystrophin, an engineered, functional form of dystrophin that stabilizes or slows Duchenne muscular dystrophy (DMD) progression. EMBARK (NCT05096221), a Phase 3, randomized, double-blind, placebo-controlled, two-part study, assesses delandistrogene moxeparvovec in DMD.

Methods:
EMBARK enrolled ambulatory males with DMD aged ≥4─<8 years (Mendell. *Nat Med*. 2024). Patients received delandistrogene moxeparvovec (single intravenous dose 1.33×10^{14} vg/kg) or placebo in Part 1 (52 weeks) and crossed over in Part 2. Due to the crossover, 2-year functional outcomes from patients treated with delandistrogene moxeparvovec in Part 1 were compared with a cohort of external controls (EC), matched using prespecified propensity-score-weighted analyses based on baseline prognostic factors that impact DMD progression.

Results:
At 2 years, patients treated with delandistrogene moxeparvovec in Part 1 (N=63) demonstrated statistically significant and clinically meaningful functional benefit versus EC (N=113–115, dependent on functional assessment). Mean change from baseline was 2.63 versus –0.25 points on the North Star Ambulatory Assessment total score (between-group difference: 2.88 points); 0.65 versus 2.71 seconds on the Time to Rise (between-group difference: –2.06 seconds), and –0.04 versus 1.32 seconds on the 10-meter Walk/Run (between-group difference: –1.36 seconds); all $P<0.01$. There were no deaths, discontinuations, or clinically significant complement-mediated adverse events (AEs). Between Weeks 52 and 104, 15 (23.8%) patients experienced 34 treatment-related treatment-emergent AEs. One patient experienced two treatment-related serious AEs of rhabdomyolysis; both resolved.

Conclusions:
2-year results indicate favorable treatment effect of delandistrogene moxeparvovec on disease progression versus a well-matched EC. Stabilized functional outcomes, prognostic for delaying loss of ambulation, demonstrate durability of treatment. Safety was consistent with prior experience.

356 Safety and Efficacy of AAV9-Mediated Gene Therapy GC301 in ERT-Stabilized Infantile-Onset Pompe Disease: An Open-Label, Single-Arm Study with Over 12 Months of Follow-Up

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Introduction
Pompe disease, a lysosomal storage disorder resulting from mutations in the GAA gene, triggers glycogen accumulation in various tissues. Enzyme replacement therapy (ERT), currently the standard treatment, requires lifelong administration and exhibits variable efficacy. In contrast, gene therapy has emerged as a promising approach to address the underlying genetic defects. This study aims to assess the safety and efficacy of adeno-associated virus serotype 9 (AAV-9) mediated gene therapy GC301 in patients with infantile-onset Pompe disease (IOPD) whose condition has been stabilized with ERT.

Methods
From April 18, 2023 to November 9, 2023, six IOPD patients who had been receiving ERT were recruited for this study. Each patient received a single-dose intravenous injection of the GC301 gene therapy at a dose of 1.2E+14 vg/kg, after which ERT was discontinued. Follow-up for all six patients has continued for over a year. Safety observations include monitoring adverse events and immunogenicity. Efficacy endpoints were frequently assessed, focusing on survival status, cardiac function, exercise ability, and GAA enzyme activity in both blood and muscle tissue. Echocardiography was utilized to evaluate cardiac function. The GMFM-88 scale was employed to evaluate motor function.

Results
A total of 6 subjects were enrolled in this study. One subject withdrew due to personal reasons 60 days after administration. Prior to enrollment, all participants had undergone ERT once or twice a month, with an average duration of 29.7 months (range: 5.0 - 49.9 months). The average time interval between the last ERT and gene therapy was 10.5± 6.1 days. Following the administration of GC301, no serious adverse events (SAEs) related to the gene therapy have been observed. Treatment-related adverse events (TRAEs) in grades 1-2 were reported, with transient fever and liver injury being the most common. Anti-GAA antibodies remained negative during the follow-up period. During the follow-up, five patients did not use ERT for an average of 525.60 ± 60.43 days. The left ventricular mass index (LVMI) was stable compared to baseline, left ventricular ejection fraction (LVEF) remained within the normal range (>55%) (Fig.1 A). Among the six subjects, three patients demonstrated significant improvements in their motor developmental milestones including 3 subjects achieving the milestone of independent walking (Fig.1 B), and their GMFM-88 scores also improved (Fig.1 C). Three subjects voluntarily underwent muscle biopsy, and their muscular GAA enzyme activities and GAA protein significantly increased after GC301 administration compared to pre-treatment levels (Fig.1 D and E).

Conclusion
This study provides evidence regarding the efficacy and safety of AAV-9 gene therapy GC301 for treating IOPD patients who have achieved disease stabilization via ERT. Throughout the follow-up period, we observed that gene therapy enables to reduce the use of enzyme while maintaining stable or improved survival rates, heart function, GAA enzyme activity, and motor function. This study suggests that AAV gene therapy has the potential to become a new therapeutic approach for IOPD.

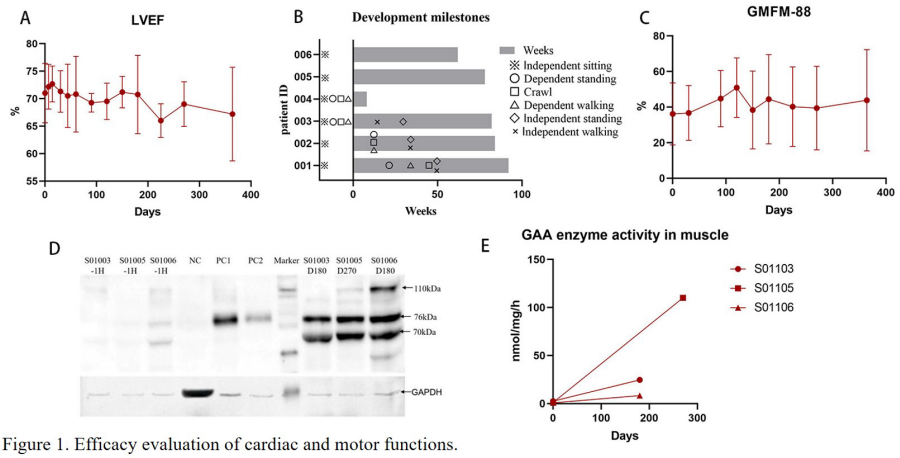

Figure 1. Efficacy evaluation of cardiac and motor functions.

A. The trend of LVEF changes in the subjects (average±SD). B. The motor developmental milestones obtained by six subjects from GC301 administration to now. C. The trend of GMFM-88 changes in the subjects (average±SD). D. Western Blot detected an increase in the expression of GAA protein after treatment. E. After administration, the GAA enzyme activities in muscle tissue significantly increased.

357 ART001: Development and Interim Clinical Outcomes of a CRISPR-Based In Vivo Gene-Editing Therapy for Hereditary ATTR

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Accuredit Therapeutics 2024

Hereditary transthyretin-mediated amyloidosis (ATTR) is a progressive disease caused by misfolded transthyretin (TTR) protein, leading to amyloid deposits in tissues and organ such as peripheral nerves or the heart. Current treatments, including TTR-stabilizing agents and RNA-based therapies, require long-term administration and primarily delay disease progression. ART001 is a novel CRISPR-based in vivo gene editing therapy designed for single-dose, long-lasting treatment by knocking out the wild-type or mutant TTR gene in hepatocytes, using a lipid nanoparticle (LNP) system. ART001's **regulatory approval for phase I clinical trials in both the United States and China** further underscores its global significance, as it is one of only two in vivo gene-editing therapies authorized for clinical trials targeting ATTR in US. In cynomolgus monkeys, ART001 demonstrated sustained, dose-dependent reductions in TTR levels by 85%, 97%, and 99% at doses of 1, 2, and 3 mg/kg, respectively, maintained for up to 360 days. Crucially, comprehensive genomic analyses detected **no off-target editing at as much as 79 times EC90, with only minimal large genomic alterations, such as large indels or translocations.** These results supported an open-label, single-center investigator-initiated trial (IIT) to evaluate ART001 in 10 patients with hereditary ATTR. Patients aged 31–65 years were enrolled across six dose cohorts (0.05–1.0 mg/kg) and received ART001 intravenously. ART001 exhibited an exceptional safety profile, **with no infusion-related reactions (IRRs), serious adverse events (SAEs), or significant immune toxicities reported. No elevations in liver enzymes beyond Grade 1** or evidence of liver toxicity was observed, reinforcing ART001's safety advantage. Adverse events were limited to mild and transient occurrences, such as hypertriglyceridemia, limb pain, and weight gain, with all resolving within the follow-up period. Efficacy results from this IIT trial have been equally impressive, with ART001 achieving dose-dependent reductions in serum TTR protein levels **at 48 weeks: 78%, 83%, and 93% in the 0.5 mg/kg, 0.7 mg/kg, and 1.0 mg/kg** cohorts, respectively. These reductions far exceeded

the clinically meaningful threshold of 60%, highlighting ART001's potential as an effective single-dose therapy for long-term disease management. ART001 represents a major advancement in gene editing, achieving sustained TTR protein knockdown with a single administration and minimal adverse effects. With regulatory approvals and promising, 48-weeks, interim clinical results, ART001 holds the potential to revolutionize ATTR treatment, reduce treatment burden, and significantly improve patient outcomes.

358 Safey and Efficacy of ATSN-201 Dose Escalation in Patients with X-linked Retinoschisis (XLRS)

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X-linked retinoschisis (XLRS) is associated with mutations in the RS1 gene and is a leading cause of juvenile macular degeneration in males that currently has no approved treatment options. XLRS is characterized by schisis, or abnormal splitting of the retinal layers, which causes impaired visual acuity that is not correctable with glasses and leads to progressive vision loss and ultimately blindness. Despite being a secreted protein, RS1 needs to be secreted from photoreceptors in order to properly function. The only effective route of administration for efficient AAV-mediated gene delivery to photoreceptors is subretinal injection. The presence of schisis cavities in the central retina poses a challenge to safe and effective delivery of AAV-RS1 to XLRS patients. The novel AAV.SPR capsid laterally spreads well beyond the margins of the subretinal injection (SRI) bleb, providing a means to perform peripheral SRI (without detaching the fovea and avoiding schisis cavities) and effectively deliver RS1 to both the peripheral and central retina. The goal of this phase 1/2 dose escalation study is to evaluate the safety and preliminary efficacy at 3 dose levels of ATSN-201, a laterally spreading subretinal gene therapy, in patients with XLRS. Nine adult patients received unilateral subretinal injections of ATSN-201. Three cohorts (N = 3 each) were treated with ATSN-201 [Cohort 1: 1.5 x 10^{10} vg/eye (low dose), Cohort 2: 5.0 x 10^{10} vg/eye (high dose), and Cohort 3: 3.0 x 10^{10} vg/eye (mid dose)], delivered in 2 or 3 blebs while avoiding foveal detachment. The primary endpoint is the incidence of dose-limiting toxicities (DLTs) and treatment-emergent adverse events (TEAEs), and key secondary endpoints include changes in retinal thickness and microperimetry (MP). Data are reported through a data cutoff date of 25 November 2024. All subjects were followed through at least Month 1 and up to Month 12 post-treatment. A total of 66 TEAEs were observed, 43 of which were related to the surgical procedure. No DLTs have been reported, and one serious TEAE that was unrelated to ATSN-201 or study procedures was reported. All 3 subjects in Cohort 2 developed TEAEs of subretinal deposits and associated retinal thickening that improved following extended steroid treatment; as a result, the dose was de-escalated to an intermediate dose in Cohort 3. All 3 subjects in Cohort 3 developed TEAEs of retinal thickening which resolved in two subjects and is resolving in one patient as of the data cutoff. One subject also experienced subretinal deposits that improved following extended steroid treatment. Of subjects with at least 3 months of follow-up, 5 of 7 subjects demonstrated closure of foveal schisis in the treated eye at the most recent visit. No untreated eyes showed foveal schisis closure. Treated eyes with structural improvements also generally demonstrated improvements in MP average threshold. In conclusion, ATSN-201 is a promising treatment approach for patients with XLRS, a disease for which no treatments currently exist.

AAV Preclinical and Proof-of-Concept Studies

359 *In Vivo* Mosaic Screens Uncover Novel Gene Therapies for Age-Related Diseases

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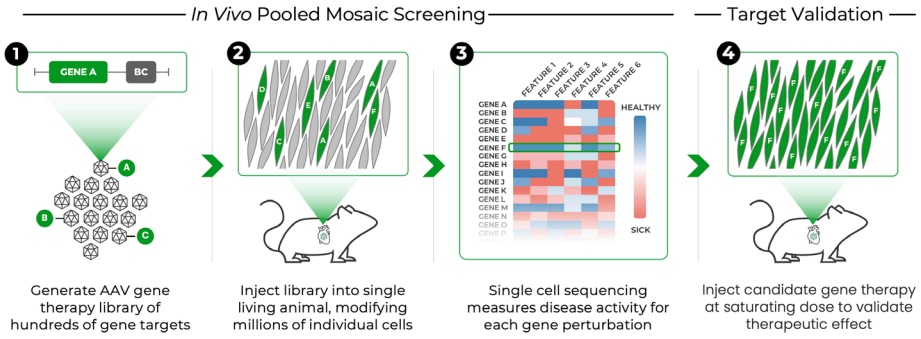
Background:
The complexity of age-related diseases, such as osteoarthritis (OA) and heart failure (HF), demands innovative strategies for therapeutic discovery. Here, we present an *in vivo* pooled mosaic screening platform that enables the identification of multiple therapeutic candidates in relevant disease models.

Methods:
Our *in vivo* drug discovery process consists of two key phases (see figure): 1) *In Vivo* Pooled Mosaic Screening – AAV delivery of barcoded therapeutic candidates into disease models, followed by single-cell sequencing and transcriptomic analysis to identify promising interventions. 2) Target Validation – Functional assessment of nominated targets in human patient samples and rodent disease models to confirm therapeutic potential.

Results:
Osteoarthritis: We screened 154 therapeutic interventions in the fetlock joints of thoroughbred horses (n=9) diagnosed with OA. Fibroblast Growth Factor 18 (FGF18), a best-in-class anabolic agent, served as a positive control, with non-targeting gene sequences as inert controls. Twenty-four top candidates were prioritized based on transcriptomic shifts indicative of therapeutic activity and were subsequently tested in ex vivo co-culture systems derived from OA patient chondrocytes and synovial cells. Four novel gene therapies demonstrated a 2.6-fold increase in glycosaminoglycan (GAG) content compared to FGF18, alongside reduced inflammatory and matrix-turnover biomarkers. The top two candidates were then administered intra-articularly into two preclinical mouse models of OA, namely, >2-year-old C57Bl6/J mice with spontaneous OA (n=16 joints per group) and monoiodoacetate (MIA) mice with inflammatory joint pain (n=10 joints per group). After six weeks, histological analysis revealed significant improvement in cartilage degeneration scores in the aged mice for both treatments, confirming structural modification potential. In the MIA pain model, both AAV gene therapies significantly restored weight bearing on the affected limb, indiciating reduction in pain.
Heart failure: A total of 151 therapeutic candidates were screened in 16-week-old female ZSF1 obese rats, a gold-standard model of heart failure with preserved ejection fraction (HFpEF). The screen incorporated positive control genes with established relevance to HF pathways or ongoing preclinical/clinical development. Transcriptomic analysis identified ten lead candidates, which were subsequently administered intravenously in two independent models of HF, namely, ZSF1 obese rats (n=12 per group) and the high-fat diet + L-NAME mouse model (n=20 per group). Here, at least one of the chosen interventions improved parameters of diastolic dysfunction on echocardiography, such as e' and E/e', and showed a reduction in hypertrophy. Further assessment was conducted using treadmill tests and pressure-volume loops to measure phenotypic improvements by the therapeutics.

Conclusions:
These findings demonstrate that *in vivo* pooled mosaic screening can uncover multiple therapeutic interventions for complex age-related diseases such as OA and HF. Several of these candidates are now progressing through IND-enabling studies. This platform is now being applied to additional indications, including Idiopathic Pulmonary Fibrosis (IPF), Metabolic Dysfunction-Associated Steatohepatitis (MASH), and obesity, further highlighting its potential for broad therapeutic impact.



360 Optidys: a Dual-AAV Gene Therapy Strategy for Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is a still incurable severe muscle wasting disease, leading to wheelchair dependence and premature death. DMD is caused by X-linked mutations in the dystrophin gene that result in a total lack of dystrophin, a crucial protein required for the preservation of muscle integrity following mechanical stress. Although DMD is a suitable disease for gene replacement therapy, the extent of Dystrophin transcript size exceeds by far the AAVs cargo capacity and only short versions of dystrophin can be accommodated. Several forms of microDys are currently being investigated in clinical trials. However, while the therapeutic effects are unequivocally met in the animal models, results from clinical trials highlighted an insufficient correction of the disease and unexpected adverse immunogenic events triggered by AAV delivery. This evidence supports the necessity to identify strategies to face the challenges of safety and efficacy.

It is still unclear to which extent microdystrophins can replace the full-length dystrophin function in human patients, as short versions lack structural and signaling domains important for the optimal function of the protein. We then generated a larger version of dystrophin (quasidystrophin-qDys EP2020/05837) including key domains for signaling protein and cytoskeleton organization, by a dual-AAV system based on homologous recombination. We evaluated the efficiency and safety parameters of our system by employing a ddPCR-based methodology demonstrating that our Dual AAV approach, systemically administered in the severe D2-mdx mice model, led to high transgene reconstitution efficiency and negligible ITRs-dependent concatemerization. This phenomenon led to remarkable protein restoration in muscles where lower fibrosis and higher muscle force strength were obtained compared to mice expressing a similar amount of microdystrophin protein.

We explored the therapeutic efficacy of our gene candidate in both DMD mouse and rat models, by employing LICA, our newly generated proprietary capsid with higher muscle tropism and liver detargeting confirmed in NHP, which allows superior efficiency in delivery and consequently the use of lower, thus safer, doses. In particular, both D2 mdx mice and DMD rat model (delEx45) treated with LICA-qDys (OPTIDYS) led to outstanding expression levels of the reconstituted dystrophin form in all muscles and to global improvement of muscle pathology and function. Moreover, the therapeutic efficacy and safety of Optidys are currently being investigated in our DMD rat model that shows a severe cardiac phenotype to evaluate the effect and safety on heart function.

In conclusion, OPTIDYS has the potential to safely increase the correction of the disease at a higher level than the current microDys approaches, keeping in mind that supportive strategies might be needed to further improve first-generation gene therapies and are currently under investigation.

361 Hepatocyte Tropism of a Bioengineered AAV3B Capsid Variant in a Machine-perfused Normal and Steatotic Human Liver

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The liver is an important target for adeno-associated virus (AAV)-mediated gene therapy not only for the correction of liver diseases but also for systemic delivery of therapeutic proteins, for example in treatment of hemophilia. Further advancing hepatic AAV gene transfer is hindered by the limitations of pre-clinical models in predicting the

transduction of human liver cells and by variability in the human population. For example, steatotic liver disease has become highly prevalent while limited data are available on the impact of steatosis on hepatocyte (HC) transduction. Previously, we identified a potential candidate for human liver gene transfer, AAV3B-v4, which was selected from a combinatorial capsid library. This capsid demonstrated enhanced transduction efficiency in mouse passaged primary human HCs as well as in humanized liver chimeric mice when compared to other AAV3B variants (Hum Gene Ther 34:289-302, 2023). To further characterize and define the transduction pattern of this vector in human liver, we administered scAAV3v4-CB-mScarlet vector to a normal and a steatotic human liver maintained on normothermic machine perfusion.

Two donor livers were sourced from the Indiana Donor Network. Donor 1, a 38-year-old Caucasian male (BMI 22.0), provided a non-steatotic liver categorized as donation after circulatory death (DCD). Donor 2, a 58-year-old African American male, provided a steatotic liver categorized as donation after brain death, exhibiting 30-40% macrovesicular steatosis with F2-3 bridging fibrosis and moderate portal inflammation. Both livers were declined for transplantation due to slow lactate clearance. To evaluate transduction, 1×10^{13} viral genomes (vg) of scAAV3v4-CBA-mScarlet were infused via the portal vein after 14 hours (donor 1) and 11 hours (donor 2) of perfusion, once lactate levels dropped below 3.0 mmol/L and HCs were analyzed at the completion of the experiment.

Liver viability was sustained for 110 hours (donor 1) and 60 hours (donor 2), evidenced by recovery in lactate clearance, glucose metabolism, pH, and bile production. Perfusion continued until lactate levels began to rise, after which the livers were harvested. Vector clearance was assessed by measuring AAV genome copies in the perfusate. For donor 1, 2.8×10^{7} vg/mL (~0.5% of total infused virus) were detected 4 hrs post-infusion, declining to 4.2×10^{6} vg/mL by the end of the perfusion. For donor 2, 6.8×10^{7} vg/mL (~1% of the total virus) were detected 2 hrs post-infusion, peaking at 1.8×10^{8} vg/mL at 17 hours before declining. Flow cytometry showed mScarlet transgene expression in ~40% of HCs in donor 1 and ~21% in donor 2, which was confirmed by fluorescent microscopy of freshly isolated HCs (with persistent expression observed during a 16-day culture period). Interestingly, mScarlet expression was minimal in non-parenchymal cells (NPCs), such as endothelial and Kupffer cells (~0.1%) in donor 1 but was higher in $CD45^{+}$ NPCs (~5%) in donor 2. The extended perfusion period for donor 1 (~4.5 days versus ~2.5 days for donor 2) likely contributed to the higher transduction of HCs (~40% vs. ~21%). Despite this, hepatocytes in the steatotic liver showed substantially brighter transgene expression. These findings are consistent with our observations in primary human HCs models that show enhanced transduction in steatotic HCs.

362 Interfering with CUG toxic repeats using AAV.U7snRNA rescue myotonia and splicing defects in myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1) is the most common form of muscular dystrophy in adults affecting the skeletal muscle, heart, and brain. It is caused by a CTG repeat expansion in the 3'UTR of the *DMPK* gene. The CUG repeats aggregates with muscle-blind proteins (MBNLs) in nuclear *foci*, leading to the impairment of several mRNA processing functions, including alternative splicing. The reversal from an adult to a fetal splicing profile alters or abrogates the function of a myriad of proteins, resulting in dysfunction in multiple organs. Currently, there is no treatment available for DM1 patients. Antisense oligonucleotides (ASOs) aiming to knock down *DMPK* expression or bind to the CUG repeats and release MBNL have been explored, with promising results in animal models. Unfortunately, a clinical trial with these drugs, while safe, failed to show improvement in patients, probably due to intrinsic limitations of ASOs. To overcome this limitation, we designed modified U7 small nuclear RNAs containing a promoter and antisense sequences targeting the 3'UTR region to promote *DMPK* knockdown or steric hindrance of the CUG repeats. We used patient-derived cell lines to test our approach, and the effect of the treatment was evaluated using RNA FISH combined with MBNL1 immunostaining for *foci* quantification and MBNL1

localization. Gene expression and splicing profiles were assessed by RNAseq and selected candidate genes were confirmed by RT-PCR. The AAV treatment reduced the *foci* number, released MBNL1 from the CUG *foci*, and shifted the splicing profile. Based on the *in vitro* results, we selected one vector candidate to test *in vivo*. We injected AAV1.U7snRNAs in the tibialis anterior (TA) and gastrocnemius (Gas) of HSA^{lr} mice, which express CTG repeats in skeletal muscles only. Eight weeks post-injection, we submitted the animals to muscle torque and electromyography (EMG) tests and collected the muscles for further analysis. The functional tests revealed reduced mechanical myotonia (a major phenotype in DM1 muscles) and even the absence of electrical myotonia in individual muscles. RNA FISH quantification showed a reduced number and intensity of *foci* in TA and Gas. The reduction in *foci* led to improved splicing of *Serca1, Mbnl1, Ldb3,* and *Clcn1* transcripts. In conclusion, U7snRNAs that interfere with the CUG-expanded mRNA can reverse DM1 pathology with the restoration of molecular features *in vitro* and *in vivo*, accompanied by significant amelioration of muscle physiological properties. This work will provide the basis for testing the first AAV-based gene therapy for DM1 in humans.

363 Insulin Replacement Gene Therapy Using a Novel Glucose- and Drug-Inducible Adeno-Associated Virus

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Objective:
To develop AAV-based gene therapies that can be activated by the FDA-approved drug doxycycline (Dox). We present proof-of-concept studies using diabetic mice that show effective delivery and pharmacological regulation of an insulin gene replacement therapy.

Methods:
Glucose-inducible promoters have been developed by incorporating the DNA sequences that bind the carbohydrate response element binding protein, ChREBP. We developed a novel promoter with multiple repeats of ChREBP binding motif 1 and motif 2 (Jeong et al., 2011). For Dox regulation we engineered a single AAV to contain both components of the 3G Dox-on system (Clontech): 1) the reverse tetracycline transactivator (rtTA), whose expression is controlled by the glucose-inducible promoter; and 2) the tet operator, where the rtTA binds in the presence of Dox to induce the expression of recombinant insulin. Recombinant AAV8 pseudovirions were injected into the tail vein of diabetic mice (streptozotocin model). Blood glucose levels were monitored after a 4 hour fast and after an intra-peritoneal glucose tolerance test (GTT). Insulin production was monitored using serum samples and an ELISA directed against the human C-peptide produced by the rAAV. Expression of recombinant insulin mRNA was also validated using RT-qPCR. AAV delivery was confirmed by qPCR on liver DNA.

Results:
Design of the AAV targeting plasmid was guided by in vitro studies measuring the expression of the fluorescent reporter, Fusion Red. In vitro experiments showed 4-fold regulation of expression by glucose and robust regulation by doxycycline with a large dynamic range (1,000-fold). All in vivo experiments were blinded and included 4 groups of mice (C57Bl/6, n=5 each): Group 1 were control (CTRL) mice (not diabetic, no AAV); Group 2 was a negative control (diabetic, null AAV); while Groups 3 and 4 tested variations of the rAAV-Insulin gene therapy. Baseline studies established that STZ-treated mice lost their ability to control blood glucose. Strikingly, administration of Dox normalized glucose handling in groups 3 & 4 to similar levels as observed in the naive group. We also performed dose response studies varying the concentration of Dox in the chow (Figure 2.). Very high doses of Dox (100 mg/kg) actually lowered blood glucose below levels observed in CTRL. Titration down to 10 mg/kg Dox in chow restored blood glucose in diabetic mice to the control level.

Significance:
The human insulin replacement market has been estimated at $42 billion dollars. Most commonly used strategies rely on the patient monitoring of their blood glucose and daily subcutaneous injections of recombinant insulin. The

present research suggests that a one-and-done pharmacologically inducible insulin gene replacement strategy can be developed.

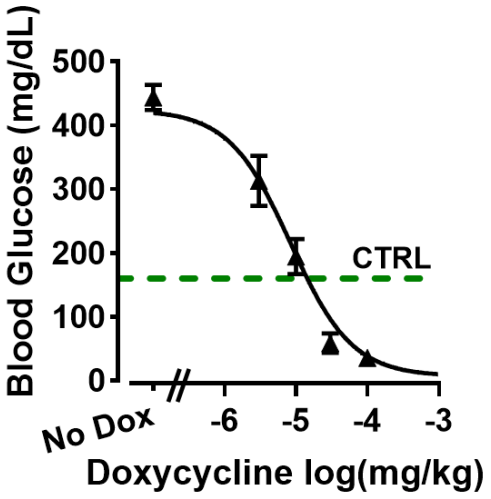

Fig. 2 Blood glucose levels in response to Dox concentrations in chow.
Titration down to 10 mg/kg Dox in chow restored blood glucose in diabetic mice to the control level.

364 Endoscopic Ultrasound-Guided Delivery of Human Glucagon-like Peptide-1 Pancreatic Gene Therapy: Safety and Feasibility in a Porcine Model

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Introduction:
Glucagon-like peptide-1 (GLP-1) is a key hormone regulator of metabolism, and GLP-1-based therapies are now an important mainstay of obesity and type 2 diabetes (T2D) care. Yet, treatment discontinuation is frequent and typically results in near-complete weight regain and loss of metabolic benefits for patients, which underscores an urgent unmet need for long-term therapeutic durability. We have developed a novel AAV-based pancreatic gene therapy (PGTx) platform with an endoscopic ultrasound (EUS)-guided delivery system to enable durable, nutrient-responsive, beta-cell-driven GLP-1 production (Smart GLP-1) toward this unmet need. In proof-of-concept murine models of metabolic disease, GLP-1 PGTx treatment improved hyperglycemia, lowered body weight, and sustained weight loss after semaglutide withdrawal. Here, we aimed to establish the feasibility and safety of delivering RJVA-001 (GLP-1 PGTx candidate for T2D) via an endoscopic transgastric route of administration (ROA) in Yucatan pigs.

Methods:
To directly target the pancreas with gene therapy, an echoendoscope with a proprietary automated fluid delivery system was inserted into the porcine stomach and a custom 27G needle was used to transgastrically infuse RJVA-001 [AAV9 vector encoding a human insulin (hINS) promoter and GLP-1 transgene, 6e13 total vector genome (VG)] into the splenic lobe of the pancreas under EUS guidance (N=5). Serum lipase and neurofilament light chain (Nf-L) were evaluated from baseline through study termination as markers of pancreatitis and dorsal root ganglia (DRG) toxicity, respectively. Pigs were necropsied at 5 weeks post-procedure and pan-pancreatic biopsies (up to 20 per pig) were assessed to establish vector copy number (VCN), transgene RNA and protein expression as indicators

of transduction efficiency and RJVA-001-driven GLP-1 production. Dual hINS/GLP-1 immunofluorescence was employed to assess beta-cell promoter specificity. Histological assessments of tissues including the pancreas, liver, heart, and DRG were carried out to evaluate inflammation.

Results:
All animals successfully underwent EUS-guided RJVA-001 delivery with correct pancreatic needle placement confirmed by ultrasound and no behavioral indicators of distress or discomfort observed. Mean lipase levels remained in the normal range [3.5±0.5 U/L standard error of the mean (SEM)] post-procedure, and no elevations in Nf-L were detected across all timepoints evaluated, with levels remaining below 10 pg/mL. Mean pancreatic VCN and RJVA-001 RNA levels were positively correlated ($r=0.85$, $p<0.0001$). Likewise, mean RNA levels correlated with RJVA-001-driven GLP-1 protein levels ($r=0.85$, $p<0.0001$). Active mean GLP-1 protein expression was five-fold higher in treated vs. endogenous levels in untreated controls (185±32 vs. 36±9 ng/g total protein SEM, $p<0.02$). GLP-1 positive immunofluorescence was observed in pancreatic islets, and co-localized with insulin, demonstrating beta-cell transduction and promoter specificity. No inflammation above background findings was observed in the pancreas, liver, heart, and DRG.

Conclusions:
Taken together, these data suggest that RJVA-001 can be safely and reproducibly delivered transgastrically to the pancreas leading to local, beta-cell specific production of GLP-1. These findings represent a major stride forward toward advancing this PGTx delivery procedure and candidate toward T2D first-in-human studies.

365 Gene Therapy of Computationally Designed IL-10 in DSS-induced Colitis Mouse Models

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Interleukin-10 (IL-10) is an immunoregulatory cytokine that can mediate both anti-inflammatory and pro-inflammatory effects. We aimed to develop IL-10 mutant drugs that have anti-inflammatory function, but no or minimal pro-inflammatory effect for the treatment of inflammatory bowel disease (IBD), which is associated with IL-10 deficiency. Using structure-based computational-design approach, we have designed several IL-10 mutants and generated recombinant adeno-associated virus (rAAV) vectors expressing wild type (IL-10W) or mutant IL-10 molecules (IL-10A, B and C). To test the therapeutic effect of these vectors, we employed dextran sodium sulfate (DSS) induced colitis mouse models. In DSS-induced acute colitis model (4% DSS in drinking water for 5 days), we showed that our rAAV8 vectors ($1x10^{11}$vg/mouse) mediated high levels of IL-10 in circulation. We observed that the expression levels decreased after DSS treatment suggesting the increase of consumptions of IL-10 during the disease development. Clinical evaluations showed the disease activity index (DAI) in IL-10C on the 7th day after DSS-induction was significantly lower than that in IL-10W group. In addition, the spleen weight in IL-10C group was significantly lower than that in IL-10W group. Flowcytometry analyses showed that our mutants have significantly lower activities in stimulating NK cells than IL-10W, while maintaining similar effects to IL-10W on monocytes indicating the mutants have reduced-pro-inflammatory effects and retained-anti-inflammatory effects. To test the long-term effect of IL-10 mutants, we performed IL-10 mutant gene therapy ($5x10^{10}$ or $1x10^{10}$ vg/mouse) in DSS-induced chronic colitis mouse model (3 cycles of DSS-induction). The results from this study showed that the cumulative DAI (AUC) of IL-10C group was significantly lower that of IL-10W. In summary, computational designed IL-10 mutants have improved function in regulating inflammation. Gene therapy of these mutants may provide a novel therapy for inflammatory bowel disease (IBD) and other chronic inflammatory diseases.

Translational Approaches: Gene Therapy of Neurological Diseases in Large Animal Models

366 Four year follow up of AAV-mediated gene replacement therapy in a large animal model of Krabbe disease.

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Krabbe disease commonly presents as a devastating, early-onset disease with rapid progression and premature death by 2 years of age. We are uniquely experienced with an invaluable large animal model of Krabbe disease. The canine model of Krabbe disease closely recapitulates the genetic, biochemical, and physiological disease in humans and provides translational advantages for evaluation of genetic therapies.

AAV9-mediated gene therapy administered directly into the cerebrospinal fluid (CSF) at the cisterna magna of 2-week-old presymptomatic Krabbe dogs increased GALC enzyme activity, normalized psychosine concentration, improved myelination, and attenuated inflammation in both the CNS and PNS in our initial reporting up to one year post-treatment (Bradbury AM et al. *Krabbe disease successfully treated via monotherapy of intrathecal gene therapy*. Journal of Clinical Investigation 2020; 130). We now report that long term follow up demonstrated that this gene replacement therapy mitigated clinical neurological dysfunction, allowing dogs to live beyond 3.5 years of age (predefined study endpoint), more than 11 times longer than untreated dogs. In two dogs, megaesophagus warranted euthanasia that was distinct from traditional Krabbe disease neurological disease progression. We speculate that the gene therapy did not completely correct the autonomic nervous system in these animals, as has been shown in other large animal models of lysosomal storage disorders. In a subset of the remaining treated dogs which lived >3.5 years of age, postmortem analysis revealed emergence of pathology in select brain regions including globoid cell accumulation, axonal degeneration, and white matter gliosis in the centrum semiovale, internal capsule, and corona radiata. While other brain regions, including the cerebellum, showed complete correction of disease pathology and intact myelination. We quantified GALC activity, psychosine levels, and inflammatory markers including GFAP for astrocytosis and CD68 for microgliosis in numerous brain regions. We corroborated these findings with clinically relevant readouts, including MRI and nerve conduction velocity testing, and longitudinal biomarker analyses of serum and CSF psychosine levels. We hypothesize that irreversible myelin loss was already present at the early 2 week treatment time point and coupled with reactivation of the disease process due to adult myelin remodeling and exhaustion of local gene correction in these focal lesions. This data will provide critical insight to both the necessary timing of treatment and the durability of AAV gene therapy, particularly for white matter diseases, using an invaluable large animal model that closely recapitulates human disease.

367 Evaluating delivery methods for treatment of neurodegenerative disease in large animal models.

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Neurofibromatosis 1 (NF1) is a neurocutaneous syndrome characterized by the development of tumors in the central or peripheric nervous system (CNS and PNS respectively), that affects 1 in 2500-3000 births. NF1 is caused by dominant mutations in the NF1 gene that encodes a protein, neurofibromin, that is a negative regulator of Ras/Raf/MEK-pathway and therefore has as a tumor suppressor activity. The loss of neurofibromin results in tumorigenesis in the skin, nerves, brain but also results in neurological signs like Autism spectrum disorder or attention deficit disorder. The lack fo Ras regulation leads to excessive cell growth provoking the development of tumors in a large verity of tissues all over the body. Examples include neurofibromas, and plexiform neurofibromas. The appears in late childhood and increase in number with age. The fibromas can vary in number form few to thousands. They can cause pain, disfigurement, local compression and loss of function of the affected area which result in a gravely affected quality of life and decreasing life expectancy. Genetically, neurofibromas are developed when the normal copy of the NF1 gene is lost in Schwann cells, a genetic mechanism known as loss-of-

heterozygosity (LOH). Gene therapy using viral vectors such as adeno-associated virus (AAV) is a promising approach for NF1 as it allow to correct the underlaying genetic defect though transporting the therapeutic gene directedly to the affected tissues. Our team has developed a NF1 minigene that halts tumor progression in NF1 mice. Given the multisystem involvement of NF1 we conducted large animal studies to determine optimal route of injection, biodistribution and safety for future human clinical trials. Cranial sub-leptomeningeal injection (Fig. 1A) of MRI contrast agent was far superior to CSF delivery, with strong enhancement of deep brain structures including the striatum and thalamus (Fig. 1B). Therefore, we tested sub-leptomeningeal injection of AAV and compared it with CSF delivery. We treated pigs and sheep with high dose AAV ~1-2 E14vg by leptomeningeal injection and compared it with cisterna magna injection or combination of multiple CSF routes (bilateral ICV, CM and lumbar intrathecal; (Fig 1C-F). Sub-leptomeningeal injection showed high vector expression in the parietal cortex near the injection site when compared to CSF injection, and therapeutically relevant delivery in deeper areas of the brain[JG1] (thalamus, striatum, parietal cortex) that were on par with the CSF delivery methods (Fig. 1C&D). This was corroborated with immunohistochemistry which showed striking staining throughout the parietal cortex and into the white matter approximately a 4mm deep from the surface of the brain near the injection site (Fig. 1F). The meninges adjacent to the injection site showed extraordinarily high vector genomes in all treatment groups with a 2x increase in the leptominengial injection. Interestingly, with sub-leptomeningeal injection we did not observe transuducion (add numbers) of the liver as compared to CSF injection (add numbers) (Fig 1E). Altogether, our data indicate that leptomeningeal injection far surpasses the cortical biodistribution of cisterna magna within the gyrus containing the injection site, with diffusion of viral particles to other brain areas similar to the CSF injection groups. In conclusion, sub-leptomeningeal injection is a promising therapeutic AAV delivery method for treatment of NF1.

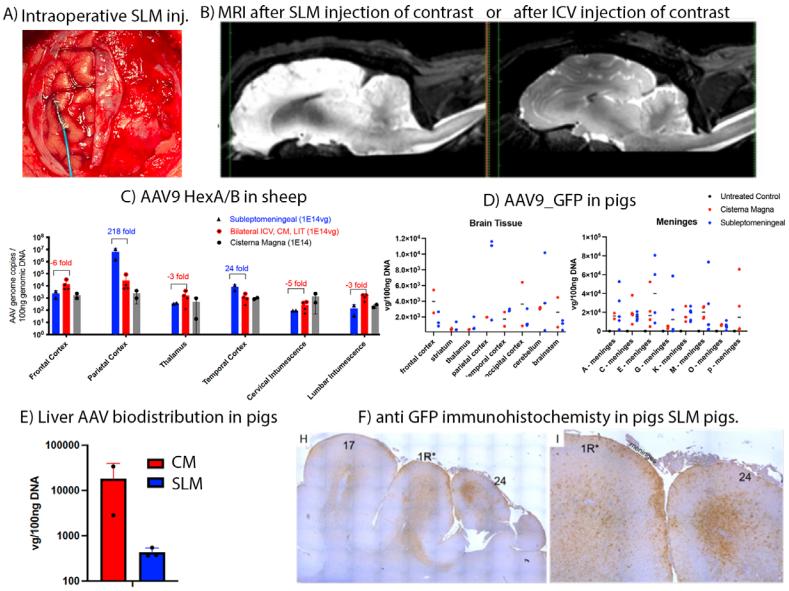

Figure 1. Sub-leptomingeal injection (SLM) compared to CSF injection delivery routes. A) Interoperative image of a pig recieving a subleptomeninges injection over the parietal cortex. B) MRI after SLM injection compared to intracerebroventricular (ICV) injection. C) vector genomes in the brain and spinal cord after SLM injection or combined bilateral ICV in sheep with 1E14vg AAV9. D) Vector genomes in the brain of pigs after 1.6E14vg of AAV9-GFP adminstered by SLM injection. E) liver biodistribution after SLM or cisterna magna (CM) injection. F) GFP immunoreactivity in the parietal cortex of pigs after 1.6E14vg AAV9-GFP.

368 AAV gene therapy for GBA-PD and Gaucher Disease

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Mutations in the *GBA1* gene, which encodes the lysosomal enzyme glucocerebrosidase (GCase), are a common genetic risk factor for Parkinson's disease (PD) and causative for Gaucher Disease (GD), a rare lysosomal storage disorder. Both diseases converge on GBA1-driven lysosomal dysfunction, leading to impaired lipid metabolism and cellular toxicity. Here, we report the development of an AAV-mediated *GBA1* replacement therapy for treating GD and GBA-PD through a one-time infusion delivered intra-CSF intravenously (for GD Type 1) or intra-CSF (for GBA-PD). We engineered human *GBA1* to be readily secretable to facilitate cross-correction across multiple tissues. We packaged our payload in AAV.GMU01, a capsid with superior biodistribution and transgene expression in both the central nervous system (CNS) and peripheral tissues. In pharmacology and efficacy studies using conduritol ß-epoxide (CBE)-induced lipid flux models in both mice and non-human primates (NHPs), our lead candidate SS3-GBA1 demonstrated robust secretion, efficient lipid clearance, and significant cross-correction. Importantly, huGBA1 protein levels in SS3-GBA1 injected NHP brain tissues reached ~50% of levels observed in healthy human donor brain tissues. Given that GBA-PD patients are carriers of heterozygous mutations in GBA1, our data indicate near-physiological restoration of GBA1 protein that is required for clinical efficacy. Intravenous dosing of SS3-GBA1 promoted effective lipid clearance across multiple peripheral tissues including liver, spleen, bone marrow and muscles, key target tissues in GD Type 1. Effective lipid clearance in the bone marrow is a notable advantage of our approach for GD Type 1 when compared to the enzyme replacement therapies (ERTs), which lack bone marrow penetration. Importantly, across all our studies, AAV.GMU01-SS3-GBA1 is well tolerated with no adverse findings, demonstrating a favorable safety profile. These findings establish AAV.GMU01 SS3-GBA1 as a promising therapeutic candidate capable of addressing the unmet needs of both GD and GBA-PD patients through a single gene therapy product. By restoring GBA1 protein levels to physiological levels and enabling effective lipid clearance across both CNS and peripheral tissues, this strategy offers a transformative approach to treating lysosomal dysfunction in these genetically defined populations.

369 AAV-mediated Delivery of an Engineered Zinc Finger Lead to Selective and Potent Repression of Nav1.7 in Human Sensory Neurons and Nonhuman Primates DRG Nociceptors Following Intrathecal Injection

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Idiopathic small fiber neuropathy (iSFN) is a type of chronic neuropathic pain that is caused by severe and permanent damage to peripheral sensory neurons. iSFN has an estimated prevalence of at least 43,000 patients in the U.S. and manifests as severe pain in lower extremities, which leads to a major impact on quality of life. Current pain medications for iSFN are inadequate; effective pain reduction occurs in less than 50% of the patients and the medications are often associated with severe side effects. In humans, the Nav1.7 sodium channel, which is encoded by *SCN9A* gene, is involved in a spectrum of inherited pain-related disorders and has emerged as a promising target for analgesic drug development. However, the development of a selective Nav1.7 inhibitor has been challenging in part due to structural similarities among other Nav channels. We have developed an engineered zinc finger repressor (ZFR) targeting *SCN9A* with high potency and selectivity as a potential treatment for iSFN. *In vitro*, adeno associated virus (AAV)-mediated delivery of the ZFR (AAV-ZFR) in human iPSC-derived GABAergic and sensory neurons resulted in a 90% reduction of *SCN9A* over a wide-dose range with no detectable off-target activity as measured by global transcriptomics analysis. Microelectrode array (MEA) recordings in human sensory neurons showed a significant decrease in the average neuronal firing rate in response to noxious heat following AAV-ZFR treatment, illustrating reduced Nav1.7 activity. In NHPs, four weeks after a single intrathecal (IT) injection of AAV-ZFR, a dose-dependent repression of *SCN9A* was observed in bulk DRG tissue at three different levels (lumbar, thoracic, and cervical). While up to 60% repression of *SCN9A* was observed across different DRG levels, other Nav channels, such as *SCN8A* (Nav1.6), *SCN10A* (Nav1.8), and *SCN11A* (Nav1.9), were not affected, demonstrating the selectivity of AAV-ZFR toward *SCN9A* in NHPs. Additionally, single-cell analysis using RNAscope revealed potent *SCN9A* repression in CGRP-positive nociceptors in lumbar DRG following IT injection. The treatment was well tolerated in NHPs, with no dose-limiting findings observed four weeks following the treatment. The results

demonstrate the potency and selectivity of a AAV-ZFR in NHPs and support further development of ST-503, an investigational therapy for the treatment of intractable pain due to iSFN.

370 Development and validation of an intravenous AAV gene therapy for mucopolysaccharidosis type IIIB in mouse and dog model of the pathology

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MPSIIIB is an autosomal recessive lysosomal storage disorder, caused by alpha-N-acetylglucosaminidase (NaGlu) enzymatic deficiency leading to accumulation of Heparan Sulfate Oligosaccharides (HSO) in tissues including the central nervous system (CNS). Patients manifest with early developmental delays followed by severe behavioral abnormalities, progressive neurodegeneration, and death before the age of 20 years. To date, there are no curative therapies for MPSIIIB. We have previously conducted an AAV5 phase I/II intracerebral gene therapy trial that has shown promising results in four MPSIIIB patients with best results being obtained in the youngest patient (18 months-old). However, disease progression in tissues as important as meninges, brain capillary walls, and choroid plexus was presumably not stopped. Therefore, treatment of patients younger than 2 years and the delivery of NAGLU both within and outside the brain was concluded.

We recently described a novel AAV gene therapy using AAVPHP.eB-CAG-NaGlu vector with intravenous delivery in mouse model and combine intracerebral and intravenous delivery in dog and non-human primates. We demonstrated a good efficacy but some local inflammation at the injection site in the brain.

We thus improved our therapeutic strategy by using last generation AAV, a serotype able of large Blood brain barrier crossing after intravenous delivery notably in large animals. We demonstrate a large CNS transduction of the vector in mouse and NHP after intravenous delivery as well as supra physiological NAGLU expression and activity and a perfect tolerance of the AAV on all tissues. Efficacy and safety studies are currently ongoing in the dog model of the pathology and data will be presented.

371 Vagus Nerve Delivery of AAV9/GAN Is Required After Intrathecal Administration for a Full Rescue of Autonomic Nervous Dysfunction in Giant Axonal Neuropathy

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Giant axonal neuropathy (GAN) is a pediatric progressive neurodegenerative disease caused by homologous loss-of-function mutations in the gene encoding gigaxonin. GAN patients manifest clinical symptoms related to the loss of function of the central nervous system, the peripheral nervous system, and the autonomic nervous system (ANS). A Phase 1 clinical trial (NCT02362438) evaluating intrathecal (IT) administration of AAV9/GAN has shown partial rescue benefits in GAN patients (Bharucha-Goebel et al., NEJM, 2024). A notable therapeutic gap from the IT administration is suboptimal targeting of key ANS neuronal populations, leading to ANS dysfunction and additional clinical unmet need. Although IT administration of therapeutic agents is widely used and has a wider applicability, IT injection of AAV9/GAN alone was insufficient to rescue the altered ANS functions in GAN model rats (ASGCT-

2020, Abstract #936). In that pilot study, a combination of IT and vagus nerve (VN) mediated AAV9/GAN delivery (same day injection) was observed to be effective compared to IT alone. Additional studies were carried out to identify an appropriate vg dose via the VN route to maximize transduction of ANS targets, and optimize the use of an FDA-approved and compatible tracking dye to monitor VN injection fidelity (ASGCT-2023, Abstract#1270). In the present study, we report a complete rescue benefit of ANS parameters in GAN rats with a combination of IT and VN administration. Notably, the IT and VN injections were spaced apart in this study, modeling a redosing scenario where patients that previously received an IT injection of AAV9/GAN would receive a later VN injection. First, at ~6.5 months old, GAN homozygous KI rats were treated IT with 20 µl of AAV9/GAN (3.6 E11 vg/rat), while GAN heterozygous KI rats (controls) were treated IT with 20 µl of vehicle. Two months following the IT injection, all rats treated with AAV9/GAN were randomly subdivided into four different subgroups: Only IT, IT+VN low dose (LD, 6.7E10 vg/rat), IT+VN middle dose (MD, 2.0E11 vg/rat), and IT+VN high dose (HD, 6.0E11 vg/rat). All subgroups, except the "Only IT" group, then received an injection of AAV9/GAN into the left VN at the cervical region. All groups of rats received a telemetry sensor implantation at 17 months old to record the ANS parameters (temperature, heart rate, blood pressure, and respiratory rates). Upon 1 mg/kg pilocarpine stimulation, all groups of rats with IT+VN (LD, MD, and HD) showed normalization of temperature, heart rate, and respiration rate compared to the "Only IT" group. No abnormal blood pressure phenotype was observed in any of the animals, compared to het control rats. These results indicate that IT administration of AAV9/GAN alone is not enough to rescue GAN ANS dysfunction, but a subsequent delivery of AAV9/GAN via the VN can rescue these deficits. Pivotal GLP toxicology studies were carried out in normal canines, which received an IT injection of AAV9/GAN followed by a VN injection of the vector or vehicle 9 weeks later. Animals received an immunosuppressive regimen matching the GAN clinical trial protocol, consisting of prednisone and rapamycin. Biodistribution to key ANS neuronal populations was achieved. No adverse effects related to AAV9/GAN were observed in dogs and rats. In January 2025, IND #31180 was cleared by the FDA to proceed, to evaluate VN administration of AAV9/GAN in GAN patients that previously received an IT injection of AAV9/GAN.

372 Sub-motor Cortex Convection-enhanced Delivery of AAV5-GDNF – a Novel Treatment Strategy for Amyotrophic Lateral Sclerosis

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Introduction
Gene therapies hold significant promise for the treatment of neurological disorders. However, achieving widespread gene expression at therapeutic concentrations in conditions characterised by degeneration of brain and spinal cord remains a significant challenge. Amyotrophic Lateral Sclerosis is characterised by degeneration of the motor pathway from cortex to spine. Extensive pre-clinical data indicate that delivery of the neutrophic factor Glial cell-line derived neurotrophic factor (GDNF) to the entire motor pathway could ameliorate symptoms and promote survival and regeneration of motor neurons. We describe an innovative method of achieving gene expression in brain and spinal cord using sub-motor cortex convection-enhanced delivery (CED) in a large animal model.

Methods
Bilateral sub-motor cortex CED of AAV5-GDNF at 2 infused titres ($2x10^{13}$ and $4x10^{13}$ Vg/mL) was performed with MRI guidance in 4 sheep using a novel acute cannula system. AAV5-GDNF was infused through a cannula targeted to the sub-motor cortex white matter through a posterior-anterior trajectory. A second more ventral cannula was inserted parallel, through which artificial CSF was infused in order to "drive" the test article into overlying motor cortex. Outcomes included MRI analysis of distribution, neurotoxicity and GDNF expression in brain and spinal cord.

Results
Gadolinium co-infusion allowed MRI-confirmation of distribution to motor cortex in all animals. No adverse surgical events were identified. No behavioural change, loss of appetite or weight loss over the 3 month study period was seen. GDNF ELISA confirmed expression at clinically-significant concentrations throughout the motor cortex of all animals. However, only the test subject receiving the lower infused titre showed GDNF expression throughout

the spinal cord. Immunohistochemical evidence of a mild immune response was identified in the high titre cohort, which was absent in the low titre test subject.

Conclusions
Sub-motor cortex CED was safe and effective, and at the lower titre achieved GDNF expression throughout the motor cortex and spinal cord at 3 months following a single infusion. This finding confirms the potential of this novel strategy for the treatment of neurological diseases characterised by widespread degeneration, with spinal cord gene expression dependent on the infused titre.

Upstream Manufacturing for AAV Vectors 1

373 Achieving Continuous Production of Recombinant Adeno-Associated Virus with Baculovirus Expression Vector System

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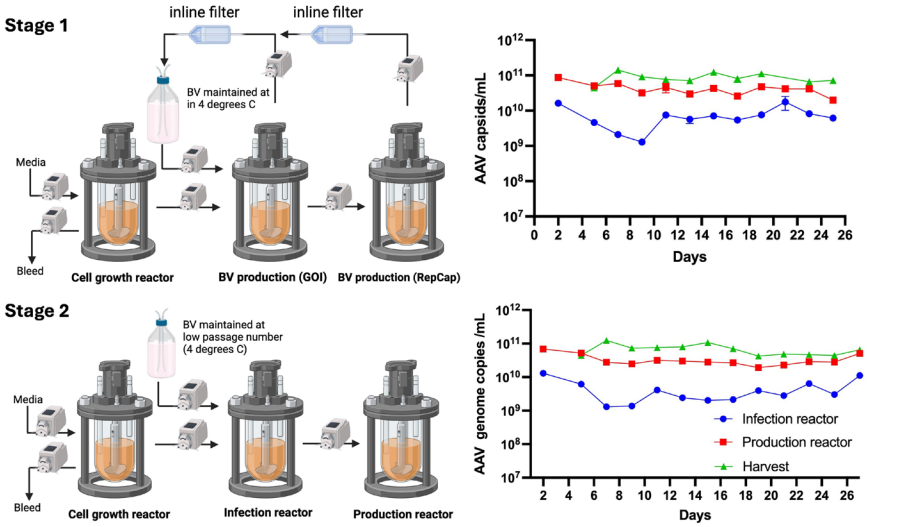
Continuous production processes offer significant advantages for biotherapeutic manufacturing, including increased productivity, improved product quality, reduced facility footprint, and decreased process turnaround time. Despite these benefits, continuous adeno-associated viruses (AAV) manufacturing has been limited due to the *in situ* formation of defective interfering particles (DIPs), which hinders continuous AAV production. This study addresses the challenge of reducing DIP propagation through the infusion of standard recombinant baculovirus and the compartmentalization of early- and late-stage infected cells, resulting in stable AAV production. AAVs were continuously produced in a series of cascading reactors, comprising a feeder reactor, an infection reactor, and a production reactor. The transfer dynamics of the inoculum across the three reactors were optimized based on the production kinetics of baculovirus and AAV derived from our mechanistic model. AAV packaging occurred in the baculovirus-infected Sf9 cells, primarily within the production reactor, which was maintained at an average residence time of approximately 60 hours. This study demonstrates the successful implementation of a continuous AAV production process, yielding over 5E10 vg/mL for 25 days. This work represents the first reported continuous AAV production process utilizing the Sf9/BEVS platform and establishes a foundation for overcoming manufacturing challenges associated with recombinant AAV (rAAV)-based gene therapies.



The schematic illustrates the continuous production of AAV using a series of cascading reactors. In Stage 1, insect cells from the growth reactor are infected with recombinant baculovirus encoding the gene of interest and AAV genes to produce a baculovirus stock. The budded baculovirus is harvested and stored at 4°C before continuous infusion into the infection reactor to maintain an MOI greater than 1 PFU/cell at Stage 2. The graphs depict successful AAV capsid and genome production in the continuous run.

374 Optimizing the TESSA® Platform for Enhanced rAAV Production

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Efficient and scalable production of recombinant adeno-associated virus (rAAV) vectors remains a significant challenge for AAV gene therapy, particularly in meeting preclinical and clinical demands. To address this, we developed the TESSA® platform, a self-silencing helper adenoviral system that delivers all the necessary components for efficient rAAV production in HEK293 cells. The system also self-inhibits expression of late adenoviral structural proteins, effectively eliminating adenoviral contamination during rAAV manufacturing.

The TESSA® platform enables rAAV production through two distinct approaches, offering flexibility for different gene therapy applications. The key difference between these models lies in how the gene of interest (GOI) is introduced into HEK293 cells:

TESSA® Duo model: A TESSA® vector encoding Rep and Cap is combined with a second TESSA® vector carrying the AAV GOI transfer genome.

TESSA® Pro model: A TESSA® vector encoding Rep and Cap is co-infected with an AAV GOI seed stock.

Previously, we demonstrated that the TESSA® platform enables cost-effective and scalable rAAV manufacturing, achieving up to a 20-fold increase in AAV vector production with productivities exceeding 1E+6 vector genome copies (GC) per cell and >1E+17 total GC from a 200L bioreactor.

To further enhance the TESSA® system, we implemented genetic engineering strategies focused on optimizing AAV Cap expression through promoter screening and evaluated hybrid AAV Rep proteins to improve rAAV yield and quality. Our assessment of rAAV9 production using both the TESSA® Pro and Duo models demonstrated vector genome titers of >2–3E+12 GC/mL of cell culture, representing a ~5-fold improvement over first-generation TESSA® RepCap vectors and a >30-fold increase compared to the conventional triple-transfection method. These

results highlight the significant impact of the TESSA® platform in overcoming key manufacturing bottlenecks in AAV gene therapy. Further optimization, including advanced promoter engineering, holds the potential to drive even greater efficiencies in rAAV production.

375 Next-Gen Recombinant Adeno Associated Virus Processes: Boosting Productivity & Quality via Process Intensification & Optimized Triple Transfection for All Common Serotypes

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Recombinant adeno-associated viruses (rAAVs) are widely used as gene therapy vectors. With the expansion of new indications, achieving higher titers and reducing manufacturing costs have become increasingly critical. In suspension-based transient transfection, low viable cell density (VCD) and fixed bioreactor volumes limit productivity. Furthermore, the absence of serotype-specific upstream optimization for transfection results in low yields and elevated levels of product- and process-related impurities, which pose significant challenges for downstream purification.

To address these limitations, perfusion using was implemented to increase cell density before triple transfection. Additionally, high-throughput process development technologies, such as Sartorius AMBR15 and AMBR250, were employed to optimize process parameters for all common rAAV serotypes (rAAV2, AAV5, AAV6, AAV8, and AAV9), enhancing overall productivity and product quality. This approach was further integrated with high-throughput analytical testing methods to assess product strength and characterize process- and product-related quality attributes post-production.

Incorporating perfusion into the upstream process led to at least a 5-fold increase in productivity and a significant reduction in the cost of goods while maintaining acceptable levels of process- and product-related impurities. Additionally, high-throughput optimization based on Quality by Design (QbD) principles significantly improved upstream titers and percent full capsids (up to 40%) for a gene of interest near the AAV vector's packaging limit (4.8 kbp). This approach provides a rapid, scalable, and cost-effective strategy across multiple serotypes, addressing the increasing demand for high-titer rAAV vectors in gene therapy. An rAAV production titer of ~1E12 vg/mL with over 40% full capsids was achieved.

376 Revolutionizing Viral Vector Production: Biodegradable Hyperbranched Poly (β-amino ester)-Based Transfection Technology Boosts Yields and Efficiency

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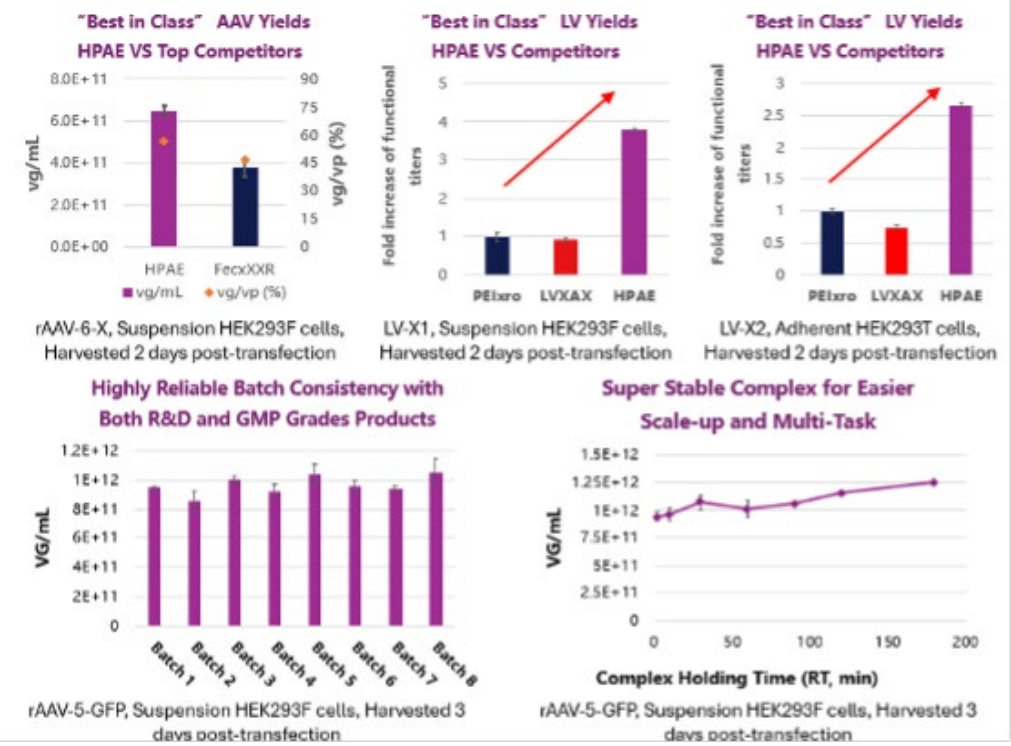
Traditional viral vector production has long relied on cationic liposomes or polyethyleneimine (PEI) as transfection reagents, despite their inherent limitations such as low efficiency, high cytotoxicity, and cumbersome scaling up processes. This study introduces a groundbreaking non-viral gene delivery platform based on hyperbranched poly (β-amino ester) (HPAE), an advancement over linear poly (β-amino ester), which presents a promising alternative to conventional transfection technologies [1,2].

The transfection reagents derived from this platform exhibit several pivotal advantages over traditional methods, including (1) Enhanced potency for efficiently transfecting large plasmid DNA (>12 kb); (2) Reduced cytotoxicity with a fully degradable polymer that leaves no residue; (3) Simplified scalability due to the stability of transfection complexes for at least 3 hours at room temperature; (4) cost-effectiveness with significantly reduced reagent consumption (＜25% of PEI); and (5) highly consistent polymer weight distribution and efficacy suitable for GMP-compliant production of viral vectors.

We demonstrate the superior effectiveness of HPAE-based transfection reagents in the production of viral vectors, including adeno-associated viruses (AAV) and lentiviruses (LV), achieving markedly higher viral yields and superior batch consistency compared to commercially available alternatives. Moreover, the versatility of the HPAE platform extends beyond viral vectors, enabling the efficient delivery of mRNA, siRNA, and CRISPR-Cas9 RNP complexes, offering a comprehensive solution that holds great potential for advancing gene therapy and cell therapy research and development [3-7].



377 Late-stage Development and Upstream Process Characterization of UX701 AAV Gene Therapy for Wilson Disease

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Introduction:
Wilson Disease (WD) is caused by mutations in the copper-transporter gene *ATP7B* which leads to toxic accumulation of copper primarily in the liver and brain. Copper chelators are the current standard of care but can result in significant side effects, do not address the underlying cause of WD, and have high rates of discontinuation. UX701 is an investigational AAV9 gene therapy designed to deliver a modified form of the *ATP7B* gene for the treatment of WD. UX701 leverages our proprietary producer cell line platform, Pinnacle PCL™, to produce rAAV in a 2000L bioreactor (Figure 1). Process characterization (PC) of UX701 is an essential aspect of the process validation lifecycle, aimed at defining manufacturing process ranges to maintain the target product profile. In this work, we have characterized the cell culture manufacturing process and demonstrate a robust and high yielding process for the manufacture of UX701. This is the first PC study for our Pinnacle PCL™ rAAV platform.

Methods/Approach:
A risk-based approach was applied to the late-stage development activities for UX701 upstream process including process parameter risk assessment, scale-down model (SDM) qualification, and PC studies, to establish process controls for the upstream unit operations in the 2000L manufacturing process. Specifically, we assessed a total of 224 upstream process parameters and raw materials on their impacts to process performance indicators (PIs) and

critical quality attributes (CQAs) and classified them into high, medium or low risk parameters. Of these, a total of 11 parameters and 2 raw materials were classified as high or medium risk and were assessed in PC studies. Upstream PC studies were conducted in a qualified SDM using 3L bioreactors coupled with high throughput purification via Tecan™ affinity chromatography and high throughput analytics including PCR-based methods for rAAV titer, host-cell DNA impurities, and helper virus impurities. High throughput AAV packaging characterization was performed using mass photometry as a surrogate for analytical ultracentrifugation.

Results:
We identified 8 process parameters to be critical (CPP) or key (KPP). Temperature and pH in the N-1 and production stages as well as production target seeding density and helper virus concentration were all found to be CPPs, which have significant impact on at least one CQA and one PI. N-1 dissolved oxygen and production harvest time relative to infection are KPPs, which have significant impact on at least one PI, but no significant impact on CQAs. PC studies also provided insights on process improvements. Delaying the harvesting time by 6hr from target can increase rAAV titer by 2-fold; increasing production temperature by 2°C can significantly increase rAAV titer and %full capsids (Figure 2).

Conclusion:
We have successfully risk assessed the UX701 upstream process, qualified a SDM, and characterized the process design space for our first Pinnacle PCL™ program. We have generated a comprehensive set of process-product relationships for UX701 as well as our rAAV platform, which will benefit other ongoing PCL™ programs in our clinical pipeline. These studies will continue to help us understand and mitigate operational and quality risks, which are critical for successful implementation of a robust commercial manufacturing process to provide consistent product quality throughout the product's lifecycle.

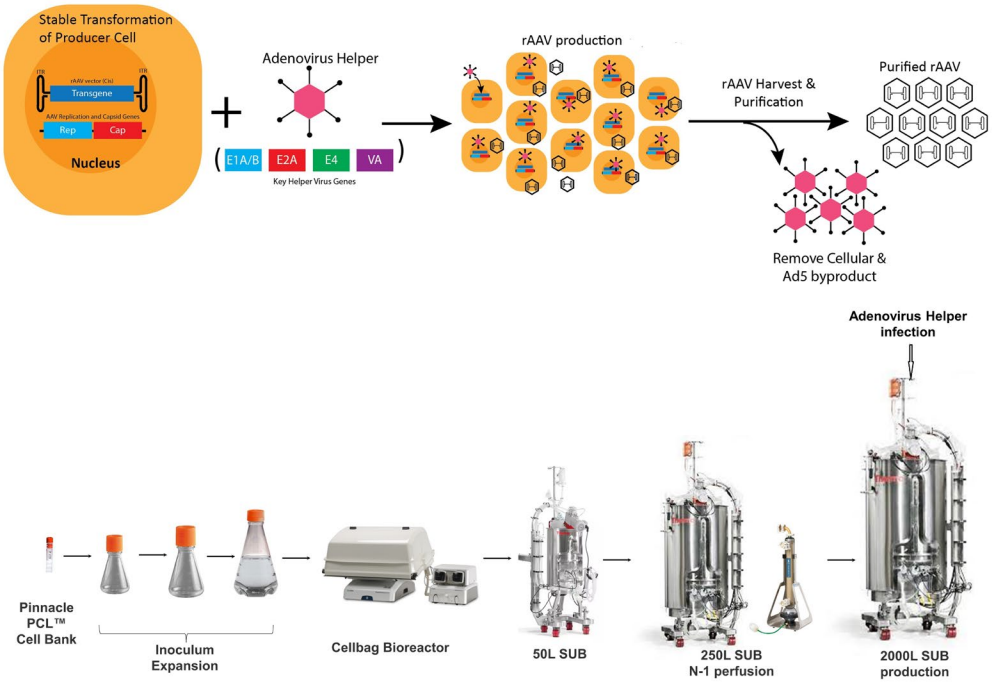

Figure 1. UX701 Pinnacle PCL™ platform for rAAV production at 2000L scale

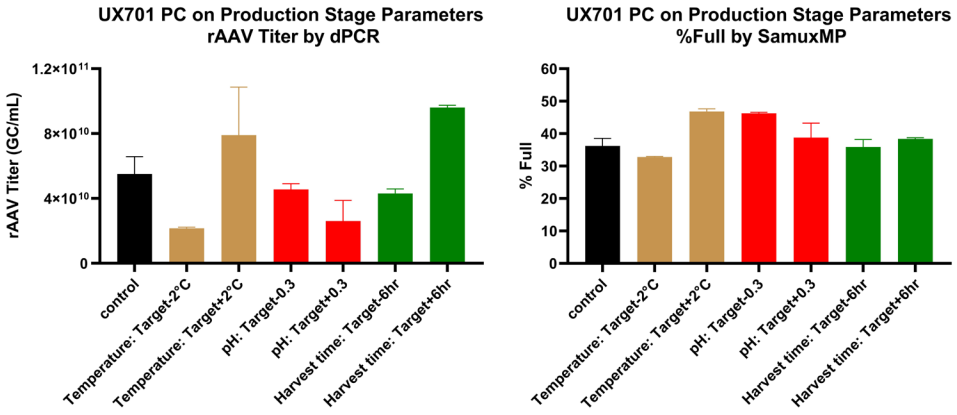

Figure 2. UX701 PC on some production stage parameters and their impacts on rAAV titer and % full capsids, bar plots by mean (SD)

378 Machine Learning-Enhanced Design of Experiments (DoE) for Optimizing AAV Plasmid Ratios in Gene Therapy Manufacturing

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Introduction:
Efficient adeno-associated virus (AAV) production is critical for the success of gene therapy programs. However, optimizing upstream processes remains challenging due to complex multivariate interactions, biological variability, and resource constraints. This study leverages a machine learning-augmented statistical approach, Self-Validating Ensemble Modeling (SVEM), to enhance traditional Design of Experiments (DoE) for plasmid ratio optimization. SVEM enables predictive modeling with limited experimental data, improving process efficiency and robustness.

Methods:
A space-filling DoE was designed to investigate the impact of varying molar fractions of pHelp, pRepCap, and pTransgene plasmids on genomic titer and empty-to-full capsid ratio in HEK293 cell-based AAV production. A total of 26 experimental conditions were tested in shake flask transfections at 30mL working volume. SVEM applied autovalidation, iterative model selection, and ensemble averaging to refine predictive accuracy and accommodate biological variability. Bayesian I-optimal design principles were incorporated to maximize model information content while minimizing experimental burden. SVEM's ability to handle supersaturated models allowed for the identification of key interactions that traditional DoE approaches might miss.

Results:
SVEM-based DoE modeling identified an optimal plasmid ratio that improved genomic titer by 42% while reducing empty capsid formation by 28% compared to baseline conditions. Traditional DoE approaches failed to capture nonlinear interactions that were resolved through SVEM's ensemble modeling, leading to more reliable predictions and faster optimization cycles. SVEM's capacity to extract insights from small datasets resulted in a 35% reduction in total experimental runs while achieving superior model robustness.

Conclusion:
SVEM enhances AAV upstream process optimization by enabling more accurate predictions from limited datasets, addressing key challenges in biomanufacturing scalability. This study demonstrates that machine learning-augmented DoE can significantly improve process development efficiency, reduce resource expenditure, and enhance reproducibility in gene therapy vector production. The findings provide a roadmap for integrating ML-driven analytics into CMC workflows, supporting accelerated clinical translation and commercial scalability of AAV gene therapies. As SVEM continues to evolve, its application in pharmaceutical and biotech industries

presents a promising approach for enhancing predictive modeling, reducing costs, and expediting development timelines.

379 Reduce ITR related Impurities in AAVone Single-plasmid System Using Secondary Structure DNAs

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Adeno-associated virus (AAV) vectors are widely used in gene therapy, but high production costs limit accessibility. Recently, we introduced the AAVone single-plasmid AAV production system, which consolidates all necessary cis- and trans-acting components into a single compact plasmid. This system streamlines AAV production and increases yields compared to traditional triple-plasmid co-transfection methods. The oversized plasmid backbone of AAVone results in fewer plasmid-related impurities in produced AAV vectors, particularly bacterial elements. However, it increases VA RNA-related impurities outside the right inverted terminal repeat (R-ITR). To address this challenge, we tested DNAs with different secondary structures to block potential ITR read-through or reverse packaging. While neither short repeat telomere sequences nor G-quadruplex DNAs reduced ITR-related impurities when placed immediately outside the R-ITR, short hairpin structure DNAs (shDNAs) significantly reduced these impurities in the AAVone produced AAV vectors. The VA-RNA related impurities were reduced from 2.5% to less than 1%. Notably, incorporating shDNAs did not significantly affect AAV production. This effect was sequence-independent but structure-related, suggesting a universal strategy for reducing backbone-related impurities in AAV vectors. Using safer shDNAs, such as lo2 shDNA derivatives, may also reduce immune responses to AAVone-produced AAV vectors. These findings validate the potential of shDNA-mediated strategies to further optimize the AAVone system, providing a safer and more cost-effective AAV production method for both preclinical and clinical applications.

On and Off-Target Method Development

380 Scalable assessment of genome editing off-targets associated with genetic variants with ABSOLVE-seq

Danilo Pellin[1], Jiecong Lin[2], My Anh Nguyen[3], Linda Lin[4], Jing Zeng[5], Archana Verma[6], Nola Neri[6], Lucas Ferreira da Silva[6], Adele Mucci[7], Kit Shaw[8], Kendell Clement[9], Scot Wolfe[10], Christian Brendel[11], Luca Pinello[12], Daniel Bauer[3]
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Genome editing with RNA-guided nucleases and nickases holds transformative potential for therapeutic applications but carries inherent risks of off-target modifications. Genetic variants may create off-target sites not found in the reference genome, but these can be difficult to identify with standard in silico, in vitro, and in cellulo nomination methods. Even after nomination, verifying these candidate variant-associated off-target sites is challenging due to the limited availability of primary cells harboring relevant genotypes. To address this challenge, we developed

ABSOLVE-seq (Assessment By Stand-in Off-target LentiViral Ensemble with sequencing), a scalable, high-throughput method for experimental verification of candidate off-target sequences that can be performed in primary cells. ABSOLVE-seq employs lentiviral delivery to integrate candidate sequences tagged with unique molecular identifiers (UMIs) into the genomes of living cells, followed by gene editing using clinically relevant protocols. This approach enables the multiplexed evaluation of dozens of candidate off-targets in a single experiment with high sensitivity and precision. Our analytical pipeline processes multiplexed sequencing data, classifies reads based on evidence of editing activity, aggregates data from multiple biological and technical replicates to estimate editing frequency by applying a penalized logistic regression model, and identifies significant editing activity using robust statistical tests based on permutation methods. We validated ABSOLVE-seq using CRISPR-Cas9 guides targeting the BCL11A erythroid enhancer employed in approved and experimental therapies for sickle cell disease and β-thalassemia. Experiments were conducted with wild-type Cas9 and high-fidelity Cas9 variants, targeting the +55 and +58 BCL11A erythroid enhancer regions. Four filtering strategies were compared, with an optimized approach reducing background indel rates to 0.002%. ABSOLVE-seq consistently identified both on-target and verified off-target sites. We observed a reduction in off-target activity with high-fidelity Cas9 compared to wild-type Cas9, as evidenced by the detection of significant off-target editing at a lower number of candidate sequences. We combined experimental data and Monte Carlo simulations to estimate statistical power at specific loci, ensuring reliable detection of rare off-target events and defining sequencing depth requirements. We demonstrated that ABSOLVE-seq maintains a high level of statistical power to detect off-target editing events as rare as 0.01% indel frequency, exceeding conventional detection thresholds. This high sensitivity enables the detection of low-frequency variant-associated off-target events. ABSOLVE-seq addresses key regulatory and scientific challenges in genome editing safety by providing a quantitative, scalable, and biologically relevant platform for preclinical off-target assessment of genetic variant-associated candidate off-target sites.

381 CHANGE-seq-BE Enables Simultaneously Sensitive and Unbiased *In vitro* Profiling of Base Editor Genome-Wide Activity

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Base editors (BEs) enable precise editing of individual DNA bases without requiring DNA double-strand breaks in the genome and holds a great promise to correct pathogenic single nucleotide variants in many genetic disorders. However, it is crucial to directly evaluate the safety and potential risks associated with BEs in clinical translation of genome-edited medicines. In this study, we introduce CHANGE-seq-BE, a novel, sensitive and unbiased biochemical approach designed to assess the genome-wide activity of base editors. This method selectively sequences genomic DNA modified by adenine or cytosine base editors (ABE or CBE) *in vitro* to identify candidate off-target sites across the genome.

To assess CHANGE-seq-BE performance, we used it to characterize ABE8e-NRCH base editor activity targeting sickle mutation (HBB^S) in the *HBB* gene to convert into non-pathogenic Makassar variant. After systematic optimization of CHANGE-seq-BE, we consistently detected all 54 validated off-targets that were previously reported by the nuclease-based CIRCLE-seq method. Moreover, CHANGE-seq-BE identified additional 29 (53% more) *bona fide* off-targets with cellular activity ranging from 0.55-13.9%. CHANGE-seq-BE also exhibited higher (2.1-fold) enrichment ratio at *bona fide* off-targets than CIRCLE-seq for HBB^S-gRNA. These results emphasize the importance of employing sensitive and unbiased approaches like CHANGE-seq-BE to directly define the genome wide off-target activity of base editors.

To evaluate the ABE8e (ABE) and eA3A-BE3 (CBE) off-target activity, we performed CHANGE-seq-BE for five therapeutically relevant loci (*B2M*, *CBLB*, *CD7*, *CIITA*, and *PDCD1*) in human primary T-cells. We selected 10,142

and 757 sites for ABE and CBE targets nominated by CHANGE-seq, CHANGE-seq-BE, and Digenome-seq and validated cellular off-target activity for 9,251 and 709 amenable sites by hybrid capture sequencing. Overall, 99 *bona fide* off-target sites nominated across all three methods exhibited significantly higher base editing activity above background. 36 ABE and 6 CBE off-target sites are exclusively identified by CHANGE-seq-BE, compared to Digenome-seq. This suggests that CHANGE-seq-BE is highly sensitive and capable of detecting *bona fide* off-targets while requiring approximately 20-fold less sequencing reads.

To understand the biological differences in genome-wide activity between Cas9 nuclease and base editors, we delivered Cas9 nuclease and ABE8e as mRNA for five therapeutically relevant ABE targets. We are currently examining *in vitro* off-target profiling of CHANGE-seq and CHANGE-seq-BE sites and corelating the differences with cellular activity validated by hybrid capture sequencing. Lastly, we applied CHANGE-seq-BE as an IND-enabling assay to support an emergency FDA-cleared IND study to treat a patient with X-linked Hyper IgM syndrome due to *CD40L* mutation. By CHANGE-seq-BE, we identified a total of 81 potential off-targets and confirmed 95.4% frequencies of on-target editing with no evident off-targets above the detection threshold of rhAmp-seq.

Overall, we present CHANGE-seq-BE as a highly sensitive and unbiased biochemical assay for characterizing genome-wide off-target activity of base editors. We envision CHANGE-seq-BE method may be used as a key assay to assess the specificity of therapeutic genome editors in biomedical research and clinical settings

382 Population-scale Cellular GUIDE-seq-2 and Biochemical CHANGE-seq-R Profiles Reveal Human Genetic Variation Frequently Affects Cas9 Off-target Activity

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Genome editing technology holds tremendous promise for treating genetic diseases, exemplified by the recent approval of a CRISPR-Cas9-based therapy for sickle cell disease in the US and Europe. However, their genome-wide activity can be altered by genetic variation at on- or off-target sites, potentially impacting both precision and therapeutic safety.

Here, we present the first population-scale study of how genetic variation affects the cellular genome-wide activity of CRISPR-Cas9, enabled by a novel, sensitive, and unbiased cellular assay, GUIDE-seq-2 with improved scalability and accuracy compared to the original broadly adopted method. We evaluated cellular on- and off-target sites associated with six gRNA targets from 95 donors across four genetically diverse populations. Our analyses identified 1,115 unique on- and off-target sites. Notably, cellular Cas9 activity detected by GUIDE-seq-2 frequently overlapped with human genetic variants. Within our study cohort, 16.6% of off-target sites (185 of 1,115) overlapped with one or more genetic variants, with individuals of ASW ancestry (African Ancestry in Southwest USA) having the highest frequencies of off-targets overlapping genetic variation (13%), and those of CHB ancestry (Han Chinese in Beijing) having the lowest (7.8%).

To validate the discovered off-target sites and identify a high-confidence set of variant effects on Cas9 editing activity, we conducted multiplexed targeted sequencing and integrated five variant effect measures based on GUIDE-seq-2 read counts, indel frequencies, tag integration frequencies, and allele-specific editing (indels or integrations). A high proportion of variants (12.6%) measured by both GUIDE-seq-2 and targeted sequencing significantly impacted off-target activity, of which 11 and 10 variants led to increased or decreased editing, respectively. Variant effects on indel percentage ranged from -25% to 32% and were corroborated by comparable changes in GUIDE-seq-2 read counts, tag-integration, and allele-specific editing frequencies. Notably, same variant

effects were confirmed in therapeutically relevant primary human T cells, with up to 3000-fold change in GUIDE-seq-2 read counts.

To understand common features of high-impact variants, we designed a massively parallel biochemical assay that measures Cas9 activity within highly diverse, partially randomized, off-target libraries which we call CHANGE-seq Randomized (CHANGE-seq-R). Each level of mismatch (up to 6) was covered by a million sequences. We further trained CHANGE-net, a deep learning model using CHANGE-seq-R data, achieving high accuracy (Spearman R=0.93) in predicting variant effects from our high-confidence set and outperforming other state-of-the-art methods. Finally, we applied CHANGE-net to predict variant effects from the 1000 Genomes Project, identifying 39 common variants that significantly impact Cas9 activity (predicted effects ranged from -64% to 4%). These findings underscore the potential of our massively parallel assay in modeling variant effects that are difficult to fully explore in cellular studies.

Our results illuminate the profound influence of genetic variation on genome editing outcomes, emphasizing the need for population-aware design in CRISPR-based therapies. Our integrated approach, combining GUIDE-seq-2, CHANGE-seq-R, and CHANGE-net, establishes a framework for rapidly assessing and mitigating variant-induced off-target genome editing risks.

383 Pooled GUIDE-seq-2 enables simultaneous assessment of cellular activity and specificity for thousands of Cas9 targets

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GUIDE-seq is a sensitive and unbiased cellular method to define the genome-wide activity of genome editors that has been broadly adopted for research and therapeutics. Although we have recently increased the scalability of GUIDE-seq with the development of a streamlined method called GUIDE-seq-2, it remains challenging to characterize sufficiently large numbers of targets to train accurate predictors of genome editing activity and specificity. This is because GUIDE-seq (and GUIDE-seq-2) are typically performed on a single target basis, limiting not only the scalability but also the comparison of relative activity. Thus, we sought to develop a version of GUIDE-seq-2 capable of simultaneously surveying activity and specificity across thousands of Cas9 gRNA targets. First, we designed a list of 500 and 4501 gRNA sequences targeting the human genome with high enough edit distance to be easily distinguishable from each other. Next, we transduced human primary T-cells with the gRNA libraries encoded in lentivirus, delivered purified Cas9 protein together with a short double-stranded oligodeoxynucleotide (dsODN) tag, and performed GUIDE-seq-2 on the pooled edited T-cells. All gRNAs were detected to have activity from the smaller pool of 500, and 99.8% (4,491 out of 4,501) was detected from the 4501 pool. Replicate experiments showed that the pooled GUIDE-seq-2 results were highly reproducible (Pearson's r = 0.95). A key advantage of this new method is that both activity and specificity can be compared and ranked across thousands of guides simultaneously. For example, 48 out of 500 (9.6%) guides were highly specific to the intended target, where only the on-target edit was detected. To determine whether pooled GUIDE-seq-2 results were comparable to individual GUIDE-seq-2, we selected a set of 20 targets for direct comparison. We found that activity and specificity were strongly correlated between pooled and individual GUIDE-seq-2 experiments (Pearson's r = 0.91, 0.89 and Spearman's R = 0.94, 0.94, respectively). Interestingly, sequence feature analysis from 500 and 4501 pools both showed high tolerability of G to A in the 11th position of the protospacer. To allow analysis of targets that do not start with a 5'G as required by standard U6 RNA Pol III promoters for gRNA transcription, we are currently collecting data from a tRNA-encoded guide library that enables targets to start with any base. This will increase the diversity of the first nucleotide of gRNAs to train deep learning models for accurate prediction of guide activity and specificity.

In summary, we have established pooled GUIDE-seq-2, a sensitive, unbiased cellular method to simultaneously evaluate the genome-wide activity of thousands of guides. Pooled GUIDE-seq-2 will support rapid selection of the

best gRNA sequences for genome editing therapies, build databases for selective guides, and may improve predictors of highly active and specific gRNA targets.

384 Single-Cell Profiling of Genome-Editing Alterations and Functional Outcomes in CRISPR-Engineered Cells

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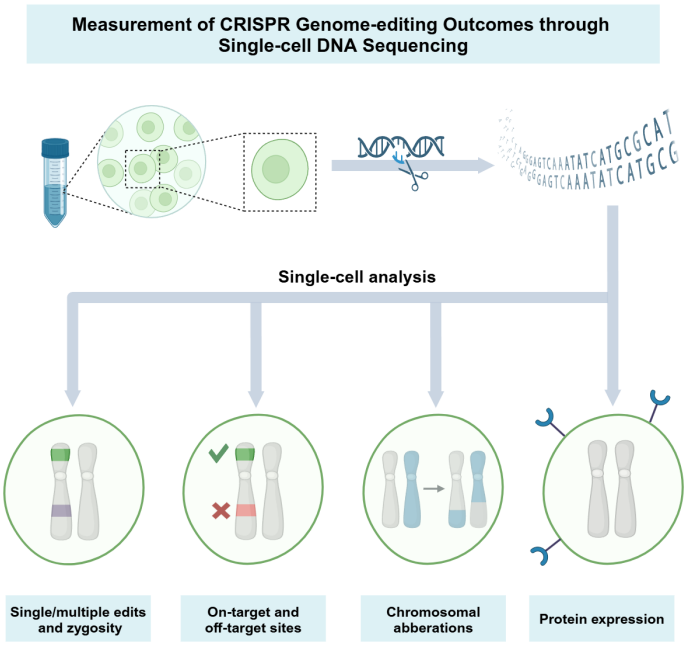
CRISPR-Cas genome-editing technology has become a cornerstone in advancing innovative clinical therapies for treating a wide range of diseases. However, the technology encounters a bottleneck in the form of off-target activity (OTA), the unintended cleavage by the Cas nuclease at non-target genomic loci. OTA introduces insertions and deletions (inDels) and structural variations (SVs), potentially driving oncogenic mutations. While many studies have characterized OTA at a cell-population level using bulk-sequencing-based methods, these approaches are limited as they fail to examine OTA at the single-cell level. Furthermore, current approaches fail to capture the functional consequences of specific genotypes in individual cells, leaving a critical gap in our understanding of the interplay between genomic modifications and functional outcomes.

To address this gap, we leveraged the Tapestri platform, a cutting-edge single-cell DNA sequencing (scDNA-seq) technology, integrated with multi-omics profiling. This approach enables precise characterization of the distribution of genotypes and phenotypes in CRISPR-engineered cells (Fig. 1). As a proof-of-concept, we engineered human primary T-cells using a multiplex of three guide RNAs (gRNAs) adapted from the first in-human CRISPR trial, designed to knock out the *PDCD1*, *TRAC*, and *TRBC* anti-cancer targets. We then created a panel of 111 putative off-target sites, amplified and sequenced these sites using the Tapestri platform, generating high-resolution data from thousands of cells per donor. Cells from the same donors were also analyzed using the rhAmpSeq targeted bulk sequencing assay. We then evaluated the inDel profile at each allele and quantified the CRISPR editing. While population-level editing levels were similar between single-cell and bulk-sequencing, single-cell analysis enabled a more detailed understanding of zygosity and the co-occurrence of specific editing outcomes. Single-cell analysis revealed a heterogeneous spectrum of CRISPR outcomes, with each cell exhibiting a unique on-target and off-target profile, highlighting the importance of thoroughly characterizing genome-engineered cell products. We also quantified translocations between on-target and off-target loci, including novel translocations previously unreported for these gRNA combinations.

Finally, to evaluate how different types of editing outcomes influence the desired phenotype, we conducted a longitudinal study on primary cells edited with the same gRNAs, sampling them over a 12-day period. Our analysis revealed a notable increase in the fraction of inDels within the population, peaking at 3 days post-editing and stabilizing thereafter through day 12. Additionally, we observed consistent downregulation of the TCRa/b and CD3 surface proteins in cells harboring bi-allelic inDels at the TCRA and TCRB on-target sites, highlighting the power of this approach for detailed characterization of genome-engineered cells.

In summary, scDNA-seq, combined with in-depth proteomic characterization, provides a robust framework for evaluating genome-engineered cells, enabling precise measurement of editing activity, detailed profiling of outcomes, and direct links between genotypes and phenotypes. This high-resolution methodology advances our understanding of CRISPR outcomes and offers a powerful platform for quality control, with the potential to enhance therapeutic applications of CRISPR-Cas genome-editing.



385 Development of a Comprehensive Framework for Assessing the Genomic Safety Profile of RNA Gene Writer Targeting Alpha-1 Antitrypsin Deficiency

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RNA Gene Writers are unique and engineerable constructs that leverage all-RNA components and harness the mechanism of target-primed reverse transcription, or TPRT, to introduce a broad range of edits to the genome, from inserting a transgene or exon to introducing single nucleotide changes. RNA Gene Writers are designed to provide a versatile toolkit for the treatment of a wide variety of genetic diseases. Here we focus on the genomic safety assessment of an RNA Gene Writer developed to correct one of the mutations leading to alpha-1 antitrypsin deficiency (AATD). Mutations in the SERPINA1 gene (Serine Protease Inhibitor, group A, member 1) lead to AATD, an inherited disorder linked to an increased risk of liver and lung disease. One of the most clinically relevant variants is the PiZ mutation, which results in a substitution of lysine for glutamic acid at amino acid position 366 (E366K). Individuals with two copies of the PiZ allele (PiZZ genotype) develop a liver phenotype due to the accumulation of toxic misfolded protein aggregates in the endoplasmic reticulum of liver cells, while lower levels of circulating AAT contribute to lung disease by impairing the inhibition of neutrophil elastase. To address this, we developed an RNA Gene Writer aimed at correcting the E366K mutation in the SERPINA1 gene, restoring it to its wild-type form. We developed an RNA Gene Writer that achieved an estimated average of 89% correction of the *SERPINA1* E366K mutation in hepatocytes of a mouse model of disease, using a single low dose of 0.25 mpk. The mouse model is a humanized mouse model with only two PiZ alleles to better approximate the efficacy of our product in patients with the homozygous PiZZ genotype. In addition, in non-human primates, a surrogate RNA Gene Writer achieved an estimated 79% editing of the SERPINA1 gene in hepatocytes of cynomolgous monkeys. One of the keys to supporting the advancement of any new genome editing technology through preclinical development is the building of a comprehensive data package assessing genomic safety. Here we present our assessment of the genomic safety profile of the RNA Gene Writer that includes a comprehensive and unbiased off-target discovery using in-silico, in-vitro and in-cell methods. We assessed nominated putative off-target sites using single and

multiplexed amplicon sequencing and our initial data showed that the RNA Gene Writer did not show any detectable off-target activity across any of the putative off-target sites enumerated during discovery. In addition, we evaluated the biodistribution of the RNA Gene Writer in the mouse model of disease and did not observe any unintended editing at putative off-target sites in any of the 31 tissues/ biofluids examined. By using this comprehensive array of orthogonal assessments of genotoxicity we characterized the genomic safety profile of the RNA Gene Writer that supports its potency and safety.

475 Joint Single-Cell Profiling of CRISPR-Cas9 Edits and Transcriptomes Reveals Widespread Off-Target Events and Affected Gene Expression

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Off-target genotoxicity is a pressing challenge in therapeutic genome engineering. A longstanding barrier has been an inability to measure the consequences of off-target events on cell function. Here we present Superb-seq, a new technology that leverages T7 in situ transcription and single-cell RNA sequencing to jointly detect genome breaks and downstream effects on gene expression. We performed Superb-seq on CRISPR-Cas9 edits in 10,000 K562 cells, targeting four chromatin remodeler genes with seven guide RNAs. Superb-seq identified 11,891 edit events in 6,230 edited cells at all seven on-target sites and an additional 36 off-target sites. Although all guides were selected for high specificity, 6/7 guides caused off-target edits, ranging in frequency from 0.03% to 18.6% of total cells. Furthermore, Superb-seq identified hundreds of differentially expressed genes associated with specific off-target events. A notable off-target within the first intron of *USP9X* disrupted the expression of this gene and over 150 downstream genes. In summary, Cas9 off-targeting is a pervasive combination of rare and common edit events, frequently damages introns of off-target genes, and can exert widespread effects on gene expression. Superb-seq uses off-the-shelf kits and standard equipment, which will enable scalable screening of preclinical candidates with reduced off-target gene perturbation

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Pharmacology/Toxicology Studies and Analytics/Assay Development Session II

386 Preclinical Models for Oncolytic Adenoviruses: Evaluating Replication and Blood Cell Interactions

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Oncolytic adenoviruses (Ads) are promising cancer therapeutics, capable of selective elimination of tumor cells while sparing healthy tissue. Beyond direct oncolysis, Ads also activate antitumor immune responses by modulating the immunosuppressive tumor microenvironment. However, systemic delivery of OAd, which is necessary for treating metastatic disease, remains a significant challenge in the field owing to the lack of animal models that support adenovirus replication (mice do not support replication of human adenoviruses). Another key challenge is understanding how adenoviral particles interact with blood components during systemic delivery, particularly red blood cells (RBCs), which can significantly impact viral half-life in circulation, biodistribution, and systemic side effects. Our recent *in vitro* and *in vivo* findings demonstrated that porcine cells and tissues effectively support the replication of both Ad5 (coxsackie and adenovirus receptor (CAR)-targeted) and Ad5/3 (retargeted to CD46 and human desmoglein 2 receptors) Ads. However, our previous work was limited by the absence of cancerous porcine tissues. To expand this, we have obtained porcine pancreatic ductal epithelial cells transformed with $KRAS^{G12D}$ and

assessed viral binding, transduction, and replication efficiency of multiple Ad types (Ad3, Ad5, Ad6, Ad11, and Ad26) as well as Ad5/3 and integrin-targeting Ad5RGD vectors, using normal and cancer human cell lines as a control. The findings suggest that porcine cells support Ad5 and Ad6 replication at levels comparable to human cells. The Ad5/3 and Ad5RGD vectors also displayed strong oncolysis and replication in porcine cells. Intriguingly, while species B Ad3 and Ad11 Ads induced cytopathic effect in porcine cells, their replication was significantly lower than in human cells, likely due to differences in viral replication machinery. Species D Ad26 showed no significant replication in either porcine or human cells. To explore systemic delivery challenges, we investigated the interaction of Ads with blood factors X (FX), a pivotal element influencing adenoviral transduction, across different mammalian species. Our results suggest that human, porcine, canine and murine FXs significantly improve the transduction of Ad5, Ad6, and Ad5/3 in CAR-, hDSG2-, and CD46-deficient CHO cells, matching human FX levels. In contrast, Ad11 transduction was enhanced only by human and porcine FXs, underscoring species-specific differences in FX-mediated transduction. Additionally, we examined hemagglutination patterns of Ad vectors with RBCs from various species (human, pig, dog, mouse, hamster, cynomolgus, rhesus macaque, and baboon). Our current results indicate that unlike human RBCs, which hemagglutinate with Ad5, Ad6, Ad26, and Ad5RGD, RBCs from pigs, dogs, mice, and hamsters did not hemagglutinate with any tested adenovirus. Interestingly, non-human primate RBCs hemagglutinated only with Ad11, highlighting species-specific viral interactions that may impact systemic vector delivery. This study enhances our understanding of preclinical models for adenoviral vector evaluation, providing valuable insights into systemic delivery mechanisms, species-specific interactions, and oncolytic efficacy, ultimately optimizing the development of more effective Ad therapies.

387 Safety and Biodistribution Assessment of Novel AAV Capsid Developed for the Treatment of Myotonic Dystrophy Type 1 (DM1)

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Recombinant adeno-associated virus (rAAV) vectors have emerged as a promising tool for gene therapy. However, high dose systemic administration of rAAV vectors pose a potential limitation for safe and effective gene therapy, particularly for dose levels >1×10^{14} viral genome per kilogram of body weight (vg/kg). Here, we tested a novel delivery platform comprising a new AAV capsid (AAV.SAN011) with high muscle transduction efficiency and a promoter with strong activity in skeletal muscles to deliver and express an artificial microRNA targeting DMPK for DM1 treatment. AAV.SAN011 was administered intravenously at 3 dose levels in cynomolgus macaques for 13-week observation period following vector administration. Pre-screened animals seronegative for AAV.SAN011 neutralizing antibodies were divided with 6 animals per group receiving vehicle formulation buffer or AAV.SAN011. Blood was assessed at different time-points to evaluate hematology, clinical chemistry, and coagulation. Minimal to mild acute and transient elevation in liver enzymes (ALT, AST) and coagulation parameters (PT, APTT) were observed in individual animals at all dosed groups that reversed to baseline following a 3-month observation period. Vector genome and artificial microRNA levels in target skeletal and cardiac muscles were dose dependent. Remarkably, no findings were observed on microscopic evaluation of liver, dorsal root ganglion, heart, skeletal muscles, and an extensive list of peripheral organs. No clinical observations or changes were reported in body weights, body temperatures, heart rates, blood pressures, respiratory rates, neurological clinical assessments, cardiac biomarkers, ophthalmology, quantitative or qualitative electrocardiology. AAV.SAN011 expressing an artificial microRNA against DMPK was well tolerated up to the highest dose level and demonstrated robust biodistribution and expression of a new gene therapy for the treatment of DM1.

388 Critical Parameters for AAV Vector Genome Titer Determination: a Multi-Laboratory Collaborative Study

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Critical Parameters for AAV Vector Genome Titer Determination: a Multi-Laboratory Collaborative Study

Adeno-associated virus (AAV) vector genome titer is a critical quality attribute that is assessed at release to ensure consistent dosing and product safety. The primary methods used are quantitative PCR (qPCR), digital PCR (dPCR), and digital droplet PCR (ddPCR), which should target a sequence in the transgene. A risk-based approach should be used to select the method, but method comparisons are difficult due to lab-to-lab differences in implementation and the absence of well-characterized reference materials. We address this challenge with a multi-platform and multi-laboratory collaborative study using a common reference material, preparation protocol, primers, and probes. To develop a suitable protocol appropriate for a collaborative study, we reviewed technical notes from instrument providers and peer-reviewed articles from product developers to determine critical method parameters. For the broadest applicability, we screened reported and novel primers and probes for the CMV promoter, SV40 poly(A) terminator, CBA promoter, ITR2 inverted terminal repeats, and GFP target sequences. We then developed a precise, accurate, specific, and robust sample preparation protocol. The well-characterized analytical reference material is based on a vector genome with a GFP reporter and capsid of the AAV5 serotype. The reference material is formulated at 5x10 10 vector genomes/mL to reduce the need for serial dilution that could compromise accuracy. This concentration is appropriate for method development, system suitability, calibration, and assay control. To ensure that an accurate genome titer value can be assigned to the reference material, we performed a collaborative study using multiple analytical platforms and independent laboratories across the world. The diverse collaborators are aligned using the common reference material, protocol, primers, and probes. We demonstrate the criticality of conditions for DNase I treatment, capsid lysis, dilution buffer composition, and MspI-mediated ITR2 digestion. Additionally, we highlight the importance of PCR target, primer design, and probe design. When conditions are aligned, the reference material's titer values are consistent across laboratories and platforms.

389 Preclinical Development of an AAV-delivered Zinc Finger Transcriptional Repressor Targeting the Prion Gene as a Novel Epigenetic Gene Therapy for Prion Disease

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Prion disease is a rapidly progressing and fatal neurodegenerative disorder that occurs when the normal cellular prion protein (PrP^{C}) in the brain misfolds to a protease resistant and pathogenic form referred to as Scrapie PrP (PrP^{SC}). Given the high unmet need and lack of effective treatment, there is an urgency to develop novel therapeutics for treatment of this disease. Several lines of evidence suggest that neuronal PrP^{SC} is required for neurotoxicity and disease progression and that overall reduction of brain PrP^{C} can protect against prion disease. To address this unmet need, we are developing a therapy using STAC-BBB – our novel blood-brain barrier (BBB)-crossing adeno-associated virus (AAV) – to intravenously (IV) deliver a highly specific engineered zinc finger repressor (ZFR) targeting the human prion gene (*PRNP*). This potential treatment is expected to achieve a widespread, rapid, and sustained reduction of brain PrP^{C}, thereby reducing the misfolded protein PrP^{SC}.

To evaluate the feasibility of this approach, an AAV-delivered ZFR (AAV-ZFR) specifically targeting the mouse prion gene (*Prnp*) was designed and assessed in mice inoculated with Rocky Mountain Laboratory prions (RML), a gold standard model of prion disease. In a 500-day study, a single dose of AAV-ZFR was delivered IV -21 (pre-inoculation), 63 (onset of disease molecular changes) or 119 (post-symptomatically) days post inoculation (dpi) at doses of 0 (vehicle), 1E+13, 3E+13 and 1E+14 vg/kg body weight. Treatment with AAV-ZFR resulted in significant extension of survival ranging from a median of 251 days to 494 days compared with the inoculated vehicle control (median of 170 days). Importantly, our data show significant extension of median survival for post-symptomatically treated RML-inoculated mice with several animals in mid- and high-dose groups surviving to the

end of study. Notably, brain slices fixed and stained for PrP revealed that AAV-ZFR treatment led to reduction of prion protein, and a dose-dependent reduction in cerebrospinal fluid (~50-80% reduction) in RML-inoculated mice. Other benefits of AAV-ZFR treatment included increase in, or maintenance of body weight and dose-dependent improvement in nesting behavior compared with the inoculated control group, supporting the premise that reduction in prion protein leads to improvement in survival and function. Furthermore, AAV-ZFR treatment was well tolerated in healthy uninoculated mice with no abnormal histopathological findings or abnormal changes in neuroinflammatory or neuronal cell markers at doses administered, demonstrating safety of AAV-ZFR. Importantly, delivery of a ZFR targeting the human/nonhuman primate prion gene using STAC-BBB was well tolerated in cynomolgus monkeys at a dose level that has been shown to be pharmacologically active. Taken together, these results support further development of our novel STAC-BBB-ZFR vector for potential treatment of prion disease.

390 Hydrophilic Interaction Chromatography for Direct Online Disruption of Lipid Nanoparticles, Intact mRNA Analysis, and Measures of Encapsulation Efficiency

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Messenger RNA (mRNA) based vaccines and therapies utilize lipid nanoparticles (LNPs) for efficient cellular delivery. Analysis of the mRNA content within a formulated LNP is an important part of drug product testing. The payloads must be assessed for integrity, concentration, and purity to ensure effectiveness and safety of this modality.

To date, LNP mRNA samples are mostly analyzed using surfactants for LNP disruption combined with capillary gel electrophoresis or reversed phase liquid chromatography. This approach requires offline sample preparation and can sometimes exhibit problematic repeatability and reproducibility. With this work, we have developed a hydrophilic interaction chromatography (HILIC) method that allows for the direct injection and on-column disruption of LNPs. This novel approach utilizes an initial mobile phase containing 60-80% acetonitrile at an elevated column temperature with a simple binary gradient and aqueous ammonium salt eluent. This technique rapidly disrupts lipid components which are not retained under HILIC conditions, and facilitates the subsequent elution, integrity checks, and quantitation of mRNA. Use of an amide-bonded ethylene-bridged hybrid stationary phase column constructed from vapor deposited hardware has facilitated high recovery measurements and straightforward implementation of the method.

On the other hand, we explore the potential of the method for measurement of unencapsulated mRNA under milder conditions, preserving the structure of LNPs and retaining only the free nucleic acid. Upon its elution and quantitation, the combination of both approaches can yield encapsulation efficiency in a high-throughput and straightforward assay.

In sum, this HILIC method simplifies the sample preparation and analytical testing protocols needed to access critical information for mRNA/LNP drugs, which makes it an especially promising new assay to consider for process development and release testing of mRNA-based treatments and vaccines.

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391 Intrastriatal AAV5.SGN.LEGB (LETI-101) administration selectively targeting mutant allele of the HTT gene resulted in broad CNS distribution and transgene expression in critical NHP brain regions associated with Huntington's disease pathology

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expanded cytosine-adenine-guanine trinucleotide repeat in the huntingtin (HTT) gene. The resultant polyglutamine-containing mutant HTT protein (mHTT) disrupts wild-type HTT function causing neuronal stress and malfunction. Because the wild-type protein supports critical neural and cellular functions, a selective strategy targeting only the disease associated allele and protein is fervently sought.

Life Edit Therapeutics, an ElevateBio company, is developing a gene editing therapeutic that selectively targets the mutant HTT allele while leaving wildtype allele intact. This approach utilizes an engineered all-in-one AAV5 expressing Life Edit's proprietary CRISPR nuclease (LEGB) and single guide RNA (SGN), termed AAV5.SGN.LEGB (LETI-101) to selectively target the mutant allele based on the presence of the PAM forming single nucleotide polymorphism (SNP) rs362331 in exon 50. Allele selective editing and associated reduction of the mHTT protein was previously demonstrated in HD patient-derived fibroblasts and clinically relevant BACHD murine model following intrastriatal injections with LETI-101.

NHPs were administered either Vehicle, 2.20E12, or 1.13E13 vg LETI-101 bilaterally into the striatum using MRI guided convection enhanced delivery using the neuroinfuse™ (Renishaw, UK) drug delivery system. Blood, CSF, saliva, and urine were collected for biodistribution with blood and CSF collected for immunogenicity assessment (ElISpot). At one-month, NHPs were euthanized, and fluids and tissues harvested for biodistribution, and/or transgene expression evaluation. Vector DNA, LEGB mRNA, SGN RNA and LEGB protein were assayed by quantitative polymerase chain reaction (qPCR), reverse transcription-qPCR (RT-qPCR), and electrochemiluminescence (ECL)-enzyme-linked immunosorbent assay (ELISA), respectively.

LETI-101 administration was well tolerated with no Test Article-related clinical signs, neurological, changes in body temperature, heart rate, or respiratory findings. There was no induction of total antibody or anti-LEGB antibody in serum or CSF following dose administration. Biodistribution evaluation demonstrated considerable dose-dependent vector and transgene expression observed in the striatum (putamen and caudate) and within the cortex, presumably via retrograde transport, and in brain regions that are particularly vulnerable in HD including the thalamus, hippocampus, and globus pallidus. Limited systemic distribution occurred in spleen, liver, and draining lymph node, with nothing detected in the gonads. These results support the translation of LETI-101 to clinical trials for selective lowering of mHTT as a potential treatment for Huntington's disease.

392 Evaluation of Quality Control Methods for Circular RNA Purity and Integrity: SEC-HPLC, CE, and RP-HPLC

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Background and Aims:
Circular RNA (circRNA) has gained significant attention in biomedical research and therapeutic development due to its unique covalently closed structure, which enhances stability and resistance to enzymatic degradation compared to linear RNA. These properties make circRNA a promising candidate for vaccines, gene therapies, and diagnostic biomarkers. However, the production of circRNA—commonly achieved through enzymatic or chemical circularization of linear precursors—poses challenges in ensuring product quality. Residual linear RNA, process-related impurities (e.g., ligases and chemical byproducts), and structural degradation (e.g., nicks and breaks) can compromise its functionality and safety. Rigorous quality control (QC) is therefore crucial to validate circRNA batches for clinical and industrial applications. Chromatographic and electrophoretic techniques, including size-exclusion chromatography HPLC (SEC-HPLC), capillary electrophoresis (CE), and reverse-phase HPLC (RP-HPLC), offer high precision but vary in their analytical strengths and limitations. This study systematically evaluates these three methods to establish a robust QC framework tailored for circRNA characterization.

Methodology:
Batches of synthesized circRNA with varying degrees of purity and integrity were prepared to simulate real-world samples. Each sample was analyzed using SEC-HPLC, CE, and RP-HPLC. In SEC-HPLC, samples were injected into a size-exclusion column, and elution profiles were recorded to evaluate size distribution and purity. For CE, samples were run in a capillary with a background electrolyte optimized for RNA separation, generating electropherograms that were analyzed to identify contaminants and degradation products. In RP-HPLC, samples were analyzed on a C18 column, and chromatograms were examined to detect impurities and degradation products. The results from each analytic method were compared based on their sensitivity to contaminants and degradation products, as well as their resolution for circRNA.

Results and conclusion:
SEC-HPLC effectively separated circRNA from small contaminants and NTPs based on size distribution but showed limited resolution for distinguishing between linear RNA and circRNA of similar sizes. CE demonstrated high resolution and sensitivity in detecting small contaminants and degradation products in circRNA samples. The electropherograms successfully resolved circRNA from linear precursor RNA and other impurities but struggled to distinguish nicked linear RNA, providing a more detailed purity profile. RP-HPLC offered complementary insights by detecting hydrophobic impurities and degradation products, revealing the presence of nicked linear RNA. Therefore, due to the strengths and limitations of various analytical methods and the complexity of contaminants and impurities in circRNA, a combination of SEC-HPLC, CE, and RP-HPLC is necessary to fully characterize circRNA products.

Targeted Gene and Cell Therapy for Cancer

393 Engineered Fas88 Receptor Provides Inducible Cytokine Signaling to Enhance Functional Persistence of CAR-Vδ2 T Cells in Xenograft Models of Leukemia and Solid Tumor

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Vδ2 T cells (Vγ9Vδ2 T cells) are a promising off-the-shelf (OTS) cell platform for CAR-mediated tumor targeting. However, short in vivo persistence of Vδ2 T cells due to the lack of cytokine support limit their therapeutic application. To overcome this, we first integrated major cytokines previously shown to promote Vδ2 T cell proliferation, namely IL-12, IL-15 and IL-18, in CAR-Vδ2 T cells. To limit cytokine diffusion and the associated toxicity, we generated bicistronic vectors coding membrane-bound (mb) cytokines with a 4-1BB-costimulated CD19.CAR (19BBz). While co-expression of mbIL-15 compromised CAR expression and mbIL-12 impeded their ex vivo expansion, mbIL-18 did not negatively impact the manufacture of CAR-Vδ2 T cells. Moreover, mbIL-18/19BBz Vδ2 T cells effectively eliminated CD19+NALM6 tumor in vitro and promoted T cell expansion (Day 6 coculture- T-cell fold change: 19BBz-0.6±0.1, mbIL-18/19BBz-3.3±0.5, $p<0.0001$). In a NALM6 xenograft mice model, mbIL-18/19BBz Vδ2 T cells outperformed the other combinations, prolonging animal survival without exogenous cytokine supplementation (median survival: control – 21 days, 19BBz – 31days, mbIL-12/19BBz – 41 days, mbIL-18/19BBz - > 70 days). Superior T cell proliferation and persistence was demonstrated by a dual-luciferase imaging system to simultaneously track tumor growth and T cell expansion.

While mbIL-18 dramatically enhanced CAR-Vδ2 T cell function, cytokine trans-presentation to the host cells poses an accelerated risk of allogeneic rejection of OTS therapeutic CAR-Vδ2 T cells. Therefore, we engineered inducible cell-intrinsic IL-18 signaling by generating a chimeric Fas88 receptor comprising of a Fas ectodomain linked to a MyD88 endodomain, a key adaptor molecule downstream of IL-18R. Fas88 leverages CAR/TCR activation-dependent surface expression of FasL in CAR-Vδ2 T cells that otherwise leads to unwanted activation-induced cell death (AICD). Upon CAR signaling, upregulated FasL engages Fas88, defusing AICD as measured by Annexin

V/7-AAD staining, and instead transmits IL-18 signaling leading to phosphorylation of NF-kB, IKKα/β and MAPK in Vδ2 T cells, on par with constitutive IL-18 signaling.

We evaluated the functional effect of Fas88 engineered CAR-Vδ2 T cells in three separate xenograft models of hematologic and solid cancers: B-cell leukemia (CD19.CAR vs NALM6), T-cell leukemia (CD5.CAR vs CCRF-CEM), and pancreatic cancer (PSCA.CAR vs CFPAC-1). In the NALM6 model, Fas88 enhanced the proliferation and persistence of CD19.CAR Vδ2 T cells, boosting tumor clearance on par with mbIL18-expressing CD19.CAR Vδ2 T cells. Similarly, the anti-tumor effect of CD5.CAR-BBz Vδ2 T cells was significantly improved by co-expressing Fas88 while unarmed CD5.CAR-BBz Vδ2 T cells failed to control tumor growth in an aggressive CD5+ CCRF-CEM T-ALL model (median survival: control – 18.5 days, CD5.CAR – 18 days, CD5.CAR/Fas88 – 56 days). Finally, in a subcutaneous CFPAC-1 xenograft model, Fas88-armed PSCA.CAR-BBz Vδ2 T cells expanded at the tumor site and controlled its growth, significantly prolonging animal survival (median survival: control – 32 days, PSCA.CAR – 38 days, PSCA.CAR/Fas88– 71 days). All experiments were performed with a single infusion of freshly thawed CAR T cells in the absence of exogenous cytokines to model conditions similar to CAR-αβT cells. Overall, Fas88 improves the functional persistence and in vivo anti-tumor effect of CAR-Vδ2 T cell across diverse tumor models.

394 STX-003: A mRNA Cancer Immunotherapy Utilizing Cancer-Selective Programmable Genetic Circuits for Systemic Tumor Control

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Cancer is responsible for roughly one in six deaths worldwide, with solid tumors accounting for approximately 90% of adult cancers. Immunotherapies have shown substantial promise for treating solid tumors, but achieving efficacy while avoiding toxicity is a key concern. Systemic administration of the immunomodulatory cytokine interleukin-12 (IL-12) has shown impressive efficacy in preclinical solid tumor models but is poorly tolerated in humans, likely due to off-target effects. To overcome this problem, we are developing STX-003, a self-replicating mRNA-LNP therapeutic that leverages the power of programmable miRNA-sensing genetic circuits to selectively express IL-12 in tumors while suppressing its expression in healthy tissues. Here, we present preclinical data demonstrating the effects of programmable genetic circuitry on our drug following intravenous injection. In mouse models, candidate genetic circuits downregulated the self-replicating mRNA in healthy organs such as spleen and heart, which was correlated with a reduction in tissue histopathological findings. Furthermore, candidate circuits dramatically reduced circulating levels of IL-12 induced by the drug in healthy non-human primates (NHPs). Additionally, we evaluated two candidate circuits in the B16.F10 syngeneic melanoma mouse model to assess the effects of these circuits on tolerability and anti-tumor efficacy over a range of drug doses. Both candidates were tolerated at all doses evaluated and exhibited anti-tumor activity. One of these candidates robustly controlled tumors in a dose-dependent manner, achieving an overall response rate of 100% at the highest dose, and this circuit was selected for our STX-003 development candidate. These studies are the first to demonstrate fully functional programmable genetic circuits with self-replicating mRNA in NHPs, the first to show the amelioration of drug-induced tissue histopathology via genetic circuitry in an animal model, and the first to provide evidence that genetic circuitry can improve the therapeutic indices of potent but poorly tolerated drugs. Our results highlight the potential of STX-003 for treating solid tumors and the promise of programmable genetic circuits for enabling targeted expression of genetic medicines and for surmounting the problem of previously “undruggable” targets through controlled expression.

395 Allele-Specific CRISPR/AAV6 Gene Correction of Dominant *JAK2*-V617F Mutation in Polycythemia Vera

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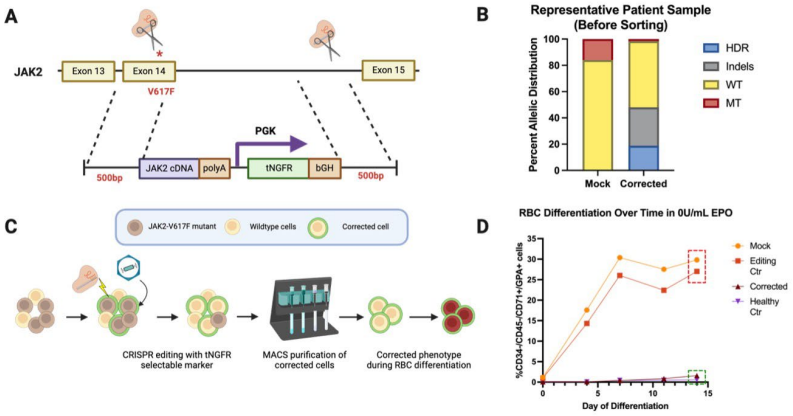
The *JAK2*-V617F gain-of-function mutation is a primary molecular driver of several myeloproliferative neoplasms, most notably occurring in ~95% of Polycythemia Vera (PV) cases. In PV, this mutation induces constitutive activation of JAK/STAT signaling, disrupting physiological erythropoietin-dependent regulation resulting in pathological erythrocytosis. There exists a need to develop more curative therapies as patients with PV are at risk of progressing to myelofibrosis or acute myeloid leukemia and current therapies only provide hematology and symptomatic benefit with variable effects on *JAK2*-V617F allele burden. As the *JAK2*-V617F mutation is autosomal dominant with high propensity for clonal expansion, curative treatment requires complete eradication of all mutant cells. To address these therapeutic limitations, we developed a dual-gRNA genome editing strategy combining CRISPR-Cas9 RNP with AAV6 to target the *JAK2*-V617F mutation in hematopoietic stem cells (HSCs). Our dual-gRNA system achieves high-efficiency mutation deactivation while maintaining allelic specificity. The first guide, targeting the V617F mutation site, achieves 51±7.9% indel formation, while a second guide directed at an intronic region ~100 base pairs downstream synergistically enhances allelic V617F deactivation to 97%. Notably, the mutation-targeting guide exhibits no activity against wild-type alleles. To enable *JAK2*-V617F correction, we engineered a homology directed repair (HDR) AAV6 vector incorporating codon-optimized downstream exons and a PGK promoter-driven tNGFR for selective enrichment (**Fig. 1A**). Electroporation of HSCs with this system yields 40.0±5.05% edited cells, with MACS enrichment achieving 97.2±1.6% edited populations. We evaluated our editing approach in HSCs isolated from PV patient bone marrow aspirates (IRB-approved) in a 14-day erythroid differentiation culture. This model replicated key PV pathophysiology, including accelerated erythroid differentiation, enhanced proliferation, and elevated mutant allele frequency. Patient-derived HSCs demonstrated spontaneous differentiation into RBCs (CD34-/CD45-/CD71+/CD235a+) at 8.0-67.8% without erythropoietin, compared to 1.3% or less in healthy controls. Under erythropoietin stimulation, patient HSCs showed accelerated differentiation (10.8-14.3% by day 4) compared to healthy controls (<0.02%). These phenotypes were validated to be *JAK2*-V617F dependent through introduction of the mutation into healthy HSCs. Application of our editing strategy without selection to these patient-derived cells achieved 81.4±12.1% elimination of mutant alleles, with 51.8±29.1% converting to wild-type sequence (**Fig. 1B**). This genetic correction significantly ameliorated the erythroid differentiation phenotypes of PV in vitro as observed by the reduction in spontaneous differentiation by 90.7±11.4% in cultures without erythropoietin (**Fig. 1C-D**), reaching levels similar to healthy controls. Similarly, erythropoietin-stimulated samples showed reduced early erythrocyte proliferation and differentiation (4.7±1.3% and 77.4±28.3% respectively) at day 4. These results provide proof-of-concept for a potential one-time gene therapy for PV, though serial transplantation studies are needed to assess impacts on clonal expansion. Moreover, our work demonstrates the potential of HDR-based gene editing for addressing autosomal dominant mutations, potentially establishing a platform for genetic correction of dominant disease alleles.

Figure 1



396 mRNA-Mediated Rescue Loss of p53 Tumor Suppressor Function as a Potent New Strategy for Cancer Therapy across Pan-p53 Alterations.

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p53 tumor suppressor mutations result in loss of function of p53 in approximately 50% of all human cancers.TP53 missense, nonsense mutations and p53 deletion are present in about 50%, 12% and 5% of all p53 driven cancers, respectively. To date there is no effective treatment for rescuing p53 function in cancers using a pan-p53 approach. We have developed ADGN-531 is a new potent strategy combining mRNA with a tumor selective peptide nanocarrier to rescue of p53 tumor suppressor function.

ADGN-531 contains proprietary p53-mRNA complexed with proprietary short amphipathic peptides in the form of nanoparticles. ADGN-531 was evaluated on 20 different cancer cell lines harboring various p53 mutations. In-vivo efficacy of IV-administered ADGN-531 nanoparticles was evaluated on colorectal cancer SW403 (p53 nonsense/$KRAS^{G12V}$), osteosarcoma SaOs2 (p53 null), and NSCLC NCI-H358 (p53 null/$KRAS^{G12C}$) mouse xenografts at a dose of 0.25-1.0 mg/kg weekly. ADGN-531/sotorasib synergy was evaluated *in vitro* on NCI-H358 (p53 null/$KRAS^{G12C}$) and Mia-PACA ($p53^{R48W}$/$KRAS^{G12C}$) cells and in vivo on NCI-H358 mouse xenograft.

ADGN-531 at low nanomolar concentration markedly inhibits the growth of a large panel of cancer cells harboring p53-missense or nonsense mutations or p53-deletion, by rescuing p53 transcriptional activity and subsequent expression of its target proteins. ADGN-531 induces cell cycle arrest in G1 due to p21 upregulation and apoptosis following BAX and PUMA activation. IV-administration of ADGN-531 resulted in dose responsive tumor growth inhibition in SaOs2, SW403 and H358 xenografts independent of KRAS status. ADGN-531 (0.5 to 3.0 mg/kg, weekly) treatment results in a tumor regression by 25 to 90%. The treatment is well tolerated as no major variation in animal weight or no major liver and kidney toxicity were detected. Tumor analysis demonstrated that ADGN-531 treatment resulted in a strong expression of p53 in the tumor, together with an activation of the apoptosis pathway in the tumor, associated to an increase of BAX expression and an up regulation of C-CAS-9 signal.

We demonstrated a strong synergy in a NSCLC H358 xenograft (tumor regression 80%) when restoring P53 function with ADGN-531 (0.5mg/kg, weekly) along with inhibition of $KRAS^{G12C}$ mutant using sotorasib (10mg/kg, daily). ADGN-531 potentiated the effect of sotorasib on sotorasib resistant cells harboring high level of $KRAS^{G12C}$ in permanent active state. ADGN-531 treatments are well tolerated, no sign of clinical toxicity was detected after single or repeated administrations.

ADGN-531 is effective in rescuing p53 function both in vitro and in vivo across a wide range of p53 alterations. Our study provides a proof-of-concept that restoration of tumor suppressor function by ADGN-531 could be used as a single agent therapy in P53 altered tumors independent of other driver mutations e.g., KRAS, or with other therapies for potent combinatorial cancer treatment.

397 CD90 Ablation on Hematopoietic Stem Cells (HSCs) Enables Targeted Leukemia Stem Cell Immunotherapy

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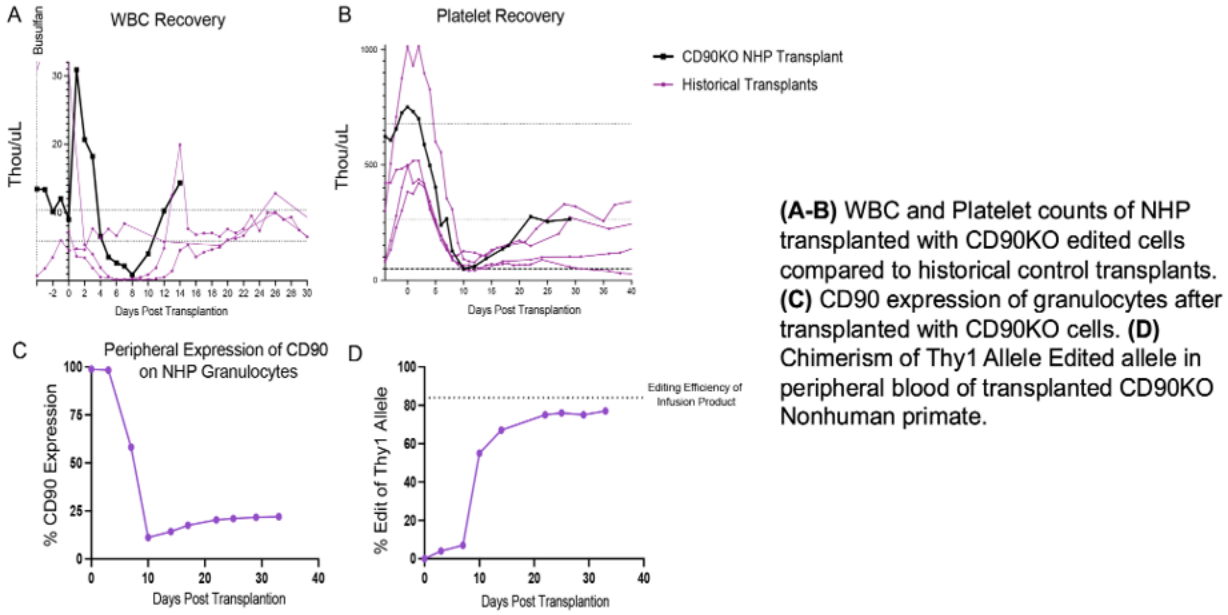
Background:
Thy1/CD90 is a known cancer stem cell marker, with high CD90 expression corresponding with increased tumor metastasis and chemoresistance. Furthermore, higher CD90 expression is found in high-risk, relapsed acute myeloid leukemias (AMLs). At the top of the hematopoietic hierarchy exists the CD34+CD90+ hematopoietic stem cell (HSC) responsible for life-long hematopoiesis. An emerging therapeutic is the ablation or epitope editing of shared cancer antigens on HSCs to protect them from anti-cancer immunotherapies. We hypothesized that modifying CD90 on healthy HSCs, combined with anti-CD90 immunotherapy, would enable a novel anti-leukemia stem cell (LSC) consolidation approach.

Approach/Results:
To study the relevance of CD90 expression on healthy HSCs, HSCs were co-transduced with either Thy1/CD90 targeted shRNA or non-targeted control shRNA and a mCherry-reporter. Transduced HSCs were then serial transplanted into primary and secondary immunocompromised mice to observe self-renewal capacity. Successful CD90-knockdown was observed with a 55% reduction of CD90+ cells in the CD34+CD38-CD133+CD45RA- subset of CD90-shRNA mice ($n=8$, $p<.05$). However, there was no significant difference in CD45 engraftment of transduced cells in either primary or secondary transplants. Furthermore, CD90-shRNA cells displayed equal myeloid and lymphoid differentiation capacity compared to control cells. We next developed an adenine base-editing strategy to remove the start codon of Thy1 (CD90-BE), allowing for complete genetic knockout of CD90. Base-edited human progenitors displayed a 22% reduction of CD90+ cells ($n=7$, $p<.05$), with no loss of human engraftment or phenotypic HSCs. Next, we applied our CD90 base-editing strategy to the nonhuman primate (NHP) autologous transplantation model. NHP HSCs were CD90-BE treated and reinfused into an autologous busulfan-conditioned recipient. Concomitant with 84% Thy1 allele editing, we observed complete platelet and neutrophil recovery at days 12 and 22 post-transplant, respectively. Furthermore, CD90-ablated NHP HSCs maintained in vitro serial re-plating capacity, confirming sustained self-renewal. These findings suggested that CD90 signaling is vestigial in adult HSC biology. We next analyzed CD90+ versus CD90- cells from bone marrow (BM) aspirates of six leukemia patients for the presence of AML driver mutations (i.e., FLT3-ITD, Runx1-ETO, NPM1). In all patients, we confirmed the presence of AML mutations in CD90+ populations. Further, Cite-Seq analysis of BM aspirates from 3 leukemia patients revealed CD90+ cells that concurrently expressed a leukemia stem cell (LSC) transcriptomic signature but did not express other known LSC markers (i.e., CD33, CD117). As such, we developed CD90-directed CAR-T (CD90-CAR-T) cells to ablate CD90-expressing malignant tumors. CD90-CAR-T cells displayed CD25 surface expression and secreted IL2 and IFN-γ only in the presence of CD90-expressing cell lines. Additionally, co-cultures of HSCs with CD90-CAR-T led to significant abrogation of serial re-plating capacity. By comparison, engrafted CD90-BE HSCs were protected against CD90-CAR-T in vivo, while CD90-expressing tumors were eliminated as measured by flow cytometry and genomic sequencing. Thus, we developed a novel LSC-targeted immunotherapy platform by ablating CD90 on non-malignant HSCs, enabling the targeting of chemoresistant AML subsets while reducing the risk of treatment-associated BM failure.



(A-B) WBC and Platelet counts of NHP transplanted with CD90KO edited cells compared to historical control transplants. **(C)** CD90 expression of granulocytes after transplanted with CD90KO cells. **(D)** Chimerism of Thy1 Allele Edited allele in peripheral blood of transplanted CD90KO Nonhuman primate.

398 Single Dose AAV-Mediated Immuno-Gene Therapy With a Dual Targeted T-Cell Engager is Superior to Mono Targeted Engagers for Multiple Myeloma

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Multiple myeloma (MM) is characterized by the abnormal clonal proliferation of plasma cells. It is the second most common hematologic malignancy, accounting for 1-2% of all cancers and 10% of hematologic malignancies worldwide. Despite many effective treatments, including chemotherapy, radiation therapy, stem cell transplants, CAR-T and T-cell engager (TCE) therapies, MM remains incurable, with a 5-year overall survival rate of around 55%. We aim to tackle MM using recombinant adeno-associated virus (rAAV)-based TCE gene therapy to improve patient outcomes due to its long-lasting, single dose, and off-the-shelf characteristics. We selected BCMA (B-cell maturation antigen) and GPRC5D (G protein-coupled receptor class C group 5 member D) as tumor immunotargets. BCMA is expressed on mature B cells and is a key target for MM immune therapies. GPRC5D, an orphan receptor typically expressed only in the hair follicle, is also highly expressed on myeloma cells. We designed several rAAV plasmids encoding CD3-targeted TCEs that bind BCMA, GPRC5D, or both targets simultaneously. In vitro, all BCMA-targeting TCE constructs effectively mediated T-cell killing of BCMA-expressing MM cell lines (MM1S & RPMI8226), but not cells lacking BCMA. The GPRC5D-targeted TCE only mediated effective killing of GPRC5D-expressing cells, but not RPMI8226, which has no detectable GPRC5D expression. Both dual-targeted TCEs (BCMA/GPRC5D) effectively mediated T-cell killing of cells expressing either target. We then selected one BCMA-targeted, one GPRC5D-targeted, and one dual-targeted TCE rAAV for in vivo assessment. We injected NSG-SGM3 mice with $1x10^6$ MM1S-LucGFP cells via tail vein and subsequently treated them with a single dose of rAAV encoding BCMA-, GPRC5D- or BCMA/GPRC5D-targeted TCEs followed by intermittent doses of human peripheral blood mononuclear cells (huPBMCs). Separate groups of mice were injected with saline or an rAAV encoding eGFP to serve as controls. Tumor burden in mice treated with rAAVs encoding BCMA- or GPRC5D-targeted TCEs was significantly reduced relative to the control mice early on, but relapse became evident in these animals as early as 4 weeks into the experiment, and all of them eventually succumbed to disease. Flow cytometry analysis of recurrent tumor cells from these TCE groups showed evidence of surface expression of both targets, suggesting that relapses were not due to antigen escape. In contrast, mice treated with the BCMA/GPRC5D dual-

targeted TCE-expressing rAAV showed a rapid decrease in tumor burden in the first 2 weeks and remained tumor-free from days 20-30 to the end of the study (days 70-77). Serum levels averaged 38.6 µg/mL in the BCMA-targeting TCE group and 22.7 µg/mL in the BCMA/GPRC5D dual-targeting group. In conclusion, our data demonstrate that tumor-targeted TCEs for multiple myeloma can be expressed via rAAV as a therapeutic strategy in vivo. Furthermore, our results suggest that dual-targeted TCEs against BCMA and GPRC5D are more effective than targeting either one alone in controlling myeloma disease burden. These data provide support for moving this construct into clinical trials.

399 Covalent Antibody Conjugation to AAV9 for Targeted Gene Therapy of Metastatic Cancer

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Adeno-associated viral (AAV) vectors are currently the preferred technology for many gene therapy applications. AAVs can target various organs, and the engineering of novel variants broadens their potential to treat many human diseases. However, their inherent tropism, typically not specific for a single cell type, makes them poorly suited for targeting metastatic tumor cells. In response, several groups are optimizing methods to restrict the broad tropism of AAVs through rational design engineering. Between the most common methods is the chemical coupling of a specific antibody to the AAV capsid, enhancing viral infection to cells expressing the corresponding antigen.

Here, we exploited a novel strategy based on the SpyTag and SpyCatcher technology for covalently binding an antibody to the capsid of the galactose-binding deficient AAV9, redirecting its tropism towards GD2 antigen highly expressed by neuroblastoma-derived metastatic cells. We named this tool AAV-STITCH to highlight its precision in "stitching" antibodies to the viral capsid.

In vitro analyses demonstrated that AAV-STITCH specifically and efficiently transduced tumor-derived cell lines but not control cells like fibroblasts or cancer cells lacking the selected antigen. We subsequently developed two models to recapitulate neuroblastoma metastasis in mice: by injecting tumor cells subcutaneously (xenograft model) or into the bloodstream (pseudometastatic model). In both models, we confirmed the specificity of our technology in targeting only tumor cells while avoiding any transduction in other organs. Then, we demonstrated that AAV-STITCH can be leveraged to deliver suicide genes to metastatic cells dampening their growth and significantly improving the survival of treated neuroblastoma animal model. Currently, this technology is under evaluation in a model closer to clinical settings using a patient-derived xenograft mouse model.

In summary, we have optimized a groundbreaking strategy to generate AAVs that precisely target cancer cells. This innovative approach has the potential to ameliorate cancer treatment by offering a highly specific and efficient method for delivering therapeutic agents directly to neuroblastoma tumor cells, paving the way for a new generation of targeted cancer therapies.

Chemistry, Manufacturing, and Controls

400 Comparative Potency Analysis of iPSC-derived and PBMC-derived NK cells for Immunotherapeutic Application

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Background & Aim
The generation of natural killer (NK) cells from induced pluripotent stem cells (iPSCs) has created new opportunities for scalable, homogeneous, and "off-the-shelf" cancer immunotherapies. iPSC-derived NK (iNK) cells offer several advantages, including uniformity, enhanced genetic engineering capabilities (e.g., CAR-NK cells), and the potential for large-scale production. However, challenges such as complex production processes and achieving functional maturity comparable to peripheral blood mononuclear cell (PBMC)-derived NK cells must be addressed. PBMC-derived NK cells, despite their high functional maturity and extensive clinical study, face limitations on donor variability, restricted expansion potential, and challenges in genetic engineering. This study aims to compare the functional potency of NK cells produced from iPSC and PBMC to determine their efficacy and acceptability for therapeutic applications.

Methods
PBMC-derived NK cells were isolated from PBMC. iPSC-derived NK cells were differentiated from cord-blood-derived iPSC using previously established differentiation methods. We conducted a comparative analysis of iNK cells, focusing on their potency through a matrix of bioassays. This included phenotypic analysis, degranulation, cytokine profiling, and cytotoxicity assessment.

Results
iPSC-derived NK cells exhibit comparable cytotoxic abilities to PBMC-derived NK cells, despite differences in surface marker expression. Both cell types demonstrate an accumulation of cytotoxic components critical for precision killing, including increased levels of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), perforin, and granzyme, in the presence of target cells. This functional activity is further supported by the analysis of cytokine secretion profiles, which confirm their potent cytotoxic response. Interestingly, iPSC-derived NK cells display a distinct pattern of surface marker expression compared to their PBMC-derived counterparts, indicating potential differences in maturation or activation states.

Conclusions
Our study provided critical insights into the relative capabilities and therapeutic potential of NK cells from PBMCs and iPSCs. These findings highlight the feasibility of iPSC-derived NK cells as a scalable, uniform, and genetically modifiable alternative to donor-derived NK cells, supporting their acceptability for clinical applications and advancing the development of NK cell-based immunotherapies.

401 Novel Nanoneedle Technology for Integrating Critical Quality Attributes: Enabling Accurate and Cost-Effective Manufacturing

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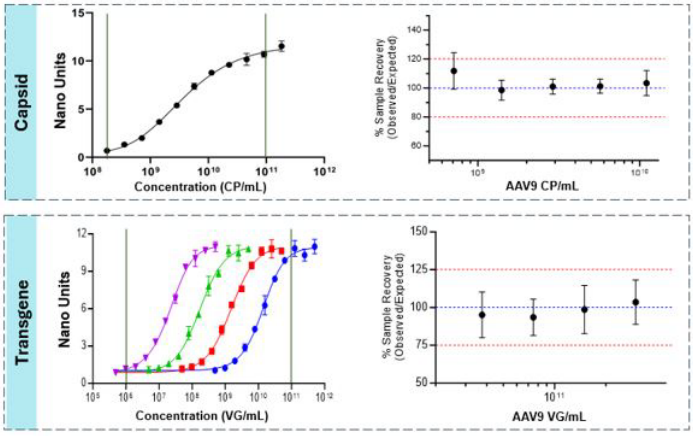
Introduction:
With the rapid growth of AAV-based therapies, various technologies are being employed throughout the manufacturing process to ensure product quality and improve process efficiency. However, there is a pressing need for the standardization of robust analytical methods alongside a deeper understanding of product heterogeneity. Standardization will foster more efficient manufacturing, reduce costs, improve scalability, increase productivity and yield, and ultimately enabling the delivery of safer, more cost-effective gene therapies.

Methods:
We have developed the multi-omic Tessie platform, based on nanoneedle technology, to quantitatively characterize the capsid titer, transgene titer, empty/full ratio, genome integrity, and contaminants (e.g. host cell DNA and proteins) in a single platform. Notably, current analytical methods either target only a short region (such as PCR) or rely on mass-based approaches (such as AUC or mass photometry) as surrogates for detecting full-length transgenes. In contrast, the Tessie platform enables precise, reproducible quantification of full-length transgenes, distinguishing them from partial genomes and bridging the gap between current analytical methods and potency assays.

Results:
We have densely integrated nanoneedles on a silicon chip using a complementary metal-oxide-semiconductor (CMOS)-compatible process developed in-house. For viral genome assays, two oligonucleotide probes are mixed with the sample and amplified into a double-stranded DNA product using the viral genome as a template. The positions of the probes define the specific transgene sub-population to be quantified. Full-length transgenes are detected using probes at opposite ends, while truncated transgenes are quantified with probes spanning the region of interest. The resulting double-stranded product is quantified on the Tessie nanoneedle sensors. For capsid assays, antibodies replace the probes, and the final sandwich complexes are quantified on the same platform. The "Empty/Full ratio," combining full-length transgene and capsid titers, can be calculated by integrating both capsid and transgene quantifications.

We have characterized the performance of the assay using standard reference well-characterized samples. Intra- and inter-assay coefficient of variation (CV), and spike-in recovery data are summarized in **Fig. 1**. The assay workflow is compatible with crude lysate samples and provides accurate quantification, which aligns with ddPCR for short-region transgene quantification and ELISA for capsid titer measurements in orthogonal comparisons. While there is currently no comparable technology for full-length transgene quantification, the data obtained show strong alignment with AUC and electrophoresis results. Additionally, we have developed an electronic signature tracking function within both the instrument and analysis software, ensuring that the system is 21 CFR Part 11 compliant.

Conclusions:
The Tessie platform offers a new methodology for integrating the gene therapy analytics into a single system, ensuring 21CFR part 11 compliance. Its ability to provide molecularly specific quantification of viral genomes and serotype-specific capsids is an advance over existing methodologies. The Tessie platform has the potential to advance AAV-based gene therapy development by providing accurate, integrated measurements, ultimately driving a more cost-effective and standardized manufacturing process.



✓ Multi-omic in one system
✓ Compatible with crude
✓ Genome Integrity
✓ Single data report 21CFR part 11compliant

Viral capsid assay spec	
Dynamic range	2E+08 – 1E+11 cp/mL
Intra- CV (duplicates)	3.3%
Inter- CV (13 plates, 3 uers, 3 instruments)	7%
Recovery-spike in rate	< 80-120%
Viral genome assay spec	
Dynamic range	9.8E+5 – 1E+11vg/ml (tunable)
Intra- CV (duplicates)	2.5%
Inter- CV (17 plates, 3 users, 3 instruments)	14%
Recovery-spike in rate	< 80-120%

Fig. 1: Top row: The capsid assay demonstrates a dynamic range of 2E8 to 1E11 cp/ml, with an intra-assay coefficient of variation (CV) of 3.3%. The inter-assay CV, tested across 13 plates, 2 users, and 3 instruments, is 7%. Bottom row: The viral genome assay offers a tunable dynamic range, depending on the number of primer extension cycles after probes are mixed with the sample. The dynamic range spans from 9.8E5 to 1E11 vg/ml, with an intra-assay CV of 2.5%. The inter-assay CV, tested across 17 plates, 3 users, and 3 instruments, is 14%. Recovery in spike-in experiments returned expected quantification within 80-120%.

402 Mastering hcDNA: A Harmonized Approach for AAV Gene Therapies

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BioPhorum's mission is to create environments where the global biopharmaceutical and device industry can collaborate and accelerate its rate of progress, for the benefit of all. To support organizations developing viral-based therapeutics, BioPhorum initiated the Impurities in Viral-Based Therapies workstream which comprised of 34 members from 20 organizations. The workstream members elected to focus on host cell DNA (hcDNA) impurities in AAV gene therapy products.

This poster will present the results of industry benchmarking surveys that provide insights as to actual hcDNA levels dosed, methods of quantitation, and strategies to control the impurity level during production. A key focus of the presentation will be the proposal of a harmonized approach to hcDNA reporting. Normalizing hcDNA levels by the number of vector genomes (vg) dosed, as well as reporting total hcDNA/dose, facilitates easier comparison within and across AAV gene therapy products.

Current FDA guidance recommends limiting hcDNA to less than 10 ng/dose and smaller than 200 base pairs. By surveying the actual number of vector genomes dosed and correlating them to the percent of development programs achieving the current hcDNA limit, we found that beyond 10^{13} vg/dose the number of programs meeting the guidance falls to less than 20 %. The wide range in vg dosed (10^{11}-10^{16} vg/dose) suggested adding vg as a normalizing factor to allow for easier comparison of hcDNA levels achieved based on the current state of technology (process and analytics). On a per 10^{13} vg basis, the 95 % confidence interval for hcDNA in drug product was found to be 39 – 235 ng hcDNA/10^{13} vg. Interestingly, in recent guidance for neurodegenerative disease gene therapies, the FDA has omitted an explicit hcDNA limit and encourages manufacturers to assess the characteristics of their production cell lines.

In conclusion, the BioPhorum workstream aims to standardize the industry approach to hcDNA reporting and serve as a barometer of the current hcDNA state of the art.

403 Breaking Boundaries in AAV Production: Exploiting High Salt Concentrations for increased AAV yields and infectivity.

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Enhancing adeno-associated virus (AAV) production while maintain highest safety standards is of utmost importance in the field of gene therapy. In this study, we have confirmed previous approaches to boost AAV production utilizing higher salt concentrations during the lysis process. Our research yielded impressive results, demonstrating that elevated salt levels, such as 500 and 1000mM, effectively enhance AAV process intensification by eliminating adhesive DNA from the AAV capsids and reducing aggregation. However, it is worth noting that high salt concentrations exceeding 200mM hamper the activity of standard endonucleases, which are virtually inactive at high salt environment. To overcome this challenge, we have engineered a cutting-edge enzyme called the Benzonase® Salt Tolerant Endonuclease, specifically designed to function optimally in high salt conditions ranging from 500-1000mM. This remarkable enzyme efficiently digests DNA fragments shorter than 10 base pairs and remains fully compatible with commonly used detergents. By incorporating >500mM salt in the lysis buffer, we have achieved remarkable outcomes. Our experimental results indicate a substantial improvement in AAV titers by 20-30% (evaluated using ELISA) and a remarkable 10-fold enhancement in viral transduction capability. These findings provide valuable insights to intensify AAV production, solving one of the key bottlenecks of the gene therapy industry: AAV production yield.

404 Scalable Hematopoietic Stem Cell Expansion in Stirred-Tank Bioreactors for Advancing Cell Immunotherapies

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Hematopoietic stem cells (HSCs) are pivotal for off-the-shelf allogeneic immune cell therapies, including natural killer (NK) cells. While HSC expansion molecules (like our proprietary IBR403) enable CD34+ cell growth, conventional 2D cell culture methods are inefficient, costly, labor-intensive, and lack scalability, limiting large-scale clinical and commercial production. To overcome these challenges, we developed a robust, scalable, and high-throughput platform using automated stirred-tank bioreactors to expand cord blood derived HSCs (CBUs) and differentiate them into NK cells.

Using a Design of Experiments (DoE) approach with MODDE® software and multivariate analysis,we optimized culture conditions by comparing CellGenix® GMP grade SCGM with RUO media, supplemented with IBR403 and key cytokines (SCF, Flt-3 ligand, TPO, IL-6). Over 70 bioreactor conditions were tested, evaluating parameters such as perfusion rates (0.5-2 VVD) and cytokine concentrations (0-100 ng/mL) across multiple donors over 28 days. CD34+ cell expansion and differentiation were performed in the Ambr® 15 bioreactor system, with functionality and purity assessed for clinical relevance.

HSCs cultured at a perfusion rate of 2 VVD produced an average of 5×10^7 CD34+ cells per mL of input cord blood in GMP-grade media, compared to 1.4×10^6 in RUO media. The DoE identified an optimal cytokine formulation that reduced costs by 50% while achieving $>6 \times 10^7$ CD34+ cells per mL of input cord blood. Media formulation and a high perfusion rate were critical for maximizing CD34+ yields per mL of input CBU. Variations in CO2 (5% vs. 10%) and dissolved oxygen setpoints (15% vs. 30%) showed no significant impact on CD34+ expansion. NK cells differentiated in bioreactors under optimal conditions showed >95% CD56+ expression (55-98% on Day 28) and >75% cytotoxicity against A549 cells at a 1:1 effector-to-target ratio, with donor variability impacting HSC expansion and NK differentiation.

This study successfully optimized CBU-derived HSC expansion and NK cell differentiation in GMP-grade media using the Ambr® 15 bioreactor system. By refining key process parameters, we achieved high-yield, cost-effective CD34+ cell expansion and scalable NK cell production. These findings establish a robust platform, transforming cord blood into a valuable resource for advancing allogeneic immune cell therapies in oncology and immune-related diseases.

405 Negative Impact of Leukapheresis Collection on Blood-derived B-cell Gene Expression — An Artificial Intelligence and Machine Learning Enhanced Study

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INTRODUCTION:
Leukapheresis (LA)-derived peripheral blood mononuclear cell (PBMC) products are the starting material for the manufacture of many T-cell, NK-cell, and B-cell adoptive cell immunotherapies. Experts have implicitly assumed that immune cells isolated from LA products are the same as those in venous whole blood (WB). This study used bioinformatics, artificial intelligence (AI), and machine learning (ML) to compare gene expression profiles of B-cells isolated from matched LA-derived PBMC and subject matched WB.

METHODS (see Figure 1):
WB was collected via venipuncture prior to LA in 3 healthy adult donors. All starting materials were transported at 4°C. B-cells were positively isolated using anti-CD19 immunomagnetic MACS® MicroBeads, without prior density gradient centrifugation or RBC lysis, within 3 hours of collection. After RNA extraction from the B-cell isolates, individual samples underwent bulk RNA sequencing. Relative proportions of immune cell subtypes were quantified using CIBERSORT *iSort™ Fractions v1.4* blood mode, AI/ML-based digital cytometry deconvolution algorithm applied to the bulk RNAseq gene expression data. CIBERSORT *iSort HiRes v1.1* was run and sequencing reads were mapped and quantified by Salmon v1.9 with Gencode v31 annotation. Significant inferred, differentially expressed genes were defined as having log2foldchange >0.5 or <−0.5 (>1.42 fold-change) and false discovery rate adjusted *p-value* <0.05. Significance vs. magnitude of expression changes were visualized via volcano plot.

RESULTS:
iSort Fractions inferred 69.7%±16.9% vs. 94.5%±1.6% B-cells, 5.9%±0.1% vs. 5.3%±1.4% T/NK cells, and 24.4%±16.9% vs. 0.2%±0.2% monocytes in the B-cell isolates from LA-derived PBMC vs. WB. Flow cytometry corroborated these findings. The 27 genes significantly upregulated after LA included ones associated with V(D)J recombination (*HMGB2*), autophagosome assembly (*ATG2A*), NF-Kappa-ℝ?-mediated transcription down-regulation (*ZCCHC9*), 4-1BB signaling regulation (*LRR1*), Wnt signaling (*SLC9A3R1*), p53-associated apoptosis (*ING1*), B-cell negative regulation (*HMGB3*), folate depletion (*MTHFS*), and several mitochondrial stress proteins. The 119 genes significantly downregulated after LA included ones associated with prevention of reactive oxygen species accumulation (*SESN1*), cell survival (*DYRK1B*), RAS signal transduction (*RAPGEF5*), B-cell reactivity and splenic localization (*KLF2*), B-cell mediated extracellular matrix remodeling (*LOXL2*), T-cell co-stimulation (*CD200*), cell cycle control (*BCAR3*), and B-cell migration (*SHCBP1*). (see Figure 2)

DISCUSSION:
We set out to determine if the gene expression profiles of human B-cells isolated from LA products are comparable to those isolated from matched venous WB. This required elimination of misleading gene expression noise from non-B-cells, mostly monocytes, found in the B-cell isolates. We used *iSort Fractions* and *HiRes* to conduct AI/ML-based digital cytometry and inferred B-cell specific gene expression. The differential gene expression profile suggests that LA-derived B-cells are significantly more mitochondrially stressed and likely less functionally active than WB-derived B-cells. This may reflect the markedly greater PBMC density and cell-to-cell contact specifically in LA products. Method of collection of B-cells, and probably other immune cells, for the manufacture of adoptive cell therapies should be carefully reassessed in order to optimize infused product performance.

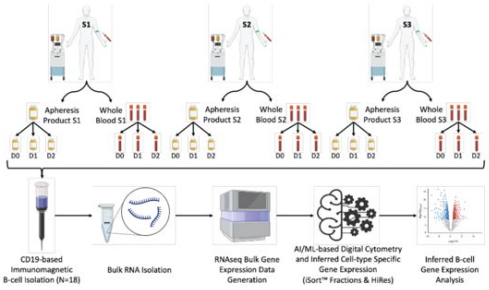

Figure 1. Study infographic illustrating the collection of source material by leukapheresis and venipuncture, isolation of B-cells, isolation of bulk RNA, gene expression data generation, AI- and ML-based digital cytometry and inferred B-cell gene expression, to generation of volcano plots for analysis.

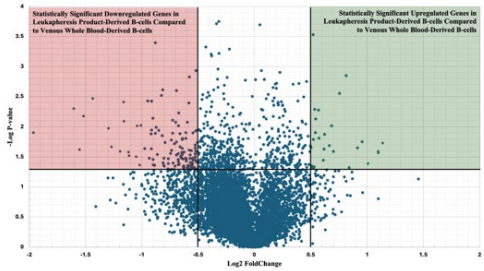

Figure 2. Volcano plot of the inferred gene expression for B-cells isolated from leukapheresis-derived PBMC products compared to the inferred gene expression for B-cells isolated from subject matched venous whole blood. The reddish and greenish shaded areas represent statistically significant and relatively downregulated and relatively upregulated genes, respectively.

406 Quantification of residual host-cell DNA fragments of the E1A gene from HEK293 via qPCR assay

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Background:
Viral vector gene therapy has made significant strides over the past decade, with numerous products, including those based on adeno-associated virus (AAV) and other viral vectors, achieving global commercialization. The HEK293 transfection platform, which employs the HEK293 cell line as the production host, is widely used for high-titer recombinant AAV (rAAV) production. These cells are genetically engineered to express the E1A adenoviral gene, enabling efficient rAAV production. However, the process inevitably generates impurities, including the E1A gene, which can transcriptionally activate other adenovirus early genes and pose potential tumorigenicity risks to patients. Accurate quantification of residual E1A DNA in viral vector drug products is therefore critical to guide process optimization and minimize E1A residuals. While process optimization can reduce E1A impurity levels, controlling the size distribution of residual E1A DNA fragments remains challenging. Robust analytical methods are essential to monitor these fragments. In this study, we developed a TaqMan real-time PCR assay to quantify E1A DNA. Although commercial E1A DNA quantification kits are available, they are often limited to measuring a single fragment length and are cost-prohibitive. To overcome these limitations, we established a cost-effective and versatile assay capable of quantifying E1A DNA fragments of varying sizes. Three primer-probe sets were designed based on the E1A gene sequence, enabling the development and validation of a tailored TaqMan real-time PCR method.

Results:
The E1A gene was synthesized and cloned into the pUC57-GW-Kan vector backbone to serve as the assay standard. The TaqMan qPCR standard curve exhibited a slope ranging from -3.60 to -3.10, with a linear correlation coefficient (R^2) of ≥ 0.98 and amplification efficiency between 90% and 110%. The assay demonstrated high specificity, with no cross-amplification signals detected. The limit of detection (LOD) was 50 copies/µL, and reproducibility testing showed intra- and inter-assay variability within 10%, indicating excellent precision. Spike recovery ranged from 91% to 113%, confirming the method's accuracy. The assay displayed a broad dynamic range, quantifying E1A DNA concentrations from 5.0×10^1 copies/µL to 5.0×10^6 copies/µL.

Conclusion:
The TaqMan real-time PCR method was successfully developed to detect residual host-cell E1A DNA by amplifying signals from three fragment lengths (100 bp, 300 bp, and 500 bp) of the E1A gene. The method demonstrates exceptional specificity, linearity, accuracy, and precision, enabling reliable quantification of residual E1A DNA fragments from HEK293 cells. Applicable to a wide range of sample types, including in-process intermediates and final drug products, this assay serves as a valuable tool for process development and quality control in gene therapy manufacturing. It provides a robust, cost-effective solution for monitoring and optimizing the production of gene therapy products, ensuring both operational efficiency and compliance with regulatory standards.

AAV Gene Transfer (C): Antibody Evasion, Cardiac & Neuromuscular Targets

407 Engineering non-mammalian chimeric AAVs for evasion of pre-existing antibodies and vector-induced immunity

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Adeno-associated virus (AAV) immunity remains a significant barrier in the gene therapy field, with 30-80% of adults harboring pre-existing antibodies against endemic AAV isolates. Further, AAV redosing is significantly limited since patients develop robust antibodies following an initial dose. One approach to overcoming AAV immunity is vectorizing non-mammalian AAV isolates. Currently, all AAV serotypes used in the clinic were derived

from human or NHP samples, leading to high similarity with endemic isolates. Non-mammalian AAVs, on the other hand, display significant capsid variability from these isolates. Typically, non-mammalian AAVs cannot transduce human cells; however, we recently overcame this barrier using a rational engineering approach that unlocks robust transduction in human cells and mouse tissue.

Here, we apply this discovery to engineer immuno-evasive AAV capsids for gene therapy applications. First, we performed a barcoded screen of diverse rationally engineered chimeric capsids from across the genus Dependoparvovirus. The top-performing capsid across cell types and tissues was derived from a Muscovy duck isolate. This novel chimeric capsid, termed AAV-div3A, demonstrates high transduction in murine liver, heart, and muscle tissue following intravenous dosing. To determine whether humans have pre-existing immunity against div3A, we screened serum from 20 donors for neutralizing antibodies (NAbs). While 6 donors had NAbs against AAV9, a clinically validated human AAV isolate, all donors were negative for NAbs against div3A. Mice were then passively immunized with serum from AAV9 NAb+ donors and dosed with div3A 72 hours later and showed robust transduction with no decrease in transgene expression compared to non-immunized controls. To validate that these results extend beyond AAV9 NAbs, we also interrogated the cross-reactivity of div3A with NAbs against ten commonly used AAV serotypes. Promisingly, div3A showed no neutralizing effects or cross-reactivity with any sera.

Next, we examined the immune evasion of div3A in the context of vector-induced immunity. Mice were initially dosed with AAV9 and then successfully dosed again with div3A six weeks later, yielding robust and unabated transduction across multiple tissue types. In order to extend our findings into a therapeutic context, we demonstrate the successful re-administration and cumulatively improved expression of a therapeutic transgene (acid alpha-glucosidase) in Pompe mice. Additional rational engineering of div3A through peptide surface display yielded a new variant, div3A-M1 with 14-fold higher transduction in murine heart tissue, 11-fold higher transduction in diaphragm tissue, and 90% reduction in liver targeting. Additional results from continued evaluation of AAV.div3A and div.3A-M1 in mouse models of disease and non-human primate models will be presented. Overall, the vectorization of chimeric AAVs promises to improve patient access to gene therapies by expanding inclusion criteria and enabling repeat dosing by evading pre-existing immunity from natural exposure and acquired immunity from previous sub-therapeutic treatment exposure.

408 It's a Zoo Out There: Analyzing the Cross-Reactivity of Human Antibodies Towards Animal AAVs to Expand the AAV Vector Toolkit for Gene Therapy Applications

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A major obstacle to AAV vectors is the presence of pre-existing neutralizing antibodies (NAbs) that circulate among the general human population, leading to vector inactivation and to a loss of treatment efficacy. One alternative to the primate-based vectors are AAVs are those found in species that may be naïve to human infections, thus exhibiting low to no pre-existing antigenicity. Such animal dependoparvoviruses include quail AAV (QAAV), bovine AAV (BAAV), serpentine AAV (SAAV), goose parvovirus (GPV), porcine AAV isolates (AAVpo.1 and AAVpo.6), rat AAV (AAVra.1), mouse AAV (AAVmo.1), bat AAV (BtAAV), and bearded dragon AAV (BDPV). Native-immuno and/or neutralization assays were used to assess the reactivity of the non-primate AAVs against human sera samples obtained from 50 healthy patients. However, with ~25-30% detection or neutralization rates, the animal AAVs were not as non-reactive to the NAbs as anticipated. To investigate the antibody-capsid interactions, anti-AAV9 and AAV2 monoclonal antibodies (mAbs) were obtained from patients treated with Zolgensma® and individuals naturally exposed to AAV2, respectively. Their binding sites to the AAV capsids were previously determined by cryo-electron microscopy (cryo-EM). While the antibodies bound to different regions, such as the 2-fold, 2/5-fold wall, 3-fold, and 5-fold symmetry axes of the capsids, only the 5-fold antibodies showed cross-reactive properties, with several non-primate AAVs. The capsid structures of the animal AAVs listed above were

determined by cryo-EM, ranging from 1.77 to 3.30 Å resolution. These structures allowed the analysis and comparison of the highly conserved 5-fold regions of the primate and the non-primate AAVs. Amino acid sequence alignments and structural superpositions of the 5-fold channel pores revealed highly conserved epitopes, most likely responsible in the observed cross-reactivity. This conservation is likely due to the AAV Rep proteins also interacting with the 5-fold region during genome packaging. Understanding the interactions of the 5-fold antibodies to the AAV capsids will aid in engineering variants capable of escaping this class of Nabs and will expand the current toolkit of AAV vectors towards human gene therapy applications.

409 AAV-CM1, a Human Receptor-Targeted Capsid with an Enhanced Tropism for the CNS, Skeletal Muscle, and Heart

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Many monogenic disorders, including neuromuscular diseases, lysosomal storage disorders, and dystroglycanopathies, affect multiple organs. To effectively treat disease manifestations across multiple organs, it is crucial to develop delivery vehicles that efficiently reach multiple cell types and tissues. We have derived several families of AAV9-based capsids that target the CNS, skeletal muscle, and heart *in vivo* by binding to human CD59 (hCD59). CD59 is a GPI-anchored surface protein widely expressed in many cell types, including vascular and parenchymal cells of most organs in mice, non-human primates (NHPs), and humans.

When administered intravenously at $5e^{12}$ vg/kg to transgenic mice ubiquitously expressing hCD59, our top capsid, AAV-CM1, achieved significantly enhanced transduction compared to AAV9 in the CNS (21x), skeletal muscle (19x), and heart (3x). Notably, AAV-CM1 exhibited reduced liver transduction (0.01x that of AAV9), a challenge with many AAV serotypes. AAV-CM1 transduces cells and crosses the blood-brain barrier by binding human or macaque CD59 *in vitro* and *in vivo* (Fig. 1), allowing evaluation in NHP models. Remarkably, after systemic delivery of an epigenetic editor by AAV-CM1 at $1.5e^{13}$ vg/kg in humanized CD59 mice, we achieved 81%, 87%, 73%, and 47% mean reductions in target gene expression in the forebrain, skeletal muscle, heart, and liver, respectively (Fig. 2). In contrast, delivery of the same epigenetic editor with AAV9 mediated reductions only in the heart (37%) and liver (78%) but not brain or muscle. Importantly, AAV-CM1 can be produced at yields comparable to AAV9 and purified using commonly used affinity resins. Studies are underway to evaluate transduction and safety profiles in NHPs.

To our knowledge, the expanded tropism of AAV-CM1 is unique among natural and engineered capsids. AAV-CM1 is a promising delivery vehicle for targeting gene therapies to the CNS, skeletal muscle, and cardiac tissue, with a human-relevant mechanism of action and the advantage of enabling evaluation in humanized mice and NHPs without the reliance on surrogate capsids.

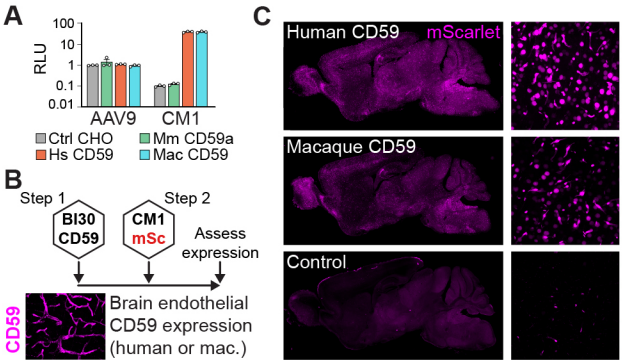

Figure 1. (A) AAV-CM1 transduces human or macaque CD59- expressing cells more efficiently than AAV9, but not control or mouse CD59-expressing cells. **(B,C)** Our "two-step" assay first mediates exogenous CD59 expression in the mouse brain vasculature using AAV-BI30, then AAV-CM1:mScarlet is administered. Brain images show transduction by AAV-CM1 in mice expressing human, macaque, or no exogenous CD59.

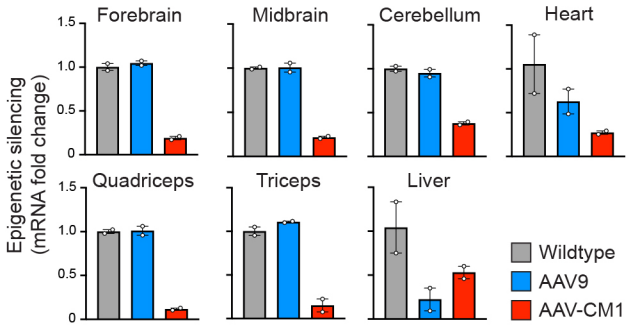

Figure 2. AAV-CM1 mediated efficient epigenetic silencing in the brain, skeletal muscle, and heart. Mice were systemically injected with 1.5e13 vg/kg of an epigenetic editor packaged into AAV9 or AAV-CM1. C57BL6/J and iCMV-hCD59 mice (C57BL6/J background) were used for AAV9 and AAV-CM1, respectively. Target mRNA fold changes in the indicated tissues at three weeks post dosing are shown.

410 Position-Specific Impact of RGD Motif in AAV Capsid Variants on Muscle Tissue Targeting and Integrin Binding Affinity

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Monogenic diseases that affect heart and skeletal muscle function are usually associated with substantial morbidity and early mortality. Several gene therapy products based on clade E and clade F capsids for these types of diseases are in the clinic with one recently approved. Most applications require high doses of intravenous adeno-associated virus (AAV) vector that are expensive to produce and associated with severe innate and adaptive immune responses. We and others have attempted to improve the performance of AAV vectors for cardiac and skeletal muscle applications by redirecting binding to integrins through the incorporation of RGD-containing peptides into exposed capsid loops. Our family of cardiac and skeletal muscle vectors, referred to as the GCap family, has been evaluated in various mouse and NHP models expressing a variety of therapeutic transgenes. These studies have produced engineered capsids with 10-to-1000-fold improvements in cardiac and skeletal muscle transduction during evaluation in NHPs. A number of themes emerged with the new capsids: 1) The position of the RGD within the engineered loop had a substantial impact on the transduction profiles, with RGD located at the N terminus of the peptide (proximal variants) showing primarily skeletal muscle transduction while RGD peptides embedded within

the loop were much more efficient in transducing heart and diaphragm with lower transduction in skeletal muscle. 2) Proximal variants showed a qualitatively different integrin binding profile than embedded variants. 3) Studies in NHPs demonstrated levels of therapeutic transduction at doses 10- to 30-fold lower than that achieved with first-generation vectors with no toxicity and up to 160-fold reductions in liver targeting. 4) Only a small fraction of the RGD library produced functional vector. Our studies demonstrate a clear but unexpected relationship between the structure of the inserted RGD peptide, its integrin binding profile, and its performance in NHPs in terms of efficiency and distribution of transduction. The repertoire of biological properties exhibited by the GCap family of vectors allows one to better address the needs of diseases affecting heart and skeletal muscle. We are advancing into the clinic a proximal RGD capsid for a muscular dystrophy and an embedded RGD capsid for a common inherited cardiomyopathy.

411 Antibody-evading AAV capsids compatible with CNS and muscle-targeting modifications

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Adeno-associated virus (AAV) capsids have been successfully engineered via surface loop modifications to target different organs such as the central nervous system (CNS), muscle, retina, and liver following systemic delivery. However, to enable the use of lower AAV doses and bring gene therapies to a larger fraction of affected patients, it will be necessary to prevent their neutralization by antibodies. The challenge is to develop AAVs that evade neutralizing antibodies (NAbs) while also maintaining their enhanced tropisms and manufacturability. To address this challenge, we generated AAV9-based libraries through the diversification of broadly distributed capsid regions, using multiple tropism-enhanced capsids as seeds. We then selected variants exhibiting resistance to intravenous immunoglobulin, while maintaining the enhanced tropism, production fitness, and compatibility with capture affinity resins of their parental capsids. When applied to BI-TFR1v2, one of our group's second generation human transferrin receptor-targeting capsids, this strategy resulted in a novel variant (BI-hTFR1v2-NAbE) that evades detectable neutralization by more than 40% of the polyclonal human serum samples that neutralized AAV9 or AAV5, while maintaining the BBB-crossing phenotype and manufacturability of its parental capsid. We tested the compatibility of our antibody-evading capsids with several cross-species RGD motif-containing capsids, and successfully obtained muscle-specific variants with improved antibody evasion. This work holds promise for expanding the pool of patients eligible for CNS and muscle-targeted gene therapies.

412 Insight into the mechanism of action of AAV-SLB101, a novel muscle-tropic capsid for neuromuscular and cardiac indications

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Adeno-associated virus (AAV) mediated gene therapy continues to be a promising therapeutic for various diseases; however, high systemic doses currently required to achieve widespread therapeutic benefit for indications like Duchenne muscular dystrophy (DMD) can pose potential safety risks. To develop a more targeted and efficacious vector, AAV-SLB101 was rationally designed to increase muscle tropism. Presently, it is utilized in Solid Bioscience's next generation gene therapy SGT-003, which is currently in a clinical study for the treatment of DMD (NCT06138639).

In multiple non-clinical studies, the novel vector AAV-SLB101 has demonstrated increased muscle tropism and decreased liver uptake in both healthy animals and disease models (wild type and mdx mice, non-human primates

and human iPSC-cardiac myocytes). Currently, the hypothesized mechanism for increased muscle uptake is through interaction of the inserted RGD peptide with integrin receptors.

To further explore AAV-SLB101's mechanism of action, a panel of studies comparing AAV-SLB101 to AAV9 in cells and in vivo demonstrated increased binding, transduction and expression of AAV-SLB101 over AAV9 in all cases. Additionally, in AAV-SLB101 binding assays the signal was reduced by the addition of anti-integrin antibody and by increasing concentration of a tight binding integrin ligand partner in both mouse and human dystrophin-deficient muscle cells. These studies provide further insight and characterization of the mechanism for AAV-SLB101.

413 A Novel Neuromuscular AAV Capsid Combines Efficient Systemic Muscle and CNS Delivery with Liver Detargeting in NHP

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Neuromuscular diseases are complex conditions that require a long-term, multisystemic approach to treat all affected tissues. Current therapeutic strategies to target both nervous and muscle tissues utilize multiple injection sites at high doses. Here we present the first demonstration of a novel AAV capsid that efficiently and specifically transduces the heart, skeletal muscle, and central nervous system (CNS) in non-human primates (NHPs). Using a single intravenous dose of 4e12 vg/kg, this first-in-class capsid can lead to wide biodistribution of skeletal muscle and enables crossing of the blood-brain barrier while maintaining a favorable safety profile by detargeting the liver. The high specificity and efficiency of this capsid in on-target tissues permits the use of lower doses, increasing the safety profile for patients and lowering manufacturing costs.

Using our AI-driven design platform, we engineered a liver detargeted neuromuscular capsid, validating its novel tropism through a single capsid study in adult NHPs. This capsid is highly compatible with large scale AAV manufacturing methods, producing 1.2-fold higher harvest yields than AAV9 with comparable purification by affinity chromatography. While the manufacturability profile of this capsid enables high doses of AAV in NHPs, even a low dose of 4e12 vg/kg systemically delivered was sufficient to effectively target skeletal muscle, heart, and CNS tissues. Across all tested skeletal muscles, including biceps, gastrocnemius, and diaphragm, we measured 57-90% of myofibers transduced and 32-66% of cardiomyocytes transduced in the heart. This capsid demonstrated blood-brain barrier crossing across cortical and deeper brain regions, transducing up to 5% of neurons. We further confirmed the specificity of this capsid to neuromuscular tissues and high safety profile by measuring low viral biodistribution in off-target tissues, such as liver (<10 vg/dg) and spleen (<0.5 vg/dg). We have identified a putative receptor for our capsid that is expressed in both brain and muscle tissue, suggesting the receptor can mediate both blood-brain barrier crossing as well as muscle transduction. This receptor is highly conserved between NHP and human, supporting potential human translatability of our capsid.

The unique characteristics of this novel neuromuscular AAV capsid enable therapeutic access to a wide range of tissues affected by neuromuscular disorders that might otherwise go untreated with the use of neuro- or myotropic capsids.

Molecular and Cellular Methods - Applications

414 Vector Insertional Mutagenesis Drives Accelerated Hematopoietic Stem Cell Aging and Acquisition of Somatic Mutations In Vivo

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Corresponding Author

Hematopoietic Stem and Progenitor Cell Gene Therapy (HSPC-GT) uses retroviral vectors such as self-inactivating (SIN) lentiviral vectors (LVs) to insert therapeutic genes into target genomes. Once reinfused, these modified HSPCs fully reconstitute hematopoiesis, maintain lifelong blood cell homeostasis, and provide therapeutic benefits. However, vector integration may inadvertently activate oncogenes, potentially causing malignancies, as seen in an LV-based trial for adrenoleukodystrophy. Moreover, ectopic oncogene activation in HSPCs induces oncogene-induced senescence (OIS), leading to proliferation arrest and the secretion of pro-inflammatory cytokines, a process known as the senescence-associated secretory phenotype (SASP). Transplantation of oncogene-expressing HSPCs in a xenogeneic model resulted in senescent cell accumulation and chronic inflammation, which triggered myeloid-restricted hematopoiesis and eventually led to bone marrow failure. These findings raise concerns that insertional mutagenesis in HSPC-GT could produce similar adverse effects. To address this issue, we monitored the hematopoiesis of wild-type (WT) mice transplanted with WT HSPCs transduced with either a genotoxic LV carrying the Spleen Focus Forming Virus promoter in its long terminal repeats (LV.SF) or a safer SIN LV with the PGK promoter in an internal position (SINLV.PGK). We applied the same approach to HSPCs from Cdkn2a-/- mice, which lack the key senescence cell cycle inhibitors p16INK4A and p19ARF. Blood composition was monitored every four weeks (up to 2.5 years), and vector integration sites (IS) were retrieved from purified B, T, and myeloid cells for clonal tracking. The genotoxic LV.SF accelerated myeloid tumor onset in mice transplanted with Cdkn2a-/- cells ($p < 0.0001$) but not in WT mice. However, WT/LV.SF mice exhibited marked myeloid skewing and a dramatic reduction in clonality across all hematopoietic lineages over time, suggesting accelerated aging compared to WT SINLV.PGK mice. Insertions activating the Braf oncogene were prevalent in tumor-infiltrated tissues of the Cdkn2a-/- group and, to a lesser extent, in WT mice. Given the link between OIS, chronic inflammation, and somatic mutations, we analyzed genomic sequences flanking each IS to assess mutation accumulation using a novel Mutation Index (MI), normalized by clone number and coverage. Analysis of over 200,000 IS covering more than 9 Gb of genomic data revealed significantly higher MI in both LV.SF groups than in LV.PGK groups ($p < 0.001$), with myeloid clones accumulating more mutations than B and T cells. This effect was exacerbated in Cdkn2a/LV.SF mice, indicating that the absence of proto-oncogene activation barriers leads to increased somatic mutations. Whole-genome sequencing confirmed a higher mutation rate in LV.SF groups compared to LV.PGK, validating that no biases were present in the flanking region analysis. Finally, a Bayesian model developed to trace the dynamics of clonal and subclonal evolution based on IS and mutations, confirmed greater mutational diversity and complexity, increased mutation burden and accelerated clonal expansion in LV.SF groups. Overall, our data show that random insertional activation of oncogenes in vivo induces accelerated stem cell aging, clonal hematopoiesis, and heightened somatic mutation burden due to persistent senescent cell accumulation in tissues and justifies further exploration of this underappreciated aspect of vector genotoxicity.

415 Decoding the Molecular Signature of Expanded Gamma Delta T Cell Products; TCR and Immune Gene Expession from Allogeneic derived Products

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Gamma-delta (γδ) T cells are a powerful cellular therapy approach due to their unique ability to differentiate between stressed/sick and healthy cells, and subsequently eliminate the sick cells, including cancer cells. γδ T cells typically comprise a small percentage of peripheral T cells (~1-5%) and can require manufacturing processes of up to 10 days to reach clinical doses. Understanding the molecular changes that occur throughout this process can provide insight into the potency, effector and trafficking capabilities of the manufactured cells as well as the reproducibility, and robustness of the process. Previous work demonstrated that our genetically engineered DeltEx drug resistant immunotherapy (DRI) γδ T cells, manufactured from diverse starting materials, exhibited similar TCR and gene expression profiles. To strengthen these findings, we analyzed our DeltEx Allogeneic (Allo) products. Here, we assessed TCR repertoire and gene expression from both (a) the apheresis starting material and (b) final manufactured γδ T cell products derived from different allogeneic donors. γδ T cell clinical products were generated from using proprietary manufacturing protocols. TCR libraries were prepared with VDJ-specific primers for α, β, δ and γ chains by dimer-avoided multiplex PCR (dam-PCR) using iRepertoire's RepSeq+. Concurrently, RNAseq libraries were prepared with iRepertoire's Immunosight, a targeted sequencing panel encoding +150 immune profiling genes. TCR repertoire and gene expression profiles were compared for the starting material and final manufactured products. Additionally, gene expression profiles of DeltEx Allo and DeltEx DRI were compared. Multigroup comparisons were performed by nonparametric statistical test while controlling for false discovery rate. While the TCR repertoire at initiation primarily consisted of αβTCR clones, the final product exhibited a shift towards γδ TCR expression, with a notable expansion of Vγ9 clones in the DeltEx Allo. Interestingly, gene expression analysis revealed a significant increase in markers of cellular activation and cytotoxicity in the final product, as previously observed in DeltEx DRI. Upregulated gene expression of cytotoxicity, proliferation and effector markers were observed related to type 1 cytokines, the TNF super family, IL7 -, IL15 -, and IL-12-family signaling pathway, as well as runt-related transcription factors, which suggest that the DeltEx cells are active and primed to kill. Similarly, the expression of immune trafficking and stimulation markers was elevated in the final product, including TBOX transcription factors, type 2 cytokines and immune cell trafficking genes, suggesting that the cells are capable of both tissue trafficking and immune cell recruitment with no observed upregulation of γδT17 and/or terminally exhausted γδ cells. Gene expression levels for both DeltEx products were similar, suggesting our manufacturing platform is robust and reproducible. Our research demonstrates that similar activated, cytotoxic γδ T cell products were successfully manufactured from different allogeneic healthy donors. Both DeltEx DRI and Allo products exhibit similar gene expression profiles at high levels and showed an upregulation of genes unique to γδ T cells. These insights help elucidate the overall gene signatures that may play a key role in the unique biology and mechanism of γδ T cell therapies.

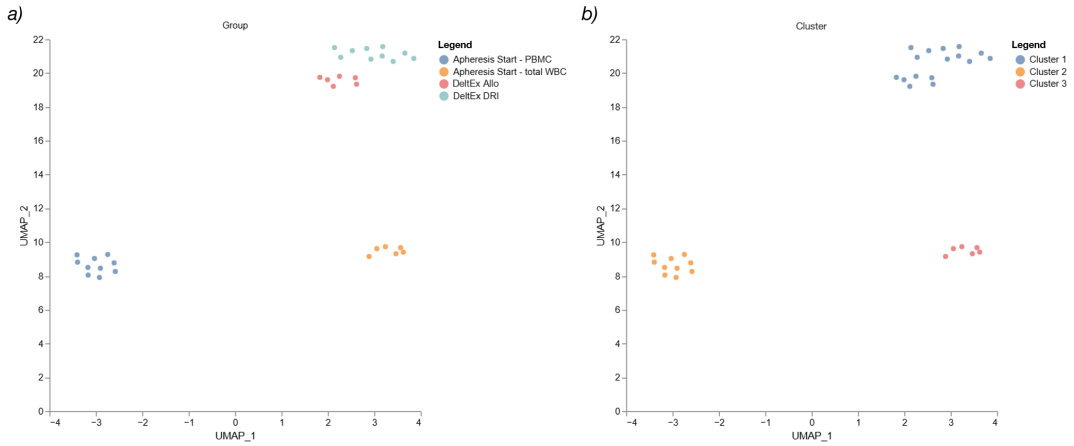

Figure 1: ***UMAP Immune Profiling Gene Expression*** *a) UMAP depicting the different groups including the manufacturing starting material: apheresis gradient separated (PBMCs) in blue, apheresis unmodified (total WBC) in yellow, and final γδ T cell products: DeltEX Allo in red and DeltEx DRI in teal. b) UMAP separated by cluster, where DeltEx Allo and DeltEx DRI form one cluster.*

416 Methods for characterizing AAV integration and genomic rearrangements *in vivo.*

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Gene therapy is an exciting and rapidly evolving field of medicine. Many viral vectors have been explored for gene delivery, including lentiviral and adeno-associated viral (AAV) vectors. Lentiviral vectors allow larger cargo to be introduced into dividing cells and mediate their effect by preferentially integrating within transcription units in the host genome. Recently, there has been some concern about potential insertional mutagenesis in lentiviral based CAR T cell therapy. However, several global reviews of large datasets show that the risk of insertional mutagenesis is very low, but warrants continued monitoring.

In contrast, typically non-integrating AAV vectors have become the vector of choice for much of gene therapy research. Despite limitations in cargo size, they have become a popular delivery vehicle for *in vivo* therapy due to their strong safety and efficacy profile. However, AAV integration does occur in many settings, and AAV vectors commonly show extensive rearrangement. This rearrangement likely reduces the efficacy of the therapeutic vector. Thus, it is crucial to characterize and understand AAV integration patterns to better inform safety and efficacy.

We developed and optimized methods for characterizing AAV vectors *in vivo*. Using ligation mediated PCR, we prime on the vector ITR to sequence either outwards (to determine host integration site) or inwards (to measure genomic rearrangements). The data is analyzed using a custom software pipeline, AAVengeR. This modular R pipeline allows analysis of integration of various vector types to determine the host-ITR/LTR junctions. Additional modules allow estimation of the percentage of sequencing reads associated with rearranged vector forms when sequencing reads face inward.

In one application, these methods were used to characterize samples from a non-human primate model of factor VIII (FVIII) gene therapy. African green monkeys were infused IV with varying doses of AAV8 containing the FVIII transgene under the liver-specific TTRm promoter. We collected liver biopsy samples at D14 and D90 post-infusion and selected 6 animals that had the highest vector copy numbers for characterization of both the integration site pattern (outward sequencing) and level of genomic rearrangement (inward sequencing). We detected high-levels (>1000 sites) of integration sites in 2/6 animals and mid-levels (>500 sites) in remaining 4/6 animals at these time points. Estimates of the frequency of genomic rearrangements ranged from 2-6%. These lower frequencies are in contrast to previously studied dogs after 10-years of treatment, where levels of rearrangement reached over 10% following the longer incubation period.

Thus, these methods are a powerful tool to characterize AAV integration patterns *in vivo* and to understand the status of the vector genome after AAV gene therapy. These methods, along with lentiviral integration site analysis and CRISPR off-target analysis, are available from the Viral Molecular High Density Sequencing Core at the University of Pennsylvania.

417 Unraveling the Dynamics of Senescent Cell and Immune System Interplay in HSPC Gene Therapy

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Hematopoietic stem and progenitor cells (HSPCs) are key targets in gene therapy (GT) due to their ability to reconstitute the hematopoietic system after gene correction and reinfusion. This approach allows the correction of genetic defects for various inherited and acquired diseases. However, vector integration near oncogenes and the acquisition of somatic mutations during hematopoietic reconstitution increase the risk of malignancies, as observed in clinical trials. Moreover, we previously showed that oncogene (BRAFV600E) expression in human HSPCs induces Oncogene-Induced Senescence (OIS), leading to irreversible cell cycle arrest and a pro-inflammatory senescence-associated secretory phenotype. This usually activates immune cells to clear senescent cells, but in immunodeficient (NSG) mice inefficient clearance leads to myeloid-skewed hematopoiesis, multi-organ histiocytosis, and bone marrow failure. Thus, understanding factors influencing senescent cell persistence or clearance is critical for improving GT safety and efficacy. Here, we investigated if a functional immune system in wild-type (C57) mice could counteract the toxic effects observed in NSG mice. Also, we explored immunological clearance as a protective mechanism to potentially identify genotoxicity biomarkers for future GT applications. We transduced mouse BM-derived HSPCs with lentiviral vectors expressing BrafV600E or GFP and transplanted them into lethally irradiated C57 mice or sub-lethally irradiated NSG mice. We monitored mice for long-term survival and performed peripheral blood (PB) analysis to assess hematopoietic reconstitution over time. NSG mice transplanted with oncogene-expressing (OE) HSPCs (NSG-V600E) exhibited rapid lethality and paracrine senescence, impairing donor and recipient PB cells. In contrast, 40% of OE-transplanted C57 mice (C57-V600E) survived long-term, showing only transient B and T cell impairment and clearing OE cells within three months. To identify biomarkers of genotoxicity, we performed RNA sequencing on donor myeloid (My) and B cells and cytokine profiling. Both models showed upregulation of multiple immune checkpoint genes in donor cells, implying immune suppression induced by OE cells. However, NSG-V600E mice had more differentially expressed genes (DEGs) in My cells, elevated IL-10, and downregulated Sirpa and Cd47, impairing donor My cell phagocytosis. This indicates an immune-suppressive environment enabling senescence cells persistence in NSG-V600E mice. C57-V600E mice displayed more DEGs in B cells, upregulated MHC Class II signaling in My and B cells, and a T-cell-related cytokine profile (IL-17A, IL12p70, IL-5), indicating recognition of OE cells by the adaptive immune system. To verify the role of adaptive immunity in the clearance of OE cells and exclude conditioning differences, we transplanted OE HSPCs into Rag1KO mice, which lack B and T cells and tolerate lethal irradiation. Rag1KO mice failed to clear OE cells and died within 40 days, proving adaptive immunity is essential for senescent cell clearance, inflammation resolution, and survival. These findings emphasize the importance of the interactions between senescent cells and the immunological status of the recipient, implying the active regulation of oncogene-induced genotoxicity by immune cells. Our results highlight the need to understand immune mechanisms governing senescent cell clearance to assess genotoxicity risks in GT and identify safety biomarkers.

418 Multiplex ddPCR Platform for Precision Monitoring of Multi-Antigen CAR T Cells in Pediatric CNS Cancer Therapy

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Background:
Pediatric central nervous system (CNS) tumors are the leading cause of cancer-related deaths in children. Targeting these tumors via surface antigens is challenging due to the antigen heterogeneity and temporal changes in tumor antigen expression. While our BrainChild-03 (BC-03, NCT04185038) demonstrated tolerability of intracerebroventricular (ICV) delivery of B7-H3 CAR T cells for diffuse intrinsic pontine glioma (DIPG), efficacy was inconsistent. To address this, we initiated BrainChild-04 (BC-04, NCT05768880), a first-in-human phase 1 clinical trial delivering ICV multi-antigen (B7-H3, EGFR, HER2, and IL-13Ra2) CAR T cells in children with recurrent/refractory CNS tumors and DIPG. Here, we present analytical methods to accurately quantify and characterize CAR T cells in patient infusion products and in post-infusion biospecimens to optimize patient outcomes.

Methods:
A fluorescence amplitude multiplex droplet digital PCR (ddPCR) assay was developed to quantify vector copy number (VCN) in infusion products and patient samples (Figure 1a). Using a probe ratio-based strategy, the two-channel ddPCR system simultaneously detected individual CARs (B7-H3, IL13zetakine, EGFR806, HER2) within a single well alongside FLAP-EF1a (total CAR) and albumin (reference gene) in a parallel well. The assay was used to analyze infusion products (n=31), as well as cerebrospinal fluid (CSF) and peripheral blood (PB) samples (n=45) post-dosing from BC-03 and -04 trials. The samples included post-flow cytometry PFA-fixed samples (CSF n=13) and fresh samples (PB n=28, CSF n=4) unsuitable for flow cytometry due to time or quality constraints.

Results:
The assay demonstrated high sensitivity, specificity, and a dynamic range of 1-100,000 copies/reaction (Figure 1b). Validation against a qPCR-based FLAP-EF1 assay showed strong concordance (R^2 =0.999), confirming assay reliability for clinical sample analysis. The assay characterized CAR representation and transduction patterns in BrainChild-04 infusion products (n=31), (Figure 1c), detecting FLAP-EF1 at 2.00 ± 0.26 copies/ng, with individual CARs detected as follows: B7H3 (0.577 ± 0.11), EGFR806 (0.366 ± 0.07), HER2 (0.536 ± 0.09), and IL13zetakine (0.440 ± 0.07). Preliminary patient sample analysis (n=45) revealed CAR T cell persistence in CSF. In BC-04 CSF samples (n=13), FLAP-EF1 was detected in 92% of samples, with CAR-specific frequencies of B7H3 (6/12), EGFR806 (9/12), HER2 (8/12), and IL13zetakine (10/12 samples). Matched BC-04 CSF and PB samples (n=13) showed higher CAR detection in CSF (12/13) versus PB (0/13), indicating localized expansion. Flow cytometry data using EGFRt (a CAR transduction tag) cross-validated ddPCR findings. In BC-04 CSF samples, all EGFRt-positive (5/5) and EGFRt-negative (5/5) samples by flow cytometry were detected by FLAP-EF1 ddPCR, demonstrating the assay's higher sensitivity. Similarly, all EGFRt-positive BC-03 CSF samples (4/4) by flow cytometry were FLAP-EF1 positive by ddPCR.

Conclusion:
Amplitude multiplex ddPCR is a robust platform for multi-antigen CAR quantification, offering critical insights into CAR T cell persistence in pediatric CNS tumors after ICV delivery. Ongoing correlative analysis from the BC-04 trial will further validate its utility. Combining VCN-based ddPCR with multiparameter flow cytometry enhances understanding of CAR T cell dynamics, guiding future therapy design, dosing strategies, and mechanistic studies for CNS tumors.

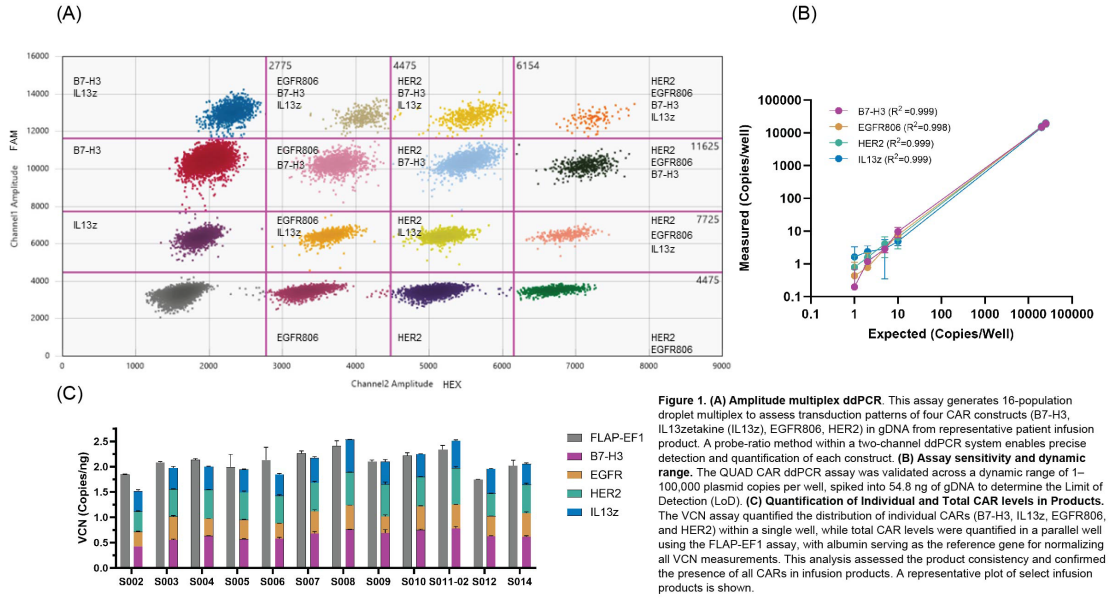

Figure 1. (A) Amplitude multiplex ddPCR. This assay generates 16-population droplet multiplex to assess transduction patterns of four CAR constructs (B7-H3, IL13zetakine (IL13z), EGFR806, HER2) in gDNA from representative patient infusion product. A probe-ratio method within a two-channel ddPCR system enables precise detection and quantification of each construct. **(B) Assay sensitivity and dynamic range.** The QUAD CAR ddPCR assay was validated across a dynamic range of 1–100,000 plasmid copies per well, spiked into 54.8 ng of gDNA to determine the Limit of Detection (LoD). **(C) Quantification of Individual and Total CAR levels in Products.** The VCN assay quantified the distribution of individual CARs (B7-H3, IL13z, EGFR806, and HER2) within a single well, while total CAR levels were quantified in a parallel well using the FLAP-EF1 assay, with albumin serving as the reference gene for normalizing all VCN measurements. This analysis assessed the product consistency and confirmed the presence of all CARs in infusion products. A representative plot of select infusion products is shown.

419 Non-*Rian* Integrations in AAV-Associated HCCs From a Murine Model of Krabbe Disease

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Background
Prior studies established that genotoxic vector integrations into the mouse *Rian* locus are associated with hepatocellular carcinoma (HCC) following systemic administration of AAV vectors. The Twitcher (Twi) mouse, a model of Krabbe disease, is deficient in galactosylceramidase (GALC) activity. While CNS-directed AAV2/9-GALC-mediated gene therapy combined with bone marrow transplantation and a substrate reduction drug results in an impressive increase in the median lifespan from ~45 days to ~400 days, >90% of the Twi mice, as well as 100% of the wild type controls, succumb to HCC (PMC7854295). HCC has never been reported in untreated Twi mice. A targeted sequence capture analysis using a tiling array with probes specific for the AAV vector was used to map ITR-containing integrations in or near *Rian*, or other cancer- and cell-growth/death-associated genes, in the HCCs. However, the integrations were not further characterized.

Methods
We developed a bioinformatics method designated as AVITA (Assembly of Viral Integrations from Tiling Arrays) to mine tiling array sequence data to reconstruct full length integrations and have applied it to both *Rian* and non-*Rian* integrations. We then used targeted PCR amplification followed by Sanger sequencing to validate the *in silico* predictions.

Results
Two non-*Rian* integrations were fully reconstructed *in silico:* one in intron 8 of the *Akap13* gene, and one in intron 5 of *Foxp1*. Both have a single truncated 3' ITR and an intact β-globin polyA signal. The integration in *Akap13* is a single 612nt fragment of the AAV vector transgene, while the event in *Foxp1* appears to be the product of a rearrangement that resulted in a ~1500nt insertion composed of two vector fragments. Using primers outside of the integration and primers designed from the assembly, we PCR amplified the left and right sides of the integrations. The PCR products were cloned into the pCR.4-Topo vector and sequenced, confirming the *in silico* integration site predictions.

Discussion
A-kinase anchor proteins like AKAP13 confine protein kinase A to specific locations within the cell; FOXP1 is a forkhead box transcription factor active in development and maturity. Both may have tumor suppressor activity. In the mouse, the integrations in *Akap13* and *Foxp1* fall near predicted enhancer-like elements. In the orthologous regions of the human genome, both integrations fall in DNAse hypersensitive regions that also contain histones with methylation patterns consistent with regulatory elements. Thus, the mouse and human genomic contexts of the two integrations suggest that they could disrupt regulation of *Akap13* and *Foxp1*. Furthermore, the integrated AAV sequence in the two non-*Rian* integrations contains the vector 3' ITR and polyA signal. This contrasts with the six *Rian* integrations that we identified by AVITA, which all contain a single truncated 5' ITR and parts of the vector enhancer. Thus, while genotoxic integrations into the *Rian* locus appear to act by transactivation of proximal microRNAs and the *Rtl1* gene (PMC4319425), the polyA signal in the non-*Rian* integrations could serve as a gene trap, disrupting the tumor suppressor activity of *Akap13* or *Foxp1*. Mechanistically, these integration events resemble some of the human genotoxic events described by Nault et al (PMID 26301494). Determining whether non-*Rian* AAV integrations can act synergistically in the formation of HCC will require further functional studies.

420 A PCR-Free Approach Combining CRISPR-Cas9 and Long-Read Sequencing Reveals Unpredictable AAV Integration in the Host Genome

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The integration of AAV vector genomes has been documented for many years. While no direct evidence has established that vector genome integration causes tumorigenicity, experts remain concerned about this theoretical risk to patients. Our understanding of vector genome integration remains limited, primarily due to sequencing technology constraints. Currently, efforts to enrich integrated AAV genomes often rely on PCR-based amplification strategies. Typically, sequencing templates are generated using a combination of AAV-ITR-specific and adaptor-specific primer amplification. However, these PCR-based methods face challenges, particularly due to the highly complex structure of the AAV ITRs, which are rich in GC content. This complexity often results in the generation of non-native sequence reads, further complicating accurate characterization of integrated AAV genomes. To address these challenges, we have introduced an amplification-free approach using a CRISPR-Cas9-based method for upstream library preparation, coupled with long-read sequencing on a nanopore platform. This strategy aims to provide a more accurate analysis of AAV vector genome integration.

The method was assessed using the HeLa cell line, which was transduced with the AAV2-CAG-GFP vector for six weeks. HeLa cells expressing GFP were harvested via flow cytometry, followed by DNA extraction and library preparation as described in Figure 1A. A total of 44181 valid, integrated vector genome reads containing both AAV and human genomic sequences were obtained. Of these, 25255 reads contain both ITR and CAG promoter. The 14588 unique sub-reads originating from CRISPR-Cas9 cleavage were aligned to the AAV genome reference sequence (Figure 1B). The plot in Figure 1C illustrates the ratio of CRISPR-Cas9 cleavage reads to total integration reads at each cleavage position, covering almost all integration sites. The integrated vector genomes primarily included (1) backbone DNA without ITR (8.10%), (2) 5′ ITR plus truncated AAV DNA (16.5%), (3) 3′ ITR plus truncated AAV DNA (22.8%), and (4) partial AAV DNA without ITR (52.2%). Typical structures of the integrated vector genomes include both the basic structures and the complicated re-arranged structures with multiple full or partial ITR elements in various directions (Figure 2B). The junction sites along the ITR element mainly locate in the “trs” and “the outside border of D’”. The reads were widely distributed across all 24 chromosomes (reference Human hg38). The lengths of integrated vector genome sequences ranged from a maximum of 6987 bases to a minimum of 29 bases. These results reveal that AAV integration events in the host genome are complicated and unpredictable.

The PCR-based amplification methods often exclude partial vector genomes lacking ITRs due to the reliance on ITR-specific primers. In contrast, this study revealed that approximately 52% of reads integrated into host genomes were from partial AAV DNA sequences lacking any ITRs and around 8% of reads were from the vector backbone sequence, even containing full-length Ampicillin gene, which will further raise concern on AAV safety. These findings suggest that this amplification-free method is a valuable tool for analyzing AAV genome integration. Additionally, it may be broadly applicable to virus integration research, facilitating and accelerating studies on vector biology.

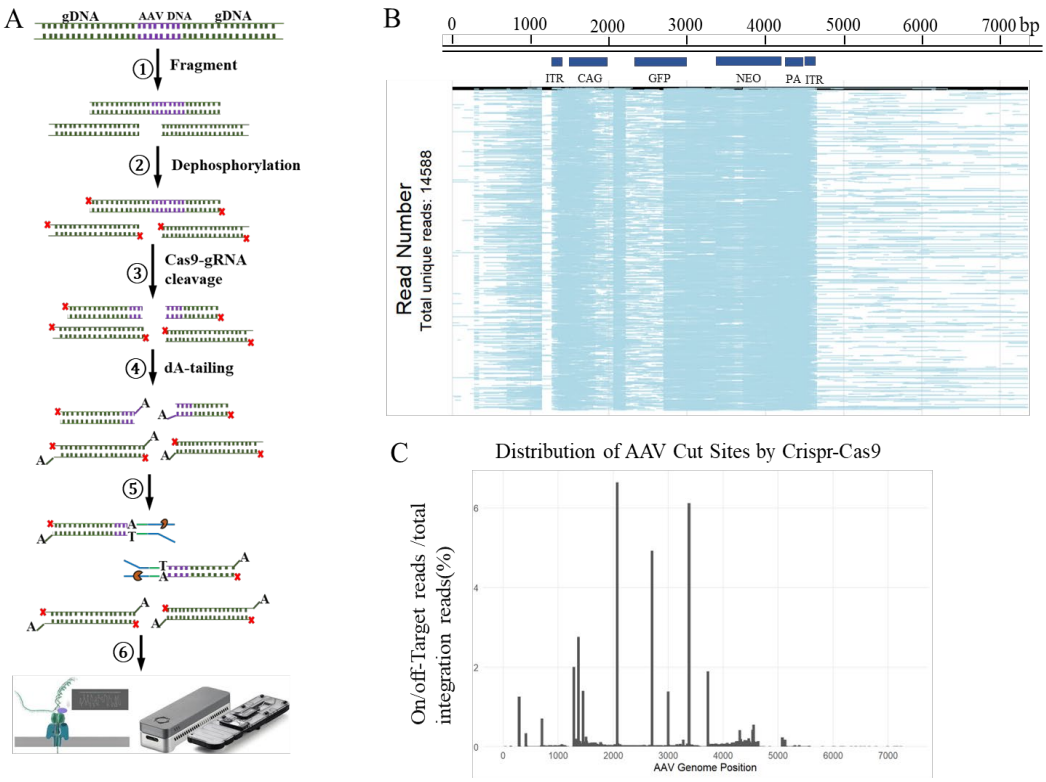
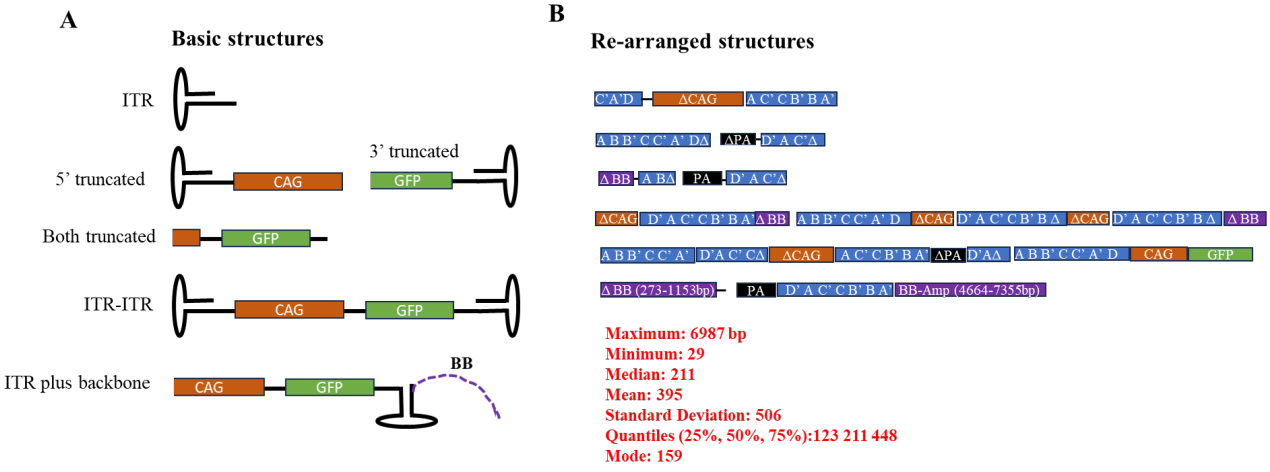


Figure 1. AAV integration analysis through PCR-free amplification based on Crispr-Cas9 cleavage combining Nanopore long-read sequencing.
A: Workflow for Nanopore long-read sequencing AAV integration DNA. The procedure starts from a randomly fragmented DNA sample (1). All ends of the fragmented DNA molecules are dephosphorylated to block adapter ligation (2). The integrated AAV DNA fragments containing gRNA binding sites are cleaved by Cas9 (3) and all 3′ ends are dA-tailed (4). Sequencing adapters are then ligated to Cas9 cleaved and dA-tailed ends (5) and these molecules are sequenced on the Nanopore PromethION P24 system (6). **B:** All Cas9-cut reads (14588, after removing barcodes and following A/Ts) were aligned to the AAV2-CAG-GFP vector genome reference sequence. The details of integration and Crispr-cas9 cleavage determination can be found in **C**. **C:** From all sequencing reads that contain both AAV and human genomic sequences (the total integration reads of 45255), we identified the subset of reads that show evidence of being cleaved by Cas9. These "Cas9 cleavage" reads are then mapped back to the AAV vector genome, and the plot displays the ratio of Cas9 cleavage reads to the total integration reads. Integration reads are determined as follows: BWA is used in Linux to align sequencing reads to both AAV and human (hg38) AAV reference genomes (pssAAV-CB-EGFP_ARM), followed by sorting and indexing using Samtools. If both human and AAV genome pieces are present in a read, then that read is an integration read. Cas9-cleavage reads are determined as follows: R is used to analyze each read for the presence of any barcode sequence (from a known list of barcode sequences). Once a barcode is found, we skip all consecutive A or T nucleotides immediately following it and then check the next portion of the read for a gRNA-related sequence. Two criteria are used to identify a crispr-cas9 cut read, allowing up to 2 nucleotide mismatches:3′-end + PAM check: The next 6 bases in the read should match the last 3 bases of any of the 10 known gRNAs, plus the associated PAM sequence. 5′-end check: Alternatively, the following 17 bases in the read should match the first 17 bases of any of the 10 known gRNAs. If either of these conditions is met (with up to 2 mismatches allowed), the read is classified as having been cut by crispr-cas9.



Supplementary Figure 3 The integrated AAV DNA molecular structure.
A: Basic structures of integrated AAV DNA. ITR: only containing full or partial ITR sequence; 5' truncated: 5'ITR plus partial vector genome; 3' truncated: 3'ITR plus partial vector genome; Both truncated: partial vector genome without any ITR sequence; ITR-ITR: full length vector genome from 5'ITR to 3'ITR; ITR plus backbone: ITR plus partial vector DNA backbone sequence. B: Typical rearranged structures of integrated AAV DNA. BB-Amp (right, bottom): the whole Ampicillin expression cassette is included. Δ means truncated.

421 Overcoming Barriers To Accessibility: Scalable, Cost-Efficient Biomanufacturing For Gene Therapies.

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The transformative potential of cell and gene therapies is evidenced by the increasing number of regulatory approvals, with over 20 products now on the market. However, pricing and reimbursement have affected **patient access**, highlighting significant commercial hurdles. These challenges, exacerbated by **high manufacturing costs** and the **constrained funding landscape**, threaten the ability of developers to address unmet medical needs effectively. In this context, achieving **cost-efficient, large-scale production** is paramount to ensuring the commercial viability of gene therapies. While the biologics industry has made remarkable strides in volumetric productivity through **upstream intensification** and **continuous processing** in stirred-tank bioreactors (STRs), gene therapy developers lag behind. This gap is largely attributed to the unique complexities of viral vector manufacturing. STR systems also face inherent scalability limitations—shear stress, mixing, and gas exchange cannot be simultaneously and linearly scaled—restricting process design flexibility. These constraints delay time-to-market, inflate costs, and limit accessibility, thus increasing risks for developers. Structured fixed-bed bioreactors (FBRs) present a compelling solution to these challenges by enabling significant upstream process intensification and facilitating continuous processing without the scalability constraints of STRs. FBRs support **low-volume, low-footprint biomanufacturing**, allowing for substantial **reductions in cost of goods (COGS)** while **expediting development timelines**. This presentation will highlight real-world case studies demonstrating the advantages of FBR technology, including

- Enhanced productivity: Achieving **2- to 10-fold higher specific productivities** for a wide range of viral products.
- Improved product quality: Delivering a **3-fold increase in AAV full-to-empty capsid ratio** and up to **5- and 10-fold reductions in host cell protein and DNA**, respectively.
- Cost efficiency: **Reducing drug substance COGS by up to 50%**.
- Accelerated scalability: **Successful transfer and scale-up** of a viral manufacturing process by a CDMO, from bench to commercial scale **in under nine months**.

These insights underscore the transformative potential of structured FBRs to **redefine scalability, cost efficiency, and accessibility** in gene therapy manufacturing.

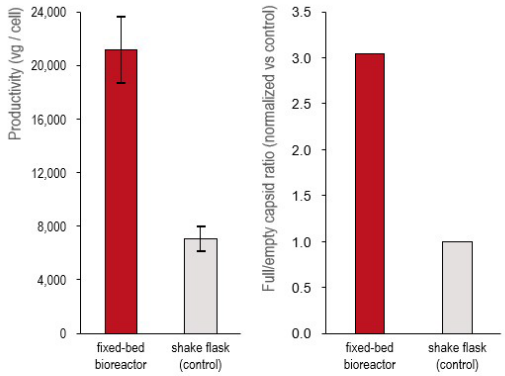

Fig 1. Fixed-bed bioreactor displays 3-fold increase in productivity and in full/empty capsid ratio for AAV2 production

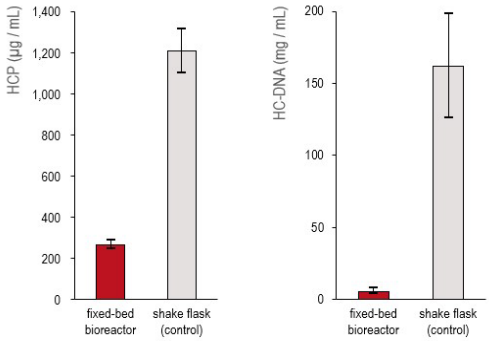

Fig 2. Fixed-bed bioreactor displays 5- and 10-fold reduction in host cell protein (HCP) and host-cell DNA (HC-DNA), respectively, for AAV2 production

422 "Improved AAV Vector Production Yields From An Optimised Manufacturing Process in Fixed-Bed Bioreactors"

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**Corresponding Author*

Introduction:
Developing reliable and efficient AAV vector manufacturing processes is a top priority in the field of gene therapy to ensure broader accessibility. AAV vector production in fixed-bed bioreactor has shown high potential due to benefits like a single-use, fully integrated system with large surface area in a small footprint as compared to suspension culture systems. The main challenges in bioreactor-based vector production are low yield and prolonged production time, which lead to higher costs for the final product. Optimising the bioreactor culture, transfection, harvest, and recovery processes can be optimised to improve the per cell vector production potential. Therefore, we tested, using a DOE approach, the effect of a set of parameters such as seeding density, plasmid DNA/cell transfected and combined harvest of both supernatant and lysate on final AAV yields.

Methods:
All bioreactor runs were performed in Cytiva® iCellis Nano bioreactor (1.06m^2). The Hek293T cells were seeded at 30,000-37,000 cells/cm^2 following aseptic conditions and expanded to reach 1.7-2x10^^6 cells/carrier strip before transfection with packaging plasmids [pRep2-Cap9, pHelper, and pAAV-ITR.EGFP] and PEIPro® (Polyplus) transfection reagent in a 1:1 to 1:2 ratio. The cell numbers, and culture conditions (pH, Dissolved Oxygen, and Media Glucose concentration) were closely monitored throughout the run. Post transfection (72 hrs and 120 hrs), culture media was harvested followed by cell lysis from carrier strips. Post-harvest, AAV vectors were captured using an Affinity chromatography-based system. Vector titration and quality checks were carried out following standard methodologies.

Results:
Our results indicate that process variables like cell seeding, DNA amount/per cell, and harvesting methods contribute most significantly towards increasing vector yields. The high cell seeding density (30,000 cells/carrier strip) resulted in a reduced cell expansion phase (3-5 days) and further compressed run time to 10 days compared to earlier reports. We have used lower amounts of total DNA (1ug/million cells) for transfection and achieved 2.5 × 10^5 vg/cell at this ratio, improving on previously published reports. Intensification of vector harvesting steps during these runs resulted in an 8-fold increase (crude yield 4.92x10^14vg) compared to the first run (crude yield 5.66x10^13vgs). This enhancement was also evident during the vector purification process, with a recovery rate of 90.26%-98.65%.

Conclusion:
To address challenges in the AAV vector production process, we have systematically optimized critical production parameters. The duration of cell expansion phase had a significant impact on vector production along with biological parameters such as pH, plasmid concentration, and metabolite concentration. We report an AAV vector production process with a significantly shorter timeline (10 days) in fixed-bed bioreactor. Our data shows that this process can serve as a scalable and cost-effective strategy for vector production. We anticipate that our optimized bioreactor-based vector production protocol can be adapted effectively for pilot-scale production processes.

423 Simplifying Large-Scale Upstream AAV Production Via a Transfection Complex Stabilizing Reagent

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Adeno-associated virus (AAV) has become the leading vector for gene therapy due to minimal pathogenicity and the ability to facilitate long-term gene expression. The success of AAV as a gene therapy has resulted in the transition from treatment of rare diseases to broad disease indications where the treatable population is sizeable; likewise, there is an increase in the development of high dose therapies. To meet the supply needs from both a therapeutic and cost prospective, the industry is moving to increasingly larger scale bioreactors for the manufacture of recombinant AAV. The production of AAV in bioreactors via transient transfection requires large volumes of liquids to be mixed, incubated, and then transferred to the bioreactor. Transfection complexes often demonstrate optimal performance after a short complex formation time (CFT), which typically range from 10-45 minutes. This small window of time can present challenges and risk in properly forming complexes and delivering them to the bioreactors quickly enough. For example, a 1000 L bioreactor would require transferring 50 L of a 5% complex, which at a pumping rate of 1 L/min would take almost an hour to deliver.

To navigate this hurdle, Mirus Bio has developed a transfection stabilization reagent which can extend the CFT for a transfection complex made with *Trans*IT-VirusGEN® from minutes to several hours, without a loss in performance. This reagent stabilizes transfection complexes across several GOIs, serotypes, DNA doses, and reagent:DNA ratios. As demonstrated through dynamic light scattering (DLS), this stabilization effect is the result of slowing the growth of transfection particles, resulting in similar sized particles at both short and long CFTs. Transfection complexes can also be stabilized by this reagent when the complex volume is reduced from 5% to 2%, which will also decrease the volume and therefore the time needed to transfer a transfection complex to a large-scale bioreactor. The complex stabilization reagent can be used successfully in a wide range of complex formation media and maintains performance after filtering, allowing it to be easily integrated into an existing workflow with the *Trans*IT-VirusGEN Transfection Reagent and the *Rev*IT™ AAV Enhancer. The transfection complex stabilizer is scalable, as stabilized transfection complexes were successfully delivered to 2 L bioreactors after 3 hours of complex formation with equivalent performance to transfection complexes formed for 30 minutes. These data demonstrate the efficacy of the transfection complex stabilizer during AAV manufacture and will substantially improve workflows and success rates in large-scale bioreactors.

424 From Bench to Bioprocess: Bridging AAV Capsid Engineering and Scalable Manufacturing for Clinical and Commercial Translation

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Introduction
The development of novel recombinant AAV vectors aims to improve tropism, transduction efficiency, and immune evasion for targeted gene delivery in clinical and commercial applications. However, capsid engineering and bioprocess optimization have traditionally been pursued separately—with capsid innovation focusing on biological function and bioprocess development optimizing production efficiency. This disconnect results in promising capsids encountering the same scalability challenges as existing serotypes, including packaging efficiency, high-titer production, and downstream purification. To address this, we present an integrated strategy aligning capsid engineering with scalable manufacturing, evaluating the production of AAV-LH9, a rationally engineered vector, and an AAV capsid library using commercially available bioprocess technologies, including the iCELLis Nano™ fixed-bed bioreactor and AAVX affinity purification. This approach ensures that vector design and optimization incorporate scalability considerations from the outset.

Methods
A systematic DoE was conducted in HEK293T adherent cultures to optimize transfection conditions, DNA:PEI ratios, and media composition for AAV-LH9 and AAV9. Optimized parameters were transferred to the iCELLis Nano, a scalable fixed-bed bioreactor designed for high-density AAV production, and an AAV capsid library was produced to assess high-throughput vector screening potential. Following harvest, AAV-LH9, AAV9, and the library were purified using AAVX affinity chromatography, a platform optimized for large-scale AAV recovery. qPCR quantified vector genome (vg) titers, and ELISA determined the full:empty capsid ratio. Transduction

efficiency was assessed in vitro and in vivo by delivering equivalent vg doses of AAV9, AAV-LH9, and selected library variants, with transgene expression analyzed to evaluate infectivity and tropism.

Results
Comparable titers were achieved for AAV9 and AAV-LH9 in small-scale adherent cultures and the iCELLis Nano, demonstrating process scalability. The AAV capsid library was successfully produced in the iCELLis Nano, confirming its suitability for large-scale capsid screening. AAVX affinity purification enabled efficient downstream processing, ensuring a robust workflow. Transduction studies in vitro and in vivo demonstrated that AAV-LH9 exhibited comparable transduction efficiency to AAV9, supporting its therapeutic potential. Library screening identified variants with similar or improved transduction profiles, reinforcing the feasibility of high-throughput capsid discovery using scalable platforms.

Conclusions
This study demonstrates the successful integration of capsid engineering with scalable AAV bioprocess development, ensuring high-yield production using commercially available technologies. The optimized process was successfully scaled from small-scale cultures to the iCELLis Nano, confirming robustness and reproducibility. The efficient production of an AAV capsid library in iCELLis Nano highlights its potential for large-scale high-throughput screening, accelerating the discovery of novel vectors. AAVX purification enabled scalable downstream processing, while AAV-LH9 showed comparable transduction to AAV9 in vitro and in vivo, supporting its therapeutic viability. By aligning capsid innovation with scalable bioprocesses, this approach facilitates the efficient translation of engineered AAV vectors for clinical and commercial applicatio

425 Enhancing AAV Production Efficiency: A Dual Approach to Design of Experiments Optimization and Economic Feasibility Analysis

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Adeno-Associated Virus (AAV)-based gene therapies show great promise, but their development is hindered by high production costs, particularly due to expensive plasmids and transfection reagents used in transient transfection. This study applies Design of Experiments (DoE) to select plasmid ratios and quantities in AAV vector production, focusing on two critical aspects: enhancing manufacturing productivity and assessing the economic viability of DoE implementation in preclinical studies. We applied DoE to improve transfection-related parameters for cost-effective AAV production scaled up for non-human primate studies. Using an R-based open-source DoE pipeline, we reduced the 3-plasmid ratio combinations to be tested from 14,553 to 28. Transfections were performed in a HEK293F-derived clonal suspension cell line, with vector titers measured by ITR2-qPCR. We identified plasmid ratios that increased productivity up to 4-fold compared to other conditions in the same experiment, achieving titers of up to $7.0x10^{14}$ VG/L. A Response Surface Model (RSM) was created and verified, demonstrating significant improvements over our previous best-performing conditions. We then tested the optimized conditions across multiple AAV serotypes (2, 3, 5, 8) and two transfer plasmids. While most serotypes showed improved productivity, with AAV2 achieving nearly 300% increase, AAV5 experienced a slight titer decrease. These results underscore the importance of serotype and construct-specific optimization in AAV production processes. To address the economic viability of DoE implementation in preclinical studies, we conducted batch size and cumulative production analyses using cost modeling. We evaluated DoE applicability across various scales ($1.0x10^{13}$ to $1.0x10^{16}$) and in scenarios of multiple independent productions. This analysis provides insights into the production scale and frequency at which DoE implementation becomes cost-effective. Our comprehensive approach not only demonstrates the benefits of DoE in AAV vector production but also offers valuable guidance for strategic decision-making in vector manufacturing. These findings have the potential to accelerate the development of more affordable gene therapies by optimizing production processes and resource allocation in preclinical studies. Moreover, this study provides a framework for balancing productivity gains against implementation costs, potentially streamlining the path from preclinical development to clinical trials in the gene therapy field.

426 Transfection Reagent Selection and Post-Transfection Feeding of HEK293 AAV Production Platforms Based on GOI and Serotype

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Adeno-associated virus (AAV) has become a prominent vector to treat life-threatening genetic diseases. As the demand for AAV therapeutics increases, so does the need for large quantities of high titer AAV. For HEK293 production platforms, transfection reagent selection, experimental optimization, and post-transfection feeding are vital to achieving the high AAV titers essential to support this rapidly growing industry. PEI (polyethyleneimine) has been the most commonly used transfection reagent for AAV viral gene therapies, and we have optimized PEI transfections with multiple HEK293 cell lines, AAV serotypes and GOIs (gene of interest) to maximize AAV titers. In addition to PEI transfection optimization, we have found a commercially available feed (Cellvento® ModiFeed Prime) that can significantly increase titers across multiple cell lines and serotypes but varies based on GOI and transfection reagent. Recently, we have begun working with non-PEI based transfection reagents from Mirus Bio, *Trans*IT-VirusGEN®+ RevIT® AAV Enhancer and the TransIT®-AAViator System. The titers achieved with the Mirus reagents are 2 logs higher than PEI-based reagents prior to transfection optimization and the ideal reagent varies with the GOI (3 evaluated) and serotype (AAV5, AAV8, and AAV9).

427 The use of novel PAT and soft sensor as process monitoring tool during AAV manufacturing

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Due to the high manufacturing costs of viral vector-based gene therapies, ensuring product quality, safety, and consistency is pivotal. In adeno-associated virus (AAV) manufacturing, in-line monitoring technologies provide real-time insights into critical process parameters (CPPs) and quality attributes (CQAs). In this work, a miniaturized process analytical technology (PAT) platform from Jobst Technologies, specifically designed for cell and gene therapy manufacturing, was applied. The novel sensor system is being developed to monitor key metabolites (glucose, lactate, glutamine, glutamate), pH, and dissolved oxygen with minimal sampling volume and without human intervention. The prototype demonstrated robustness and accuracy with calibration solutions and under process conditions. For AAV production, this type of sensor is essential for understanding cell culture dynamics, particularly during transfection and virus production. Data sets were generated using the exoReady manufacturing platform in a Bioblu 1c stirred tank bioreactor and compared with offline and historical data (Cedex Bio HT, Roche). Novel PAT tools are enablers for the development of process soft sensors. Lactate, ammonium (metabolic byproducts), and LDH (cell health marker) have been identified as key indicators of shifts in the cell culture phases and AAV yield. Correlations between metabolic rates and AAV accumulation were calculated. The validity of the correlations is being further evaluated in a perfusion process. Prediction of AAV titers with a model allowed us to use them as soft sensor of process performance. When soft sensors alone were insufficient to explain low or instable titers, we evaluated the potential of novel transcriptomics PAT platform (Gensensor) to analyze gene expression, uncovering cellular stress pathways and production bottlenecks.

Real-time metabolite tracking, combined with transcriptomic when deeper insights is needed, allows early detection of process deviation. Looking towards the future, the application of soft sensors is expected to evolve further to include the monitoring of CQAs, such as product-related impurities (e.g., empty capsids, host cell proteins, and nucleic acids). The development of soft sensors can revolutionize AAV manufacturing with proactive quality control strategies.

B-cell and Solid Organ Therapies

428 CD19 CAR EngTreg Effectively Modulate Activated Human B Cells and Antibody-Secreting Plasma Cells

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Preliminary results from Phase I clinical trials of adoptive CD19 CAR T cell therapy in treatment-refractory systemic lupus erythematosus (SLE) demonstrated rapid and sustained therapeutic benefit despite limited CAR T cell persistence. However, CAR T therapies face potential challenges, including cytokine release syndrome (CRS) and limited ability to modulate the pathogenic memory T cell subsets that drive autoreactive B cell proliferation and differentiation and additional disease manifestations. To address these potential limitations, we developed CD19 CAR-engineered regulatory T cells (EngTreg), which leverage the CD19-targeting specificity of traditional CAR T cells but are anticipated to operate through multiple mechanisms including modulation of both pathogenic B and T cells, and therefore hypothesized to provide broad and sustained bystander immune tolerance with an improved safety profile. We generated and tested EngTreg expressing both CD19 CAR and a chemically inducible IL-2 receptor (CISC) using homology directed repair (HDR)-based gene editing. As previously described by our group, this approach overcomes the scarcity of antigen-specific natural Treg by converting bulk CD4+ T-cells to a Treg phenotype via enforced expression of FOXP3. To produce CD19 CAR EngTreg, human $CD4^+$ T cells were isolated and transduced with lentivirus encoding the CD19 CAR. The *FOXP3* locus was HDR-edited using an AAV repair template to enforce FOXP3 expression along with expression of the CISC cassette for enrichment. We also employed an alternate method where CD4+ T cells were dual-HDR edited at *FOXP3* and *TRAC,* using AAV donor cassettes targeting both loci. Successful engineering with either strategy resulted in constitutive expression of CD19 CAR, endogenous FOXP3, and the synthetic CISC receptor, thereby permitting enrichment of therapeutic cell products to high purity in the presence of rapamycin. Cytotoxicity assays indicate that CD19 CAR EngTreg are less cytotoxic than CD19 CAR T cells and bear the hallmarks of a Treg phenotype with high FOXP3, CD25, and CTLA-4 expression. CD19 CAR EngTreg also feature reduced production of key pro-inflammatory cytokines in response to CAR stimulation when compared to CD19 CAR T cells. Next, using a robust *in vitro* human plasma cell (PC) differentiation model capable of generating engraftable, long-lived human PCs, we found that CD19 CAR EngTreg effectively block the differentiation of B cells into $CD38^{hi}$ $CD138^+$ plasma cells and inhibit IgM and IgG antibody production. PC differentiation blockade could be induced both during B cell expansion, when CD19 levels are high, and during PC/plasmablast differentiation, as CD19 is progressively downregulated. These findings underscore the versatility and broad applicability of this approach. Finally, in a humanized mouse model, adoptive transfer of autologous CD19 CAR EngTreg ablated splenic human B cells with efficacy comparable to CD19 CAR T cells. This outcome was achieved despite markedly reduced levels of pro-inflammatory cytokines including IFN-γ and CXCL10 at 7 days post T-cell transfer. Comparable numbers of CD19 CAR+, CD4+ T cells were present in the spleen at endpoint for both CAR T and EngTreg groups, indicating sustained engraftment for both products. Taken together, these findings suggest that CD19 CAR EngTreg may offer a promising therapeutic avenue for SLE and other B cell-mediated autoimmune disorders, where precise immune modulation is paramount.

429 Synthetic Signaling to Repurpose Inflammation-Associated with Arthropathy Toward Disease Resolution

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INTRODUCTION:
Pro-inflammatory cytokines IL-1β and IL-6 are pro-inflammatory cytokines that play major roles in the pathology of arthropathies.1 Several IL-1β and IL-6 blockers have been clinically approved for use in treating arthropathies, though systemic administration of these drugs can have severe side effects. This motivates the need to develop

avenues to antagonize cytokine signaling selectively and specifically. Our prior work has demonstrated that cells engineered with synthetic Notch (synNotch)2 receptors display spatially regulated responses to soluble inputs captured by a programmable biomaterial surfaces.3 Here, we build off that work and developed methods that use IL-1β and IL-6 as inputs to activate engineered cells to respond via synthetic gene circuits to spatially constrain expression of pro-resolution transgenes. We hypothesize that combining a regenerative medicine approach with synthetic biology principles will allow for programmable responses to inflammatory cytokines.

METHODS:
We used a synthetic receptor platform synNotch, which is based on the native Notch receptor, to recognize IL-1β and IL-6 and activate downstream gene circuits. By leveraging synNotch in conjunction with ligand-capturing biomaterials targeted to cytokines of interest, we can localize soluble IL-1β and IL-6 to desired regions and rredirect cytokine signaling into transgene expression via synNotch signaling. We developed IL-1β and IL-6 responsive mouse mesenchymal stem cell (mMSC) lines that express both a cytokine-specific synNotch receptor and a reporter mCherry and luciferase transgene payload.[BJ1] Both synNotch cell lines were tested using a functionalized cell culture plate coated with a monoclonal antibody that binds to an epitope of IL-1β or IL-6 that does not overlap with the epitope recognized by the cognate synNotch receptor (Fig. A). This allows for the immobilization of either IL-1β or IL-6 and subsequent activation of synNotch cells, which is measured via plate reader-based luminescence assays. For macrophage polarization experiments, mMSCs were engineered to express mouse IL-10 and IL-4 in response to synNotch activation. Macrophage polarization was measured via flow cytometry of surface marker expression of CD163, CD206 and CD86. Cytokine levels were measured using ELISA.

RESULTS:
Here, we demonstrate that mMSCs engineered with IL-1β and IL-6 responsive synNotch receptors can significantly activate reporter luciferase transgenes with cytokine concentrations as low as 1 ng/ml (Fig. B-C). Since inflammatory macrophages are commonly found at the site of joint injury, we hypothesize that polarizing macrophages toward an anti-inflammatory phenotype will reduce local inflammation that contributes to arthritis. After co-culturing engineered mMSCs with RAW 264.7 macrophages[BJ2] , we saw significant upregulation in both CD163 and CD206 in response IL-1β supplementation, indicating a more anti-inflammatory phenotype (Fig. D) compared to control reporter cells. Quantification of mIL-10 and mIL-4 showed up to 10 ng/mL of therapeutic cytokine produced in response to synNotch activation (Fig. E). We also demonstrated a significant increase in CD206+ CD163+ primary macrophages using this system (Fig. F).

CONCLUSION:
These data suggest the potential for our engineered cells to contribute toward inflammation resolution via re-routed cytokine signaling. Cytokine-responsive cells can be programmed with anti-inflammatory transgenes to

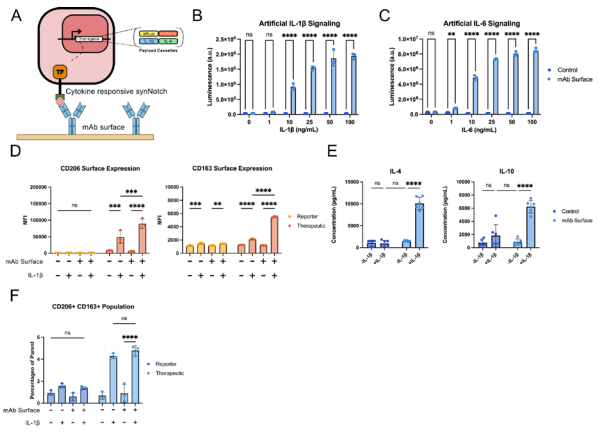

Figure 1. A) Schematic of engineered cell-surface system. B) Dose dependent activation of IL-1β responsive synNotch receptor C) Dose dependent activation of IL-6 responsive synNotch receptor. D) CD206 and CD163 surface expression of RAW 264.7 macrophages co-cultured with IL-1β responsive synNotch mMSCs. Reporter cell lines were outfitted with a luciferase and mCherry transgene and therapeutic was outfitted with a mouse IL-10 and IL-4 cassette. E) mIL-10 and mIL-4 concentrations at 48 hours. F) CD206 and CD163 double positive primary bone marrow derived macrophage expression after co-culture with mAb surface and engineered cells. A two-way ANOVA analysis was conducted on all groups with Tukey's post hoc test for multiple comparisons. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

430 *Ex Vivo* Gene Editing of Autologous B Cells Produce Sustained Levels of Tissue Nonspecific Alkaline Phosphatase *In Vivo* for the Potential Treatment of Hypophosphatasia

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Hypophosphatasia (HPP) is a rare inherited metabolic disease caused by deficient tissue-nonspecific alkaline phosphatase (TNSALP) which leads to defective bone mineralization and other systemic complications. HPP results from loss-of-function mutations in the *ALPL* gene that encodes the TNSALP enzyme. Deficient TNSALP activity leads to the accumulation of the enzyme's substrates, namely inorganic pyrophosphate (PPi), an inhibitor of bone mineralization, resulting in disease manifestation.

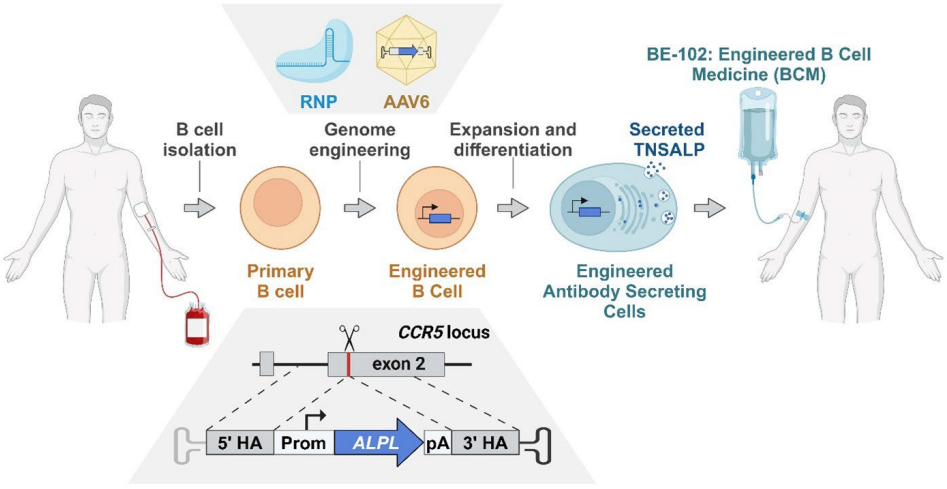
Enzyme replacement therapy is the only approved treatment for HPP which requires frequent lifelong administration accompanied by injection site reactions that can significantly affect a patient's quality of life. To address these limitations, we have developed BE-102, a B cell-based cell therapy. BE-102 is manufactured from primary human B cells by isolating, activating, and precision engineering with CRISPR/Cas9 followed by AAV-mediated homology directed repair mediated insertion of human *ALPL* gene into CCR5 locus (a safe harbor locus). The *ALPL* transgene expression cassette was designed as a soluble glycoprotein composed of two polypeptide chains. Each chain contains three segments: (1) the catalytic domain of human TNSALP, (2) a human IgG4 fragment crystallizable (Fc) domain, and (3) a deca-aspartate peptide (D10). Engineered cells were then further expanded and differentiated towards the plasma cell lineage, resulting in TNSALP-producing cells, with the properties of long-lived plasma cells. BE-102 is expected to continuously secrete active TNSALP to enable reduction of elevated levels of the enzyme's substrates that accumulate in patients with HPP.

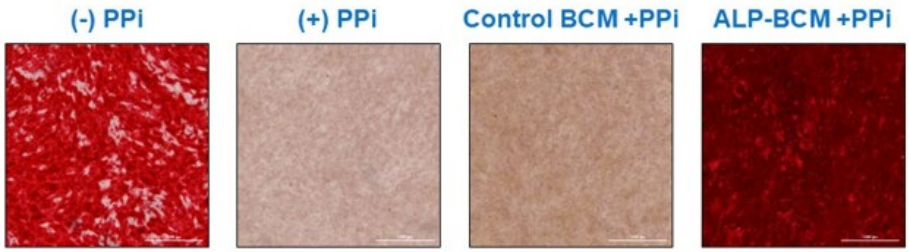
BE-102 cells were manufactured from six healthy donors and were characterized for viability (84.9 ± 3.5%), CD38+ expression (93.3 ± 4.3%), *ALPL* transgene integration (45.1 ± 7.2%) and TNSALP secretion rate (9.5 ± 10.2 ng/mL/hour). *In vitro* pharmacology results demonstrate that BE-102 secretes active TNSALP (409.7 ± 10.2 μUnits/mL using 4-MUP as the fluorometric substrate), which is capable of rescuing calcium deposit inhibited by PPi in a cell-based *in vitro* test system.

Having demonstrated the *in vitro* function of BE-102, *in vivo* studies were conducted in immune-deficient NOG-hIL6 mice. The animals received a single intravenous (IV) injection of 2 x10^7 viable cells. Human TNSALP was detected in serum at every time point starting at Day 1 and up to 6 months following a single dose of BE-102 using a human specific immunocapture assay. Consistent with BE-102 pharmacology, serum ALP activity from clinical chemistry assessment of terminal blood collection (month 5 or 6) showed statistically significant increase of ALP in BE-102 treated animals as compared to vehicle-treated animals. The *in vivo* data confirm long-term engraftment and continuous production of active TNSALP *in vivo* following a single IV injection of BE-102.

Finally, general clinical chemistry and hematology results at the end of study were all within acceptable ranges. These data together with the absence of adverse clinical observations and unscheduled animal deaths over the entire course of study support that BE-102 was well-tolerated *in vivo*.

In summary, pharmacology and safety data establish non-clinical proof of concept that BE-102 has the potential to be a disease-modifying therapy by providing sustained level of active TNSALP following a single IV infusion for patients with HPP.





The murine osteoblast cell line, MC3T3, was cultured under osteogenic conditions (ascorbic acid + β-glycerophosphate) with or without inorganic pyrophosphate (PPi) for 21 days. Supernatant from BCM control and BCMs engineered with the optimized ALP-Fc protein construct were added to cells treated with PPi. Cells were stained with Alizarin Red S to visualize calcium deposition. ALP secreted by ALP-BCMs rescued PPi-induced mineralization inhibition. Bar = 1000 μm. Control BCM: electroporated control cells.

431 Compact Transcription Factor Cassettes Generate Functional, Engraftable Motor Neurons by Direct Conversion

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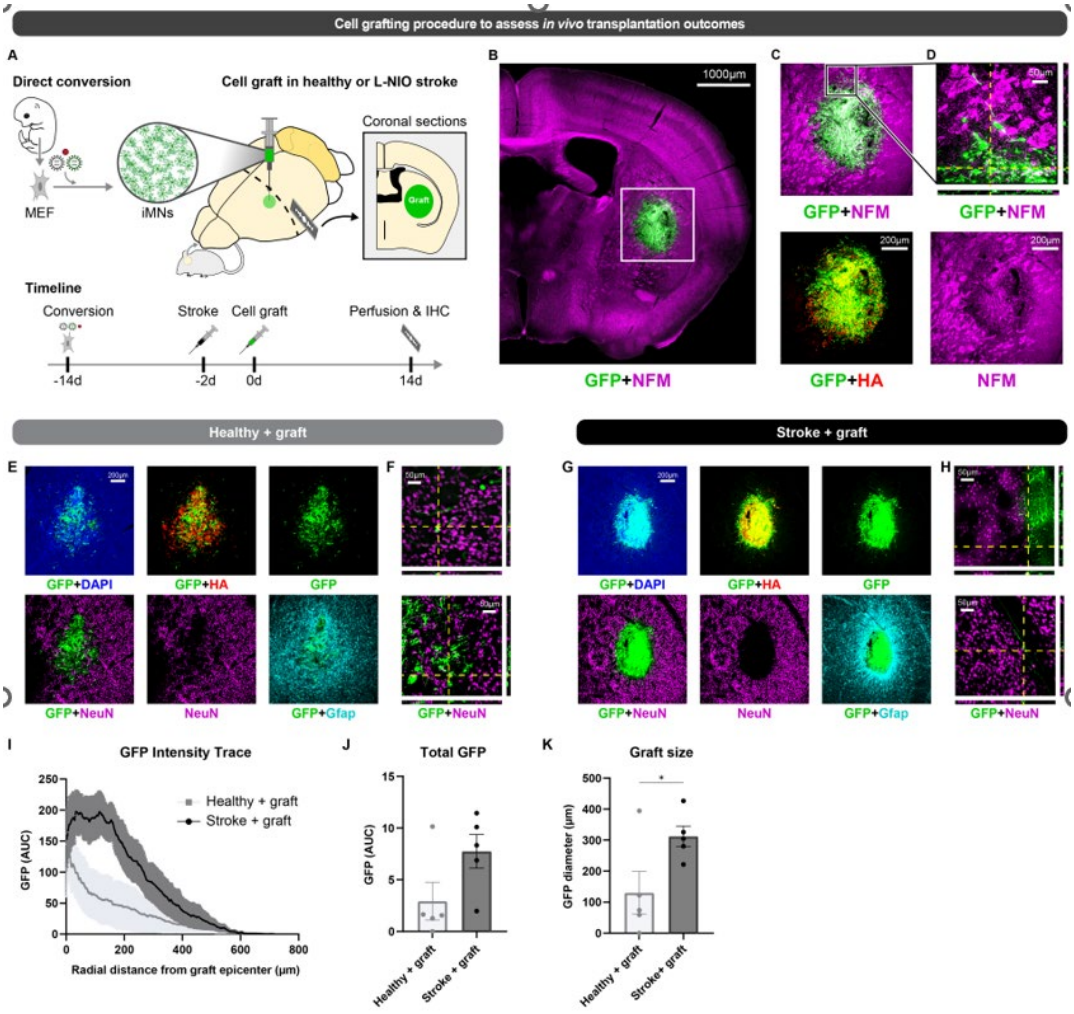
Introduction:
Cell transplantation to promote neural regeneration holds great promise to restore neurological functions. However, rapidly sourcing, and deriving, and purifying sufficient numbers primary- or iPSC-derived neurons for neuronal grafts remain a substantial challenge. By bypassing the intermediate pluripotent reprogramming step, direct conversion more quickly generates therapeutically-relevant, autologous cells from readily accessible patient cells such as those from a skin biopsy. Rapid sourcing of therapeutic cells for delivery within the short therapeutic window is critical to support optimal recovery. Yet, low rates of direct conversion and complex protocols limit scalability and thus the potential of direct cell-fate conversion for biomedical applications. In this work, we set out to develop a robust, scalable, direct conversion process to generate functional motor neurons for neural cell therapies.

Results:
We optimize the direct conversion protocol across molecular and cellular scales by examining process parameters including transcript design, delivery via AAV, retrovirus and lentivirus, cell seeding density, and the impact of media conditions. Using systems and synthetic biology approaches, we report a compact, portable conversion process that boosts proliferation and increases direct conversion of mouse fibroblasts to induced motor neurons (iMNs) to achieve high conversion rates of above 1,000%, corresponding to more than 10 motor neurons yielded per cell seeded which we achieve through expansion. We confirm electrical activity of the induced motor neurons by high-density multi-electrode assay (MEA) and genetically encoded calcium indicators (GECI). Finally, we demonstrate proof-of-principle for iMN grafting for spinal cord injuries by injecting iMNs generated by direct conversion of primary mouse embryonic fibroblasts into the central nervous system of mice.

Conclusion:
Our optimized, direct conversion process generates functional motor neurons from primary mouse embryonic fibroblasts at scales relevant for cell therapies (>10^7 cells) that graft with the mouse central nervous system. High-efficiency, compact, direct conversion systems will support scaling to patient-specific, neural cell therapies. Though there remain questions on the long-term stability, safety and efficacy of directly converted neurons when transplanted *in vivo*, we demonstrate that differentiated motor neurons generated by direct conversion can serve as a well-defined neural cell source for neural regenerative applications.



432 Broadly-neutralizing Anti-HIV Antibody Production in Rhesus Macaques Transplanted with B Cells Engineered at the IgH Locus.

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Antibodies induced by either natural infection or current vaccines are unable to provide long-term control of HIV. In contrast, a small number of broadly neutralizing antibodies (bnAbs) have been identified that suppress virus replication when delivered as recombinant proteins or expressed from AAV vectors. As an alternative approach for long-term production of bnAbs *in vivo*, we are using genome engineering at the constant region of the immunoglobulin heavy chain (IgH) locus to reprogram human B cells to express custom heavy-chain only antibodies (HCAbs). The HCAbs mimic a natural antibody type found in camelids and can be engineered with a variety of anti-HIV recognition domains such as VHHs, scFvs, or other protein domains. Human B cells expressing HCAbs retain critical B cell functions *in vitro*, including clonal expansion in response to antigen stimulation. We hypothesize that transplantation of HCAb-engineered autologous B cells could provide long-term HIV control, including by providing B cell memory that could be boosted over time. To test HCAb-engineered B cells as a prevention or therapy against HIV, we adapted the editing approach for the IgH locus in rhesus macaques. B cells were engineered to express anti-HIV HCAbs based on either a humanized J3 VHH domain or an optimized scFv derived from the well-characterized bnAb, PGT121. Six animals received autologous infusions of 4.5–20 × 10^4 edited cells/kg, and were immunized with HIV gp140 at weeks 0, 4, and 8 post-infusion. HCAbs in serum and edited cells were detected up to 14 weeks in 4 out of 6 animals, with one animal showing a transient increase after the week 4 booster immunization. In an attempt to improve HCAb levels, we re-transplanted the four responding animals 9 months later with B cells expressing the alternate HCAb, using 3-5.8-fold higher numbers of edited cells, and including cells with reduced time *in ex vivo* culture. In this second study, we detected higher overall levels of HCAb secretion that persisted for a longer time, but were still unable to achieve stable, long-term HCAb secretion that responded to vaccination. Interestingly, the patterns of expression in individual animals were remarkably similar from one study to the next, despite the expression of two different HCAbs. Together these data show that HCAb engineered B cells can survive and secrete HCAbs in vivo over several weeks. However, the lack of response to antigen suggests the need to further optimize *ex vivo* selection and culture conditions to better support B cell subtypes that can give rise to long-lived antibody-secreting cells and memory phenotypes *in vivo*.

433 Expandable, Functional Hepatocytes Derived from Primary Cells Enable Liver Therapeutics

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Introduction
Liver disease impacts millions annually in the US, with orthotopic transplantation as the only curative option for many patients. The scarcity of donor organs underscores an urgent need for alternative therapies. Hepatocyte-based approaches hold promise due to the cells' synthetic, metabolic, and detoxifying functions, but critical barriers remain, including the lack of a scalable source of functional hepatocytes and poor engraftment. We developed a scalable PHH expansion process that preserves hepatic identity and function. By leveraging heterocellular aggregation with stromal cells, we generated cryopreserved seed constructs that maintained viability and function post-thaw. Seeds demonstrated enhanced metabolic and detoxification functions and robust engraftment across multiple sites outside the liver. Our approach addresses key limitations of hepatocyte-based therapies, offering a clinically viable platform for liver cell therapy.

Methods

We utilized PHHs from multiple donors and subjected them to our expansion protocol to yield expanded human hepatocytes (EHHs). We confirmed hepatic identity during expansion through immunofluorescent co-staining of HNF4α and albumin and bulk RNA sequencing. For in vivo studies, we injected EHHs, PHHs, and iPSC-derived hepatocytes (iHeps) into Fah$^{-/-}$Rag2$^{-/-}$Il2rg$^{-/-}$ (FRG) mice and evaluated engraftment using plasma biomarkers like human albumin. NTBC was withdrawn to assess survival and liver injury biomarkers. To generate seeds, we combined EHHs with NHDFs in suspension bioreactors and assessed function across synthesis, metabolism, and detoxification axes. Cryopreserved seeds were evaluated for viability and function post-thaw. Finally, we tested engraftment at ectopic sites, including the spleen, fat pad, and kidney capsule.

Results

Our expansion protocol enabled PHH expansion across multiple donors, with 46% achieving >20-fold expansion at passage 0 and a mean fold expansion of 4,373 by passage 1. The highest-expanding donors maintained cuboidal morphology and exceeded 2,000-fold expansion per donor. EHHs retained hepatic identity, with >99% co-expressing HNF4α and albumin, and showed no significant differences in key liver-specific genes compared to PHHs ($p > 0.05$). In the FRG model, EHHs successfully engrafted and repopulated the liver, producing 1.4 - 2.2 mg/mL human albumin and improving survival ($p = 0.02$). In contrast, iPSC derived hepatocytes did not engraft (0 mg/mL albumin) (Fig 1). To further enhance engraftment, EHHs were aggregated with NHDFs into seeds. We compared EHH seeds to PHH seeds and found comparable or superior albumin and A1AT secretion in vitro, and CYP3A4 activity showed a 5.5-fold induction upon rifampin treatment. Our cryopreservation process maintained seed stability with 90% post-thaw recovery, preserved size distribution, and comparable viability (85.0% vs. 86.3%, $p = 0.43$). To assess engraftment, we tested NHDF-seeded, fibroblast-free, and MSC-seeded constructs in NSG mice, with NHDF seeds demonstrating superior engraftment (AUC: 258 µg·day/mL vs. 48 µg·day/mL for MSC, $p <$ 1E-4). NHDF-seeded constructs successfully engrafted across extrahepatic sites, confirmed by stable albumin levels and OTC staining (Fig 2).

Conclusions

PHH expansion and aggregation into seeds address key limitations of hepatocyte-based therapies, providing a scalable and clinically viable solution to advance liver cell therapy and broaden treatment options for liver disease.

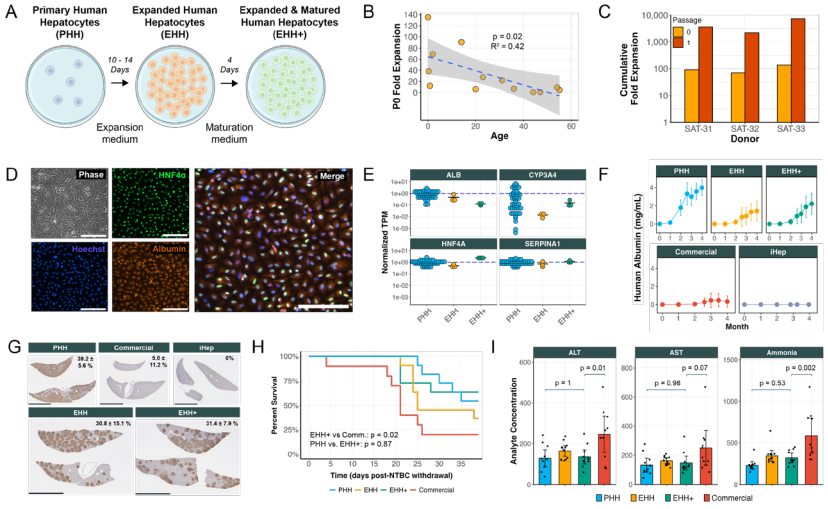

Figure 1: Primary hepatocyte expansion and maturation yield functional cell populations for therapeutic applications. (A) Illustration of hepatocyte culture, expansion, and maturation phases. (B) Correlation analysis showing a negative correlation between donor age and passage 0 (P0) fold expansion ($p = 0.02$, $R^2 = 0.42$). (C) Cell count analysis demonstrating >2,000x cumulative fold expansion with a two-passage scheme across three donor lots. (D) Imaging analysis demonstrating the characteristic cuboidal morphology of expanded hepatocytes (Phase) and robust co-expression of HNF4α (green), albumin (orange), and Hoechst (nuclei). Over 99% of the expanded hepatocytes exhibited triple positivity, confirming their hepatic identity (scale bars = 200 µm). (E) Gene expression analysis showing comparable levels of liver-specific genes (ALB, CYP3A4, HNF4A, SERPINA1) across PHH, EHH, and EHH+ samples. Each dot represents a unique donor (n = 46 for PHH, n = 3 for EHH, n = 3 for EHH+). Transcripts per million (TPM) values are normalized to the mean of the PHH group. (F) Human albumin levels in mouse plasma (mean ± 95% CI) demonstrate successful engraftment and repopulation in PHH, EHH, and EHH+ groups, with no engraftment observed in iPSC-derived hepatocytes and 'commercial' cells. (G) Liver explant staining with human fumarylacetoacetate hydrolase (FAH) confirms engraftment patterns consistent with human albumin levels (scale bar = 5 mm). Percentages within each micrograph indicate repopulation rates, quantified from FAH-stained sections (mean ± 95% CI). (H) Kaplan-Meier survival curves showing enhanced survival rates in animals receiving PHH and EHH+ cells compared to 'commercial' cells. The p-values are calculated using the log-rank test. (I) Endpoint liver function tests (ALT, AST) and plasma ammonia levels indicate reduced liver injury in PHH, EHH, and EHH+ groups compared to 'commercial' group (mean ± 95% CI). The p-values are calculated using a one-way ANOVA followed by Tukey's HSD post hoc test for pairwise comparisons. Units for ALT and AST are U/L, while the units for ammonia are µM.

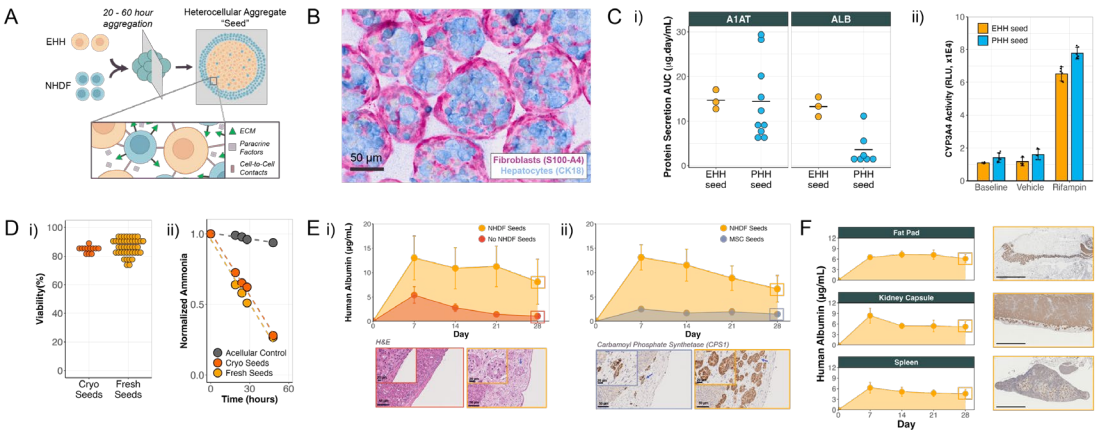

Figure 2: Expanded hepatocyte aggregation into heterocellular constructs leads to a cryopreserved drug product with enhanced functional maturation. (A) Schematic depicting the assembly of hepatocyte-fibroblast seed constructs, supported by extracellular matrix (ECM), paracrine, and cell-cell interactions. (B) Histological staining (S100-A4 for fibroblasts, CK18 for hepatocytes) highlights the integration of the two cell types within a core-shell seed structure. (C) (i) A1AT, albumin production levels (n = 3 donors for EHH seeds and n = 7-10 donors for PHH seeds, and (ii) CYP3A4 induction in EHH seeds versus PHH seed control groups, indicating comparable function to primary cells (n = 1 donor, n = 6 technical replicates). (D) Demonstrating successful cryopreservation of seeds, evidenced by comparable (i) cell viabilities (n = 12 batches for cryopreserved seeds and n = 44 batches for fresh seeds) and (ii) ammonia clearance capacity between fresh and cryopreserved seeds (ammonia values are normalized to t = 0 hours for each group). (E) Heterocellular aggregation of EHHs with dermal fibroblasts significantly enhances engraftment within the kidney capsule, as evidenced by increased plasma albumin levels (mean ± 95% CI) and histological comparisons showing reduced engraftment in (i) the absence of dermal fibroblasts or (ii) when using bone marrow-derived mesenchymal stromal cells (BM-MSCs). (F) Time-course of human albumin levels (mean ± 95% CI) and immunohistochemical analysis of ornithine transcarbamylase (OTC) across implantation sites (fat pad, kidney capsule, spleen) shows that seed constructs support stable engraftment and sustained hepatocyte activity across various extrahepatic locations (scale bar = 1 mm).

434 Curative Allogeneic Hepatocyte Transplantation without Immune Suppression

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Hepatocyte transplantation is a cell therapy method with distinct advantages relative to gene therapy. One main advantage is that hepatocytes from a healthy donor can be used as a universal therapy for a multitude of genetic conditions without customization to each disease indication. However, the use of hepatocytes from an allogeneic donor requires immunosuppression. An ideal protocol for liver cell therapy would allow sustained engraftment of allogeneic cells without the need for immunosuppression. Others have reported methods to render hematopoietic and iPSC cell therapies immune stealthy by a combination of three or more genetic edits: β_2 microglobulin (*B2m*) knockout to prevent MHC class 1 presentation, Ciita knockout to prevent MHC class 2 expression, and transgenic expression of immune-inhibitory ligands to prevent NK cell responses. We have previously reported that knockout of *B2m* alone is sufficient to allow allogeneic engraftment of murine hepatocytes. This result suggests that creation of an off-the-shelf allogeneic liver-directed cell therapy product may be feasible with minimal genetic modification. Here, we report correction of two murine models of human metabolic disease: hereditary tyrosinemia type 1 (HT1) and phenylketonuria (PKU). Hepatocytes singly modified to be immune stealthy by *B2m* knockout were used and no immune suppression was needed. HT1 is a disorder resulting from a deficiency in fumarylacetoacetate hydrogenase, leading to the hepatotoxic accumulation of protein metabolites in affected hepatocytes. Congenic wild-type hepatocytes transplanted into the HT1 liver will expand in vivo as they are not susceptible to toxicity and thus have a selective advantage. We created *B2m*-deficient primary mouse hepatocytes from a wild-type donor of the C57BL/6 strain by CRISPR gene editing in vitro. When transplanted into allogeneic HT1 mice on the 129S4 strain background, *B2m*-deficient hepatocytes engrafted and expanded to repopulate the liver to >95% replacement. This corresponded with weight stabilization for over 100 days and correction of liver injury markers and amino acid levels in blood. The same result was seen when the experiment was repeated in the opposite direction, with *B2m*-knockout 129S4 hepatocytes transplanted into HT1 C57BL/6 recipients. PKU is a disorder resulting from a deficiency in phenylalanine hydroxylase, which causes increased blood phenylalanine (Phe) and neurotoxicity. Unlike HT1, PKU does not result in a selective advantage for transplanted hepatocytes. To overcome this limitation, creation of a genetic deficiency in a drug metabolism gene (*Cypor*) in hepatocytes results in a selective advantage for gene-edited transplanted cells when treated with acetaminophen (APAP). We have previously published that, in a congenic setting, transplantation and APAP selection of Cypor-deficient hepatocytes results in complete correction

of Phe levels with ~10% liver repopulation. To apply this in the allogeneic setting, we created knockouts of *Cypor* and *B2m* in wild-type cells from a 129S4 mouse using CRISPR gene editing in vitro. Following transplantation into PKU mice of the C57BL/6 background and APAP selection, this led to 15% liver repopulation with transplanted hepatocytes and a correction of blood Phe to wild-type levels. Taken together, these results indicate that *B2m*-knockout hepatocytes represent a potential off-the-shelf allogeneic cell therapy with applicability to a variety of disease indications.

Gene Therapy for Muscle Diseases

435 Recurrent Rhabdomyolysis and Fatality Following Administration of AAV Microdystrophin Gene Therapy

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A 4-year-old boy with dystrophinopathy died 14 months following systemic administration of fordadistrogene movaparvovec, an AAV microdystrophin gene therapy. The discussion of his exceptional clinical course is imperative to understanding potential complications associated with gene replacement therapies.

At 3 years of age, the patient was assessed in a tertiary neuromuscular clinic. His phenotype was consistent with Duchenne muscular dystrophy with a predicted in-frame deletion of *DMD* exons 45-48.

The patient was enrolled in an early phase, open-label clinical trial and received $2x10^{14}$ vector genomes per kg of fordadistrogene movaparvovec with high-dose steroid coverage.

He presented seven weeks post gene therapy administration with his first episode of rhabdomyolysis (severe muscle pain, myoglobinuria and acute critical hyperCKaemia). Varying immune modulation regimens were trialled with minimal efficacy and the patient experienced 16 episodes of rhabdomyolysis over the following 12 months.

Despite the initial hypothesis of immune mediated myositis and consistent responses to corticosteroids acutely, there was no clear clinical, serological or tissue evidence of immune dysfunction or inflammation. Anti-dystrophin antibodies remained unremarkable. Muscle biopsy taken during a rhabdomyolysis crisis did not support an immune cause and showed significant levels of endogenous dystrophin, in keeping with the genotype. The only additional finding on genome sequencing was a heterozygous variant of uncertain significance in *DAG1*.

During the 17th episode of rhabdomyolysis, the patient experienced acute extreme hyperkalaemia and a fatal cardiac arrest, having never had elevated potassium in previous episodes. Postmortem muscle analysis demonstrated multiple areas of necrotising myopathy that appeared to have occurred at various timepoints.

The timing of the onset of clinical symptoms, consistent with the expression of the microdystrophin transgene and his subsequent clinical course presents several learnings and key questions regarding AAV gene therapy, including the implications of in frame deletion on patient selection, approaches to investigation and management of potential immune mediated adverse events and considerations of the risk versus benefit ratio for AAV gene therapy in individual patients.

436 Treatment of Myotonic Dystrophy Type 1 (DM1) by GNDM-mediated Suppression of the DMPK Gene

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Myotonic Dystrophy Type 1 (DM1) is a neuromuscular disorder that affects over 100,000 people in the United States and Europe. The primary cause of DM1 pathology is a genetic expansion of CTG repeats in the 3' untranslated region of the DMPK gene. These expansions are believed to cause formation of DMPK mRNA aggregates that sequester and inhibit splicing factor proteins required for normal muscle function. Therapeutic treatments for DM1 have focused on the prevention or elimination of these pathogenic aggregates, though an effective treatment has not been approved for the clinic.

Modalis Therapeutics has pioneered CRISPR-based epigenetic editing, and our therapeutic platform, termed CRISPR-GNDM®, enables activation and suppression of compensatory and toxic gene expression, respectively. This platform is composed of genetic elements encoding an engineered Cas9 protein fused with a transcription modulator (GNDM) and a gRNA expression cassette, which can be packaged in a single AAV vector. Herein, we describe how CRISPR-GNDM® technology can be used to suppress the expression of DMPK mRNA for the treatment of DM1 (Figure 1). We first screened gRNAs in the regulatory region of the human DMPK gene (Figure 1a). Candidate gRNAs were varied in size to optimize potency and specificity, and, to facilitate preclinical development, they were designed to be compatible for both human and non-human primate genomes. Co-expression of GNDM and the candidate gRNAs that can suppress DMPK gene expression more than 80% in human cells were further evaluated for local specificity by measuring expression perturbation in neighboring genes. Candidate gRNAs were then tested for global gene expression alterations, allowing identification of potent and specific gRNAs as candidates for further studies (Figure 1b). Next, we tested the effect of three gRNAs for rescue of molecular phenotypes in DM1 patient-derived cells. Indeed, we found that GNDM and gRNA expression led to an almost complete removal of nuclear DMPK mRNA aggregates and reversal of aberrant splicing phenotypes (Figure 1c), which are likely the root cause of DM1 pathologies. We next optimized packaging of CRISPR-GNDM® components into an AAV vector for in vivo delivery (Figure 1d). To facilitate robust delivery to the target muscle tissue, genomes were packaged using muscle-tropic capsids. Packaged AAV genomes were also isolated and long-read sequencing approaches were used to ensure complete packaging of the CRISPR-GNDM® and gRNA components. These muscle-tropic AAVs were used to treat DMSXL mice, a mouse model for DM1, to test for in vivo potency. Treated mice showed reduced transgenic DMPK levels by >80% in the heart, and 40% to 60% DMPK suppression was achieved in all skeletal muscles tested (Figure 1e). Taken together, these results showed a promising avenue for CRISPR-GNDM® technology for treatment of DM1. Epigenetic editing holds great promise for suppressing disease-causing genes. However, its current application is limited to liver-related conditions due to the feasible access provided by non-viral particles. The CRISPR-GNDM® technology, in combination with the recently developed muscle-tropic AAV capsids, offer a promising avenue for treating various genetic muscle diseases, such as DM1.

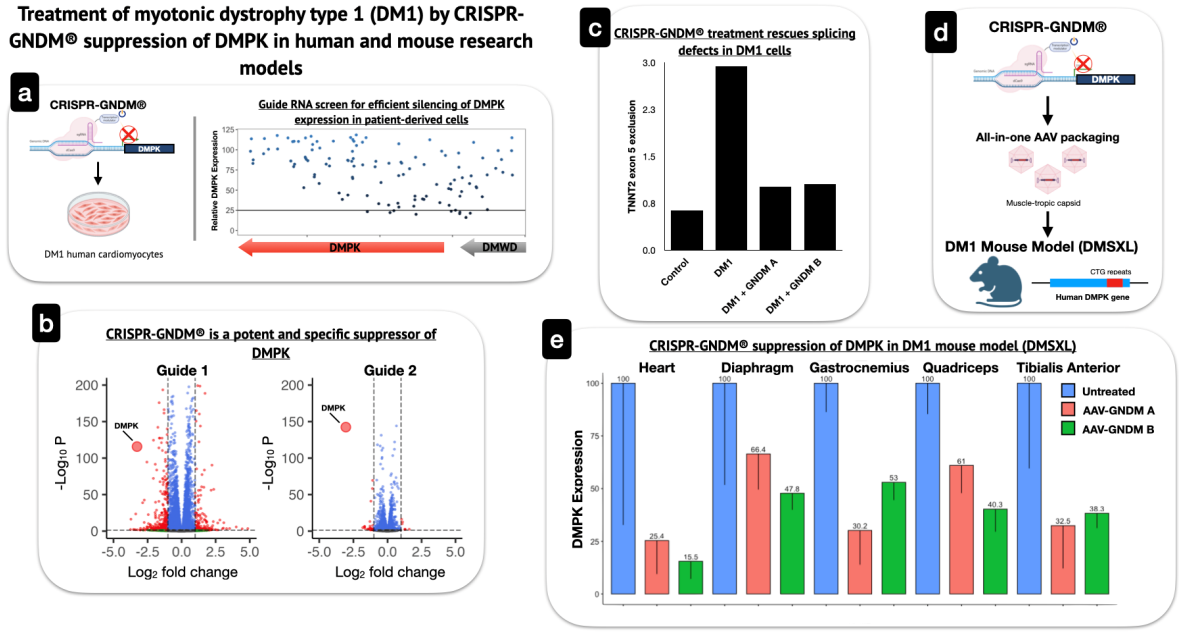

Figure 1: CRISPR-GNDM® suppression of DMPK mRNA. CRISPR-GNDM® compatible gRNAs were designed near the DMPK promoter and tested for potency **(a)** and specificity **(b)**. Candidate constructs were able to reduce splicing defects in DM1 patient-derived cells **(c)**. Muscle-tropic AAV packaging of CRISPR-GNDM® components allowed delivery **(d)** and suppression of DMPK **(e)** in target tissues of DMSXL mice.

437 A clinical DMD cytosine base editing drug

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Duchenne muscular dystrophy (DMD) is the most severe form of muscular dystrophy affecting 1 in 3500 to 5000 boys at birth globally. Patients experience a progressive wasting of both skeletal and cardiac muscles attributable to the loss of dystrophin protein encoded by the DMD gene on chromosome Xp21. The milder symptoms in Becker muscular dystrophin (BMD) patients, who have an in-framed truncated but still functional dystrophin protein, provide a hereditary basis for exon skipping therapy against DMD.

GEN6050X is a clinical investigational drug which utilized Targeted AID-mediated Mutagenesis (TAM) cytosine base editor (CBE) to induce DMD exon 50 skipping via editing the 5'SS of human DMD IVS50. GEN6050X is an intravenous dual AAV product containing two AAV9 drug products. One encodes muscle-specific promoter-driven TAM CBE protein while the other encodes 3 copies of hE50 sgRNA targeting human DMD IVS50 5'SS. Additionally, the sgRNA vector carries a human gamma-actin gene, which can bind de novo dystrophin to rapidly facilitate costamere and dystroglycan-associated complex remodeling and provide a synergistic therapeutic effect. Single dose of GEN6050X i.v to 21-day-old humanized Dmd mouse model *Dmd*^{hE50delE51} greatly restored dystrophin protein expression in heart and skeletal muscles. Correspondingly, GEN6050X treatment significantly improved the muscle function and histopathology in both heart and skeletal muscles of *Dmd*^{hE50delE51} mice. No high-risk sgRNA-dependent or independent DNA off targets, chromosome structure variation and transcriptome off targets were found *in vitro*. In 26 weeks of cynomolgus monkey toxicology study, minimal liver toxicity was observed at Week 13 while liver toxicity was resolved at Week 26 in both 5E13vg/kg and 2E14vg/kg GEN6050X group. The Highest Non-Severely Toxic Dose (HNSTD) was identified as 2E14vg/kg in NHP. An IIT study was initiated in Aug 2024 to evaluate the safety and tolerability of GEN6050X (NCT06392724). Two patients have received 5E13vg/kg GEN6050X till Oct 2024. The first patient has finished 3-month follow-up. The safety profile is good. The first muscle biopsy will be conducted in Feb 2025. All clinical manufacture has been finished, and IND filing will be expected at the end of 2024. Collectively, GEN6050X is a safe and effective gene editing drug candidate and may provide a cure for DMD exon 50 skipping patients.

438 GNT0004, Genethon's AAV-based gene therapy for Duchenne muscular dystrophy: long-term follow-up of ambulatory boys enrolled in the dose-escalation phase of GNT-016-MDYF.

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Duchenne muscular dystrophy (DMD) is a severe disease caused by mutations in the dystrophin gene that leads to progressive muscle degeneration and early death.

GNT0004 is a gene therapy utilizing an adeno-associated virus serotype 8 (AAV8) vector, containing a shortened, functional dystrophin gene (hMD1) under the control of the Spc5.12 promoter, which is specifically designed to target skeletal and cardiac muscle tissues. GNT0004 is being evaluated in the international, all-in-one (phase 1/2/3) clinical trial GNT-016-MDYF. This trial includes a first-in-human dose escalation (Part 1), a placebo-controlled pivotal Phase 3 (Part 2), and a long-term follow-up (Part 3). The study considers ambulatory boys with DMD, aged 6 to 10 years, who have a stable or early decline in their North Star Ambulatory Assessment (NSAA) score. Participants are on stable corticosteroid treatment and have been followed for at least 6 months in the natural history (NH) study GNT-014-MDYF. Long-term data from Part 1 patients receiving Dose 2 ($3x10^{13}$ vg/kg, selected for the pivotal phase) are presented. A comparison with an external control group from the NH study was conducted to explore clinical efficacy. Five patients were enrolled in Part 1, three of whom received Dose 2. At week 8, biopsy results showed a mean of 53% hMD1-positive fibers in the Dose 2 group. A significant decrease in serum creatine kinase (CK) levels was observed post-dose, with levels remaining low from week 8 and up to 2 years, based on available follow-up data. In the Dose 2 group, at one year, the mean creatine kinase (CK) level was 5,306 IU/L, reflecting a mean reduction of 68% compared to baseline (mean 19,175 IU/L). Clinically, functional outcomes were stable or improving, clearly differentiating from the NH progression in favor of GNT0004-treated patients across all efficacy parameters analyzed (e.g., in the Dose 2 group, a delta of +4.7 points was observed on the NSAA scale, along with an improvement of 0.1 m/s in the Stride Velocity 95th Centile (SV95C) at one year) (Figure 1). At Dose 2, no serious adverse events (SAEs) were reported, nor were there any opportunistic or severe infections. Three adverse drug reactions (ADRs) were reported, all of which were mild and resolved without complications. GNT0004 at Dose 2 was well-tolerated and provided long-term stabilization of the sarcolemma, as evidenced by the notable and sustained low levels of CK, with a clear clinical benefit compared to the natural disease progression. These effects will need to be confirmed in Part 2, the pivotal phase of GNT-016-MDYF.

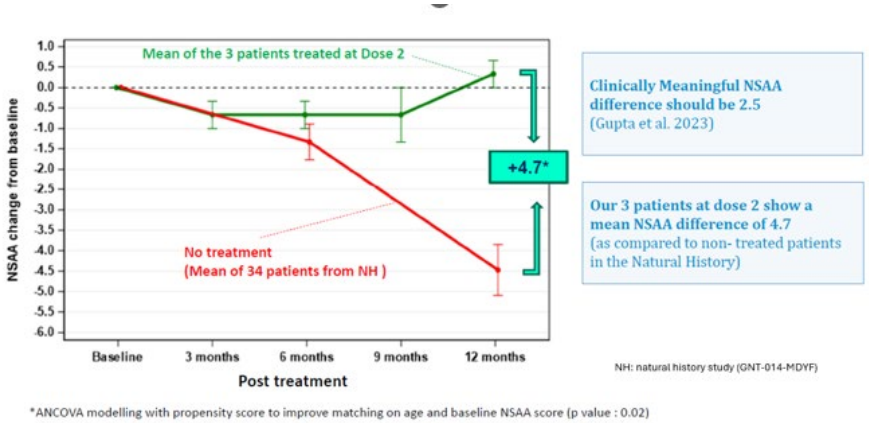

Figure 1. Early evidence of clinical benefit (Part 1, GNT-016-MDYF)

439 Determining the Effectiveness of a *FKRP/FST* Single AAV Dual Gene Vector for the Treatment of Limb Girdle Muscular Dystrophy type R9 (LGMDR9) at Different Ages

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Limb Girdle Muscular Dystrophy Type R9 (LGMDR9) is an autosomal recessive dystroglycanopathy in which α-dystroglycan is not properly glycosylated due to loss of function mutations in the Fukutin-Related Protein (FKRP) gene. Patients with LGMDR9 have skeletal muscle damage causing weakness concentrated in the pelvic and shoulder girdles. LGMDR9 patients may also experience loss of ambulation, decreased respiratory function, and cardiomyopathy. FKRP gene replacement inhibits disease progression in animal models but will likely not recover muscle strength that has already been lost. We have developed a single AAV vector with dual FKRP/FST gene therapies to couple FKRP gene replacement with a muscle building follistatin (FST) therapy to rebuild lost muscle strength in LGMDR9 patients while simultaneously preventing subsequent disease. Here we compare the dual gene therapy to FKRP or FST therapy alone to determine the potential benefits of coupling these genes in a single AAV treatment. We also compare outcomes of treatment at different stages of the disease. $FKRP^{P448L}$ mutant mice were injected with AAV via tail vein at 4 weeks of age for necropsy at 28 weeks of age, or 52 weeks of age for necropsy at 70 weeks of age. One week before euthanasia ambulation was tested on a treadmill. Tetanic force and force drop during repeated eccentric contractions were measured in the TA muscles in situ. Using a custom automated analysis workflow, measures of muscle histopathology, including myofiber diameter, percent central nuclei, and non-muscle area were quantified. This workflow also calculated the intensity of matriglycan expression in IIH6C4-immunostained sections. Matriglycan is the glycan reduced in the disease state, thus allowing visualization of AAV-affected FKRP expression. At 28 weeks, all wild-type mice successfully completed the treadmill protocol, while ambulation time of PBS-treated $FKRP^{P448L}$ mice was reduced. Treatment with 5E+13 vg/kg rAAV.MHCK7.FKRP.pA.CMV.FST (FKRP.FST) restored ambulation to normal, while mice treated with rAAV.MHCK7.FKRP (FKRP) had some improvement. Mice treated with rAAV.CMV.FST (FST) had no improvement. FKRP.FST- and FST-treated mice showed an increase in absolute tetanic force, but only FKRP.FST-treated mice maintained a force deficit near wild type in repeated eccentric contractions. The percent myofiber central nuclei was increased in PBS-treated $FKRP^{P448L}$ mice compared to wild type, which FST treatment did not significantly improve. FKRP and FKRP.FST treatment reduced central nuclei. Myofiber diameter was significantly increased in mice treated with both FST and FKRP.FST. Immunostaining with IIH6 antibody showed significant increases in matriglycan expression in FKRPP448L mice treated with FKRP and FKRP.FST. At 70 weeks, no mice in any group were able to complete the treadmill protocol, and there was no significant difference in walking times between any of the groups. While muscles in mice treated with FKRP.FST were significantly larger than other groups, the absolute tetanic force was not improved. Myofiber diameter was only significantly increased in $FKRP^{P448L}$ mice treated with FKRP.FST. These results demonstrate the clinical usefulness of a single AAV dual gene therapy for treatment of LGMDR9 when treated at a young age. We continue to explore treatment with other promoters to optimize these effects and to dose mice at different ages and intervals to better understand the full potential of this therapy.

440 Cross-Species Comparison of AAV8, 9, rh74, and Six Myotropic AAV Variants in Murine and Canine Models Following Systemic Delivery

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Recent advances in AAV capsid bioengineering have provided novel variants with promising performance in striated muscle and reduced liver tropism in mice and nonhuman primates. Several of the most potent muscle-directed, or "myotropic" AAV capsids feature a surface exposed Arg-Gly-Asp (RGD) motif. Myotropic AAV variants hold great promise to treat neuromuscular diseases such as Duchenne muscular dystrophy (DMD). Murine

and canine models are the most common animal models for DMD. To determine the best myotropic AAV capsids for DMD preclinical studies, we compared six myotropic AAV capsids with three AAV serotypes currently used in clinical trials (AAV8, 9, and rh74) in murine and canine models using a novel exonic barcode system. Unlike conventional short barcode systems, the exonic barcode system allows precise quantification of AAV delivery (vector genome (vg) copy number) and AAV-mediated expression (transcript copy number) by barcode-specific TaqMan qPCR. Barcoded but otherwise identical reporter AAV genomes expressing EGFP driven by the ubiquitous CAG promoter were packaged in each of the nine capsids. Barcoded AAV vectors were mixed in equal proportions and delivered intravenously to adult BL10 mice (n=10), adult mdx mice (n=10), and 1-week-old (n=3) and 1-month old (n=2) dogs. Mouse studies were performed in two different doses (3.0E+12 and 1.0E+13 vg/kg/AAV, n=5 mice/dose). Dog studies were performed using one dose (3.0E+12 vg/kg/AAV). Tissues (heart, liver, diaphragm, and various limb muscles) were collected at 1-month post-injection. DNA and mRNA were extracted. We first validated the barcode-specific TaqMan PCR by next-generation sequencing of RNA extracted from the quadriceps of BL10 mice. Linear regression analysis revealed a robust correlation between the two methods (r=0.988). Next, we quantified vg and transcript copy number in all tissues by TaqMan qPCR. In mice, we obtained consistent results from BL10 and mdx mice at both doses. Specifically, AAV8 and AAV-RGD-1 had the highest and lowest vg copy number in the liver, respectively. AAVrh74 and AAV-RGD-1 had the highest and lowest transcript copy number in the liver, respectively. AAV-RGD-3 had the highest vg copy number (in most samples) and the highest transcript copy number in cardiac and skeletal muscle. In dogs, 1-week-old and 1-month old dogs yielded consistent results. Specifically, AAV8 and AAVrh74 had the highest vg copy number and transcript copy number in the liver, respectively. Interestingly, while AAV-RGD-5 had the highest vg copy number in most samples, AAV-RGD-1 consistently yielded the highest transcript copy number in cardiac and skeletal muscle. Our results will help guide selection of the most appropriate AAV capsids for preclinical studies in murine and canine DMD models. (Supported by NIH NS90634, NS131416, and AI177600).

Engineered Immune Effector Cells for Solid Tumors

441 T cell engineering using V(D)J recombination allows tumor growth inhibition in mice

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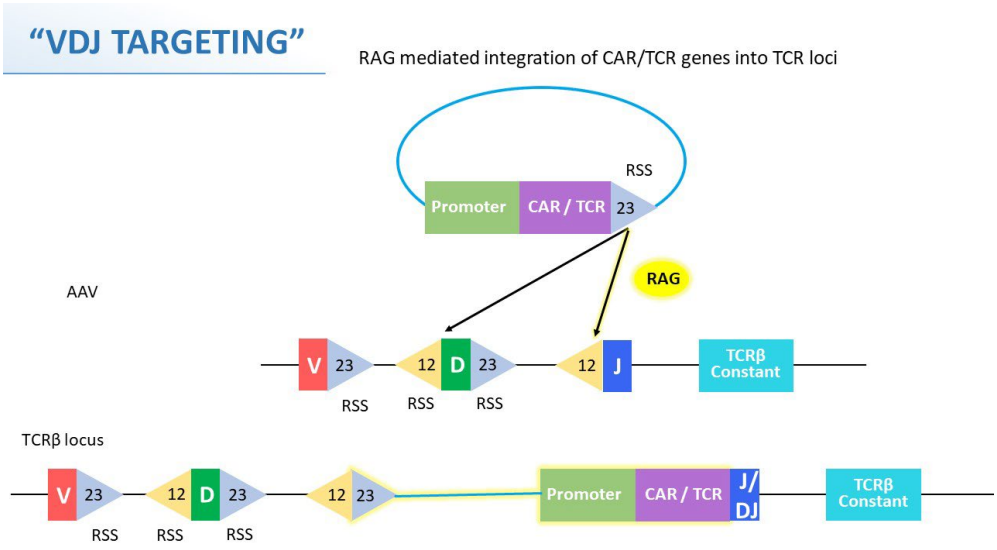
Introduction:
T cell therapies have shown clinical success in hematological malignancies, but they rely on prolonged, expensive *ex vivo* manipulations and their efficacy is hampered by early T cell exhaustion, promoted by the prolonged culturing required for the scarcely retrieved cells. We propose “VDJ targeting”: a novel immunotherapy approach targeting AAV-delivered immune genes into the genome using V(D)J recombination in developing T cells.

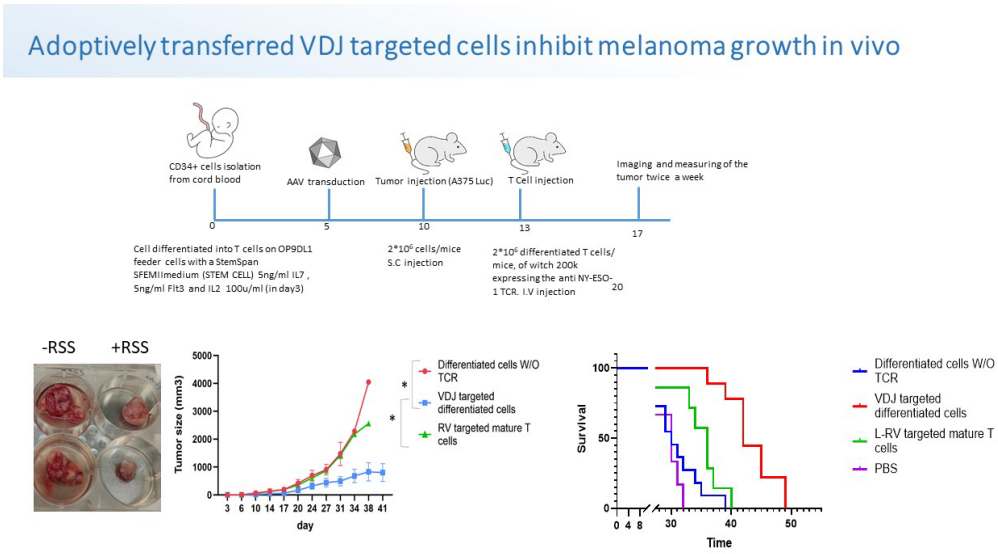
Material and methods:
We use AAV vectors coding for a transgenic TCR/CAR gene followed by a recognition signal sequence (RSS) for the recombination activating gene (RAG) complex, which is active only during T cell development) Fig 1). We targeted *ex vivo* differentiated T cells from human cord blood and bone marrow as well as from mouse bone marrow. Mouse bone marrow cells were grown on OP9DL1 feeder cells in IL-7 and FLT-3 containing medium for 5 days, and then transduced with 1e6 vector genomes per cell of an AAV-DJ vector coding for a CAR against CD19. Human cord blood or bone marrow derived HSPCs were grown on feeder cells with an SPEM-2 medium for

5 days and then transduced with a 7e5 vector genomes per cell of an AAV6 vector coding for either a CAR anti-CD19 or a TCR targeting an NY-ESO1 peptide presented on HLA-A*02.

Results and discussion:
Following transduction, mouse bone marrow-derived hematopoietic stem and progenitor cells (HSPCs) $CD3^+$ were found to express the CAR and were activated by CD19-expressing cancer cells. Similarly, human cord blood and bone marrow derived HSPCs $CD3^+$ cells successfully expressed the TCR and were activated by NY-ESO-1 expressing cancer cells. Importantly, human T cells, engineered by VDJ recombination to express the anti-NY-ESO-1 TCR, inhibited tumor growth and prolonged survival in immunodeficient mice implanted with a human NY-ESO-1^+ melanoma. Moreover, our VDJ-targeted T cells vastly outperformed conventionally engineered T cells, transduced with a lentiviral vector coding the anti-NY-ESO-1 TCR. (Fig 2)

Conclusion:
VDJ targeting produces potent naïve T cells that may be transplanted in lower numbers and have increased persistence compared to T cells engineered with state-of-the-art technologies. VDJ targeting is thus an efficient T cell engineering technology applicable in the autologous, allogeneic and *in vivo* engineering settings.





442 Sensitive HLA-independent T cell Receptors Overcome Tumor Antigen Heterogeneity in Solid Tumors

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Background:
Overcoming tumor antigen heterogeneity in solid tumors is a major challenge for cancer immunotherapies including chimeric antigen receptor (CAR) T cells. Unlike CD19 for B-cell malignancies(1), no target with pan-cellular expression in solid tumors and absence in normal vital cells has been identified(2–5). Thus, CAR T cell efficacy in solid tumors is compromised by heterogeneous target antigen expression and unacceptable toxicities in normal tissues. CD70 is a potential candidate due to its aberrant upregulation in a variety of solid tumors(6–12), whilst its physiological expression is restricted to immune cell subsets(13–15). However, CD70 expression in tumors is typically heterogeneous, with some tumor cells exhibiting high expression and others, often accounting for the majority of tumor cells, appearing low/negative(16–19). Not surprisingly, the clinical responses obtained in patients who were treated with CD70-targeted CAR T cells have been modest(20, 21).

Methods:
We developed patient-derived xenograft (PDX) orthotopic models of clear cell renal cell carcinoma (ccRCC), high grade serous ovarian carcinoma (HGSOC), and pancreatic ductal adenocarcinoma (PDAC), which recapitulate CD70 tumoral antigen heterogeneity observed in patients, to study the limitations of CD70-CAR T therapy. Flow cytometry, confocal/STED microscopy, transcriptional and chromatin accessibility profiling were used to characterize CD70 expression in PDX models. CD70 transcriptional and chromatin accessibility in ccRCC primary patient tumors were assessed by 10x Multiome. CD70 gene scores as a proxy of targetable expression were determined using a single cell atlas(22) of chromatin accessibility for 222 cell types adult normal human tissues.

Results:
We found that within CD70-heterogeneous tumors, apparent CD70-negative tumor cells do in fact express low levels of CD70, though not at a level high enough to be eliminated by conventional CAR T cells. Thus, we could leverage this pan-tumor cell expression of CD70 by adapting CAR design for more sensitive detection by using an HLA-independent T cell (HIT) receptor, which substitutes the antigen recognition domain of a T cell receptor with that of a CAR, thereby co-opting the downstream signaling of the CD3 complex. Unlike CD70-CAR T cells, CD70-HIT T cells completely eradicated CD70-heterogeneous tumor models of renal cell carcinoma, ovarian and pancreatic cancer. We further found that the CD70 locus in apparent-negative tumor cells is epigenetically silenced by Enhancer of Zeste Homolog 2 (EZH2)-mediated histone H3 lysine 27 trimethylation (H3K27me3), still allowing for very low expression. Given conventional methods are at their limit of sensitivity to detect very low CD70 expression, we harnessed chromatin accessibility to identify low-level but HIT targetable expression in our PDX models. We could then infer an epigenetic signature that predicted low-level expression in a cohort of primary patient samples and limited expression in normal tissues.

Conclusions:
Our works opens up the possibility that tumors comprising only a fraction of CD70-positive tumor cells may express CD70 in all or most tumor cells, and therefore may be targeted by a sensitive immunotherapy such as CD70-HIT T cells. Our findings position CD70 as a pan-cancer target and provide a model for uncovering additional stealth targets amenable to sensitive immunotherapeutic approaches in the face of apparent tumor antigen hete

443 IL-18 Metabolically Reprograms CAR-expressing Natural Killer T Cells and Enhancing Antitumor Activity Against Neuroblastoma

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Invariant natural killer T cells (NKTs) have intrinsic anti-tumor properties that make them promising candidates for CAR-based immunotherapies. GD2-specific CAR (GD2.CAR) NKTs co-expressing IL-15 have shown promising results in patients with relapsed/refractory neuroblastoma (NB, NCT03294954). We also recently demonstrated that CAR-NKTs armed with a membrane-bound form of the proinflammatory cytokine IL-12 further enhance CAR-NKT antitumor activity without added toxicity in mice. Herein, we hypothesized that co-expressing IL-18, another proinflammatory cytokine, alone or with IL15 would further boost the therapeutic potential of CAR-NKTs. To test this hypothesis, we generated retroviral constructs expressing IL-15 and/or IL-18 with the inducible caspase 9 (iC9) safety switch and co-expressed them in human NKTs with a GD2.CAR using dual retroviral transduction. After repeated *in vitro* tumor challenge, IL-18 or IL-15.IL-18 co-expressing GD2.CAR-NKT retained higher functionality (proliferation, cytotoxicity, and cytokine secretion) compared with IL-15 or no cytokine controls. Transcriptomic analyses showed significant enrichment of lymphocyte activation as well as proliferation- and metabolism-related processes in IL-18-expressing CAR-NKTs when compared to IL-15 expressing cells. Targeted metabolomics analyses revealed that GD2.CAR/IL-18 NKTs produced significantly elevated levels of glycolysis (glyceraldehyde 3-phosphate and phosphoenolpyruvate) and citric acid cycle (cis-aconitate and succinate) metabolites compared to GD2.CAR/IL-15 NKTs. *In vivo*, GD2.CAR-NKTs co-expressing IL-18 or IL-15.IL-18 demonstrated superior antitumor activity in a xenograft model of metastatic NB, though mice treated with IL-15.IL-18 cells showed signs of toxicity, accompanied by an increased CAR-NKT expansion in blood and elevation of human cytokine concentrations in serum. These effects were ameliorated by triggering the iC9 safety switch. In contrast, mice treated with GD2.CAR/IL-18 cells did not show signs of toxicity and survived significantly longer than all other groups ($p<0.001$). These results provide support for using IL-18 for clinical development of the next generation of cytokine-armed CAR-NKT cancer immunotherapy.

444 Adoptive cell therapy with genetically engineered T cells for Epithelial Ovarian Cancer

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The limited clinical benefits of immunotherapy in epithelial ovarian cancer (EOC) highlight the urgent need for innovative treatment strategies to improve outcomes. This study employs a dual-pronged strategy to promote EOC immunotherapy, focusing on tumor-specific T-cell receptor (TCR)-redirected T cells.

Cancer cells derived from the ascites of EOC patients were analyzed to determine the expression of selected tumor-associated antigens (Mesothelin, HER2, NY-ESO-1, and WT1) and membrane-bound inhibitory receptor (IR) ligands. These antigens, critical for tumor progression, were selected as targets for our T cell products. Using TCR gene editing techniques, T cells with HLA-A*02:01-restricted receptors specific for these antigens were generated, drawing from both our laboratory's library and relevant literature. In clinically relevant models using patient ascites-derived primary cultures (PC), T cells engineered with WT1-, MSLN-, and NY-ESO-1-specific TCRs exhibited potent and specific elimination of target cells, with WT1-TCR emerging as the most promising candidate for further development. We hypothesized that disrupting IR pathways could shield engineered T cells from

immunosuppression within the tumor microenvironment (TME), thereby enhancing their therapeutic efficacy. To explore this, T-cell products were engineered to express WT1-TCR while incorporating gene disruptions of specific IRs (TIM3 or LAG3) (IR_{KO} WT1-TCR). To assess the impact of IR disruption on anti-tumor efficacy, WT1-TCR and IR_{KO} WT1-TCR T cells were tested against WT1-positive, HLA-A*02:01-positive PC and tumor organoids (PDOs). Evaluations included cytokine secretion, T cell activation using flow cytometry, and apoptosis measured via Incucyte live cell imaging. IR_{KO} WT1-TCR T cells demonstrated superior cytotoxicity compared to WT1-TCR T cells, with $TIM3_{KO}$ WT1-TCR showing heightened effectiveness against tumor cells expressing TIM3 ligands. Co-culture experiments with PDOs further confirmed the increased induction of apoptosis mediated by $TIM3_{KO}$ and $LAG3_{KO}$ WT1-TCR T cells compared to their IR-competent counterparts. Finally, in vivo experiments were conducted to monitor tumor growth using IVIS live imaging and ultrasound. Considering the results of in vitro experiments, our primary focus was to analyze the efficacy of $TIM3_{KO}$ WT1-TCR T cells in comparison to $IR_{COMPETENT}$ WT1-TCR T cells. Both T cell products significantly inhibited tumor growth compared to control groups that received no T cell therapy. Ultrasound imaging revealed a reduction in the size of abdominal masses, with $TIM3_{KO}$ WT1-TCR treatment achieving the most pronounced decrease in mass diameter, particularly in the intermediate size range (4-8 mm).

These findings support the hypothesis that TIM3 knockout enhances the anti-tumor efficacy of WT1-TCR-engineered T cells for EOC.

445 Multimodal T cell immunotherapy CAR-TA (B7-H3 CAR and PRAME Tumor Antigen cells) for pediatric brain and solid tumor patients

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While tumor-associated-antigen cytotoxic T-cell (TAA-T) therapies have shown a robust safety profile and a signal of efficacy in pediatric patients with solid tumors, previous clinical trials did not observe any objective clinical responses. This lack of response is likely due to potent tumor-induced immune evasion tactics. We hypothesized that gene engineering TAA-T cells with a dominant negative TGF-b receptor (DNR) and combinatorial approaches with a B7H3-targeted chimeric antigen receptor (CAR) T-cell product would overcome multiple tumor immune evasion strategies including: antigen loss (targeting intracellular and extracellular antigens with TAA-T and CAR-T), MHC downregulation (with a CAR) and cytokine (TGF-B) suppression. Hence our objective was to evaluate whether this multimodal therapeutic (CAR-TA) would elicit enhanced anti-tumor responses in vitro compared with the single modalities.

Methods:
We engineered T cell products (CAR-TA) from two donors that comprised B7H3 CAR-engineered T-cell and DNR-transduced TAA-T products at a 1:1 ratio. Experiments were performed with the combined product, single products, and untransduced (Un-T) and/or non-specific T cells as controls.

Results/Discussion:
We first demonstrated that TAAT targeting PRAME elicited potent anti-tumor activity against tumor cells (36.92% ± 1.07% target lysis at 48 hours of co-culture). In contrast, the co-culture of tumor cells with non-specific T-cells resulted in tumor proliferation by 24.33% ± 3.109% (Mean tumor growth difference TAAT vs Un-T: 67.76% ± 6.94%; p=0.0006). We also confirmed that DNR-transduced T-cell products obviated TGF-b downstream signaling in vitro (addition of exogenous TGF-b to DNR-T cells resulted in only modest upregulation of 38.65% ±8.56% in pSMAD2/3 expression compared to 66.25% ± 4.03% upregulation in Un-T). We next determined whether CAR-T-cells upregulate MHC on tumor cells. Using D556 medulloblastoma tumor lines, we observed an upregulation of MHCI (57.4% increase when co-cultured with CAR-T cells compared to 14% when co-cultured

with Un-T cells). We also observed a 90.3% increase in MHCII expression in D556 tumor cells when cultured with CAR-T cell compared to a 17.4% in the Un-T cell co-culture condition. Finally, we determined whether the combined CAR-T & DNR-TAAT product (CAR-TA) elicited enhanced anti-tumor activity in vitro compared to the individual components alone. CAR-TA products elicited 53.52% ± 5.86% clearance of HLA-matched tumor significantly higher than 36.13% ± 2.87% for CAR-T alone (p=0.0099) within 21 hrs of co-culture while we observed no tumor clearance for DNR-TAAT, TAAT or Un-T alone. Additionally, we observed antigen spreading when CAR-TA products (n=2) were repeatedly stimulated with HLA-matched tumor cell lines (Ewing Sarcoma and D556 cells). Specifically, after repeated stimulation, CAR-TA products targeting PRAME and B7H3, promoted expansion of T cells targeting other tumor associated antigens (MAGE3/4, WT1, Survivin, and SSX2) in vitro. This "antigen-spreading response" was not observed with the CAR-T or TAA-T or non-specific T cell products. In summary, we have developed a novel T-cell therapy (CAR-TA) that addresses multiple tumor immune evasion strategies in a single product for the treatment of children with relapsed/refractory solid tumors. We are currently testing more donors and will validate this T-cell therapeutic in preclinical in-vivo models before translating to clinical trials

Figure 1.A

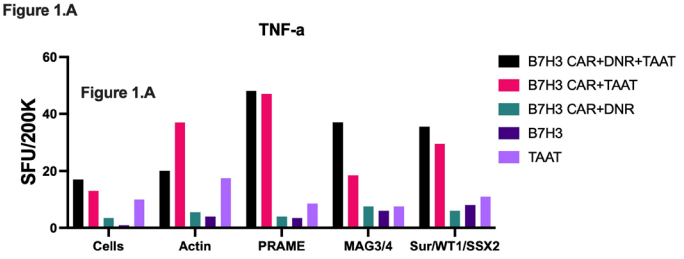

Figure 1.B

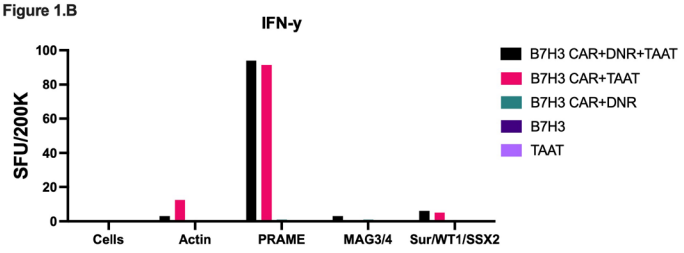

Figure 1. **Only CAR-TA products cocultured with HLA-matched tumors promote the expansion of TAA-specific T cells "antigen-spreading" in vitro.** D556 cell line is used as the target. Individual points represent the mean of replicates. Figure 1.A TNF-a spot count after repetitive tumor stimulation. Figure 1.B IFN-y spot count after repetitive tumor stimulation.

Figure 2

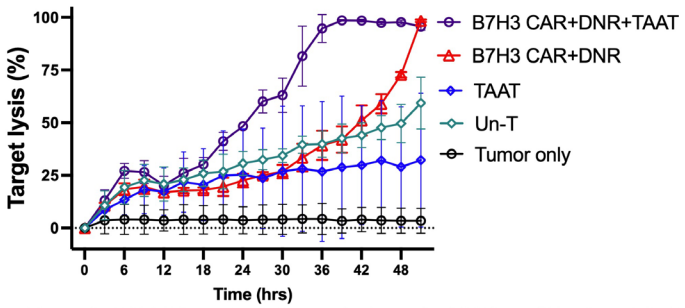

Figure 2. **CAR-TA product demonstrates potent killing of HLA-matched tumors.** HLA-matched tumor cell line (Ewing Sarcoma) is used as the target in xcelligence-based cytotoxicity assay (E:T 5:1 ratio). Individual points represent the mean of replicates, and the error bars represent the standard deviation.

446 Allogeneic GPC3 CAR-iNKT cells show robust anti-tumor activity in hepatocellular carcinoma mouse models

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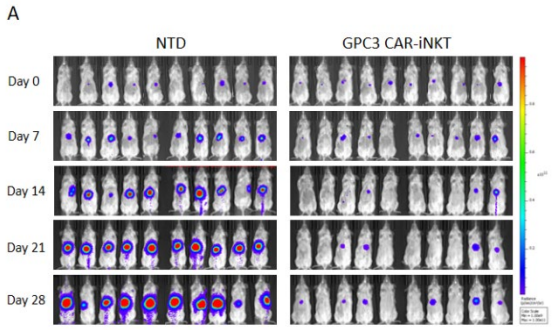
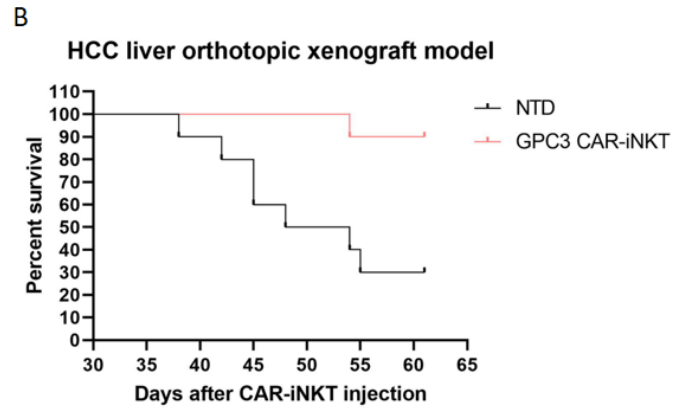
Background:
Hepatocellular carcinoma (HCC) is recognized as the sixth most common malignant tumor worldwide, with a five-year survival rate of only 22%. Invariant natural killer T (iNKT) cells, a rare subset of innate-like immune cells, exhibit unique features: they kill targets by co-expressing natural killer (NK) lineage receptors and a semi-invariantly rearranged T cell receptor (iTCR) that recognizes glycolipids presented by CD1d, modulating the immune microenvironment in both TCR-dependent and independent manners, all without the risk of Graft versus Host Disease (GvHD).

Methods:
Allogeneic CAR-iNKT cells were generated from peripheral blood mononuclear cells (PBMCs) that derived from healthy donors. After purification, iNKT cells were genetically engineered to express a third-generation CAR targeting GPC3 through genome editing. The CAR construct also includes secretory components IL-15 and IL-21 to increase the proportion of memory cell subsets and enhance anti-tumor activities. The CAR template was integrated into the β2M locus through ribonucleoprotein (RNP)-based CRISPR/Cas editing. HCC intraperitoneal (IP) xenograft model, HCC liver orthotopic xenograft model and xenogenic GvHD model engrafted with human CD3- PBMCs in B-NDG mice were employed to evaluate the efficacy and the safety of cell products.

Results:
The CAR-iNKT cells demonstrated a final product purity averaging around 95%, β2M knockout efficiency around 90%, and CAR expression varying between 60% and 90%. In the IP xenograft model, three weeks after the injection of CAR-iNKT cells, complete clearance of tumor cells was observed and there were no signs of tumor recurrence up to the seventh week (week 3: NTD vs. GPC3 CAR-iNKT, $P < 0.001$; week 7: NTD vs. GPC3 CAR-iNKT, $P < 0.001$). Additionally, CAR-iNKT cell product showed superior anti-tumor activity in liver orthotopic xenograft model (Fig. A & Fig. B): treatment with GPC3 CAR-iNKT cells significantly suppressed tumor growth (week 6: NTD vs. GPC3 CAR-iNKT, $P < 0.001$) and extended the survival time of mice (medium survival: NTD, 51 days; GPC3 CAR-iNKT, undefined). At last, iNKT cells demonstrated higher safety compared to T cells in the xenogenic GvHD model, with almost no infiltration of human T cells in vital organs (week 6: histological analyses of lung, liver and spleen) and cytokine secretion levels similar to those in the negative control group (ELISA analyses of IFN-γ production from week 6: T cells vs. iNKT cells, $P < 0.001$; PBS vs. iNKT cells, $P = 0.4263$; PBS vs. T cells, $P < 0.001$).

Conclusions:
Our GPC3 CAR-iNKT cells demonstrated exceptional anti-hepatocellular carcinoma potency in mouse models. These findings strongly support the continued preclinical and clinical development of this innovative cell therapy for patients with relapsed or refractory hepatocellular carcinoma.





447 ADI-270, an Armored Allogeneic Anti-CD70 CAR γδ T Cell Therapy, Demonstrates Robust CAR-Directed and -Independent Anti-Tumor Activity Against Hematologic and Solid Tumor Models Compared to Conventional CAR αβ T Cells

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Background
CAR-engineered γδ T cells represent a promising therapeutic platform for addressing the challenges associated with the treatment of refractory solid and hematologic malignancies. Unlike conventional αβ T cells, γδ T cells possess both innate and adaptive immunity that enable targeting and killing of tumors in an MHC-independent manner. Together with their natural tissue tropism and anti-tumor activity, γδ T cells have the potential to effectively traffic to tumor sites and reduce antigen escape. To leverage these distinct effector functions of γδ T cells, we developed ADI-270, a novel allogeneic γδ T cell product engineered with a third-generation CAR targeting CD70 using its natural receptor (CD27). In addition, ADI-270 is armored with a dominant negative TGFβ receptor (dnTGFβRII) to resist the immunosuppressive effects present in solid tumor microenvironments.

Methods
Vδ1 T cells engineered to express an anti-CD70 CAR and dnTGFβRII were generated from healthy donor human PBMCs. To expand on the potential of ADI-270 to treat solid and hematologic malignancies, we compared the functionality of ADI-270 to clinically relevant benchmarks representing allogeneic scFv-based anti-CD70 αβ CAR T cell approaches. Cell-based in vitro assays were used to evaluate the cytotoxicity of these products against hematologic and solid tumor cell lines with low to negative CD70 expression. CITE-seq was used to profile gene

and surface expression levels from single ADI-270 cells. Human tumor xenografts in NSG mice were used to assess in vivo trafficking and efficacy of ADI-270 against various cancer models.

Results
ADI-270 exhibited potent in vitro cytotoxicity against a panel of cancer cell lines expressing varying levels of CD70 and maintained effector function in the presence of TGFβ1. In contrast to αβ CAR T cell benchmarks, ADI-270 was associated with increased potency against low CD70 expressing cells and in $CD70^{+/-}$ cell mixtures, and demonstrated activity against $CD70^{-}$ tumor cells, suggesting both CAR-directed and CAR-independent killing mechanisms. Despite this increased potency, ADI-270 demonstrated a lower inflammatory cytokine profile when co-cultured with $CD70^{+}$ cells compared to αβ CAR T benchmarks. Additionally, ADI-270 showed rapid tumor homing in vivo and significantly inhibited tumor growth in all $CD70^{+}$ human tumor xenografts tested, including those with low and heterogenous CD70 expression. Analysis of transcriptomes obtained by scRNAseq (10X) using UMAP dimensionality reduction and cluster analysis confirmed ADI-270 cell population homogeneity.

Conclusion
These results support the enhanced potency of ADI-270 against multiple CD70-low to CD70-negative-expressing tumor cell models compared to relevant scFv-based αβ CAR T cell benchmarks. The anti-tumor activity of ADI-270, which involves CAR-directed and CAR-independent mechanisms against both hematologic and solid tumors, highlights the potential to overcome the challenges associated with antigen heterogeneity. These favorable preclinical efficacy and safety characteristics support the clinical development of ADI-270 to address multiple $CD70^{+}$ cancers.

Lipid Nanoparticles III

448 Multi-Objective Machine Learning-Guided Optimization of mRNA Lipid Nanoparticles for Cell-Selective Transfection

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Statement of Purpose:
Lipid nanoparticles (LNPs) have emerged as promising non-viral gene delivery vectors. However, they require extensive development across a vast and complex design space—a task where heuristic and brute-force screening approaches have proven inefficient. Here, we build and deploy a machine learning (ML)-guided iterative screening platform to accelerate the optimization of LNP compositions for cell-selective gene delivery. Starting from sparse initial library screens of LNP transfection against on-target and off-target cell types, we select, train, and refine high-performing ML models that predict transfection outcomes as a function of LNP design features. We then leverage multi-objective (MO) search algorithms and experimental validation to create a build-test-learn-design iterative loop that generates de novo LNP compositions aimed at *maximizing on-target and minimizing off-target transfection*. Our *in silico*-guided approach accelerates the design of LNP formulations with high cell-selective transfection properties.

Methods:
Human RAMOS-B cell line, a canonically hard-to-transfect cell type, were chosen as the *on-target* cells. Human THP-1 monocytes were used as the *off-target* cells. To generate a foundational LNP library for initial ML training, we utilized DLin-MC3-DMA as the ionizable lipid (IL), along with cholesterol, DMG-PEG2000 (PEG), and DSPC as the helper phospholipid (HL). We varied the combined molar percentage of IL and HL, the molar ratio of cholesterol to PEG, the molar ratio of IL to HL, and the molar ratio of lipids to mRNA. 81 LNP formulations were screened separately in each cell for *in vitro* transfection efficiency using luciferase reporter mRNA. For each cell, a panel of tree-based, linear, and partial least squares regression ML models were trained to predict LNP transfection and were optimized using the scikit-learn Python package (1.2.2). For each cycle of MO iterative optimization, our

Gaussian process-based Bayesian Optimization via Scikit-Optimize (0.9.0), fed with our best Ramos B and THP-1 ML models, suggested 12 *de novo* LNPs that maximized Ramos B and minimized THP-1 transfection, utilizing expected improvement (EI) to balance exploration and exploitation of the design space.

Results:
LNPs designed to maximize transfection in *on-target* B cells and minimize *off-target* THP-1 cells (B-skewed LNPs) were developed through an ML-guided Build-Test-Learn-Design loop (Fig. 1A). After just 4 iterations, B-skewed LNPs achieved 50-fold higher transfection in B cells (RLU~ e^7) compared to THP-1 cells (RLU~ e^4) at the same dose (Fig. 1B). In a 50:50 coculture of B and THP-1 cells treated with GFP encoding mRNA LNPs, we observed that our B cell-skewed LNPs transfected 60% of B cells and were highly selective for B cells, with over 75% of the transfected cells being B cells (Fig. 2A–C). Compared to commercially available and top grid-search LNPs, B-skewed LNPs achieved significantly higher GFP expression per cell and a higher percentage of GFP+ cells in the B cell population than in the THP-1 cell population (Fig. 2D, E).

Conclusion:
An iterative automated ML-guided platform was developed to accelerate the MO optimization of LNPs. Our platform successfully identifies LNPs that achieve better and more selective transfection in *on-target* B cells than in *off-target* THP-1 cells *in vitro* compared to both conventional grid-searched and commercially available LNPs.

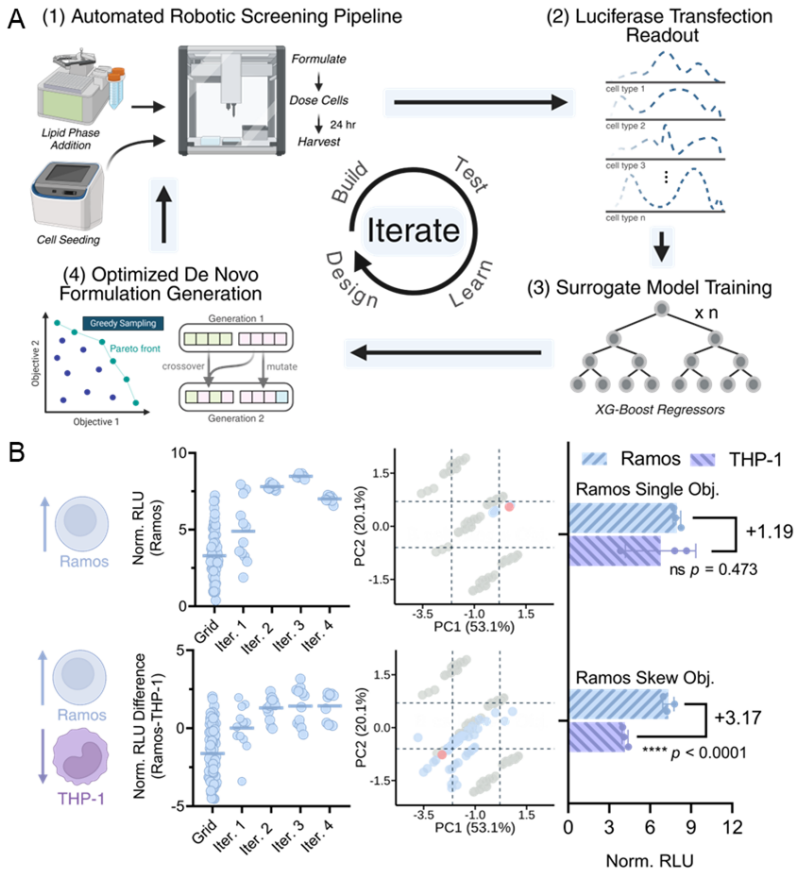

Figure 1. Iterative strategy for machine learning (ML)-driven optimization of cell-type selective mRNA-LNPs. (A) The Design-Build-Test-Learn loop for iterative screening. (B) Four rounds of iterative screening were to either only optimize Ramos B cell transfection alone (single objective) or Ramos B cell selective transfection by maximizing the difference between Ramos B and THP-1 cell transfections (multi-objective).

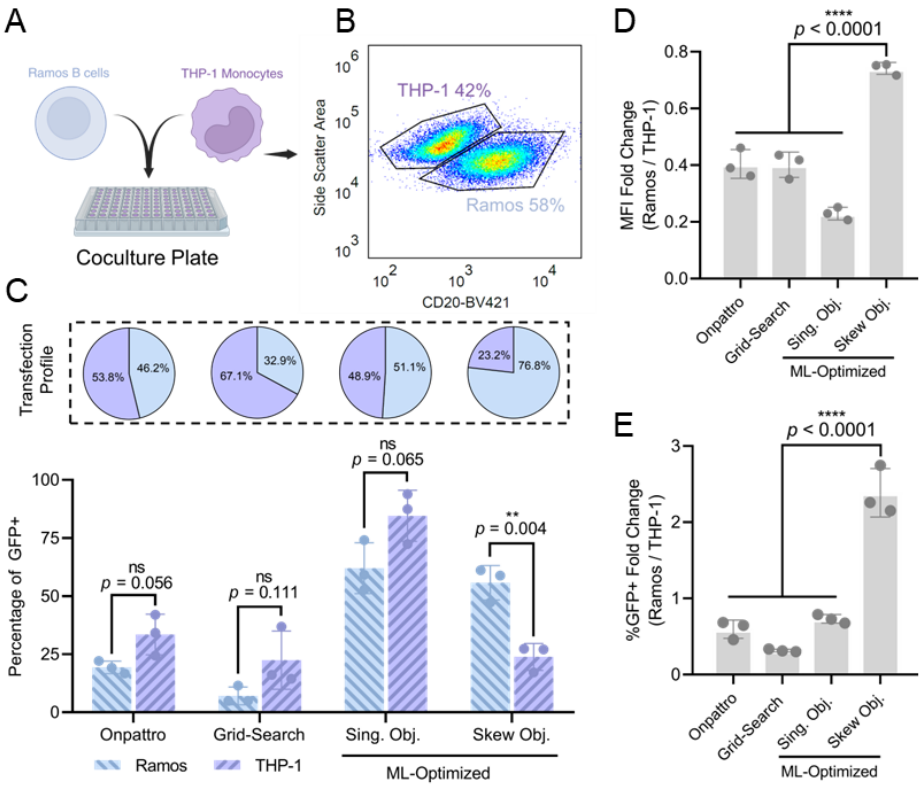

Figure 2. A) Ramos B cells and THP-1 monocytes were seeded together at equal cell concentration and treated with 1) Onpattro formulation, 2) the top grid-searched LNP, and the top ML-optimized LNPs from 3) single objective and 4) dual-objective skewed approaches. LNPs were formulated using GFP reporter mRNA. **B)** Representative flow gating of cell populations in the coculture via CD20 stain. **C)** Overall transfection profile and GFP+ percentage of Ramos and THP-1 cells after 24 hours. **D–E)** Transfection fold change (Ramos/THP-1) in both GFP+ percentage and mean fluorescence intensity (MFI) of transfected cells.

449 Gene Replacement Therapy for Pulmonary Lymphangioleiomyomatosis

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Pulmonary lymphangioleiomyomatosis (LAM) is a rare genetic lung disease that is characterized by aberrant mTORC1 hyperactivity caused by inactivating mutations of the tumor suppressor genes tuberous sclerosis complex 1 or 2 (Tsc1 or Tsc2) genes. Rapamycin (sirolimus), an mTORC1 inhibitor, is FDA-approved for the treatment of LAM, although ongoing loss of lung function and need for lung transplantation can still occur. Thus, there is an urgent need to develop new therapies for the treatment of LAM. Given that LAM is caused by mutations in a single gene, either TSC1 or TSC2 (more common), gene replacement therapy presents a potential therapeutic strategy by introducing functional TSC1 or TSC2 genes into affected cells. Achieving safe and effective systemic delivery of messenger RNA (mRNA) to specific organs and cells in vivo remains a significant challenge in the development of mRNA-based gene replacement therapy. To date, systemically administered lipid nanoparticles (LNPs) co-formulated with mRNA have predominantly targeted the liver and spleen. Through a library screening approach, we identified the specific modifications on the functional lipids and route LNP to the lung. Furthermore, by modifying the head-group structure of LNPs, different pulmonary cell types can be precisely targeted.

In this study, we demonstrate the efficacy of LNP-based mRNA therapy in a preclinical model of LAM. Our lung-targeting LNPs efficiently delivers mouse Tsc2 mRNA to restore TSC2 tumor suppressor function in tumor nodules,

resulting in a significant reduction in tumor burden. This research establishes mRNA-loaded LNPs as a promising therapeutic platform for the treatment of LAM.

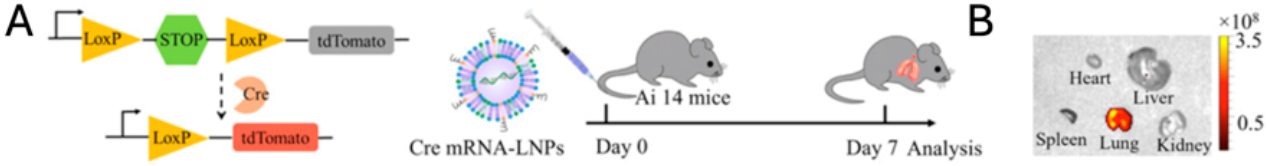
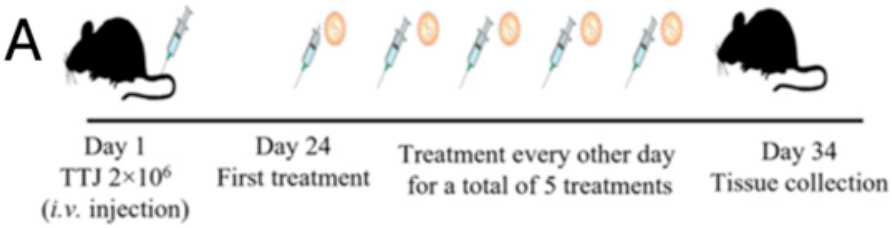


Fig. 1. Lung-targeting LNP. (A) Schematic illustration of the delivery of Cre mRNA to the lung to activate tdTomato expression via Cre-mediated genetic deletion of the stop cassette in tdTomato transgenic Ai14 mice. (B) Representative *ex vivo* image of tdTomato fluorescence in edited Ai14 mouse organs.



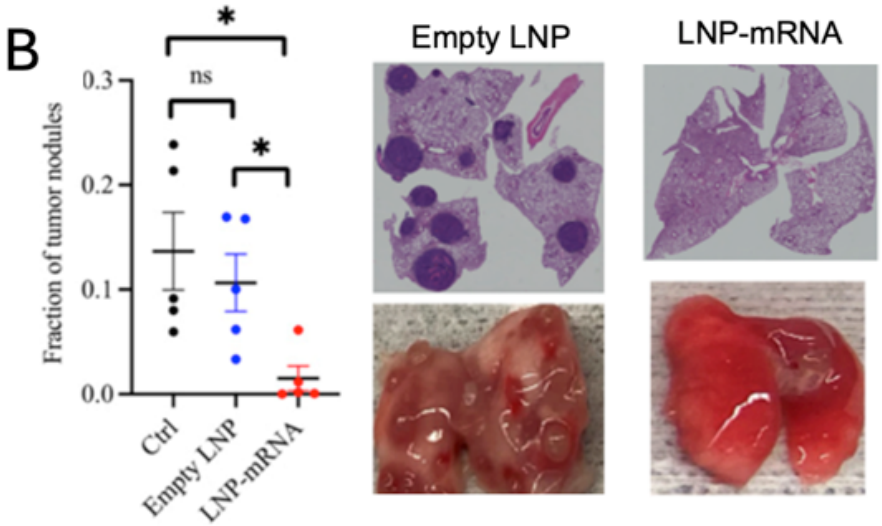


Fig. 2 Therapeutic effect of Tsc2 mRNA-loaded LNP in antitumor growth. (A) Treatment design. Syngeneic C57BL/6J mice were tail vein injected with 2 million Tsc2-deficient cells to form tumor nodules in the lung. On day 24 after tumor cell inoculation, mice were randomly assigned to three groups: untreated control, empty LNP treatment, and LNP-mTsc2 treatment. Mice were treated every other day for a total of five times (n = 5). (B) Fraction of tumor nodules per lung in the three treatment groups (left), and representative H&E and image of lungs collected from empty LNP and LNP-mTsc2 treated groups (right).

450 Ionizable Lipid Development and LNP Formulation Optimization Enable the Use of RNA Gene Writers for *In Vivo* Treatment of Genetic Diseases in Liver

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In recent years, RNA-encapsulated lipid nanoparticles (LNPs) have been developed into FDA-approved siRNA therapeutics and prophylactic mRNA vaccines. Yet, while several clinical trials are in progress, the application of LNPs for *in vivo* gene editing remains a challenge, owing to both the development of complex payloads which are harder to deliver at a therapeutically relevant dose and the need for well-tolerated lipid compositions. To address this challenge and enable *in vivo* gene editing for genetic diseases in the liver via an intravenous (IV) administration, we have developed novel ionizable lipids (iLipids) and LNPs to safely and efficiently deliver RNA Gene Writers to hepatocytes.

We applied our RNA Gene Writer to address phenylketonuria (PKU) by editing the human *PAH* gene. To achieve high levels of gene writing along with a desirable safety profile, we screened known iLipids and LNP formulations, while independently developing our own proprietary iLipid library and LNP formulations in parallel. We designed, synthesized, and tested novel iLipids from various cores to identify multiple promising core structures, then optimized iLipid structures for each genus via iterative screenings based on our structure-activity relationship (SAR) model. We successfully discovered a highly potent proprietary iLipid and LNP formulation that delivered RNA Gene Writer to an estimated average of 54% of hepatocytes in a *hPAH* transgenic mouse model, while exhibiting a desirable tolerability profile based on serum chemistry assessments. Upon evaluating potent LNP formulations, we observed that the iLipid identity has a large influence on the safety profiles of the resulting LNPs.

We also applied our RNA Gene Writer to edit the disease-causing PiZ allele in Alpha-1 antitrypsin deficiency (AATD) through a similar iLipid discovery campaign. After screening our own iLipids and LNP formulations, we identified multiple proprietary iLipids and LNP formulations that exceeded the anticipated curative levels of *SERPINA1* gene correction in the hepatocytes of a transgenic mouse model (*hSERPINA1*$^{E366K/E366K}$ mouse). We then further optimized a lead LNP formulation that achieved an estimated average of 79% of hepatocytes in non-human primates (NHP) after a single IV administration, resulting in the production of 84% of the correct mRNA transcripts on average. When we evaluated the biodistribution profile of our RNA-LNP formulation in *hSERPINA1*$^{E366K/E366K}$ mice at a saturating dose, we observed high levels of intended gene editing in the liver and minimal to undetectable levels of gene editing in off-target tissues, suggesting a high degree of liver tropism of our LNP formulation. We further evaluated the safety of the LNP formulation by investigating its pharmacokinetics (PK) profile, where we observed that the LNP formulation was cleared from the blood quickly relative to other LNPs and iLipids. This profile of high on-target delivery and relatively rapid clearance is highly desirable for further translation to the clinic.

Taken together, we demonstrated that our LNP delivery platform can be applied to efficiently and safely correct different genes in hepatocytes at therapeutically relevant doses with a single IV administration. Through successful iLipid discovery campaigns and LNP formulation optimizations, we enabled the use of our RNA Gene Writers for editing multiple disease targets in liver hepatocytes.

451 Targeted Gene Editing in the Fetal Brain via In Utero Intracerebroventricular LNP mRNA Delivery

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Many genetic disorders affecting the central nervous system (CNS) begin during prenatal development, resulting in permanent damage. CRISPR-Cas9 gene editing has the potential to provide a one-time curative treatment, provided the therapeutic agents reach the appropriate cells and tissues. Despite the potential, obstacles like the blood-brain barrier (BBB) and the limited regenerative ability of neurons have slowed the advancement of therapies aimed at the CNS. While non-viral ionizable lipid nanoparticles (LNPs) are recognized for their safety and effectiveness in mRNA delivery to the liver, data on the cell-type-specific biodistribution throughout the CNS following ICV administration are limited. Fetal gene editing via ICV administration addresses disease processes before they manifest and leverages the distinctive biological characteristics of the developing fetus to enhance therapeutic delivery to the brain. To optimize fetal ICV mRNA delivery to neurons, we evaluated three different LNP formulations containing CRE recombinase mRNA in a GFP reporter mouse model. To quantify mRNA delivery, GFP expression was analyzed in neurons, endothelial cells, and microglia in the cortex, cerebellum, and spinal cord via flow cytometry. Among the tested formulations, one LNP (LNP2) demonstrated notable efficiency in delivering mRNA to endothelial cells (~80%), macrophages (~15%), and neurons (~35%) in fetal recipients when compared to negligible mRNA delivery observed in postnatal recipients. Similar results were observed in the cerebellum and spinal cord. Together, these results indicated that the fetal environment provides unique conditions that enhance the uptake and distribution of LNP-delivered mRNA in the CNS. Building on these findings, we quantified the differential binding of LNP2 to proteins commonly found in cerebrospinal fluid, which provided insights into strategies for enhancing brain-specific drug delivery. Finally, we used LNP2 to conduct CNS gene editing in a mouse model of Friedreich Ataxia (FRDA). To this end, we delivered SpCas9 mRNA and single guide RNA (sgRNA) targeting the mutation via ICV at the 14th gestational day. We observed low levels of gene editing in the brain of FRDA fetal recipients, and no quantifiable editing in the postnatal recipients. These preliminary results highlight the feasibility of in utero gene therapy to address the genetic basis of FRDA and provide a foundation for further exploration. Overall, this study highlights the potential of leveraging fetal biology to optimize LNP-mRNA delivery for CNS-targeted therapies, paving the way for innovative in utero treatments for neurological disorders.

452 Multi-stage-mixing to create a core-then-shell structure improves DNA-loaded lipid nanoparticles' transfection by orders of magnitude

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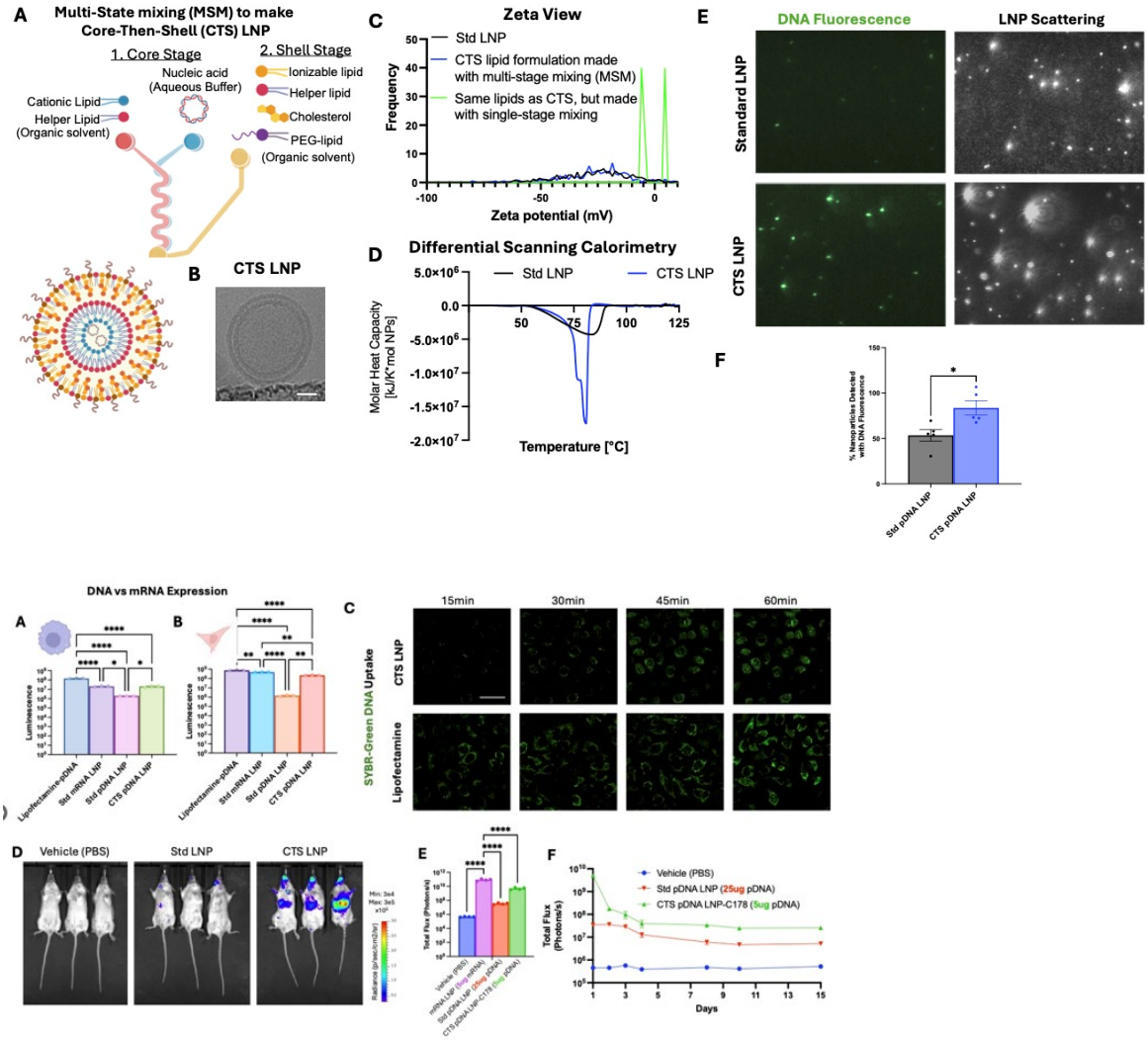
DNA-loaded lipid nanoparticles (LNPs) offer unique advantages for treating chronic diseases, including months-long expression and cell-type-specific targeting through genetic promoters. However, their clinical translation has been limited by poor protein expression levels that are orders of magnitude lower than mRNA LNPs. Here, we introduce multi-stage mixing (MSM) microfluidics to create core-then-shell (CTS) structured DNA LNPs that dramatically improve transfection efficiency. In MSM synthesis, DNA is first mixed with cationic and zwitterionic lipids to form a compact core, which is then immediately encapsulated by standard LNP lipids before aggregation can occur, forming a stabilizing shell (**Fig 1A**).

Physical characterization reveals that CTS LNPs possess a distinct architecture from conventional LNPs that are produced by rapid mixing aqueous phase containing nucleic acids and organic phase containing lipid mixture. Cryo-electron microscopy shows a well-defined central region with two distinct outer shells, contrasting with standard LNPs' amorphous structure (**Fig 1B**). Single-particle zeta potential measurements show similar zeta distribution of CTS LNPs vs. standard LNPs (**Fig 1C**). Differential scanning calorimetry confirms the unique internal organization of CTS LNPs (**Fig 1D**). Notably, CTS LNPs exhibit two sharp thermal transitions (75°C and 85°C) versus one broad transition in standard LNPs, indicating more ordered structural domains. Nanoparticle tracking analysis demonstrates superior nucleic acid loading, with 83% of CTS LNPs containing detectable DNA compared to 53% of standard LNPs (**Fig 1E-F**).

In vitro studies show that CTS dramatically improves DNA transfection efficiency. CTS LNPs achieve >10-fold higher expression than standard LNPs in RAW macrophages (**Fig 2A**) and ~150-fold higher in HeLa cells (**Fig 2B**), reaching levels comparable to mRNA LNPs. The delivery of DNA into nucleus by CTS LNPs is on a very similar time course to that of Lipofectamine (**Fig 2C**). The enhancement extends to traditionally hard-to-transfect primary cells, with CTS LNPs outperforming the gold standard Lipofectamine in mouse embryonic fibroblasts and primary cortical neurons.

In vivo, CTS DNA LNPs demonstrate remarkable improvements in both efficiency and safety. When co-loaded with the STING inhibitor C-178, CTS DNA LNPs reduce DNA induced mortality to from 100% to 0%, and achieve peak expression levels approaching those of mRNA LNPs but with sustained expression over months rather than hours. Notably, this is accomplished with a 5-fold lower DNA dose compared to standard LNPs while eliminating toxicity concerns (**Fig D-F**).

This work advances both materials science and therapeutic applications by demonstrating how microfluidic control over nanoparticle assembly kinetics can access otherwise unattainable particle architectures. The development of CTS DNA LNPs represents a significant step toward realizing the potential of DNA-based therapeutics for chronic diseases, offering the unprecedented combination of high expression levels, sustained duration, and cell-type specificity in a single delivery system. This innovation opens new possibilities for treating numerous common chronic diseases where current genetic medicine tools face limitations.



Vector Product Engineering, Development and Manufacturing (excluding AAV)

453 Optimization of Cell Expansion Phase Parameters for a Cost-Effective Lentiviral Vector Perfusion Process

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Impact/Novelty
This pioneering study optimizes cell expansion phase conditions to enhance the efficiency of lentiviral vector (LVV) production via perfusion process, employing a Design of Experiments (DoE) methodology.

Introduction
The demand for high-quality, high-yield LVV production processes is growing rapidly. Perfusion technology emerges as a promising solution, offering reduced processing time, enhanced vector quality and lower costs. Despite its potential, the establishment of an effective perfusion process remains underexplored, particularly in the cell expansion phase. A typical perfusion process comprises two phases: the cell expansion phase, aimed at achieving a target cell density, followed by a steady-state production phase. While existing literature predominantly focuses on optimizing the production phase, this study addresses the overlooked potential of optimizing the cell expansion phase. By refining key parameters in this phase, we ensure the entry of high-quality, productive cells into the production phase, while minimizing media consumption and reducing process time.

Methods
A DoE approach was employed to investigate the interactions amongst three critical parameters: batch duration, seeding cell density, and induction reagent concentration. A three-level factorial design was used and analyzed with JMP 18 software. Inducible producer HEK293T cells, adapted for serum-free, suspension growth, were employed for LVV production. A total of 29 experimental runs were conducted in shake flasks under pseudo-perfusion conditions through daily centrifugation over 8-11 days. Optimal conditions identified through the DoE were subsequently validated in benchtop stirred-tank reactors using an ultrasonic cell retention device under fully continuous perfusion conditions.

Results and discussion
Our findings indicate that a lower induction reagent concentration in the N-1 shake flask led to earlier induction, reducing cell growth due to VSV-G cytotoxicity but enhancing early cell productivity. Additionally, the timing of perfusion initiation, in conjunction with appropriate seeding cell density, proved critical. Premature initiation of perfusion could hinder cell growth, while delayed initiation could result in glucose depletion and reduced cell productivity. Principal Component Analysis (PCA), conducted using DoE software and Python, provided deeper insights into the interactions between cell expansion parameters and their impact on key responses such as cell growth rate, specific productivity, expansion phase duration, glucose consumption, and space-time yield. By optimizing these parameters, we achieved higher cell productivity, reduced media consumption, and a shorter overall process time, ensuring the health and productivity of cells entering the production phase. This optimization was successfully validated in benchtop stirred-tank reactors, demonstrating a significant improvement in perfusion efficiency and cost reduction.

Conclusion
This study underscores the critical importance of optimizing the cell expansion phase in LVV perfusion manufacturing. By fine-tuning key parameters, we enhanced process efficiency, reduced media usage, and shortened processing time, thereby laying the foundation for more cost-effective and scalable LVV production.

454 Advancing Lentiviral Vector Manufacturing: a Platform for Commercial Cell Therapy Success

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The demand for efficient strategies to advance lentiviral vector (LV)-based therapies from early development to commercialization is growing. LVs are efficient tools for delivering genetic material to cells and are widely used in *ex vivo* cell therapy clinical trials, but their commercial success depends on robust, well-characterized LV manufacturing processes. We have previously reported the development and scale-up of a transient transfection, suspension culture-based LV production process that consistently achieves upstream infectious titers exceeding 1E8 TU/mL and purified final titers of 1E9 TU/mL, with particle-to-infectious titer ratios below 200 and 2–3 log reductions in residual DNAs and host cell proteins (HCPs). These outcomes were demonstrated using TCR (NYESO) and CD19 CAR model cargos and have since supported clinical manufacturing of additional cargo constructs.

GMP manufacturing data demonstrates the platform's scalability, with a single batch capable of producing enough vector to treat up to several hundred patients, depending on titer, transduced cell numbers, and MOI dosing. Process development studies investigated the expanded operational ranges for parameters such as buffer pH in chromatography, cell density, plasmid quantities during transfection, and in-process hold times, to enhance process flexibility. Additionally, a GMP-compliant HEK 293 working cell bank (WCB) was established, demonstrating comparability to the master cell bank (MCB). To accelerate commercial readiness, a 2L scale-down model (SDM) was evaluated across multiple LV products to mirror the 25L production scale used in process development (for Tox-grade material) and GMP manufacturing (for clinical material). A Quality Target Product Profile (QTPP) with product-independent quality criteria for ex vivo applications was established, along with risk assessments for Critical Quality Attributes (CQAs), raw materials, and process parameters, providing a foundation for process characterization. This comprehensive approach integrates robust process design, clinical insights, and risk-based analysis, providing a clear pathway for commercializing LV-based therapies.

455 Serum Free Suspension HEK293 Cells Stably Expressing Gag/Pol /Rev /VSVG for Efficient Production of Lentiviral Vectors

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Several groups have generated serum free suspension HEK293T cells with regulated expression of the integrated helper plasmids. The same is not true of the HEK293 parent cells without the SV40 Large T antigen. Anticipating future regulatory requirements that may include avoidance of bovine serum, Large T antigen, and antibiotics, we have developed a new lenti producer cell line with tightly regulated expression of VSVG.

First, HEK293 cells were selected based on their ability to proliferate as suspension adapted serum-free cells. Promising oligoclonal cells were further adapted to highly efficient proliferation in serum free suspension culture. This was followed by screening for transfection efficiency and efficient production of LV after transfection with the vector and helper plasmids (4 plasmids). The most promising of these oligoclonal populations of serum-free suspension cells also proliferate rapidly in adherent culture and can readily transit between a suspension and an adherent phenotype.

The most efficiently transfectable suspension populations were expanded adherently and transfected to express stable Gag/Pol and Rev in a single step. To achieve this, Gag/Pol encoding pMDL.RRE was linearized and paired

with a novel Rev/Puromycin construct. The linearization provided cohesive ends that were compatible with the linear pMDL.RRE. Different Gag/Pol to Rev ratios were concatemerized and transfected into the serum-free suspension HEK293 cells. Selection of these cells in low concentration of puromycin enabled the isolation of a mixed population of cells that stably expressed a broad range of different levels and ratios of Gag/Pol and Rev. This original population was used to sort cells with the optimum levels of Rev expression. This population was used in turn used to identify individual clones of cells able to support the production of high titre lenti by transient transfection of plasmids encoding VSVG and the vector. These clones were further modified with a tightly regulated and inhibitable VSVG cassette. The new cells grew in the presence of appropriate inhibitors to regulate both Protease and VSVG toxicity. Following the removal of Protease and VSVG inhibitors, infectious LV could be readily produced by transfection of the vector plasmid. This strategy, based on the expression of optimised levels and ratios of Gag/Pol and Rev, in combination with a tightly regulated VSVG, which is rapidly degraded in the absence of a small molecule stabilizer, allows the generation of stable LV producer cells. Clonal populations of stable, high-titre, producer cells are now being characterised.

Data presented here supports our hypothesis that we could generate a stably expressed Gag/Pol/ Rev, partnered with a novel regulated VSVG. The VSVG can be constitutively expressed based on the addition of the desired inducer and removal/dilution of the stability molecule. The resulting vector is non-infectious until the "stop signal" is removed and the protein is stabilised. This cell line combines the flexibility of suspension or adherent culture, with Stable Gag/Pol, Rev, and a tightly regulated VSVG expression.

456 A to Z of replication-competent free adenovirus production from gene to manufacturing process intensification

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Adenoviral vectors are versatile tools used for their high transduction efficiency, immunogenic properties and easy manufacturing across various applications. Non-replicating adenoviruses are widely used in gene therapy and vaccination, delivering therapeutic genes without the risk of viral replication. However, safety concerns remain regarding unintended recombination and creation of replication-competent adenoviruses (RCA). This study examines the intensified production of non-replicating adenoviruses using CAP® cells (Cytiva's amniocyte production cells), a suspension-adapted human cell line engineered for efficient and safe viral vector manufacturing.

Transfection conditions of the adenovirus cosmid vector were optimized to generate adenovirus seed stock in suspension CAP cells. To accelerate screening, we developed a rapid identification method using flow cytometry, initially with a GFP reporter gene and later extended to the therapeutic gene of interest. We achieved a transfection efficiency of 21% and a virus yield of 1.93×10^9 VG/flask surpassing our 1×10^5 VG/flask needed for clonal selection.

To ensure correct gene insertion, we used standard plaque picking for adherent HEK293 cells. For suspension CAP cells, we developed a cloning method using limited dilution in plate format to ensure single viral particle infection per well (0.1 ifu/well) and screening the clones by infecting CAP and adherent HEK293 cells analyzed by qPCR and cytopathic effect, respectively. . Selected clones were then expanded under optimized conditions over multiple generations and tested for RCA absence.

With the virus seed, we established a scalable production process. Optimization of key parameters—including cell density at infection (CDI), multiplicity of infection (MOI), and medium exchange strategies—significantly improved adenovirus yields. Perfusion-based fed-batch systems were evaluated to sustain cell viability and productivity, increasing the final yield.

We tested two perfusion-enabled bioreactor technologies: the iCELLis nano fixed-bed bioreactor and the BioBlu 1c stirred-tank bioreactor connected to an ATF system. The perfusion rates were studied measuring key metabolites, such as glucose consumption. This approach increased cell density and led to at least a 5-fold improvement in titer compared to an optimized batch process.

These results highlight CAP cells as a versatile and efficient platform for adenovirus production across different upstream technologies, particularly in meeting the growing demand for gene therapy and vaccine manufacturing. The intensified adenovirus production process using CAP cells represents a major advancement in bioprocessing, offering a scalable, high-yield, and RCA-free platform.

457 Establishing a Versatile Platform for Intensified Lentiviral Vector Manufacturing

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Lentiviral vectors (LVVs) have become important tools in cell and gene therapy, valued for their ability to efficiently modify both dividing and non-dividing cell types. This, combined with their ability to integrate into the host genome for long-term expression, have made LVVs increasingly attractive for treating a wide range of diseases. However, current LVV production methods face significant challenges, including low titers, limited scalability, and high manufacturing costs – leading to a high cost of goods (COGs) and limiting adoption of these vectors. Through this work, we aim to develop a flexible, intensified LVV manufacturing process by systematically evaluating key process parameters and implementing effective process intensification strategies such as TFDF perfusion to improve LVV production performance.

In this work, we first evaluated LVV production via transient transfection in suspension at a small scale to develop a baseline production platform. This entailed the screening of several commercial LVV production systems, and the selection of the system which performed best on the basis of LVV functional titer and quality. We further refined the packaging plasmid system used, and transfection conditions were optimized to increase LVV productivity, resulting in a 10-fold increase in functional titer. This optimized process was then successfully scaled to 2L benchtop bioreactors, maintaining comparably high productivity to the small-scale runs, with titers exceeding 2.0x108 transduction units per milliliter (TU/mL).

Further, process intensification was achieved through the implementation of Repligen's Tangential Flow Depth Filtration (TFDF) technology. TFDF combines both tangential flow and depth filtration principles through the use of a tubular depth filter with a 2-5 µm pore size operated in tangential flow mode. Use of this system resulted in a greater than 3-fold increase in cell density at time of transfection, increasing from 4.0x106 cells/mL in our batch process to over 13.0x106 cells/mL in perfusion while maintaining high cell viability. TFDF operation also enabled the continuous harvest of LVV for the duration of the study while retaining cells and cell debris within the bioreactor volume. Overall, this intensified process resulted in a significant increase in yield compared to our non-intensified process, with total titers harvested from the 2L perfusion bioreactor exceeding 1.9x1012 TU. Space yield in the bioreactor increased more than 4-fold from 2.0x108 TU/mLBR in batch operation to nearly 9.0x108 TU/mLBR in TFDF-mode operation, indicating an increase in cell-specific LVV production. This intensified process demonstrates significant potential to improve access to high-quality lentiviral vectors, allowing for equivalent LVV production at smaller scales and potentially obviating the need to scale beyond 200L, thereby reducing overall manufacturing costs and time. Future work will focus on further optimization of the TFDF perfusion process and its integration with downstream purification to develop a fully continuous LVV manufacturing platform.

458 Efficient and Scalable Chromatographic Purification of Clinically Relevant Lentiviral Vectors

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Lentiviral vectors are an important tool in gene and cell therapy due to their ability to transduce both dividing and nondividing cells. However, manufacturing challenges hamper the application of these vectors, particularly due to the lack of an efficient, scalable purification method. Historically, yields of over 50% have been achieved using a 5 mL Mustang Q membrane, but this yield decreases to 10-15% when a larger Mustang Q membrane is used for purification of lentiviral vector produced from suspension cells in serum free media. In this work, we compare chromatographic purification methods for a clinically relevant lentiviral vector including a Sartobind D membrane, Capto DEAE, and tangential flow filtration (TFF) to increase the load over a 5 mL Mustang Q membrane. We also compare differences in yield when using a process development focused AKTA Avant or a GMP appropriate AKTA Ready 450.

A 5L shake flask is inoculated with $1x10^6$ cells/mL in 1.5 L of media (80% Freestyle™ 293 Expression Media, 20% LV-MAX™ Production Media). Transfection occurs one day after inoculation, and Benzonase is added on the following day. The culture is harvested 48 hours after transfection and filtered through a 0.45 µM final filter before proceeding to chromatography. All methods conclude with a TFF step to bring the final volume to 2% of the culture volume and buffer exchange the purified product into X-VIVO 15 media.

While purification of lentiviral vector over a 5 mL Mustang Q membrane using an AKTA Avant 150 generated a yield of 55%, purification using the GMP appropriate AKTA Ready 450 gave a yield of 30% (857 mL load). Using TFF (500 kDa Pellicon Mini) to concentrate 4.5 L of supernatant to 500 mL before loading on the AKTA Ready 450, gave a similar yield of 27% showing that tangential flow filtration prior to chromatography does not significantly decrease lentiviral vector yield and increases the potential load volume of the membrane. This yield is significantly higher than the yield of 10-15% seen using the Mustang Q 10 mL during previous runs.

Lentiviral vector was also purified with an AKTA AVANT using a 3 mL Sartobind D membrane (50% yield), and Capto DEAE resin (11x90 mm, 21% yield). While further work is needed to verify scalability of these methods, they each show an improved lentiviral yield in a theoretically scalable format. As the need for clinical lentiviral vector has increased, efficient, scalable chromatography methods have lagged. This work presents potentially scalable methods for purification of lentiviral vectors.

459 Novel Lung Cell Penetrating Peptides Target Airway Epithelial Type 2 Cells

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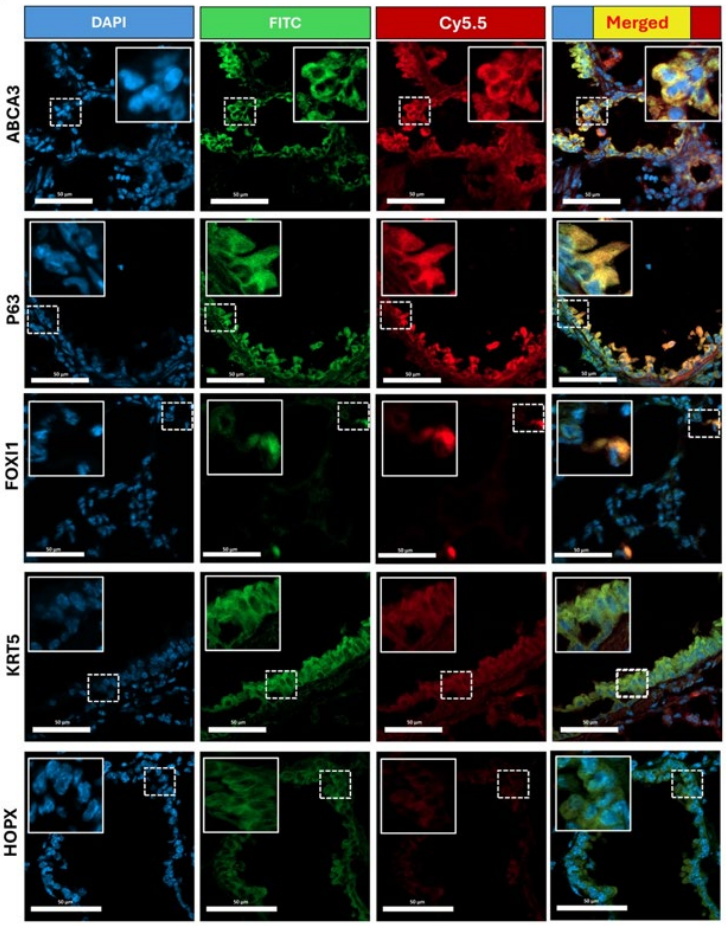
Background:
Cell-penetrating peptides (CPPs) are 5-30 amino acid peptides capable of crossing cell membranes without transfection reagents while delivering large cargoes in an intact, functional form. Our previous phage display studies identified a unique, non-naturally occurring 12-amino acid peptide, termed the cardiac-targeting peptide. Through alanine scanning, we identified two robust lung-targeting peptides (LTPs): S7A and R11A. This study details the identification, biodistribution, comparison of linear and cyclic version, uptake mechanism, and targeted cell types of LTPs. We further demonstrate LTP as delivery vectors for siRNA targeting SARS-CoV-2 proteins in an air-liquid interface model of viral infection using human lung cells.

Methods:
Wild-type CD1 mice were injected with Cy5.5-labeled LTPs, and the biodistribution was analyzed via imaging at various circulation times. Lung uptake was assessed via cross-staining of lung sections post-injection, as well as single cell isolation followed by immunostaining with various lung cell markers followed by FACS. Human bronchial epithelial cells (HBECs) were incubated with linear or cyclic LTPs, and FACS measured uptake intensity. Endocytosis mechanisms were explored via FACS analysis of cyclic R11A (cR11A) uptake in HBECs treated with endocytosis inhibitors (Pitstop2, Amiloride, and Nystatin) and via confocal microscopy tracking co-localization with transferrin or the lysomal marker LAMP1. For siRNA delivery, cR11A was conjugated to siRNA duplexes using a DTME linker and confirmed by MALDI-TOF analysis. Anti-viral efficacy against SARS-CoV-2 was tested in an air-liquid interface model of human lung infection.

Results:
Alanine scanning identified S7A (APWHLSAQYSRT) and R11A (APWHLSSQYSAT) as potent LTPs with minimal heart uptake. Intravenous injection of LTPs into mice demonstrated robust lung accumulation of both S7A and R11A, with minimal liver and kidney uptake and a favorable lung-to-liver ratio. Peak lung uptake occurred at 15 min, with fluorescence intensity decreasing in the liver over time (Fig.2). cR11A significantly enhanced cellular uptake (~100-fold increase) compared to linear R11A. FACS analysis showed Pitstop2 significantly reduced cR11A uptake, while Amiloride and Nystatin had no effect, suggesting clathrin-mediated endocytosis. Confocal imaging showed early lysosomal localization of cR11A, followed by cytosolic release within 120 min, confirming lysosomal escape, which was further validated by the lack of co-localization between LTPs and Alexa-488-transferrin in serum starved conditions. Further, FACS analysis of lung single-cell and confocal images revealed that cR11A selectively targeted ATII cells, p63+ basal cells, and ionocytes. Notably, cR11A-siRNA conjugates targeting SARS-CoV-2 effectively inhibited viral replication in an air-liquid interface model of human lung cells, with EC_{90} values of 0.64 ± 0.2 μM (cR11A-S1) and 1.04 ± 0.2 μM (cR11A-E2), compared to 0.019 μM for Remdesivir.

Conclusions:
We identified two novel LTPs, R11A and S7A, that targets ATII, basal cells and ionocytes after an intravenous delivery in as little as 15 min. Cyclization enhanced transduction efficiency in vitro and in vivo by enhancing serum stability. Uptake occurs via clathrin-mediated endocytosis, followed by endosomal escape. Finally, we successfully conjugated cR11A to siRNA and demonstrated a potent anti-viral effect in an air-liquid interface bronchial epithelial cell culture.

Figure .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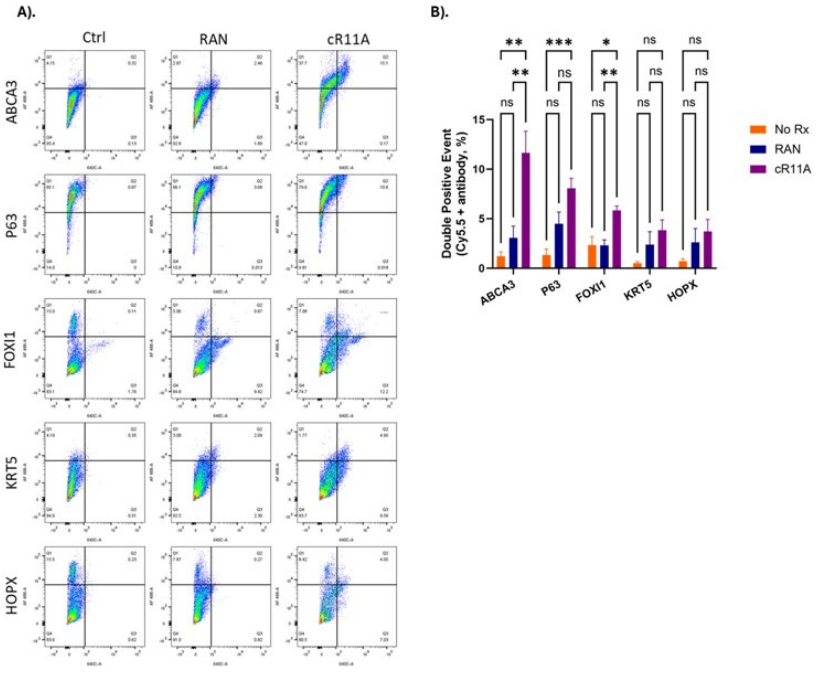
FITC
Cy5.5
Merged
ABCA3
P63
FOXI1
KRT5
HOPX


A).
Ctrl
RAN
cR11A
ABCA3
P63
FOXI1
KRT5
HOPX
B).
Double Positive Event (Cy5.5 + antibody, %)
No Rx
RAN
cR11A
ABCA3
P63
FOXI1
KRT5
HOPX

Oncolytic Virus Therapies

460 CRAd657-CD40L as a Potent Oncolytic and Immune-stimulatory Treatment for Hepatocellular Carcinoma

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The αPD-L1 antibody atezolizumab – as an Immune Checkpoint Blockade (ICB) - in combination with the vascular endothelial growth factor (VEGF)-targeting antibody bevacizumab, has been approved as standard-of-care (SOC) for hepatocellular carcinoma (HCC). However, only a subset of patients responds to ICB therapy, possibly associated with tumors which already have an immune infiltrated ("hot") Tumor Micro Environment (TME) and a weak anti-tumor T cell response which can then be expanded and de-repressed by ICB. Oncolytic viruses (OV) are a promising method to convert poorly immune infiltrated tumors ("cold") into "hot" tumors by inflaming the TME with virus induced cytokines which may also lead to the priming of anti-tumor T cells. In this respect, we developed CRAd657-CD40L, a live, conditionally replicative adenovirus derived from the species C human adenovirus serotype 6 (Ad6). CRAd657-CD40L encodes the CD40L transgene. CD40, a member of the tumor necrosis factor superfamily, is a costimulatory factor on antigen-presenting cells (APCs) which, when bound by its ligand CD40L triggers APC maturation and enhances the generation of anticancer cytotoxic T lymphocytes. CRAd657-CD40L is conditionally replicative because it carries two deletions in the E1A region, *dl*1101/1107, and the hyper variable region (HVR) involved in viral capsid neutralization has been replaced with the HVR of the adenovirus serotype 57 (Ad57). Here, we tested the efficacy of CRAd657-CD40L against a Sleeping Beauty model of HCC in which anti-PD-L1 ICB is partially effective. *In vitro* studies showed that replication of CRAd657-CD40L is tightly restricted to human tumor cells. Direct iv delivery of the virus alone improved significantly upon ICB treatment alone despite a relatively poor ability of the virus to replicate in murine tumor cells. Therapy was dependent upon CD8+ T cells. To alleviate concerns regarding adenovirus delivery to the liver, we also showed that the virus could be effectively delivered to HCC tumors using pre-loading of GPC-targeting CAR T cells – thereby allowing us to reduce the dose of virus given i.v. Finally, as a prelude to clinical translation of CRAd657-CD40L into patient trials for both HCC and melanoma, we have performed extensive toxicity studies in an adenovirus permissive Syrian hamsters, which have shown an excellent safety profile for this virus.

461 Mechanisms of synergy between TGF-beta inhibitor and IL12 expression from the systemically deliverable clinical oncolytic immunotherapy VET3-TGI

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Oncolytic vaccinia virus therapies actively recruit T-cells towards the tumor microenvironment. These can be combined with IL-12 expression to help enhance the immune response by stimulating Th1 cells. However, IL-12 mediated induction of IFN-gamma was also found to increase TGF-beta signaling within the tumor microenvironment.

TGF-beta mediated resistance is one of the major mechanisms of immune suppression utilized across multiple solid tumor types. Blockade of TGF-beta (TGFBi) will therefore likely improve response to immunotherapy. Combining TGF-beta inhibition with IL-12 expression can maximize therapeutic benefits through simultaneously reducing immunosuppression and enhancing anti-tumor immune response. Current studies describe a vaccinia-based immunotherapy, VET3-TGI, combining enhanced systemic virus delivery to tumors and locally expressed IL-12 and TGFBi within the tumor microenvironment, for efficient control of multiple tumor models. *In vivo* mouse studies demonstrated potent therapeutic activity, including many CRs, even at doses several logs below equivalent clinical

doses after systemic (intravenous) delivery. In addition, toxicity studies demonstrated the safety of VET3-TGI in mouse models. This data led to the selection of VET3-TGI as a lead clinical candidate and clinical testing has begun.

462 Fusogenic onclytic vaccinia virus armed with immunostimulatory cytokines improves tumor immune microenvironments systemically and induces complete response in mice bearing bilateral tumors and orthotopic tumors.

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Oncolytic virus has great immunotherapeutic potential for treatment of various refractory tumors. It induces systemic antitumor immune response following local oncolysis. We have developed two distinct oncolytic platforms, MAPK-dependent recombinant vaccinia virus (MDRVV) and fusogenic oncolytic vaccinia virus (FUVAC). MDRVV achieved tumor-specific virus replication by deleting two viral growth factors, VGF and O1L. FUVAC has been isolated from the MDRVV as a mutant clone which induces cell-cell fusion due to the nonsense mutation of viral fusion inhibitor, K2L. Cell-cell fusion of FUVAC enhances viral oncolysis leading to higher antitumor immune response compared to non-fusogenic MDRVV. FUVAC changed tumor immune microenvironment from “cold” to “hot”, through decreasing tumor associated immunosuppressive cells locally and increasing cytotoxic T cells systemically.

However, it was insufficient to treat distant untreated tumors such as metastatic tumor region. Most oncolytic viruses are treated directly via intratumoral route; then it decreases antitumor effects against untreated tumors because of the lack of direct oncolysis. To remodel tumor immune microenvironment systemically, we have developed FUVAC-IL12/CCL21 armed with two immunostimulatory cytokines, interleukin 12 (IL12) and C-C motif chemokine ligand 21 (CCL21). In mice bearing bilateral subcutaneous CT26 colorectal tumors, unilateral intratumoral injection of FUVAC-IL12/CCL21 more prolonged the survival of mice than it of FUVAC and MDRVV-IL12/CCL21. FUVAC-IL12/CCL21 achieved 72% complete response in both tumors compared to 0% for FUVAC and 29% for MDRVV-IL12/CCL21. Pharmacokinetic analysis showed that viral replication, IL12 and CCL21 productions were mostly accumulated in the treated tumors while IFN-γ was systemically elevated by FUVAC-IL12/CCL21. FUVAC-IL12/CCL21 significantly increased the IFN-γ secretion of splenocyte T cells stimulated with MMC-treated CT26 tumor cells rather than UV-irradiated vaccinia viruses. Furthermore, tumor immune microenvironment was analyzed by single cell RNA-seq. FUVAC-IL12/CCL21 clearly improved the immune microenvironment of distant untreated tumors, by increasing effector memory $CD8^+$ T cells while decreasing the exhausted T cells. TCR repertoire analysis revealed that FUVAC-IL12/CCL21 increased the TCR diversity through its cell-cell fusion induction. Thus, FUVAC-IL12/CCL21 elicited robust antitumor immune responses by infiltrating effector memory T cells including diverse tumor-reactive clones. FUVAC-IL12/CCL21 was also evaluated in the orthotopic pancreatic ductal adenocarcinoma (PDAC) KMPC tumor model. KMPC cells were cloned from PDAC tumor tissue of Pft1a-Cre/LSL-KrasG12D mouse and induced clinically relevant stromal infiltration in cold pancreatic tumors by intrapancreatic transplantation. Intraperitoneal injection of FUVAC-IL12/CCL21 significantly prolonged the survival of mice compared to mock or FUVAC treatment. Moreover, the tumor microenvironment was analyzed by spatial transcriptomics and immunostaining. FUVAC-IL12/CCL21 enhanced infiltration of $CD4^+$ and $CD8^+$ T cells while decreasing tumors and stromal infiltration.

In conclusion, fusogenic oncolytic vaccinia virus armed with IL12 and CCL21 augmented the antitumor immunotherapeutic potential through improving tumor immune microenvironments systemically. FUVAC-IL12/CCL21 would have the potential to overcome oncolytic virus-resistant tumors which limit the clinical benefits.

463 Stroma Modulating Recombinant Oncolytic Vesiculovirus Shrinks Pancreatic Tumor and Increases Immune Response

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Vesicular stomatitis virus is one of the most studied oncolytic virus candidates used in preclinical and clinical cancer research. A newly developed synthetic chimeric vesiculovirus, VMG, has been tested across multiple cancer types including pancreatic ductal adenocarcinoma (PDAC) in vitro and in vivo. While the VMG virotherapy seems promising, the presence of hyaluronic acid (HA) and collagen (CN) in the tumor stroma elevates tumor stiffness and interstitial fluid pressure, which incapacitate the effectiveness of oncolytic virotherapy by hindering the penetration of oncolytic viruses/drugs or immune cells into the tumor. We have engineered and evaluated the efficacy of VMG expressing collagenase (CNase) or mouse hyaluronidase (mPH20) or special enzyme (Enz) having both CNase- and PH20-like effect in preclinical PDAC model. The genetic modification of VMG virus did neither compromise its oncolytic/cytotoxic activity nor its replication ability in vitro which are evident from the MTS cell viability assay and viral kinetics assessment respectively. We then tested the efficacy of VMG vectors in the Pan02 in vivo PDAC mouse model. The result show that out of all the synthetic VMG vectors, the VMG-Enz significantly reduced tumor volume. IHC staining and QPLI data demonstrate that the hybrid VMG vectors effectively infected the tumor and were able to digest stromal components such as HA and CN to help viruses duplicate within the tumor. Furthermore, flow cytometry data confirm us that there was an increase in $CD8^+$ T cells, granzyme B^+ T cells, dendritic cells, natural killer cells and a reduction in M-MDSCs which are the hallmarks of successful onco-immunotherapy. However, there was also an increase in immune checkpoint markers including PD-1, PDL-1, PDL-2 and anti-viral resistance genes for IFN-α, IFN-β and Mx-1 which provide us an evidence of resistance built up against the VMG virotherapy. We conclude that, the VMG-Enz was able to increase immune response and significantly abate tumor growth by curtailing stromal hyaluronic acid and collagen level. In future, studies should focus on evaluating the potential of VMG-Enz as an arm of combination therapy with immune checkpoint inhibitors or type I IFN antagonists in increasing survival benefits in experimental animal.

464 Redressing the Balance Between Immunodominant Antiviral and Immunosubdominant Antitumor T Cell Responses to Enhance the Efficacy of Oncolytic Viroimmunotherapy

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Oncolytic viruses (OVs) are engineered specifically to replicate in, and kill, tumor cells. The current paradigm proposes that OV tumor cell killing induces immunologic cell death which triggers innate responses culminating in the priming of adaptive T cell responses against tumor associated antigens (TAA). However, the distracting effect of the (immunodominant) antiviral T cell response upon generation of an effective (immuno-subdominant) antitumor response is a major potential impediment to efficacy of OV immunotherapy. Therefore, here we directly addressed the impact of oncolytic virotherapy on the host antitumor T cell response. We show that the immune response stimulated by highly immunodominant viral antigens immunologically distracts from the priming of antitumor CD8 T cells recognizing weak immuno-subdominant TAA and consequently restricts therapeutic efficacy. Thus, in the syngeneic B16 and CT26 models the highly inflammatory VSV was unable to stimulate an antitumor T cell response in C57 mice unless armed with a TAA (VSV- TAA). Moreover, treatment with the virus led to the ablation of pre-existing active antitumor T cells, and a shift of the tumor-reactive T cell landscape toward exhaustion. Furthermore, combination with anti-PD1 ICB therapy significantly improved mouse survival only in mice treated with VSV-TAA compared to VSV-wt. However, by expressing mutated, altered-self versions of a TAA we identified a goldilocks affinity threshold of TAA recognition by T cells which generated T cells which cross reacted with unaltered, SELF TAA expressed by the tumor and correlated with improved tumor control. Taken together, our data challenge the paradigm that immunogenic viruses alone function as *in situ* tumor vaccines. However, we offer a potential strategy to enhance antitumor T cell responses which retains the acute antitumor therapy provided by antiviral innate and T

cell responses, whilst alleviating the antiviral T cell distraction that diminishes the induction of curative tumor-specific T cells.

465 Oncolytic Vaccinia Virus Expressing Non-Secreted Decoy-Resistant IL-18 Mutein Elicits Potent Antitumor Effects with Enhanced Safety

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Cytokines are promising for cancer treatment but face challenges like short half-lives, high dose requirements, and systemic toxicity. Oncolytic viruses provide an ideal platform to overcome these limitations by delivering cytokines directly to tumor sites, enabling high local concentrations within the tumor microenvironment (TME). This study utilized an oncolytic vaccinia virus (oVV) to deliver interleukin-18 (IL-18) and its variants and assessed their antitumor effects across different tumor models. The non-secreted mature IL-18 mutein (nsmDR-18)-expressing oVV demonstrated potent antitumor efficacy with reduced systemic toxicity. It increased the expression of antitumor factors, including IL-2, IFN-γ, granzyme B, and perforin, while expanding tumor-reactive $CD39^+CD103^+CD8^+$ T cells and reducing severely exhausted $PD\text{-}1^+TIM\text{-}3^+CD8^+$ T cells, thereby transforming immunologically "cold" tumors into "hot" tumors. However, nsmDR-18-expressing oVV treatment also elevated regulatory T cells (Tregs) and upregulated immune checkpoint molecules (PD-1, PD-L1 and CTLA-4). Combining nsmDR-18-expressing oVV with anti-CTLA-4 antibody significantly improved overall survival and induced systemic, tumor-specific antitumor immunity. These results highlight the potential of nsmDR-18-expressing oVV as a therapeutic strategy, supporting further exploration in clinical trials.

466 Enhanced Tumor Microenvironment (TME) Immunomodulation Through Repeat Dosing with a Synthetic Vesiculovirus Library

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Intravenous (IV) delivery is the ideal route of administration for oncolytic virotherapy for the treatment of advanced metastatic cancers. However, IV delivery yields diminished vector to the tumor due to neutralizing antibodies and absorption by the reticuloendothelial system. As such, most efforts in the clinic have not been successful at attaining clinical regressions. Vesicular stomatitis virus (VSV), a negative strand oncolytic RNA virus, has yielded anti-tumor activity in <10% of patients in clinical trials. In most patients, replication competent virus is cleared within 48 hours and long term anti-tumor immunity is established in varying degrees. Given the delivery limitations with single administrations, extended dosing could provide an approach that parallels other anti-tumor modalities. Towards this end, we have developed a library of replication competent synthetic vesiculovirus vectors displaying distinct glycoproteins that evade antibody cross neutralization. Herein we show an *in vivo* model in which tumor bearing animals with pre-existing anti-vesiculovirus immunity can be treated with IV oncolytic vesiculovirus therapy, efficiently delivering virotherapy to the tumor and inducing anti-tumor immunity.

Utilizing seven novel vesiculovirus glycoproteins we generated chimeric vesiculovirus vectors using the clinically characterized oncolytic VSV vector backbone. We established *in vitro* replication kinetics, high titer production, and oncolytic capacity on a panel of tumor cell lines. We confirmed the vectors' ability to evade antibody cross neutralization by employing patient derived polyclonal antibody populations obtained from a clinical study with VSV-IFNβ (NCT01628640). We applied *in silico* modeling to identify antibody epitope regions to two monoclonal antibodies, 8G5F11 and 1E9F9, and noted sequence hypervariability amongst vesiculovirus glycoproteins in these regions, suggesting a mechanism of immune evasion. Next, we used an *in vivo* model of a syngeneic B16-OVA-$IFNAR^{-/-}$ melanoma tumor in C57BL/6 mice. Following IV immunizations with our novel vectors we treated mice with IV doses of VSV expressing OVA antigen. We confirmed delivery of the virus to the tumor by isolating live

virus as shown by plaque assays and further validated with viral genome qPCR. We assessed antibody responses and demonstrated unique antibody populations against each viral vector. Evaluation of inflammatory cytokines indicated antigen re-exposure only in our murine group both vaccinated and treated with the identical VSV vector. Examination of the tumor microenvironment indicated significant improvement of immune cell infiltration in groups immunized and then treated with alternative vesiculovirus vectors over repeated VSV vector delivery. Antigen specific tetramer staining identified both anti-viral and anti-OVA tumor antigen specific T cell populations, with tumor antigen specific T cell responses aligning with overall survival and decrease in tumor burden. In conclusion, we provide evidence that a library of novel vesiculovirus vectors both evade prior antibody generated immunity and allows delivery of a therapeutically significant secondary dose of an oncolytic vesiculovirus vector. This study lays the groundwork for further development of repeat dosing strategies in the clinic with oncolytic vesiculovirus vector libraries.

Innovation in Alternative Cell Therapy Sources

467 CAR-Engineered HSCs for Treating B-cell Acute Lymphoblastic Leukemia

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The adoptive transfer of T and NK cells modified to express chimeric antigen receptors (CARs) has emerged as a promising therapy for the treatment of B-cell acute lymphoblastic leukemia (B-ALL). However, challenges such as poor proliferation, loss of engineered cells, and cell exhaustion limit its efficacy. To address these issues, we hypothesized that transducing CAR in hematopoietic stem cells (HSCs), instead of mature immune cells, could provide a continuous supply of CAR-modified cells. To test this approach, NSG (NOD/SCID/IL2Rγ null) mice were humanized with HSCs transduced with a CAR targeting CD22 under the control of the human elongation factor-1 (EF1)-α promoter. We demonstrated that CAR expression in T-cell progenitors disrupted T-cell development, resulting in the absence of peripheral CAR-T cells. Additionally, constitutive CAR expression across the hematopoietic compartment increased the risk of proto-oncogene activation. To address these challenges, we developed a bioinformatic pipeline to identify enhancer regions of the genome that can function as cell-specific promoters when paired with minimal promoter elements. Leveraging this pipeline, we designed a synthetic T/NK-specific promoter to restrict CAR expression to mature T and NK cells. Using a Green Fluorescent Protein (GFP) reporter gene under the synthetic promoter's control, we validated its specificity in T and NK cells after in vitro and in vivo HSC differentiation. Next, we humanized mice with HSCs transduced with a CAR-CD22 driven by the T/NK-specific promoter. Substituting the EF1-α promoter with the T/NK promoter overcame the CAR-T differentiation blockade, enabling the generation of HSCs derived mature CAR-T cells.

When challenged with B-ALL cells, mice engrafted with CAR-engineered HSCs under the T/NK promoter exhibited delayed leukemia progression and significantly improved survival. Interestingly, mice engrafted with HSCs under the EF1-α promoter also showed improved survival, despite the absence of CAR-T cells, suggesting a role for CAR-NK and/or CAR-macrophages in leukemia control. This raises the question of which cell types should be targeted for CAR expression in a context of CAR HSCs, when persistence of CAR-expressing cells is no longer an issue. To investigate, we have developed a high-throughput method to screen a large number of lineage-specific promoters. A lentiviral library of fifty promoters controlling GFP expression was used to transduce T, B, NK cells, and macrophages isolated from human peripherical blood. GFP^+ populations, containing cells with active promoters, were sorted *via* fluorescence-activated cell sorting. Long-read nanopore sequencing were then used to determine the enrichment of each promoter sequence in the GFP^+ T, B, NK cells, and macrophages. The sequencing results showed a strong correlation with those obtained from individual promoter transduction experiments in human peripheral blood mononuclear cells, confirming the robustness and reliability of the approach. To extend this

strategy, we are applying it to HSCs by transducing them with the lentiviral promoter library and generating humanized mice for *in vivo* validation.

The improved survival observed with the T/NK-specific promoter highlights the potential of CAR-HSC therapy. Expanding the repertoire of available cell-specific promoters will allow further evaluation of each cell type's contribution to B-ALL control, paving the way for more efficient CAR-HSC strategies.

468 Revitalizing aged CAR T cells with anti-inflammatory cytokine IL-37

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Chimeric antigen receptor (CAR) T cell therapies have shown remarkable clinical efficacy against B-lineage leukemias and lymphomas, but over 50% of patients receiving CD19-targeted CAR T cell therapy will relapse within the first two years of receiving treatment. As all FDA-approved CAR T cell products are autologous, the fitness of a patient's T cells is a critical factor in final efficacy of the drug. It has been proposed the poor fitness of elderly patients, coupled with the biological decline of T cell function with age, can impact CAR T cell therapy outcomes. Therefore, there is a clinical need to improve CAR T cell therapies for older patients diagnosed with B-lineage hematological malignancies. Interleukin-37 (IL-37) is a newly discovered anti-inflammatory cytokine in the interleukin-1 family. IL-37 reduces inflammation by inhibiting downstream activation pathways and reducing the production of proinflammatory cytokines. We hypothesize that modifying aged CAR T cells to constitutively express IL-37 cytokine will reinvigorate these cells and improve anti-tumor outcomes.

Published studies have shown supplementation of systemic recombinant IL-37 (rIL-37) in vitro restores proliferative capacity and cytokine production as well as reduces surface expression of immunoinhibitory proteins, like PD-1 and TIGIT. We engineered CD19-directed CAR T cells to secrete IL-37 using healthy donor samples over the age of 60. Construct validation revealed successful transduction of primary human T cells, cytotoxicity against CD19-expressing tumor cells, and secretion of functional IL-37. Aged healthy donor CAR T cells armored with the anti-inflammatory IL-37 cytokine exhibit more stem-like phenotype by CD62L surface expression compared to second-generation CAR T cells. Aged IL-37-secreting CAR T cells express fewer inhibitory molecules and secrete less pro-inflammatory cytokines compared to parental CAR T cells after CAR manufacturing. Further, engineered IL-37-expressing CAR T cells show robust proliferative capacity compared to second-generation CAR T cells after repeated antigen stimulation in vitro. Previously published work demonstrated that treatment with systemic rIL-37 improves the anti-tumor efficacy of aged CD19-targeting CAR T cells in both syngeneic and xenograft mouse models of B-cell acute lymphoblastic leukemia (B-ALL). We found aged, > 65 years, IL-37-secreting healthy donor CAR T cells significantly decrease tumor burden and improve overall survival of tumor-bearing mice in a xenograft mouse model of B-cell lymphoma. To address these findings in a clinically relevant model, we manufactured armored CAR T cells using T cells from elderly patients, > 65 years, diagnosed with chronic lymphocytic leukemia (CLL). Like data collected from healthy donors, CLL patient-derived IL-37-expressing CAR T cells are characterized by a decreased exhausted phenotype and demonstrate enhanced cytotoxicity against CD19-expressing tumor cells compared to parental CAR T cells.

Ongoing work will determine how IL-37 expression alters aged patient-derived CAR T cell function as well as investigate the expression of IL-37 by aged CAR T cells in syngeneic mouse models of B-cell malignancies. We anticipate the findings of our research will improve our understanding of aging-associated immune function in the context of cellular immunotherapy and enhance the development of novel CAR T cells specifically for patients with advanced age or chronic inflammation.

469 In Vivo Self-Renewing HSC-Based CAR-NK Cell Factory

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Despite high response rates, target-antigen positive relapse remains the largest challenge post-CAR T cell therapy in adult oncology patients, mostly following progressive decline over time of the infused gene-modified cells. CAR NK cells potentially have a larger therapeutic window given absence of cytokine release syndrome toxicity, but the limitations of persistence are even more severe. We hypothesized that genetic engineering of the self-renewing pluripotent hematopoietic stem cells (HSCs) will enable constant production of in vivo “CAR-effector cell factories", thus overcoming challenges of persistence in cell therapy. To avoid potential toxicity and potential pro-tumor immunosuppressive effects of a pan-hematopoietic CAR expression precluding clinical applicability, *we developed a gene-modified HSC platform in which CAR expression is precisely controlled and induced only upon NK cell differentiation*.

We generated an integrated computational pipeline using primary human immune cell subset ATACseq/RNAseq datasets to identify candidate NK-specific regulatory DNA elements. From 198 non-redundant potential NK-specific elements, we designed 117 unique assemblies – each combining 1 to 3 elements with a minimal promoter. Assemblies were synthesized and cloned upstream of a GFP reporter in a third-generation self-inactivating (SIN) lentiviral (LV) vector, thus lacking endogenous viral LTR transcriptional activity upon transgene integration in the genome, and dependent on our synthetic regulatory element assemblies (Fig. 1A–E). We screened this library in primary NK and $CD34^+$ cells purified from adult peripheral blood mobilized donors in vitro. Most combinations resulted in similar NK and $CD34^+$ cell reporter expression (Fig. 1F-G, e.g. assembly NK005); however, we identified a small number of lead regulatory element combinations driving massive preferential expression in NK cells (Fig. 1F–G, e.g. NK022, NK026). The in vitro screen was independently repeated four times, from different donors, every time identifying the same lead candidates. Importantly, in vivo regulated expression in NK cells was confirmed following mouse humanization with LV-transduced HSCs and comparison of various differentiated immune subsets by multicolor Flow cytometry analysis (Fig. 2). We further combined 5 lead elements and identify an optimized, compact NK-specific regulatory element combination. Finally, we demonstrated that this NK-specific assembly could induce strong and functional expression of the Cilta-cel (Ciltacabtagene autoleucel) anti-BCMA CAR. Developmentally controlled CAR expression through an HSC platform represents a promising strategy to generate unprecedented CAR persistence that we hypothesize will facilitate long-term disease control. Combining HSC self-renewal with NK cell-specific expression uniquely enhances prospects for safety, therapeutic window, and feasibility of translation. We envision that this regulated HSC-CAR approach will pave the road to novel durable synthetic immune cell therapies.

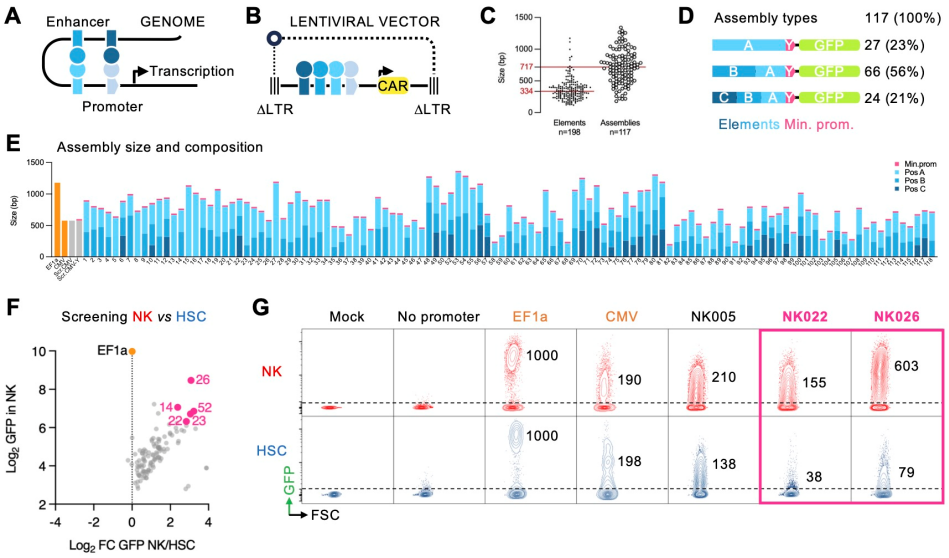

Figure 1. Identification of NK-specific promoters. (**A**) Schematic of distant regulatory elements in the genome (enhancers and promoter) driving specific gene transcription. (**B**) Schematic of a compact assembly of regulatory elements in a 3rd generation lentiviral vector to drive specific expression of a CAR. **Δ**LTR signifies the deletion in the long terminal repeat that define a self-inactivating (SIN) LV and render transcription activation dependent on the synthetic regulatory elements/minimal promoter, and not a viral element. (**C**) Size of 198 selected NK-specific elements selected from ATAC-seq data and 117 assemblies (success of production 117/118 assemblies; 99%). (**D**) Diagram of the different assembly types with 1, 2 or 3 elements associated with a 25-bp minimal promoter Y (YB_TATA). The annotations A, B and C indicate the relative position of each element from the minimal promoter Y. (**E**) Size and element composition of each assembly compared to constitutive promoters (EF1a and CMV) and control scrambled CMV sequences. (**F**) Screening results representative of four independent experiments comparing the activity (GFP expression) of each assembly in NK cells and peripherally mobilized and isolated CD34+ cells (HSCs). Data were normalized to EF1a for each cell type. Five assemblies showing the highest expression in NK cells (Y-axis) and highest fold-change compared to HSCs (X-axis) were selected (highlighted in the graph). (**G**) Representative Flow cytometry analysis of GFP expression in NK cells and HSCs. Enumerated values indicate normalized geometric mean fluorescence (MFI) of GFP+ cells (relative to EF1a=1000). NK005 is an example of a non-specific candidate, representative of a majority of tested assemblies, whereas NK022 and NK026 represent a minority of assemblies exhibiting preferential expression in NK cells. Of note, background HSC expression of GFP for control and NK regulatory assembly candidates is likely to be artificially increased in this in vitro assay by the short-term nature of this approach, proximity to LV transduction, and differences in HSCs in cell culture with required cytokine support *vs* in vivo results. This difference in vitro is noted *vs* post-HSC engraftment and hematopoietic differentiation where lower "leakiness" was observed in non-NK cells in vivo (Figure 2).

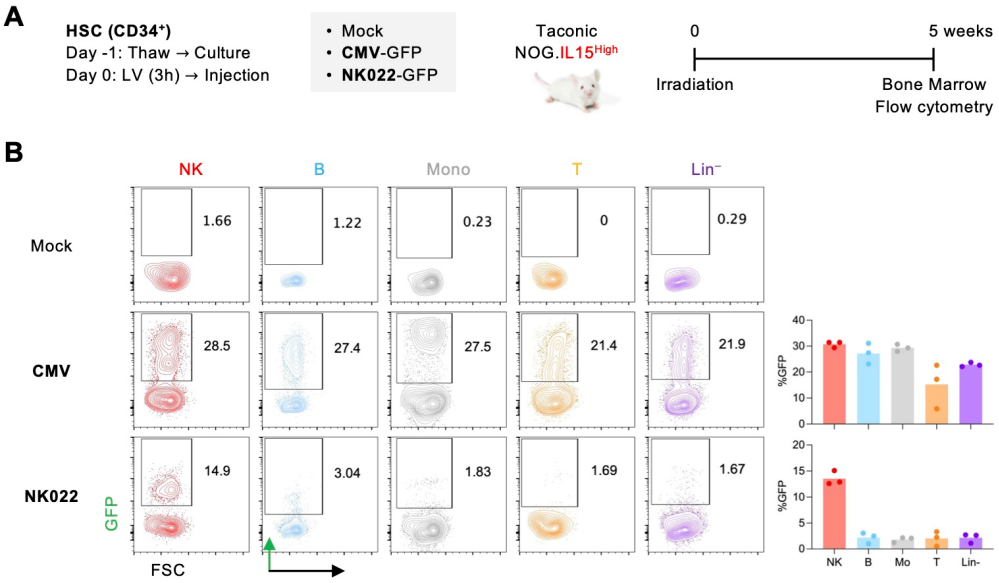

Figure 2. In vivo NK-specific promoter activity of NK022 candidate. (**A**) Schematic of the experimental procedure for the testing of in vivo NK-specific promoter activity of the NK022 assembly. Human HSCs were lentivirally transduced with CMV-GFP or NK022-GFP constructs and injected into irradiated (200 Rad) NOG-hIL15 (Taconic Biosciences®, supra-physiologic IL-15) mice (n=3 per group). After five weeks, mice were euthanized, and the bone marrow was harvested for flow cytometry analysis. (**B**) Plot of the percentage of GFP$^+$ cells in differentiated NK (CD3$^-$ NKp46$^+$), B (CD19$^+$), monocytes (CD14$^+$), (emerging) T cells* (CD3$^+$ NKp46$^-$) and lineage-negative (Lin$^-$) cells. *Of note, in this model, T cells could only be observed in a few experiments and might not represent fully matured T cells at this timing. Quantification in individual mice is shown on the right panel.

470 Phase 1 Translational Assessment of an Off-The-Shelf CAR NK Cell Armed with Alloimmune Defense Technology for Conditioning-free Therapy

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Autologous chimeric antigen receptor (CAR) T-cell therapy has revolutionized the treatment of hematological malignancies; however, its scope of application is limited by manufacturing complexity, cost of treatment, and patient accessibility. Allogeneic strategies to overcome such limitations include the use of induced pluripotent stem cell (iPSC)-derived CAR T or NK cell therapies that can be uniquely manufactured "off-the-shelf" for broad patient access. Unlike donor-derived allogeneic approaches, iPSC-based cell therapies are highly amenable to genetic engineering, can be consistently produced in a cost-effective manner, and reliably available for on-demand treatment.

Intense conditioning chemotherapy (CCT), often consisting of cyclophosphamide and fludarabine, is commonly administered to patients prior to treatment with CAR therapies, although CCT is associated with severe toxicities. We developed FT522, a next generation iPSC-derived CAR NK cell therapy, that is designed to confer therapeutic benefit without requiring the administration of CCT. FT522 builds off the success of our previous CAR NK cell therapy (Ghobadi et. al., 2025) and incorporates five synthetic controls of cell function: a CD19-targeted CAR; a high-affinity non-cleavable CD16 (hnCD16) Fc receptor to maximize antibody-dependent cellular cytotoxicity; an IL-15/IL-15 receptor fusion protein to offer cell-intrinsic cytokine support and enhanced function; a

CD38 knockout to enhance metabolic fitness; and an alloimmune defense receptor (ADR) targeting 4-1BB to promote functional persistence in the absence of CCT.

Preclinical studies were performed to evaluate whether FT522 could maintain anti-tumor activity while under threat by alloreactive immune cell populations. FT522 effectively culled allogenic responses *in vitro,* and uniquely eliminated tumor cells better than control CAR NK cells without ADR *in vivo* (2000-fold lower tumor burden at study endpoint, p=0.016). A Phase 1 multicenter clinical study of FT522 in relapsed/refractory B cell lymphoma (NCT05950334) is currently ongoing, where FT522 is administered either with or without CCT. Preliminary clinical data showed that when combined with CCT, FT522 induced deep and lasting CD19+ B cell depletion, and clinical responses in both indolent and aggressive lymphoma were observed. In addition, FT522 attenuated activated (4-1BB+) T-cell recovery (7-fold fewer CD4 T cells at day 8 post infusion, n=3), suggesting that treatment with FT522 can target both tumor cells as well as activated host alloreactive T cells. Importantly, when administered without CCT, FT522 did not result in the expansion of activated (4-1BB+) T cells. Furthermore, a dose-dependent increase in FT522 persistence, which outpaced that of prior CAR NK cells without ADR, was observed (16-fold greater AUC at 50% the dose n=3). No evidence of either cellular or humoral responses against FT522 was detected, suggesting that ADR can protect FT522 from host immune response in the absence of CCT. Collectively, our preclinical and early-stage translational data from our Phase 1 study of FT522 demonstrate that ADR is capable of enabling anti-tumor activity, promoting functional persistence, and avoiding immune rejection without requiring administration of CCT to patients.

471 Generation of iPSC-derived CD4$^+$ and CD8$^+$ CD19 CAR αβ T cells with *in vivo* tumor control and cell expansion comparable to healthy donor T cells.

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**Corresponding Author*

Successful development of off-the-shelf CAR-T cell therapies requires complex engineering to i) prevent graft vs host disease (GvHD) and rejection by the patient's immune system, and ii) equip the cells with anti-tumor capabilities, such as a chimeric antigen receptor (CAR). Induced pluripotent stem cells (iPSCs) provide an ideal platform; engineered clones can be characterized and banked for future differentiation due to the unique ability of these cells to both self-renew and retain the potential to make any cell in the adult body. Using a scalable feeder-free method, we report the generation of allogeneic iPSC-derived CAR T cells with comparable function to primary T cells. iPSCs were engineered with a CD19 CAR and allo-evasion edits designed to combat cellular and humoral responses. A fully characterized clone was selected and differentiated using a novel process that generated a mix of CD4$^+$ and CD8$^+$ T cells. scRNA-seq analysis of cells at different stages of this process confirmed that cells follow a trajectory matching normal thymocyte development to αβ T cells. Upon in vitro tumor challenge, these iPSC CAR T cells displayed Ag-dependent cytotoxicity, proliferation, and cytokine production, including IL-2. A single infusion of Century iPSC-CAR-T cells demonstrated tumor control in an established NALM6 tumor model without the need for exogenous cytokines or cytokine engineering. Importantly, iPSC-CAR-T cells persisted in peripheral blood for at least 21 days, trafficked to the bone marrow, and measurably proliferated upon tumor rechallenge in an in vivo model. Both in vivo proliferation and tumor control were at least comparable to primary T cell controls. These data are a significant advance in the field of allogeneic iPSC CAR T cells and support the use of this platform for the development of scalable, accessible CAR T cell medicines.

472 Combinational "Off-the-Shelf' CAR T Cells: A Unified Front Against AML Heterogeneity

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Most chimeric antigen receptor (CAR) T cell trials to date have employed autologous T cells and have avoided the hurdles of allogeneic immunity. As an alternative to autologous CAR T cells, effectors can be generated from donors through an "off-the-shelf" approach and used without HLA matching following appropriate genome editing steps. Cytidine deaminase editors mediate irreversible C>U>T edits for multiplexed knockout of αβ T cell receptor (TCRαβ) to avoid graft-versus-host disease (GvHD), shared antigens to evade self-targeting through CAR, and CD52 to confer resistance to serotherapy or HLA to directly avoid rejection. In the context of heterogeneous disease such as acute myeloid leukaemia (AML), multiple targeting moieties are required for complete coverage of blast populations and an approach using combinations of "off-the-shelf" CAR T cells was investigated.

We generated 'universal', TCRαβ depleted BE-CAR7, BE-CAR33 and BE-CARCLL-1 CAR T cells and evaluated their application against highly heterogeneous and aggressive AML. BE-CAR33 and BE-CARCLL-1 demonstrated potent anti-leukaemic effects against HL-60, MOLM-14, U-937 and Kasumi-3 cell lines and CLL-1^{+}CD33^{+} primary AML samples *in vitro*. To model disease heterogeneity, NSG mice engrafted with CRISPR-engineered CLL-1^{-} CD33^{+} and CD33^{-}CLL-1^{+} HL-60 cells were treated with BE-CAR33 and BE-CARCLL-1 either alone or in combination. Disease progression could be inhibited more effectively in combined BE-CAR infusions over monotherapy strategies. Compatibility of BE-CAR7 and BE-CAR33 after removal of CD7 was also confirmed in a humanised patient derived xenograft (PDX) model of AML with effective clearance of CD7^{dim}CD33^{bright} disease.

In addition, BE-CAR T cells against CD70 were developed as an emerging candidate in immunotherapy for haematological malignancies such as AML. BE-CAR70 cells quadruple base edited for TCRαβ, B2M, RFX5 and CD70, to minimise self-targeting effects, and magnetically depleted of residual TCR, HLA-I and II, were able to eliminate AML cell lines *in vitro* and sustain durable disease control over a period of 38 days in a humanised murine model of AML, demonstrating uncompromised functionality compared to conventional single TCRαβ-edited versions. Importantly, a clear advantage in evading host rejection, attributed to quadruple editing of BE-CAR70 was overserved in *in vitro* mixed lymphocyte reactions. Therapeutic strategies envisage 'pick and mix' applications of base edited universal CAR T cells in combination, ultimately determined by disease specific antigen profiles for individual patients. Pre-manufactured and ready to use allogeneic combinations should enable timely interventions against AML where disease can otherwise evolve and progress rapidly.

473 Development of a Feeder-Free Maturation Method for TCR$^{-/-}$ CAR iT Cells

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Induced pluripotent stem cells (iPSC) are a renewable source for the generation of allogeneic, off-the-shelf, chimeric antigen receptor (CAR) T cell therapy and are currently under evaluation in clinical trials for both $CD19^+$ hematological malignancies as well as systemic lupus erythematosus. However, large scale T cell differentiation is challenged by the reliance on xenogeneic reagents, including bovine sera and murine feeder cells, and their requirement to grow in adherent cultures. Protocols have been established to support T cell differentiation utilizing immobilized recombinant proteins, with positive selection mimicked through TCR engagement with antibodies or mitogens. For allogeneic application, avoidance of GvHD through elimination of the TCR at the iPSC level requires TCR-independent maturation protocols. We previously reported that stimulation of double-positive (DP), $TCR^{-/-}$ anti-CD19 CAR^+ iT cells (*TRAC*-1XX-iT) with CD19 and 4-1BBL expressed on irradiated feeder cells facilitates positive selection and proliferation, generating single-positive (SP) CD8ab *TRAC*-1XX-iT cells. We hypothesized that antibody-mediated CAR and 4-1BB stimulation could support a xenograft-free maturation method of functional SP antigen-specific cytotoxic iT cells. To induce CAR signaling we developed anti-idiotype (anti-ID) antibodies, which were selected for CAR specificity and ability to induce CAR activation. Two antibodies were identified, one with anti-CD19 CAR specificity and the other with pan-CAR specificity. Immobilized but not soluble anti-ID antibody was able to induce iT cell maturation in a dose-dependent manner. However, CAR engagement alone was not able to induce proliferation. Similar to the feeder-based method where proliferation required additional 4-1BB engagement. To provide 4-1BB costimulation, we tested both soluble and immobilized recombinant protein and agonist antibodies. Proliferation only occurred with the addition of a soluble 4-1BB agonist. The specificity and potency of the plate-bound matured iT cells combined with 4-1BB engagement was demonstrated using in vitro cytotoxicity and intracellular cytokine assays. Additionally in vivo, the resulting *TRAC*-1XX-iT cells showed enhanced tumor control compared to feeder-based maturation. To eliminate the requirement for anti-ID antibody immobilization, we aimed to generate soluble methods for CAR stimulation through bead coating or antibody crosslinking to promote clustering of CAR molecules on the iT cells' surface. Antibody crosslinking combined with soluble 4-1BB agonist resulted in T cell maturation and robust proliferation. In vitro, the feeder-free matured iT cells display a phenotype and cytotoxicity similar to feeder-matured iT cells. In a systemic mouse model of leukemia, a single dose of feeder-free matured iT cells demonstrated potent tumor control and significantly increased survival when evaluated against feeder-matured iT cells. In sum, we established a soluble feeder-free and TCR-independent method for $TCR^{-/-}$ CAR^+ iT cell maturation through antibody-mediated CAR and 4-1BB engagement that produces potent cytotoxic CAR iT cells for therapeutic application.

HSC Transplantation and Gene Therapy

476 Dilanubicel Improves Single Cord Blood Transplantation: Updated Results with a Larger Cohort

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**Corresponding Author*

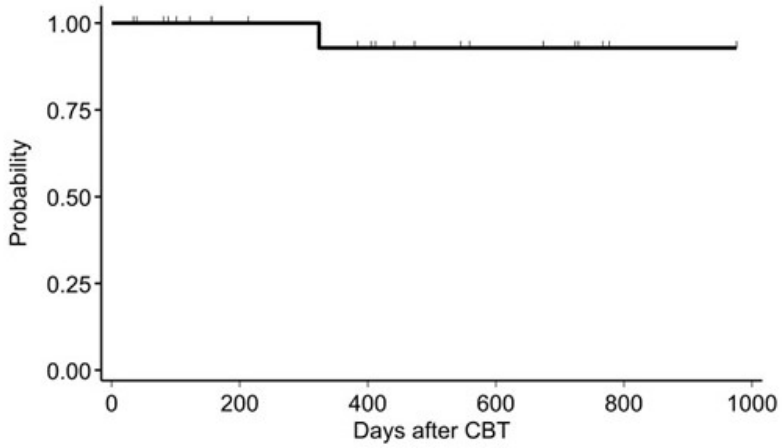
Background:
Recent clinical data suggest that third-party donor granulocyte transfusions can enhance donor CD8 T cell expansion and induce remission in relapsed AML patients undergoing cord blood transplant (CBT). Accessibility to CBT could improve by eliminating the need for a second CB unit without added risk. Dilanubicel, a cryopreserved, non-HLA-matched progenitor cell therapy product that is derived from the proprietary ex vivo expansion of cord blood CD34+ cells isolated from 6-8 pooled CB units, has shown clinical safety, early myelomonocytic recovery support, and contains antigen-presenting cells. This study evaluates dilanubicel as an adjuvant, transient graft to enhance graft-versus-leukemia effects and remove the need for a second CB unit.

Methods:
From March 2022 to January 2025, 22 patients with hematologic malignancies were enrolled in a single-center Phase II trial to assess the safety of dilanubicel in augmenting single CBT. Conditioning regimens included FLU/TBI/CY (n=11) or FLU/TBI/CY/Thiotepa (n=11). CB units required ≥4/6 HLA matching, while dilanubicel was used off-the-shelf without matching. The selected donor CB unit had to contain ≥2.5×10^7 TNC/kg and 1.7×10^5 CD34/kg pre-freeze. Dilanubicel infusion followed CB infusion by 4 hours, delivering 800×10^6 total CD34+ cells. Chimerism studies were performed from day 7 to one-year post-HCT. GVHD prophylaxis included cyclosporine and mycophenolate mofetil.

Results:
The median age was 33 years (range, 10-63), and 50% were ethnic minorities. Most patients had acute leukemias (AML=10, ALL=10, CML=1, MPAL=1), with 6 (27%) having minimal residual disease pre-HCT. Most (n=16) received a 4/6 HLA-matched CB unit. Median infused TNC and CD34 per kg were 3.14×10^7 and 2.53×10^5, respectively. All patients engrafted neutrophils at a median of 18 days (range, 14-30) and platelets at 31 days (range, 26-42). Early (day 7) myelomonocytic recovery was derived from dilanubicel confirmed by chimerism analysis and by day 14 myelomonocytic recovery transitioned to the primary CB donor (unit). A transient early lymphocyte expansion was observed in all patients from day +8/9 (median 855/μL, range 100-1316/μL), preceding ANC recovery, and was derived exclusively from the primary CB unit. With a median follow-up of 13 months (range, 1-32), all patients remain alive with a single case of relapse (Figure 1). The infusion of a non-HLA-matched pooled donor product did not increase GVHD risk, in fact there have been no cases of grade III-IV or chronic GVHD observed to date.

Conclusion:
Relapse remains one of the main causes for treatment failure following stem cell transplant. The addition of Dilanubicel as an adjuvant transient graft in single CBT was found to be safe and led to excellent outcomes, including low relapse rate and no grade III-IV acute or chronic GVHD. Importantly, as a cryopreserved and off-the-shelf, non-HLA-matched allogeneic cell therapy, dilanubicel readily accessible for all patients and easily integrated into standard CBT. Further, pooled donor manufacturing eliminates the issues of donor-to-donor variability and is a highly scalable process, associated with very low cost of goods. With significant follow-up, the absence of relapse may be linked to third-party donor APCs activating donor T cell responses. These promising findings warrant further biological investigations and larger trials.

Figure 1. Disease-Free Survival at 3 years post Cord Blood Transplant. Median follow-up time 13 months.



Pharmacology/Toxicology Studies and Analytics/Assay Development Session I

477 Enhancing TCR Off-Target Potency and Safety Through In Vitro and In Silico Approaches

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T-cell antigen receptor (TCR)-based therapies utilize TCRs that specifically recognize distinct peptide-Human Leukocyte Antigen complexes (pHLA). If the TCR sufficiently binds to a given pHLA, the engaged T cell becomes activated and kills the target cell. A crucial concern following TCR discovery is assessing the potential for cross-reactivity to unintended targets. There are no standardized methods for preclinical safety assessment of TCRs. Prior failures of preclinical safety assessments of TCRs have led to severe toxicities and mortalities in the clinic. Each failure has prompted retrospective assessments, resulting in improvements in preclinical safety methodologies. However, most studies have focused on a single TCR, or a small panel of TCRs often with the same pHLA target, lacking a robust strategy applicable to a wide range of TCRs. Therefore, urgent need for improved methods to predict and identify potential epitopes recognized within healthy tissues in humans is highlighted.

We have developed a robust *in silico*/*in vitro* model to predict and validate TCR cross-reactivity by utilizing a diverse panel of TCRs. This panel of TCRs includes three primary categories: TCRs that have exhibited prohibitive clinical toxicities, TCRs that have been demonstrated to be clinically safe with some efficacy, and TCRs without clinical experience but expected to be innocuous. In order to produce a robust model that captures the diversity of the TCR intrinsic cross-reactivity potential, our panel of TCRs includes two pairs of naturally occurring TCRs that recognize the same pHLA and two pairs of affinity enhanced TCRs along with their wild-type counterparts, from which they were derived, that recognize the same pHLA.

In our predictive model, we deployed the X-scan technique to assess TCR-T cell activity following exposure to a panel of altered peptide ligands (APLs). This panel of APLs was derived from the target peptide by systematically substituting each amino acid position with all 19 alternative amino acids, while all other positions match the original target peptide. This technique allows us to evaluate the specificity of the TCR for the original pHLA and predict TCR degeneracy and cross-reactivity. We utilize primary human T cells engineered to express the TCR and assess whether each peptide in the X-scan assay triggered TCR-T cell activation by evaluating expression of activation markers, cytotoxic activity, and effector molecule secretion. The Xscan data combined with experimental testing of a list of candidate peptides identified within the human proteome was used to develop a machine learning model. We deployed XGBoost and random forest algorithm to predict cross-reactive peptides.

Our findings indicate the model was able to classify peptide cross reactivities and quantitatively predict peptide functionalities. The machine learning model achieves a high accuracy of 0.83 indicating the model can provides an effective computational tool to identify T cell responses against mutated epitopes.

The culmination of this dataset is to establish a robust lab-in-the-loop model to predict TCR cross-reactivity and help guide the development of a next-generation platform for derisking TCRs. The model advance personalized, cost-effective, and efficient TCR-based therapies. This approach has the potential to enhance TCR potency and safety, revolutionizing therapeutic applications in oncology and beyond.

Poster Talk Presentations

483 Mobilize LNPs: A Novel Platform for In Vivo Targeted Delivery of RNA Therapeutics to T Cells

Justin Fang[1], Amit Singh[2], Zhiwei Luo[3], Anthony Cura[2], Ting Lan[3], Feng Lin[3], Sanpeng Li[4], Bob Crooker[2], Fei Jiang[5], Wei Qin[5], Roxi Mitrut[2], Cassidy Purrington[6], Nikhita Khanna[2], Jack Norton[6], Alina Zhankulov-Nguyen[6], KieuDuyen Tran[2], Qiujie Cai[3], Rujia Wang[3], Annie Xue[6], Andrea Graham[6], Maria-José Araneda Rubio[2], Yongting Huo[3], Alex Wesselhoeft[2]
[1]Mote, Cambridge, MA, [2]Mote Therapeutics, Lexington, MA, [3]Mote Therapeutics, Dongguan, Guangdong, China, [4]Mote Therapeutics, Dongguan, Anhui, China, [5]Mote Therapeutics, Dongguan, China, [6]Mote Therapeutics, Lexington

Introduction:
Lipid nanoparticles (LNPs) have cemented their utility as efficient and effective delivery systems for RNA-based therapeutics with successful clinical translation as COVID vaccines (CORMIRNATY® and Spikevax) and hepatic delivery (ONPATTRO®). However, their usefulness in delivering to extra-hepatic tissues suffers several technological challenges and warrants further innovation. *Mobilize* is a versatile and modular extra-hepatic delivery platform that is designed to resolve these challenges through a simple but unique approach.

Methods:
Mobilize as a platform technology enables surface functionalization of LNPs with tissue specific ligands by inline micromixing as a single step to generate targeted delivery system (*Mobilize* LNPs). As proof of concept, we used Green Fluorescent Protein (GFP) encoding circular RNA (circRNA) as model reporter cargo for targeted delivery to T cells. The tLNPs were characterized for their size, polydispersity index (PDI), surface charge, encapsulation efficiency (EE), and stability. We evaluated the delivery efficiency of *Mobilize* LNP in multiple cell-based systems (Jurkat cells, human PBMC and T cells) before confirming *in vivo* targeted delivery capability in humanized mouse model and non-human primates (NHP).

Results:
Mobilize LNPs consistently demonstrated excellent physicochemical properties (size <100 nm, PDI <0.1 and EE >95%) and colloidal stability under refrigerated as well as frozen conditions. We validated activity of these LNPs in Jurkat, human PMBCs, and human T cells achieving ~100%, ~70% ~35% delivery respectively. We then tested *in vivo* delivery efficiency of the *Mobilize* LNPs in humanized mice and achieved nearly 55% positive quiescent T-cells at a very low dose. We finally dosed the *Mobilize* LNPs to non-human primates (NHPs) to achieve successful delivery in ~20% quiescent T-cells through a single dose with no overt toxicity at any tested dose.

Conclusions:
The *Mobilize* platform consistently demonstrates the ability to both (1) detarget the liver, (thus avoiding passive LNP sinks), and (2) specifically deliver to T-cells *in vitro* and *in vivo* in multiple models. The inherent design of the *Mobilize* platform elegantly addresses the challenges in functionalizing LNPs with targeting ligands for extra-hepatic delivery. The process behind *Mobilize* technology maintains platform versatility, stability, robustness, and delivery efficiency. Our data indicates that Mobilize has strong potential as an *in vivo* delivery platform of RNA therapeutics to extrahepatic tissues.

484 Treating Pancreatic Cancer with "Armed" Oncolytic Adenoviruses and Adoptive T-Cell Therapy

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Introduction:
Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related mortalities and is projected to become second by 2030. The survival rate for PDAC has not improved in nearly four decades. Two primary features make PDAC refractory: 1.) the highly immunosuppressive tumor microenvironment (TME) suppresses anti-tumor

immune cells and 2.) desmoplasia, which creates a fibrotic physical barrier for infiltration of anti-tumor immune cells and therapeutics. The goal of this project is to develop viro-immunotherapies against PDAC that target the immunosuppressive TME and desmoplasia by utilizing "armed" oncolytic adenoviruses (OAds) and adoptive T-cell therapy (CAR T-cells). Four transgenes were selected to "arm" the OAd constructs: i.) TGF-beta blocker, ii.) interleukin-7 (IL-7), iii.) interferon gamma (IFN-gamma), and iv.) unnamed effector. The top two transgenes were selected to be combined and analyzed further.

Methods:
"Armed" replication-deficient adenovirus (Ad) constructs were first generated to isolate transgene function from oncolytic activity. Anti-tumor effects were measured using a syngeneic mouse model harboring subcutaneous KPC cell tumors that were intratumorally treated with virus. Tumors were harvested at various time points and stained for immune cell infiltration and fibrosis. The top two transgenes that elicited the greatest anti-tumor effects (TGF-beta blocker and IFN-gamma) were combined into a single vector and analyzed for anti-tumor effects in a similar manner. Mesothelin-directed CAR T-cells (meso-CARTs) were injected (tail vein) into NSG mice bearing subcutaneous Panc1 tumors after intratumoral injection of Ad constructs.

Results:
Tumor growth was significantly inhibited in syngeneic mouse models in the presence of each "armed" Ad construct. Furthermore, significant tumor infiltration of anti-tumor immune cells was induced upon treatment as well as a decrease in fibrosis. When combining the top two transgenes into a single adenovirus vector (TGF-beta blocker and IFN-gamma), this vector induced a significant anti-tumor effect compared to all vectors harboring a single transgene. Abscopal effects were observed on contralateral tumors after injection of ipsilateral tumors. The vector expressing TGF-beta blocker and IFN-gamma showed the greatest abscopal effects as well as an increase in tumor infiltration of anti-tumor immune cells. Finally, combining meso-CARTs with IFN-gamma-expressing Ad induced the greatest anti-tumor responses compared to monotherapies.

Conclusions:
PDAC is very resistant to conventional therapeutics, so utilizing combination strategies seems essential. The findings in this study have broad implications in the field of pancreatic cancer by shedding light on how the TME can be manipulated to enhance anti-tumor effects to aid in the development of more efficient therapeutics against PDAC. Combining transgenes that enhance the immune system (IFN-gamma) and decrease the immunosuppressive features (TGF-beta blocker) of PDAC is a superior approach by enhancing therapeutic efficacy from multiple angles. Moreover, therapeutics inducing abscopal effects should be highly valued because most PDAC patients die from metastasis.

485 Supramolecular Assembly of Polycation/mRNA Nanoparticles and In Vivo Monocyte Programming

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Introduction.
Conventional drug delivery vehicles employ nanoparticle sizes below 200 nm to reduce loss to phagocytic cells. Our work, instead, takes advantage of this size effect to achieve preferential messenger ribonucleic acid (mRNA) delivery to monocytes by engineering colloidally stable particles larger than 200 nm. Utilizing surface charge modulation as a generalizable strategy, we report assembly of poly(β-amino ester) (PBAE)/mRNA nanoparticles into supramolecular assemblies that are kinetically tunable between 200 and 1,000 nm and reveal an optimal size of 400 nm for safe and efficient transfection of monocytes following intravenous administration.

Methods.
When mixing the ethanol-dissolved PBAE with mRNA in a mildly acidic (pH 5.0) aqueous solution, PBAE/mRNA nanoparticles formed as a result of polyelectrolyte charge neutralization and hydrophobic interactions. Their

colloidal stability depends on competition between long-range electrostatic repulsion of residual positive charges on the surface and short-range van der Waals attractions among hydrophobic groups on PBAE. To allow further assembly of these initially stable nanoparticles to form submicron-and micron-sized particles, the excess positive charges of PBAE amines on the nanoparticle surface can be reduced by an increase in pH (to 7.4) that deprotonates amine groups and an increase in solution ionic strength (I = 0.205 M) that decreases the range of the electrostatic forces *via* Debye screening (Figure 1A). The assembly can be quenched (Figure 1B) by hydrophobic insertion of DMG-PEG2000 lipid onto the particle surface to stabilize any desired particle size, such as growing from 100 nanoparticles to 400 nm in size (Figure 1C).

Results.
Following *i.v.* injection of the 100- or 400-nm particles carrying Cy5-mRNA in C57BL/6J mice, they were quickly taken up by circulating Ly6C^{high} inflammatory monocytes. However, the 400-nm particles yielded a 10-fold higher uptake than the 100-nm particles (Figure 2A). To assess the gene expression profile, the assembled particles carrying mRNA encoding Cre recombinase (mCre) were *i.v.* injected into Ai-9 mice. At 72 h, a greater abundance of mature macrophages (F4/80^{+}CD11b^{+}) was detected in the liver and spleen compared to the untreated control mouse. Evaluation of transfection efficiency indicated that monocyte particle uptake and their subsequent differentiation were linked to successful transfection of the resulting macrophages, with 400-nm particles producing the highest level of tdTomato expression (Figure 2B). We tested the assembled 400-nm particles in a proof-of-concept therapeutic vaccine model for treating subcutaneously (*s.c.*) implanted ovalbumin (OVA)–expressing melanoma (B16-OVA). Statistically significant extensions of survival and delays of tumor growth were observed in the group that received the 400-nm particles loaded with mOVA or ODN as the sole functional payload but not in the group treated with the 100-nm particles (Figure 2C).

Conclusions.
Efficient transfection of monocytes by tumor antigen mRNA offers opportunities for cancer vaccines due to the ability of monocytes to differentiate into mature antigen-presenting cells that can mediate an antitumor response.

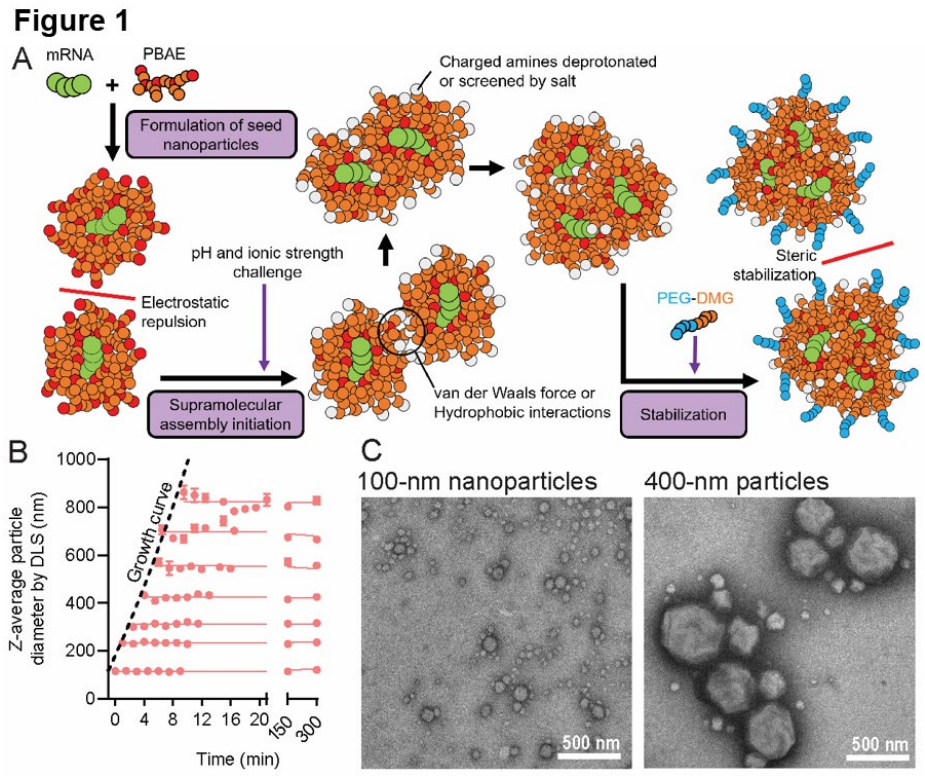
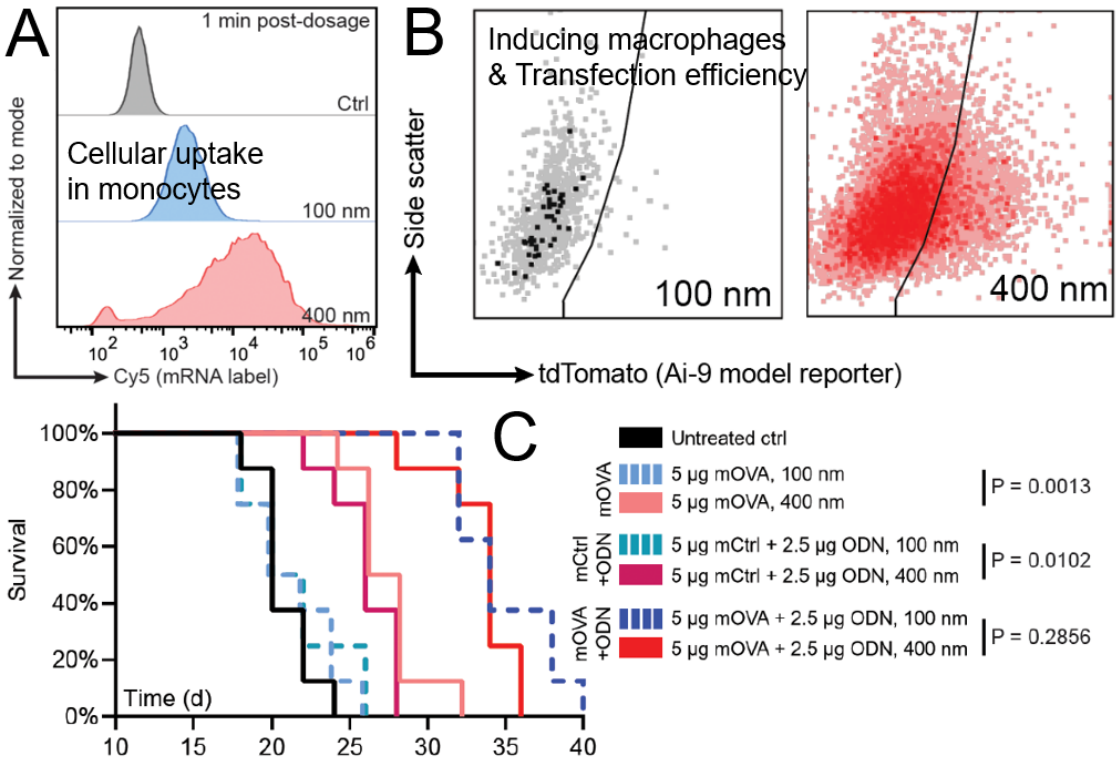


Figure 2



486 CS1 CAR Binds to Multiple Myeloma-Specific Isoform to Enable Precise Tumor Targeting

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Multiple myeloma (MM) is a common hematological malignancy, with approximately 36,000 new cases diagnosed annually in the United States. Although novel therapies such as chimeric antigen receptor (CAR) T cells have shown promise in treating MM, achieving durable remission remains challenging. We developed CAR T cells targeting SLAMF7 (CS1) using a single-chain variable fragment (scFv) derived from the humanized monoclonal anti-CS1 antibody, HuLuc63 (elotuzumab). Although elotuzumab is a well-established therapy for MM, developing a CS1-targeting CAR platform has been hindered by fratricide during CAR T cell manufacturing due to expression of CS1 by a subset of T cells. However, our CS1-CAR T cells did not show evidence of fratricide, given their comparable expansion to mock-transduced T cells, and had potent anti-MM activity. Moreover, CD4/CD8 ratios remained stable, and a soluble version of our CS1 scFv did not bind to or induce cytotoxicity of T and NK cells from healthy donors or patients with MM. To investigate the specificity of our CS1-CAR, we incubated our soluble CS1 scFv with neoplastic MM cells from patient bone marrow and found that the CS1 scFv selectively bound to dominant light chain-positive MM tumor cells. We confirmed via co-immunoprecipitation that our soluble CS1 scFv bound to the MM cell line MM.1S, but not to other CS1-expressing cells, including peripheral blood mononuclear cells (PBMCs), CD19-targeted CAR T cells, or CS1-CAR T cells. Notably, there are 10 isoforms of CS1 generated by alternative splicing that vary in expression of constant (C-) and variable (V-) domains. Using a cellular microarray assay, we determined that our CS1-CAR specifically bound to CS1 isoform 6, which uniquely contains both C- and V-domains and is the isoform expressed by MM.1S cells but not PBMCs or T cells. For functional validation, we used CRISPR technology to knock out CS1 in MM.1S cells and subsequently re-expressed various isoforms. Our CS1-CAR T cells selectively targeted MM.1S cells expressing isoform 6 but not those expressing other CS1

isoforms. Through protein modeling, we determined that our CS1-CAR specifically binds to the C-domain of isoform 6 and that removing the V-domain causes conformational changes that prevent binding.

In conclusion, our CS1-CAR is highly selective for a tumor-specific CS1 isoform and does not induce fratricide during manufacturing. By targeting a tumor-specific CS1 isoform, this CAR spares normal T cells and enables the successful generation of clinical doses without CS1 knockout, which we are currently testing in a phase 1 clinical trial (NCT03710421). These findings provide valuable insights into CS1-CAR function, isoform-specific targeting, and broader considerations for CAR design in other cellular therapies.

487 A human cell-based platform for testing olfactory ensheathing cells as vectors for cancer gene therapy in hiPSC-derived brain-glioma assembloids

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Olfactory ensheathing cells (OECs) are a hybrid glial cell population enriched in the olfactory bulb and nasal mucosa. Previous studies have reported that OECs may traffic through the brain and accumulate at sites of tumorigenesis, suggesting their potential to serve as vehicles for delivering therapeutic transgenes to CNS neoplasms. The goal of this study was to further characterize these properties in mucosal OECs, as these cells could potentially be obtained from patients and engineered for personalized cell-based therapies. As a first step, we confirmed the potential migratory capacity of endogenous mucosal OECs in a mouse model bearing glioma cells implanted within the brain. To selectively label mucosal OECs, Ai9 mice bearing a Cre-activated reporter received nasal injections of an AAV vector encoding Cre recombinase under the transcriptional control of *MPZ*, which encodes a canonical marker of OECs. Brains were collected and sectioned for analysis after 2 weeks post-inoculation. Within sections of brain parenchyma, we detected significant clustering of *MPZ*-Cre(+) cells surrounding the implanted tumors. Based on this observation, we next established a cell culture platform for evaluating human mucosal OECs in a human brain tissue model. Nasal biopsies from the superior turbinate were obtained from de-identified discarded tissue from human subject volunteers, and tissue was dissociated to generate mixed cultures of mucosal cells. The isolation protocol was validated in parallel by culturing olfactory bulbs dissected from 8–10-week-old mice. Immunostaining of the primary cultures from both human mucosa and mouse olfactory bulb revealed cells co-expressing known markers of OECs, including the canonical OEC markers, p75NGFR and MPZ, as well as S100beta, N-Cadherin, NCAM, and FAPB7. To isolate putative OECs, cultures were expanded over 2 weeks and then subjected to immunopanning with antibodies against either p75NGFR or MPZ. Both strategies successfully isolated mucosal and olfactory bulb cell populations that were double positive for both OEC markers. These primary OECs exhibited robust expansion in culture, successful cryopreservation and recovery, and efficient transduction with lentiviral vectors bearing transgene reporters of interest. To develop a human brain tissue model in which the effects OECs could be evaluated, a human induced pluripotent stem cell line derived from a healthy control individual was differentiated and matured over two months to generate cerebral organoids (Cos). At day 52, COs were co-cultured with glioma cells stably transduced with mCherry. After six days, the resulting assembloids were fixed and sectioned for image analysis, revealing significant invasion of the cortical organoid structure by the mCherry (+) glioma cells. Taken together, these brain-glioma assembloids provide a human cell-based platform for characterizing the time course by which mucosal OECs traffic through cortical tissue to accumulate around tumor cells, the nature of OEC:tumor interactions, and the efficacy of OEC-delivered therapeutic factors in limiting tumor growth. Functional studies to assess these parameters in OECs are currently ongoing.

488 Surfaceome CRISPR Activation Screens to Delineate Determinants of Natural Killer Cell Killing towards Cancer Cells

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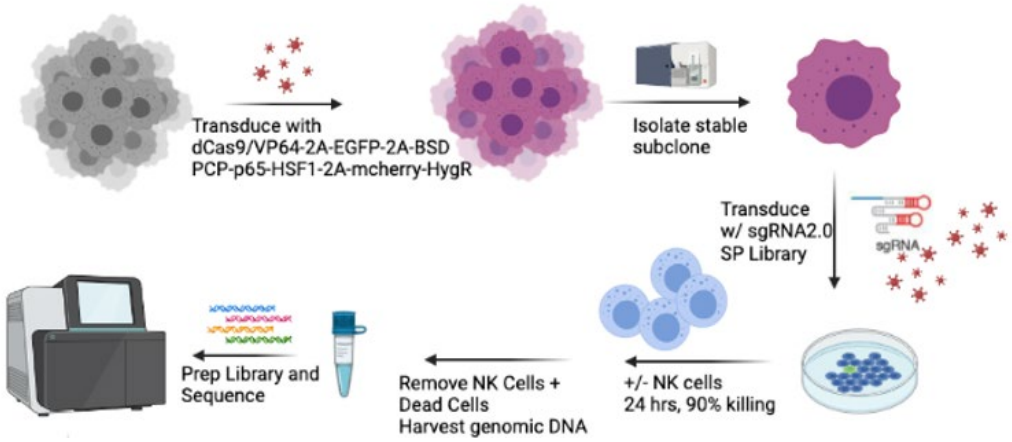
Major advances in tumor control have recently been achieved by targeting immune inhibitory signaling pathways. Natural killer (NK) cells are crucial for cancer immune surveillance due to their robust function and intrinsic anti-cancer activity. The responses of NK cells to cancer or infected cells are determined by integration of signals from germline encoded inhibiting and activating receptors engaged with cognate ligands on cancer cells. While NK cells offer protection against emerging tumors, they can also become dysregulated in response to signals from cancer cells and associated stroma in the tumor microenvironment. CRISPR screens have been applied to identify factors that control the sensitivity of cancer cells killing by NK cells. However, these studies were usually conducted on genome-wide scale, which is noisy to identify real hits, and in a loss-of-function way, which can only detect effect of genes that are expressed in the cell lines used.

Here we perform gain-of-function CRISPR screens in K562 and HGC27 cancer cells co-cultured with NK cells, using a surface protein gRNA library to uncover novel ligands across human cancers that can increase resistance or sensitization of tumor cells to NK cell killing. The Synergistic Activation Mediator (SAM) system was utilized to offer a high level of gene activation, and 3567 genes that have been shown through systemic analysis to comprise the cancer surfaceome were included in the library. We validate genes from our screen using arrayed cDNA overexpression in addition to knockouts in cell lines with endogenous factor expression and demonstrate a role for *SPN*, *CD4*4, *CD24*, and *PDPN* in mediating cancer cell resistance. Using NSG mice with transgenic expression of IL-15 (NSG-IL15) we further show that knocking out the above genes can enhance the NK cell killing towards tumor cells *in vivo*. Taken together, our results demonstrate the power of gain-of-function screening to identify novel targets for improved NK cell-based immunotherapy.



489 Attenuating Inflammation in Midbrain Dopaminergic Neurons through TNF-Responsive Synthetic Receptors

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Introduction:
Parkinson's disease is a pervasive and damaging neurodegenerative disease, affecting more than 10 million worldwide, with treatment costs totaling to nearly $52 billion a year in the U.S. The main cause of Parkinson's disease is the death of dopamine producing cells, midbrain dopaminergic (mDa) neurons, in the brain. Current treatments involve Levodopa, a dopamine precursor medication that helps to replace this vital neurotransmitter, but over time, this medication loses its effectiveness. To address this, researchers have begun investigating transplantion of mDa neurons derived from induced pluripotent stem cells (iPSCs) to replace disease-related neuron loss. This

strategy has shown promise, but up to 90% of mDa neurons die within a week after transplantation due to inflammation caused by the transplantation process. A major cause of this is increased susceptibility of mDa neurons to the pro-inflammatory cytokine TNF-a (TNF). Here, we present an approach to ameliorate mDa neuron death by engineering them with TNF-sensitive synthetic receptors to combat inflammatory TNF in the environment through production of therapeutic proteins, like cytokine antagonists.

Materials and Methods:
Our system leverages a synthetic intramembrane proteolysis receptor (SNIPR) platform to program cellular responses to cues in their microenvironment. SNIPR is a synthetic receptor that regulates ligand-dependent transcriptional programs in cells through user-defined recognition motifs and transcription factors. By exchanging extracellular and intracellular domains, we can produce custom receptors that can sense a variety of ligands and respond in an orthogonal manner. Here, we engineered differentiated mDa neurons with SNIPR receptors to respond to TNF and produce therapeutic proteins.

Results and Discussion:
KOLF2.1J iPSCs were differentiated into mDa progenitor neurons through an established 11-day protocol. They were then lentivirally transduced with a TNF-sensitive synNotch receptor and a response element for either reporter outputs, like mCherry protein and firefly luciferase, or therapeutic outputs, like soluble TNF receptor 1 (sTNFR1), a TNF antagonist. After 72 hours of TNF treatment, engineered mDa neurons were fixed and stained for progenitor markers (Fig. 1A). Engineered neurons showed hallmarks of mDa progenitor differentiation after lentiviral transduction. The SNIPR receptor demonstrated potent reporter activation, in the form of firefly luminescence and mCherry fluorescence, in response to TNF treatment (Fig. 1B and 1C). Significant production of the therapeutic protein sTNFR1 was measured as well, demonstrating that a cytokine antagonist can be produced in response to a native inflammatory marker by an engineered mDa neuron (Fig. 1D).

Conclusions:
Here we present an innovative method to address transplantation-induced inflammation in mDa neurons through a TNF-sensitive synthetic receptor. We successfully engineered neurons that retained mDa progenitor markers and showed significant activation in response to a native cytokine. Up to 30 ng/mL of a cytokine antagonist was produced, which, based on prior work, could provide a measurable benefit. Further work will involve *in vitro* testing to quantify reductions in inflammation and cell death via inducible TNF antagonism as well as *in vivo* implantation of engineered mDa neurons.

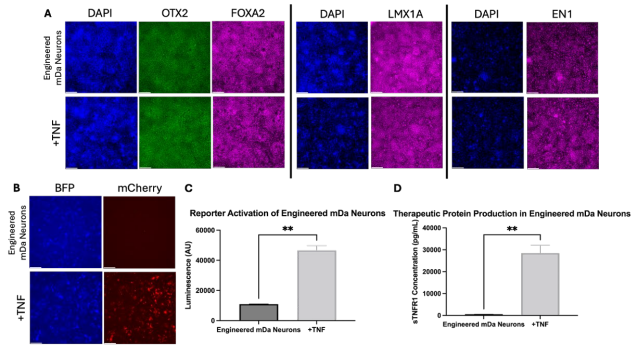

Fig. 1: A. Staining for the mDa neuron progenitor markers, OTX2, FOXA2, LMX1A, and EN1, for engineered mDa neurons with and without treatment with 50 ng/mL of TNF. A DAPI nuclear counterstain is included for visualization of cells. LMX1A and EN1 markers were stained and imaged in separate wells. B. mCherry reporter activation of engineered mDa neurons exposed to TNF. Neurons are engineered with a constitutive BFP marker for ease of visualization. C. Engineered mDa neurons treated with TNF show a significant increase in reporter firefly luminescence compared to control engineered neurons. D. Therapeutic protein, sTNFR1, production is significantly increased in engineered mDa neurons treated with TNF. Scale bar = 200 µm. (**p<0.01, Student's t-test with Welch's correction).

490 Oncolytic Herpes Simplex Virus and Radiation Therapy Synergize with IGF1R-Targeted Therapy to Enhance Glioblastoma Treatment

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Purpose:
We recently uncovered a novel mechanism of resistance to oncolytic herpes simplex virus-1 (oHSV) therapy, where infection stimulates the secretion of Insulin-like Growth Factor 2 (IGF2), subsequently activating Insulin-like Growth Factor-1 Receptor (IGF1R) signaling. This activation contributes to resistance against radiotherapy (RTx), establishing IGF1R as a key marker of poor RT response. In this study, we investigated the therapeutic potential of combining IGF1R blockade with oHSV and RTx to overcome resistance and enhance treatment efficacy.

Experimental design:
Western blotting, MTT assay, colony formation assay, and flow cytometry were performed to investigate the mechanism of IGF1R activation and its impact on cytotoxicity. Viral replication was assessed using Cytation imaging and plaque formation assays. The synergistic interaction of the triple combination therapy—including oHSV, RTx, and IGF1R blockade (e.g., OSI-906 or PPP)—was quantified using SynergyFinder analysis. Orthotopic mouse models were utilized to evaluate antitumor efficacy, with survival outcomes analyzed using Kaplan-Meier curves and the two-sided log-rank test.

Results:
oHSV treatment robustly activates IGF1R signaling in vitro and in vivo, thereby sensitizing tumors to IGF1R blockade. While IGF1R-targeting therapies show limited efficacy as monotherapy, their combination with oHSV significantly enhances cytotoxicity in all tested breast cancer and glioma cell lines and primary GBM cells compared to either agent alone. This effect is accompanied by a dose-dependent increase in PARP cleavage and caspase-3 activity. Additionally, RTx-induced IGF1 secretion further amplifies IGF1R activation, leading to increased phosphorylation of eIF2α and upregulation of YAP1 in combination with oHSV. The triple combination of oHSV, RTx, and IGF1R blockade exhibits strong synergy in breast cancer and glioma cells in vitro. It demonstrates enhanced anti-tumor efficacy in orthotopic breast cancer and GBM tumor models in vivo.

Conclusions: Our study provides preclinical evidence for the efficacy of a triple combination therapy of oHSV, RTx, and IGF1R blockade, supporting its potential for future clinical evaluation in patients.

491 Adenine Base Editing Rescues Dravet Syndrome in Mice

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**Corresponding Author*

Dravet syndrome (DS) is a severe neurodevelopmental disorder characterized by drug-resistant epilepsy, temperature-sensitive seizures, cognitive impairment, and a high incidence of sudden unexpected death in epilepsy (SUDEP). DS is caused by loss-of-function variants in *SCN1A*, which encodes the voltage-gated sodium channel α subunit Nav1.1. Current approved treatments for DS are focused on symptom management and do not address its root cause. Here, we describe the use of adenine base editing (ABE) to directly correct $SCN1A^{R613X}$, a mutation documented in multiple DS patients. In cell culture, we identified ABE strategies to efficiently correct R613X in engineered homozygous $SCN1A^{R613X}$ HEK293T and Neuro-2a cells (72% and 92% correction, respectively). We used a dual-AAV9 approach to deliver an optimized ABE system to $Scn1a^{R613X/+}$ mice, which

recapitulate key DS pathologies. AAV9-ABE treatment resulted in high editing efficiency at both the genomic (59%) and transcriptomic (97%) levels in bulk brain cortices of P0 treated mice. Brain slice electrophysiological assays of ABE-treated mice revealed that parvalbumin-expressing inhibitory neurons displayed restored excitability and function, with an average 1.7-fold improvement in maximum instantaneous firing rate over untreated controls. Untreated *Scn1a*$^{R613X/+}$ mice undergo febrile seizures at P18-21 at an average core body temperature of 40°C, whereas ABE-treated mice do not experience febrile seizures up to 42.5°C (108.5°F; **Figure 1**). In a 45-day survival study, we observed substantial rescue from SUDEP in ABE-treated mice, with 90% survival at P45 compared to 30% in vehicle-treated control mice (**Figure 2**). These findings represent the direct correction of a common genetic cause of DS, establish a platform to correct *SCN1A* mutations with ABE, and suggest the potential of future precision genome editing treatments for DS.

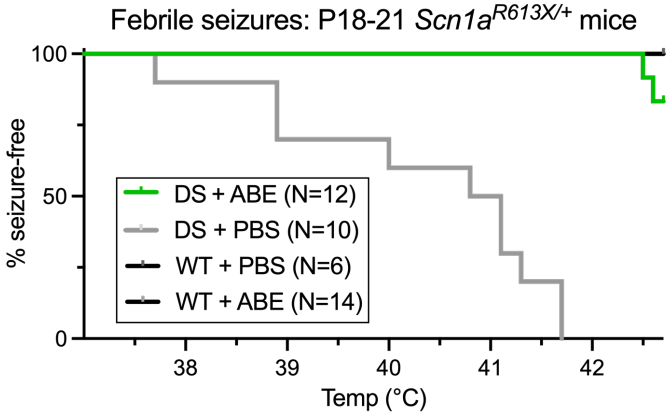

Figure 1 - Febrile seizures are ameliorated by ABE treatment. In heated cage assays, 10 of 12 ABE-treated DS mice did not experience seizures despite elevated core body temperatures. All vehicle-treated DS mice experienced febrile seizures at an average core body temperature of 40°C.

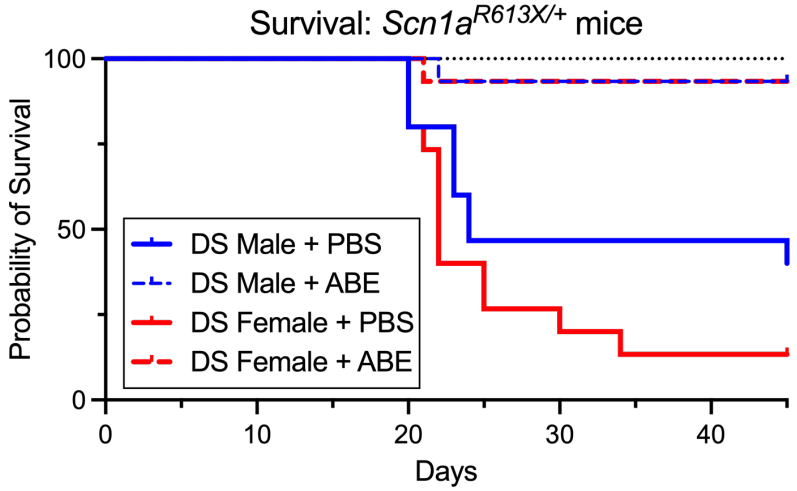

Figure 2 - ABE treatment rescues DS mice from SUDEP. Mice treated with AAV9-ABE or PBS at P0 by intracerebroventricular injection (N=15/group/sex).

492 Enhancing Truncation Event Prediction in AAV Vector Genome Designs Through Advanced Deep Learning

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Truncation events in recombinant adeno-associated virus (rAAV) vector genomes remain a significant obstacle in gene therapy, leading to reduced therapeutic efficacy, increased toxicity, and higher production costs. These events are driven by complex sequence features, secondary structures, and replication dynamics, yet the underlying molecular determinants remain poorly understood. We hypothesized that a deep learning (DL)-based model leveraging diverse genomic features could predict truncation hotspots in rAAV genomes with high precision, enabling the rational design of more efficient and reliable vectors. To test this hypothesis, we curated a comprehensive dataset of rAAV sequences obtained from single molecule, real-time (SMRT) and nanopore platforms used to captured high-resolution, genome-wide truncation events. The vectors encompass single-stranded (ssAAV) and self-complementary (scAAV) vectors, diverse promoters, transgenes, and sequence lengths spanning from 2.5 kb to 5 kb.

A robust bioinformatics pipeline was employed to normalize sequencing data, filter palindromic reads that result from truncation events, and extract critical features such as GC content, free energy states of DNA secondary structures, and nucleotide composition. These refined inputs provided a strong foundation for training the predictive model. We developed a multi-output DL model integrating convolutional neural networks (CNNs) and bidirectional gated recurrent units (Bi-GRU) to simultaneously predict normalized truncation counts (regression) and classify truncation signals (classification). Rigorous evaluation demonstrated that the model was accurate at identifying truncation-prone regions across diverse vector designs, with predictions closely aligning with observed patterns in benchmark constructs.

Importantly, this work has the potential of highlighting molecular drivers that can be targeted for rational optimization of rAAV vectors. By advancing our understanding of truncation mechanisms, this study establishes a transformative approach to streamline vector development and enhance the reliability of next-generation rAAV-based therapies.

493 A New AAV Manufacturing Platform with *in Cellulo* Replication of Plasmid DNA in HEK293 Cells

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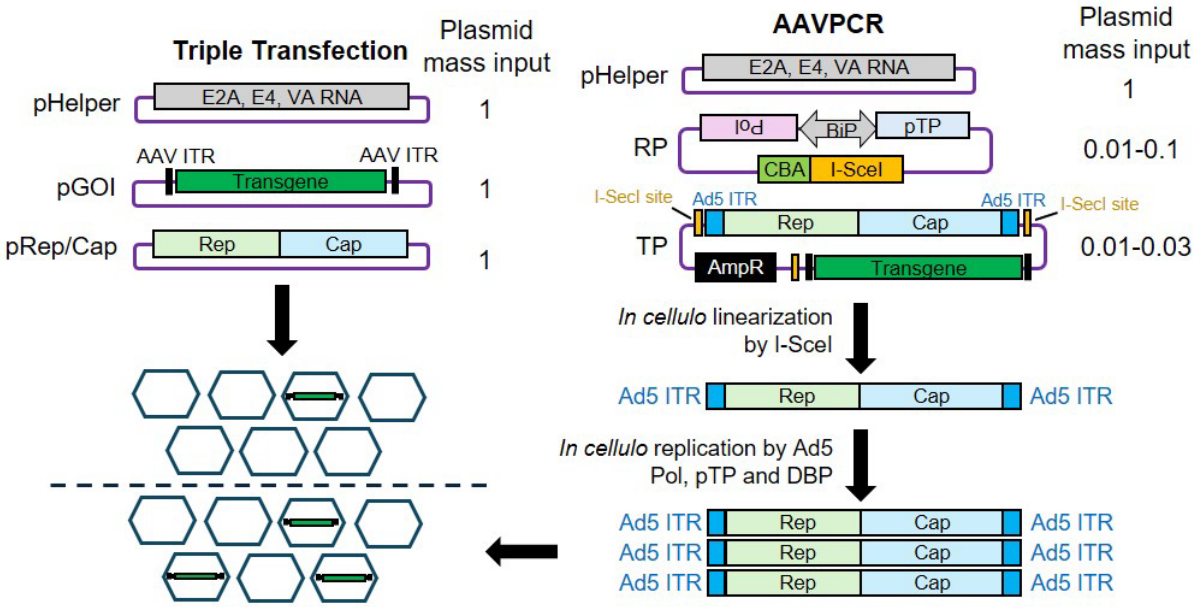
Introduction:
Triple transfection of HEK293 cells is the most widely used method for producing recombinant adeno-associated virus (rAAV), a leading vector for human gene therapy. This process involves co-transfecting three plasmids at approximately equal molar or mass ratios: pHelper, which provides the human adenovirus serotype 5 (Ad5) helper genes; pRep/Cap, which expresses the AAV *Rep* and *Cap* genes; and pGOI, which harbors a therapeutic gene-of-interest (GOI) cassette flanked by AAV inverted terminal repeats (ITRs) **(Figure)**. Despite its tremendous success, this method faces several challenges, including high plasmid demand, suboptimal rAAV titers, and alarming vector impurities. Here, we introduce a new **AAV** manufacturing platform featuring **P**lasmid DNA *in* ***C****ellulo* **R**eplication in HEK293 cells (**AAVPCR**). AAVPCR comprises three plasmids: the same pHelper used in triple transfection; RP (replication plasmid), which expresses Ad5 polymerase (Pol) and precursor terminal protein (pTP) under the control of a bi-directional promoter (BiP), along with the meganuclease I-SceI directed by the CBA promoter; and TP (template plasmid), which carries I-SceI restriction site and Ad5 ITR-flanked *Rep/Cap* along with GOI **(Figure)**. Upon co-transfection into HEK293 cells, TP is linearized by I-SceI and *Rep/Cap* is replicated by the

Ad5 replication machinery consisting of Ad5 Pol, pTP, and DNA-binding protein (DBP) from pHelper *E2A* gene (DNA cannot be replicated without being linearized or flanked by Ad5 ITRs). By leveraging this *in cellulo* replication process, only a minimal amount of TP is needed to generate abundant *Rep/Cap* copies that support robust rAAV production, thereby greatly reducing the plasmid requirement **(Figure)**.

Results:
Using *EGFP* as a model transgene, we demonstrated that AAVPCR substantially reduced plasmid demand and improved vector purity compared with triple transfection: (1) *Rep/Cap* genome was amplified approximately 200-fold during the rAAV production phase, allowing a 33- to 100-fold reduction in TP mass input while still generating comparable rAAV titers; (2) The full capsid ratios in crude lysates, determined by ddPCR of the genome titer normalized to ELISA capsid titer, increased from 9.8% to 40.7% for rAAV2 and from 16.3% to 34.9% for rAAV5; (3) Prokaryotic backbone DNA encapsidation was reduced from 3.8% to 0.48% for rAAV2 and from 6.5% to 0.32% for rAAV5. Notably, AAVPCR effectively packaged non-permissive transgenes, such as *GPR78*, which are incompatible with triple transfection. AAVPCR boosted the AAV2.GPR78 titer by 16.4-fold, reaching 44% of *EGFP* control. Moreover, the full capsid ratio of AAV2.GPR78 at harvest was increased by around 10-fold, from 6.1% in triple transfection to 59.4% in AAVPCR.

Conclusion:
AAVPCR first demonstrates the proof-of-concept that plasmid DNA segments can be engineered to be selectively replicated by reconstituted Ad5 replication machinery during rAAV production phase. The strategy of *Rep/Cap* replication enables high-purity, high-titer rAAV production with an unprecedented low plasmid requirement. Since pHelper and RP are universally used in all rAAV preparations, and TP requires 33- to 100-fold less material than its triple transfection counterparts, AAVPCR significantly accelerates the rAAV production process and paves the way toward economical and robust off-the-shelf AAV manufacturing. HL and AD: co-first authors; GG and DW: co-corresponding authors.



494 Deep learning-trained codon optimization algorithms identify novel tissue-derived codon usage patterns and enhance transgene expression *in vivo*

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Gene therapy involves introducing, removing, or modifying genetic material within a patient's cells to treat or prevent disease. Often the genetic material being delivered is a protein-coding transgene aimed to correct expression of a missing or mutated gene. One of the key challenges in gene therapy is achieving optimal expression of the protein-coding transgene, which can be partially addressed by codon optimization. This technique employs modifying the nucleotide sequence of the encoded transgene to reflect the codon usage patterns of the host species. Such modifications can enhance the efficiency and effectiveness of the gene therapy. While codon look up tables based on tRNA abundances have been historically used for determining codon usage, we aimed to address this issue using deep learning-based recurrent neural networks. We hypothesized that highly expressed genes might display the most efficient codon usage patterns. Algorithms were trained with coding sequences of >2,000 top expressed genes from mouse brain, liver, and muscle. Additional algorithms were also trained with transcripts of secretory genes to develop a codon-optimization pipeline for secreted transgenes. Once trained, these algorithms were then utilized to predict optimized coding sequences of randomly selected benchmark genes for evaluation of model performance. Predicted sequences showed novel codon usage patterns as compared to wildtype sequences and those obtained using a publicly available optimization tool. We observed that sequences predicted by our algorithms have an improved codon adaptation index, decreased usage of rare codons, and a selective pool of effective codon pairs. Additionally, we observed that our models result in sequences with lower CpG dinucleotide content, an unintended outcome that could be beneficial for therapeutic designs. Our algorithms also matched codon utilization with tRNA abundances without being trained with this information, pointing towards another hidden feature in codon usage of highly expressed genes. AAV9-serotyped vectors with DL-optimized EGFP versions enhanced transgene expression following intravenous and intramuscular administration in mice. Elevated expression and augmented biodistribution was observed in the liver, skeletal muscle, brain, heart, lung, and kidney. Codon optimization also improved expression in neurons and astrocytes of brains and in glomeruli of the kidney cortex and medulla. When applied to SMN1, the therapeutic transgene for spinal muscular atrophy (SMA), our muscle-optimized vector displayed higher expression as compared to the wildtype design, or those defined using publicly available optimization tools. For secreted factors, such as A1AT and factor IX, our algorithms produced similar enhancements in expression. Retaining the wild-type signal sequence, while codon-optimizing only the mature peptide, led to increased transgene production. These findings highlight the application of machine learning-based approaches to independently identify novel codon usage patterns merely from mRNA coding sequences, resulting in improved transgene expression that can be beneficial to the field of gene therapy.

495 Delivery of miRNA by Self-Complimentary AAV Provides ATXN1 Knockdown in SCA1 Mice and Supports Therapeutic Translation

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Spinocerebellar ataxia type 1 (SCA1) is a neurodegenerative disease caused by an expanded CAG trinucleotide repeat in the ATXN1 gene. The accumulation of mutant ATXN1 leads to progressive Purkinje cell degeneration and motor deficits. As a therapeutic approach, we have developed miS1, a microRNA (miRNA) targeting ATXN1 transcripts. Direct delivery of AAV encoding miS1 to the deep cerebellar nuclei has been shown to prevent and reverse disease phenotypes in multiple mouse models of SCA1[1-3]. In support of an IND application, we performed a

long-term study using AAV-miS1 in non-human primates. Unlike in mice, we observed cerebellar toxicity that was attributed to inverted terminal repeat (ITR) promoter activity. Interestingly, this activity was abolished in a dual therapy AAV where miS1 was co-expressed with the ATXN1 homolog, ATXN1L, supporting the remediation of toxicity by including a transgene driven by an RNA polymerase II promoter[4]. Here, we looked to optimize our approach further, testing miS1 in a self-complimentary AAV (scAAV) driven by an RNA polymerase II promoter and in multiple combinations with ATXN1L. Validation of scAAV-miS1 *in vitro* showed similar levels of knockdown and miRNA production to previous constructs. In SCA1 mice, scAAV-miS1 showed an improved reduction of ATXN1 compared to our previous AAV-miS1 virus and a reduction of inflammatory markers AIF1 and GFAP. Importantly, scAAV.miS1 packaged efficiently and generated high virus titers, reducing cost and mitigating any constraints on dosage. Combined with our extensive prior work, these results support a translatable SCA1 therapeutic and warrant further studies. References: 1. Keiser, M. S et al., Neurobiology of Disease 2013. 2. Keiser, M. S et al., Molecular Therapy 2014. 3. Keiser, M. S et al., Annals of Neurology 2016. 4. Keiser, M. S. et al., Nature Medicine 2021.

496 AAV-mediated gene therapy for Pyruvate Dehydrogenase Complex Deficiency

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Pyruvate dehydrogenase (PDH) complex deficiency (PDHD) is a severe debilitating childhood mitochondrial disorder. PDH complex plays an integral role in glucose metabolism and comprises of three enzymatic subunits with the gene for the E1 α-subunit is located on the X-chromosome (*PDHA1*). Patients display developmental delay, hypotonia, hypertonia, dystonia, ataxia, and seizures. There are currently no disease-modifying therapies, leading to poor outcomes with mortality and morbidity. A brain-specific mouse model for PDHD has been reported, where the *Pdha1* KO mice display, neurological delay, ataxia, seizures, rapid weight loss, premature death by P21, neuropathology, abnormal metabolite levels, and reduced PDH enzyme activity.

AAVs have been used successfully in pre-clinical and clinical studies for severe neurological disorders. We have developed a novel gene therapy treatment for PDHD, using an AAV vector encoding human *PDHA1* gene driven by a CAG promoter. In this study we aimed to test AAV9 and AAV-F in a clinically relevant *Pdha1* KO mouse model, to assess the effects of AAV-mediated gene therapy on amelioration of the disease phenotypes. We administered titre-matched AAV9 and AAV-F gene therapy to newborn *Pdha1* KO mice via intracerebroventricular delivery. We observed 75% survival for AAV-F (n=8), 30% for titre-matched AAV9 therapy (n=10), and 0% for PBS control-treated *Pdha1* KO (n=10) mice over 100 days of development. In addition, we administered AAV9 at a higher dose (one log higher) and observed 80% survival, compared to 0% for PBS control-treated *Pdha1* KO (n=10) mice over 100 days of development. Behavioural assessment at P18 and P30 revealed no significant difference to wild-type (WT) controls however, by experimental endpoint (P100), the remaining AAV-F or AAV9 treated mice displayed a significant reduction in locomotion function compared to WT mice. Furthermore, we observed comparable PDH complex enzyme activity and relative metabolites in the brain for AAV-F and high dose AAV9 to WT control mice. Neuropathology assessment of the treated brains revealed a significant improvement in inflammation and neuronal loss in the olfactory bulb, prefrontal cortex and cortex in both groups. Cortical thinning was also significantly reduced after AAV-F and high dose AAV9 treatment. In addition, AAV-F revealed significantly lower transgene expression in the liver compared to high dose AAV9. We have shown by administering AAV-F one log lower to AAV9, we can observe a similar therapeutic effect in the CNS, whilst de-targeting the liver and therefore reducing potential off-target toxicities. Our proof-of-concept study so far has shown promising data for AAV-mediated therapy and as a potential treatment for patients with PDHD.

497 Safe and Efficient Editing of Novel CRISPR-Cas Ribonucleoprotein Complexes Delivered by Engineered Protein Delivery Vehicle

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Clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR-Cas) system holds great promise to revolutionize genome editing and therapy in basic and translational settings. Various CRISPR-Cas systems have been explored and engineered, such as CRISPR-Cas9 and CRISPR-Cas12a that belong to Class 2 of CRISPR-Cas system. However, therapeutic applications of these tools are still limited due to potential off-target effects.

To address this limitation, we developed a novel Cas discovery platform combining a tailored protein-based delivery vehicle system. With this new platform, we identified and characterized dozens of novel type II Cas nucleases with diverse PAM restrictions and protein sizes. We next packaged high-fidelity Cas ribonucleoprotein (RNP) complex with an in-house tailored Protein Delivery Vehicle (PDV). The PDV encapsulated nucleases showed generally higher editing potency than plasmid modality delivered by lipofectamine at over ten endogenous loci, and with higher fidelities at tested sites as evaluated by GUIDE-seq. *In vivo* experiments demonstrated that the Cas RNP-PDV achieved saturating editing efficiency in mice's liver. Moreover, off-target profiling revealed a significant reduction in off-target effects in a hepatoma cell line compared to the conventional plasmid-based method. The transient nature of RNP delivery by PDV eliminated prolonged Cas9 exposure, reducing the risk of overloaded DNA double-stranded breaks. Biodistribution study in mice confirmed targeted delivery to the designated liver, with negligible accumulation in off-target tissues. This platform represents a significant advancement in *in vivo* genome editing, offering a safer and more efficient alternative to existing non-RNP-based delivery strategies for real clinical settings. Furthermore, our modular combination allows adaptation to diverse Cas nucleases encapsulated by PDV, broadening applications in cell and genome editing therapy.

498 Addressing the Manufacturing Bottleneck in Gene Therapies Through AAV Production in Whole *Nicotiana benthamiana* Plants

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Introduction:
The demand for adeno-associated virus (AAV) vectors is increasing due to their role in gene therapies for various genetic disorders. However, current production methods using mammalian and insect cell systems are costly, slow, and hard to scale, limiting accessibility. High production costs result in multi-million-dollar price tags for approved gene therapies, which are often unaffordable for patients, especially in low- and middle-income countries. With thousands of gene therapies in development, traditional cell culture-based production struggles to meet the growing demand, as it requires specialized facilities and lengthy scale-up times. Moreover, inconsistent vector yields and quality impact clinical reliability, especially for progressive genetic disorders where timely treatment is critical. To address these challenges, we have developed an innovative, plant-based AAV production system that is cost-effective, scalable, reliable, and faster, ensuring wider accessibility to life-saving treatments. We utilize a split-Rep and split-Cap system, where the VP1, VP2, and VP3 capsid proteins, along with the Rep78 and Rep52 proteins, are split across separate plasmids. This approach enhances AAV production efficiency, genetic stability, and scalability while minimizing the toxic effects of high Rep expression levels.

Methods:
Plasmids for AAV9-CMV-GFP production were generated by splitting Cap and Rep proteins across separate plasmids. These plasmids were introduced into *Agrobacterium tumefaciens*, which was used to deliver the vectors into *Nicotiana benthamiana* plants through a transient expression method. The infiltrated leaves were harvested five days later and homogenized. The expression levels of VP1, VP2, VP3, Rep78, and Rep52 were analyzed using western blot and Jess for stoichiometry and relative expression. The performance of the plant-based system was compared to the traditional HEK-293 cell-based method, where Rep and Cap were expressed from a single plasmid.

Capsid titer was measured using ELISA, and Dynamic Light Scattering (DLS) was performed on the purified material using Stunner.

Results:
The natural stoichiometry of AAV capsid proteins is 1:1:10 (VP1:VP2:VP3), but achieving this ratio from a single plasmid is challenging due to competing transcription and translation. By separating VP1, VP2, and VP3 onto different plasmids, their expression levels can be independently controlled, ensuring proper capsid assembly. We observed that fine-tuning the expression of VP1, VP2, VP3, Rep78, and Rep52 from separate plasmids optimized capsid formation, leading to higher AAV vector production efficiency. We consistently produced more than 1e15 AAV capsid particles from just 1kg of plant biomass (approximately 2 liters of plant homogenate for downstream processing).

Conclusion:
This work demonstrates for the first time that plant-based AAV production using a split-Cap and split-Rep plasmid system significantly enhances yield and scalability. This method preserves the natural stoichiometry of AAV vectors, prevents toxicity from overexpression, and reduces recombination issues, resulting in more consistent vector production. Moreover, the split-Cap system potentially allows for the creation of hybrid AAV vectors by mixing and matching VP1, VP2, and VP3 proteins from various serotypes, which could improve tropism and immune evasion in future applications.

499 AAV9 Neutralizing Antibody Seroconversion in Household Contacts Of Participants With Duchenne Muscular Dystrophy Receiving Fordadistrogene Movaparvovec

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Introduction:
Fordadistrogene movaparvovec was a candidate disease modifying AAV9 gene-replacement (mini-dystrophin) therapy for Duchenne muscular dystrophy (DMD). Viral vector shedding has been reported in trials of AAV-based gene therapies, but the risk of developing neutralizing antibodies (NAbs) to AAV9 for household contacts of clinical trial participants receiving fordadistrogene movaparvovec is unknown. This study aimed to determine the rate of AAV9 NAb seroconversion in previously seronegative household contacts of participants in phase 2 and 3 trials of fordadistrogene movaparvovec (NCT05429372 and NCT04281485, respectively).

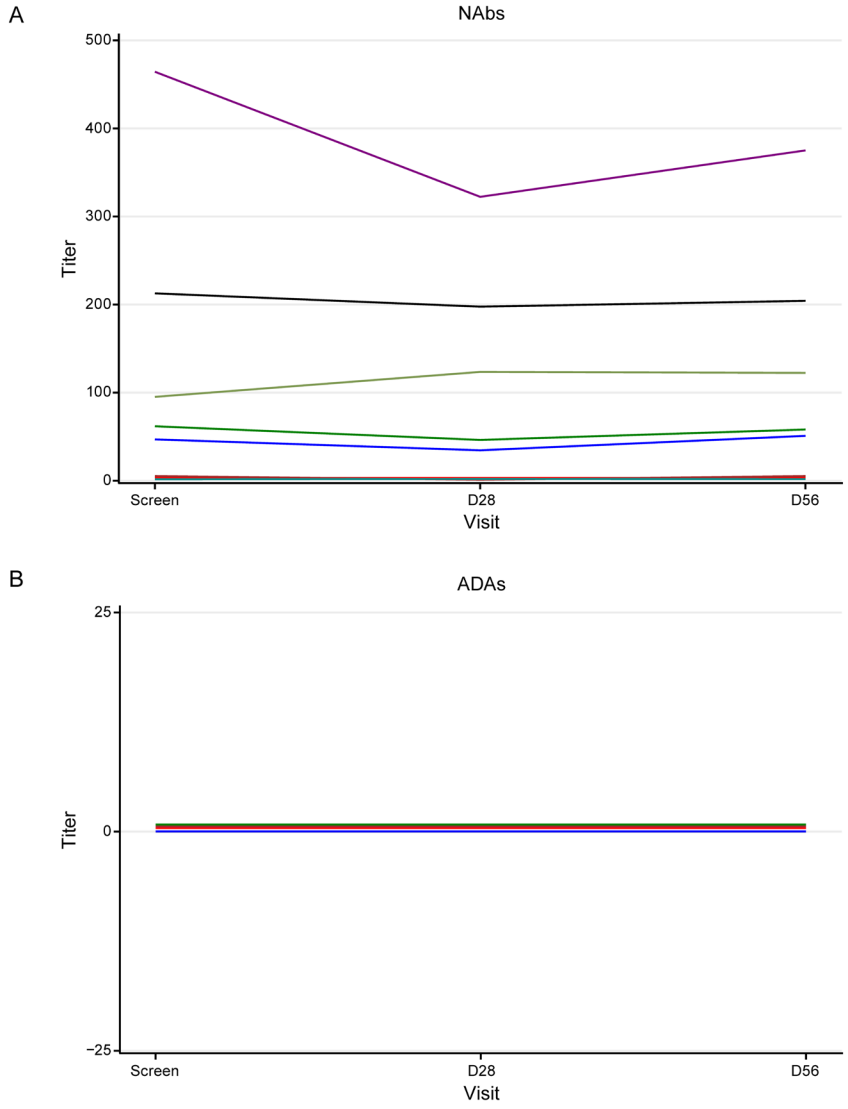
Methods:
The primary endpoint was the development of NAbs to AAV9 in household contacts defined by an increase ≥6-fold above baseline titer after a detectable but negative test (titer ≥1 to <4) at baseline, or development of a titer of ≥6 in those with an undetectable titer (<1) at baseline. The secondary endpoint was development of total binding antibodies (anti-drug antibodies; ADAs) to AAV9 in household contacts defined by a titer of ≥300 in those with a negative test (titer <50) at baseline. Blood samples from household contacts were collected at baseline and at 28 (±8) days and 56 (±10) days after fordadistrogene movaparvovec dosing (single intravenous dose of 2E14 vg/kg) in the clinical trial participant.

Results:
8 household contacts of 5 clinical trial participants receiving fordadistrogene movaparvovec were assessed. All household contacts (8 of 8) had direct contact with the fordadistrogene movaparvovec recipients for each of the first 14 days after infusion. 3 of 8 household contacts had negative NAb titers at baseline, and none of them seroconverted. None of the other 5 household contacts had ≥6-fold increases from their baseline NAb titers. Further, 7 of 8 household contacts had negative ADA titers at baseline and none of them seroconverted. The remaining household contact was unevaluable for ADAs due to a missing baseline sample. The rates of NAb and ADA

seroconversion were therefore 0% (0 of 3 and 0 of 7, respectively). Changes in NAb and ADA immunogenicity over time for household contacts are shown in Figure 1A and 1B, respectively.

Conclusions:
The results of this study indicate that the risk of developing NAbs and ADAs to AAV9 through contact appears to be low in a setting where household contacts have been advised to practice good hygiene. The study results should be interpreted with caution due to the small sample size of 8 household contacts. Specifically, the finding that 0% of household contacts displayed NAb seroconversion was based on results from only 3 individuals, since only 3 household contacts were negative for NAbs at baseline. This study was sponsored by Pfizer. ClinicalTrials.gov: NCT04543357.

Figure 1. Change in NAb and ADA immunogenicity over time.



Each line represents an individual household contact. (**A**) NAb titers below the limit of quantification were imputed as 0; the lower limit of quantification was 1. (**B**) ADA titers below the limit of quantification were imputed as 0; the lower limit of quantification was 50. Although all recorded values were below the lower limit of quantification and considered to be 0, the titer values plotted have been offset between 0 and 1 to allow for visual discrimination between household contacts. ADA, antidrug antibody; D, Day; NAb, neutralizing antibody

Poster Presentations

500 Novel Liver-Targeted Gene Therapy Platform vs. Brain-Targeted Approaches for Inherited Metabolic Liver Diseases

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Inherited metabolic liver diseases (IMLDs) represent a diverse group of genetic disorders caused by enzyme deficiencies that disrupt essential metabolic pathways, resulting in systemic complications, including severe neurological manifestations. Although neonatal liver transplantation is used to treat certain IMLDs, it presents significant risks for patients, challenges in finding suitable donors, and does not fully address neurological impairment. These limitations highlight the urgent need for alternative therapies targeting both the liver and the brain.

Liver gene therapy holds significant promise; however, its efficacy is limited by risks such as DNA cargo depletion over time and potential insertional mutagenesis. To overcome these challenges, we developed the LTRX lentiviral vector, which improves safety by removing viral cis-regulatory elements. We tested LTRX in a mouse model of Maple Syrup Urine Disease (MSUD), an IMLD characterized by the absence of the mouse Dihydrolipoamide Branched Chain Transacylase (mDBT) gene, a critical subunit of the Branched-Chain Alpha-Keto Acid Dehydrogenase (BCKDH) Complex. This deficiency impairs the metabolism of branched-chain amino acids (BCAAs), leading to their toxic accumulation and resulting in neurological deficits.

Neonatal MSUD mice were treated with LTRX-mDBT at doses of 1.4×10^{11} and 1.0×10^{10} TU/kg, using a liver-specific promoter to restore BCKDH enzymatic activity in the liver. This intervention extended lifespan, improved body weight, and normalized plasma BCAA levels in a dose-dependent manner. Brain metabolomic analysis further demonstrated the correction of BCAA metabolism, including the normalization of neuroactive metabolites and sphingolipids.

In addition, we explored brain-targeted therapy by intracerebroventricular injection of an AAV-mDBT vector (PhP.eB serotype) at a dose of 7.5×10^{9} vg/pup. Unexpectedly, brain-targeted gene therapy alone led to prolonged survival and amelioration of specific CNS metabolites in MSUD mice, despite persistently elevated plasma BCAA levels. This approach also prevented delayed neuromotor development and mitigated the ataxic and hyperactive phenotypes characteristic of the MSUD model. Comparative analysis of brain metabolites from brain-targeted versus liver-targeted gene therapies, as well as untreated mice at different ages, revealed distinct effects of brain DBT expression on redox homeostasis, gamma-glutamyl amino acids, and lactoyl amino acid metabolites. These findings underscore the role of BCKDH in brain metabolism.

In conclusion, LTRX offers a promising approach for liver gene therapy in IMLDs, while brain-targeted interventions may hold promise in addressing neurological manifestations of MSUD. Together, these approaches hold the potential to pave the way for combinatorial therapies that target both hepatic and neurological dysfunctions.

501 Long-term correction of metabolic impairment in a mouse model of Glycogen Storage Disease type III after rAAV gene transfer reveals disease-specific host-vector interactions.

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Glycogen Storage Disease type III (GSDIII) is a rare metabolic disorder caused by mutations in the *AGL* gene, leading to the accumulation of abnormally structured glycogen in the liver, heart, and muscles. The hepatic impairment associated with this disease is characterized by chronic liver damage, fasting hypoglycemia, hepatomegaly, elevated liver enzymes, and progressive fibrosis. Disease management is currently limited to dietary restrictions, underscoring the need for effective long-term treatments.

Adeno-associated virus (AAV) gene therapy has demonstrated promise in numerous metabolic disorders, including GSDIII. We recently reported the complete correction of muscle impairment in GSDIII mice and rats using a truncated transgene. However, correcting the metabolic impairment remains a challenge in GSDIII mice. While short-term, partial correction has been achieved, further development of gene transfer technologies is necessary for long-term disease correction.

This study supports the potential of recombinant adeno-associated virus (rAAV)-mediated gene therapy to correct the hepatic phenotype in GSDIII mice ($Agl^{-/-}$), a faithful model of the human liver disease. By tailoring the rAAV vector approach to the model's specific characteristics—such as capsid selection and transgene expression cassette optimization—we achieved sustained correction of hypoglycemia and glycogen accumulation.

Our data indicated that the short-term, complete correction achieved in $Agl^{-/-}$ mice with advanced liver disease was not maintained in the long term. However, an optimized transgene expression cassette, devoid of CpG dinucleotides in both the transgene and promoter sequences, resulted in prolonged transgene expression and metabolic correction at nine months post-injection. Interestingly, despite these advancements, a small subset of mice still exhibited a loss of transgene expression regardless of the optimization.

To investigate potential mechanisms contributing to this loss, we examined hepatocyte proliferation—an established disease manifestation in GSDIII livers—and identified a disease-specific immune response within liver-infiltrating immune cells. Notably, AAV-injected mice showed higher frequencies of immune cells infiltrating the liver including effector and memory lymphocytes, along with increased frequencies of activated CD69+ T cells. . Importantly, this heightened immune activation was observed exclusively in $Agl^{-/-}$ mice and only when the transgene was expressed, suggesting a previously uncharacterized immune activation mechanism in the GSDIII liver, likely influenced by its inflammatory background.

These findings highlight the importance of understanding host-vector interactions to enhance the durability of AAV gene therapy for liver diseases. The GSDIII mouse model serves as a valuable tool for studying these interactions. Furthermore, this research underscores the challenges of achieving stable correction in the presence of chronic liver disease and reinforces the need for continued optimization of gene therapy approaches to ensure lasting therapeutic benefits for patients with liver metabolic disorders.

502 mRNA therapy for Glycogen Storage Disease type 1b.

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Glycogen storage disease type 1b (GSD1b) is an inherited metabolic disorder due to deficiency of glucose-6-phosphate transporter (G6PT) that result in recurrent episodes of life-threatening hypoglycemia, growth retardation, osteoporosis and long-term risk of renal failure and liver cancer. Current treatments focus on managing hypoglycemia by diet. In contrast to GSD1a, GSD1b patients also show neutropenia that causes recurrent infections and inflammatory bowel disease currently treated with Granulocyte colony stimulating factor (G-CSF) injections or sodium–glucose cotransporter 2 inhibitor (Empagliflozin). However, treatments for glucose metabolic defect remain unsatisfactory and more effective therapies are needed. mRNA encapsulated into lipid nanoparticles (LNP) has great potential for therapy of inherited metabolic liver diseases and several clinical trials for various disorders, including GSD1a, are ongoing. In a novel preclinical murine model of GSD1b knock-out for the gene encoding G6PT ($G6pt^{-/-}$ mice), we investigated the efficacy of mRNA replacement therapy. $G6pt^{-/-}$ mice showed a severe phenotype with high perinatal mortality and extensive liver glycogen storage. Glucose therapy by intraperitoneal injections only minimally improved neonatal survival whereas intravenous (IV) injections of mRNA encoding G6PT encapsulated into lipid nanoparticles (LNP-G6PT mRNA) significantly increased survival of $G6pt^{-/-}$ mice. Moreover, IV injections of LNP-G6PT mRNA rescued the fasting-tolerance of $G6pt^{-/-}$ adult mice. Normal blood glucose concentrations were achieved in mice injected with LNP-mRNA as early as at 6 hours post-dosing and the metabolic correction lasted for at least one week after the injection of the LNP-mRNA. In conclusion, our study provides for the first time LNP-G6PT mRNA as potential therapy for GSD1b and might pave the way for a clinical trial in GSD1b patients.

503 3D-printed Highly Porous PLG-based Scaffolds as a Platform for Therapeutic Cell Delivery

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Introduction: Cell-based therapies have gained significant attention in treating several diseases, including Type I Diabetes (T1D) and liver disease. Therapeutic cells have traditionally been delivered via direct infusion or hydrogel encapsulation; however, these strategies have been hindered by the immunological rejection of injected cells, insufficient cell engraftment, and the use of biomaterials that limit cell attachment and nutrient and oxygen diffusion, each of which greatly impact cell viability and function. To address these limitations, scaffolds fabricated from highly porous biomaterials 3D-printed in open-architecture designs may provide a more effective method to deliver therapeutic cells and maintain their function long enough to restore organ function. In this approach, a 3D-printed PLG-based scaffold can be fabricated with an intrafiber porosity that promotes cell attachment, tissue infiltration, and vascularization within the scaffold macropores where cells are seeded. Here, we evaluated the ability of these scaffolds to support the viability and function of seeded therapeutic cells for the treatment of T1D and liver disease.

Materials: Tissue scaffolds were 3D-printed to produce open pore structures with printed fibers having ~80 vol% porosity and a total scaffold porosity of >95 vol%. Primary human islets (PHIs; Prodo Labs) or hepatocytes (PHHs; Thermo Fisher Scientific) were seeded onto scaffolds, evaluated for viability, and implanted into mice to assess the therapeutic function of the cell-seeded scaffolds. For T1D studies, scaffolds seeded with 2750 islet equivalents of PHIs were implanted into the epididymal fat pad of streptozotocin-induced diabetic nude mice. Glycemic control by the implant was assessed across 30 days via changes in blood glucose levels, body weight, and human c-peptide serum levels. For liver disease studies, PHH-seeded scaffolds were implanted into NOD SCID mice for 30 days. Blood was collected weekly to assess viability and synthetic function via quantification of human albumin. In both studies, implants were retrieved at study termination and processed for histology.

Results: For the T1D studies, all PHI-seeded implants showed functional responses to glucose challenge on Day 27, and human c-peptide was present across all timepoints. Furthermore, the PHI-seeded scaffolds reduced blood glucose to normoglycemic ranges for up to 30 days. Following histological processing, PHIs stained positively for

human insulin, indicating the implanted PHIs remain viable and functional within the scaffolds for at least 30 days. For the liver disease studies, hAlbumin was detected in mouse blood at levels equal to those of *in vitro* controls for at least 30 days. In addition, extensive vascular networks within scaffolds were identified ($CD31^+$ and presence of circulating red blood cells) suggesting significant graft vascularization.

Conclusion: The data from this study clearly demonstrate that both PHIs and PHHs can be successfully seeded into highly porous PLG open-scaffolds, maintaining viability and therapeutic functionality for at least 30 days *in vivo*. Notably, the scaffold's intrafiber porosity promotes vascular engraftment and tissue infiltration—critical factors for ensuring long-term cell viability and function. These compelling results pave the way for further exploration of this innovative delivery system and advance the treatment of Type 1 Diabetes (T1D), liver disease, and beyond.

504 Characterization of KRIYA-839, an AAV-based Gene Therapy Providing Insulin and Glucokinase, in Streptozotocin-induced Diabetic Mice

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Type 1 diabetes (T1D) is a chronic, autoimmune disorder characterized by profound insulin deficiency resulting from destruction of insulin-producing β-cells in the pancreas. T1D results in persistent hyperglycemia and increases risks for severe, debilitating complications. Historically, life-long insulin therapy has been the only treatment for T1D. This approach requires multiple daily insulin injections and careful monitoring of blood glucose, which presents a significant patient burden. More recent approaches, including islet transplantation, have shown promise but face significant challenges and often require life-long immunosuppression, which increases risks for opportunistic infections and cancers.

KRIYA-839 is a novel AAV1-based gene therapy providing human insulin (AAV1.hINS) and glucokinase (AAV1.hGCK) that is administered via intramuscular injection and is being developed to treat T1D. Skeletal muscle is responsible for the disposal of around 70-80% of circulating glucose after meals but only around 20-25% during the fasted state (Petersen 2018). In muscle, glucose uptake is regulated by insulin-stimulated and concentration-dependent glucose transport through glucose transporter type 4 (GLUT4) and phosphorylation of glucose by hexokinase II (Printz 1993, Kahn 1996). In patients with T1D, GLUT4 translocation to the plasma membrane and glycolytic flux through hexokinase II activity are both suboptimal due to a lack of circulating insulin, which presents a therapeutic opportunity for KRIYA-839.

Prior investigations have demonstrated improved glycemic control in streptozotocin (STZ)-induced diabetic mice and dogs following intramuscular injection of AAV1-based vectors expressing insulin and glucokinase, with robust glycemic control durably maintained in dogs for up to 8 years (Mas 2006, Callejas 2013, Jaen 2017).

The constituent vectors of KRIYA-839 have been modified from these earlier constructs to optimize safety and efficacy. Injection of KRIYA-839 into skeletal muscle in STZ-induced diabetic mice resulted in improved glycemic control across multiple clinically relevant parameters. After 8 weeks of treatment, all KRIYA-839 treatment groups had significantly improved fasting blood glucose ($p<0.001$) and HbA1c levels (>2% reduction; $p<0.001$) and improved response to oral glucose challenge compared to the STZ-control group. Serum triglyceride levels were also significantly reduced in treated mice compared to STZ and non-STZ controls ($p<0.001$), suggesting potential secondary benefits of the therapy. Furthermore, symptomatic hypoglycemia was not observed in mice treated with KRIYA-839 at any dose or timepoint over the duration of the 8-week study. This study demonstrates the potential of KRIYA-839 for T1D and lays the foundation for future clinical studies.

References: Callejas et al. (2013) Diabetes 62(5): 1718-1729. Jaen et al. (2017) Mol Ther Methods Clin Dev 6: 1-7. Kahn (1996) Diabetes 45(11): 1644-1654. Mas et al. (2006) Diabetes 55(6): 1546-1553. Petersen et al. (2018) Physiol Rev 98(4): 2133-2223. Printz et al. (1993) J Biol Chem 268(7): 5209-5219.

505 Towards the Development of Adeno-Associated Viral Gene Therapy for the Treatment of Patients with Propionic Acidemia Resulting from a Deficiency of PCCB

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Background: Propionic acidemia (PA) is rare autosomal recessive disorder of metabolism caused by defecits in the mitochondrial localized enzyme propionyl-CoA carboxylase (PCC). PCC is composed of six α- and six β-subunits with causative genetic variants occurring at equal frequencies in either the nuclear encoded *PCCA* or *PCCB* genes. Patients with PA can suffer from poor growth, lethal metabolic decompensations, and cardiomyopathy despite current medical management, which has led to the pursuit of gene therapy as a new therapuetic option for patients. Here, we create several new murine models of PA caused by PCCB deficiency and generate pre-clinical proof of concept data as part of the Accelerating Medicine Partnership (AMP®) Bespoke Gene Therapy Consortium (BGTC). Led by NCATS, the BGTC partnership relies upon the FDA, FNIH, 10 NIH Institutes and Centers (ICs), including NHGRI, several pharmaceutical companies, and nonprofit organizations. The BGTC is intended to bring together partners from the public, private, and non-profit sectors to foster development of gene therapies for the treatment of rare genetic diseases.

Results: To enable the testing of gene delivery as a treatment for PA, CRISPR-Cas9 gene editing of the murine *Pccb* gene and transgenesis were used to create several new murine models of PCCB deficiency and recapitulate the spectrum of clinical severity observed in PA. Neonatal lethal, juvenile lethal and hypomorphic PCCB PA mice, representative of the spectrum of genetic mutations and disease phenotypes reported in PA patients, were treated systemically via retroorbital injection of AAV9-hPCCB at doses ranging between $5x10^{12}$ to $1x10^{14}$ vg/kg. Based on disease severity, murine models of PA were treated as neonates, juveniles, or adults, which is reflective of the age range of the PA patient population.

AAV9-hPCCB treatment resulted in significantly increased survival (Figure 1), reduced disease related metabolites, improved growth and protection against metabolic stress in comparison to the respective untreated mutant mice. All the doses tested improved survival and reduced disease related biomarkers in comparison to untreated PA mice, but the response in $5x10^{12}$ vg/kg dose cohort was suboptimal and temporal in nature. AAV9-hPCCB gene therapy resulted in increased *PCCB* mRNA and PCCB protein expression in liver, heart, skeletal muscle, brain and the kidney, which are tissues impacted by PA.

Conclusions: The preclinical efficacy data presented here was used successfully to obtain both Orphan and Rare Pediatric Drug Designations from the FDA. PA patients experience early mortality and lethal metabolic decompensation in response to stressors, therefore the significant improvement in survival and biochemical parameters seen in all models treated with AAV9-hPCCB therapy strongly supports the active pursuit of an IND and advancement toward clinical translation.

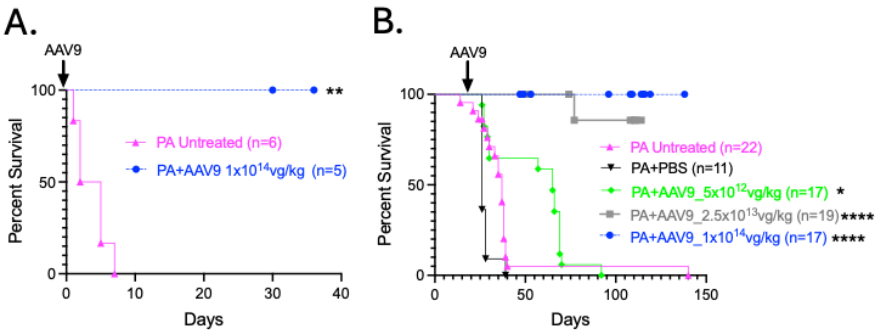

Figure 1. Survival Curve of Neonatal Lethal (A) and Juvenile Lethal (B) Models of PA Caused PCCB Deficiency. PBS=phosphate buffered saline

506 In Vivo Pooled Mosaic Screening for the Discovery of Drug Targets in Adipose Tissue

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Reducing obesity is an important clinical objective because it's a risk factor for many diseases, including diabetes, cardiovascular disease, and cancer. Adipose tissue is involved in many important aspects of human health from metabolic to immune. Recently, GLP-1 receptor agonists have shown dramatic effect in diseases such as obesity and diabetes, but indirectly regulate the adipose tissue that contributes to these diseases. Our in vivo pooled screening methodology, termed mosaic screening, is able to assay hundreds of genetic perturbations in a cell type specific manner. Using a pool of barcoded AAV interventions, we can deliver single gene therapy interventions to our cell type of interest in an animal model of clinical relevance. The tissue is then processed with 10x single-cell system and Illumina sequencing, and readouts are assessed using single-cell transcriptomics. Gene therapy candidates that show improvement of gene pathways associated with disease are nominated and further tested in vivo to confirm improvement of various physiological readouts. This technique has been applied to a variety of complex diseases such as osteoarthritis and heart failure to good effect and herein we show the ability to apply our method to the adipose tissue.

Several AAV serotypes were delivered to the adipose tissue to mice fed an obesogenic high fat, cholesterol and fructose diet, also known as the Gubra-Amylin (GAN) diet. We compared three methods of administration: intravenous injection (IV), intraperitoneal injection (IP), and intra-depot injection. Transduction was observed in all three routes with IP delivery chosen as the preferred method due to its higher adipose transduction than IV and ease of administration as compared to intra-depot. In order to prevent off-targeted transduction in the liver, we engineered short adipocyte-specific promoters, in contrast to existing adipocyte-specific promoters that are too large to fit into AAVs. These promoters expressing red fluorescent protein were barcoded and produced in a pool and injected in GAN mice at three different doses (n = 3 per dose). Transduced nuclei from white adipose tissue were isolated and the promoter with the highest expression as determined by sequenced barcodes was nominated for further use. With confirmation of feasibility of screening in adipocytes and preadipocytes, we further tested an AAV pool of barcoded therapeutic interventions in GAN diet-fed SpCas9-Tg or female C57BL6/J mice (n = 11 or 20 mice). Results of the screen were analyzed by single-nucleus RNA-sequencing and compared to non-targeting controls. With the ability to see cell-type specific transcriptomic perturbations in the adipose tissue, we hope to nominate further therapeutic targets tailored to adipose-associated diseases.

507 An Ultra-Low Dose of a Localized CNS Gene Therapy for Severe Pediatric Obesity

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Brain-derived neurotrophic factor (BDNF) is a secreted growth factor that promotes neuronal health throughout the central nervous system and is a key signaling component of metabolic homeostasis. In the ventromedial hypothalamus (VmH), elevated leptin signals melanocortin 4 receptor (MC4R) expressing neurons to release BDNF, which then acts through tropomyosin receptor kinase B (TrkB) to signal fullness and reduce food intake. Patients with loss-of function mutations along this pathway develop severe early-onset obesity syndromes characterized by hyperphagia and food-related distress as young as six months old. Current therapeutic approaches such as bariatric surgery and glucagon-like peptide 1 (GLP1) agonists may be effective for generalized obesity, but do not result in significant, durable treatment for individuals with MC4R or BDNF deficiency. By directly delivering BDNF to the VmH through an adeno-associated virus (AAV)-based gene therapy, we aim to treat these individuals early in

childhood in a safe, effective, and lasting way. Treating these children early could significantly improve their physical and emotional well-being and prevent the development of irreversible sequelae such as cancer and diabetes.

To achieve this, we developed a more potent vector which could decrease the AAV gene therapy dose required to maintain effective weight loss, potentially mitigating adverse effects associated with high levels of AAV administration. Our optimized BDNF clinical construct, designed by altering various cis-regulatory components, expresses BDNF up to 143-fold greater than basal levels *in vivo*, significantly higher than the original academic construct. This is well in excess of the levels shown to prevent weight gain in the diet-induced obesity (DIO) wild-type mouse model. This highly potent AAV-BDNF vector showed efficacy in DIO mice with significant dose-dependent weight loss observed across a wide therapeutic window, with a 60-fold-dose range. Delivery of AAV-BDNF to a single hemisphere of obese mice on high-fat diet resulted in dramatic weight loss up to approximately 40%, which plateaued at the level of wild-type lean controls. In comparison, weight loss in DIO animals injected daily with a GLP-1 agonist, semaglutide, plateaued at approximately 12%. Weight loss in AAV-BDNF treated mice has been maintained for at least three months and continues to be monitored to further assess the durability of AAV-BDNF. In addition to lower body weight, AAV-BDNF-expressing mice showed significantly improved glucose tolerance compared to control AAV-GFP-expressing obese mice.

To assess the safety of AAV-BDNF gene therapy administered directly to the hypothalamus, we performed biodistribution, hematology, serum chemistry, and brain histology. Because the hypothalamus controls additional behaviors besides feeding, we also assessed animals using the open field, novelty-suppressed feeding, food intake measurements, and standard opponent tests to survey other behavioral attributes. As previously reported, locomotion increased in AAV-BDNF-treated animals. More importantly, animals did not display heightened aggression after AAV-BDNF treatment and exhibited normal pro-social behaviors. Furthermore, AAV-BDNF treatment did not ablate the ability of animals to feed. Overall, our data suggest that AAV-BDNF is an effective and safe therapeutic strategy for severe early-onset pediatric obesity syndromes.

508 A Novel Non-Viral DNA Gene Therapy Ameliorates Disease in a Mouse Model of Hypophosphatasia

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Hypophosphatasia (HPP) is an inborn error of metabolism caused by mutations in the gene encoding tissue non-specific alkaline phosphatase (TNALP). Reduced expression of functional TNALP leads to defective bone mineralization, and depending on the age of onset, can result in a wide spectrum of skeletal abnormalities, respiratory failure, seizures, premature tooth loss, and musculoskeletal pain. In infantile-onset HPP, failure to thrive, delayed motor development, and 50% mortality are observed. Currently, the standard-of-care is enzyme replacement therapy with asfotase alfa (STRENSIQ®). While effective, asfotase alfa has a short half-life and requires almost daily subcutaneous injections leading to painful site reactions in most patients.

To overcome this requirement, we have assessed a novel gene therapy platform, HALO™, for the treatment of HPP. HALO is a circular DNA vector with unique structural DNA elements designed to mediate nuclear uptake, nuclear retention, and immunomodulation, which greatly increases efficiency, durability, and the ability to readminister the treatment *in vivo*. We constructed a HALO DNA vector encoding a secreted form of human TNALP fused to a human IgG Fc domain and a bone-targeting peptide (HALO TNALP). This DNA construct was formulated in an MC3-based lipid nanoparticle (HALO TNALP-LNP) for systemic administration and expression of TNALP from the liver. To evaluate its potential therapeutic application, we utilized an established mouse model of HPP (Akp2$^{-/-}$, *Alpl*^{tm1Jlm} on B6129S background, constitutive knock-out of the murine gene encoding TNALP, *ALPL*). This model is characterized by premature mortality (median survival of ~19 days), seizures, and impaired bone development, recapitulating major aspects of the infantile form of the human disease.

In Akp2$^{-/-}$ mice, a single intravenous dose of HALO TNALP-LNP shortly after birth on post-natal day (PND) 3 enabled complete rescue of mortality until scheduled study termination on PND 30. Untreated or PBS-treated Akp2$^{-/-}$ mice died prematurely, just prior to weaning, as expected. In treated Akp2$^{-/-}$ mice, skeletal development, as measured by long bone (femur and tibia) length via calipers, was the same as observed in Akp2$^{+/+}$ (wild-type littermates). Examination of vertebrae and metatarsal spacing in radiographic images further supported full phenotypic rescue in this model. Rescue was associated with marked and durable increases in plasma ALP activity compared to untreated Akp2$^{-/-}$. HALO DNA vector copies were detectable in the liver, as expected with MC3-based LNP delivery, with no concomitant indication of hepatoxicity through measurement of serum liver transaminase activity.

These data show that a HALO DNA vector encoding TNALP can be formulated within an LNP for systemic delivery to support full rescue of premature mortality and impaired skeletal development in Akp2$^{-/-}$ mice following a single dose. These data provide proof-of-concept for HALO as a viable and durable non-viral DNA platform for the next generation treatment for HPP.

509 Homology-Independent Targeted Integration-Based Strategy Restores Canine FVIII Activity from FVIII Intron 22 Inversion Mutation

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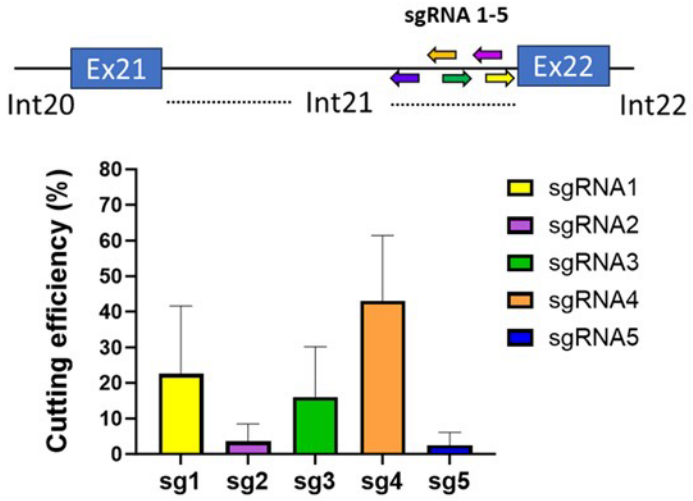
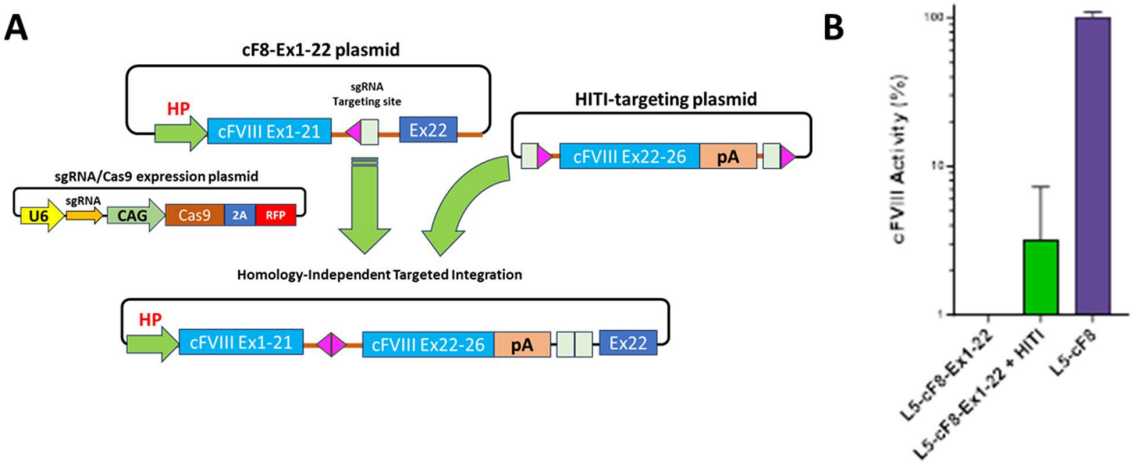
Over 50% of severe hemophilia A (HA) cases are caused by the intron 22 inversion (Int22inv) mutation in the factor VIII (FVIII) gene. This mutation disrupts the transcription downstream of exon 22, resulting in a truncated canine FVIII transcript. The Chapel Hill HA dog model carries the similar FVIII Int22inv mutation, making it a valuable model for developing therapeutic gene editing strategies for a long-term cure of this mutation. Homology-independent targeted integration (HITI) strategy is a CRISPR/Cas9-based strategy that enables DNA integration at sgRNA-targeting sites. In this study, we applied the HITI approach at the canine FVIII (cF8) gene locus, inserting exon 22-26 upstream of the inversion site to restore normal cF8 transcription.

To evaluate the efficiency of intron 21-targeting sgRNAs, we constructed a plasmid containing the cF8 exon 20-intron 22 sequence (cF8-ex20-in22). Plasmids encoding the Cas9 protein and cF8 intron 21-targeting sgRNA (**Cas9/cF8In21sg**) were co-transfected into 293T cells. The insertion and deletion (Indel) efficiency of sgRNA was assessed, with the selected cF8 intron 21-targeting sgRNA demonstrating an average gene editing efficiency of 43% *in vitro*.

To demonstrate HITI efficiency in *vivo*, we designed a truncated cF8 plasmid containing cF8 exon 1-22 and intron 21 (**cF8-ex1-in21**) and a HITI-targeting construct carrying cF8 exon22-26 (**HITI-cF8-ex22-26**). HA mice were hydrodynamically injected with a plasmid mixture, including Cas9/cF8In21sg, cF8-ex1-in21 and HITI-cF8-ex22-26. Rescue of the truncated cF8 protein expression was confirmed through activated partial thromboplastin time (aPTT) assay. After hydrodynamic injection of the plasmid mixture into HA mice, **cFVIII expression levels were restored to 3.2% on day 7 relative to the levels achieved from a wild-type cFVIII plasmid,** whereas mice injected with the cF8-ex1-in21 plasmid alone exhibited no detectable activity.

HITI Efficiency was further evaluated using NGS sequencing, confirming approximately 2.4% of successful integration of the exon 22-26 gene cassette into intron 21. This HITI strategy achieved successful integration of the exon 22-26 gene cassette into intron 21 and restored the truncated cFVIII transcripts, leading to functional cFVIII activity *in vivo*. This HITI approach will be evaluated to repair cFVIII Int22Inv mutation in HA dogs.





510 *A Novel Agent-based Computational Model for Liver-targeting, AAV-based Gene Therapies Could Predict Response Durability in Hemophilia B Patients Treated with Etranacogene Dezaparvovec*

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Many gene therapies use adeno-associated viral (AAV) vectors to deliver ITR-flanked therapeutic transgene cassettes that persist as non-integrated and non-replicating episomes in target cells. The liver, known to be self-renewing, is a common target organ for AAV based gene therapies, including etranacogene dezaparvovec, developed for the treatment of hemophilia B (congenital factor IX (FIX) deficiency).

The assumption of regular cellular turnover causing transgene loss has led to speculations that therapeutic protein expression could not be sustained in liver-targeting gene therapies. Differential equation-based models have previously been developed to describe AAV uptake and protein production. However, episomal transgene loss associated with target cell turnover has proved difficult to capture in these models. To overcome this shortcoming,

an agent-based model (ABM) was developed and described herein, enabling simulation-based analyses on the effects of several assumptions around hepatocyte cell biology and patient physiology on the long-term durability of etranacogene dezaparvovec.

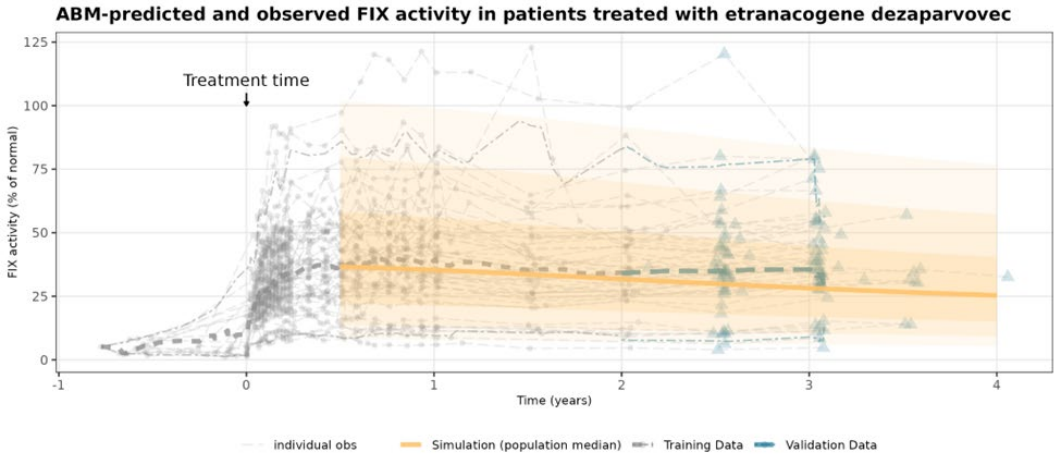
In this ABM, each individual hepatocyte, diploid or polyploid, was represented by an agent, capable of cell division and death, changing ploidy, and of being transduced with one or several AAV particles. Upon transduction, the AAV genomes form stable episomes, enabling the expression of therapeutic protein by the host's transcriptional and translational machinery. During cell division, all episomes were passed from mother and equally shared among daughter cells. Episomes were only lost when transduced cells died. The agents' behavior over time was specified by kinetic parameters describing cell division, diploid to polyploid differentiation, death, and associated effects on FIX production. One ABM consisted of 1000 agents, with the initial proportion of diploid and polyploid hepatocytes depending on patient age. The initial fraction of transduced cells and the initial episome count per transduced cell were estimated from preclinical studies.

To understand the expected variability in treatment outcomes, a population of 160,000 virtual patients was set up. One anticipated source of variability was the impact of patient age - and its implications for cellular composition of the liver. Less quantifiable sources of potential inter-patient differences included the fraction of cells transduced immediately after treatment, the number of episomes per transduced cell, and the maximum number of actively expressed episomes per cell.

The ABM and virtual population were parameterized based on liver physiology, endogenous coagulation FIX biology reported in literature, and an early subset of FIX activity data (training data) following the first two years after treatment of hemophilia B patients with etranacogene dezaparvovec (CSL222; NCT03569891).

Virtual population ABM simulations of FIX activity in patients treated with this therapy were consistent with the range of clinical observations over three years (incl. validation data; Figure 1), capturing the median, population variation, and dynamics of FIX expression. Prospective virtual population simulations out to 20 years established confidence in long-term outcomes and highlighted the impact of target cell turnover rates.

While the model was applied here in the context of treating hemophilia B, the modeling and simulation-based framework should be adaptable to many other AAV-based gene therapies.



Light grey dots: training data set individual observations.

Thick light grey dashed line: median of training data.

Light blue triangles: validation data set individual observations.

Thick blue dash line: median of validation data.

Thin dashdot lines: outlining 95% CI of the observed FIX activity.

Yellow solid line: ABM-predicted median FIX activity, increasingly lighter shaded areas representing 50%, 80% and 95% CIs.

511 Identification of bioengineered AAV capsids with enhanced targeting of liver sinusoidal endothelial cells (LSECs)

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Most coagulation factors (factor IX, factor V, etc.) are synthesized in hepatocytes within the liver. However, coagulation factor VIII (FVIII) is primarily synthesized in liver sinusoidal endothelial cells (LSECs). Deficiencies of FVIII cause the X-linked bleeding disorder, hemophilia A (HA). Current gene therapy approaches using AAV vectors to treat HA target hepatocytes for transgene expression by using AAV serotypes that efficiently target hepatocytes in combination with hepatocyte-specific promoter elements. While several naturally occurring AAV serotypes target LSECs, they do not target them efficiently. Through an in vivo screen of an AAV capsid library, we identified novel AAV capsids that efficiently transduce LSECs. To identify novel AAV capsid variants that efficiently target mouse LSECs, we used AAV capsid libraries based on AAV2. Previous studies in our laboratory have demonstrated that the parental AAV2 does not efficiently target LSECs. Random 7-mer peptides were inserted at a surface accessible region in the capsid VP3. Three rounds of screening were performed to identify capsids with improved LSEC-targeting. The top 2 capsid candidates (AAV^{LSEC1} and AAV^{LSEC2}) were selected for validation of improved targeting of LSECs. HA/CD4KO mice were intravenously administered $1x10^{11}$, $1x10^{10}$, or $1x10^{9}$ vector genomes/mouse of either AAV^{LSEC1}-CAG-eGFP, AAV^{LSEC2}-CAG-eGFP, or a positive control AAV8-CAG-eGFP. Four weeks after vector administration, mice were perfused using a modified two-stage collagenase perfusion to harvest hepatocytes and liver NPCs for FACS analysis of $CD31^{+}CD146^{+}CD45^{-}$ LSECs. At $1x10^{11}$ vg/mouse, FACS quantification of $CD31^{+}CD146^{+}CD45^{-}GFP^{+}$ LSECs showed 94 and 97% GFP^{+} LSECs, respectively, for AAV^{LSEC1} and AAV^{LSEC2} compared to AAV8 (89%), suggesting that at this dose the vector may be saturating the cellular receptors required for entry into the cell. At a 10-fold lower vector dose ($1x10^{10}$ vg/mouse), AAV^{LSEC1} transduced 64% and AAV^{LSEC2} transduced 48% of LSECs and compared to 11% with AAV8, demonstrating 4-6-fold enhanced targeting of AAV^{LSEC} compared with the AAV8 serotype. At the lowest vector dose ($1x10^{9}$ vg/mouse), AAV^{LSEC1} (7% GFP+ LSECs) and AAV^{LSEC2} (3% GFP+ LSECs) compared to 2.8% GFP+ LSECs with AAV8. Based on these data, we then compared AAV^{LSEC1} and AAV^{LSEC2} targeting of LSECs at the $1x10^{10}$ vg/mouse dose in another experiment to validate these findings. FACS quantification of $CD31^{+}CD146^{+}CD45^{-}GFP^{+}$ LSECs showed 58% and 63% GFP+ cells respectively, for AAV^{LSEC1} and AAV^{LSEC2}, confirming the improved LSEC targeting of these capsids. Thus, AAV^{LSEC} vectors significantly improved (~6-fold) targeting of LSECs compared to AAV8 at $1x10^{10}$ vg/mouse. Overall, these studies identify bioengineered AAV capsids that can target LSECs more efficiently than naturally occurring serotypes. These LSEC-targeted capsids provide an innovative strategy for a second-generation approach to hemophilia A gene therapy by targeting FVIII expression to the endogenous site of FVIII synthesis.

512 Superior Efficacy of AAV Gene Therapy for Hemophilia A Using Engineered Factor VIII Variants and Transient B Cell Depletion for Effective Vector Readministration

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Liver-directed adeno-associated viral (AAV) vector-based gene therapy drugs have recently received regulatory approvals for treatment of the X-linked bleeding disorder hemophilia. However, large vector doses are used to treat hemophilia A (deficiency in factor VIII, FVIII). Furthermore, FVIII is not normally expressed in hepatocytes, may induce cellular stress responses, and its expression has substantially declined over time in many patients. We developed a total of 16 FVIII vector cassettes expressing CpG-depleted, codon-optimized, B domain-deleted (BDD) human FVIII. These vectors include clinically tested liver-specific promoters (HLP and TTR) and 8 different FVIII

variants (WT, F309S for reduced aggregation and ER stress and improved secretion, X5 for improved secretion, R336Q/R562Q or "QQ" for improved stability in circulation, ΔSQ linker deletion, and combinations F309S-X5, QQ, X5-QQ, ΔSQ-X5). AAV8 vectors were administered IV into hemophilia A mice (C57BL6/129 F8e16-/-) at the dose of 1.0E+11 vg/mouse, and FVIII activity in plasma was measured at week 2 using a chromogenic assay. For most FVIII variants, TTR promoter constructs outperformed the HLP constructs by ~2 fold. Overall, the highest performers were HLP-X5-QQ (on average 539% of normal human FVIII plasma activity), TTR-X5-QQ (468%), and TTR-F309S-X5 (631%). These levels were >1 log better than those from the basal HLP-FVIII cassette. Biochemical assays using in vitro transduced primary human hepatocytes showed that superior variants not only exhibit improved secretion but also enhanced biosynthesis. Human FVIII is highly immunogenic in hemophilic mice. For example, hepatic gene transfer in the hemophilia A mouse strain BALB/c F8e16-/- induces inhibitory antibody and CD8+ T cell responses (Mol Ther 30:3552, 2022). We administered AAV8-TTR-FVIII vector (expressing WT BDD-FVIII) at 1.0E+11 vg/mouse to this strain in combination with our recently developed transient immune suppression protocol, which combines anti-CD20 and anti-BAFF treatments for effective transient B cell depletion (Mol Ther Methods Clin Dev 32:101216, 2024). With this combination therapy, we achieved sustained expression in the normal human FVIII range. FVIII expression reached a peak (383%) at week 4, decreased by 8 weeks, then remained within the normal range (45-133%) for the next 5 months, which is 4 months after B cells had reconstituted. Notably, after 6 months, we administered another AAV8 vector (expressing human FIX) at identical dose and achieved expression levels in the normal FIX range, similar to naïve control mice. Using this strain and B cell depletion, we tested our so far most promising variants at a 5-fold lower dose (2.0E+10 vg/mouse) in the same experimental condition as above. Surprisingly, X5 and X5-QQ variants expressed much lower than expected for a 5-fold reduction in vector dose. In contrast, F309S-X5 variant maintained efficacy and expressed within the normal range (~62-73%) during the first month (we are now following up for long-term expression). These results indicate: i) an unexpected threshold effect; and ii) the ability of the F309S mutation to enhance X5 secretion when FVIII expression is low. In totality, our experiments establish a benefit for a combination of FVIII variants. Inclusion of X5 yielded superior results, and F309S-X5 emerged as the most optimal combination, thus representing strong candidate for improved clinical AAV-FVIII gene therapy for hemophilia A.

513 Homology-Independent Targeted Integration in a Humanised Mouse Model of Haemophilia A

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Haemophilia A (HA) is the most common X-linked bleeding disorder caused by lack of factor VIII (FVIII), an essential cofactor of the coagulation cascade. Almost half of the severe HA cases are due to an inversion within intron 22 of the *F8* gene, which results in the inversion and translocation of the first 22 exons and consequent absence of FVIII expression and activity. In order to correct this common *F8* inversion by genome editing, we generated a humanised mouse model missing *F8* exons 23 to 26 and instead carrying the first 4 kilo bases of the human *F8* intron 22. We then used adeno-associated viral (AAV)-mediated homology-independent targeted integration (HITI) directed to liver to insert the last missing *F8* exons (23 to 26) downstream of the exon1-22 translocation, thus restoring FVIII expression. To do this, 2 different AAV8 vectors were used: one vector encodes *Staphylococcus pyogenes* CRISPR/Cas9 (*SpCas9*); another vector encodes the HITI donor containing the mouse *F8* exons 23 to 26 flanked by two inverted gRNA sites, and the U6 promoter expressing the gRNA specific for the human *F8* intron 22. We confirmed positive donor integration of all gRNA-treated animals by PCR amplification of DNA extracted from liver samples. In contrast, mice administered with tested constructs show undetectable FVIII activity levels and prolonged coagulation times at various time points post-injection, regardless of the promoter used to express SpCas9. These findings suggest that the donor integration at the target site is taking place but not at sufficient levels and/or in the correct liver cell type, particularly in liver sinusoidal endothelial cells (LSECs) which are the physiological producers of FVIII. We are currently setting up a platform to

deliver *SpCas9* mRNA with lipid nanoparticles with high tropism to LSECs. Restoration of FVIII expression in a relevant portion of the LSEC population could result in therapeutic efficacy in HA mice.

514 Enhancing the Potency of *In Vivo* Lentiviral Gene Therapy to Hepatocytes

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Lentiviral vectors (LV) are attractive vehicles for *in vivo* liver-directed gene therapy, representing a complementary strategy to adeno-associated viral (AAV) vectors. In contrast to AAV, LV integrate into the genome of target cells, potentially ensuring transgene maintenance during liver growth. We have previously shown stable gene transfer to the liver in mice, dogs, and non-human primates by LV administration. However, high quantities of highly purified LV are required for clinical translation. Increasing the potency of gene transfer remains a crucial goal in gene therapy since lowering the dose required to achieve therapeutic efficacy would result in a reduction of dose-dependent toxicities and immunogenicity, besides reducing the costs associated with manufacturing. We focused on LV-mediated in vivo gene transfer to hepatocytes of coagulation factor VIII (FVIII) and coagulation factor IX (FIX), the genes mutated in hemophilia A and B, respectively. We tested strategies to increase the corrected liver mass *a posteriori* or *a priori*. To increase the potency *a posteriori*, we provided, together with the coagulation FIX or FVIII transgene, a short hairpin RNA downregulating Cypor, the redox partner of all microsomal cytochromes P450s (CYP450s), a previously shown strategy. Since paracetamol is partially metabolized by CYP450s generating a toxic metabolite, the transduced hepatocytes become resistant to toxic doses of the drug, replacing the damaged ones. We confirmed and expanded the feasibility of this strategy, applying it to hemophilia A and B. We achieved positive selection of transduced hepatocytes, turning FVIII and FIX transgenes amounts from sub-therapeutic to fully therapeutic. Notwithstanding the clonal expansions, we did not detect any enrichment of LV integrations near oncogenes in the mice that underwent the selection procedure, suggesting that hepatocyte expansions were not driven by LV integrations but due to the selective advantage of LV-transduced hepatocytes. To increase potency *a priori*, we evaluated several procedures (fasting, inhibition of interferon, IFN, or proteasome pathways) to implement before LV administration. Specifically, fasting mice or administering a single dose of an anti-IFNα receptor (IFNAR) antibody or of the proteasome inhibitor Bortezomib before LV increased the circulating amounts of coagulation FVIII or FIX ~5-fold, indicating their role as transduction enhancers. When testing these transduction enhancers in combination, we achieved up to 13-fold higher circulating FIX amounts compared to LV only, paralleled by an increase in LV copy number in hepatocytes. Finally, we assessed the possible effect of transduction enhancers on LV enriched, on their surface, with CD47, a phagocytosis inhibitor (CD47high LV). These LV have been shown to be more protected from macrophage uptake following intravenous administration to NOD mice, which can cross-recognize the human CD47. We administered CD47high LV to NOD mice, with or without pre-treatment with the combination of the previously identified enhancers. We observed >40-fold higher FIX circulating amounts in the combination group, suggesting a synergistic effect in maximizing hepatocyte transduction when phagocytosis-shielded LV are combined with transduction enhancers. Here, we successfully enhanced the potency of *in vivo* LV-mediated hepatocyte gene transfer, thus paving the way to expand its in vivo applicability and ease its clinical translation.

515 Liver-Humanized Mice Repopulated with Hepatocytes From a Hemophilia B Donor Exhibit Delayed Blood Clotting and Have a Physiologic Response to Gene Therapy Correction of Human FIX

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Hemophilia B (HEMB), a single gene disorder affecting Factor IX, is an ideal target for gene therapies. In order to establish a relevant model for evaluating such therapeutics, primary hepatocytes from a patient with HEMB were evaluated for clotting defects and gene therapy correction in vivo in liver-humanized mice. For this, primary hepatocytes from a male HEMB donor were transplanted into male FRG® mice on the NOD background (FRGN). Primary hepatocytes from a non-hemophilic human male donor were transplanted into male FRGN mice as a control. Mice with human albumin levels of >4000 µg/mL, indicative of livers repopulated with >70% human hepatocytes, were compared. Humanized mice repopulated with control donor cells (n=4) had average circulating hFIX levels of 895 ±96.6 ng/mL, whereas mice repopulated with the HEMB donor (n=5) had 9-fold lower average circulating hFIX levels of 98.9±26.9 ng/mL. Measurement of partial thromboplastin time (PTT) in plasma samples from liver-humanized mice created with the control donor cells (n=9) revealed a mean PTT of 28.49 seconds. In HEMB liver-humanized mice (n=9), the average PTT was increased by 24% to 35.34 seconds. Thus, physiologically relevant defects in clotting could be modelled in liver-humanized mice reconstituted with a HEMB donor. To demonstrate that this model could be used to assess efficacy of gene therapies, HEMB mice were injected i.v with $2.5x10^{11}$ copies per mouse of liver-tropic AAV expressing hFIX (AAVDJ-CAG-hFIX). Two weeks after injection, increased expression of hFIX (343±66.6 ng/mL) and a significant decrease in PTT (30.25s, $p<0.005$) was observed compared to untreated HEMB liver-humanized mice. Even without significant optimization of AAV-mediated correction, there was no significant difference in clotting time between HEMB liver-humanized mice that received hFIX gene therapy and mice that were liver-humanized with hepatocytes from a control donor. Our findings demonstrate that primary human hepatocytes from donors with genetic disease such as hemophilia B can successfully repopulate FRG mice, recapitulate physiologic disease pathologies, and be used to evaluate relevant in vivo therapeutic approaches.

516 Gene therapy of hemophilia a: identification of the most efficient aav serotype vector for transduction of human liver sinusoidal endothelial cells

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Gene therapy of hemophilia A has been performed with several AAV serotype vectors, but none has proven to be optimal. For example, although a BioMarin-sponsored clinical trial with AAV5 vectors at a high dose of $6x10^{13}$ vg/kg led to ~100-200% levels of expression of human clotting factor VIII (hFVIII), over a three-year follow up period, the hFVIII levels declined to ~8% (Pasi *et al*., *N Eng J Med*, 382: 29-40, 2020). Nontheless, given the overall improvement in disease severity (such as multi-year substantial deduction in annual bleeding rates), this drug was approved by the FDA in 2023 as Roctavian, priced at $2.9M/dose. In a Takeda-sponsored clinical trial with AAV8 vectors at a dose of $3x10^{13}$ vg/kg, all patients lost expression of hFVIII within 1 year (Chapin *et al*., *Haemophilia*, doi: 10.1111/hae.15121, 2024). More recently, despite encouraging results from two Phase I/II clinical trials, one with AAV-LK03 vectors (George *et al.*, *N Engl J Med.,* 385: 1961-1973, 2021) and one with AAV6 vectors (Leavitt *et al*., *Blood*, 143: 796-806, 2024), two Phase III hemophilia A clinical trials sponsored respectively by Spark-Roche with AAV-LK03 vectors, and Sangamo-Pfizer with AAV6 vectors, were halted. Although some patients treated with Roctavian or other vectors have maintained FVIII levels in the normal range, it has generally been difficult to achieve lasting high levels of FVIII expression (i.e. near or within the normal range). While all these vectors target hepatocytes for transgene expression, FVIII is normally made in liver sinusoidal endothelial cells (LSECs), which are able to efficiently secrete FVIII by co-expression of von Willebrand factor. None of the aforementioned AAV capsids have been shown to efficiently target human LSECs. In our present studies, we evaluated the transduction efficiencies of AAV5, AAV6, and AAV3 vectors in human hepatic cells as well as in human LSECs under identical conditions. These results, shown in **Figure 1A**, document that AAV6 vectors are ~623x more efficient than AAV5 vectors, and AAV3 vectors are ~2,600x more efficient than AAV5 vectors in human hepatic cells. Interesingly, AAV3 vectors also transduce human LSECs most efficientlty, ~7x more efficiently than AAV6 vectors, and ~51x more efficiently than AAV5 vectors (**Figure 1B**). Taken together, these results suggest that AAV3 vectors may prove to be efficacious as well as cost-effective in gene therapy for hemophilia A by directing FVIII expression to the natural site of synthesis, thereby improving secretion and avoiding mechanisms that downregulate expression as a result of intracellular FVIII accumulation.

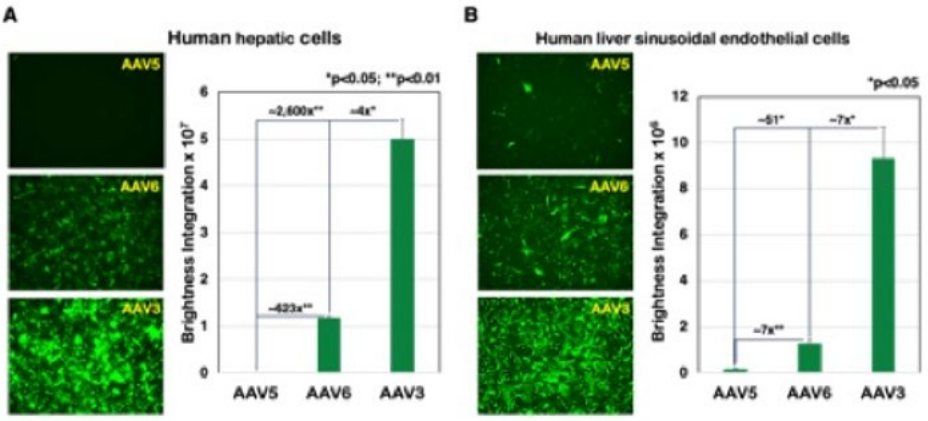

Figure 1: Transduction efficiencies of AAV5-, AAV6-, and AAV3-EGFP vectors in human hepatic **(A)** and human liver sinusoidal endothelial cells **(B)**. Cells were transduced in triplicates with each vector at $3x10^3$ vgs/cell. Transgene expression was determined 72 hrs post-transduction and quantitated using a Keyence microscope.

517 Gene therapy of hemophilia b: identification of the most efficient aav serotype vector for transduction of human hepatic cells

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In 2022, the United States Food and Drug Administration (USFDA) approved Hemgenix, an AAV5-based vector from uniQure for gene therapy of hemophilia B. A relative high dose of 2x1013 vgs/kg of this vector leads to up to ~12% expression of the human clotting factor IX (hFIX). In 2024, the USFDA also approved Beqvez, an AAVrh74-based vector from Pfizer for gene therapy of hemophilia B, which leads to up to 21% expression of hFIX at a dose of 5x1011 vgs/kg. Both Hemgenix and Beqvez are priced at $3.5M/dose. However, neither AAV5 nor AAVrh74 serotype vectors possess selective tropism for human liver. More than a decade and a half ago, we observed that of the 10 most commonly used AAV vectors, AAV3 is the most efficient in transducing primary human hepatocytes *in vitro* (*Molecular Genetics & Metabolism*, 98: 289-299, 2009). Subsequently, we reported AAV3 vectors to be 82x more efficient than AAV5 vectors in transducing primary human hepatocytes in humanized mice in vivo (*Molecular Therapy*, 24: 1042-1049, 2016) (**Figure 1A**). In our current studies, we compared the transduction efficiencies of AAV5, AAVrh74, and AAV3 vectors in human hepatic cells under identical conditions. As can be seen in **Figure 1B**, AAVrh74 vectors are ~6x more efficient than AAV5 vectors, whereas AAV3 vectors are ~1,100x more efficient than AAV5 vectors. AAV3 vectors are also ~6x more efficient than AAVrh74 vectors. We have previously reported the remarkable tropism of AAV3 vectors to be mediated by the human hepatocyte growth factor receptor (huHGFR), which AAV3 utilizes as a cellular co-receptor to gain entry into human hepatic cells (*Human Gene Therapy*, 21: 1741-1747, 2010). We have also reported that AAV3 vectors mediate therapeutic levels of hFIX expression in humanized mice (*Human Gene Therapy*, 31: 1114-1123, 2020) as well as in non-human primates (*Molecular Therapy Methods & Clinical Development*, 23: 98-107, 2021). All of these proof-of-concept studies make a compelling case for the use of AAV3 vectors with lower immunogenicity, improved safety, ensuring translation to the clinic with higher probability of success for gene therapy of hemophilia B. Furthermore, the reduced vector production costs as well as lower cost per patient should allow the eligible patient population worldwide to benefit from AAV3 vector-mediated gene therapy of hemophilia B in particular, and human liver diseases in general, in which other AAV serotype vectors have proven to be less than optimal.

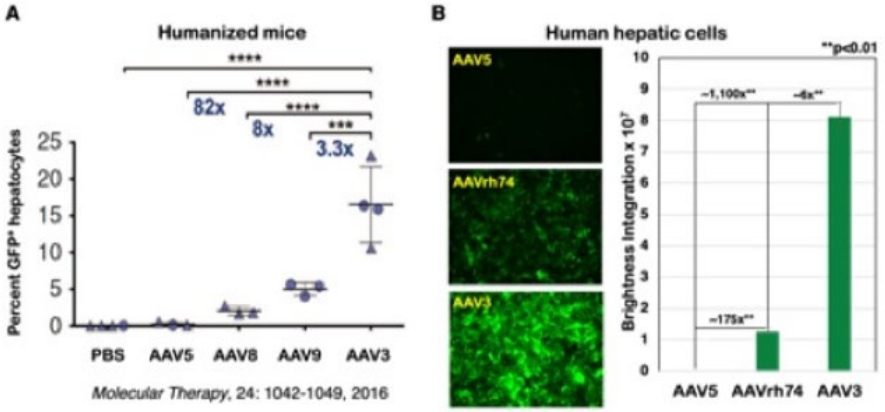

Figure 1: (A) Transduction efficiencies of AAV5, AAV8, AAV9, and AAV3 vectors in human hepatocytes in humanized mice *in vivo*. **(B)** Transduction efficiencies of AAV5, AAVrh74, and AAV3 vectors in human hepatic cell line, Huh7. Cells were transduced in triplicates with $3x10^3$ vgs/cell with each of the vectors expressing the EGFP reporter gene. Transgene expression was visualized and quantitated 72 hrs post-transduction using a Keyence microscope.

518 Novel intravenous AAV gene therapy for symptomatic mucopolysaccharidosis type IIIA in canine model of the pathology

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MPSIIIA is an autosomal recessive lysosomal storage disorder, caused by N-sulfoglucosamine sulfohydrolase (SGSH) enzymatic deficiency leading to accumulation of Heparan Sulfate Oligosaccharides (HSO) in tissues and notably in the central nervous system (CNS). Patients display progressive central nervous system degeneration that manifest as severe intellectual disability, developmental regression, and other neurologic manifestations including autism spectrum disorder (ASD), behavioural problems, and sleep disturbances. To date, there are no curative therapies for MPSIIIA. Several trials have been initiated with intracerebral or intravenous but with poor CNS targeting and thus no real clinical improvement.

Based on our strategy developed for MPS3B, we used a last generation AAV, a serotype able of large Blood brain barrier crossing after intravenous delivery notably in large animals. Here we described for the first time symptomatic delivery at 18M in the canine model of the pathology. In vivo monitoring demonstrates significant MRI improvement with regression of all brain abnormalities, prevention of cerebral loss, neurobehavior improvement as well as reduction of GAG accumulation. Efficacy and safety studies are ongoing and data of molecular and anatomopathological read outs will be disclaimed.

519 Dual site administration of AAV gene therapy for treatment of feline GM1 gangliosidosis

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GM1 gangliosidosis is a fatal neurodegenerative disease caused by a deficiency of lysosomal β-galactosidase (βgal). There are currently two ongoing clinical trials for the treatment of GM1 gangliosidosis using adeno-associated viral (AAV) gene therapy administered either intravenously (IV) or in the cerebrospinal fluid (CSF) via the cisterna magna (CM). Preclinical trials of either administration route show that single site treatment significantly increases lifespans in a feline model of GM1 gangliosidosis. We hypothesize that deficiency in efficacy caused by either single site injection will be addressed by the additive effect of combinatorial administration. GM1 cats received $6.0x10^{13}$ vector genomes/kg of AAV9 split equally between IV and CM administration at approximately 2 months of age. Four animals will be followed to humane endpoint and four animals were evaluated 16 weeks following treatment. Untreated GM1 animals survive approximately 8.0 ± 0.6 months while treated animals have a significant increase in average lifespan to 19.9 ± 7.8 months, with 2 animals in the long term cohort still alive at 28.7 and 26.5 months of age (Figure 1A). Clinical assessments include neurological exams, CSF biomarkers, and 7T magnetic resonance imaging (MRI) and spectroscopy (MRS). Postmortem analysis included βgal and AAV distribution. Neurological deficits, which in untreated GM1 animals lead to an inability to stand and humane endpoint by 8 months of age, were attenuated in all treated animals (Figure 1B). Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), biomarkers of central nervous system damage when measured in the CSF, were both reduced in treated animals 16 weeks after treatment and maintained to 1 year in comparison to untreated animals. 7T magnetic resonance imaging (MRI, Figure 1C) revealed a delay in progression of neurodegeneration and preservation of brain architecture. While animals are ongoing we are unable to fully determine if CM+IV administration of AAV is better than either single administration route. Further analysis indicates that the combined treatment outperforms intravenous delivery using the same dose and AAV backbone.

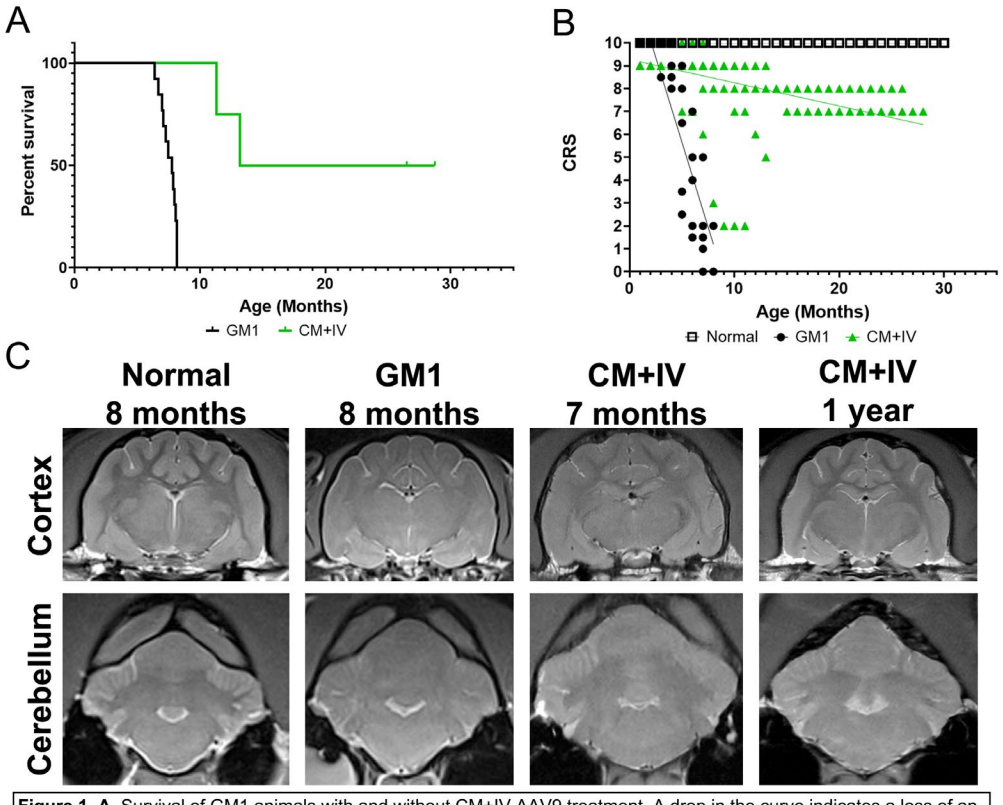

Figure 1. A. Survival of GM1 animals with and without CM+IV AAV9 treatment. A drop in the curve indicates a loss of an animal in the cohort. P=0.0018. **B.** Clinical rating score (CRS) of treated animals. A decrease in value designates progression of neurological disease. A line of best fit is graphed for each cohort. **C.** Magnetic Resonance Imaging (MRI) of cortex and cerebellum of treated animals.

520 Dosage Effects on Circulating GAA as a Pharmacodynamic Biomarker for systemically delivered rAAV9-coGAA Gene Therapy in Pompe Disease

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Pompe disease (PD) is a lysosomal storage disorder caused by a deficiency in acid α-glucosidase (GAA), resulting in glycogen accumulation in cardiac, skeletal muscle, and central nervous system cells. The gene therapy product rAAV9-coGAA (GC301), a recombinant adeno-associated virus (rAAV) vector carrying the codon-optimized gene for GAA (coGAA), has demonstrated promising preclinical efficacy, including prolonged lifespan and significant reductions in tissue glycogen accumulation in Gaa-/- mice after a single intravenous administration. To advance the clinical development of GC301, it is crucial to identify reliable pharmacodynamic biomarkers to confirm the drug's efficacy and monitor its biological effects over time. In this study, we evaluated circulating GAA activity as a potential pharmacodynamic biomarker to assess the therapeutic effects of GC301. Wild type Balb/c mice were treated with 1.2E+14 vg/kg of GC301 and monitored for 60 weeks. During this period, animals were regularly sacrificed for sampling (Fig 1.A) to measure vector genomes, GAA expression and GAA activity. Plasma and hemocytes (prepared as dried blood spots, DBS) were separated to determine whether their contributions to circulating GAA differed. Mice treated with GC301 exhibited widespread biodistribution and efficient GAA expression. Compared to the GAA enzyme activity in each tissue of the control group, the fold increase results were presented in Figure 1.B. The heart showed the highest fold elevation, followed by the liver, diaphragm and quadriceps. Regarding circulating GAA activity, the maximal values in hemocytes and plasma peaked at week 6, reaching 4-6 times, and remained approximately twice that of the control mice for up to 60 weeks. Interestingly, while the GC301 vector genome became undetectable in the blood by 12 weeks, it persisted in other tissues, accompanied by sustained GAA expression (Fig 1.C). These findings suggest that the elevated GAA activity in plasma following GC301 administration primarily reflects a "spillover" from treated tissues, thus making it a promising pharmacodynamic biomarker for monitoring the ongoing efficacy of GC301. In Gaa-/- mice treated with GC301, efficient glycogen clearance was observed, along with a dose-dependent increase in GAA activity across multiple tissues and in the blood at dosages of 3E+13 and 6E+13 vg/kg. In the low-dose cohort (3E+13 vg/kg), GAA activity increased by 1.5- to 58-fold in various tissues compared to untreated wild-type mice. However, blood GAA activity in the low-dose cohort showed minimal elevation, remaining comparable to untreated Gaa-/- mice throughout the 16-week study period. (Fig. 2)

Our findings highlight that circulating GAA activity could serve as a potential non-invasive biomarker for monitoring the long-term pharmacodynamic or therapeutic effects of GC301 in Pompe disease, particularly in high-dose treatments. However, for individuals receiving low-dose treatment, blood GAA enzyme activity is unlikely to be a reliable biomarker, while muscle GAA enzyme activity appears to provide a more accurate indicator of pharmacodynamic. Early clinical trial results further support these findings, demonstrating that in IOPD patients treated with GC301, the increase in muscle GAA enzyme activity significantly exceeded that observed in the blood.

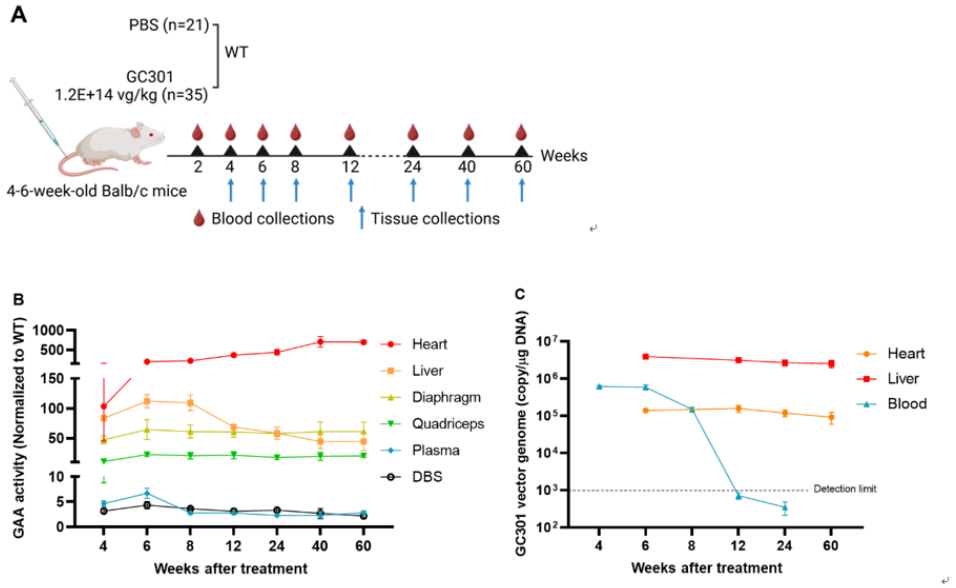

Figure 1. Schematic study design (A), GAA Activity (B), and Vector Genome Kinetics (C) in Tissues and Blood Following High-Dose GC301 Delivery

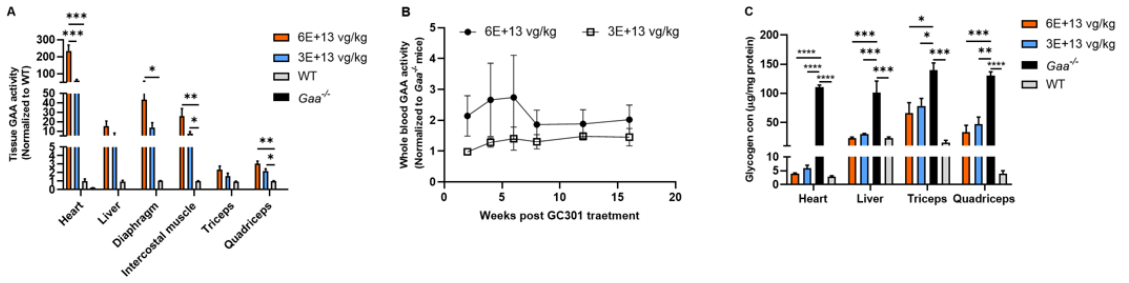

Figure 2. Dose-Dependent GAA Activity in Tissues (A) and Blood (B) with Effective Glycogen Clearance (C) in Pompe Disease Mouse Models Treated with Medium- and Low-Dose GC301

521 A Non-GLP Dose Range Finding Study in Cynomolgus Macaques Evaluating the Biodistribution, Expression, and Safety of LTS-101, a Novel Preclinical Gene Therapy Candidate for the Treatment of CLN2 Batten Disease

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Introduction:

Latus Bio, Inc. is developing LTS-101, a gene therapy for the treatment of neuronal ceroid lipofuscinosis Type 2 (CLN2), a form of Batten disease caused by deficiency in the tripeptidyl peptidase 1 (TPP1) protein, leading to lysosomal dysfunction and neurodegeneration. LTS-101 is an intracerebral ventricular (ICV)-delivered gene therapy utilizing a novel capsid, AAV-Ep+, packaging the *hTPP1* gene, which transduces the ependymal cells that line ventricles that can produce and secrete TPP1 protein into the CSF. AAV-Ep+ was identified by a capsid

screening campaign in multiple non-human primate (NHP) species as a potent transducer of ependymal cells and neurons. A non-GLP dose range finding (DRF) study in cynomolgus macaques was conducted to evaluate the biodistribution, and transgene expression, and safety of LTS-101 at three doses. This study demonstrated supraphysiological, potentially therapeutic, levels of TPP1 protein in the CSF at all three doses of LTS-101 and identified a maximum tolerated dose of LTS-101.

Results:
Juvenile macaques were administered a vehicle, or a low-, mid- or high-dose of LTS-101 via MRI-guided bilateral ICV administration for an in-life study duration of 6 weeks. Safety endpoints included in-life safety observations, neurological exams, clinical pathology, and histopathology. AAV biodistribution and expression were quantified by qPCR, RT-qPCR and ELISA. The low and mid doses of LTS-101 were well tolerated, with no significant in-life safety observations. However, animals treated with the high dose of LTS-101 developed motor deficits that spontaneously improved slightly before scheduled necropsy. Histopathology results supported that LTS-101 was well tolerated at all doses except for dose-dependent DRG inflammation and neurodegeneration. Based on these safety data, the mid dose of LTS-101 was identified as the maximum tolerated dose. Biodistribution of LTS-101 viral genomes in the brain was generally dose-dependent and was highest in ependyma. Very low levels of viral genomes were detected in peripheral organs, with the highest levels in the liver and spleen. Brain human *TPP1* mRNA expression was detected in all dosed groups. High concentrations of TPP1 protein were detected in the CSF at all doses, with no clear dose response. Finally, elevated levels of TPP1 enzyme activity were detected in brain parenchyma, indicating cross correction and mature processing of TPP1 protein within cells.

Conclusion:
In a dose-range finding study investigating three doses of LTS-101 in NHPs, bilateral ICV administration produced brain-wide TPP1 activity, high levels of TPP1 in the CSF, a favorable safety profile, and low systemic exposure at logs-fold lower doses that than the conventional capsids which have been used in clinical gene therapy programs. High levels of TPP1 protein in the CSF at the low dose suggest that even lower doses should be explored in future studies and underscore the potency of the novel AAV-Ep+ capsid. In conclusion, LTS-101 is a promising gene therapy candidate with the potential to treat CLN2 Batten disease patients with a one-time ICV administration providing stable, long-term expression of TPP1.

522 SPR301: Dose Dependent Reduction of Inflammation and Substrates in a Surrogate Model for GBA1-linked Neuroinflammation

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Parkinson's disease (PD) is one of the most common neurodegenerative diseases, affecting nearly two million people across US and Europe. An association between Gaucher disease (GD), *GBA1* mutations which attenuate or abrogate the activity of the lysosomal enzyme glucocerebrosidase (GCase) and PD has been established, with approximately 5-15% of PD patients carrying a pathogenic or non-pathogenic *GBA1* mutation. There is also evidence for reduced GCase activity in PD patients *without* a known *GBA1* mutation (idiopathic PD, iPD), with factors impacting GCase stability, activity, lipid clearance and α-synuclein aggregation. Although most *GBA1*^{mut} patients do not develop PD, *GBA1* mutation status is still the most predictive genetic factor for the development of PD and is associated with early onset PD and rapid progression.

SPR301 is an AAV9 based gene therapy in development for GBA1-linked PD, containing an expression cassette encoding a novel engineered GCase variant (GCase85) with improved stability compared to GCase. Initial proof of principle was established in an SH-SY5Y *in vitro* model of dopaminergic neurons. Untreated or α-synuclein aggregate seeded cells were pre-treated with SPR301 or AAV9-GBA1 WT. SPR301 demonstrated a significant and

superior GCase activity in treated cell supernatants and a more efficient intracellular α-synuclein reduction when compared with AAV9-GBA1 WT treated cells.

In vivo proof of principle was established in a surrogate murine model for GBA1-linked neuroinflammation. Wildtype C57BL/6J mice treated daily for 16 days with conduritol-β-epoxide (CBE), a brain penetrant irreversible inhibitor of GCase, established a model of *GBA1* deficiency. AAV vectors were administered by unilateral stereotactic injection to the right caudate putamen, 24h post first CBE dose, at doses 5.2 x 10^8, 2.6 x 10^9, 1.3 x 10^{10} vg for SPR301, while positive (AAV9-GBA1 WT) and negative (AAV9-'stuffer') vector controls were at 1.3 x 10^{10} vg only. GCase activity and substrates glucosylceramide and glucosylsphingosine were analysed in the cerebrospinal fluid, treated and untreated hemispheres. Semi-quantitative immunohistochemistry for GBA, inflammation markers (e.g. IBA-1) and co-location of GBA to dopaminergic neurones (tyrosine hydroxylase) was also performed.

SPR301 was effectively distributed within the treated hemisphere from the striatum, the site of injection, to target dopaminergic neurones in the substantia nigra and other key areas of the brain associated with PD. A dose response was observed for SPR301 for GCase activity and substrate reduction. SPR301 at low to mid dose, ≥ 25-fold lower dose than that required for AAV9-GBA1 WT, successfully reduced CBE induced inflammation to near naïve levels in the thalamus, hypothalamus and substantia nigra and did not stimulate inflammation at the site of injection.

523 A Humanized *RBM20* Mouse Model Exhibits Dilated Cardiomyopathy Phenotypes and Enables Development of *In Vivo* Prime Editing for Treating Human *RBM20* Cardiomyopathy Patients

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RNA-binding Motif Protein 20 (*RBM20*) encodes a splicing regulator and mutations in *RBM20* can cause dilated cardiomyopathy (DCM), displaying high penetrance and aggressive progression with high rates of heart failure, arrhythmias, and sudden cardiac death. The majority of *RBM20* disease-causing mutations cluster in a 5-amino-acid arginine/serine-rich region, which mediates nuclear localization of *RBM20* protein in cardiomyocytes. While no disease-specific therapy targeting the underlying genetic cause of *RBM20* cardiomyopathy is available, we have developed an in vivo cardiomyocyte prime editing (PE) strategy which may be suitable for correcting the mutations in many *RBM20* patients, as PE can mediate all 12 types of base-to-base conversions and various mutations in the *RBM20* hotspot may be addressed by the same PE therapeutic.

To model the disease and enable *in vivo* testing of human *RBM20* targeting PE therapeutics, we engineered a humanized *RBM20* mouse model with one allele carrying the human wildtype (WT) DNA sequence and one allele carrying the human R634Q (equivalent to R636Q with mouse numbering) mutant DNA sequence in the hotspot and flanking regions ($hRBM20^{R634Q}/hRBM20^{+}$). The total humanized region was designed to be large enough to accommodate binding of guide RNAs (prime editing guide RNA and nicking guide RNA) in potential human *RBM20* PE therapeutics, while being restricted to avoid deviating from the native mouse protein coding outcome. While echocardiogram monitoring detects no abnormal heart function in the control ($hRBM20^{+}/hRBM20^{+}$) mice, $hRBM20^{R634Q}/hRBM20^{+}$ mice display a DCM phenotype, mimicking the heart function defects in the corresponding mouse endogenous *Rbm20* R636Q heterozygous model and in human patients. Additionally, $hRBM20^{R634Q}/hRBM20^{+}$ mice show abnormal splicing of *RBM20* downstream target genes, consistent with findings in the corresponding mouse endogenous model and human patients. Finally, we developed PE therapeutics targeting the human *RBM20* hotspot region and screened them in the humanized *RBM20* mouse model for efficiency of correcting the pathogenic R634Q mutation *in vivo*. Together, we present a novel mouse DCM model with humanized *RBM20* sequences, exhibiting expected heart function and gene-splicing phenotypes of *RBM20* cardiomyopathy and enabling *in vivo* testing of human *RBM20* PE therapeutics. (WL and EE contributed equally to this work.)

524 Development of a Novel AAV Gene Therapy for the Treatment of Dilated Cardiomyopathy

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Dilated cardiomyopathy (DCM) is a complex cardiovascular disorder characterized by dilation and impaired contractility, leading to systolic dysfunction, reduced cardiac output, and congestive heart failure. DCM is the leading cause of heart failure and the most prevalent indication for heart transplantation. Therefore, novel therapeutic strategies are urgently needed to prevent and treat the cardiac dysfunction associated with DCM. Previously, we showed that small molecule-mediated induction of the activating transcription factor 4 (*ATF4*) rescued the contractile dysfunction in induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) carrying DCM-associated mutations[1]. In this study, we aimed to investigate the therapeutic potential of *ATF4* gene augmentation using novel AAV vectors. To optimize expression, enhance safety and specificity, human *ATF4* (h*ATF4*) was codon-optimized and several human cardiac-specific promoters were tested. The novel vectors were initially assessed for manufacturability and subsequently they were tested *in vitro* in human iPSC-CMs. The candidate that exhibited the highest mRNA and protein expression levels was selected for further investigation in a proof-of-concept gene therapy *in vivo* study using a preclinical model of DCM, the TM54 transgenic mouse. TM54 mice and wild type littermates were dosed at 6 weeks of age, when DCM is evident, with 3E+13 vg/Kg of the candidate AAV via tail vein injection. After dosing, cardiac function was assessed by echocardiography at different time points (2, 6, and 8 weeks) to evaluate heart function. At study termination (8 weeks post-dosing), molecular analysis was performed in relevant tissues (heart, liver, skeletal muscles) demonstrating selective expression of the codon-optimized h*ATF4* transcript in the heart. Importantly, biomarker analyses revealed no significant toxic effects in the liver or the kidneys. This study underscores ATF4 as a promising novel therapeutic target for DCM and supports the potential of *ATF4* gene augmentation as a viable therapeutic strategy for DCM. Further studies in larger species are warranted to evaluate the safety, efficacy, and potential for clinical translation.

[1]Perea-Gil et al. (2022). European Heart Journal, Volume 43, Issue 36, pages 3477-3489.

525 Preclinical Efficacy and Safety of ALXN2350, a rAAV Gene Therapy for BAG3-Associated Dilated Cardiomyopathy

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Bcl2-associated athanogene 3 (BAG3) is a ubiquitously expressed molecular chaperone that regulates protein homeostasis. In cardiomyocytes, BAG3 modulates apoptosis, autophagy, sarcomere integrity, and contractility. Mutations in *BAG3* lead to haploinsufficiency and are associated with dilated cardiomyopathy (DCM). A gene therapy to augment BAG3 protein expression in cardiomyocytes can be a disease-modifying therapy for patients with *BAG3*-mutation-associated DCM. To model BAG3 deficiency and evaluate the potency of rAAV, BAG3 knocked-down human iPSC-derived cardiomyocytes were transduced with rAAV encoding transgenic BAG3 under a cardiac-specific promoter. Knockdown of endogenous BAG3 resulted in the depletion of BAG3 and its partner protein HSPB8, which was stabilized by AAV-mediated BAG3 expression. The impact of BAG3 on cardiac function *in vivo* was assessed using a cardiac-specific BAG3 knockout (*Bag3* cKO) mouse model. Homozygous *Bag3* cKO mice develop progressive LV systolic dysfunction and dilatation. rAAV expressing human BAG3 (ALXN2350) was administered intravenously after the onset of DCM. ALXN2350 demonstrated dose-dependent improvements in LV systolic function and dilation, halting disease progression at 3E13 vg/kg and above. By 12-weeks post-dosing, ALXN2350 progressively improved LV ejection fraction and significantly reduced *Nppa* and *Nppb* expression in the heart. Biodistribution and safety of ALXN2350 were assessed in cynomolgus macaques at 4E13 and 1.3E14 vg/kg for a 6-week period following intravenous administration and with daily methylprednisolone. ALXN2350 treatment was well tolerated and there were no test-article-related histopathology findings. ALXN2350 treatment resulted in dose-dependent increases in human BAG3 mRNA and protein expression in the NHP heart. Collectively, ALXN2350 demonstrated dose-dependent efficacy in the *Bag3* cKO mouse model of DCM and preclinical safety in non-human primates, supporting its potential as a treatment for patients with BAG3-associated DCM.

526 Ad-VEGF-B186R127S gene therapy induces angiogenesis in mouse heart by recruiting endothelial progenitor cells

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Myocardial ischemia is the most common clinical consequence of cardiovascular disease, due to reduced blood supply to the affected region(s) of the heart. Subendocardial blood flow is frequently insufficient despite reperfusion therapies (traditional bypass surgery or catheter-mediated procedures). Hence, there is a strong need for new therapeutic approaches to encourage capillary and collateral artery formation. Vascular endothelial growth factor B186 (VEGF-B186) is known to promote coronary vascular development in both healthy and damaged myocardium. However, prior research also suggests that VEGF-B186 has a serious adverse effect that prevents its use in cardiac gene therapy, which may be caused by binding to neuropilin 1 (NRP1). We have designed adenoviral vector (Ad) based VEGF-B186 variant, VEGF-B186R127S, which is resistant to proteolytic processing and unable to bind to NRP1. Here, we investigated the mechanism of VEGF-B186-induced vascular development as well as its impact on cardiac function by studying its effects on the mouse heart. Without having any negative consequences, such as arrhythmias, the proteolytically resistant VEGF-B186R127S greatly stimulated vascular development in the mouse heart by recruiting endothelial progenitor cells.

527 Novel Combination Gene Therapy to Prevent Rejection after Solid Organ Transplantation

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INTRODUCTION:
Administration of targeted gene therapy to allografts prior to transplantation has the potential to decrease morbidity associated with rejection and chronic systemic immunosuppression. We have previously demonstrated the ability of a modified programmed death ligand 1 (PDL1) single-gene therapy to prolong graft survival in a murine heart transplant rejection model when provided with a brief course of concomitant low-dose systemic co-stimulation

blockage (CTLA-4 Ig). A combination of multiple immunosuppressive transgenes into one construct is currently an untested alternate approach. We evaluated six novel combinatory transgene constructs in two distinct *in vivo* models.

METHODS:
Six novel constructs utilizing PDL1 in combination with other immunosuppressive genes were engineered (ASC-1 through ASC-6). We first engineered an immunogenic rejection model of ovalbumin (OVA)-expressing E0771 triple-negative breast cancer cells which are normally rejected in an adaptive immunity-dependent manner in immune-competent mice within three weeks of implantation. Cells were transduced with each of the six novel constructs. After confirmation of gene expression, 500,000 cells were then injected into the flanks and mammary fat pads of immune-competent mice. Tumor engraftment was monitored through biweekly caliper measurements. The best performing constructs from this model were then packaged into a proprietary Solid Biosciences AAV capsid (AAV-SLB101) and tested in an MHC-mismatched murine heterotopic heart transplant model. BALB/c mice were each injected with one of the viral vectors two weeks prior to transplant. Donor hearts were transplanted into the abdominal position of C57BL/6 recipients with and without concomitant CTLA-4 Ig. Grafts were assessed for function by abdominal palpation and echocardiography. Fulminant graft rejection, defined as complete cessation of allograft function on exam and by echocardiography, was the endpoint of the study.

RESULTS:
When implanted into the flanks of immune-competent mice, immunogenic cell lines with gene constructs 2 and 5 consistently outperformed all other constructs in tumor growth and engraftment. Anti-OVA response was intact when measured by quantification of OVA-specific IgG antibodies and in vitro T cell responses against the immunodominant OTI/II peptides. ASC-2, ASC-5, and an unmodified PDL1-only construct were tested in the murine heart transplant model. In the absence of concomitant immunosuppression, transplanted control and therapeutic grafts were fully rejected within a median of 7-9 days. With the addition of concomitant CTLA-4 Ig, all treated allografts demonstrated higher graft survival at 100 days compared to grafts with CTLA-4 Ig alone (ASC-2 and PDL-FL 66.7% vs ASC-5 50% vs CTLA-4 Ig alone 22%), though overall survival distributions between groups were not statistically different.

CONCLUSIONS:
Overexpression of PDL1 in combination with other key immunomodulatory pathways has the potential to prevent or delay acute cellular rejection locally without abrogation of systemic immune function. Further work is required to determine the optimal combination, transgene order, and dose to achieve therapeutic biodistribution.

528 Preclinical Efficacy and Safety Evaluation of FT-017: a Novel AAV-Based Gene Therapy for *MYBPC3*-associated Hypertrophic Cardiomyopathy

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Background:
Hypertrophic cardiomyopathy (HCM) is a common inherited heart disease, primarily caused by mutations in the cardiac myosin-binding protein C (*MYBPC3*) sarcomere-associated gene, which encodes the cMyBP-C protein. Most pathogenic mutations in *MYBPC3* arise via frameshift, nonsense, or conserved RNA splice site mutations on a single allele, resulting in lower levels of total cMyBP-C (haploinsufficiency). Reduction of cMyBP-C protein in cardiac myocytes leads to sarcomeric dysfunction and deregulation of contraction and relaxation. Restoration of cMyBP-C haploinsufficiency offers a viable therapeutic approach for the treatment of HCM. We have designed an AAV gene therapy, FT-017, for *MYBPC3*-associated HCM. Here, we further investigated the preclinical efficacy and safety of FT-017 for its therapeutic use.

Results:
FT-017 is a novel and unique AAV9 vector expressing a codon-optimized human *MYBPC3* gene under the control of a cardiac-specific promoter. *In vitro*, FT-017 efficiently transduced C2C12 cells to express human cMyBP-C in a

dose-dependent manner. *In vivo*, the efficacy and biodistribution of FT-017 were evaluated in neonatal, juvenile, and adult *Mybpc3*-KO mice, respectively. Following a single intravenous injection of FT-017 at doses of 1×10^{13} to 3×10^{14} vg/kg, the vector effectively and specifically transduced cardiomyocytes in the hearts to express cMyBP-C protein, restoring its level to that of wild type. FT-017 significantly reduced myocardial fibrosis and the severity of myocardial pathology, improved left ventricular (LV) systolic function, and decreased the serum cardiac biomarker levels in *Mybpc3*-KO mice. The data suggest that FT-017 has the potential to delay or prevent the progression of *MYBPC3*-related HCM. In a GLP-compliant toxicology study in non-human primates (NHPs), FT-017 was well tolerated up to 1.2×10^{14} vg/kg. Apart from a transient elevation of ALT/AST levels in some individuals within two weeks post-injection, no test article-related systemic, immunological or histopathological abnormalities were observed during the 13-week observation period.

Conclusion:
Overall, these preclinical studies demonstrated the robust efficacy of FT-017 in non-symptomatic and symptomatic *Mybpc3*-KO HCM model mice, with a favorable safety profile showing no hepatotoxicity, thrombotic microangiopathy (TMA), cardiotoxicity, or neurotoxicity observed in NHPs. This provides strong support for the clinical translation of this approach in patients with *MYBPC3*-associated HCM.

529 *An Effective Viral Vector for Treatment of Myocardial Infarction.*

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Ischemic diseases, including myocardial infarction and ischemic stroke, are due to blocked arteries or compromised vasculature. In the last 15 years and despite advances in pharmacologic care, they have remained as the leading cause of global mortality necessitating further development of adjunct therapies. To this end gene therapy treatments utilizing viral vector delivery of angiogenic factors, intended to stimulate new blood vessels from existing ones and thereby rescue the remaining viable tissues, were developed. Some of these demonstrated efficacies in increasing the vasculature but were associated with problems such as oedema. These gene therapy treatments have yet to yield success in Phase III clinical trials. Our recent work with novel cell-specific viral vectors and transgene supports a unique therapeutic strategy. We first developed a lentiviral vector (LV) to activate transgene expression specifically in the vessel-forming endothelial cells (ECs), and which was regulated by hypoxia, a low oxygen condition occurring in ischemia. This development was based on a screen of enhancers derived from endothelial specific super-enhancers, from which one enhancer, named E2 was selected due to its superior endothelial activation and specificity. We then combined this vector with a modified vascular endothelial growth factor receptor 2 (VEGFR2mod) which is the main signaling molecule for the potent VEGFA. Once activated VEGFR2 can stimulate endothelial cell proliferation, migration and permeability and is absolutely required for vascular formation. The modification of VEGFR2, abrogates a particular phosphorylation site in VEGFR2 that was previously shown to control adherence junctions and subsequently endothelial permeability but maintains all other functions, therefore we expected that this transgene would facilitate new blood vessel formation with reduced oedema. We tested this novel vector in treatment of ischemic disease by direct delivery into mouse myocardium following induced infarction. We observed survival of all the mice in LV-E2-VEGFR2mod group in contrast to control groups, where mortality was observed. Specifically, as opposed to 100% survival of the mice in LV-E2-VEGFR2mod group (n=5/5), in wild-type VEGF-R2 (LV-E2-VEGFR2) treated mice only 39% (n=7/18) of the mice survived. Likewise, in mice treated with control vector incorporating stuffer DNA in place of E2 (LV-SD-VEGFR2) only 64% (n=7/11) of the mice survived. Consistent with the poor survival of wild-type VEGFR2-treated mice we also observed significantly reduced left ventricular ejection fraction (EF) in this group. Our ongoing studies of the mechanisms of this vector are focused on assessment of capillaries, oedema, inflammation and fibrosis to determine its precise activity. Overall, our studies to date indicate that this vector may provide a breakthrough in angiogenic and regenerative gene therapy for myocardial infarction.

530 Investigating the Mechanisms of Acute Preservation of Cardiac Function Post-Myocardial Infarction Following Angiotensin-(1-9) Gene Therapy

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Introduction
Adeno-associated viral vector serotype 9 (AAV9)-mediated gene therapy expressing Angiotensin-(1-9) (Ang-(1-9)), a peptide of the counter-regulatory renin angiotensin system axis, preserved left ventricular systolic function in mice 8 weeks post-myocardial infarction (MI)[1]. We now demonstrate preservation of systolic function as early as 24h post-MI with AAV9-Ang-(1-9) gene therapy.

Aim
Investigate molecular mechanisms underlying preservation of cardiac function 24h post-MI following Ang-(1-9) gene transfer *in vivo* and *in vitro*.

Methods
AAV9 and adenoviral vectors encoding Ang-(1-9) with mCherry or mCherry alone (control) were constructed. Wild-type (*At2r*$^{+/+}$) and angiotensin type 2 receptor (AT_2R) knockout (*At2r*$^{-/-}$) male mice underwent coronary artery ligation and were intravenously administered AAV9-Ang-(1-9) or AAV9-Control at MI onset. Cardiac fractional shortening was measured at 24h and 14 days post-MI via echocardiography. Bulk RNA-Sequencing of infarct border zone tissue was performed 24h post-MI in wild-type C57BL/6J mice to compare sham + AAV9-Control, MI + AAV9-Control, and MI + AAV9-Ang-(1-9) groups. Ingenuity Pathway Analysis was conducted on differentially expressed genes. Isolated neonatal rat cardiomyocytes (NRCMs) and fibroblasts (NRCFs) were transduced with adenoviral vectors encoding Ang-(1-9) and mCherry (AdAng-(1-9)) or mCherry alone (AdControl) 24h before pathological stimuli treatment. NRCFs were stimulated with 100nM Angiotensin II (Ang II) and 20ng/mL Transforming Growth Factor Beta-1 (TGF-β1), with proliferation measured by MTT assay at 48h. NRCM hypertrophy was induced with 500nM Ang II for 48h and measured via immunofluorescence microscopy. Ang-(1-9) peptide served as a positive control.

Results
Fractional shortening decreased post-MI in wild-type mice without (Sham vs MI: 24h, $P<0.01$; 14 days, $P<0.01$) or with AAV9-Control (Sham vs MI + AAV9-Control: 24h, $P<0.05$; 14 days, $P<0.05$). AAV9-Ang-(1-9) preserved fractional shortening 14 days post-MI (Sham vs MI + AAV9-Ang-(1-9): 42.2%±1.5 vs 39.8%±2.3, $P>0.05$) and as early as 24h post-MI (Sham vs MI + AAV9-Ang-(1-9): 42.1%±2.80 vs 38.4%±3.79, $P>0.05$). Fractional shortening also decreased in *At2r*$^{-/-}$ mice post-MI without (Sham vs MI: 24h, $P<0.05$; 14 days, $P<0.05$) or with AAV9-Control (Sham vs MI + AAV9-Control: 24h, $P<0.05$; 14 days, $P<0.05$). Unlike wild-type mice, *At2r*$^{-/-}$ mice transduced with AAV9-Ang-(1-9) had reduced fractional shortening 14 days post-MI (Sham vs MI + AAV9-Ang-(1-9): 44.0%±1.9 vs 32.0%±3.1, $P<0.05$) and at 24h post-MI, albeit non-significant (Sham vs MI + AAV9-Ang-(1-9): 46.1%±1.6 vs 34.4%±2.4, $P=0.088$). RNA-Sequencing of border zone tissue at 24h post-MI detected transgene expression in AAV9-administered mice. Differentially expressed genes and pathways were identified for further analysis. *In vitro*, adenoviral-mediated Ang-(1-9) gene transfer abolished Ang II-induced NRCF proliferation ($P<0.01$) and reduced TGF-β1-induced proliferation by 20.1% ($P<0.05$). Ang II-induced NRCM hypertrophy (60.1% cell area increase, $P<0.05$) was prevented by AdAng-(1-9).

Conclusions
AAV9-mediated Ang-(1-9) gene therapy preserves cardiac function 24h post-MI in mice, with RNA-Sequencing revealing accompanied border zone transcriptome changes. *In vitro* Ang-(1-9) gene transfer mitigated pathological fibroblast proliferation and cardiomyocyte hypertrophy, underscoring its therapeutic potential for cardiac disease.

(1) Fattah, C et al. JACC 68, 2666 (2016)

531 Gene Therapy for Cardiac Conduction Disorders and Muscular Dystrophy in POPDC2 Null Mice by Systemic MyoAAV4A.POPDC2 Delivery

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POPDC2 is a member of the Popeye domain-containing gene family, predominantly expressed in skeletal muscle and the heart, particularly in the sinoatrial and atrioventricular nodes. It plays a crucial role in cardiac pacemaking and conduction. A recent study identified a heterozygous nonsense mutation in the POPDC2 gene (POPDC2W188∗) in patients with familial atrioventricular block (AVB), causing a loss-of-function and impaired modulation of the TREK-1 channel. This mutation highlights POPDC2 as a novel susceptibility gene for cardiac conduction disorders.

Here we developed a new POPDC2 null mouse model that exhibits sinus bradycardia, sinus pauses, and tachybradycardia. These mice also show decreased body weight as early as three months of age, vocal sounds, and reduced muscle weight. Histological analysis reveals muscular dystrophy, elevated creatine kinase levels indicating muscle injury, and decreased contractile strength. These findings underscore the multifaceted impact of POPDC2 mutations on both cardiac and skeletal muscle function.

Currently, no therapeutic treatments exist for POPDC2 mutant patients. Our work explores the potential of gene therapy to address this gap. We are evaluating the efficacy and safety of MyoAAV4A-mediated systemic delivery of human POPDC2, driven by a muscle-specific promoter MHCK7, in POPDC2 null mice. Previous studies have shown that this delivery system can efficiently transduce cardiac and skeletal muscle tissues when intravenously injected. Our ongoing experiments aim to develop the first systemic MyoAAV4A.POPDC2 delivery that ameliorates both cardiac conduction disorders and muscular dystrophy in mouse models of POPDC2 mutants. This work holds significant promise for advancing therapeutic strategies for patients with POPDC2-related disorders.

532 FT-018, a Novel AAV Gene Therapy for ARVC, Improves Heart Function and Rescues Survival of Cardiac Pkp2-Deficient Mice

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Introduction: Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a hereditary cardiac disorder characterized by early electrical disturbances in the right ventricle, followed by fibrofatty replacement of myocardial tissue. These pathological changes often lead to ventricular arrhythmias and sudden cardiac death. ARVC affects approximately 1 in 1,000 to 5,000 individuals globally. Most cases of ARVC are linked to mutations in desmosomal genes, with mutations in plakophilin 2 (PKP2) accounting for nearly 45% of cases (>750K worldwide). Current treatment options for ARVC are mainly palliative, focusing on symptom management and arrhythmia prevention but failing to address the underlying genetic cause. Given the haploinsufficiency associated with mutations in the PKP2 gene, which has a small coding sequence, AAV-mediated gene therapy emerges as a promising therapeutic approach.

Methods: Utilizing the tailored recombinant AAV capsid and transgene for optimal response (TRACTOR) vector engineering platform, we designed various human cardiac PKP2 expression cassettes aiming for optimal and durable expression specifically in cardiomyocytes. The transduction and effectiveness of these AAV-PKP2 vectors were evaluated in a cardiac Pkp2 conditional knockout (cKO) mouse model, which recapitulates major clinical features of ARVC in human patients. Survival, cardiac function, myocardial fibrosis, and transgene and biomarker expression

were assessed to identify the best design as the lead development candidate. The safety of the lead candidate was evaluated in WT mice.

Results: All vehicle-treated Pkp2-cKO model mice died between 5 and 9 weeks after induction of Pkp2 knockout. All AAV-PKP2 vectors could significantly improve survival and cardiac function while reducing myocardial fibrosis in the Pkp2-cKO mice. Five of the 9 AAV-PKP2 vectors achieved 100% survival rescue at 13 weeks post-knockout induction (study termination). Among which, PA271, a vector containing a unique payload, showed superior performance in terms of left ventricular ejection fraction (LVEF) and cardiac output (CO) and significantly reduced expression of biomarkers of fibrosis (Col1a1), tissue remodeling (Mmp2), and heart failure (Nppa), while maintained human PKP2 high expression specifically in the heart. Immunohistochemistry confirmed efficient cardiomyocyte transduction and proper human PKP2 localization to the junction of the cardiomyocytes. Based on its novel design and best performance, AAV-PA271 was selected as the lead development candidate (FT-018). In vitro pharmacology testing of FT-018 revealed a dose-dependent expression of PKP2 in C2C12 myocytes. Preliminary toxicity studies in wild-type mice treated with 3E13 vg/kg and 1E14 vg/kg of FT-018 showed dose-dependent on-target PKP2 expression in the heart and no detectable expression in the liver and skeletal muscle. Serum clinical chemistry panel and tissue pathology results showed no overt liver toxicity.

Conclusions: FT-018 containing a unique and sophisticated payload demonstrated robust cardiac PKP2 expression, restored cardiac function, reduced myocardial fibrosis, and significantly improved survival of Pkp2-cKO mice. The innovative vector design of FT-018 offers the potential for optimal efficacy and great therapeutic durability and safety. Further preclinical studies are warranted to advance FT-018 towards clinical development and address the unmet medical needs of patients with PKP2-associated ARVC.

533 Gene Therapy Targeting Atrial Fibrillation Using Mouse Models: A Novel Approach to Cardiac Rhythm Regulation

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Background: Atrial fibrillation (AF) is the most prevalent cardiac arrhythmia, characterized by rapid and irregular atrial contractions, significantly increasing the risk of stroke and heart failure. Current therapeutic options, including pharmacological agents and catheter ablation, exhibit limited efficacy and significant adverse effects. Gene therapy represents a unique approach, offering the potential to correct molecular aberrations underlying AF. A common hallmark in AF progression is the hyperactivation of CaMKII, a serine/threonine kinase that phosphorylates multiple ion channels and intracellular calcium regulators, leading to calcium overload, increased afterdepolarizations, and atrial arrhythmogenesis. Elevated CaMKII activity has been directly linked to electrical and structural remodeling in AF, making it a promising target for therapeutic intervention. Here, we investigate the efficacy of adeno-associated virus (AAV)-mediated gene therapy targeting CaMKII activity in two mouse models of AF.

Methods:

1. **Gene Therapy Vector:** A multimerization of a CaMKII peptide inhibitor was developed through serial optimization to attenuate calcium dysregulation associated with atrial cardiomyocyte hyper-excitability.
2. **Model Development:** Two models of spontaneous AF were utilized: mice with atrial specific deletion of liver kinase B1 (LKB1) or T-box protein 5 (TBX5). Cre recombinase expression in LKB1flox/flox or TBX5flox/flox mice, driven by an atrial-specific promoter and delivered via AAV9 at postnatal day 3 (P3), resulted in nearly 100% penetrant spontaneous AF with atrial enlargement and fibrosis, mimicking human disease progression.
3. **In Vivo Administration:** To mimic a clinically relevant scenario, the effectiveness of gene therapy was tested after AF onset. An inducible AAV system was employed to overcome adaptive immune responses to multiple AAV doses. Mice were injected with AAV-Cre alongside a Tet-inducible AAV-TRE-AIPx5-mNeonGreen vector at P3. The Tet-on system restricted CaMKII inhibitor expression to atrial tissue upon doxycycline administration, ensuring targeted therapy.

4. **Electrophysiological Analysis:** AF was assessed using in vivo electrocardiography. Variability in the n-to-n interval was measured, with higher variability indicating dysregulated atrial activity consistent with AF.
5. **Molecular Characterization:** Ion channel phosphorylation changes were evaluated via western blot. Viral transduction and therapy expression were confirmed using immunohistochemistry (IHC).

Results:

- AAV-mediated CaMKII inhibition resulted in a significant reduction in atrial CaMKII activation, confirmed by western blot. Expression of the CaMKII inhibitor was localized to the atria, preventing systemic off-target effects.
- AF inducibility was markedly decreased in both treated models of AF. LKB1-knock out mice saw a 15.4% reduction in beat-to-beat variability while TBX5 knock out mice saw a 24.13% reduction just one month after induction of therapy.

Conclusion: This study demonstrates that AAV-mediated gene therapy targeting atrial CaMKII remodeling effectively reduces AF burden in mouse models. By addressing CaMKII-driven calcium dysregulation and electrical remodeling, this approach presents a promising, minimally invasive therapeutic strategy for AF. These findings emphasize the potential of gene therapy as a precise, impactful treatment, warranting further preclinical development and translation to human studies.

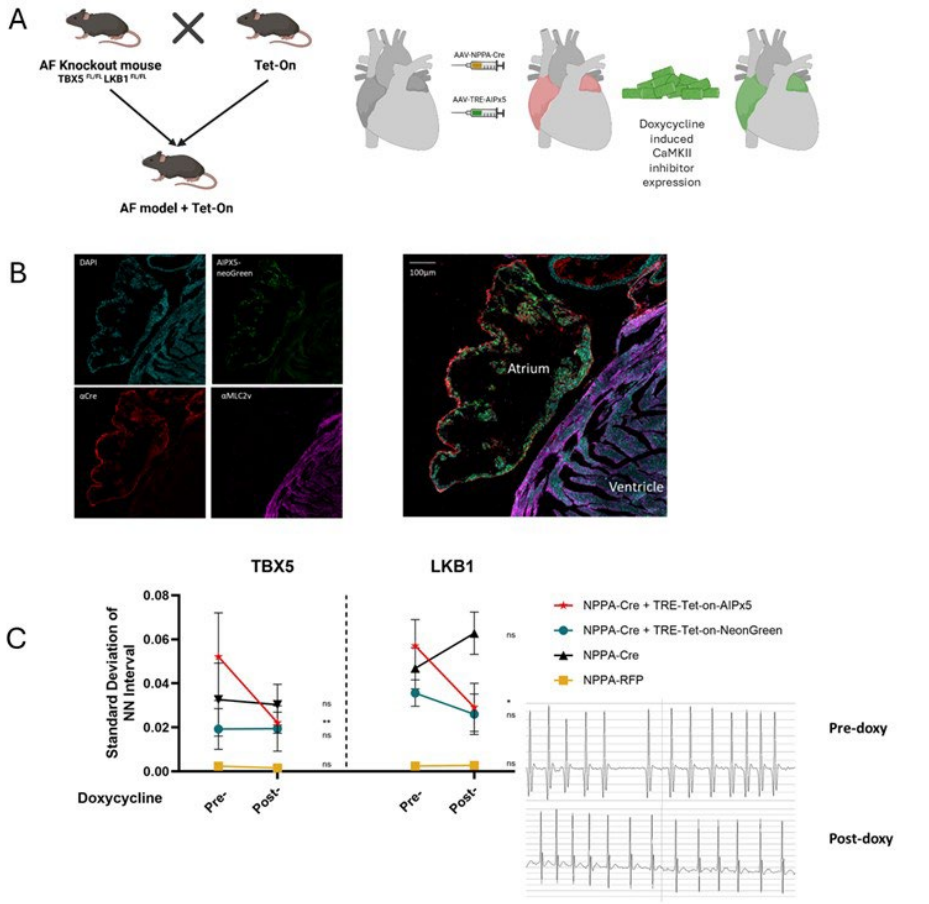

Figure 1. (A) Study design. TBX5 fl/fl and LKB1 fl/fl mice were crossed with a tet-on inducible mouse. The progeny were injected with NPPA-Cre and TRE-CaMKII inhibitor at P3, limiting expression to the atria only. After mice had developed AF (confirmed via EKG), mice were administered a diet with doxycycline, thus activating the therapy. (B) Histology confirming the presence of Cre and CaMKII-inhibitor only in the atria. (C) Results show a significant decrease in the beat-to beat variability in EKG recordings. TBX5 showed a reduction of 24.13% while LKB11 saw a reduction of 15.4%.

534 Engineered Epicardial Hydrogels for Cardiac-Specific Gene Therapy

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Introduction: Gene therapy is a promising approach to treat cardiovascular disease. However, clinical trials using adeno-associated virus (AAV) are challenged by immune-mediated responses to AAV and poor transduction of cardiomyocytes. The field has relied on increasing vector doses, but this has led to the discovery of dose-limiting toxicities. Local delivery provides a plausible solution. Here we investigate an epicardial hydrogel delivery of AAV as a potential approach to overcome limitations to cardiac gene transfer. We hypothesize that hydrogels delivered to the epicardium will increase cardiac gene uptake, minimize off-target effects, and circumvent immune-mediated neutralization, while supporting reduced doses needed for efficacy.

Methods: AAV9-CMV-mCherry-luciferase at a dose of 1E12 vector genomes (vg) was delivered to male Sprague-Dawley rats by epicardial hydrogels, intravenous (IV) injection, intramyocardial (IM) injection, and intrapericardial (no gel) delivery (N=5-9/group). Another cohort received AAV at a dose of 1E11 vg via epicardial hydrogels (N=5). All animals were randomized to receive a single AAV dose. Hydrogels were synthesized with 4-arm poly(ethylene glycol) maleimide-terminated (PEG-4MAL) macromer reacted with a crosslinker. Bioluminescence imaging was performed after 28 days. Cardiac and hepatic AAV expression was quantified with regions of interest drawn around the heart and liver from *in vivo* images. Total anti-AAV9 antibody titers were measured by ELISA. Histology evaluated mCherry expression in cardiac tissue.

Results: Epicardial hydrogel delivery displayed local cardiac expression with minimal off-target transduction, whereas the other routes led to systemic vector distribution. In the high dose groups, epicardial hydrogels resulted in 8.9-fold higher cardiac expression compared to IV rats ($p<0.0001$) without significantly differing to IM injections. Epicardial hydrogels showed 2.6- and 3.4-fold lower hepatic expression with respect to IV and IM groups, respectively. Delivery to the pericardial space (with or without gel) efficiently targeted the heart; however, hydrogels generated 12-fold lower hepatic expression compared to no gel delivery ($p<0.05$). Epicardial hydrogels blunted immune responses to AAV with 2.4- and 2.6-fold lower anti-AAV9 antibody titers compared to IV and IM injections ($p<0.05$), respectively, but was not significantly different from intrapericardial delivery. Epicardial hydrogels supported a 10-fold dose reduction with cardiac expression comparable to high dose systemic delivery. Low dose epicardial hydrogels resulted in 49- and 63-fold lower hepatic expression ($p<0.0001$) as well as 3.4-fold reduced anti-AAV9 antibody titers compared to high dose IV and IM groups ($p<0.01$), respectively. High dose epicardial hydrogels localized mCherry expression to the epicardium and myocardium with an average 2.7-fold higher fluorescent intensity across the thickness of the heart compared to IV groups which showed diffused transmural transduction. IM injections resulted in mCherry expression limited to the needle track.

Conclusion: These results suggest epicardial hydrogels are a promising delivery platform to effectively localize vector cargo to the heart, while minimizing systemic circulation, attenuating AAV immune responses, and reducing the dose required for efficient cardiac transduction. Future directions will focus on optimizing epicardial AAV hydrogels for cardiac therapeutic applications.

535 HG204 CRISPR-Cas13 RNA-Editing Therapy for *MECP2* Duplication Syndrome: Preclinical Success to First-in-Human Study

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Background: Methyl-CpG binding protein 2 (*MECP2*) is an X-linked, dosage-sensitive gene critical for neurodevelopment. Its gain-of-function leads to *MECP2* duplication syndrome (MDS), a severe fatal

neurodevelopmental disorder. To address this, we developed HG204, a CRISPR-Cas13-based RNA-editing therapy utilizing high-fidelity Cas13Y (hfCas13Y) and *MECP2*-targeting gRNAs (g*MECP2*) packaged into a single AAV vector (AAVhfCas13Y-g*MECP2*). Preclinical studies in MECP2 transgenic mice demonstrated the long-term safety and efficacy of HG204 in reducing MeCP2 protein levels and reversing disease phenotypes. These findings laid the foundation of clinical translation, and HG204 is now being evaluated in the HERO clinical trial to assess its safety, tolerability, and therapeutic potential in MDS patients.

Methods: Preclinical studies evaluated the editing efficiency of HG204 in reducing *MECP2* mRNA in the brain and the MeCP2 and GDF11 levels in the cerebral spinal fluid (CSF) of the adult wild-type (WT) non-human primates (NHPs) following intracerebroventricular (ICV) injection. Safety assessments included histopathology, MRI, serum biochemistry, CSF analysis, and immunological indicators.
HG204's clinical evaluation is ongoing in the **HERO** (a first-in-**H**uman clinical trial to **E**valuate the safety, tolerability, and efficacy of a single ICV injection of HG204 **R**NA-editing therapy in MDS, a rare **O**rphan disease) study in six boys aged 2 to 18 years with genetically confirmed MDS and stable pattern of seizures within 8 weeks before screening. The primary objectives are the safety and tolerability of HG204 at different doses. Secondary objectives include change from baseline in motor behavior, adaptive behavior, seizure frequency, and other assessment scales within 52 weeks post-injection.

Results: In preclinical studies, HG204 effectively edited *MECP2* mRNA and significantly reduced MeCP2 protein levels in the brains of WT NHPs at low and high doses, with sustained effects observed at 13 and 26 weeks post-ICV injection. High-dose HG204 also reduced CSF's MeCP2 and GDF11 protein levels from D29 to D203 post-ICV injection. MRI scans at D92 or D203 showed no brain injuries in treated NHPs. Minimal histopathological changes were observed in the brain across all dose groups and in the dorsal root ganglion (DRG) and spinal cord of the high-dose group, with no microscopic findings in the liver, kidney, and other tissues. Other safety evaluations, including functional observation battery (FOB), serum biochemistry, CSF cytology/biochemistry, and immunological assessments, confirmed the safety of both low- and high-dose HG204 in adult WT NHPs.
In the HERO trial (Outlined in **Figure 2**), the first subject with MDS was successfully dosed with HG204, demonstrating favorable safety and tolerability. No elevations in liver enzymes or bilirubin were observed, and there were no signs of thrombotic microangiopathy or abnormalities on the brain MRI or electroencephalogram. Early clinical data from the low-dose cohort suggested potential improvements in certain adaptive behaviors.

Conclusions: Our preclinical and early clinical findings support the safety and efficacy of HG204 as a novel CRISPR-Cas13 RNA-editing therapeutic strategy for MDS. The U.S. FDA and the EMA's Committee for Orphan Medicinal Products (COMP) have designated HG204 as an orphan drug and rare pediatric disease (a total of 3 designations), underscoring its potential as a transformative treatment for MDS.

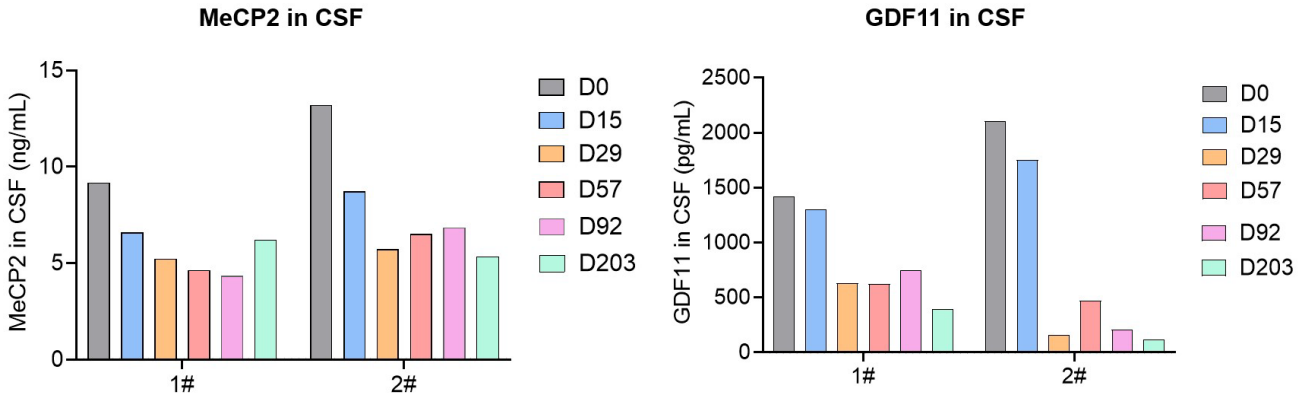

Figure 1 The MeCP2 and GDF11 content in CSF of NHPs after high dose of HG204 ICV dosing. 1# and 2# represent two monkeys.

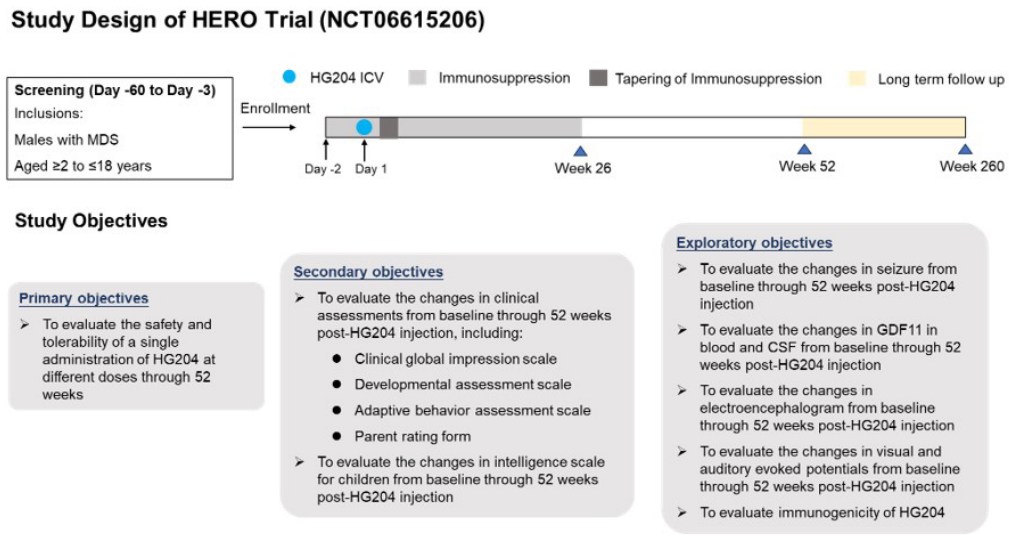

Figure 2: HERO clinical trial study design

536 RNA Editing Rescues SUDEP and Seizure Phenotype in a Mouse Model of Dravet Syndrome

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Dravet syndrome (DS) is a severe, early-onset genetic epilepsy, caused by *de novo* loss-of-function mutations of *SCN1A*, resulting in haploinsufficiency of Nav1.1, a voltage-gated sodium ion channel expressed throughout the brain and at low levels in the heart. Patients exhibit symptoms at approximately 6 months of age, including tonic-clonic and febrile seizures, ataxia and sudden unexpected death in epilepsy (SUDEP) in 15% of cases. Importantly, symptoms are often refractory to pharmacological intervention and no therapies targeting the genetic cause have been licensed.

Here, we describe the first use of a novel, humanised RNA editing technology known as CRISPR-Cas-inspired RNA targeting system (CIRTS) in a therapeutic setting for DS. We designed 21 guide RNAs (gRNAs) targeting a long non-coding RNA within the natural antisense transcript of *Scn1a* (*Scn1anat*) to inhibit its function, thereby reducing negative regulation of *Scn1a* and increasing levels of the endogenous *Scn1a* transcript. *In vitro,* 4 gRNA constructs resulted in a significant upregulation of endogenous *Scn1a*. gRNA9 and 21 led to 1.9- and 2.6-fold increases in *Scn1a*, respectively, restoring approximate wild-type expression levels, whilst also exhibiting high homology (>90%) to human *SCN1ANAT.* As such, these gRNAs were chosen as candidates for the *in vivo* study. Using the highly efficient, CNS-tropic AAV-F capsid, we produced AAV-F-CIRTS-gRNA vectors. We delivered AAV-F-CIRTS-gRNA9 to a clinically relevant heterozygous DS mouse model via neonatal intracerebroventricular and intravenous administration to target the brain and heart. Results revealed an increase in survival, reduced spontaneous seizure frequency, reduced severity of febrile seizures and increased endogenous *Scn1a* mRNA in the brain and heart. Neuropathology and RNAseq were also used to assess the safety of this technology.

Overall, in our proof-of-concept preclinical study, we show that our novel AAV-F mediated RNA editing treatment has promise as a potential therapy for DS.

537 Long-term expression of transgene in targeted cortical neurons following inoculation in the mouse striatum of a non-replicative HSV-1 vector.

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Efficient targeting of cortical neurons in central nervous system (CNS) remains a significant challenge for gene therapies. While direct intracerebral injection efficiently targets focal cell populations, this approach is unsuitable for covering broad structures like the cortex. Administration in the cerebrospinal fluid offers broader coverage but is often hampered by poor penetration of viral vectors in deep cortical structures. Systemic administration in the blood stream has been used to overcome these limitations. However, it requires high vector doses that often impact non-CNS tissues like the liver and DRG neurons. This can elicit immune responses, compromising safety and efficacy of the therapeutic product.

Herpes simplex virus type 1 (HSV-1) is a human neurotropic virus that establishes productive infections in epithelial cells and latent infections in neurons. Non replicative (nr) HSV-1 based vectors have been developed to exploit their therapeutic potential by leveraging their neurotropism, safety, and long-term transgene expression abilities. In contrast to AAVs, nrHSV-1 vectors can efficiently transduce neurons of the CNS at very low doses and can be efficiently transported retrogradely to connected brain regions. Here we administrated a nrHSV-1 vector in the striatum to broadly target, by retrograde transport, cortical neurons projecting in this area. This vector (EG143A) contains deletions in ICP4 and ICP27 genes and expresses the mGreenLantern reporter gene under the control of the CAG promoter. When stereotactically injected into the striatum of mice with no more than 4E+06 PFU (Plaque Forming Unit), EG143A demonstrated high-level transgene expression in layer V cortical neurons. Transgene expression was seen in many cortical areas such as the anterior cingulate, orbital, somatomotor, somatosensory and entorhinal cortex. Co-staining with neuronal marker NeuN showed that cells expressing mGreenLantern were exclusively neurons. Transgene expression kinetics were assessed at one week and six weeks post-administration using fluorescence microscopy and digital-droplet PCR. Both methods demonstrated stable transgene expression levels over time (Figure 1A). Digital PCR on isolated cortices showed stable levels of vector episome (on DNA) and levels of mGreenLantern transcripts (on RNAs after reverse transcription) (Figure 1, B and C).

Our results suggest that intra-striatal administration of low amount of EG143A broadly targets cortical neurons by retrograde transport achieving stable long-term transgene expression for at least 6 weeks. This vector is a promising tool for gene therapies targeting cortical neurons for the treatment of diseases of the CNS (such as Parkinson's disease, Huntington's disease, or other neurodegenerative diseases with cognitive impairment).

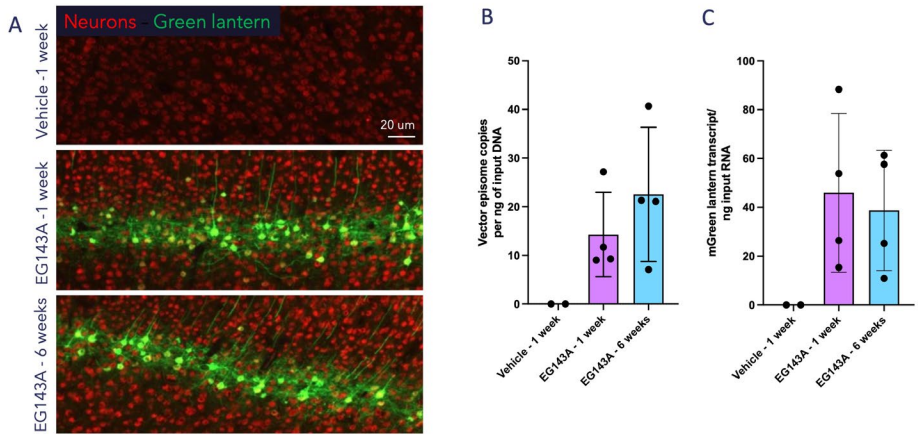

Figure 1. Long-term transgene expression after intrastriatal injection of EG143A in mouse. A. Fluorescence microscopy showing cortex of vehicle-injected animal at one week and EG143A-injected animal at one and 6 weeks. Sections were stained for mGreen lantern (green) and the neuronal marker NeuN (red). B. ddPCR showing quantification of number of vector episomes in the cortex. C. Reverse transcription ddPCR showing quantification of number of mGreen lantern transcripts in the cortex. Mean +/- SD. Each dot represents one injected hemisphere.

538 Successful RNA Therapy for Schinzel-Giedion Syndrome Demonstrated in Patient-Derived Stem Cell Models and a Clinically Relevant Mouse Disease Model

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Schinzel-Giedion Syndrome (SGS) is a devastating neurodevelopmental disorder causing premature death. SGS is caused by increased SETBP1 protein due to heterozygous missense mutations in a degron motif of SETBP1 required for protein degradation. To lower SETBP1 protein levels characteristic of SGS, we developed antisense oligonucleotides (ASOs) to reduce SETBP1 mRNA and so lower SETBP1 protein levels. ASOs are able to downregulate SETBP1 in four independent SGS patient iPSC models with different disease-causing mutations at two neurodevelopmental stages. We calculate an IC50 of approximately 70nM suggesting that ASOs are efficient, with no evidence of off-target effects based on whole transcriptomic studies. These anti-SETBP1 ASOs behave similarly when assessed in SGS neuroepithelial primary tumor cells, SGS engineered cell lines, or healthy primate iPSC-derived neurons. We developed a brain-specific SGS mouse model that recapitulates disease, showing failure to thrive with hydrocephalus and death within 8 months. Intracerebral ventricular injection of anti-SETBP1 ASOs in brain of SGS mice all survive long term, gain weight normally, and have significantly reduced hydrocephalus. ASOs reduce SETBP1 protein in vivo, reverse clinically relevant pathological phenotypes, and show no major adverse events. This work highlights a novel RNA therapeutic for the treatment of Schinzel-Giedion Syndrome.

539 Convection-enhanced delivery of gene therapies to the pons – a novel method of achieving transgene expression throughout the Central Nervous System

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Introduction
Convection-enhanced delivery describes the direct delivery of drugs to the brain through intraparenchymal microcannulas, thus bypassing the blood-brain barrier. CED facilitates safe and targeted drug distribution through clinically-relevant brain structures. This method is particularly relevant to the delivery of gene therapies which cannot effectively cross the blood-brain and CSF-brain barriers, and where systemic administration can be associated with dose limiting toxicity. To date, the majority of gene therapy CED trials have aimed to treat disorders with a localised structural target such as the putamen in Parkinson's Disease or caudate and putamen in Huntington's disease. However, many CNS disorders are characterised by widespread neurodegeneration including Amyotrophic Lateral Sclerosis, Alzheimer's Disease, enzyme deficiencies and metabolic disorders. We hypothesised that due to the unique and extensive connectivity of nuclei and tracts within the pons and cerebellum, infusion of a retrogradely and anterogradely transported viral vector into this relatively small CNS volume, has the potential to transduce all the major neuronal circuits participating in sensory-motor processing including the pyramidal and extrapyramidal motor pathways as well as the limbic, somatosensory, visual and auditory circuits that modulate motor activity.

Methods
Bilateral trans-middle cerebellar peduncle cannula trajectories were used to deliver 250uL of AAV9-mCherry into the right hemipons, whilst 250uL of AAV5-mCherry was delivered into the left hemipons in a large animal (ovine model). Both vectors were infused at a concentration of 1E12vg/mL. In a second test subject, a unilateral pons infusion of 300uL of AAV9-mCherry was delivered at an infused concentration of 5E12vg/mL. GFP and mCherry expression throughout the brain and spinal cord was analysed using ELISA, fluorescence and DAB immunohistochemistry.

Results
GFP and mCherry expression measured by ELISA confirmed expression in the ipsilateral and contralateral motor cortex and spinal cord at 1 month post unilateral pontine infusion. At this early timepoint, GFP and mCherry was detected at 25cm along the spinal cord. Comprehensive analysis following unilateral AAV9-mCherry infusion

demonstrated mCherry expression measured by ELISA throughout all components of the motor and sensory subsystems of the brain and spinal cord, the limbic system and throughout the spinal cord. Fluorescence and DAB immunohistochemistry confirmed mCherry expression through widespread areas of the cerebral and cerebellar cortex, brainstem nuclei (including hypoglossal and inferior olive) and anterior horn cells of the spinal cord.

Conclusions
These data demonstrate that a single CED infusion of an antero- and retro-gradely transported adeno-associated viral vector to the pons through a transcerebellar trajectory results in gene expression in multiple components of the cerebral cortex, motor system and sensory and limbic systems – the motor cortex, basal ganglia, sensory, auditory, visual and visual association cortex, hippocampus and amygdala, spinal cord, cranial nerve nuclei, inferior olivary nucleus, thalamus, cerebellum including dentate nucleus, as well as the anterior horn cells of the spinal cord. This novel gene therapy method opens the door to the treatment of disorders characterised by widespread degeneration of multiple subsystems of the CNS with a single, low volume infusion.

540 AAV mediated APOE4 Silence and Replace Therapy to Treat Homozygous APOE4-associated Alzheimer's Disease

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Variants of APOE, a 299 amino acid lipid transport protein that functions as a major carrier of cholesterol in the CNS, are the major genetic risk factors for sporadic late-onset Alzheimer's disease (AD). APOE has 3 common variants: APOE3 (C112/R158), APOE4 (R112/R158) and APOE2 (C112/C158). Epidemiologic, clinical and experimental evidence demonstrate that APOE3 is associated with an average risk for AD, APOE4 is pathogenic, conveying a high risk and APOE2 is protective, reducing risk. Inheritance of the APOE4 allele is linked to dysfunction in cholesterol efflux, cerebral hypometabolism, synaptic defects, mitochondrial dysfunction, blood-brain barrier dysfunction, enhanced neuroinflammation, cognitive impairment, increased Aβ production and aggregation, decreased Aβ clearance and deposition, neurotoxicity, increased tau phosphorylation and oligomerization and tau-related neurofibril tangles. In the context that APOE4 is toxic while APOE2 is protective, the ideal therapy for APOE4 homozygotes is to reduce CNS levels of APOE4 and to substitute APOE4 by addition of APOE2. To accomplish this goal, we have developed a "silence and replace" therapy, where suppression of CNS APOE4 is achieved using adeno-associated virus (AAV) expression of microRNAs (miRNA) designed to hybridize to and "silence" the endogenous human APOE4 mRNA, together with simultaneous "replacement" with an APOE2 gene resistant to the miRNAs. The endogenous APOE4 suppression was achieved by identification of miRNAs that suppress human APOE4 expression in TRE4 mice with humanized APOE4 gene when delivered with AAV.S2, a novel neurotropic AAVrh.10 variant that has broad CNS distribution following intraparenchymal CNS administration. By combining 2 different APOE-directed miRNAs, over 70% suppression of endogenous APOE4 mRNA level could be obtained (Karan KR et al Hum Gene Ther 2024; 35:904). We then engineered a silencing resistant (SR) APOE2 gene (APOE2-SR) that was expressed at high levels even in the presence of the 2 miRNAs that suppress endogenous APOE4 expression. AAV.S2 vectors expressing APOE2-SR with and without the miRNAs were administered to the hippocampus of human APOE4 (TRE4) mice and APOE protein levels assessed 2 weeks later by ELISA in coronal sections of the brain. Relative to endogenous APOE4 levels in TRE4 mice PBS control, both APOE2-SR vectors increased total APOE protein by 2.0±1.1 fold for APOE-SR and 2.3±1.0 fold for APOE-SR with miRNAs ($p<0.05$ compared to PBS control, $p>0.5$ comparing APOE2-SR with or without the miRNAs). TaqMan allelic discrimination assay was used to assess APOE2 to APOE4 ratio. As expected, both vectors with APOE2-SR increased the E2/E4 ratio relative to controls by 3.5±0.6 fold for APOE2-SR and 6.3±0.4 fold for APOE2-SR+miRNAs ($p<0.05$ comparing APOE2-SR with and without miRNA). We conclude that a single AAV vector can deliver a cassette that has the dual functions of suppressing expression of the toxic APOE4 variant while also delivering the protective APOE2 variant. This dual approach may provide a more potent gene therapy for APOE4-dependent Alzheimer's disease than either alone.

541 One-Time Delivery of a Vectorized Anti-Amyloid Antibody for Increased and Sustained CNS Expression and Target Engagement

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Alzheimer's disease (AD) is characterized by the accumulation of amyloid plaques, leading to cognitive decline. Clinically, passive administration of anti-amyloid antibodies has shown promise in reducing amyloid plaque burden and improving cognitive function in patients with mild cognitive impairment. However, these therapies are limited by poor blood-brain barrier (BBB) penetration and require frequent administration, which can be burdensome for patients and care partners. Alternatively, the use of an adeno-associated viral (AAV) vector to deliver a transgene encoding an anti-amyloid antibody could enable sustained expression of the antibody in the brain after a single injection, especially if the vector is delivered via a BBB-penetrant capsid.

Here, we show that in wildtype mice a vectorized anti-amyloid antibody packaged in a BBB penetrant capsid has over 15-fold greater brain:plasma ratios as compared to a passively administered antibody over 4 weeks. In a transgenic mouse model of AD, we observe broad biodistribution of vector genomes and sustained antibody expression in the brain over a 2-month period. Immunohistochemical and immunofluorescence analysis of the cortex and hippocampus reveal that the vectorized antibody co-localizes with human amyloid-beta plaques, indicating successful target engagement.

Together, these results demonstrate that vectorization of an anti-amyloid antibody using a BBB penetrant capsid offers a promising strategy for sustained and enhanced, brain-wide antibody expression, overcoming the limitations of passive antibody therapies in AD.

542 Retargeting of AAV to CNS Using Anti-AAV X TFR1 Bispecific Antibodies

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Adeno-associated virus (AAV) vectors are widely used in gene therapy due to their safety, efficiency, and ability to deliver therapeutic genes into target cells. However, most naturally occurring AAV serotypes are inefficient at crossing the blood brain barrier (BBB), making them suboptimal for delivering therapeutic cargo to the central nervous system (CNS) when administered systemically.

At Regeneron, we have developed platforms to target AAV to specific tissues using antibodies, which can bridge AAVs to target receptors specifically expressed on tissues of interest. Previously, we demonstrated enhanced AAV targeting to the CNS in both mice and non-human primates using a protein tagging system to covalently attach anti-Transferrin receptor 1 (TFR1) antibodies to the viral capsid. Here, we demonstrated that efficient TFR1-based BBB crossing can also be achieved using anti-AAV x TFR1 bispecific antibodies, thereby further expanding the applications of the antibody retargeted AAV platform.

By conducting extensive screening of antibodies and creating innovative antibody formats, we have developed bispecific antibodies that can effectively target AAV to the entire brain, achieving over a 10-fold increase in on-target transduction compared to AAV alone, measured by reporter mRNA expression level. Furthermore, the bispecific platform is shown to be highly modular, flexible, and adaptable. For instance, AAV and bispecific antibodies can be combined and administered using various dosing strategies while maintaining comparable efficacy. Also, by utilizing a broad serotype binding anti-AAV arm, we demonstrate that the same bispecific

antibody can improve CNS transduction of multiple different serotypes. In addition to CNS delivery, we have previously demonstrated that our bispecific platform can enhance AAV retargeting to skeletal muscle in mouse models, this highlights the potential to utilize bispecific antibodies to enhance transduction for a wide range of target tissues. Moving forward, bispecific antibodies can serve as a "plug and play" system that can combine with existing AAV programs to improve on-target transduction efficacy.

543 Investigating Focused Ultrasound Enhancement of CSF-Delivered AAV to the Brain

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Gene therapy with recombinant adeno-associated virus (rAAV) is used across a variety of disorders to achieve long-term expression of delivered DNA in postmitotic cells. For neurological disorders, AAV9 is commonly used due to its high neuronal and glial tropism, as well as its ability to cross the blood-brain barrier (BBB). While AAV9 can be targeted to the brain intravenously, this approach includes risks of hepatotoxicity and immune responses. Intrathecal CSF administration largely avoids these concerns and has improved CNS transduction, although it is limited by poor distribution to deeper brain and forebrain areas, especially in aged animals. Transcranial low-intensity focused ultrasound (FUS) can be used for transient BBB opening and more efficient targeting of AAV to deep brain structures. Previously, our group showed that FUS can enhance transduction of CSF-injected AAV to deep brain structures, such as the hippocampus and striatum. However, it remains unclear whether FUS enhances CSF-delivered virus by redirecting AAV that escapes to the blood back into the targeted brain or via other mechanisms, such as CSF-brain diffusion under FUS-stimulated glymphatic flux.

As part of the optimization of CSF-FUS delivery, we first established the timing of AAV9 CSF-blood escape in mice. Adult (6 months old) and aged (18 months old) C57Bl/6 mice were intrathecal intra-cisterna magna (ICM) injected with $1x10^{11}$ vg of AAV9-GFP and then blood was collected at a series of time points. Plasma DNA was tested for AAV genomes by qPCR. Virus concentrations rose in the blood within 30 minutes of injection, peaking at stable levels 1-4 hours post-injection and mostly clearing after 3 days. No significant differences in circulation kinetics were seen between age groups. These results indicate early post-injection FUS-BBB opening may be optimal for AAV brain entry. Since CSF-delivered virus peaked in the blood in 1-4 hours of injection, we hypothesized that FUS at 1 or 4 hours after AAV injection results in the highest transduction of targeted regions. To investigate optimal CSF-FUS delivery, we compared adult C57Bl/6 mice treated with FUS 1 hour before, or 1, 4, or 24 hours after ICM injection of $1x10^{11}$ vg AAV9-GFP. A microbubble-free control group was included to test the necessity of BBB opening. Mice received systemic infusion of Definity microbubbles and FUS-BBB opening of 3 targets in the left hippocampus. Systemically infused gadolinium was used to confirm BBB opening by T1-weighted MRI. Three weeks post-injection, brain transduction was assessed by GFP IHC and analysis is ongoing. If higher transduction is seen with BBB opening 1- or 4-hours post-injection this could indicate that blood circulation of CSF-delivered AAV contributes to its enhancement via FUS. Overall, these findings suggest CSF-delivered virus escapes to blood within 30 minutes of injection, with peak levels 1-4 hours post-injection and that FUS-BBB opening in this time frame should allow for maximal AAV transduction. The results of this work could enable improvements in AAV-mediated therapeutic delivery to the brain.

544 Targeted Bacteriophage Gene Delivery to Astrocytes Using Intelligent Phagemid-Assembled Gene Expression (iPhAGE) Technology for Neuronal Regeneration

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Neurodegenerative diseases are the leading cause of disability and remain largely incurable as current treatments focus on symptom management rather than slowing disease progression. As part of the iNeuron project, this research develops Intelligent Phagemid-Assembled Gene Expression (iPhAGE), a novel platform utilizing M13 bacteriophage-derived miniphagemids to deliver Neurogenic Differentiation Factor 1 (NeuroD1) to astrocytes, reprogramming them into neurons. This system addresses key limitations of traditional gene delivery methods, such as restricted cargo capacity, high immunogenicity, and inability to cross the blood-brain barrier.

M13 bacteriophages offer unique advantages, including their rod-shaped structure that facilitates blood-brain barrier penetration, large DNA cargo capacity, and lack of mammalian cell tropism, enhancing safety. The bacterial host, *Escherichia coli* JM109, packages NeuroD1 into miniphagemids lacking bacterial backbone elements, significantly reducing immunogenicity. The highest bacterial backbone contamination in the produced miniphagemids was detected to be $1x10^{-8}$. Miniphagemid production was optimized using a specialized helper plasmid, SW8, which ensures precise excision and circularization of the transgene cassette.

Targeting specificity can be enhanced through the incorporation of IRL1620 and EGF ligands into the M13 system. IRL1620, an endothelin B receptor agonist, selectively binds reactive astrocytes, while EGF targets the epidermal growth factor receptor, facilitating efficient gene delivery. To validate this approach, miniphagemids encoding NeuroD1 were transfected into A7 astrocyte cells. Transfection efficiency was assessed using a luciferase reporter gene, and neuronal differentiation was confirmed via PSA-NCAM1 immunostaining. At day 7 post-transfection transfected A7 cells showed close to half the level of expression compared to positive control – a neuronal cell line, HT22.

This research demonstrates a safe, efficient gene therapy approach that enables precise astrocyte targeting and reprogramming, providing a promising strategy to regenerate neurons and treat neurodegenerative diseases. Future directions include developing a test to mimic hypertrophic astrocytes. One key milestone will be to establish a protocol for 'activating' resting astrocytes via proinflammatory cytokines, oxygen deprivation, or LPS treatment to resemble hypertrophic astrocytes following a stroke event. The iPhAGE system's versatility suggests potential applications in other neurological and regenerative medicine contexts, marking a significant advancement in gene therapy technology.

545 Gene replacement therapy reverses behavioral and molecular phenotypes in succinic semialdehyde dehydrogenase deficiency (SSADHD) mice

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Introduction: Succinic semialdehyde dehydrogenase deficiency (SSADHD) is a rare autosomal recessive neurodevelopmental disorder due to loss of function mutations of *ALDH5A1* gene essential for degradation of the inhibitory neurotransmitter g-aminobutyric acid (GABA). In SSADHD, pathologic GABA and g-hydroxybutyrate (GHB) accumulation results in early life encephalopathy, refractory epilepsy, and high mortality. Emerging evidence also suggested that elevated GABA/GHB levels underlie late-life disease risks in heterozygous SSADHD carriers (~0.3% of the population). Targeted drug treatments for patients with SSADHD and carriers are absent. Gene replacement therapy for SSADHD has not been developed.

We recently demonstrated in a Cre-dependent SSADH mouse model, *Aldh5a1*$^{lox\text{-}STOP}$, that SSADHD symptoms and mortality are reversible. As a step toward clinical translation of SSADH gene replacement, we developed a novel vector encompassing a human *ALDH5A1* full length native promoter (FLnP) tethered with a human *ALDH5A1* coding sequence, namely AAV-FLnP-hALDH5A1, and observed complete phenotype reversal when packaged in the blood-brain barrier (BBB)-penetrating capsid AAV-PHP.eB.

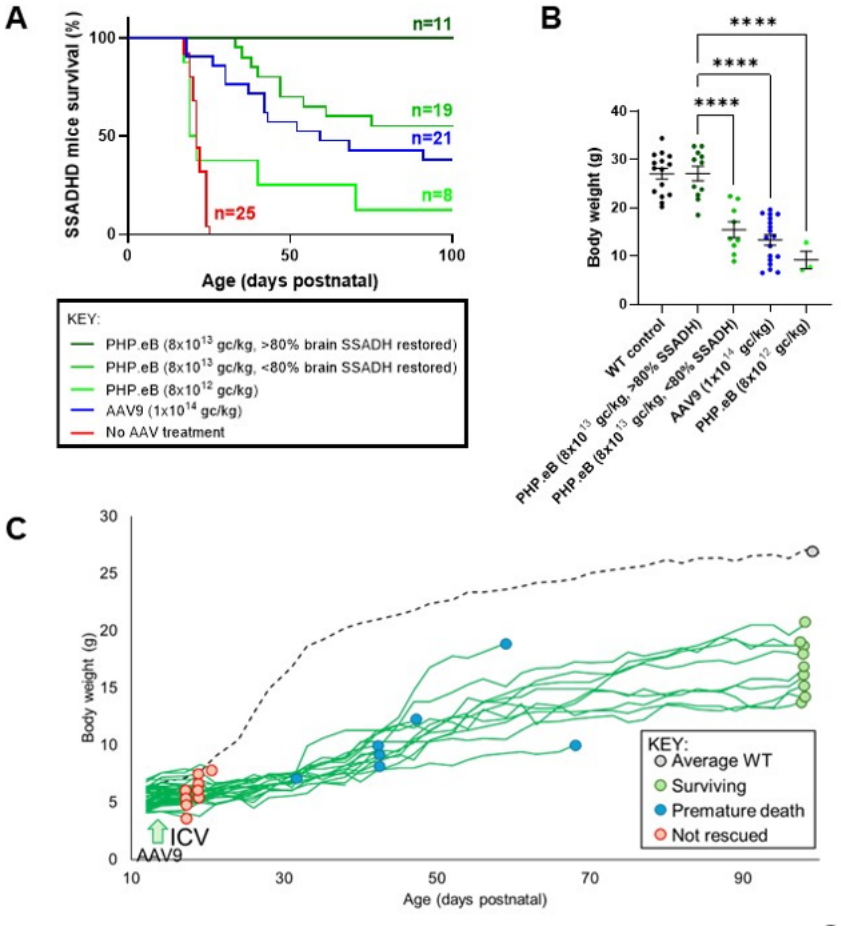
Since AAV-PHP.eB is not clinically translatable, we now test AAV-FLnP-hALDH5A1 encapsulated in AAV serotype 9 (AAV9). As an exploration of gene replacement in SSADHD carriers, we also treated AAV-PHP.eB-FLnP-hALDH5A1 in heterozygous SSADHD mice and measured SSADH content and GABA/GHB levels.

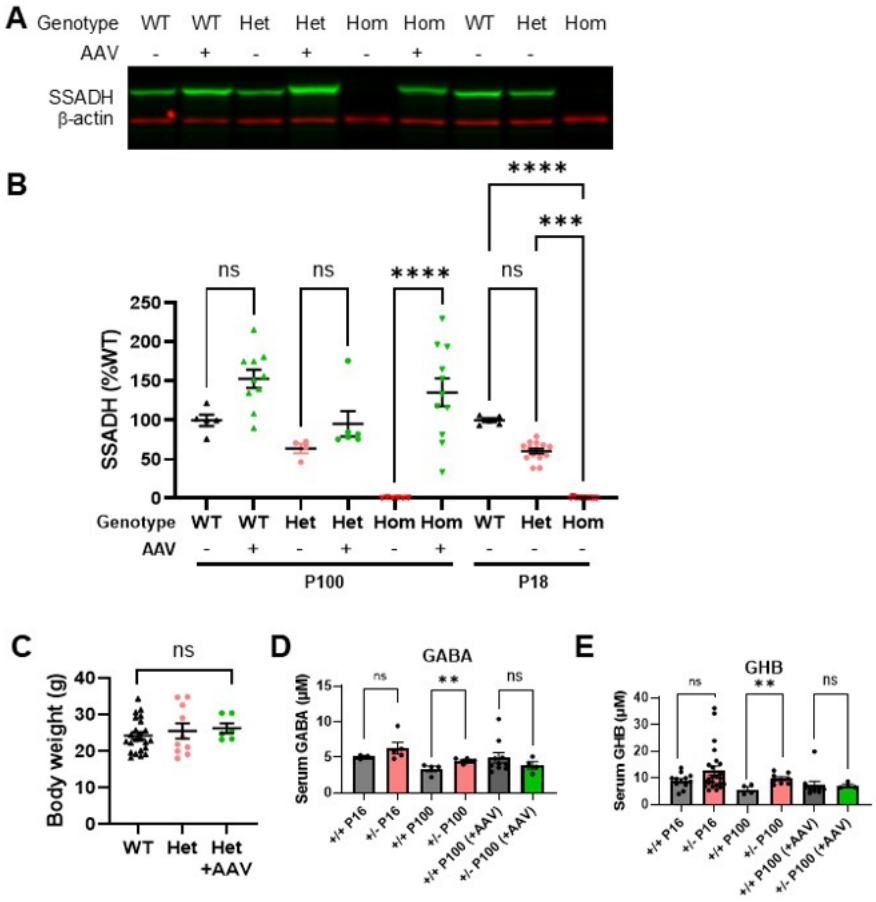
Methods: We packaged AAV-FLnP-hALDH5A1 into AAV9 and injected into homozygous mutant (Hom) *Aldh5a1*$^{lox\text{-}STOP}$ mice via retro-orbital (RO), intracerebroventricular (ICV), and intraperitoneal (IP) injection through a dose de-escalation paradigm at a symptomatic stage P14. Body weight and survival were tracked until P100, when blood and brain tissues were collected for metabolites and protein analyses. We also injected AAV-PHP.eB-FLnP-

hALDH5A1 into heterozygous (Het) mice and check for cortical SSADH expression and blood GABA/GHB content at P100.

Results: Relative to AAV-PHP.eB treatment, AAV9 resulted in incomplete rescue among injection routes (IP:1x10^{14} gc/kg; RO:1x10^{14} gc/kg; ICV:2x10^{13} gc/kg). At P100, the survival rate of AAV9-treated mice was 38%, comparable to a de-escalated dose of PHP.eB (54% survival in 8x10^{13} gc/kg, 13% survival in 8x10^{12} gc/kg) (Fig. 1A). AAV9 treatment also resulted in sub-optimal body weight restoration (WT:27±4g; Hom+AAV-PHP.eB (8x10^{12} gc/kg):9±3g; Hom+AAV9 (1x10^{14} gc/kg):13±5g) (Fig. 1B) and impaired growth trajectory (Figure 1C). AAV-PHP.eB-FLnP-hALDH5A1 resulted in SSADH overexpression in the cortex of WT (153±12% of un-injected WT), Het (un-injected:64±6% WT; injected:95±16%), and Hom (un-injected:1±0.2% WT; injected:136±18%) (Fig. 2A-B). AAV-PHP.eB did not affect body weight of Het mice (un-injected:26±2g, injected:26±1g) (Fig. 2C). AAV-PHP.eB reduced blood GABA (WT:3±0.4µM; Het:5±0.1µM; Het+AAV-PHP.eB:4±0.5µM) and GHB (WT:6±0.9µM; Het:10±0.7µM; Het+AAV-PHP.eB:7±0.5µM) in Het mice (Fig. 2D-E).

Conclusions: We found that SSADHD mice are partially rescued with AAV9-FLnP-hALDH5A1. AAV9 therapeutic effects are slightly better than a sub-optimal dose of AAV-PHP.eB, suggesting room for optimization and testing next-gen capsids with higher BBB penetrance. AAV-FLnP-hALDH5A1 is well-tolerated in Het mice, with reduced blood GABA/GHB content suggesting target engagement, paving the way for experimental SSADH-targeted gene therapy for SSADH carriers.





546 Translational Devices for Simultaneous Neural Recordings and Drug Delivery in the Brain: From Small to Large Animals

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The evaluation of drug effects on neural activity is essential for the development of brain-targeted therapies. Real-time neural recordings are useful in assessing drug efficacy, dosing, mechanism of action, and potential toxicity. Typically, drug effects on neural activity are assessed using in vitro systems (e.g. cell cultures, brain slices) or small animal models (mice, rats), with less frequent testing in large animals or humans. This gap in continuity between small and large species often contributes to drug failure in late developmental stages. This disparity may arise from a mismatch in technological capabilities between devices used in small versus large species and, specifically, the lack of a device able to simultaneously record neural activity and deliver drugs.

We are developing a drug delivery (DD) system with integrated neural recordings, utilizing an FDA-cleared stereoencephalography (sEEG) electrode. This system is intended for convection enhanced delivery (CED) in large brains (human and large animals) and has successfully been tested in swine. Here, we introduce a miniaturized version of the device tailored for use in small rodents. A first prototype consisting of a modified human-size sEEG with 0.8 mm outer diameter and an inner silica infusion cannula (320 µm inner diameter) inserted through the sEEG lumen, was tested in the rat brain. The device was implanted stereotactically into the hippocampus, and recordings were made prior to and following the administration of a drug that modulates neural activity (penicillin, 5000

units/µl; 5 µl; 0.5 µl/min). Histological analysis of the brain tissue using hematoxylin and eosin staining enabled the estimation of the diffusion volume.

While the modified human-size prototype demonstrated effective CED along with the ability to record neural activity in rats, its size and design limit its suitability to acute experiments only. Our most recent prototype is scaled down to better match the brain size of rodents and accommodate the small space available in headstages typically used for survival/chronic studies. The sEEG length is shortened by a factor of 10, its diameter reduced to 0.6 mm and the recording contacts shortened to 0.5 mm, a quarter of the length used in the human-sized version. The inner diameter of the infusion cannula is reduced by a factor of 3 to minimize the dead volume of the infusion line, a crucial aspect to cut drug expenses at early stages of drug development.

The integration of drug delivery and real-time neural recording into a single device, suitable for both small and large species, ensures consistency across species and streamlines the drug discovery process from rodents to humans. This approach enables more precise assessments of drug efficacy, dosing, toxicity, and mechanisms of action across species, potentially reducing the failure rate of drugs in later development stages and improving clinical outcomes.

547 ETX201: An AAV9-based Vectorized miRNA Therapeutic Candidate for Angelman Syndrome

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Angelman syndrome (AS) is a severe, neurodevelopmental disorder caused by loss of neuronal ubiquitin E3 ligase, *UBE3A*. Currently available treatments may address certain symptoms, but there are no approved disease-modifying therapies. Adeno-associated virus (AAV)-based vectorized knockdown of the paternal long non-coding RNA, UBE3A anti-sense transcript (UBE3A-ATS), has the potential to target the underlying cause of AS by increasing *UBE3A* expression specifically in neurons, with a single administration. Moreover, given UBE3A's involvement in the duplication disorder dup15q, an unsilencing approach mitigates the potential toxicity risk associated with UBE3A overexpression by limiting maximum expression to the capacity of the paternal allele. We previously showed that a mouse-specific miRNA candidate targeting Ube3a-ATS was capable of upregulating Ube3a RNA and protein. We also established that partial *Ube3a* reinstatement can rescue multiple disease phenotypes in an AS mouse model. We then developed an AAV9-based vectorized miRNA to knockdown human UBE3A-ATS and durably unsilence paternal *UBE3A* for potential long-term genetic correction. Screening of >120 human miRNA sequences identified top candidates capable of near-complete paternal *UBE3A* unsilencing in human iPSC neurons, with lead candidate, ETX201, emerging as the most potent human candidate. Here, we evaluate safety, biodistribution, and *UBE3A* target engagement for ETX201 at two doses in wild-type (WT) non-human primates (NHPs) following unilateral intracerebroventricular (ICV) administration. In this study, we observe broad vector distribution and miRNA transcript expression in the brain, particularly in disease-relevant forebrain regions important for learning, memory, and cognitive functioning. The miRNA product is also detected in CSF and serum, with expression stabilizing by day 8 post-administration and remaining sustained throughout the 60-day study. ETX201 is well-tolerated in NHPs, with significant UBE3A-ATS knockdown and dose-dependent paternal *UBE3A* upregulation of 15–29% across multiple cortical and hippocampal brain regions post ICV delivery. These paternal *UBE3A* levels are within the efficacy range induced in our prior in vivo studies in an AS mouse model. In addition, ETX201 significantly increases total UBE3A RNA and protein in multiple cortical regions, surpassing the normal background levels in WT NHPs. Collectively, these data establish the feasibility of a one-time, AAV9-based approach for the treatment of AS and support further development of ETX201.

548 AAV-delivered *GRIN1* gene improves synaptic NMDA receptor function in mouse models of *GRIN* disorder

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N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor that plays an important role in brain development, learning and memory. NMDARs are encoded by *GRIN* genes: *GRIN1*, *GRIN2A-D*, and *GRIN3A-B*. Rare *de novo* mutations in one of the *GRIN* genes lead to *GRIN* disorder, a neurodevelopmental disorder with symptoms of profound intellectual disability, epilepsy, and visual cortical impairment. Most pathogenic variants are missense heterozygous mutations that leads to over- or under-activation of NMDAR. As a result, pharmacological interventions directly targeting NMDAR often show mixed results in patients. Development of gene-based therapies are desired. A functional NMDAR is a heterotetrametric complex consists of two obligatory GluN1 subunits (encoded by *GRIN1* gene). The other two subunits are a combination of any of the GluN2 or GluN3 subunits. We hypothesize that delivery of exogenous *GRIN1* gene is beneficial for missense *GRIN1* variants. The exogenous *GRIN1* gene will lead to excess expression of functional 'wildtype' GluN1 proteins that competes with variant proteins for the assembly into NMDARs. As a result, more NMDARs will contain only wildtype GluN1 subunits and function normally (Figure 1). To test our hypothesis, we developed two genetic constructs. Both constructs contain i) human *GRIN1* cDNA (hGRIN1); ii) a V5 tag sequence at the N-terminus; iii) 5' and 3' inverted terminal repeats (ITRs) for AAV transfer. We used two promoters that drive ubiquitous or neuron-specific expression. The constructs are packaged into an AAVHSC capsid that can cross the blood brain barrier and transduce neurons and glial cells. We generated two mouse models possessing the equivalent heterozygous missense *GRIN1* patient variants, the Grin1$^{Q536R/+}$ and Grin1$^{Y647S/+}$ mice. Electrophysiological studies revealed that, remarkably, AAVHSC-delivered hGRIN1 showed significantly improved NMDAR-mediated synaptic transmission and restored long-term potentiation (LTP) in the hippocampus of both Grin1$^{Q536R/+}$ and Grin1$^{Y647S/+}$ mice (Figure 2). Importantly, the treatment did not negatively impact normal synaptic functions. For systemic transduction, we also administered the vectors intravenously (i.v.). hGRIN1 expression was detected at mRNA and protein levels in the brain of Grin1$^{Q536R/+}$ mice four- and twenty-week after i.v. injection. Efforts are made to determine the optimal dose, promoter, and route of administration for behavioural rescue. In summary, our strategy has translational potential for clinical applications and may serve as a novel one-time treatment for patients with pathogenic *GRIN1* variants.

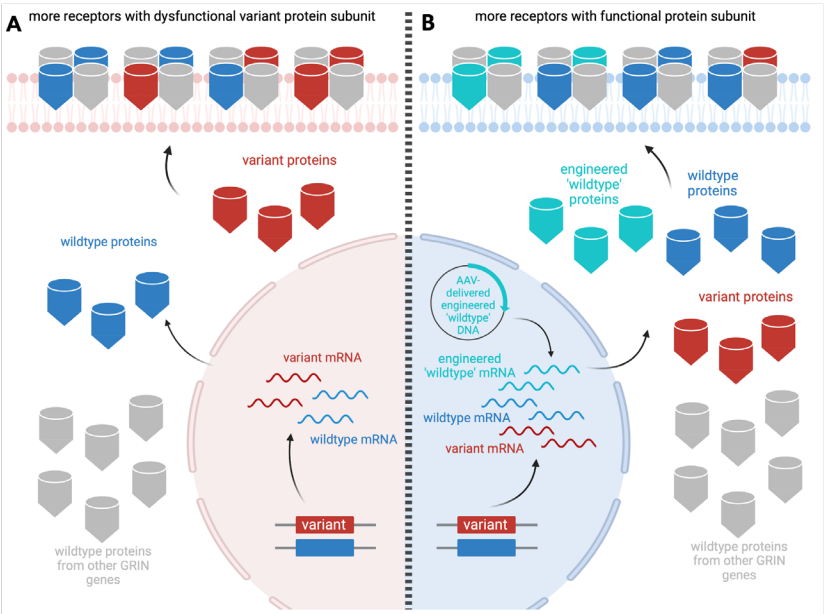

Figure 1. Rationale of *GRIN1* replacement therapy for patients with heterozygous missense variants in *GRIN1* gene, regardless of the locus. (A) Schematic showing a cell from a heterozygous *GRIN1* variant patient that expresses both wildtype (blue) and variant (red) GluN1 protein. **(B)** Delivery of exogenous *GRIN1* cDNA coding for 'wildtype' protein (teal) that competes against variant GluN1.

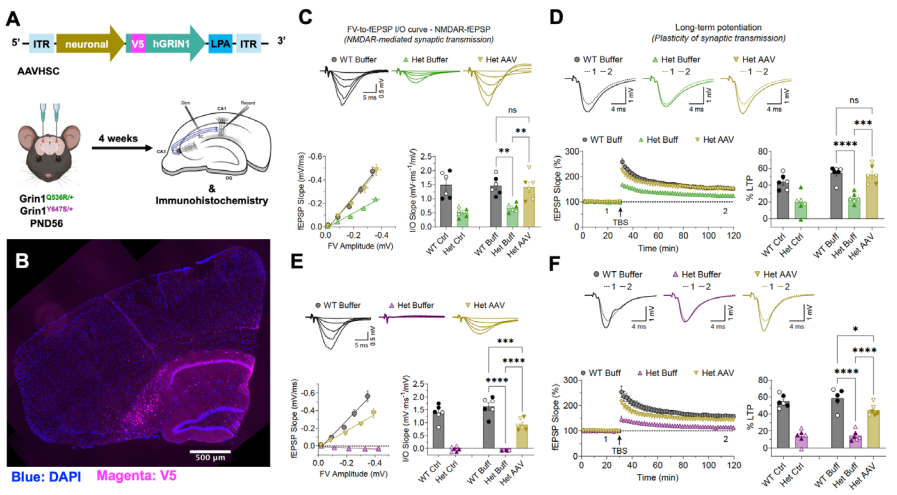

Figure 2. Rescue of NMDAR function following AAVHSC-hGRIN1 expression in the hippocampus of two distinct mouse models of GRIN1 disorder. (A) Design of bilateral intrahippocampal AAVHSC-hGRIN1 injection in Grin1$^{Q536R/+}$ mice (Het, green), and Grin1$^{Y647S/+}$ mice (Het, purple) followed by electrophysiology and immunohistochemistry. WT and Het mice receiving buffer are used as controls. **(B)** V5 immunofluorescence pattern in the hippocampal and surrounding cortical regions. Scale bar=500µm. **(C-F)** Electrophysiological results revealed significant improvement of synaptic NMDAR function in the hippocampus of both Grin1$^{Q536R/+}$ (C-D) and Grin1$^{Y647S/+}$ mice (E-F). Stimulation electrode (Stim) was placed at Schaffer collateral/commissural fibers (SC) and recording electrode (Record) were placed in CA1 stratum radiatum. Baseline recording dataset (no injection) included on the left side of each graph for comparison. (C&E) Representative NMDAR-mediated field excitatory postsynaptic potential (fEPSP) traces, input/output (I/O) function of NMDAR-fEPSP against fiber volley (FV) amplitude, and slope of the I/O curve calculated as a measure of NMDAR-mediated synaptic transmission. NMDAR-mediated responses were recorded in low magnesium (0.1mM) with AMPARs blocker NBQX; (D&F) Representative AMPAR-fEPSP traces before (dotted line) and after (solid line) induction of long-term potentiation (LTP). fEPSP slope (as a percentage of baseline) plotted over time before and after theta burst stimulation (TBS) of LTP. % LTP difference calculated between time points 1 and 2. Filled symbols: males; open symbols: female. One-way ANOVA. Mean±SEM. Tuckey's post hoc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.

549 Recovery in a Mouse model of Tyrosine Hydroxylase Deficiency by Triple Transduction of Striatal Cells with AAV Vectors Expressing Dopamine-Synthesizing Enzymes

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Background: For efficient dopamine synthesis, three enzymes are necessary: tyrosine hydroxylase (TH), the rate-limiting agent, first converts tyrosine to l -3,4-dihydroxyphenylamine (l-dopa). Aromatic-l-amino-acid decarboxylase (AADC) then converts l-dopa to dopamine. Guanosine triphosphate cyclohydrolase I (GCH) is the rate-limiting enzyme for synthesizing essential TH cofactor tetrahydrobiopterin (BH_4). TH deficiency caused by variants in *TH* gene presents with dystonia, oculogyric crisis, loss of voluntary movements, developmental delay, and autonomic symptoms. Motor symptoms are treated with l-dopa, but the onset of dyskinesia and hyperactivity requires repeated dose reductions and interruptions, making treatment challenging. We have shown that gene therapy injecting AAV-AADC into the putamen is effective for AADC deficiency, which has symptoms similar to TH deficiency. We have also developed a gene therapy for Parkinson's disease in which AAV vectors expressing TH, AADC, and GCH, respectively were injected into the putamen. **Methods:** We used Dat-Cre×Th-flox mice as a TH deficiency model. In these mice, *TH* gene was deleted in dopamine transporter (Dat) expressing neurons, and the level of dopamine in the striatum was reduced by less than 5 % of those in the wild type mice. The dopamine deficient mice showed hypoactivity in the open field test. These mice were stereotaxically injected with AAV-TH, AAV-AADC, and AAV-GCH (7×10^9, 4×10^9, and 1×10^{10} vector genomes, respectively) in the caudo-putaminal unit (CPU). Control mice were received AAV-GFP. Brain tissues were harvested four weeks after injection for biochemical and histological analyses. **Results:** Three weeks after injection of a mixture of AAV-TH, AAV-AADC, and AAV-GCH into the CPU, treated mice showed a marked behavioral improvement in the open field test, including an increase in locomotion. Immunohistochemistry showed almost all transduced cells were medium spiny neurons. **Conclusions:** We have shown motor symptoms in TH deficient mice are reversible by striatal injection of AAV-TH/GCH/AADC. This approach avoids the surgical risks associated with brain stem infusion. Transduced striatal neurons efficiently synthesize dopamine. These findings support the notion that AAV-mediated TH/GCH/AADC delivery could be a promising therapy for individuals suffering from TH deficiency.

550 GABAergic-Selective AAV Gene Therapy for SLC6A1-Related Disorder

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SLC6A1-related disorder is a severe developmental epileptic encephalopathy with an urgent need for an innovative disease-modifying therapy. The disease is caused by the loss of the primary GABA transporter in the brain, GAT1. Endogenous GAT1 neuronal expression is restricted to inhibitory neurons and promotes the clearance of GABA from synaptic and extra-synaptic sites. AAV-mediated gene delivery of *SLC6A1* could provide a one-time curative treatment to patients that suffer from this disease, yet the importance of targeting expression to defined brain cell types is unknown. Here we developed AAV-based therapeutics to express human *SLC6A1* either selectively in inhibitory neurons or in all neurons to test if neuronal subclass-selectivity is essential for the safe rescue of phenotypes. We found that inhibitory neuron-specific expression was better tolerated and reversed major disease-relevant phenotypes, including GABA uptake activity, inhibitory current kinetics, EEG epileptiform activities, seizures, motor dysfunction, and cognitive deficits. In contrast, pan-neuronal expression of *SLC6A1* resulted in increased mortality, significant side effects, and very limited phenotypic rescue. Thus, our findings suggest that the

inhibitory-circuit selective expression of *SLC6A1* is necessary for safe and efficacious gene therapy for *SLC6A1*-related disorder.

551 A Platform for Engineered Cross-Correction of AAV-Expressed Transgenes

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A limitation of many AAV gene therapy approaches is insufficient transduction of target cells and tissues to achieve the necessary therapeutic effect. In some cases, this limitation can be mitigated through capsid engineering and/or increased AAV dosing; however, certain tissues and cell types remain difficult to efficiently transduce. Moreover, increased AAV dosing presents both safety risks and increased manufacturing costs.

To address this challenge, we took inspiration from proteins which are naturally "cross-corrective"; that is, which are produced and secreted from one cell and taken up to exert their action in another. For example, the Arylsulfatase A (ARSA) enzyme - which is implicated in Metachromatic Leukodystrophy (MLD) - is naturally secreted into the extracellular matrix where it is taken up by neighboring and distant cells via the mannose 6-phosphate receptor (M6PR). We have shown that the recombinant human protein can leverage normal ARSA secretion and reuptake mechanisms, facilitating enzyme spread to secondary, non-transduced cells. This results in "cross-correction" in a broad range of cells, including oligodendrocytes and microglia (which are not efficiently transduced in vivo by AAV vectors).

To engineer cross-correction, we first carried out in silico screens to identify possible signal peptides (SPs) for efficient protein secretion as well as efficient cleavage of the SP. In one example, SP optimization of an AAV-delivered human GBA1 transgene (implicated in diseases like GBA-PD and Gaucher Disease) resulted in greatly enhanced GCase protein spread compared to the footprint of AAV-transduced cells; like ARSA, this protein can then be taken up by non-transduced cells, resulting in a functional restoration of enzyme activity in non-transduced cells in vivo. In cases where the secreted protein is not naturally taken up by cells, further modification via the incorporation of a cell penetrating peptide (CPP) may be used to enable uptake. For example, SP- and CPP-modified frataxin protein (for the treatment of Friedreich's Ataxia) demonstrated cross-correction and evidence of cellular uptake across tissue types. Together, these results highlight the exciting potential of engineered cross-correction to overcome AAV transduction limitations across a range of indications.

552 Gene Replacement Therapy for TECPR2-Related Hereditary Sensory and Autonomic Neuropathy

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Background
TECPR2-related hereditary sensory and autonomic neuropathy with intellectual disability (HSAN type 9) is a rare autosomal recessive disorder caused by biallelic pathogenic variants in the gene named TECPR2 (tectonin beta-propeller repeat containing 2). The TECPR2 gene encodes a protein crucial for autophagy regulation, sensory processing, and motor coordination. Clinical manifestations include developmental delay, motor deficits, and autonomic dysfunction, with limited life expectancy. Despite the critical impact of this disorder, no disease-modifying therapies are available, highlighting the need for innovative therapeutic interventions.

Aim
These studies aimed to investigate the feasibility, safety, and effectiveness of adeno-associated viral 9 (AAV9)-mediated TECPR2 gene replacement therapy in a preclinical mouse model.

Methods
For the efficacy study, the AAV9/TECPR2 vector was administered to TECPR2 knock in (KI) and WT mice at 7–10 days (P7-10) or 0-2 days (P0-2) of age. Mice were administered with vehicle or a low (1.25e11 vg), middle (2.5e11 vg), or high (5e11 vg) dose of AAV9/TECPR2 via intrathecal (IT) injection. Efficacy was assessed using body weights, survival and behavioral tests. Behavioral tests including startle response, hot plate, open field, rotarod, hindlimb clasping, and nest building were performed at 3-, 6-, and 9-months post-injection. For the safety study, AAV9/TECPR2 was administrated to WT mice at P7-10 and animal safety was monitored through serum toxicology panels, histopathological and survival analysis.

Results
No obvious adverse effects in body weight and survival were observed in efficacy groups. In behavior testing, p7-10 treatment demonstrated a dose-dependent rescue in startle response. Temperature hypersensitivity at 6-month post injection was rescued with high dose treatment at P7-10. In the open field test, distance and the time spent in the center parameters were partially rescued. Increased front paw lifted times and decreased front right luminance were partially rescued dose dependently. Interestingly, in the mouse cohort treated at P0-2, less therapeutic benefits were observed. No significant adverse effects in body weight, survival, or histopathologic analysis were observed in the safety study.

Conclusion
AAV9-mediated TECPR2 gene replacement therapy shows some promise as a therapeutic approach for TECPR2-related HSAN type 9. Both the efficacy and safety results highlight the potential for clinical translation.

553 Gene Replacement Therapy for MPS IIIC with scAAV9/HGSNAT Vector

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Background:
Mucopolysaccharidosis type IIIC (MPS IIIC), or Sanfilippo syndrome type C, is an ultra-rare autosomal recessive lysosomal storage disorder caused by mutations in the heparan-α-glucosaminide N-acetyltransferase (**HGSNAT**) gene. This results in lysosomal accumulation of heparan sulfate, leading to progressive neurodegeneration. Clinical manifestations include neuropsychiatric symptoms, developmental delay, and childhood dementia, with most patients succumbing to the disease before adulthood. Current treatment options remain palliative, highlighting the urgent need for therapeutic strategies to address the **HGSNAT** enzyme defect.

Objective:
This study aimed to evaluate the efficacy and safety of an intrathecally delivered **scAAV9/HGSNAT (JLK-247**) vector to treat MPS IIIC in a mouse model.

Methods:
The gene therapy vector was administered to **HGSNAT P304L** mutant mice at postnatal 7–10 days (early dosing) or 150 days (delayed dosing) of age. Efficacy was assessed using behavioral tests, enzymatic activity assays, and histopathological analyses. Safety was monitored through serum toxicity panels, organ weights, and survival analysis.

Results:
Early dosing rescued the urinary retention phenotype significantly better compared to delayed treatment, with a partial restoration of **HGSNAT** enzymatic activity in key organs. Improved survival was noted in treated mice, primarily driven by the need to euthanize the untreated mice for animal welfare due to extreme urinary retention. In behavior testing, impaired novel object recognition was rescued in treated mice irrespective of their age at dosing. No significant adverse effects in body weight, survival, or open-field behavior were observed in **JLK-247** treated mice irrespective of their age at dosing.

Conclusion:
The findings highlight that CNS-directed gene replacement therapy with **JLK-247** is a promising strategy to treat MPSIIIC. Further studies will assess postmortem histological and biomarker changes in these mice. GLP toxicology studies will assess the safety of **JLK-247** to advance this experimental gene therapy approach towards clinical application.

554 Novel Peptide Therapy Targeting Pro-Inflammatory Microglia for Neonatal Hypoxic-Ischemic Encephalopathy in Mice

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Introduction: Hypoxic-ischemic encephalopathy (HIE) is a neonatal brain disorder caused by perinatal complications such as placental abruption. Neonates born with HIE are at high risk of severe neurological and developmental impairments; however, no effective treatments are currently available. HIE is aggravated by intracerebral inflammation caused by pro-inflammatory microglia. We investigated the therapeutic effects of an apoptotic peptide on HIE due to depletion of pro-inflammatory microglia.

Methods: MG1 homing peptide, which selectively targets and binds pro-inflammatory microglia, was conjugated with the pro-apoptotic peptide $_{D}$[KLAKLAK]$_2$ (KLA), resulting in MG1-KLA. The therapeutic effects of MG1-KLA for HIE were examined *in vitro* and *in vivo*. We administered MG1-KLA into pro- and anti- inflammatory microglia derived from primary cultures of microglia, and examined binding ratio, cell viability and apoptosis assay. Next, HIE model mice were induced in P9 (postnatal day 9) , which corresponds to full-term birth in human, using carotid artery ligation followed by hypoxia. We performed intracerebroventricular (ICV) injection of MG1-KLA or phosphate-buffered saline (PBS) 24 hours after-hypoxic ischemic surgery, and evaluated the therapeutic effects. After conducting behavioral tests such as rota-rod test and open field test on P23–25, histological analysis using MAP2 staining was conducted on P26 to evaluate tissue damage. Additionally, histological analysis using IBA-1 and CD86 was performed on P12 to evaluate pro-inflammatory microglial accumulation, and biochemical analyses with quantitative polymerase chain reaction (qPCR) were performed on P10, 8 hours after ICV injection, and on P11 to examine pro- and anti-inflammatory cytokine changes over time.

Results: MG1-KLA selectively bound to 81.8% of pro-inflammatory microglia and decreased cell viability in a dose-dependent manner. Annexin V apoptosis assay showed that MG1-KLA induced apoptosis in 24.2% of pro-inflammatory microglia. The effect of MG1-KLA was less shown on anti-inflammatory microglia. Next, HIE mice treated with MG1-KLA showed significant improvement in the rota-rod test and open field test, and MAP2 staining results showed significantly reduced brain atrophy compared with the PBS-administered non-treated mice with HIE. MG1-KLA administration improved HIE-induced neuronal and behavioral dysfunction in mice. Furthermore, IBA-1 and CD86 staining showed that accumulation of pro-inflammatory microglia in ipsilateral side of MG1-KLA treated mice was suppressed compared with PBS-administered non-treated mice. The results of qPCR showed that pro-inflammatory cytokines such as TNF-α and IL-1β were suppressed in the ipsilateral hemisphere of MG1-KLA treated mice compared with PBS-administered non-treated mice. This cytokine suppression corresponded with decreased microglial activation.

Conclusions: MG1-KLA selectively depleted pro-inflammatory microglia both *in vitro* and *in vivo*. These findings suggest that the novel MG1-KLA peptide therapy has a high potential for treating neonatal HIE through the selective induction of apoptosis in pro-inflammatory microglia.

555 Extracellular Vesicle Trafficking of Gangliosides Boosts rAAV-Based Correction for GM3 Synthase Deficiency

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GM3 synthase deficiency (GM3SD) is a severe infantile epilepsy syndrome with debilitating neurological phenotypes. GM3SD is a monogenic disorder that is caused by mutations in the *ST3GAL5* gene, permitting rAAV-based gene therapy as a promising approach. Using a neuron-specific promoter to drive *ST3GAL5* expression in an AAV9 vector, we have successfully restored both cognitive and motor functions in mouse models for GM3SD. Immunofluorescence staining showed widespread correction of gangliosides across the cortex beyond what is typically achieved by AAV9-based vectors. Widespread expression of gangliosides across the brain suggested that some brain structures may be cross-corrected following gene therapy. We hypothesize that gangliosides use extracellular vesicles (EVs) as a mechanism to cross-correct cells that are not directly transduced by gene therapy vectors. Tunable resistive pulse sensing (TRPS) quantification of EV size and concentration showed that *ST3GAL5* expression influences EV biogenesis. Specifically, loss of *ST3GAL5* resulted in smaller vesicles and lower production of vesicles overall. This result suggests that *ST3GAL5* expression may influence the overall lipid profile of EV membranes. LC/MS measurement of ganglioside content in EVs also confirm that GM3 and GD1a/b gangliosides are present in EVs isolated from brains. The presence of simple and complex gangliosides in these EVs allows them to be viable vehicles for ganglioside trafficking, which enables the EVs to correct nearby cells and other brain structures. Additional experiments to confirm our findings will allow research into increasing rAAV efficiency for the treatment of GM3SD and other neurological disorders.

556 Harnessing AAV-RNAi Technology for the Treatment of Neurodegenerative Diseases

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Neurodegenerative proteinopathies including Huntington's, Parkinson's, and Alzheimer's disease face significant unmet medical needs. These disorders are driven by the accumulation of toxic proteins, and the structural heterogeneity of protein aggregates complicates therapeutic targeting. RNA interference (RNAi) provides a straightforward method to overcome this complexity, reducing all toxic protein isoforms with a single drug for each proteinopathy. Here, we present three examples of adeno-associated virus (AAV)-mediated RNAi for the sustained reduction of pathogenic proteins, illustrating a platform with transformative potential for neurodegenerative proteinopathies. By employing artificial microRNAs (amiRNAs) against specific targets of interest, AAV-RNAi delivers precise, scalable, and durable gene silencing to address the underlying genetic and molecular pathologies of these diseases.

Huntington's disease (HD) is a fatal, monogenic neurodegenerative disease caused by expanded trinucleotide repeats in the huntingtin gene (*HTT*) resulting in the accumulation and aggregation of huntingtin protein and subsequent neurotoxicity. Partial suppression of *HTT* mRNA in the brain has been shown to be safe and efficacious in animal models, validating *HTT*-lowering as a therapeutic strategy agnostic to allele type. We designed amiRNAs targeting *HTT,* implementing a novel optimization workflow to resolve liabilities associated with inefficient amiRNA strand processing. Potent amiRNAs were identified with robust *HTT* lowering *in vitro* and *in vivo* in a rat model of HD highlighting the promise of AAV-RNAi for the treatment of HD.

Synucleinopathies are a family of diseases, including Parkinson's disease and multiple system atrophy (MSA) characterized by the accumulation of alpha-synuclein protein. Using a brain-penetrant AAV capsid, 50% wild-type *Snca* knockdown in the pre-formed fibril mouse model prevented alpha-synuclein pathology, protected dopaminergic neuron in the substantia nigra, and halted the spread of pathological aggregates. Potency of our gene

therapy candidates was improved through amiRNA concatenation, showcasing scalable design improvements and clinical translation potential.

Similarly, for tauopathies such as Alzheimer's disease and progressive supranuclear palsy (PSP), targeting microtubule-associated protein tau (*MAPT*) with AAV-RNAi reduced tau aggregates, phospho-tau species, and neurodegeneration biomarkers in a tauopathy mouse model. Off-target effects were minimized through rigorous *in vitro* and *in vivo* screening, identifying a lead amiRNA with a wide therapeutic window that achieved clinically relevant target knockdown with no off-target effects. Pharmacology studies in nonhuman primates further demonstrated proof-of-concept for neuronal *MAPT* knockdown.

These studies underscore the versatility and transformative power of AAV-RNAi technology for the treatment of neurodegenerative proteinopathies. Ongoing advancements in vector engineering and capsid optimization, supported by robust *in vivo* preclinical validation, promise to accelerate clinical translation and bring hope to patients facing devastating neurodegenerative diseases.

557 A Computational Model Predicts Treatable Huntington's Disease Patient Population and Minimum Therapeutic Efficacy Requirements for Gene Therapies Targeting Somatic Instability

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Precise delineation of a treatable patient population is a challenge across indications targeted by gene therapies. Because effective gene therapy intervention requires a viable target cell population, patients must be treated sufficiently early in disease prior to irreversible cell damage or loss. Therapeutic outcomes are determined by the number of viable target cells and efficacy characteristics of the therapy (transduction rate, and transgene functionality). This complex relationship between disease, patient, and therapeutic intervention defines clinical success or failure. Therefore, enrollment of patients who are too advanced in disease may doom an efficacious therapy in clinical trials. This challenge is particularly apparent in Huntington's disease where somatic instability (SI), the stochastic increasing of CAG repeat length in medium spiny neurons, advances with patient age and underlies cellular health and death.

Somatic instability (SI) in the mutant HTT allele is a high value therapeutic target for Huntington's disease (HD). However, rates of SI and the speed of HD progression vary widely with inherited germline CAG repeat lengths across the HD patient population. Understanding this variability and projecting the treatable range of patients for novel SI-directed therapies is critical to their design, preclinical development, and for conferring optimal benefit to HD patients.

Latus has developed a computational model that tracks the percentage of healthy medium spiny neurons (MSNs) across the lifetime of HD patients; the model further predicts changes in MSN survival based on a SI-directed interventional gene therapy. Leveraging the previously reported rates of SI in human MSNs (Handsaker et al., 2025), and inputs describing therapeutic efficacy, the model projects the range of patients treatable using LTS-201 - an MSH3-targeting gene therapy preclinical candidate in development by Latus Bio.

The model predicts that for a hypothetical patient, presenting with a germline CAG length of 40, treated at age 30, a SI-targeting gene therapy with conservative efficacy characteristics achieves substantial therapeutic benefit: a 15 year delay in the onset of motor changes, and 12 and 15 year delays in the onset of HD-ISS Stage 2 and Stage 3, respectively.

To evaluate the treatable range of HD patients, the model has been expanded to predict therapeutic benefit across a spectrum of germline CAG lengths and ages. This analysis predicts that LTS-201 could provide a minimum therapeutic benefit of > 5 years in delay to onset of motor symptoms for the majority of HD patients and that it has the potential to confer the greatest benefit when delivered early, prior to the onset of motor symptoms.

The flexible framework provided by this computational model enables therapeutic performance prediction across the HD patient population and serves as a valuable tool for defining the target populations for SI-directed therapies.

558 Long term safety and efficacy of BGT-DTDS in the DAT-knockout mouse model of Dopamine Transporter Deficiency Syndrome

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Dopamine transporter deficiency syndrome (DTDS) is an inherited, life-limiting childhood neurotransmitter disease caused by biallelic loss-of-function mutations in the solute carrier family 6 member 3 (*SLC6A3)* gene encoding the dopamine transporter (DAT), integral to dopamine homeostasis and neurotransmission. There is an unmet clinical need for paediatric patients presenting with a progressive parkinsonism-dystonia to adults with early-onset Parkinson's disease, who do not respond to currently available medical or neurosurgical treatments. DTDS is likely misdiagnosed as cerebral palsy and underreported worldwide. We previously reported our gene therapy development for DTDS in patient-derived iPSC neuronal model and DAT-knockout (DAT-KO) mice to 3 months post gene therapy.

In the present study, we assessed the long-term efficacy and safety of BGT-DTDS (AAV2.SLC6A3*)* gene therapy: an AAV2-mediated gene therapy delivered to the midbrain of DAT-KO mice to model clinical delivery application. We observed a dose-dependent therapeutic effect demonstrated by normalisation of locomotor behaviours in open field, vertical pole and foot fault tests. Brain immunohistochemical analysis showed sustained DAT expression in the midbrain of treated animals. Neuropathology analysis at 12 months post gene therapy demonstrated safety and no neurotoxic effects with Hematoxylin & Eosin, Nissl, and glial fibrillary acidic protein (GFAP) staining. Our data affirms that stereotactic midbrain-targeted BGT-DTDS gene therapy provides long-term correction of DAT function and safety in a clinically relevant animal model of DTDS. The parallel success of a similar gene therapy approach for aromatic L-amino acid decarboxylase (AADC) deficiency further corroborates the promise of this approach. With this body of preclinical data, BGT-DTDS received Orphan Drug Designation from the European Commission (EC) and the U.S. Food and Drug Administration (FDA) as well as Rare Pediatric Disease Designation (RPDD) from the FDA for the treatment of DTDS and provides further support for clinical trial application.

559 Cross-Species BBB-Penetrant IV-Delivered AAV Gene Therapy Provides Broad and Robust CNS Tau Lowering in Tauopathy Mouse Models and Non-Human Primate

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Progression and spread of tau pathology in the human brain correlates well with cognitive decline in Alzheimer's disease (AD). Pan-tau lowering in the central nervous system (CNS) was recently shown to have favorable clinical

outcomes for the treatment of AD. However, anti-sense oligonucleotides which reduce tau expression and tau pathology in both preclinical and clinical studies require repeated administration via intra-ventricular or intrathecal administration. In contrast, a single intravenous (IV) administration of a blood-brain barrier (BBB)-penetrant, self-complementary adeno-associated virus (scAAV) gene therapy can durably and effectively reduce CNS levels of human tau in tauopathy mouse models, which we previously reported (Bao et al, #1602, ASGCT 2024).

In the present study, we utilized a second-generation, cross-species BBB-penetrant TRACER™-derived capsid to intravenously deliver a vectorized primary artificial microRNA (pri-amiRNA) containing a potent tau siRNA, VY-1706, in tauopathy mouse models as well as non-human primates (NHP). In addition, to further optimize our AAV gene therapy targeting tau, pri-amiRNA driven by two different promoters with distinct cellular tropisms were evaluated head-to-head in rodent and NHP pharmacology studies to compare tau lowering. The results demonstrated a dose-dependent increase in vector genome (VG) and concomitant tau mRNA knockdown, in multiple brain regions of VY-1706 treated hTau and P301S mice at 8 weeks post-injection. In hTau mice, which express all six human isoforms, tau mRNA levels were reduced by 60-80%, and total tau protein levels decreased by 70-80% in the high dose group compared to the vehicle-treated controls across multiple brain regions. Similarly, in P301S mice, a disease model carrying a human tau mutation, reductions in tau mRNA and total tau protein levels in the high-dose group were comparable to those observed in hTau mice within the same brain regions. Additionally, pathological tau protein levels were reduced by up to 80% in these P301S mice, highlighting the promising efficacy of this approach. In the NHP study, broad biodistribution of vector genomes was observed in multiple critical AD related brain regions. Tau mRNA levels were reduced by greater than 50%, and total tau protein levels were reduced by 40-55% at 11 weeks post-injection. In addition, there was no associated perturbation of the endogenous microRNA transcriptome. Importantly, treatment with VY-1706 was well-tolerated, with no notable test article related adverse effects. The overall pharmacological activity and safety profiles were similar between the two promoters driving the pri-amiRNA in both rodent and NHP studies. These findings demonstrate that the combination of a potent and well-tolerated tau targeting pri-amiRNA with a novel cross-species BBB-penetrant capsid holds promise as a one-time, IV treatment option for AD and other tauopathies.

560 Selective targeting of trans-synaptic tau using chimeric antibodies receptors tethered to the synaptic membrane

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Trans-synaptic transmission of pathological tau contributes to the spread of tau aggregates and neurofibrillary formation in Alzheimer's disease (AD). Reducing the prevalence of pathogenic tau species within the synaptic cleft may therefore prevent tau propagation and attenuate AD progression. Passive immunotherapy using monoclonal IgG antibodies has been used to target extracellular tau, however these therapies have not achieved clinical success suggesting a failure to target propagating tau species. Additional challenges such as antibody selectivity towards spreading species, availability within the CNS and physical limitations in accessing the narrow synaptic cleft may further prevent immunotherapies from accessing and identifying trans-synaptic propagating tau. To address these limitations, we purified synaptosomes from AD and pathologically negative control patients and used phospho-proteomics to identify tau epitopes present in the AD post-synaptic terminals. Comparison of the AD and normal post-synaptic fractions identified 17 phospho-tau epitopes enriched in the AD fraction, including the PHF1 tau epitope pS396/pS404. Using this information, we developed a novel approach to increase effective anti-tau antibody concentration at the synapse by using AAV9 to deliver a tethered synaptic-chimeric antibody receptor (synCAR) to the post-synaptic membrane. To achieve this aim, we fused the PHF1 small antibody fragment (scFv) with the post-synaptic membrane component neurolignin-1 (NLGN1). Using both murine and hiPSC neuronal models, we show that our PHF1 synCAR effectively expresses at the synaptic membrane and localizes with the post-synaptic marker PSD95 without impacting neuronal viability. Finally, we show that PHF1 synCAR effectively targets pathogenic tau species by observing that tau aggregation in seeded mouse primary neurons increased by ~73%. This indicates that PHF1 synCAR captures and concentrates tau seeds resulting in accelerated seeding pathology. Overall, these data

suggest a novel mechanism for targeting proteins either in the synaptic cleft or within the post-synaptic membrane and provides an effective approach for isolating and identifying propagating tau species.

AbbVie Disclosure Statement:

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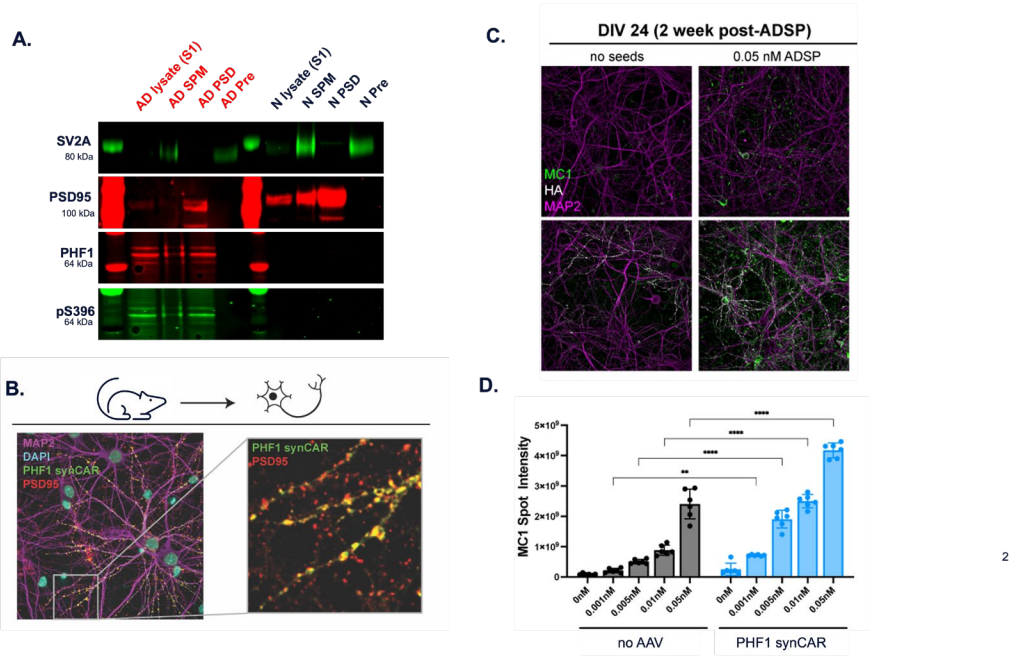

2

561 Tuberin Replacement Therapy in Cultured, Patient-Derived Neural Progenitor Cells and a Cerebral *in vivo* Mouse Model of Tuberous Sclerosis Complex Type 2

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Tuberous sclerosis complex (TSC) is an autosomal dominantly inherited neurologic disease caused by loss of function of tuberin or hamartin. Affected individuals inherit one defective allele of either the *TSC2* or *TSC1* gene, respectively, with a somatic mutation in cells in a susceptible tissue during development or later life leading to benign tumors (hamartomas) and focal dysplastic lesions affecting the brain, skin, eye, heart, lungs, and kidney. Most serious consequences result from second hits in the brain leading to cortical tubers, subependymal nodules, subependymal giant cell astrocytomas, demyelination, astrogliosis, and epilepsy. There has been some success in treating neurologic and non-neurologic manifestations in symptomatic TSC patients with inhibitors of the mammalian target of rapamycin (mTOR) signaling pathway such as sirolimus/everolimus (rapalogs). Rapalogs have to be continuously taken, compromise immune functions, may cause psychobehavioral problems and are less effective in treating epilepsy than other symptoms. While ketogenic diet and treatment of infantile spasms with vigabatrin can attenuate seizure burden, treatment-resistant seizures remain the major disease burden for patients throughout their life despite the current standard of care. In a previous study we showed that systemic delivery of an adeno-associated virus (AAV) vector (serotype 9) encoding a condensed form of human tuberin (cTuberin) in a

cerebral mouse model of *TSC2* led to extended lifespan and reduction in brain pathology, including normalization of subependymal overgrowths with a decrease in cell proliferation around the ventricles and reduced neuropathology.

Here we show that in these cerebral TSC2-KO mice, AAV-cTuberin rescues ventricular overgrowths and demyelination by magnetic resonance imaging (MRI), as well as decreased proliferation of subventricular cells, reduced size of neurons, and pS6 staining intensity by immunohistochemistry. Chronic *in vivo* electrocorticography (ECoG) recordings revealed that both AAV-cTuberin therapy and everolimus reduced seizures in this animal model, but gene therapy was more effective in normalizing the interictal ECoG in mice, compared to everolimus. These findings support use of AAV-cTuberin as gene replacement to increase survival, normalize brain morphology and reduce seizures in the cerebral TSC2 mouse model.

562 AAV-SynCav1 Administration to the Hippocampus of Cuprizone Mouse Model of Multiple Sclerosis Preserves Hippocampal Myelination

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Introduction: Multiple Sclerosis (MS) is a chronic autoimmune demyelinating disease of the brain and spinal cord that causes axonal injury and cerebral atrophy. There exist few interventions that specifically target neuroprotection, neurorepair, and remyelination. Cuprizone ingestion in mice induces white matter pathology similar to MS lesions and thus serves as an excellent model to test potential remyelinating therapies. Six weeks of cuprizone (0.2%) diet results in demyelination of the corpus callosum (CC) and hippocampus (Hpc). Withdrawal of cuprizone results in early-stage re-myelination at 2-3 weeks. We have previously shown that brain delivery of the membrane/lipid raft (MLRS) scaffolding protein caveolin-1 (Cav-1) using a neuron-specific synapsin promoter (synapsin-Cav-1, SynCav1) increased hippocampal axonal myelination of CA3 Schaffer collaterals and augmented cognitive function in aged mice. Moreover, when delivered to the brain of Alzheimer's disease (AD, PSAPP) mice, SynCav1 preserved hippocampal axonal myelination, dendritic arborization, synaptic ultrastructure, and hippocampal-dependent fear conditioning (FC) learning and memory. These findings provide strong support for the potential therapeutic applicability of *SynCav1* in treating de-myelinating neurodegenerative diseases like MS. Current study tested AAV.CAP-MAC, an engineered variant that achieves broad CNS biodistribution in multiple non-human primate species.

Methods: C57Bl/6 mice at the age of 8-week were fed cuprizone diet (0.2%) for 6 weeks. Mice were randomly allocated to normal diet group, cuprizone exposure (CE) sham group, and CE+ SynCav1 group. For CE sham group and CE+SynCav1 group underwent surgery 1 week prior to the CE initiation. 3.0 µL of either 0.9% saline or CAP.MAC SynCav1 was injected at 6 locations (3 locations/hippocampus). The tissue collection was performed at the age of 14 weeks. For the CE+ SynCav1 group, the 2.1E10 (genome copies, g.c.)/animal were given. Fear conditioning (FC) test was performed at the end of diet to evaluate memory function. After CE time point mice were euthanized and the brains were harvested. Black Gold staining was used for myelin evaluation for the hippocampus. **Results**: There was no difference in FC learning (Day 1) between the normal diet group and the CE sham group. In both Contextual recall (Day 2) and Auditory-cue recall (Day 3), the freezing time (%) were significantly lower in CE sham group with the p-value of 0.02 and 0.03 (Unpaired t-test), respectively. Black-Gold myelin histology analysis showed significant decreased signal in the CE hippocampus compared to normal diet ($p < 0.0001$, Unpaired t-test). In FC, CE impaired both context ($p=0.058$) and cue (Figure 1) memory recall. In CE mice that were pre-treated with SynCav1, Black-Gold staining showed obvious myelinated fibers in the hippocampus when compared to the untreated CE mice (Figure.2 A-C). Black Gold density analysis showed significant increased myelin signal in the hippocampus of SynCav1-treated CE mice (mean: 7.4) compared to untreated CE mice (mean: 2.5, Unpaired t-test) (Figure 2D)

Conclusion: Cuprizone diet for 6 weeks induces prominent hippocampal demyelination and resultant fear memory deficits. Direct hippocampal gene delivery of SynCav1 preserved hippocampal myelin in cuprizone mice, suggesting that SynCav1 may be an effective gene therapy to mitigate neurodegeneration in MS.

Figure.1

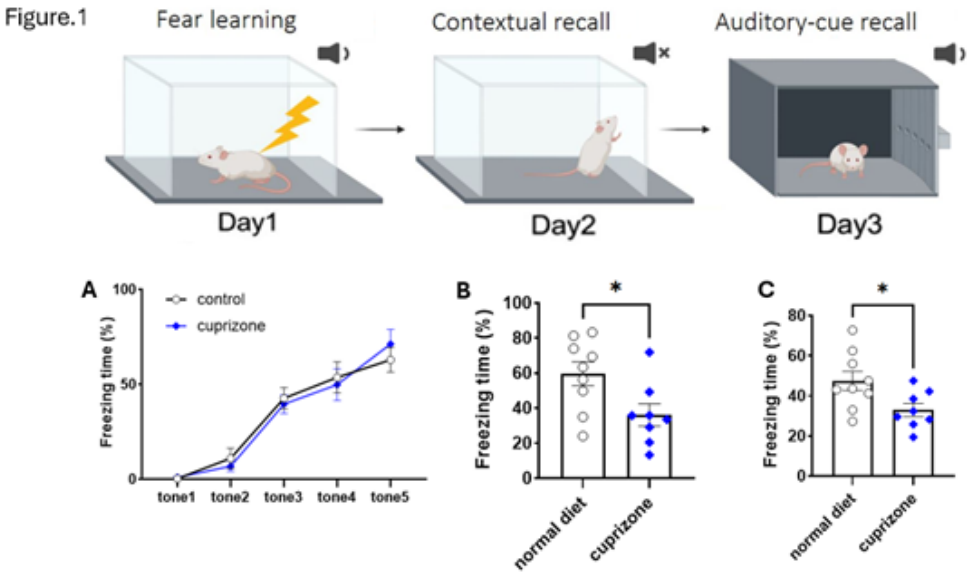
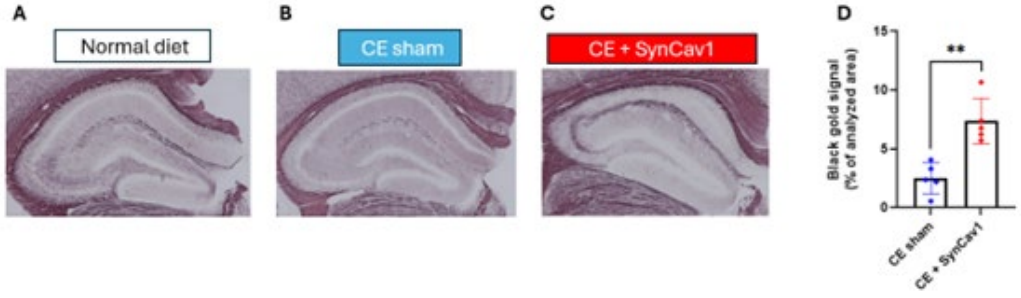


Figure.2



563 Adeno-Associated Virus Based Therapy to Treat Disease and Reduce Demyelination in an Animal Model of Multiple Sclerosis

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Multiple Sclerosis (MS) is the prototypical neuro-inflammatory demyelinating disease. In MS, inflammation can result in the development of focal lesions, axonal damage, and eventual axonal death resulting in progressive disability. However, there are currently no clinically available treatments specifically for combating demyelination. Thus, current MS treatments only effectively treat a portion of the disease.

Remyelination is the regenerative process where new myelin sheaths are formed and can result in the restoration of axonal function and prevent axonal death. Within MS, remyelination occurs but is inefficient and ultimately fails in most lesions. Thus, induction of remyelination represents an effective treatment modality for MS, either alone or in conjunction with current therapies.

Astrocytes have been identified as key players in the demyelinating lesion. These cells are responsible for creating an inflammatory environment conducive to demyelination, suppress remyelination by inhibiting oligodendrocyte progenitor cells (OPCs) proliferation and differentiation, show increased GFAP expression, and form glial scars.

Other than astrocytes, OPCs and microglia are considered the two other major cell types directing the demyelinating lesion. Astrocytes are unique in that they can affect both OPCs and microglia in a paracrine fashion through the secretion of neurotrophic factors, cytokines, and other molecules. Thus, it is our opinion that astrocytes represent an ideal cell target to induce remyelination.

Adeno-associated virus (AAV) serotype 9 (AAV9) represents a novel platform to develop a therapy for demyelination as it crosses the BBB and provides widespread biodistribution within the central nervous system (CNS). Though peripheral injections suffer from inefficient BBB crossing, poor CNS transduction, and off-target transduction/expression they are preferred to direct injections as direct injections are invasive and provide less than ideal biodistribution within the CNS.

In this work we show that the AAV platform can be utilized to specifically target activated astrocytes for transgene expression in an animal model of MS. Further, we show that in our model a single i.v. injection of AAV results in robust transgene expression and transduction throughout the CNS of animals. Finally, here we show the first AAV vector to treat demyelination and reduce disease severity in an animal model of MS (Fig 1). The reduction in disease severity was mediated via down-regulation of cytokines transcription levels known to play a role in disease onset and severity (Fig 1A-C). Finally, we show that our vector is capable of significantly reducing the amount of demyelination that occurs in the corpus collosum of treated animals (Fig1D & E). This work represents the first step in developing an effective AAV therapy to treat demyelination in patients with MS.

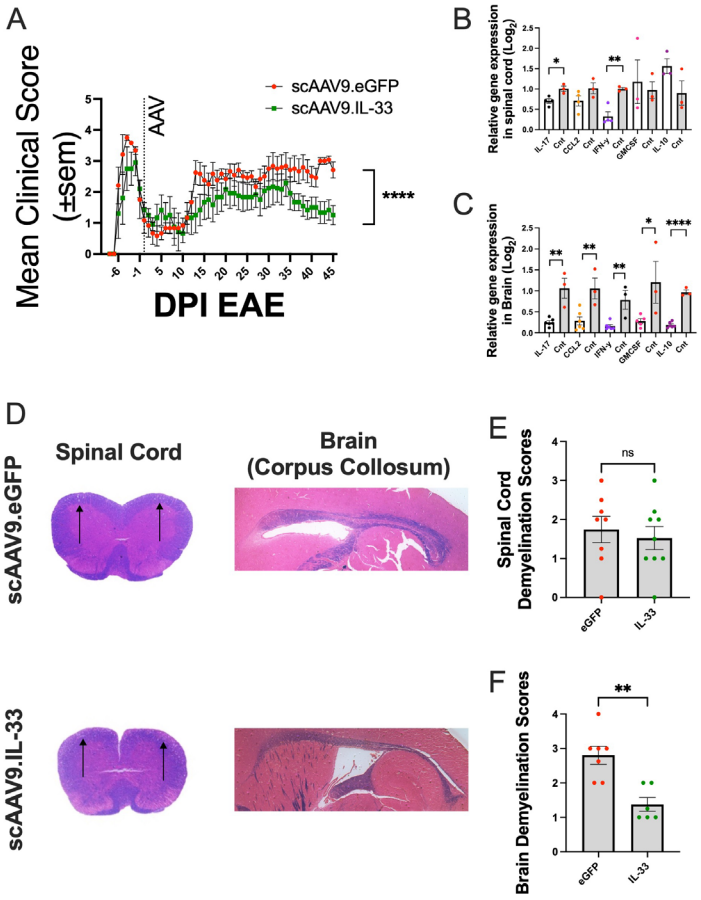

Figure 1: Therapeutic efficacy of IL-33. Either an scAAV9.IL-33 or scAAV9.eGFP vector was administered to animals suffering from RR-EAE. A) The scAAV9.IL-33 vectro resulted in reduced disease severity as compared to the control. B) scAAV9.IL-33 vector resulted in significant down-regulation of IL-17 and IFN-γ mRNA transcript in the spinal cord. C) scAAV9.IL-33 vector resulted in significant down-regulation of IL-17, CCL2, IFN-γ, GMCSF, and IL-10 in the brain. D) Luxol fast blue stains showing demyelination in spinal cord and brain. E) scAAV9.IL-33 vector did not result in significant differences in demyelination in the spinal cords of animals. F) scAAV9.IL-33 vector resulted in significantly less demyelination (lower demyelination scores) in the brains of treated animals as compared to controls. N=3-7, * ≤ 0.05, ** ≤ 0.005, ***≤ 0.0005, **** < 0.0001

564 *A 4-Week Study of Rodent Multiple Sclerosis (MS) Model*

Mele Avilla[1], Alexander Doering[2], Zachery Fetterhoff[3], Megan Rohde[3], Megan Speich[3], Megan Ender[3], Erick Gjertson[3], Stacey Schumacher[3], Nicole June[3], Susan Lynk[3], Vaithinathan Selvaraju[4], Aaron Peterson[3], Jay Herman[2], Brian McIntosh[2]

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Introduction: Multiple sclerosis (MS) is a debilitating, chronic, neurological autoimmune disorder affecting 1 in 3,000 people caused by the loss of myelin, and manifests in symptoms ranging from mild to severe paralysis. The primary event leading to symptomatic pathology is the infiltration of peripheral immune cells that have been primed against components of myelin sheath. Peripheral immune cells, primarily T and B lymphocytes and macrophages, infiltrate into the central nervous system (CNS) parenchyma leading to demyelination and neuroaxonal degeneration. The complex nature of MS makes it difficult to fully replicate the human disease. Therefore, the aim of our study was to establish a reproducible MS rodent that mimics serum activity and anatomic pathology of the human disease. We induced MS by administering C57BL/6J female mice with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG)/Complete Freund's Adjuvant (CFA) and pertussis toxin (PTX); which the result is an

experimental laboratory model of myelin destruction, capturing the pathophysiology of autoimmunity and neuroinflammation.

Methods: The CFA is a solution comprised of the antigen, MOG, emulsified in mineral oil composed of inactivated and dried mycobacteria (i.e. mycobacterium tuberculosis) and is used as an immunopotentiator (booster). Pertussis toxin is a toxin produced by the bacteria Bordetella pertussis and is used as an adjuvant to enhance the development of the disease by disrupting the blood-brain barrier and facilitating immune cell infiltration into the CNS; it helps trigger the autoimmune response in the MS model. The study was conducted for 4-weeks and entailed evaluating four groups: Groups 1 and 2 were the control groups and groups 3 and 4 consisted of the test groups. Test articles MOG/CFA and PTX dose levels were chosen to alter and/or reduce ambulation activities. Group 1 was treated with only PBS. Groups 2, 3, and 4 were treated with 200 µg of MOG/CFA; followed by 1x PBS treatment twice for Group 2; 50 ng of PTX twice for Group 3 or 100 ng of PTX twice for Group 4. 100 µL of MOG/CFA (1.0×10^6 ng/mL) or 1xPBS was subcutaneously injected twice on Day 1, followed by 100µL of PTX (500ng/mL or 1000 ng/mL) or 1x PBS intraperitoneally injected once on Day 1 and once on Day 2. To capture changes in locomotor activities, MotorMonitor (Kinder Scientific) was used to evaluate/characterize movement activities. Immunohistochemistry, histology, serum chemistry and cytokine analyses were also deployed to evaluate the MS model.

Conclusion: The development of a consistent rodent MS model, such as that evaluated in this study, will aid in evaluating new candidate cell or gene therapy products for efficacy and human safety. The overarching goal of providing this model to the community is aimed in providing the millions afflicted with MS potential for new autoimmune focused therapies and a healthier life.

565 Endo-Tek™: A Tissue-Engineered Alternative to DMEK/DSAEK for Corneal Endothelial Dysfunction

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Introduction: Corneal endothelial dysfunction is a leading cause of corneal transplantation worldwide, traditionally treated by deceased donor tissue transplantation methods such as Descemet Membrane Endothelial Keratoplasty (DMEK) and Descemet Stripping Automated Endothelial Keratoplasty (DSAEK). However, these procedures are limited by donor tissue shortages and associated complications. This study introduces Endo-Tek™, a tissue-engineered graft designed as an alternative to DMEK/DSAEK, utilizing *in vitro* cultured human corneal endothelial cells (HCECs) on a novel hydrogel scaffold.

Methods: Endo-Tek™ grafts were engineered using a transparent, thin collagen hydrogel combined with *in vitro* cultured HCECs. A molded design was developed and added to the scaffold to introduce curvature approximating the posterior surface of the cornea. Cell viability on the grafts was assessed using calcein-AM staining, and corneal endothelial markers were evaluated through immunofluorescence staining. In a feline model with induced corneal endothelial dysfunction, Endo-Tek™ grafts were transplanted to assess *in vivo* functionality. Postoperative outcomes, including corneal clarity and thickness, were monitored over 12 weeks using optical coherence tomography (OCT). *In vivo* confocal microscopy and histological analysis were performed on the enucleated eyes to confirm cell and graft attachment.

Results: The engineered hydrogel scaffold was approximately 15-25 µm in thickness and maintained a high transparency over 90% in the visible light spectrum. A confluent monolayer of HCECs on the Endo-Tek™ graft exhibited typical morphology and marker expression with a cell density of >3000 cells/mm^2. The hydrogel demonstrated superior mechanical properties, facilitating easy handling without breakage, and was compatible with loading into the Straiko-modified Jones tube for injection into the anterior chamber, reopening post-injection on every attempt. By week 6 post-transplantation, treated eyes exhibited substantial corneal clearing. Pachymetry indicated a reduction in corneal thickness towards baseline levels. *In vivo* confocal microscopy confirmed the presence of a healthy endothelial cell layer, and OCT, along with histological analysis, verified firm graft attachment

to the stroma with a graft thickness of less than 25 μm. Additionally, the curved design of the scaffold could potentially reduce detachment rates post-implantation.

Conclusions: Endo-Tek™ presents a promising tissue-engineered alternative to traditional DMEK/DSAEK procedures, addressing limitations related to donor tissue availability and surgical complications. The novel hydrogel scaffold's properties include optimal thickness, high transparency, mechanical strength, biocompatibility, and curved shape.

566 SELECTION OF QUALIFIED TREATMENT CENTRUM FOR CELL THERAPY

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Introduction: Unlike established pharmaceutical companies, start-ups lack local presence and structured launch activities, limiting awareness and patient access to treatments. In ophthalmology, Holoclar, a tissue-engineered therapy for moderate-to-severe limbal stem-cell deficiency (LSCD) due to trauma, is an example of a start-up innovation, which lacks wider acknowledgement. Holoclar can be administered only in qualified treatment centers (QTCs) per EMA and Transplantation Centre regulations. Selecting appropriate QTCs is critical to ensuring broad patient access, but challenges include complex certification processes and fragmented referral pathways. To ensure the best outcome for cell therapy, standardized processes need to be established, including the selection of patients based on complex eligibility criteria, the certification and qualification of the Holoclar® treatment center in line with the Transplantation Centre (TC) and EMA regulations and the standardization of biopsy, manufacturing and implantation processes with follow-up. At present, cornea trauma patient treatment is individual. These patients are treated at regional centers, their traumas and treatment outcomes are specific and their long-term tracking is individual. In most cases, patients are referred based on personal requests by attending doctors *via* an established personal contact with the doctor from the Qualified Treatment Center (QTC).

Methods: We mapped current practices to identify patient distribution and referral trends. Key criteria included patient volume, center expertise, specialization, infrastructure, and institutional support. Additional factors such as legal, financial, and logistical readiness were considered.

Results: From 10 centers, three were shortlisted: Prague VFN, Prague Vinohrady, and FN Brno. Despite a lower patient volume, FN Brno was ultimately selected due to its advanced qualification status, strong institutional support, and readiness to complete legal and financial agreements. To address Prague's higher patient concentration, a novel patient-doctor tandem model was implemented, facilitating treatment access at Brno QTC.

Conclusions: Effective QTC selection requires evaluating treatment capabilities, patient pathways, and institutional support. Strong center management and adaptability are essential for overcoming logistical and operational challenges. The structured coordination by Medasol facilitated a streamlined approach to patient access and treatment continuity.

567 Screening of Matrix Gla promoters to drive efficient expression of AAV-delivered Engineered Mechanosensitive Channel in trabecular meshwork for Enhancing Outflow Facility and lowering IOP in TGFβ2 Animal Model of Glaucoma

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The current standard of care for ocular hypertension associated with Glaucoma relies on daily eye drop medications to lower intraocular pressure (IOP), however, patients exert poor compliance. Previously, we have shown that the intracameral gene therapy using an engineered mechanosensitive channel (EMC) targeted to the trabecular meshwork (TM), provided a sustained IOP lowering through control of the outflow facility. Here, we present the optimization of the specific TM Matrix Glia Protein (MGP) promoter to enhance the EMC expression in human TM cells.

EMC was packaged in self-complementary (sc)AAV2 and produced using the triple transfection method. EMC expression in TM was driven by the Matrix Gla Protein (MGP) promoter upstream, while mCherry downstream served as a biomarker. MGP promoter was modified with proprietary elements, and five MGP promoter derivatives were assessed in NTM7820 cells (a TM cell-line). Our results show successful transduction of human TM cells by scAAV2-EMC, visualized by fluorescence microscopic imaging of mCherry reporter. MGP-3 and MGP-5 promoters were superior to wild-type MGP promoter in driving EMC expression.

Intravitreal Ad5-TGF-β2 (transforming growth factor beta) injection, known to increase IOP, was carried out in adult C57BL/6J mice. IOP was measured by TonoLab. Intracameral injection of scAAV2-EMC was carried out to determine changes in the aqueous outflow facility and IOP due to EMC transduction of TM. The AH outflow measurement was conducted using manometry in EMC-treated and control groups. Visually evoked potential (VEP) measurement was conducted to evaluate the effect of EMC treatment on the health of retinal neurons.

Intracameral delivery of scAAV2-EMC led to EMC expression in TM, lowered the elevated IOP, and restored the AH outflow in the TGFβ2 mouse model. VEP measurement showed a 50% higher amplitude in the scAAV2-EMC treated group as compared to the control.

The number of retinal ganglion cells (RGCs) was quantified by immunohistochemistry analysis using rabbit anti-RBPMS (RNA-binding protein with multiple splicing) and mouse anti-tubulin III on flat-mounted retinas. The observed higher survival of RGCs based on RBPMS and tubulin III labeling, suggests a neuroprotective effect on retinal ganglion neurons owing to reduced ocular hypertension.

Transduction of EMC, a pressure sensor channel, in TM restored its capacity to regulate IOP. EMC, upon activation by increased IOP, enhanced the outflow facility and facilitated pressure relief through the conventional TM pathway. The optimized MGP promoter should enhance the efficacy of the IOP-lowering EMC gene therapy.

568 Local delivery of inflammation-inducible vectorized anti-TNF biologics to treat non-infectious uveitis

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Introduction: Non-infectious immune-mediated inflammation of the eye (Uveitis) accounts for significant visual loss in adults. A key goal for immunotherapy is to minimize patients' systemic steroid use, which requires more selective, local and targeted approaches. Gene therapy using adeno-associated viral (AAV) vectors offers a step change opportunity to provide sustained and controlled depot delivery of therapeutics in the eye. Our preclinical evaluation targets a critical immune pathway, testing AAV constructs encoding anti-TNF biologics, given their tractable evidence of systemic efficacy in man.

Methods: AAV7m8 constructs, encoding anti-TNF biologics under control of constitutive (CMV) or inflammation-responsive (AP1—NFkB) promotors, were designed and produced. *In vitro*, HEK-293T cells were transfected [MOI 1E5vg/ml] to evaluate transgene expression (ELISA), inducibility (IL-1b stimulation) and bioactivity (HEK-Blue™TNF-α reporter assay). *In vivo*, therapeutic AAV vectors were injected intravitreally [2E9 vg/eye] in mice, with contralateral fellow eye receiving control AAV (NULL). Experimental autoimmune uveoretinitis (EAU), a preclinical model of uveitis was induced to assess inducibility and efficacy in individual eyes by imaging (fundus & optical coherence tomography) and FACS (CD45+ infiltrates).

Results: We were able to detect expression of clinically relevant anti-TNF biologics, including human TNFR1-IgG and Adalimumab, after AAV transfection of HEK-293T with both constitutive and inducible promoters. These products were demonstrated to be bioactive, neutralizing TNF mediated signalling and NFkB activation *in vitro*. *In vivo*, constitutive expression of a murine version of the TNFR1-IgG biologic, which potently neutralizes mouse TNF signalling, reduced clinical inflammation and immune cell infiltrate to the eye during peak disease of the EAU model. Furthermore, we demonstrate effective inducibility of our inflammation responsive promotor to express a bioactive anti-TNF therapeutic transgene, with detectable expression in ocular supernatants (retina and vitreous) at peak inflammation.

Conclusions: Intravitreal delivery of AAV permits expression of clinically relevant, bioactive anti-TNF biologics in mouse eye, and are efficacious in a preclinical model of uveitis. An inflammation responsive promotor driving expression of therapeutic transgenes in response to early clinical signs of inflammation offers a potential for regulated gene therapy, delivering targeted biologics within the eye as and when required during disease for uveitis treatment.

569 AAV-based Gene Replacement Therapy Improves Visual Behavior in a Murine Model of Usher Syndrome Type 1C (USH1C)

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**Corresponding Author*

Usher Syndrome (Usher) is the leading cause of combined deaf-blindness in the world. There are no current treatments available to address the visual loss in Usher. We created a mouse model of Usher that contains the *USH1C* c.216G>A (216A) mutation responsible for USH1C in patients. The 216A mutation produces truncated harmonin, a key scaffolding protein in the retina and inner ear, leading to dysfunctional photoreceptors and cochlear hair cells. We showed that antisense oligonucleotides targeting this mutation transiently improves gene and protein expression, and retinal function in USH1C mice. To improve upon these results, we developed a gene replacement strategy using adeno-associated virus (AAV) for long-term expression of USH1C in photoreceptors and restoration of visual function. Previously, subretinal injection (SRI) of AAV8-*Ush1c* resulted in transduction of photoreceptors within the injection bleb; however, visual function was not improved. Here, we aim to drive USH1C expression in photoreceptors within and beyond the surgical margins and confer long-term improvements in visual function and behavior using AAV44.9, a novel AAV vector capable of spreading from the site of injection throughout the retina. USH1C and control mice were treated by a single SRI at postnatal day 21 (P21) with AAV44.9(E531D)-hGRK1-*Ush1c-a1*-P2A-GFP vectors (5e11 vg/mL) or saline as vehicle. To assess the extent of the treated area, GFP expression was measured by fundoscopy, fluorescent confocal scanning laser ophthalmoscopy, and immunohistochemistry (IHC). Over a 1-year time-course, *Ush1c* transgene expression was measured using RT-PCR, and retinal function and visual behavior were assessed using electroretinography (ERG) and visual cliff test, respectively. On fundoscopy, AAV44.9(E531D)-mediated GFP expression was observed in >80% of USH1C and control eyes 8-weeks post-injection, and IHC analyses at 3-months-of-age showed transduction of primarily photoreceptors, with robust GFP signals in the inner/outer segments. At 3-months-of-age, RT-PCR analysis showed increased transgene expression in AAV-treated USH1C mice compared with saline-treated USH1C littermates. At 6-months-of-age, visual cliff analysis showed improved visual behavior in AAV-treated USH1C mice compared with saline-treated USH1C littermates. ERG analyses are currently underway. Mice are being aged to determine the

duration of treatment benefit. These results suggest the therapeutic potential of early AAV44.9(E531D)-*Ush1c* intervention to improve gene expression and visual behavior in USH1C mice.

570 Mini-PCDH15 Gene Therapy for Blindness in Usher Syndrome Type 1F

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Purpose: Usher syndrome 1F (USH1F), an autosomal recessive disorder caused by mutations in the *PCDH15* gene, is characterized by profound congenital deafness, balance dysfunction, and progressive vision loss due to retinitis pigmentosa. Cochlear implants can compensate for deafness but there are no therapies for blindness. Gene addition therapy is attractive, yet the *PCDH15* coding sequence of ~5.8 kb is too large to fit into a single AAV capsid. We engineered mini-PCDH15 genes in which 5 of the 11 extracellular cadherin repeats were deleted, and delivery by AAV to inner ears successfully rescued hearing in mouse models of USH1F. To evaluate the efficacy of mini-PCDH15 gene therapy to rescue blindness, we used zebrafish and mouse USH1F models.

Methods: With transposon insertion, we introduced *mini-pcdh15b* into USH1F zebrafish. Rescue of visual function was assessed with electroretinogram (ERG) and optokinetic reflex (OKR). Mini-Pcdh15b localization and photoreceptor morphology were analyzed and quantified with immunofluorescence and electron microscopy. In mouse experiments, we injected adult mice subretinally with AAV8-mini-hsPCDH15. Retinas were collected for histological analysis to evaluate the mini-gene expression and localization in photoreceptors.

Results: Untreated *Pcdh15b*-mutant zebrafish exhibited early and progressive defects in photoreceptor morphology and visual function: immunofluorescence and electron microscopy revealed abnormal calyceal processes and distorted outer segments in untreated mutants, and reduced ERG and OKR. In contrast, the treated group showed correct localization of the mini-Pcdh15b, preserved photoreceptor morphology, and improved vision with ERG and OKR restored to wild-type levels. In mouse, immunofluorescence of retinas injected with AAV8-mini-hsPCDH15 confirmed efficient expression and proper localization of mini-PCDH15 in photoreceptors.

Conclusion: *Mini-pcdh15b* expression restores vision in a zebrafish model of USH1F and mediates expression and normal localization of Pcdh15b in zebrafish photoreceptors. In mice, mini-hsPCDH15 is efficiently expressed and correctly localized in photoreceptors. These findings offer a promising approach for treating progressive blindness in humans with USH1F. More generally, they show that shortening proteins based on atomic structure may overcome the packaging limitations of AAV vectors to treat certain forms of vision loss.

571 AAV Compatible Delivery of Full-Length PCDH15 for Treatment of USH1F via RNA End Joining

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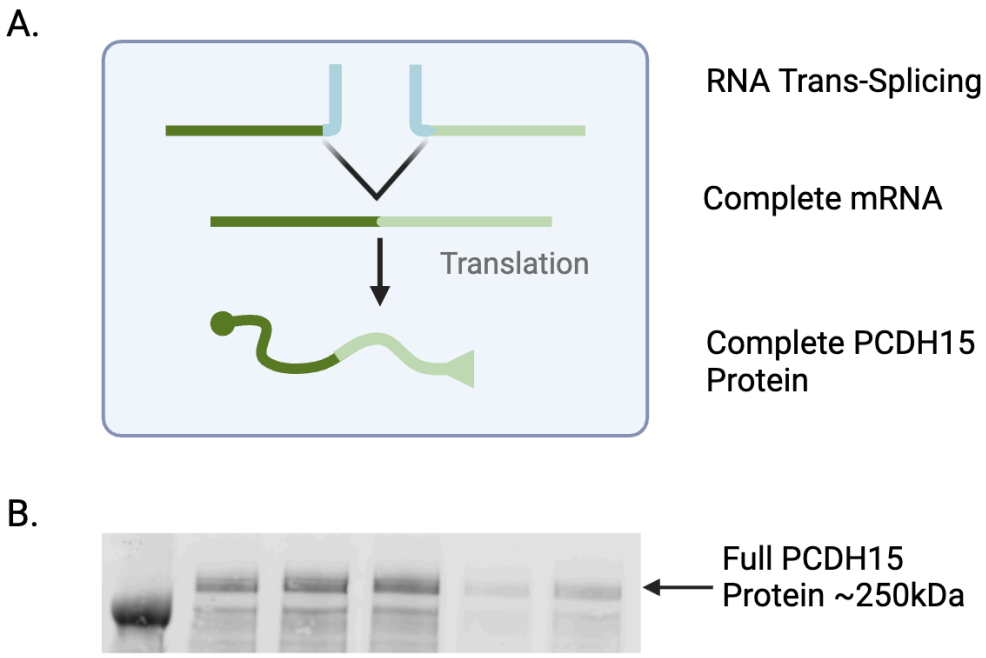
Usher Syndrome 1F (USH1F) is a debilitating genetic disease where patients experience congenital, bilateral, profound sensorineural hearing loss, vestibular areflexia, and adolescent-onset retinitis pigmentosa. USH1F is caused by mutations in protocadherin-15 (PCDH15) making it an ideal candidate for gene replacement or gene editing strategies for treatment. PCDH15 is a cell-membrane binding protein that is primarily present in stereocilia and the retina and its coding sequence is approximately 5.8 kb, which exceeds the maximum packaging capacity of AAVs. To overcome the size limitations of AAVs for delivery of PCDH15 in a gene replacement strategy, we utilized our novel RNA End Joining (REJ) technology and created five different constructs to optimize the expression of full-length PCDH15 in vitro. REJ is a highly efficient mRNA trans-splicing process, which can be

used in the delivery of multiple DNA constructs, which will scarlessly recombine transcribed RNAs in the nucleus, resulting in a full-length mRNA of the desired protein. The REJ variants were transfected into HEK293T cells and the expression of the 250kDa PCDH15 dimer was validated by Western blot. All screened variants exhibited full-length PCDH15 at varying levels with minimal fragment expression, and an optimal design was packaged into AAV8 driven by the photoreceptor specific GRK1 promoter. Subretinal injections into mice demonstrate robust protein expression and promising histological results. These findings illustrate a promising gene replacement strategy for USH1F.

Figure 1. (A) Schematic of REJ-enabled PCDH15 delivery. RNA dimerization domains are in blue and the split coding sequence is in green. (B) Western Blot depicting PCDH15 successfully reconstituted in wildtype mouse retinas.



572 A novel dual-target gene therapy strategy design with balanced gene expressions

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Purpose: Contributors to dry AMD is multifactorial. Dual-target therapeutic strategies have the potential for superior therapeutic outcomes over the single-target approach. Currently, the design of dual-target gene therapy constructs typically employs either a self-cleaving 2A peptide or a multi-promoter tandem construct. However, the 2A peptide structure is plagued by incomplete self-cleavage issues (Fig.1b) and tandem structure design often leads to compromised expression of the transgenes. The purpose of the current study is to develop a dual target gene therapy construct to overcome the above issues.

Methods: A series of dual-target constructs expressing Gefurulimab and sCD59 were constructed (Fig.1a). These constructs were transfected into ARPE19 cells, and the expression efficiency for each construct was evaluated by detecting the target genes' expression using an ELISA method. The high expression single-target constructs were used as benchmark.

Results: Benchmark construct T252 expressed sCD59 level of 106.4 ng/mL, T273 expressed Gefurulimab level of 3969.1 ng/mL. In contrast, the multi-promoter tandem structures of T306 (sCD59: 16.1 ng/mL, Gefurulimab: 2843.7 ng/mL) and T307 (sCD59: 106 ng/mL, Gefurulimab: 175.5 ng/mL) demonstrated imbalance in expression. While the construct with insulator framework of T356 achieved a balanced dual-target expression, with 66 ng/mL and 2349.7 ng/mL for sCD59 and Gefurulimab, respectively (Fig.2).

Conclusion: By employing the insulator framework, we identified a vector construct capable of balancing the expression of dual-target genes. Both target gene exhibited relatively high expression levels. Such a vector design can be utilized for gene therapy of multi-target diseases, such as dry AMD.

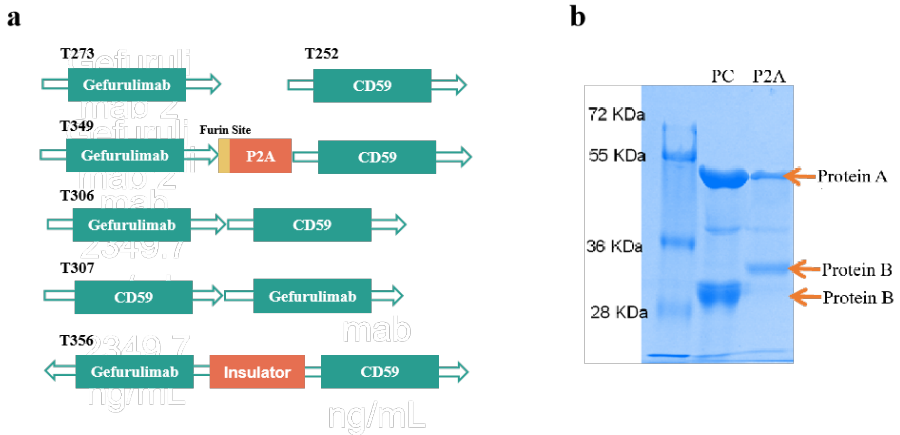

Fig.1: Dual-target gene therapy cassettes.
a Schematic Representation of Construct with Diverse Designs. T349 embodies the 2A peptide structure, T306 integrates an upstream Gefurulimab fused in-frame with a downstream sCD59 expression cassette, T307 is a similar design but with different position of target genes. T356 illustrates the incorporation of an insulator structure in between target genes. **b** Comparative SDS-PAGE analysis of proteins expressed from the self-cleaving 2A peptide construct versus a positive control. PC: positive control, P2A: protein products expressed through a self-cleaving 2A peptide construct.

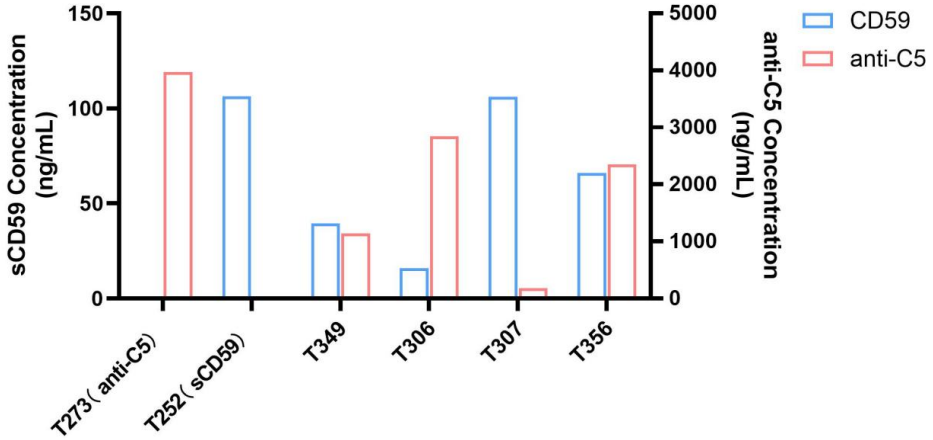

Fig.2 Protein Expression Levels of different constructs in ARPE19 cells.

573 A Bicistronic AAV-Based Gene Therapy for Wet Age-Related Macular Degeneration Combining VEGF and CTGF Inhibition to Target Angiogenesis and Fibrosis

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Introduction
The formation of macular scars is strongly associated with poor visual outcomes in eyes receiving anti-VEGF treatments. Connective tissue growth factor (CTGF), which plays a crucial role in fibrosis, is highly expressed in eyes with wet age-related macular degeneration (wAMD). To address both neovascularisation and fibrosis, we have developed a bicistronic gene therapy vector that targets VEGF, through VEGF Capture 2 (VC2), and CTGF using an anti-CTGF single-chain variable fragment (anti-CTGF ScFv). This study assesses the therapeutic efficacy of this bicistronic vector.

Methods
In vitro co-culture angiogenesis assays (HUVEC/human fibroblast) and *in vivo* laser-induced choroidal neovascularization (CNV) and subretinal fibrosis models were used to evaluate the vectors.

Results
In the co-culture assay, the bicistronic vector (VC2/anti-CTGF ScFv) significantly reduced VEGF-induced angiogenic tubule length and branch points relative to a Null control (3661µm vs 12678µm tubule length, 2.25 vs 33.5 branch points, n=3). However, the anti-CTGF ScFv monocistronic vector was unable to significantly affect tubule length or branching (11157µm and 39.75 branch points, n=3). In the laser CNV model, 90% of lesions were classed as leaky in the Null-treated eyes compared to 4% in the bicistronic vector treated eyes (n=25). Interestingly, the anti-CTGF ScFv monocistronic vector also had a significant effect on vascular leakage with only 36% of the lesions classed as leaky (n=28). In the fibrosis model, the bicistronic vector significantly decreased leakage and fibrosis markers such as fibronectin and α-SMA). Impressively, the anti-CTGF ScFv monocistronic vector also showed a notable reduction in fibrotic markers, highlighting its effectiveness in mitigating fibrotic progression.

Conclusions
The bicistronic VC2/anti-CTGF ScFv vector has been shown to reduce angiogenesis, vascular leakage, and subretinal fibrosis in preclinical models of wAMD, underscoring the therapeutic potential of inhibiting both VEGF and CTGF. Additionally, the anti-CTGF ScFv monocistronic vector demonstrated significant anti-fibrotic effects in vivo, highlighting the importance of targeting CTGF to prevent fibrosis progression. This dual-target strategy represents a promising advancement in wAMD treatment, as it addresses both neovascularization and fibrosis to enhance visual outcomes.

574 IKC159V: A bicistronic AAV gene therapy for Geographic Atrophy delivering dual protein expression and multifaceted protection against retinal damage

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Background
Age-related macular degeneration (AMD) persists as a predominant cause of blindness on a global scale. The

current standard of care involves frequent, burdensome ocular injections, which exhibit limited success in maintaining vision. Emerging therapies seek to address the intricate pathology of geographic atrophy (GA), a severe form of AMD, with the aim of improving therapeutic outcomes. This research introduces findings on IKC159V, a bicistronic AAV gene therapy designed to co-express two secreted, therapeutically significant proteins: a neuroprotective protein, pigment epithelium-derived factor (PEDF), and anti-complement protein, soluble CD46 (sCD46).

Methods
Human induced pluripotent stem cell-derived retinal pigment epithelium (iPSC-RPE) cells were transduced with the bicistronic AAV-PEDF/sCD46 construct (IKC159V), or monocistronic AAV constructs expressing either PEDF or sCD46, and used to assess expression and the protective effects of PEDF and sCD46 by ELISA and immunohistochemistry (IHC). IKC159V was administered subretinally in the murine eye and secreted PEDF and sCD46 protein levels were quantified from vitreal samples. Successful transduction of the retina was confirmed by RNAscope and IHC on ocular sections. To recapitulate degeneration of photoreceptors and RPE cells, a chemically-induced model of retinal injury (using N-methyl-N-nitrosourea (MNU)) was employed, with the impact on visual function determined using electroretinography (ERG) and histology.

Results
In response to chronic A2E exposure and blue light challenge, transduced human iPSC-RPE cells maintained stable protein expression and exhibited improved RPE cell survival compared to controls (n=6). Subretinal delivery of IKC159V in mice resulted in dose-dependent increases in vitreal concentrations of both PEDF and sCD46 protein (n=10). Transduction of both RPE and photoreceptor cells was confirmed at the DNA and protein level. In the MNU injury model, IKC159V protected against photopic b-wave loss compared to a Null control (IKC159V = 21% loss, Null = 92% loss; n≥11), which was supported by histological data showing preservation of cone cells (IKC159V = 235 cells/FOV, Null = 26 cells/FOV; n≥6), and RPE morphology (IKC159V = 36% RPE damage, Null = 77% RPE damage; n≥6).

Conclusions
IKC159V effectively supports cellular preservation critical for vision in both rodent and human models, highlighting its potential to improve clinical outcomes in AMD-associated GA. IKC159V is a promising gene therapy candidate that may address multiple pathological hallmarks of GA.

575 KRIYA-825 (AAV2.CR2-CR1) for Geographic Atrophy: Characterization of Biological Activity and Biodistribution

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Therapies that modulate an overactive complement system have shown clinical benefit for retinal diseases including geographic atrophy (GA). Current treatments for GA require intravitreal injection every 1 to 2 months to sustain complement inhibition, resulting in a high treatment burden. KRIYA-825 is an optimized complement receptor 2-complement receptor 1 fusion protein (CR2-CR1) designed to inhibit C3 and C5 convertase activity, encoded and packaged into an AAV2 gene therapy for delivery by suprachoroidal injection. Here, we report KRIYA-825's in vitro complement inhibition, rodent efficacy and non-human primate (NHP) suprachoroidal biodistribution to support clinical development.

KRIYA-825's CR2-CR1 fusion protein was designed based on previously reported CR2-CR1 proteins (Fridkis-Hareli et al. 2019) but modified for number of CR1 activity domains and size of domain linkers and evaluated for

alternative and classical complement inhibition in Wieslab assays. For efficacy assessment of KRIYA-825, C57BL/6 mice (N=10/group) were randomly assigned to bilateral subretinal treatment groups: naïve, vehicle, and KRIYA-825 (8E8 or 3E9 vg/eye). Retinal damage was induced by intraperitoneal sodium iodate ($NaIO_3$) 4 weeks after AAV treatment. Evaluations 10-days after $NaIO_3$ included optimal coherence tomography (OCT), vector genomes (qPCR), transgene mRNA (RT-qPCR), and protein of transgene and complement fragments (immunoassay) in ocular homogenates. Biodistribution of KRIYA-825 was performed in NHPs (cynomolgus monkeys) receiving bilateral suprachoroidal injections (3E11, 1E12, or 3E12 vg/eye; 2-5 animals per group) using the Everads Injector. Animals were monitored for ocular health and at 4- and 12- weeks post injection for ocular (retina, RPE-choroid and sclera) and systemic biodistribution of vector genomes, transgene mRNA, and transgene protein. KRIYA-825 demonstrated classical and alternative pathway complement inhibition with <1 nM IC_{50} values. In mice, KRIYA-825 led to dose-dependent preservation of retinal thickness, vector genomes and transgene mRNA in ocular homogenates, and detectable CR2-CR1 protein levels. Retinal damage by $NaIO_3$ increased C3b and iC3b fragment levels, while treatment with KRIYA-825 reduced iC3b protein to levels comparable to those seen in healthy animals. The Everads Injector was compatible with KRIYA-825 and in NHPs, suprachoroidal delivery was well-tolerated with no unanticipated ocular findings. Vector genomes and transgene mRNA were detected at high levels in ocular tissues including sclera, choroid-RPE and retina, with minimal detectable systemic transgene mRNA. KRIYA-825 preserved retinal structure in an acute retinal degeneration mouse model, and suprachoroidal administration with the Everads Injector achieved targeted biodistribution in NHPs. This CR2-CR1 protein demonstrated complement inhibition in vitro, reduced complement fragment levels in vivo and detectable biodistribution across NHP and mouse ocular tissues. These findings support further clinical development of KRIYA-825 as a potential one-time sustained therapy for GA.

576 Generation of a non-human primate model of Geographic atrophy, Dry Age-related macular degeneration

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Introduction: Age-related macular degeneration (AMD) is a leading cause of vision loss in adults over 60 years of age, with prevalence expected to grow to 288 million in 2030 as the population ages. As AMD progresses, the macula deteriorates and leads to advanced forms of disease – either wet AMD, which is characterized by neovascularization and vascular leakage, or dry AMD [also known as geographic atrophy (GA), which is characterized by the death of photoreceptor and RPE cells. There is a lack of NHP GA models that recapitulate lesion growth- an important therapeutic efficacy readout in patients. Primates are the only species with a macula; therefore, rodent models cannot accurately recapitulate human disease features. Here, we created an NHP model that recapitulates key features of progressive geographic atrophy by inducing outer retinal lesions in the macula.

Methods: In 3 cohorts of cynomolgus macaques, we induced retinal atrophy in specific regions of the macula (sparing the fovea) using different laser settings by altering the power, spot size and duration of laser exposure. The first 2 cohorts of animals were used to optimize the laser settings in 4-week studies. In the 3rd cohort, we induced atrophic lesions using the optimal laser settings identified from cohorts 1 and 2 and tracked laser-induced lesion growth in an 8-week study. We used in-life imaging – cSLO, OCT and FA – to track retinal and macula changes over time and conducted proteomic analyses of aqueous humor collected at baseline, 2 weeks and 4 weeks post laser treatment.

Results: We identified a specific laser setting that reproducibly produced lesions with localized atrophy of the RPE and photoreceptor layers, the main cell types affected in geographic atrophy. Additionally, we observed a significant increase in lesion surface area and decrease in retinal thickness over time, indicating a loss in retinal cells. Importantly, the model did not develop vascular leakage or neovascularization seen with the laser induced CNV model. We also observed an increase in complement proteins in the aqueous humor after laser induction.

Conclusion: This NHP model is a potential efficacy model for dry AMD therapeutics wherein the features of RPE and photoreceptor atrophy and lesion growth in the macula can be assessed.

577 A novel AAV vector delivering sCD59 for the treatment of dry AMD in mice model

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**Corresponding Author*

Purpose: Abnormal activation of the complement system is considered a major cause in the development of dry age-related macular degeneration (dry-AMD). sCD59 is a protective protein that shields the retina from damage mediated by the complement activation, and it holds potential for the treatment of dry AMD. The purpose of the current study is to develop a gene therapy approach that enables sustained and effective expression of sCD59 to mitigate the progression of dry AMD.

Methods: A low-dose laser induced dry-AMD mice model was used in this study to evaluate our therapeutic vectors. A soluble CD 59 (sCD59) was packaged into an engineered AAV capsid variant with high transduction efficiency for ocular tissues and into AAV2 serotype as a control. These two vectors were injected intravitreally into eyes of each animal. Laser induction was performed 2 weeks post injections. Stage 4 lesions were measured for each eye by fluorescein angiography to assess disease severity. Retinal tissues were harvested at the end point and choroidal flat mount was conducted to show the retinal damage for each eye.

Results: the fluorescein angiography data and choroidal flat-mount images indicated that both vectors treated groups greatly reduced the stage-4 lesions in animals' retina, and the AAV-variant-sCD59 group had superior therapeutic effect than AAV2-CD59 group (Fig.1).

Conclusion: Using a novel AAV variant to effectively express sCD59 showed pharmacological effect in low-dose laser induced dry AMD model, providing potential for clinical translation in the treatment of dry AMD.

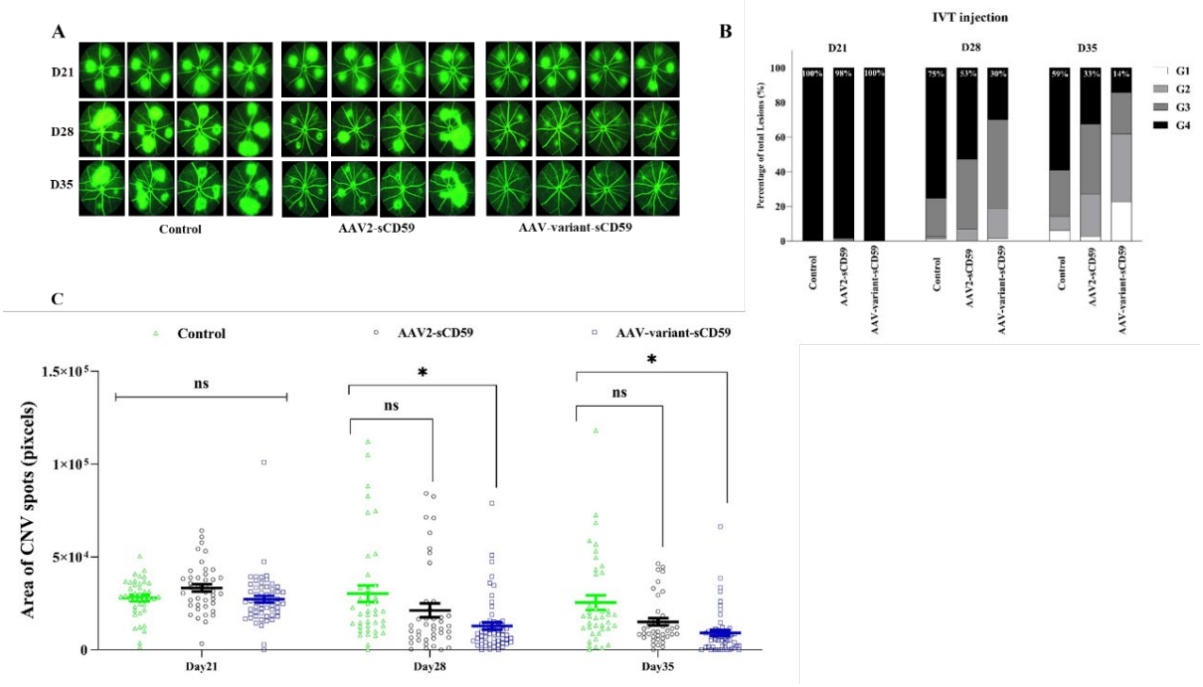

Fig.1 Fluorescein Angiography Results Following Intravitreal Injection of two vectors in Model Mice

A) Representative fluorescein angiography images obtained at various time points in model mice (n=80 per group); **B**) Percentage of stage 4 lesion counts at different time points in model mice (n=80 per group); **C)** Stage 4 lesion areas at different time points in model mice (n=80 per group).

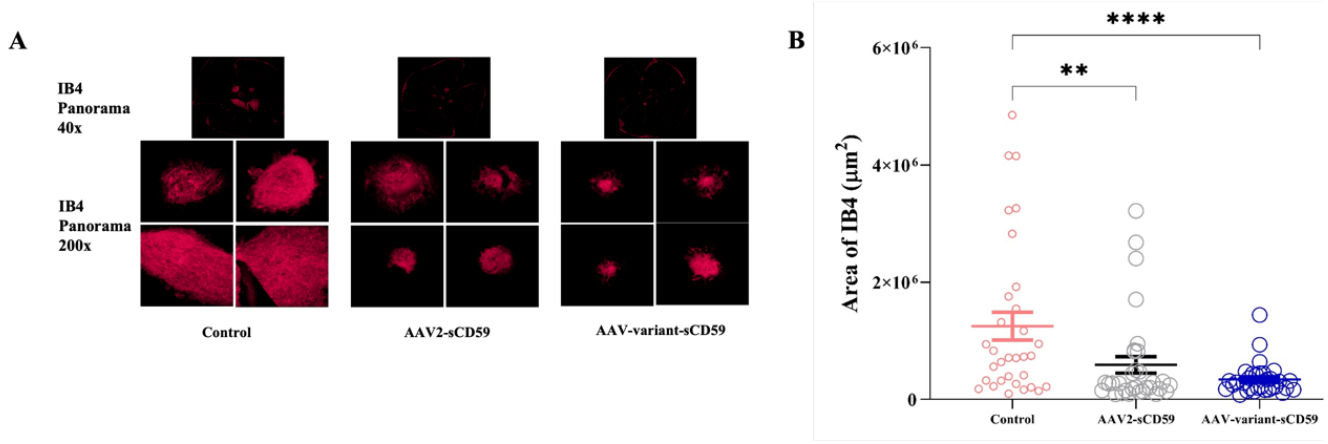

Fig.2 Choroidal Flat-Mount Results After Intravitreal Injection of Different Vectors in Model Mice

A) Representative fluorescent images of choroidal flat-mounts from model mice after intravitreal injection of different vectors (n=36 per group); **B**) Agglutinin staining results from model mice after intravitreal injection of different vectors (n=36 per group).

578 Identification of cell-type-specific *cis*-regulatiory modules (CRMs) in the primate retina

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**Corresponding Author*

Identification of cell-type-specific *cis*-regulatory modules (CRMs) in the primate retina:
Globally, at least 2.2 billion people suffer from vision impairment, with over 300 million facing irreversible blindness due to neurodegenerative retinal diseases. Specific retinal diseases are often linked to the degeneration of particular cell types, each associated with unique genetic mutations. For instance, in age-related macular degeneration and retinitis pigmentosa, photoreceptors are primarily lost, while retinal ganglion cells (RGCs) degenerate in glaucoma. Adeno-associated virus (AAV) has emerged as the gold standard vector for gene augmentation- and gene editing- based treatments for retinal disease. However, a key challenge lies in developing AAV tools capable of targeting and manipulating specific cell types or subtypes. Here we report a workflow using *cis*-regulatory modules (CRMs), such as enhancers, promoters, and the 5' UTR region, which exhibit high cell-type specificity, to guide AAV-mediated gene expression in specific retinal cell types.

In this study, we first focused on identifying cell-type-specific CRMs in individual cell types. To achieve this, we generated chromatin accessibility data using scATAC-seq assays and identified differentially accessible regions for the enriched genes in distinct retinal cell types. We refined the potential CRM candidates by applying thresholds to screen these regions, focusing on those with higher conservation scores and greater densities of cell-type-specific

transcription factor binding sites. Next, we inserted these candidate CRMs, each paired with a unique barcode, into AAV vectors to drive the expression of Cre, and packaged them into AAV2-based capsids. We then intravitreally injected mice with a pool of AAVs carrying these CRMs, along with AAVs expressing a Cre-dependent fluorescent reporter (tdTomato). Subsequently, we assessed expression of barcodes uniquely associated with each CRM across different retinal cell types through Fluorescence-Activated Cell Sorting and scRNA-seq analyses. After identifying the CRMs that demonstrated cell specificity, we injected them individually into marmoset retinas to further validate their ability to guide gene expression in specific retinal neurons. Immunofluorescence staining and immunohistochemistry were used to examine the expression pattern of tdTomato.

In conclusion, our research aims to contribute to the development of a versatile toolbox for targeting specific retinal cells by incorporating cell-type-specific CRMs into AAVs, with significant potential for advancing therapeutic treatments for retinal diseases.

579 AAV-delivered engineered suppressor tRNA rescues visual function in mice with an inherited retinal disease

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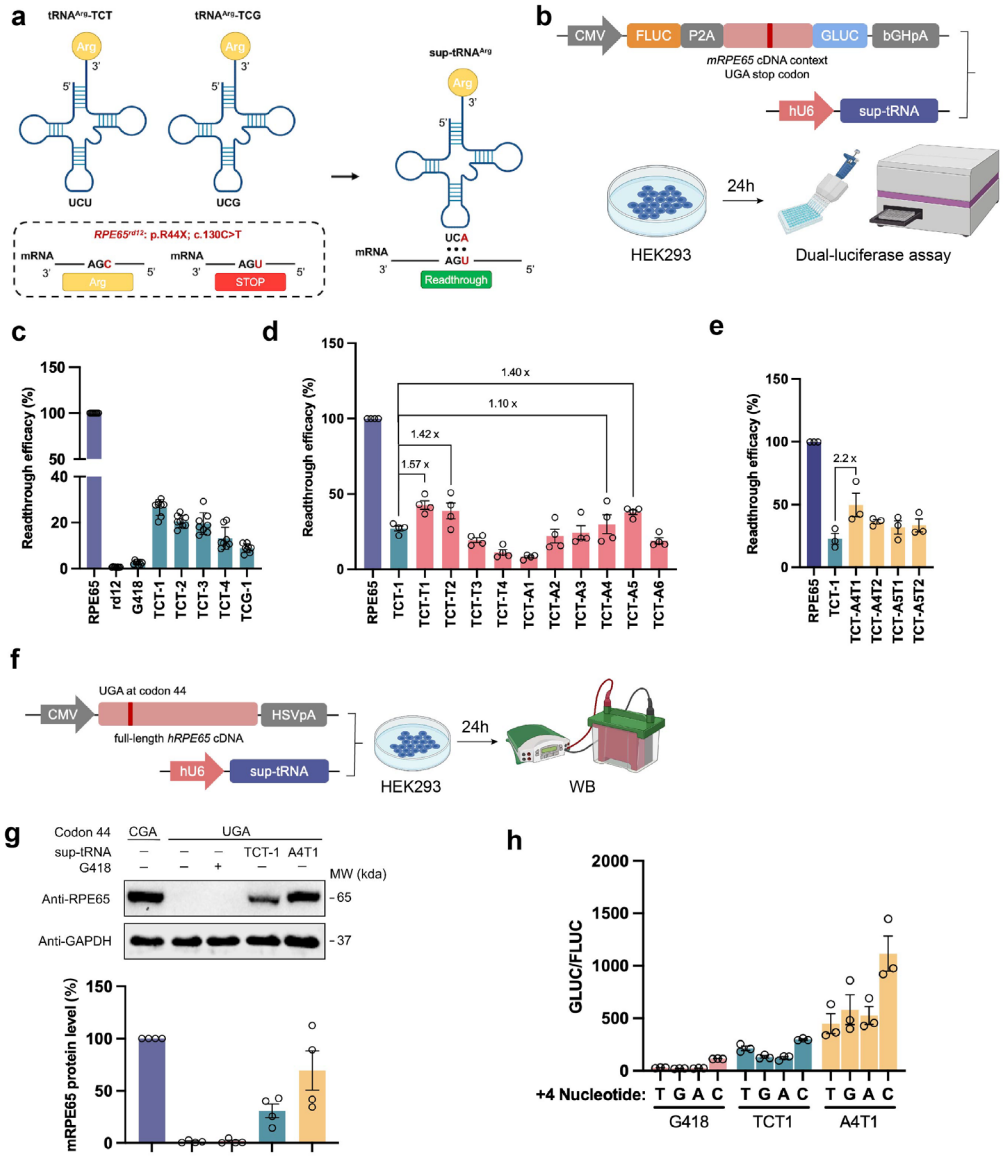
Nonsense mutations account for 11% of human pathogenic mutations and result in the synthesis of truncated proteins. Nonsense suppression therapies by small molecular drugs or suppressor tRNAs (sup-tRNAs) can introduce an amino acid at premature termination codon, thereby, promote the production of full-length protein. Although nonsense suppression therapies by sup-tRNAs demonstrate promising results in cell culture models, the in vivo delivery still faces the challenges related to efficacy, stability and safety. Here, we engineered the body sequence of sup-tRNA^{Arg} to achieve a 2.2-fold readthrough capacity. The sequence engineered sup-tRNA^{Arg} (SE-sup-tRNA^{Arg}) outperforms the original one at PTCs with all four types +4 nucleotides. By using the recombinant adeno-associated virus (rAAV) delivery platform, we successfully restored production of full-length protein and rescued visual function in a mouse model carrying a nonsense mutation in *RPE65* gene. rAAV.SE-sup-tRNA^{Arg} had limited retinal toxicity and minimal effects on the activation of unfolded protein response pathways, which is related to readthrough on normal stop codons. This study highlights the therapeutic potential of rAAV.SE-sup-tRNA^{Arg} on endogenous transcripts carrying clinically relevant PTC. Moreover, our findings encourage the further application of rAAV.SE-sup-tRNA^{Arg} on other inherited retinal disease models carrying nonsense mutations.


a
tRNA^Arg-TCT
tRNA^Arg-TCG
sup-tRNA^Arg
UCU
UCG
UCA
RPE65^rd12: p.R44X; c.130C>T
mRNA
AGC
Arg
AGU
STOP
Readthrough
b
CMV
FLUC
P2A
GLUC
bGHpA
mRPE65 cDNA context
UGA stop codon
hU6
sup-tRNA
24h
HEK293
Dual-luciferase assay
c
Readthrough efficacy (%)
RPE65
rd12
G418
TCT-1
TCT-2
TCT-3
TCT-4
TCG-1
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1.10 x
1.42 x
1.57 x
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TCT-A5T2
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UGA at codon 44
full-length hRPE65 cDNA
HEK293
WB
g
Codon 44
CGA
UGA
sup-tRNA
G418
TCT-1
A4T1
MW (kda)
Anti-RPE65
Anti-GAPDH
65
37
mRPE65 protein level (%)
h
GLUC/FLUC
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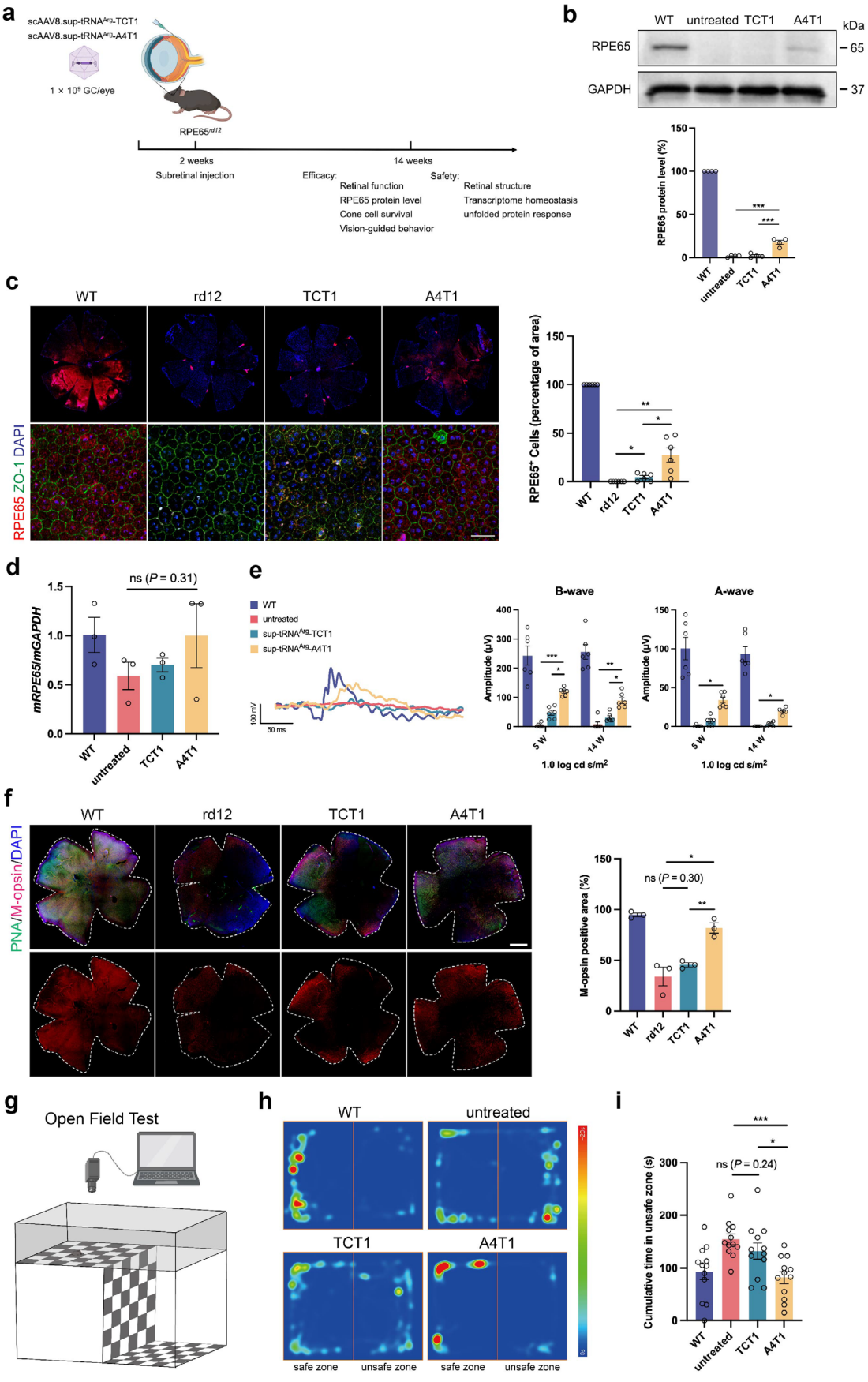
G418
TCT1
A4T1


a
scAAV8.sup-tRNA^Arg-TCT1
scAAV8.sup-tRNA^Arg-A4T1
1 × 10^9 GC/eye
RPE65^rd12
2 weeks
Subretinal injection
14 weeks
Efficacy:
Retinal function
RPE65 protein level
Cone cell survival
Vision-guided behavior
Safety:
Retinal structure
Transcriptome homeostasis
unfolded protein response
b
WT
untreated
TCT1
A4T1
kDa
RPE65
GAPDH
65
37
RPE65 protein level (%)
c
WT
rd12
TCT1
A4T1
RPE65 ZO-1 DAPI
RPE65+ Cells (percentage of area)
d
ns (P = 0.31)
mRPE65/mGAPDH
e
WT
untreated
sup-tRNA^Arg-TCT1
sup-tRNA^Arg-A4T1
100 mV
50 ms
B-wave
A-wave
Amplitude (μV)
5 W
14 W
1.0 log cd s/m^2
f
PNA/M-opsin/DAPI
M-opsin positive area (%)
ns (P = 0.30)
g
Open Field Test
h
WT
untreated
TCT1
A4T1
safe zone
unsafe zone
i
Cumulative time in unsafe zone (s)
ns (P = 0.24)

580 Molecular Stability of Human Donor Retinal Gene Expression and Protein Abundance Post-Mortem: Implications for Cell and Gene Therapy Applications

Cerys Easton[1], Megan Criss[2], Phillip Wilmarth[3], Ashok Reddy[3], Mark Ellison[1], Megan Straiko[4], Khoa Tran[1]
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Purpose: The integrity of human post-mortem retinal tissue is critical for advancing retinal cell and gene therapy. This study evaluates transcriptional and proteomic stability in donor retina over two distinct post-mortem intervals to assess their effects on tissue quality and stability for therapeutic research and biomarker discovery.

Methods: Whole eyes were recovered from post-mortem donors (n=7 pairs) under standard US eye banking protocols, with ocular cooling maintained post-mortem. Eyes were stored at 2–8°C until dissection and flash freezing in liquid nitrogen. The OD retina was collected at 12 hours and the OS retina at 18 hours post-mortem. The macular region superior to the fovea was used for RNA sequencing (RNA-seq), while the inferior region was processed for TMT-mass spectrometry. Library preparations were performed simultaneously, and the sequencing of all samples were conducted on the same chip (Floragenex, Inc., Tigard, OR). Gene alignment was performed using the GRCh38 (GENCODE v32/Ensembl 98) human reference genome. Proteins were isolated and labeled with one of 14 TMTPro isobaric tags (OHSU Shared Proteomics Resources), and all 14 samples were analyzed in a single TMT-mass spectrometry run to minimize experimental variability. RNA-seq data were processed using DESeq2 (Bioconductor), and proteomic data were analyzed using the PAW pipeline.

Results: RNA-seq identified 22,446 unique genes, with differential expression analysis revealing only six genes with significant changes between the 12- and 18-hour post-mortem intervals (adjusted p-value < 0.1). Four of the six genes were upregulated and only two were down-regulated in the 18-hour time point compared to the 12-hour. No enriched pathways were represented by the differentially expressed genes. Proteomic analysis identified 6,109 unique proteins (each confirmed by the identification of at least two distinct peptides, 1% FDR), with no significant differences in protein abundance between time points (all $P > 0.05$). Molecular markers for retinal cell types, including those relevant to cell and gene therapy applications, remained detectable and stable in both datasets. Furthermore, genes and proteins associated with retinal diseases including AMD and glaucoma were detectable and exhibited no post-mortem degradation between the two time points.

Conclusions: Retinal gene expression and protein abundance remain stable between 12- and 18-hours post-mortem, ensuring the molecular integrity required for therapeutic development. These findings support expanding the post-mortem interval cut-off from 12 to 18 hours for the use of donor retinal tissues in transcriptomic and proteomic studies relevant to regenerative medicine, gene therapy target validation, and the development of cell-based interventions for retinal diseases.

581 A novel spontaneously immortalised Non-Human Primate Müller glia cell line reveals a population of Müller glia cells with stem cell-like qualities, a potential source for an in situ retinal regeneration

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Purpose
Retinal degenerative diseases are considered the leading cause of blindness worldwide. Cell therapies for ocular diseases aim to replace or restore the degeneration of the light-sensitive cells in the retina, the photoreceptors. Such therapies are often challenging due to low rate of proliferation and/or differentiation of cells in vitro, and poor migration and integration in vivo. Therefore, a stable source of cells within the retina to replace lost photoreceptors is promising for cell replacement therapies.

Methods
A spontaneously immortalised Müller glia cell line was derived from a primary culture of neural retina of a Macaque monkey by dissociation with papain dissociation reagent. The cells were cultured, passaged and eventually immortalised without the presence of growth factors. A differentiation cocktail containing fibroblast growth factor 2 (FGF2), taurine, retinoic acid, and insulin-like growth factor 1 (IGF-1) was used to induce differentiation, followed by characterisation with immunocytochemistry and reverse-transcriptase (RT-PCR) analyses to examine the expression of specific Müller glia, stem cell and neuronal cell markers.

Results
Here we report ~~a~~ spontaneously immortalised Müller glia cell line isolated from rhesus macaque monkey, grown under normal culture conditions and could be expanded indefinitely without the presence of growth factors. The cell line exhibits normal morphology and expressed Müller glia markers such as VIMENTIN, GLUTAMINE SYNTHASE, glutamate-aspartate transporter (GLAST), and CD44. As well as expressing Müller glia specific markers, they also express stem cell-specific markers such as paired box 6 (PAX6) and SRY (sex determinant region)-2 (SOX2). The cell line lacked expression of postmitotic retinal neurons including RHODOPSIN and CD73, however, in the presence of factors that induce differentiation, these cells show a shift in gene expression patterns and start to express retinal-specific markers such as RHODOPSIN, and RECOVERIN, as well as neuronal markers including bIII TUBULIN and 160kDa neurofilament (NEFM). In addition, the cells maintained expression of stem cell markers such as PAX6 and NOTCH1.

Conclusion
Our observations indicate that the non-human primate retina harbours a population of cells that express both Müller glia and stem cell markers, which can undergo a state of de-differentiation, a phenomenon known in reprogrammed Müller glia cells. They also suggest that these cells can be useful to further understand the regeneration potential in the mammalian retina, which represents a major obstacle for potential therapies to restore retinal function in humans.

582 Injury-responsive gene therapy for neuroprotection of retinal ganglion cells

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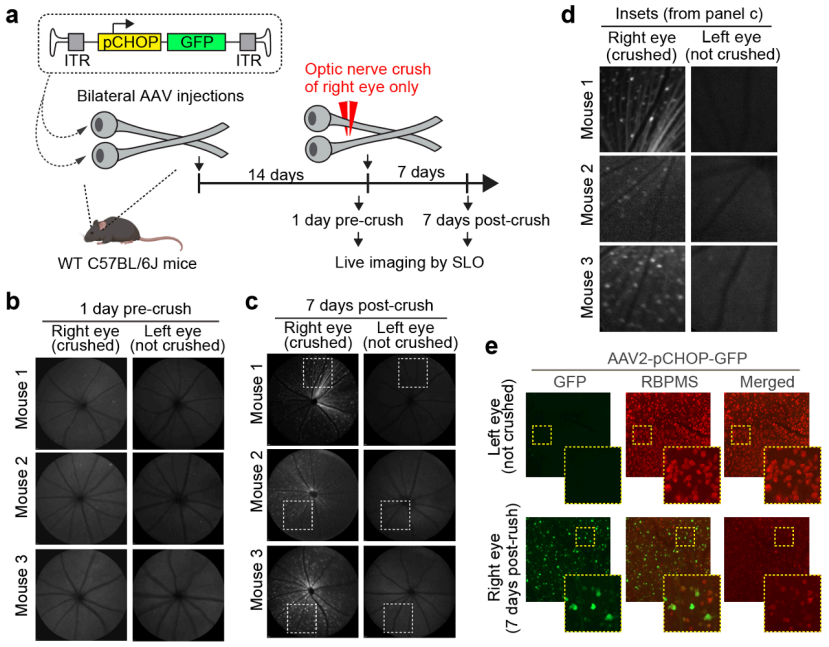
[1]Stanford University, Stanford, CA, [2]Palo Alto, CA, [3]Stanford, Palo Alto, [4]Stanford University, [5]Stanford University, Stanford, [6]Stanford University, Stanford, CA, [7]Stanford University, Palo Alto, CA
**Corresponding Author*

Purpose: Precise control of gene expression is crucial for safety and efficacy of gene therapies. While AAVs enable long-term gene expression in non-dividing tissues such as the neural retina, uncontrolled expression risks adverse effects. We hypothesize that using injury-specific regulatory elements, such as the endoplasmic reticulum (ER) stress-responsive CHOP promoter ("pCHOP"), can enable "smart" gene therapies to allow cells to sense disease conditions and autoregulate therapeutic gene expression.

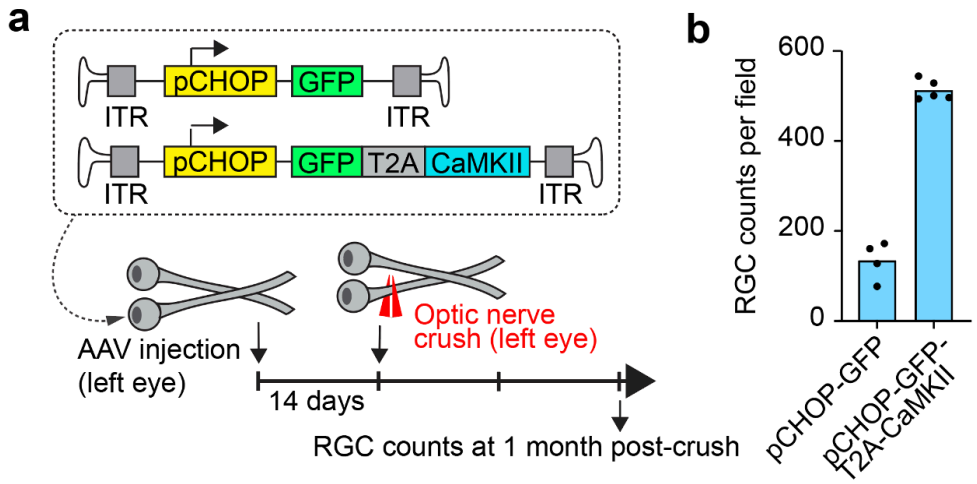
Methods: AAV2 expressing pCHOP driving GFP was intravitreally injected into C57BL/6 mice (N=3 per group). Optic nerve crush was performed on one eye per animal, leaving the contralateral eye uninjured. Live fluorescence imaging with scanning laser ophthalmoscopy (SLO) was conducted at 3- and 7-days post-crush using the Heidelberg Spectralis system. Additionally, AAV2 expressing pCHOP driving Ca2+/calmodulin- dependent protein kinase II (CaMKII) T286D—a gene shown to promote neuroprotection—was tested in C57BL/6 mice (N=5 per group). Optic nerve crush was performed 2 weeks after injection, and retinal ganglion cells (RGCs) were quantified via RBPMS immunostaining 1 month later.

Results: GFP expression was minimal in uninjured eyes but was strongly induced in injured eyes following optic nerve crush. Cryosections revealed GFP expression localized predominantly in the ganglion cell layer. Mice that received AAV2-pCHOP-CaMKII T286D exhibited significantly higher RGC survival compared to those injected with AAV2-pCHOP-GFP ($p<0.05$).

Conclusions: These findings demonstrate that the ER stress-responsive CHOP promoter can drive injury- specific gene expression and confer neuroprotection following optic nerve injury. Given the central role of ER stress in various ocular pathologies, pCHOP offers a promising strategy for developing gene therapies with controlled, injury-responsive expression, potentially confining exogenous gene expression to injured or degenerating cells, and reducing the risks of constitutive overexpression.



a) Mice are injected with AAV2-pCHOP-GFP in both eyes, and optic nerve crush was performed in the right eye only. b-d) Live imaging by SLO of each retina at 1 day before crush (b) and 7 days after crush (c-d). e) Immunohistochemistry on retinal wet mounts, of RGCs (with RBPMS) and GFP.



a) Schematic of mice injected with AAVs before optic nerve crush. b) Quantitation of RGC counts on retinal whole mounts.

583 Intein-based Dual AAV Gene Therapy Restores ABCA4 Function in Stargardt Disease

Xing Wang[1], Weiwei Wu[2], Guixiang Yang[3], Liying Chen[2], Shuna Cui[3], TJ Cradick[4], Alvin Luk[5], Linyu Shi[2], Hui Yang[6]

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Background: Loss-of-function mutations in the *ABCA4* gene result in the accumulation of toxic bis-retinoid compounds, leading to photoreceptor degeneration and central vision loss in Stargardt disease (STGD1). Due to the large size of the *ABCA4* gene, its delivery via AAV requires a dual-vector approach. We developed an intein-based dual AAV gene replacement therapy (HG005) designed to express the full-length human ABCA4 (FL-ABCA4) protein via protein trans-splicing (PTS). This study evaluates the therapeutic potential of HG005 in a mouse model of STGD1.

Methods: We employed a dual-plasmid system in HEK293T cells to co-express truncated ABCA4 proteins fused with inteins, facilitating PTS production of FL-ABCA4 (Outlined in **Fig. 1A**). Nine split sites and 14 intein types were screened for optimal efficiency, comparing the results with previously reported versions, $C1150^{Npu}$ and $C1150^{Rma}$ (Ruiting 2023). Western blotting (WB) assessed FL-ABCA4 expression and fusion efficiency. Based on the in vitro findings, dual AAV vectors were constructed with promoters, introns, and codon optimization. Subretinal injections of these dual AAV vectors were performed in the *Abca4*-deficient mice ($Abca4^{-/-}$). Expression and localization of ABCA4 protein were assessed by WB and immunofluorescence (IF) at 8 weeks post-injection, using C57BL/6J mice as controls (**Fig. 2A**). The recovery of ABCA4 protein was measured at different HG005 injection doses. Therapeutic efficacy was evaluated by quantifying A2E accumulation in the retina using liquid chromatography-mass spectrometry (LC-MS) at 12 weeks post-injection (**Fig. 2D**).

Results: The WB analysis of HEK293T cell screening identified Site 2 as the most efficient split site for the fusion efficiency of FL-ABCA4, with a significantly higher full-length to truncated protein ratio (**Fig. 1B**). Among the intein types, Intein 4 combined with Site 2 yielded the highest FL-ABCA4 protein expression, exceeding $C1150^{Npu}$ by more than twofold (**Fig. 1C**). In vivo studies showed robust and dose-dependent functional human ABCA4 (hABCA4) protein restoration in the retina of $Abca4^{-/-}$ mice treated with HG005 8 weeks after subretinal injection. WB analysis revealed that HG005 achieved ABCA4 protein expression levels up to 150.4% of those in wild-type (WT) mice, significantly outperforming the previously published $C1150^{Rma}$ expression (**Fig. 2C**). Immunofluorescence results confirmed specific localization of hABCA4 protein to the photoreceptor outer segments (OS), consistent with the WT retinal biodistribution (**Fig. 2B**). Notably, LC-MS analysis demonstrated a dose-dependent reduction in A2E accumulation, with the high-dose group of HG005 achieving 74.5% reduction in A2E compared to controls at 3 months post-injection (**Fig. 2D**).

Conclusions: This study demonstrates the feasibility of using the PTS mechanism to successfully express the FL-ABCA4 protein via a dual-plasmid system. Through rigorous optimization and screening of split sites, intein types, and vector design, HG005 is mediated by Site 2 and Intein 4 and has emerged as a superior candidate. HG005 achieved much higher ABCA4 protein expression at equivalent dosages and significantly reduced the A2E accumulation than previously reported $C1150^{Rma}$ approaches, markedly enhancing the therapeutic potential. These findings establish HG005 as a promising gene therapy for Stargardt disease and support its advancement toward clinical development.

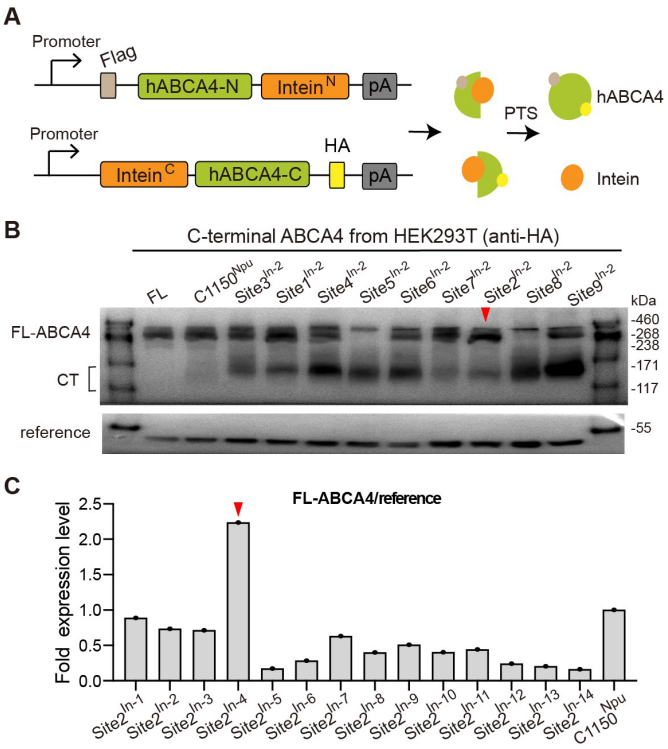

Figure 1. *In vitro* screening in HEK293T cells.
A. Split intein-mediated protein ligation model diagram.
B. Screening results of 9 different split sites.
C. Screening results of different intein types based on Site 2. N=1.
$C1150^{Npu}$ from Ruiting Li, et al. Hum Gene Ther. 2023. PTS, Protein trans-splicing; FL-ABCA4, full length ABCA4; CT, C truncated protein; In, Intein.

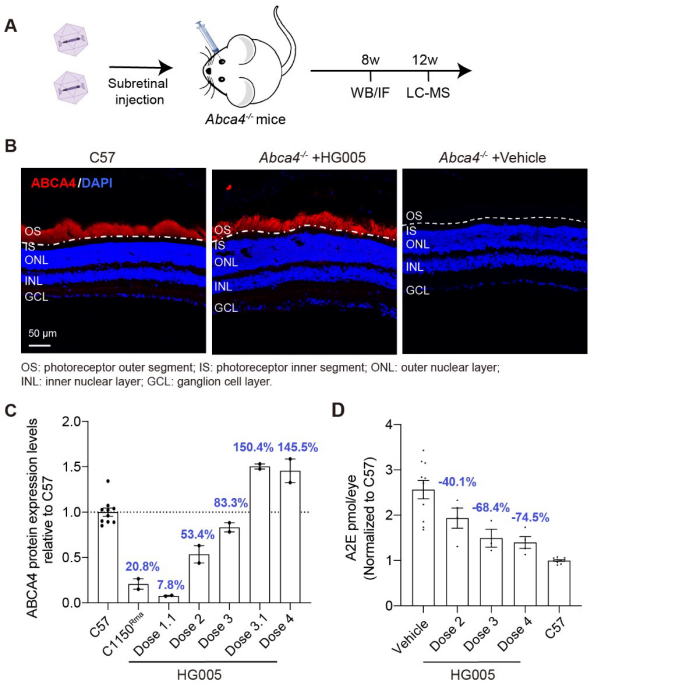

Figure 2. The *in vivo* therapeutic effects of HG005 on *Abca4*$^{-/-}$ mice.
A. Schematic diagram of *in vivo* treatment of *Abca4*$^{-/-}$ mice.
B. Immunofluorescence results of *Abca4*$^{-/-}$ mice after 8 weeks of injection.
C. The recovery of ABCA4 protein *in vivo* mediated by HG005 at different injection doses.
N=10 for C57, N=2 for others.
D. The reduction of A2E accumulation *in vivo* mediated by HG005 at different injection doses.
N=10 for Vehicle and C57, N=3 or 4 for others.
C1150^{Rma} from Ruiting Li, et al. Hum Gene Ther. 2023. The dosage of C1150^{Rma} is Dose 4.
WB, western blot; IF, immunofluorescence; LC-MS, liquid chromatography-mass spectrometry.

584 Human Retinal Cell Delivery of a Bioengineered, Synthetic Protein Sensitive to Multiple Wavelengths of Light in Patients with Stargardt Disease

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Background
Multi-characteristic opsin (MCO), a triprotein fusion complex from non-mammalian sources, has demonstrated high ambient light sensitivity over a broadband light spectrum without compromising kinetics. As an optogenetics approach targeting higher-order retinal neurons (e.g., ON bipolar cells), the initial safety and efficacy of viral-vector-delivered MCO (MCO-010) was clinically evaluated in Stargardt disease (SD), a progressive inherited retinal disease that leads to severe and irreversible vision loss through photoreceptors and retinal pigment epithelial (RPE) cell degeneration.

Methods
A single intravitreal injection of MCO-010 was administered in the worse-seeing eye of SD patients in STARLIGHT, phase 2 open-label study conducted over 48-weeks at two US sites. Six adults (mean age 50 years, range 32-71 years, 4 males) received 1.2E11 genome copies (gc)/eye of MCO-010. The primary outcome was the incidence, nature, and severity of treatment-emergent adverse events (TEAEs). Secondary endpoints included change in best-corrected visual acuity (BCVA), vision-guided mobility, and shape determination accuracy. Visual field perimetry and Michigan Retinal Degeneration Questionnaire (MRDQ) were exploratory endpoints.

Findings
All 6 participants had at least 1 ocular TEAE; non-ocular TEAEs occurred in 3 participants. The most common TEAEs were conjunctival hemorrhage, ocular hypertension, and vitreous cells (2 subjects). There were no deaths, hospitalization, loss of eye, retinal detachment, endophthalmitis, or TEAEs leading to study discontinuation. The mean BCVA of the 6 treated eyes at baseline and 48-week follow-up was 22.8, and 28.3 ETDRS letters respectively. The BCVA change from baseline was 7.2, 4.2, and 5.5at 12, 24, and 48 weeks respectively. With a wearable magnifier (low-vision glasses), the mean BCVA change was 17.8, 15.7, 13.3 at 12, 24, and 48 weeks respectively. The improvement in mean defect in visual field perimetry was 1.02, 2.47, and 2.63 dB at 12, 24, and 48 weeks respectively. Specific improvements were noted in reading, and color and contrast domains of the MRDQ.

Interpretation
MCO is the most bioengineered non-mammalian protein that has been heterologously expressed in humans. Intravitreal MCO-010 was safe, and improvement in on(eye)-chart vision of SD participants with severe low vision was observed. MCO-010 optogenetic phase 2 results support the further investigation for treatment SD.

585 AAV-Mirtron Treatment Preserves Retinal Structure and Function in the Human P23H Knock-In Autosomal Dominant Retinitis Pigmentosa Mouse Model

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[1]University of Oxford, Oxford, Oxfordshire, United Kingdom, [2]University of Oxford, Oxford, United Kingdom, [3]Universtity of Oxford, Oxford, Oxfordshire, United Kingdom
**Corresponding Author*

Introduction
Mutations in the rhodopsin (RHO) gene are the leading cause of autosomal dominant retinitis pigmentosa (ADRP). The heterogeneity of RHO mutations presents a significant therapeutic challenge, necessitating precise suppression of mutant RHO transcripts while ensuring optimal replacement of codon-modified rhodopsin. Mirtrons, a subclass of short hairpin introns, are processed into functional RNA interference effectors following splicing from precursor mRNA. This study investigates an adeno-associated virus (AAV)-delivered triple mirtron vector to achieve balanced knockdown of mutant RHO and replacement of codon-modified RHO in a humanised ADRP mouse model.

Methods
The lead candidate triple mirtron AAV vector targeting human RHO (RHOp.3xM.coRHO) was administered at a dose of 5×108E genome copies (gc)/eye via subretinal injection in Rho+/−, hP23H+ mice at 3–4 weeks of age. Post-injection assessments included optical coherence tomography (OCT) at 1, 3, and 6 months, electroretinography (ERG) at 4 and 6 months and RNA extraction from harvested retinas for transcript assessments.

Results
In P23H+ mice, the photoreceptor layer thickness in both the superior and inferior retinas remained consistently greater in treated eyes compared to paired uninjected controls at 1, 3, and 6 months post-injection. Functional improvements were also evident, as demonstrated by enhanced scotopic and photopic ERG b-wave amplitudes at both 4 and 6 months post-injection. Quantitative PCR revealed significant reductions in mouse rhodopsin and human P23H rhodopsin transcript levels, with concurrent detection of coRHO transcripts, maintaining total rhodopsin levels at ~50% of uninjected eyes at 6 months. Biodistribution analysis confirmed retinal specificity of transgene expression.

Conclusions
AAV-delivered triple mirtron vector achieved effective knockdown of native rhodopsin transcripts and balanced rhodopsin replacement in the human P23H knock-in mouse model. Structural improvements correlated with enhanced retinal function, supporting the potential of mirtron-based RNA knockdown and replacement as a novel approach for gene therapy of ADRP.

586 12-Month Safety and Efficacy Evaluation of Subretinal Gene Therapy (FT-002) for the Treatment of RPGR Gene Mutation Associated X-linked Retinitis Pigmentosa

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[1]Eye and Ent Hospital of Fudan University, Shanghai, Shanghai, China, [2]Eye and Ent Hospital of Fudan University, Shanghai, China, [3]Peking Union Medical College Hospital, Beijing, Beijing, China, [4]Frontera Therapeutics, Shanghai, Shanghai, China, [5]Frontera Therapeutics, Shanghai, China
**Corresponding Author*

Background: X-linked retinitis pigmentosa (XLRP) is a severe form of retinitis pigmentosa (RP) characterized by early onset and rapid progression of vision loss or even blindness. The majority of XLRP cases are caused by mutations in the retinitis pigmentosa GTPase regulator (RPGR) gene, and currently there is no effective treatment. FT-002 is an investigational gene therapy that uses the adeno-associated virus serotype 5 (AAV5) vector to deliver a codon-optimized human RPGRORF15 gene into retinal photoreceptor cells through subretinal injection. This gene therapy aims to express active RPGR protein, potentially rescuing photoreceptor cell loss due to RPGR gene mutation, thus improving visual function or delaying the progression of visual impairment in XLRP patients.

Methods: A pilot clinical study (NCT05874310) conducted in China is an open-label, dose-escalation trial enrolling males aged 18-45 years with RPGR mutation-associated XLRP who receive a single subretinal injection of FT-002. The safety and tolerability, immunological responses, and efficacy of FT-002 are evaluated. All participants are encouraged to enter long-term follow-up for up to 5 years. This study complied with ICH-GCP, was approved by the IC of each site, and all participants signed the ICF.

Results: As of January 15, 2025, 18 participants enrolled in the pilot study received FT-002 at doses of 5×10^{10}, 10×10^{10} and 20×10^{10} vg/eye and completed 1 year follow-up. FT-002 was well-tolerated, with no DLT or FT-002 related SAE observed. Most AEs were related to the surgical procedure, and signs of FT-002 related ocular inflammation observed in 8/18 (44%) participants but were controllable via topical use of steroids. Vitreous cells were the most common ocular inflammation of grade 1-2 in severity. OCT imaging revealed no retinal pigment epithelium (RPE) atrophy, and pigmentation observed at the retinotomy sites. Notably, it is the first time to report that no significant progression of ellipsoid zone (EZ) loss was noted at 12 months post FT-002 injection compared to baseline, especially for participants who had well preserved EZ. Additionally, immunological findings did not indicate safety concerns. Improvement in microperimetry mean sensitivity across 68 loci was observed in 10×10^{10} vg/eye (1.32 dB) and in 20×10^{10} vg/eye (0.83 dB) at month 12, with 50.0% and 33.3% of participants maintained ≥7 dB improvement in ≥5 loci, respectively. Updated follow-up data in all participants will be presented.

Conclusion: Subretinal injection of FT-002 at doses of 5×10^{10} to 20×10^{10} vg/eye was generally well-tolerated with no RPE changes noted. FT-002 has the potential to provide long-term clinical benefits for XLRP patients with defects in the RPGR gene, including improvements in visual acuity and retinal sensitivity.

587 Optogenetic Therapy for the Treatment of Photoreceptor Degeneration Diseases

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[1]U-genex, Shanghai, Shanghai, China, [2]Ugenex Therapeutics Ltd.Co, shanghai, Shanghai, China, [3]Ugenex Therapeutics Ltd.Co, shanghai, China, [4]UgeneX, Shanghai, China

Photoreceptor degenerative diseases such as retinitis pigmentosa and age-related macular degeneration ultimately lead to photoreceptor degeneration and irreversible blindness. We aim to restore vision in affected patients through optogenetic therapy, specifically by expressing light-sensitive ion channel proteins in the remaining retinal ganglion cells (RGCs).

We have designed light-sensitive ion channels and their variants with high photosensitivity and excellent photodynamic properties, such as low light sensitivity thresholds, fast response speeds, and high maximum response frequencies. Through extensive screening of various ion channel variants, we selected the most optimal version. It covers the entire visible spectrum, achieves optimal absorption at 550 nm, and exhibits exceptionally low phototoxicity. By combining our in-house developed adeno-associated virus (AAV) capsid vectors and RGC-specific promoters, we successfully delivered the constructs into blind C3H mice and induced full-field electroretinograms (ERGs) under ordinary white-light stimulation. Concurrently, visual evoked potentials (F-VEPs) were recorded in the visual cortex, indicating complete restoration of the visual pathway. Furthermore, the AAV vectors we developed exhibited excellent infection efficiency in the retinas of non-human primates (NHP), effectively targeting multiple regions, including the macula and surrounding areas. In addition, promising light-sensing properties were recorded in NHP retinas.

In conclusion, we have developed an optogenetic therapy candidate with high sensitivity, fast kinetics, exceptional safety profiles, coupling with highly efficient AAV capsid vector, which is now progressing into clinical investigation in photoreceptor degenerative patients.

588 Therapeutic Evaluation of a Gamma-Globin Gene Addition Vector System With a Large LCR in Sickle Cell Disease Mice by *In Vivo* HSC Transduction

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[1]University of Washington, Seattle, Washington, [2]University of Washington, Sandy, UT, [3]University of Washington, [4]Protein Advances Inc., Seattle, WA, [5]University of Washington, Seattle, WA
**Corresponding Author*

We developed an *in vivo* hematopoietic stem cell (HSC) gene therapy strategy by directly targeting mobilized HSCs inside the body by intravenously delivered helper-dependent adenovirus (HDAd) vectors. This strategy does not require *ex vivo* culture of HSCs and transplantation. It holds the potential for broad applications due to its simplified procedure and affordability. Based on *in vivo* HSC transduction in a sickle cell disease mouse model (CD46/Townes), we have previously reported that *in vivo* prime editing mediated the correction of the sickle cell disease mutation and the amelioration of the disease phenotype. However, the *in vivo* use of prime editors could cause editor enzyme-related adverse effects or/and off-target editing. Here, we therefore evaluated the therapeutic efficacy of a HDAd vector that contained a 29kb g-globin expression cassette including 26kb of the b-globin locus-control region (LCR). After *in vivo* transduction with the HDAd-long-LCR-g-globin vector plus HDAd-SB (for vector integration) and *in vivo* selection with low doses of O^6BG/BCNU to expand transduced HSCs by utilizing an $MGMT^{P140K}$ gene incorporated in the vector, we observed that more than 90% of peripheral red blood cells (RBCs) expressed g-globin. Levels of added g-globin protein were >20% of human b-globin present in CD46/Townes mice. The treated animals demonstrated significantly reduced percentages of sickling RBCs compared to untreated controls. Other disease indicators including reticulocyte counts and histological readouts also showed significant amelioration. No noticeable side effects were observed after *in vivo* transduction/selection. We isolated lineage-negative cells from the *in vivo* transduced mice and transplanted them into irradiated C57BL/6 mice. As of week 12 after engraftment, the gamma-globin expression remained stable. We are following the secondary recipients for one more month for terminal analyses. In summary, the endogenous long LCR in the HDAd-long-LCR-γ-globin vector drove therapeutic levels of gamma-globin expression in SCD mice and demonstrated advantages over mini-LCRs which are widely used in the development of *ex vivo* gene therapy products for sickle cell disease.

589 Novel Lentiviral Vectors Enable Controlled Expansion of Transduced Hematopoietic Stem Cells: Beta-Hemoglobinopathies as disease models

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Introduction: Gene therapy holds a significant promise for treatment of beta hemoglobinopathies, although its full therapeutic potential is constrained by several challenges. Clinical approaches employ lentiviral vectors with the truncated beta-globin Locus Control Region (uLCR) driving high-level transgene expression in erythroid cells. However, the large size of the uLCR limits the vector's capacity for multiplex purposes.

Aim: To overcome current limitations and optimize globin vectors for better safety and efficacy, we developed a new lentiviral vector featuring a compact, erythroid-specific enhancer that is significantly smaller than the uLCR, while maintaining strong transgene expression. This modification allows for the integration of two additional functional components; (1) a dual-functioning chromatin insulator, serving both enhancer-blocker and barrier functions to improve transgene expression stability and protect against insertional mutagenesis, and (2) a positive selection ($FKBP^{F36V}$-MPL) cassette that can expand genetically modified cells in vivo upon stimulation in the presence of AP20187. As a therapeutic transgene we have used a short-hairpin RNA (shRNA) targeting the HbF-suppressor BCL11A while a GFP reporter gene was added for normalization and tracking purposes. The $FKBP^{F36V}$-MPL cassette encodes for a fusion protein that initiates proliferation when exposed to its ligand, the chemical inducer of dimerization AP20187.

Methods: After low-MOI transduction, CD34+ HSCs from healthy donors, β-thalassemia and SCD patients were cultured ±AP20187. Transduction efficiency, proliferation during erythroid and myeloid (granulocytic-monocytic) ex vivo differentiation and γ-globin expression in transduced erythrocytes were evaluated by flow cytometry and HPLC, respectively.

Results: Transduction efficiency ranged between 1-30%, depending on the MOI. AP20187-treatment at the end of ex vivo erythropoiesis and granulocytic/monocytic differentiation led to a 2.8±1.6-fold and 58,5±26,5-fold expansion of transduced cells, respectively [GFP+ cells (%): 19,1±9.3 +AP20187 vs 7.5±2,5 -AP20187, p=0.001 and 36.4±24.9 +AP20187 vs 0.67%±0.42 -AP20187, p=0.0001, respectively). Notably, the expanded cells in the granulocytic/monocytic culture exhibited a distinct erythroid signature (CD235a+/CD36+) and were negative for myeloid markers (CD33-/CD13-), indicating that AP20187-mediated expansion is erythroid-specific even in the absence of erythropoietin. Finally, HPLC evaluation of the globin chains at the end of the erythroid differentiation showed a significantly increased γ-globin/β-like-globin ratio after AP20187 treatment in all cultures (healthy donors: 0.30 vs 0.18 (p=0.05), SCD: 0.6 vs 0.41 (p=0.05), thalassemic: 3.49 vs 2.25 (p=0.0001), vs -AP20187).
Conclusions: Our insulated, erythroid-specific lentiviral vector, combined with the FKBPF36V-MPL cassette, provides an optimized system for safe and effective viral vector gene therapy targeting beta hemoglobinopathies. Additionally, it offers the potential for selective expansion of target cells in other hematopoietic stem cell (HSC) gene therapy approaches.

590 Split Intein Lentiviral Vector Hematopoietic Stem Cell Gene Therapy for the Treatment of DOCK8 Deficiency

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Introduction: Dedicator of Cytokinesis 8 (DOCK8) Deficiency is a rare autosomal recessive primary immune deficiency caused by loss of function mutations of the *DOCK8* gene including deletions of various sizes, missense

and nonsense mutations. DOCK8 is expressed primarily in cells of the hematopoietic stem cell lineage and encodes a guanine nucleotide exchange factor (GEF) protein responsible for actin cytoskeletal rearrangement and association and translocation of phosphorylated STAT3. While likely underdiagnosed, roughly 1 in 1 million individuals have DOCK8 Deficiency affected by recurrent and often severe infections involving the skin and respiratory tract along with recalcitrant viral infections leading to dysplasia and cancers. Currently, allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative therapy but can be associated with many complications. Therefore, the goal is to provide safe and improved curative treatments for DOCK8 Deficiency patients by utilizing autologous HSCT and *ex vivo* gene correction strategies. To achieve this goal, we have designed and tested multiple lentiviral vectors. Given the large size of *DOCK8*, we developed split intein constructs which independently undergo genomic integration. Upon translation, two protein halves undergo spontaneous protein splicing *in trans* to generate the desired DOCK8 protein.

Methods: To study candidate vectors *in vitro*, we transduced Jurkat, DOCK8 KO Ramos, DOCK8 KO primary peripheral blood stem cells (PBSCs) and DOCK8 KO CD4 T cells from peripheral blood mononuclear cells (PBMCs) with candidate intein vectors in comparison to constructs containing the entire *DOCK8* cDNA. DOCK8 deficient cell lines and primary cells were generated using CRISPR/Cas9 knockout. VCNs achieved by each candidate vector were determined by ddPCR. Expression and pSTAT3 function of DOCK8 were assessed through flow cytometry and Western Blots, respectively.

Results: When transducing cells with each candidate vector, the single lentiviral vectors achieved VCNs below 0.05 whereas the split intein lentiviral vectors achieved VCNs of ~1 in primary PBSCs. Furthermore, split intein vectors had improved DOCK8 expression at lower doses compared to the single vectors. Mock-treated Jurkats do not express DOCK8, but when transduced and stimulated with hIL-21, there is increased pSTAT3 expression via Western Blot. Ongoing studies focus on functional restoration of DOCK8 in KO CD4 T cells.

Conclusion: Thus far, we have been able to overcome the large size of the DOCK8 gene using a split intein lentiviral vector approach. We have demonstrated the promising potential nature of these intein constructs to express a functional and full length DOCK8 protein *in vitro*. Our next steps include characterization of the candidate constructs in *in vivo* murine models to further validate our lentiviral approach. Our split intein approach could serve as a viable alternative option to gene modify other monogenic diseases due to defects in large genes.

591 Comparing Efficacy and Safety of Prenatal versus Neonatal AAV5 Gene Therapy for Hemophilia A in a Sheep Model

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Persons with severe hemophilia A (HA) lack functional factor VIII (FVIII) protein and experience severe and spontaneous bleeding. Clinical trials in adult HA patients receiving AAV gene therapy (GT) have underscored the risk of hepatoxicity and genotoxicity and demonstrated waning FVIII levels post-treatment. Moreover, the presence of pre-existing anti-AAV antibodies in circulation restricts treatment eligibility and negatively impacts clinical efficacy. We hypothesized in utero AAV-based GT (IUGT) would enable correction of HA prior to birth, circumventing both AAV- and FVIII-related immune hurdles. Sheep fetuses at 60-65 gestation days (human 18-20 g.weeks) received an AAV5 vector encoding a codon optimized, bioengineered fVIII variant (ET3) (AAV5-lcoET3) by ultrasound-guided intraperitoneal injection, at a dose of $2x10^{12}$ vg/kg (n=4) (IUGT recipients). Neonatal lambs (3 days old) received the same dose of AAV5-lcoET3 (n=3) via cephalic vein (NNGT recipients). Both groups were evaluated for hematological and hepatic alterations. All IUGT recipients evaluated monthly post-birth had normal complete cell counts (CBCs) and normal liver enzymes, while all NNGT recipients had normal CBCs but increased alanine aminotransferase (ALT) levels by 7 days post-GT, returning to normal by 30d. In addition, one NNGT

recipient maintained elevated gamma glutamyltransferase (GGT) levels up to 30 days post-GT, which subsequently returned to normal (60d) without treatment. ET3 protein in circulation was quantified using an ET3-specific ELISA. IUGT recipients had mean ET3 levels of 7.3+/-3.03 ng/mL at 120d post-IUGT corresponding to 30 days post-birth. Intriguingly, 1 of 3 IUGT recipients exhibited circulating ET3 levels for up to 270d post-IUGT. Specifically, it had plasma ET3 levels of 8.79+/-1.13 ng/mL at 120d, 3.9+/-0.44 ng/mL at 150d, 2.07+/-0.08 ng/mL at 210d, 3.49+/-0.39 ng/mL at 240d, and 24.25+/-0.36 ng/mL at 270d post-IUGT. Circulating ET3 levels in the other two IUGT recipients dropped below the level of detection by 150d post-IUGT. In contrast, all NNGT recipients exhibited an initial spike of ET3 (5.26 +/- 1.6 ng/mL) at 7d post-NNGT, with ET3 levels declining by day 30 to 0-1 ng/mL. ET3 transcripts were detected in circulating mononuclear cells by RT-dPCR in both IUGT and NNGT recipients up to 210 and 120d post-GT respectively. To determine immunogenicity of ET3 and of the AAV vector, ELISAs were performed for anti-ET3 IgG and anti-AAV5 IgG antibodies, respectively. While no anti-ET3 antibodies were detected at any timepoint in the IUGT recipient with sustained ET3 levels, the other IUGT recipients developed anti-ET3 antibodies detectable at 240 and 270d post-IUGT respectively, and two of the NNGT recipients developed anti-ET3 IgG at 120d and 180d post-NNGT. Of note, anti-AAV5 IgG antibodies emerged at similar time points as the anti-ET3 IgG antibodies and were detected in the same IUGT and NNGT recipients that developed anti-ET3 IgG; in one of the animals, a wild AAV-type infection was confirmed prior development of anti-AAV antibodies. Studies are ongoing to elucidate the: 1) molecular mechanism by which therapeutic ET3 persists long-term in the IUGT recipient and 2) cause of the development of anti-ET3 antibodies after waned circulating ET3. These studies will improve our understanding of efficacy and of potential toxicities of IUGT and NNGT using AAV vectors and will pave the way for safer and more effective treatments for HA.

592 Development of a Humanized Immune system mouse supporting primary AML PDX engraftment for drug efficacy studies

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Acute Myeloid Leukemia (AML) is a devastating hematological malignancy with a global incidence of approximately 120,000 new cases annually and a five-year survival rate below 30%. Despite advances in therapeutic strategies, treatment outcomes remain poor. The complexity of AML, characterized by genetic heterogeneity and clonal evolution, presents significant challenges for the development and testing of novel therapeutics in clinically relevant settings.

AML research relies heavily on cell lines and syngeneic rodent models, or immunodeficient rodent models without the inclusion of a human immune system. While these models provide valuable insights, they fail to fully recapitulate the genetic and phenotypic diversity of primary human AML tumors. Traditional models often lack the tumor microenvironment and immune interactions critical for therapeutic evaluation and successful translation to the clinic. Furthermore, existing models generally rely on extensively passaged samples, which lose the heterogeneity and clonal diversity intrinsic to AML, undermining their clinical relevance.

To address these limitations, we developed an in vivo model supporting a primary AML PDX and a humanized immune system derived from CD34+ hematopoietic stem cells (HSCs). The NOG-EXL mouse was selected as the preferred in vivo model as it has been successfully engrafted with primary AML PDXs, albeit not also with a humanized immune system component. The NOG-EXL, transgenic for human IL-3 and GM-CSF, supports engraftment of both human myeloid and lymphoid lineages, making it suitable for diverse therapeutic applications. Additionally, stem cell donors were selected so that HLA-matching to specific AML PDXs was optimized. The model was generated by first engrafting HLA-matched HSCs into NOG-EXL mice followed by engraftment of the primary AML PDX. Human immune cell engraftment and AML cancer progression were assessed at different timepoints via flow cytometry on peripheral blood and bone marrow. A custom flow cytometry panel was used to identify the human immune cells present as well as the main AML sub-populations including the AML progenitors, AML blasts and monoblasts.

This novel approach bridges a critical gap in AML research by offering a robust, clinically relevant platform that reflects real-world disease biology by demonstrating an in vivo model with primary, never-passaged AML PDX in the context of a human immune system. The AML humanized model sets a new standard for preclinical evaluation, promising to accelerate the development of personalized therapies and improve outcomes for AML patients worldwide.

593 Human Liver Tissue Equivalent (hLTE) Models for Studying the Safety and Efficacy of AAV-based Gene Therapy for Patients with Hemophilia A

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Gene therapy (GT) promises long-term-treatment for hemophilia A (HA), a bleeding disorder caused by various mutations in the F8 gene. Ongoing clinical trials with AAV-based GT in adults showed that most patients experienced unexpected hepatoxicity and required subsequent immunosuppression with FVIII levels dropping over time, forcing patients to revert to factor replacement. These data underscore key differences compared to data from pre-clinical animal models and emphasize the need for more accurate models enabling the assessment of the safety and efficacy of GT products in a fully human system. To investigate the impact recipient age has on these variables and whether GT could be safely extended to younger populations, we developed human liver tissue equivalent (hLTE) models to test the efficacy and potential toxicity of AAV-mediated GT at different ages. Using adult-derived hLTE, we have previously shown impaired self-aggregation of hLTE, and a significant dysregulation of genes involved in hepatotoxicity and inflammation. Here, we used pediatric human donors' hepatocytes and stromal cells (Kupffer, stellate, and sinusoidal endothelial cells) to develop a 2000-cell organoid that recapitulates the structure, biology, and functions of the native liver. The cells were transduced using AAV5 or AAV6 encoding GFP in suspension and were plated in U-bottom 96-well plates to self-aggregate into hLTEs, alongside non-transduced (NT) controls. The hLTEs were monitored for 10 days; GFP expression was monitored daily via fluorescence imaging, DNA and RNA were collected on days 5 and 10 for dPCR, viability assays (Live/Dead, ATP) were performed on days 5 and 10, a functionality assay (CyP450) was performed on days 7-10, and media was collected on days 5-10 to perform ELISAs for urea, albumin, transaminases (ALT, AST), and Alpha-1 antitrypsin (AAT). Data from 3 biological replicates of pediatric hLTEs indicate transduction with AAV5 or AAV6 had no significant impact on hLTE function or viability as determined by Live/Dead, ATP, and Cyp450 assays when compared to NT groups. Fluorescence imaging of the GFP transgene demonstrated a continuous increase in GFP signal in both AAV5 and AAV6, with significantly higher levels of GFP expression in AAV6 beginning on day 4 post-transduction. dPCR results indicated significantly higher levels of GFP mRNA in the AAV5-transduced hLTEs compared to AAV6. Interestingly, there was no significant difference in transgene DNA copy number between transduced groups. No significant differences in urea and albumin production were found; however, we observed a large variation in AST and ALT levels between biological replicates, indicating that patient profile plays a significant role in transaminase response to transduction. Additional comparison studies using adult hLTEs to define the impact of recipient age on efficacy and hepatic response to GT are currently underway.

594 An *In Vitro* Air-Liquid Interface Lung Model of Alpha1 Antitrypsin Deficiency for Assessing an Adeno Associated Virus Based Genetic Medicine

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Alpha1 antitrypsin deficiency (A1ATD) is a rare genetic disease caused by mutations in the SERPINA1 gene, resulting in deficient levels of A1AT. Obstructive lung disease develops when the lack of A1AT in the alveoli leads to uninhibited elastase activity, causing connective tissue breakdown and the development of emphysema in the lungs. Treatment options for individuals with lung diseases associated with A1ATD are very limited and largely mirror those available for patients with non-A1ATD-related chronic obstructive pulmonary disease (COPD), including bronchodilators, steroids, antibiotics and other anti-inflammatory therapies. The only regulatory agency-approved disease-specific therapy for A1ATD is intravenous augmentation therapy with plasma-purified A1AT. Adeno-associated virus (AAV)-based genetic medicine targeting the lung parenchyma has the potential to serve as a long-term approach for the treatment of A1ATD lung disease, by expressing the wild type (WT) allele M-AAT gene in pulmonary cells, which will then secrete the protein into the airway, thereby augmenting the lung levels of A1AT and preventing alveolar proteolytic destruction.

To facilitate assessment and development of translatable therapeutic candidates, we developed an air-liquid interface (ALI) lung model using iPSCs derived from PiZZ A1ATD patient cells. PiZZ and control WT iPSCs were expanded and differentiated into lung progenitor cells, which were then seeded onto transwell membranes to differentiate and mature into an ALI system. The ALI cultures were characterized through immunofluorescent staining targeting biomarkers for key airway epithelial cells, including ciliated, goblet, basal, alveoli and club cells. Disease markers were characterized by protein analysis using gel electrophoresis (PAGE) on cell lysate and enzyme-linked immunosorbent assay (ELISA) on cell culture basal medium.

The PiZZ iPSC-ALI disease model exhibited a decreased secretion of A1AT compared to the WT iPSC-ALI model and showed an increased accumulation of aggregated intracellular A1AT in the total cell lysate. The cells were transduced apically using an AAV carrying the A1AT transgene payload, and the basal medium was collected over time to assess A1AT expression and functionality of exogenously produced A1AT following AAV transduction. Both WT and PiZZ iPSC-ALI models were successfully transduced with AAV, resulting in A1AT secretion in a dose-dependent manner at varied multiplicities of infections (MOIs). In both contexts, functional A1AT was produced and secreted, as demonstrated by the ability of the cell culture basal medium to inhibit neutrophil elastase activity.

In conclusion, a PiZZ iPSC-ALI lung model was developed that replicated key disease characteristics. This A1ATD disease model can serve as a valuable tool for evaluating genetic medicine candidates during their product development process.

595 A Novel Strategy for Treating Gefitinib-Resistant Cancer: Correction of Multiple pathogenic single-nucleotide polymorphism (SNP) Using Adenine Base Editor- expressing adenovirus

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This study presents an innovative strategy for addressing drug-resistant cancers by targeting specific oncogenic mutations. The research focuses on editing targeted mutations. Here we have targeted EGFR-T790M and TP53-R273H, single-nucleotide missense mutations associated with gefitinib resistance in cancer by utilization of an adenine base editor (ABE) coupled with specific single-guide RNAs, delivered via adenovirus. The ABE system demonstrated high accuracy and efficiency in correcting oncogenic mutation sites both in vitro and in vivo. Co-treatment with the ABE system targeting EGFR and TP53 SNPs, combined with gefitinib, showed superior results compared to gefitinib alone. Notably, this combination therapy increased drug sensitivity and more effectively suppressed abnormal tumor growth. Collectively, this approach not only addresses the issue of drug resistance but also offers a more targeted and potentially more effective treatment option for cancer patients.

596 Design and Development of Improved LNP Targeting Ligands for *In Vivo* Hematopoietic Stem Cell Editing

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Lipid nanoparticle (LNP) technologies have emerged as effective vehicles for *in vivo* delivery of therapeutics for a variety of diseases. The ability to deliver nucleic acids via LNPs presents an opportunity to leverage CRISPR/Cas-based gene editing machinery *in vivo* to correct genetic diseases with a minimally invasive approach. Although significant progress has been made in adjusting LNP composition to target organs of interest, the specific targeting of subcellular compartments within a given organ remains a challenge. In particular, the hematopoietic stem cell (HSC) compartment represents a minority fraction of the total bone marrow that generates the entirety of an organism's blood system. Enabling LNP transfection of the bone marrow can be broadly met through adjusting lipid composition, however targeting the HSC compartment remains a challenge. Here, we describe the development and optimization of a targeted LNP (tLNP) strategy to enable editing of HSCs *in vivo*.

We designed a screening platform to select targeting ligands that maximize the internalization of cell-surface targets while maintaining cellular specificity for HSCs. This approach utilizes a combination of available omics datasets paired with a flow cytometry-based measurement with the cell target of interest. Potential targeting candidates are further assessed using a tLNP format, which enables selection of an optimal targeting ligand. Using this platform *in vitro*, we identified an improved targeting ligand, Ligand 2, for HSCs that yielded 7 times greater internalization compared with Ligand 1, our first iteration of targeting ligand, as measured by the mean fluorescence intensity of the target. These *in vitro* findings directly translated to LNP efficacy in the NBSGW mouse model engrafted with human hematopoietic and stem progenitor cells (HSPCs). Upon infusion of tLNPs containing our proprietary editing cargo, targeting Ligand 1 achieved 0.25% editing of HSPCs in humanized mice, while Ligand 2 produced 26% editing. While the core LNP remains the same, these data indicate that the targeting ligand is a significant contributor to achieving abundant transfection directed to a discrete cell compartment of the bone marrow. The validation of this *in vitro* testing platform in an *in vivo* context underscores the need for targeting elements to actively promote the uptake of LNPs in the HSC population of the bone marrow, providing an opportunity for target cell-specific editing for the durable treatment of β-hemoglobinopathies.

597 Comprehensive Analysis of ABL1 Variant Resistance to Multiple Kinase Inhibitors via Prime Editing

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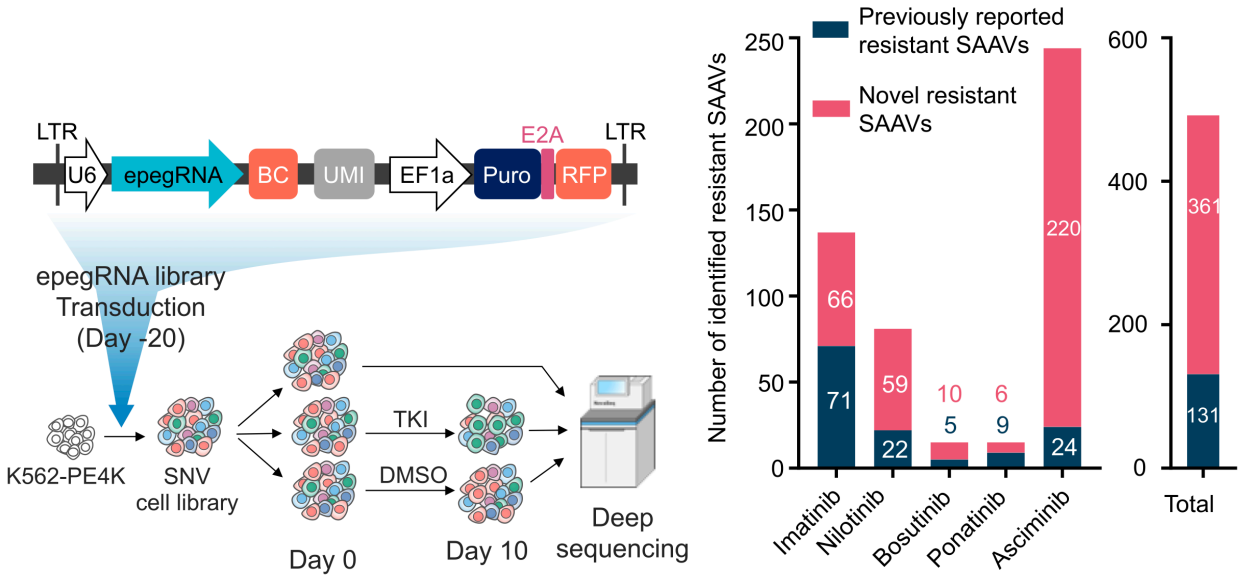
Uncertain significance variants (VUSs) pose a challenge to the clinical utilization of genetic data, particularly in the context of precision medicine. Chronic myeloid leukemia (CML) exemplifies this issue, as treatment relies on tyrosine kinase inhibitors (TKIs) spanning four generations, yet predicting TKI resistance for patients harboring ABL1 mutations remains challenging, especially when these mutations are not accounted for in existing clinical guidelines.

In this study, we employed prime editing to systematically introduce 97% (2,802/2,892) of all potential single nucleotide variants within the ABL1 kinase domain, resulting in the expression of 98% (1,954/1,998) of all possible single amino acid variants (SAAVs). We then assessed the resistance profiles of these SAAVs against a panel of five TKIs—imatinib, nilotinib, bosutinib, ponatinib, and asciminib—representing all four TKI generations, using CML-relevant K562 cells.

Through this approach, we identified 361 previously uncharacterized SAAV-TKI resistance pairs, significantly expanding the current knowledge of mutation-driven drug resistance. The comprehensive resistance landscape generated in this study serves as a valuable resource to refine clinical decision-making and enhance precision

medicine strategies for CML patients by providing an evidence-based framework for drug selection based on ABL1 mutation status.



598 Fixing the Balance: A Dual-Targeted Base Editing approach to reduce α-globin and reactivate Fetal Hemoglobin to treat β-hemoglobinopathies

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Beta-hemoglobinopathies are caused by a reduced or abnormal synthesis of the adult hemoglobin (Hb) β-chain. In sickle cell disease (SCD), a point mutation in the β-globin gene results in β^S-chain production and leads to Hb polymerization, causing red blood cell (RBC) sickling and organ damage. In β-thalassemia, mutations in the β-globin causes a reduced (β^+) or absent (β^0) β-chain production, α-globin precipitation and death of erythroid precursors. Transplantation of autologous, genetically modified hematopoietic stem cells (HSCs) is an attractive therapeutic option.

Patients affected by these disorders in association with α-thalassemia traits have improved clinical phenotypes. In β-thalassemia, the co-occurrence of α-thalassemia, characterized by a reduced α-globin expression, ameliorates the α/β globin ratio and decreases free α-chains, thus reducing death of erythroid precursors. In SCD, reduced α-globin levels lower Hb concentration decreasing Hb polymerization. Moreover, the lower affinity of β^S-chain for α-chain compared to the wt β-chain generates excess of toxic α-chains, which is diminished with the co-inheritance of α-thalassemia. In parallel, mutations inducing reactivation of fetal β-like γ-globin and expression of fetal Hb

(HbF) can also ameliorate the β-thalassemic and SCD phenotypes by improving the α-/β-globin ratio and preventing Hb polymerization, respectively. Base editing (BE) allows specific DNA mutations (C>T, cytidine base editors, CBEs; A>G, adenine BEs, ABEs) without generating dangerous double strand breaks, thus allowing the multiplexed editing of several targets. We developed a common BE strategy that concomitantly (i) reduce α-globin production by targeting MCS-R2 enhancer, the most critical regulator of the α-globin *HBA* genes and (ii) reactivate HbF expression by disrupting enhancers of BCL11A, a master repressor of γ-globin expression.

First, we used ABEs and CBEs to target either the GATA1 or NFE-2 activator binding sites (BS) present in MCS-R2 *HBA* enhancer. Screening in K562 erythroid cell line showed that disruption of different BS resulted in significant differences in α-globin production, suggesting that some BSs within MCS-R2 enhancer are more critical for α-globin expression. Based on our screening results, we were able to partially down-regulate α-globin synthesis to 50% of its normal levels in both SCD and β-thalassemic RBCs derived from HSCs carrying up to 80% of edited enhancers. This led to a better α-globin/β-like globin ratio in β-thalassemic RBCs. While modulation of α-chains levels may be sufficient for β^+-thalassemic patients, it might be limiting for β^0-thalassemic or SCD patients, as these patients require the additional expression of a β-like chain. Thus, we simultaneously targeted the MCS-R2 enhancer and the GATA1 or ATF4 activators binding motifs within the erythroid-specific *BCL11A* enhancers (+58-kb and +55-kb regions) to reactivate HbF using ABEs. Targeting both regions in HSCs did not alter editing efficiencies at each locus that reached up to 80%. A colony forming cell assay showed no impact on progenitors' viability. Besides reduced α-globin levels, RBCs displayed high HbF levels that were sufficient to ameliorate the pathological phenotype. Overall, we reactivated γ-globin expression and downregulated α-globin, resulting in beneficial effects resembling those observed in patients with co-inheritance of α-thalassemia.

599 eePASSIGE Knock-In of *F8* Exons 23-26 cDNA for Intron 22 Inversion Mutations

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Introduction:
Hemophilia A, the most common severe congenital bleeding disorder, is caused by *F8* mutations resulting in deficient function of the coagulation factor VIII (FVIII) protein. Given modest FVIII function imparts large phenotypic improvement, meaning that even low-efficiency *F8* gene correction or integration is expected to be clinically significant. The *F8* intron 22 inversion mutation accounts for nearly 50% of severe hemophilia A cases. In this intrachromosomal recombination event, a gene within intron 22 of *F8* recombines with one of two copies upstream and telomeric to the *F8* sequence. This rearrangement splits *F8* into two parts, separating exons 1-22 from exons 23-26. The unaffected *F8* promoter continues to transcribe the first 22 exons, resulting in a truncated, nonfunctional protein. Knock-in of exons 23-26 cDNA into intron 22, before the site of recombination, could rescue transcription of the full *F8* gene through the sustained use of the endogenous promoter and subsequently restore hemostatic function. Due to the size of exons 23-26 cDNA (627bp), few tools are available for the insertion of a DNA fragment this size without double-stranded breaks. Currently, there are no published findings using twin prime editing, PASSIGE, or any similar derivatives for any *F8* disease-causing mutation, including the intron 22 inversion. We aim to use recently enhanced eePASSIGE (Pandey et al. Nat Biomed Eng 2025) to efficiently insert codon-optimized exons 23-26 cDNA using single-stranded breaks, thus minimizing the risk of genotoxicity, to rescue complete transcription of *F8* and restore FVIII hemostatic function.

Methods and Results:
63 combinations of prime editing RNA guides (pegRNAs) were designed in the first 500bp of intron 22 of *F8* to insert the *attP* sequence. The guides were transfected in Huh7 cells with the prime editor (PE)6d variant. Screening results were analyzed through Next-Generation Sequencing (NGS) to determine precise *attP* insertion, imperfect edits, and non-homologous end joining (NHEJ) indels. Five combinations of guides, varying only by primer binding site lengths, achieved >18% precise insertion with <1.4% imperfect edits and <0.3% NHEJ indels (Figure 1). NHEJ indel percentages were consistent with untransfected control cells and thus considered negative. The highest achieving pegRNA pair, which demonstrated 20.13% precise insertion, 1.27% imperfect edits, and 0.21% NHEJ indels, was used in the following transfection. PE6d, eeBxb1, pegRNAs, and a donor plasmid containing *F8* exons 23-26 cDNA, splice acceptor, and *attB* sequence, were co-transfected in Huh7 cells to assess integration of the

donor. Insertion was evaluated by junction PCR of the region between endogenous intron 22 and the newly introduced exon 23. Sanger sequencing results showed recombination of *attP* and *attB* forming *attL* as well as the correct insertion and orientation of the splice acceptor and the *F8* exons 23-26 cDNA.

Conclusion:
These data indicate the possibility of using eePASSIGE to restore loss of FVIII function for hemophilia A patients with intron 22 inversion mutations. The use of the endogenous *F8* promoter and single-stranded breaks provide significant benefits from a safety and efficacy perspective. Work is ongoing to evaluate the phenotypic outcomes of these methods in an in vitro stable cell line and an in vivo mouse model.

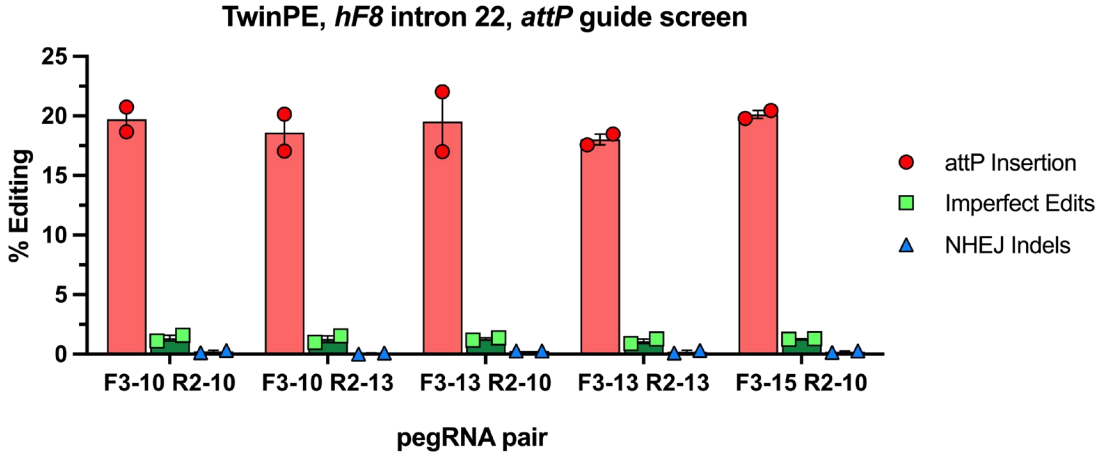

Figure 1: Twin prime editing guide screen leading candidates. Data are mean ± SEM (n=2).

600 Base Editing Rescue of *SOD1* associated ALS

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Background: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with a median survival of 2-5 years after diagnosis. Mutations in the cytosolic superoxide dismutase (*SOD1*) gene, which encodes an antioxidant enzyme, account for ~20% of familial ALS and 2-3% of sporadic ALS. Misfolded SOD1 mutants form toxic aggregates that drive motor neuron degeneration and ultimately lead to respiratory failure. Despite extensive research, treatment options remain limited. Antisense oligonucleotides (ASOs) targeting SOD1, known as tofersen, have been promising in reducing mutant SOD1 protein levels but require repeated administration, limiting their long-term feasibility. Base editors offer a potentially more durable genetic therapy by precisely modifying pathogenic SOD1 transcripts without inducing double-stranded breaks. In this study, we screened a panel of Nme2 base editors to identify one capable of disrupting SOD1 expression via splice site editing.

Methods and Results: We screened a panel of Nme2 base editors with variations in deaminase positioning and linker length to identify the most efficient editor for targeting a splice site in the *SOD1* gene at exon 2. Various linker lengths and deaminase positioning can change the editing window and hence change the editing efficiency at the target adenine. Successful editing induces exon skipping, leading to decreased SOD1 transcript levels. Our most effective editor achieved ~35% editing efficiency. Verification at the endogenous site via plasmid transfection confirmed similar editing efficiency (~35%). An all-in-one version showed ~17% editing, which correlated with a two-fold reduction in total SOD1 RNA by qRT-PCR and a four-fold decrease in protein levels. Analysis of bystander edits showed that the amino acids remained conserved.

Conclusions: A gene-editing therapy that reduces the SOD1 transcript levels could serve as a one-time treatment for ALS patients with *SOD1*-linked disease. Our study demonstrated that Nme2 base editors are promising candidates, effectively reducing both mRNA and protein levels. By targeting a splice site, this approach is mutation-agnostic, making it broadly applicable to a larger ALS patient population. Future studies will focus on optimizing delivery and assessing long-term therapeutic potential in ALS disease models.

601 Base Editing for Precise and Specific Elimination of Lipoprotein(a)

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Lipoprotein(a) (Lp(a)) is a causal risk factor for cardiovascular disease (CVD), and current therapeutic options are limited. Commercial efforts are currently underway to develop one-time gene editing interventions aimed at permanent inactivation of the *LPA* gene in the livers of high risk patients. In our previous work, we demonstrated that adeno-associated virus (AAV) delivery of CRISPR/Cas9 could reduce liver and plasma apolipoprotein(a) (apo(a)) to undetectable levels in an *LPA* transgenic mouse model. However, due to the highly repetitive nature of the targeted genomic region, we observed frequent chromosomal rearrangements in the treated animals - including large deletions, inversions, and AAV genome insertions. Consequently, we hypothesized that cytosine base editing (CBE) could be used to more precisely introduce premature stop codons in *LPA* to decrease Lp(a) levels, while circumventing double-strand DNA breaks and the associated deleterious chromosomal rearrangements.

Guide RNAs (gRNAs) were designed with BE-Designer to target exon 2 of the *LPA* gene and the latest TadA-derived CBE (CBE6) was utilized to install a premature stop codon. *In vitro* testing in HEK293T cells revealed successful editing by several gRNAs, with guide #1 and #2 exhibiting the highest editing efficiency (23 ±1.9 and 44 ± 1.4 % respectively). gRNAs were also tested in cell lines that stably express apo(a) or an inactive plasminogen protein (PLG-S741C). Both gRNAs reduced secreted apo(a) levels by 50%, and only guide #2 decreased secreted PLG-S741C levels, indicating off-target activity, which was confirmed by DNA sequencing. To test this approach *in vivo,* CBE6 was packaged into helper-dependent gutless adenovirus driven by a liver specific promoter (HDAdV-CBE6). gRNAs 1 and 2 were packaged into AAV and injected separately into *LPA* transgenic mice via tail vein at a dose of 5×10^{11} genome copies (GC), in conjunction with 3×10^{11} viral particles (VP) of HDAdV-CBE6. An 80% reduction of circulating apo(a) was measured 10 days after viral injection.

Ongoing efforts aim to use lipid nanoparticles (LNP) to deliver the CBE6 and evaluate the capacity of this LNP-CBE approach to reduce apo(a) and Lp(a) levels *in vivo*. This approach offers a promising avenue for reducing Lp(a) levels and addressing a significant unmet need in CVD management.

602 High Efficiency Prime Editing of Alzheimer's Disease High Risk APOE4 Allele by Brain-directed AAV Vectors

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Common variants of the apolipoprotein E gene (APOE) have a major impact on the risk of developing Alzheimer's disease (AD). Relative to homozygotes with the common E3 allele, the APOE4 variant (C112R) increases risk by 3.5-fold in E3/E4 heterozygotes and 13.5-fold in E4 homozygotes. Since the E3 and E4 alleles differ only by a single nucleotide, gene editing is a potential route to reducing AD risk in E4 homozygotes. Because the apolipoprotein E pool in the brain is separate from systemic APOE, editing would ideally be directed to the brain without disrupting the systemic APOE pool. Following *in vitro* optimization of editing guide RNAs, efficient prime editing expression cassettes were inserted in the optimized 6th generation prime editor PE6 split-intein plasmid and

packaged into pairs of adeno-associated viruses (AAV) for *in vivo* editing. TRE4 transgenic mice with replacement of the mouse ApoE gene by the human APOE4 variant were administered AAV vectors and APOE4 to APOE3 editing efficiency was assessed after 4 weeks. We compared editing efficiency in the central nervous system (CNS) using three different delivery routes (intrahippocampal, intracisterna magna and intravenous) and three AAV capsids: AAVrh.10 a clinically tested vector for CNS transduction (Sondhi D et al, Sci Transl Med 2020; 12:eabb5413); AAV.S2 an engineered AAVrh.10 derivative with improved distribution following intraparenchymal brain administration (Karan K et al, Hum Gene Ther 2024; 35:904); and AAV-CAP.B10, an engineered AAV9 derivative designed to enhance transport through the blood brain barrier (Goertsen et al., Nature Neurosci. 2022; 25:06). The same dose (10^{12} genome copies) was used for all vectors and routes. Intrahippocampal administration of AAVrh.10 vector achieved an average of 18.6±5.5% editing across the brain but with higher editing in cortex (28.1±6.1%) than cerebellum (3.4±2.1%). A higher average editing efficiency was obtained by IHC injection of AAV.S2 with an average of 26.7±3.1% editing across the brain with high editing both in cortex (21.2±10.8%) and in cerebellum (41.5±2.3%). Intravenous delivery of AAV-CAP.B10 provided 24.5±4.8% across the brain. Vector DNA levels were the same for all 3 groups ($p>0.05$). However, CNS delivery of either AAVrh.10 or AAV.S2 led to significantly higher vector copies in the liver [1.5±0.6 vector copies/haploid genome (vc/g) for AAVrh.10; 1.47±0.1 vc/g for AAV.S2] while intravenous CAP.B10 mediated only 0.1±0.1 vc/g in the liver. We conclude that the high efficiency APOE4 to APOE3 editing in the brain is feasible using intrahippocampal administration of the clinically proven AAVrh.10 vector. However, a wider distribution of editing in the brain was achieved by both IHC administration of AAV.S2, an engineered AAVrh.10 derivative, and by intravenous delivery of brain-penetrant AAV-CAP.B10. In future studies, these novel engineered capsids may provide the best therapeutic option.

603 In vivo base editing extends lifespan of a humanized mouse model of prion disease

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Prion disease is a fatal neurodegenerative disease caused by the misfolding of prion protein (PrP) encoded by the *PRNP* gene. While there is currently no cure for the disease, depleting PrP in the brain is an established strategy to prevent or stall templated misfolding of PrP. We developed *in vivo* cytosine and adenine base strategies delivered by adeno-associated viruses (AAVs) to permanently modify the *PRNP* locus to achieve PrP knockdown in the mouse brain. Systemic injection of dual-AAV PHP.eB encoding BE3.9max and sgRNA installing *PRNP* R37X resulted in 37% average installation of the desired edit, 50% reduction of PrP in the mouse brain, and 52% extension of lifespan in transgenic human *PRNP* mice inoculated with pathogenic human prion isolates representing the most common sporadic and genetic subtypes of prion disease. We further engineered base editing systems to achieve improved *in vivo* potency and reduced base editor expression in non-targeting tissues, resulting in 63% average PrP reduction in the mouse brain from a 6.7-fold lower viral dose, with no detected off-target editing of anticipated clinical significance observed in either human cells or mouse tissues. These findings support the potential of *in vivo* base editing as one-time treatment for prion disease.

604 Targeted Gene Correction Using AAV-Delivered Base Editing Improves Smooth Muscle Cell Function in the Eye of an ACTA2^{R179H} MSMDS Mouse Model: A Promising Surrogate Endpoint For Future Clinical Trials

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Introduction: Multisystemic smooth muscle dysfunction syndrome (MSMDS) is a severe, ultrarare disorder caused by pathogenic variants in ACTA2, leading to widespread smooth muscle dysfunction, vascular abnormalities, and life-threatening complications such as early-onset stroke and aortic dissection. A key early diagnostic indicator is congenital mydriasis caused by impaired contractility of iris smooth muscle cells (SMCs). In this study, we evaluated the therapeutic efficacy of CRISPR-Cas9 adenine base editors (ABEs) delivered via a novel smooth muscle–tropic adeno-associated virus (AAVPR-AB8e-VRQR) in rescuing pupillary contractility and retinal vasculature defects in an MSMDS mouse model. Concurrently, we utilized a Cre-inducible tdTomato reporter system (AAVPR-Cre) to assess the *in vivo* tropism and cellular specificity of our AAVPR construct.

Methods: An MSMDS mouse model developed in-house was retroorbitally injected with AAVPR-AB8e-VRQR at postnatal day 3. At 8 weeks, we analyzed pupillary reflex to light and performed conventional histology and immunofluorescence of SMC markers. To validate the targeting specificity of our AAVPR vector, we utilized Cre-inducible tdTomato reporter mice injected with AAVPR-Cre, with retinal tissues harvested 5 weeks post-injection for biodistribution analysis.

Results: Untreated MSMDS mice exhibited dilated, non-reactive pupils and a marked reduction in α-SMA expression in the iris and retinal vasculature. Retinal analysis revealed abnormalities in arteriole branching, vessel caliber, and autonomic innervation. AAVPR-AB8e-VRQR-treated mice demonstrated significant restoration of α-SMA expression, normalization of vascular morphology, and, in a subset of mice, partial recovery of pupillary reflexes—supporting the therapeutic potential of base editing in smooth muscle diseases. To confirm the targeting efficiency of our AAVPR vector, Cre-inducible tdTomato mice injected with AAVPR-Cre exhibited widespread tdTomato expression in retinal smooth muscle cells, endothelial cells, and pericytes, validating the construct's tropism for smooth muscle-rich vasculature.

Conclusions: These findings provide the first evidence that systemic delivery of AAVPR-AB8e-VRQR can restore smooth muscle contractility and vascular integrity in MSMDS mice through CRISPR-based gene correction. The partial rescue of pupillary function suggests that gene therapy may effectively reverse aspects of MSMDS pathology, although residual innervation deficits or dose-response effects may influence outcomes. Importantly, the use of a Cre-inducible tdTomato reporter system confirms the tropism of our AAVPR vector, supporting its potential as a platform for future therapeutic applications. Given the systemic nature of MSMDS, the restoration of smooth muscle function in the anterior chamber and retinal vasculature provides a critical, noninvasive surrogate endpoint for evaluating broader treatment efficacy in clinical trials.

605 Precise Correction of the *Pde6b*-L659P Mutation Causing Retinal Degeneration via Base and Prime Editing

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Purpose: Retinitis pigmentosa (RP) is a group of inherited retinal disorders characterized by the progressive degeneration of photoreceptor cells, ultimately leading to blindness. The *Pde6b* gene, which encodes the β-subunit of rod cGMP phosphodiesterase, has been identified as a causative gene for autosomal recessive retinitis pigmentosa (RP40). Currently, no effective treatments are available for this condition. Genome editing technologies offer promising potential for treating inherited genetic disorders. Among these, recently developed base editing (BE) and prime editing (PE) technologies allow precise and efficient genome editing while minimizing the risks of double-strand DNA breaks and associated toxicity. This study explores the potential of BE and PE technologies to rescue retinal degeneration in a mouse model of RP carrying the *Pde6b*-L659P mutation (c.1976T>C, p.L659P).

Methods: A Neuro-2a (N2a) cell line harboring the *Pde6b* (c.1976T>C, p.L659P) mutation was generated using BE-mediated modeling, serving as the N2a cell model. BE or PE vectors were transfected into the N2a cell model, and editing efficiencies were assessed via Sanger sequencing. Plasmids expressing the optimized BE or PE systems were electroporated into the retinas of *Pde6b* mutant mice on postnatal day 0 (P0), with co-expressed EGFP used as a marker for successful electroporation. Electroporated retinas were collected at P30, and the rescue effect was evaluated through sanger sequencing and immunofluorescence (IF) analysis.

Results: We tested four CBE systems, including rA1-CBE, A3G-CBE, CDA1-CBE, and AID-CBE, along with seven sgRNAs in the N2a cell model. Sequencing results revealed that only the A3G-CBE system was able to precisely correct the L659P mutation, while the other three CBE systems generated a high proportion of bystander mutations. The optimal combination of A3G-CBE and sgRNA4 achieved a 15% targeted correction rate. For PE, we tested five PE systems, five epegRNAs, and six nicking gRNAs. The optimal combination of PE7, e2, and n1 achieved an 20% precise correction rate in the N2a cell model. In vivo electroporation of BE or PE plasmids achieved approximately 10% targeted editing, and IF results confirmed the restored expression of the PDE6B protein.

Conclusions: This study provides a proof-of-concept demonstration for precisely correction of Pde6b-L659P mutation causing retinal degeneration by BE and PE tools, paving the way for future translational approaches.

606 Improving the Safety and Efficacy of *In Vivo* Genome Editing in HSCs by Using Strong HSC-Specific Promoters and a Pomalidomide-Induction-System to Regulate the Level and Duration of Editor Expression after HDAd Gene Transfer.

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We are using HDAd vectors for *in vivo* genome editing in HSCs with a focus on therapies of hemoglobinopathies and HIV/AIDS. Our approach requires improvements both in its safety and efficacy. While our HDAd vectors preferentially transduce primitive HSCs after intravenous injection into mobilized CD46-transgenic mice, humanized mice and rhesus macaques, off-target transduction (e.g. other blood cell lineages and cells in the spleen, liver, and lung) was also found. Furthermore, the comparison of the PGK and the relatively strong EF1a promoters to drive ABE8e expression has shown that the editor expression level critically determines the editing rates/percentage of edited cells, specifically in multiplex editing vectors. Our first goal was therefore to develop highly active HSC-specific promoters. First, we generated GFP reporter plasmids containing ~2kb of proximal promoters (encompassing the TATA box, transcription start site and 5'UTR) derived from genes that are expressed in HSCs at high levels (CD164, cKit, DSG2, PROM1, and PROCR). Nucleofection of these plasmids containing these promoters into human $CD34^+$ cells showed the highest GFP expression levels for the cKit and PROCR promoters in $CD34^+$ subsets enriched for HSCs ($CD34^+/CD45RA^-/CD90^+$). We then linked these promoters to distal HSC enhancers that are likely involved in the activation of c-kit (2 enhancers), CD164 (4 enhancers), and PROM1 (2 enhancers). These enhancers were identified using an ENCODE/Hi-C data-based approach. After nucleofection into $CD34^+$ cells, GFP expression levels from the HSC-plasmids in $CD34^+/CD45RA^-/CD90^+$ cells were 3- to 4-fold higher than the those from the EF1a promoter, with the highest GFP levels found for the combination of the proximal cKit promoter with CD164-3, PROM1 or PROM2 enhancers. This activity was specific for $CD34^+/CD45RA^-/CD90^+$ cells. We have generated HDAd vectors using HSC- promoter/enhancers driving an AB8e base editor for reactivation of g-globin to study specificity and efficacy of *in vivo* base editing in in humanized mice and in CD46/bYAC-transgenic mice.

The second problem we tried to address was the prolonged activity of genome editors in slowly dividing/quiescent HSCs after *in vivo* HDAd transduction. Editors are expressed and active as long as the episomal HDAd genomes are present in HSCs, which, on the one hand, increases the rate of biallelic edits, but on the other hand increases the risk of off-target editing. We used a pomalidomide-based "switch-on" approach to control the half-life of an (HBG1/2 promoter-specific) ABE8e base editor and a SCD mutation specific prime editor via a degron-linked to an anti-CRISPR peptide. (Pomalidomide is an anti-cancer drug.) The system was incorporated into an HDAd vector and

transduction/editing studies in cell lines and $CD34^+$ cells were performed. Without pomalidomide no editing of the target site was detectable by next-generation sequencing (NGS) indicating tight suppression in the absence of pomalidomide. Editing rates increased in a pomalidomide concentration-dependent manner. The plateau of editing is reached on day 2 after exposure to pomalidomide. The editor is active for 3 days after removal of pomalidomide. *In vivo* studies are ongoing. Eventually, we will combine the inducible and HSC-specific expression systems for genome editors.

607 Base Editing for Generating a Gain-of-Function Variant in Hemophilia B

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Background: Hemophilia B, a congenital bleeding disorder caused by pathogenic variants in the *F9* gene, is often the result of single-nucleotide variants. Although genome editing to correct the disease-causing variant offers a promising strategy, developing a unique therapeutic reagent for each individual variant is impractical.

Aim: We investigated whether base editing could induce a gain-of-function variant in blood coagulation factor IX (FIX), thereby enhancing FIX activity as a strategy for treating hemophilia B.

Methods: We selected several *F9* variants derived from Hemophilia B patients and established HEK293 cells and knock-in mice harboring the patients' variants. The plasmid containing SpCas9 (D10A) conjugated with several cytosine deaminases was transfected into the cells. Base-editing constructs were packaged into adeno-associated virus (AAV) vectors or lipid nanoparticles (LNPs) and administered to the mice. Editing efficiency was determined by next-generation sequencing, and off-target effects were assessed using GUIDE-seq and R-loop assays. FIX activity and antigen levels were measured by one-stage clotting assays and ELISA, respectively.

Results: We engineered a G:C to A:T substitution at c.1151 of the *F9* by cytosine base editing to generate the R338Q variant, known as the Shanghai *F9* variant, which markedly enhances coagulation factor activity. This gain-of-function variant significantly boosted FIX activity in the supernatant of HEK293 cells expressing various pathogenic *F9* variants. Among the base editors tested, the wild-type SpCas9 (D10A) fused to TadCBEd provided the highest C-to-T conversion with minimal bystander edits and negligible off-target effects. AAV-mediated delivery achieved more than 60% conversion of the target G:C to A:T, not only in HEK293 cells carrying different *F9* variants but also in knock-in mice harboring human *F9* cDNA. Additionally, LNP-based delivery of base editor mRNA and guide RNA increased FIX activity in vivo, and repeated dosing further enhanced the therapeutic benefit.

Conclusion: Our results demonstrate that cytosine base editing to introduce the R338Q variant in the *F9* gene represents a promising genome editing strategy for treating various forms of hemophilia B. This approach holds potential for long-term therapeutic benefits and overcomes several limitations of conventional AAV-based gene therapy, including challenges related to re-administration and limited applicability in pediatric populations.

608 A Novel RNA Splicing Therapy for USH2A-Associated Retinitis Pigmentosa

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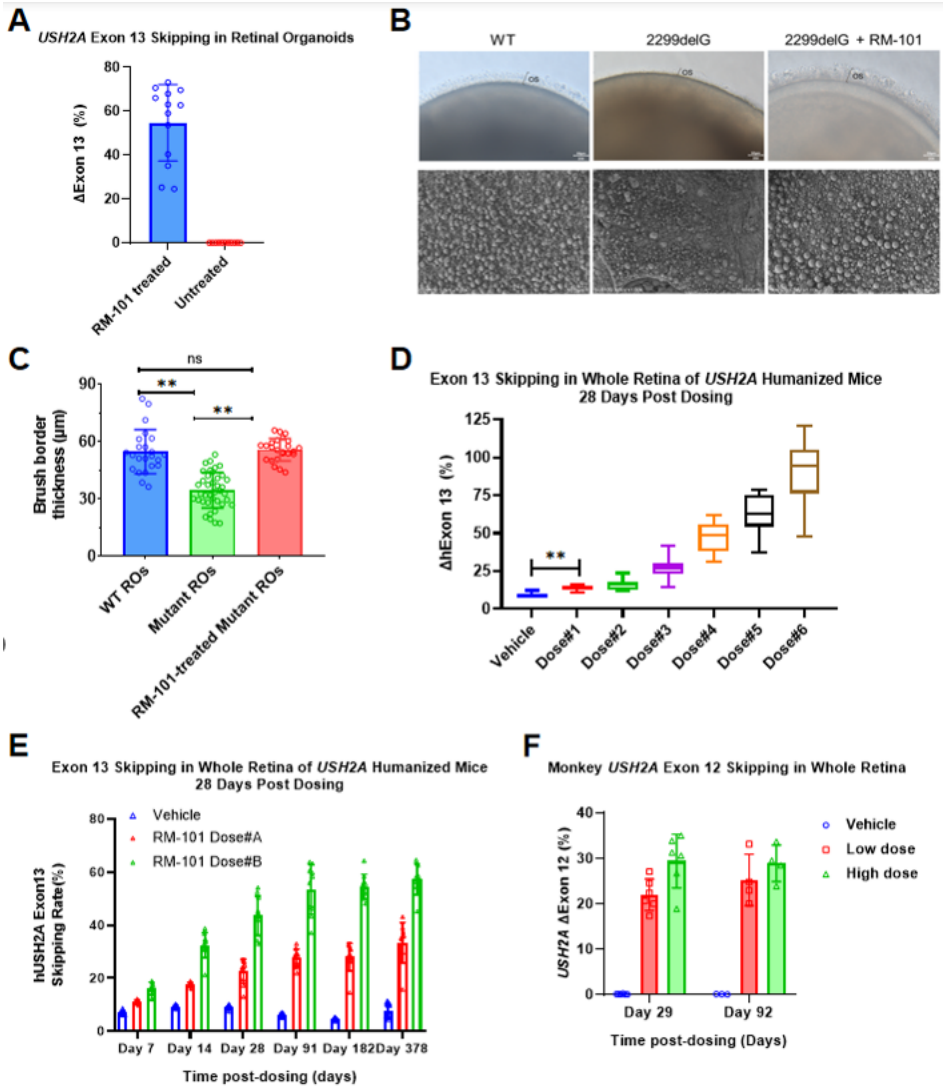
Purpose: Currently, no approved therapies exist for retinitis pigmentosa (RP) associated with the USH2A gene. Antisense oligonucleotide-mediated transient exon skipping has shown promise in treating RP linked to mutations in USH2A exon 13. This study reports on RM-101, a single administration exon-skipping therapy based on adeno-associated virus (AAV) and engineered U7 snRNA.

Methods: Retinal organoids (ROs) with the c.2299delG mutation in both alleles, USH2A humanized mice harboring human USH2A exon 13, and non-human primates were treated with RM-101 to evaluate exon skipping efficiency and efficacy. To assess the safety of RM-101 administration, both biodistribution and toxicology studies were performed. RNA sequencing was conducted on ROs, monkey retina, and choroid samples to analyze off-target local splicing variants (LSVs). Potential binding of RM-101 in surrounding regions was predicted using in silico approaches to evaluate the correlation of LSVs with RM-101.

Results: RM-101 transduction resulted in an average of 52.8% exon skipping in mutant ROs (Fig. A), restoring Usherin protein expression. The brush border, indicative of the photoreceptor outer segment, was restored to normal thickness, as confirmed by microscopy (Fig. B & C). In USH2A humanized mice, subretinal injections of RM-101 induced targeted exon skipping in a dose-dependent manner, with the highest efficiency of 91% observed (Fig. D), peaking at approximately 28 days post-dosing and remaining stable for 378 days (Fig. E). In monkeys, 25% exon skipping was detected in intact retinas (Fig. F). Biodistribution studies in monkeys and rabbits demonstrated that RM-101 was predominantly distributed in the retina and choroid. Ophthalmic examinations in toxicology studies revealed no product-related abnormalities in monkey eyes over a 13-week observation period. RNA-Seq identified 406 unique LSVs. For those with an absolute change in splice frequency >10%, further application of Blastn did not reveal any potential binding sites with >13 bp matches to RM-101 antisense, suggesting an insignificant correlation between these LSVs and RM-101 antisense. Based on these results, the Investigational New Drug (IND) applications for RM-101 were approved by the U.S. Food and Drug Administration and the China National Medical Products Administration.

Conclusions: RM-101 can stably and efficiently reprogram USH2A mRNA splicing, restore functional Usherin expression and rescue retinal outer segment development. This has the potential to improve visual function in patients with USH2A-associated RP. RM-101 is now ready to enter a Phase 1 trial.



609 An RNA Trans-Splicing Strategy to Rewrite mRNAs Implicated in Cardiomyopathy

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RNA trans-splicing is currently being evaluated to edit large sections of defective mRNAs with a corrected sequence. Our lab and others have developed both CRISPR-assisted (CRAFT) and CRISPR-free methods to edit mRNAs implicated in different diseases characterized by multiple mutations. Clinical trials using this platform for treatment of ocular diseases such as Stargardt disease caused by mutations in the *ABCA4* gene and inherited retinitis pigmentosa are either planned or underway.

In earlier work, we introduced CRISPR Assisted mRNA Fragment Trans-Splicing (CRAFT) to edit mRNAs that underly distinct monogenic diseases: *DMD* (Duchenne muscular dystrophy, *DMPK* (myotonic dystrophy type I), *LMNA* (dilated cardiomyopathy), *RYR2* (catecholaminergic polymorphic ventricular tachycardia), and *FXN* (Friedreich's ataxia). Here, we report the design, engineering and characterization of next generation CRISPR-free RNA trans-splicing constructs (simply referred to as RAFT) for rewriting large RNA stretches. To achieve such, we

replaced the Cas13 direct repeat in the trans-splicing RNA with a panel of unique RNA structures and assessed the performance of these CRISPR-free editors using a split fluorescent reporter as a proxy for trans-splicing efficiency. Across all target mRNAs tested (*RYR2*, *DMPK*, and *LMNA*), two of these structures achieved editing levels on par with CRAFT, consistently enhanced trans-splicing efficiency by 3-4-fold over conventional spliceosome mediated RNA trans-splicing (SMaRT). Importantly, RAFT improved both 5' as well as 3' trans-splicing in all targets.

We then utilized the RAFT platform to rewrite *MYH7,* which encodes the cardiac beta-myosin heavy chain. Mutations in *MYH7* are one of the most common inherited causes of Hypertrophic cardiomyopathy (HCM), a prevalent form of genetic heart disease which affects 1:500 individuals with a majority exhibiting autosomal dominant inheritance. For this reason and due to the large gene size of MYH7, conventional AAV gene replacement is not feasible. Using our fluorescent reporter split across a target *MYH7* intron, we individually screened more that 100 unique trans-splicing RNAs, to identify optimal RNA architectures that enable rewriting of either the first (5' trans-splicing) or second half (3' trans-splicing) of the *MYH7* open reading frame. Optimized RAFT constructs achieved editing rates of 35% with the 5' editor and 38% with the 3' editor. Further assessment with AAV-RAFT yielded 8% and 10% for 5' and 3' approaches respectively. Experiments focused on assessing the performance of AAV-RAFT in cardiomyocytes differentiated from normal donors and HCM-patient derived induced pluripotent stem cells (iPSC-CMs) are currently underway. Efficiency of *MYH7* mRNA trans-splicing and functional impact in patient derived iPSC-CMs will be presented. The RAFT approach may be particularly impactful towards editing large genes and/or diseases with autosomal dominant inheritance.

610 Improving mRNA Trans-Splicing Strategies to Repair Extensive Genetic Mutations

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Spliceosome-mediated RNA trans-splicing (SMaRT) technology represents a versatile tool for the correction of disease-causing mutations at the pre-mRNA level for genes that are too large for most viral vectors. However, therapeutic application of mRNA trans-splicing has been hindered by low efficiency and difficulty in accurately detecting trans-splicing events. Seeking to enhance the efficiency and accurate detection of SMaRT-mediated repair of transcripts containing large disease-causing mutations, we used *HUNTINGTIN* (*HTT*) transcript as a template. Huntington's Diseases (HD) is a neurodegenerative disease caused by a CAG repeat expansion in exon 1 of the *HTT* gene. Using *in silico* tools, we identified introns 8, 9, and 10 of the *HTT* gene as candidates for trans-splicing. A fluorescence-based reporter was used to screen for optimal trans-splicing modules. Quantitative sequencing methods revealed that our WT-*HTT* pre-mRNA trans-splicing molecules (wtHTT-PTMs) achieved up to 16% splicing with the endogenous *HTT* transcript in HEK293s. Further enhancement was achieved by replacing the self-cleaving ribozyme in our initial wtHTT-PTMs with a *MALAT1* terminator, increasing trans-splicing efficiency up to approximately 30%. These findings represent significant progress toward using trans-splicing as a platform to reprogram large mutated genomic regions, expanding the potential for therapeutic applications.

611 Intronization of Splice Editors Expands Trans-Splicing Toolkit

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Classical gene therapy approaches have faced significant challenges due to the size limitations of the cargo that can be effectively delivered into target cells. These limitations have restricted the potential therapeutic applications of gene therapy, particularly for disorders that require the replacement of large transcripts. Multi-kilobase RNA editing (trans-splicing) is an emerging therapeutic strategy which offers a promising solution to these challenges. Splice Editor systems co-opt the endogenous splicing machinery to facilitate multi-kilobase RNA edits via trans-splicing. Splice Editors enable repair of much larger mRNAs by allowing delivery of only a portion of the target mRNA. Additionally, Splice Editor systems mitigate the risks of toxic overexpression by ensuring the protein product is expressed at levels consistent with the endogenous RNA. Another key advantage of trans-splicing is that it does not

make permanent changes to the genome, theoretically making it a safer alternative to traditional gene editing techniques.

Despite its potential, Splice Editors face several unique challenges that need to be addressed for successful therapeutic application. These challenges include: 1) the stability of Splice Editors, 2) the efficient localization of Splice Editors to the target RNA, 3) the overall efficiency of the Splice Editing process, 4) off-target trans-splicing, and, 5) translation of Splice Editor RNA resulting in expression of a truncated peptide. These novel challenges necessitate novel solutions. Recent studies have highlighted the role of intronization in enhancing transgene expression. Intron excision has been previously identified as a key factor for efficient nuclear export of mRNAs. Furthermore, evidence suggests that intronization is a more effective strategy than codon diversification for minimizing the usage of cryptic splice sites in transgene constructs. Additionally, the deposition of exon junction complexes following splicing has been shown to protect exons from post-transcriptional modifications, including m6A methylation.

In this work, we investigate the incorporation of small introns into Splice Editors and explore the context-dependent nature of intron usage. Using machine learning-based tools, we are able to predict optimal placement of introns within a Splice Editor. Furthermore, we examine the impact of intronization on Splice Editing efficiency. Our work expands the toolkit for developing Splice Editing therapeutics.

612 Machine Learning Guided Design of Drug-Inducible Splicing Elements for Gene Therapy

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Inducible regulation of gene expression represents an important opportunity to enhance gene therapy, because the ability to modulate therapeutic transgene expression could significantly expand treatment options and precision. We previously demonstrated a novel regulatory system utilizing drug-induced mRNA splicing to modulate gene expression in vitro. To optimize this system for therapeutic applications, we developed a machine learning approach to understand and predict sequence features that govern drug-induced splicing. Our analysis leveraged a comprehensive RNAseq dataset of over 500,000 diverse RNA sequences (50-2000bp, median 235bp) measured across five mammalian cell lines, capturing both de novo splicing induction and modulation of baseline splicing activity in response to a small molecule (log2 fold-change between -7.4 and 10.8).

We designed a deep convolutional regression network incorporating interpretable filters to predict multiple aspects of sequence-dependent splicing behavior. For each input sequence, the model predicts both baseline splicing levels (rank correlation >0.2) and the magnitude of drug-induced splicing changes (rank correlation >0.3). Additionally, the model provides sequence-specific uncertainty estimates for induced splicing levels (rank correlation >0.35), enabling identification of sequences with predictable splicing responses. The neural network architecture was designed to capture both local sequence motifs and longer-range motif interactions that influence whether and to what degree the drug induces or modulates splicing at these sequences. Importantly, the interpretable nature of the model could allow identification of specific sequence patterns and structural elements that determine splicing induction and regulation, thereby potentially providing mechanistic insights into drug-mediated control.

Beyond prediction and optimization of experimentally obtained sequences, the trained regression network can be used to screen large libraries of computationally generated sequences to identify those with promising inducibility and low-baseline profiles. This computational pipeline provides a framework for enhancing the dynamic range and reducing required drug doses in future therapeutic applications of inducible splicing systems.

613 CRISPR assisted RNA trans-splicing for controlled and transient activation of T cells

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Adoptive T cell therapies for various cancers and autoimmune diseases are being used in the clinic. The potency and duration of the T cell response are key to successful outcomes of these therapeutic modalities. Several factors have been implicated in altering T cell state and improving function both broadly and in the context of CAR-T therapies. Amongst these, certain naturally occurring mutations and gene fusions in T cell associated cancers seem to improve the overall robustness and fitness of T cells. However, leveraging these targets through over-expression, stable genomic insertion using lentiviral vectors and other approaches to increase activation and potency of T cell therapies can prove challenging due to oncogenicity risks.

One such example is *VAV1*, a cytoplasmic guanine nucleotide exchange factor (GEF) that is activated upon downstream signaling of T cell receptor activation. *VAV1* plays a role in the activation of certain transcription factors, TCR clustering, calcium flux and polarization of the T cell. Recent studies have identified various activating mutations and fusions in *VAV1* in different T cell cancers such as peripheral T-cell lymphoma (PTCL-NOS), ALK-negative anaplastic large cell lymphoma (ALCL), and angioimmunoblastic T-cell lymphoma (AITL).

Here, using two RNA editing approaches, CRAFT (CRISPR assisted Fragment Trans-splicing) and RAFT (RNA Assisted Fragment Trans-Splicing) recently developed by our lab, we introduce certain activating mutations and fusions into endogenously expressed *VAV1* mRNA. Importantly, our trans-splicing approach limits the maximum number of edited mRNA copies to that of the endogenous transcript in the cell. We first designed a split-GFP reporter for one of the target introns in *VAV1* and carried out guide selection and parameter optimization for high efficiency trans-splicing of exogenous RNA fragments into *VAV1* pre-mRNA. Trans-splicing was quantified by percent of GFP positive cells and mean fluorescence intensity (MFI) through flow cytometry. Lead CRAFT and RAFT constructs were validated in HEK293, Jurkat (human T cell lymphoma) and in primary human T cells, where we achieved up to 56% and 62% GFP positive cells for CRAFT and RAFT respectively. Next, we utilized optimized CRAFT and RAFT constructs to edit endogenous *VAV1* pre-mRNA and achieved efficient trans-splicing as evidenced by molecular analysis. As proof-of-concept for T cell applications, we rewrite *VAV1* mRNA to generate a *VAV1-STAP2* fusion swapped for the C-terminal SH3 domain of VAV1. The latter chimeric protein is thought to increase downstream signaling and T cell activation. Correspondingly, editing *VAV1* mRNA and generation of the trans-spliced product in primary T cells was confirmed using Sanger sequencing and resulted in increased T cell activation as validated by different T cell surface markers. Overall, our work provides the foundational basis for exploring RNA trans-splicing as a strategy to transiently rewrite and tune T cell transcriptomic signatures.

614 Massively Parallel RNA Barcode Screens for CRISPR-Assisted Trans-Splicing

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We recently introduced CRISPR Assisted Fragment Trans-splicing (CRAFT), a novel RNA editing approach that leverages CRISPR-Cas13 systems to introduce exogenous RNA sequences into mRNA transcripts by trans-splicing. CRAFT is an attractive modality for gene therapy, as only a single editing reaction enables precise multi-kilobase rewriting while maintaining the expression profile of the target transcript. We developed a split fluorescent reporter-based assay to screen parameters that can impact trans-splicing efficiency. While this assay provides a simple unambiguous readout, the throughput is limited by the need to individually clone and test each parameter.

To overcome this bottleneck, we developed an RNA barcode-based screening pipeline to efficiently probe the large parameter space across several endogenous transcripts in vitro. To discriminate trans-spliced transcripts arising from distinct variants, we introduced a “barcode” of single nucleotide polymorphisms (SNPs) in the trans-spliced exons that was unique to each trans-splicing RNA. By linking the barcode to the parameter of interest, we successfully applied this platform to first identify efficient trans-splicing RNAs for *LMNA* and *DMPK* from a large, pooled library of guide RNA sequences (415 for *LMNA*, and 172 for *DMPK*) tiled across each intron target within the

transcript of interest. A pool of oligos containing an antisense "spacer" sequence, hemi-intron, as well as a unique spacer that was operably linked to SNP based barcodes located within a fragment of the next downstream exon was generated. This oligo pool embedded within the CRAFT RNA editing construct was transfected along with catalytically dead *Rfx*Cas13d in vitro and targeted amplicon sequencing on the RNA across the splice junction was carried out to determine the relative trans-splicing efficiency of each guide. The latter was calculated as the ratio of abundance of the associated barcode in the mRNA to the corresponding abundance within the plasmid pool delivered to the cells. Using this approach, we identified guide RNA sequences capable of achieving 25% trans-splicing efficiency for *LMNA* and 24% trans-splicing for *DMPK* endogenous transcripts, which corroborated trends observed from the earlier reporter-based assay. Overall, the barcode screen described allows rapid probing of CRAFT construct design parameters that can impact trans-splicing efficiency across diverse endogenous target RNAs, and may be applied for the engineering of other trans-splicing platforms.

615 Exploration of Trans-Splicing Methodologies in AAV Therapeutic Development Targeted at Treating Neurofibromatosis Type-I and Associated Tumors

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Neurofibromatosis Type 1 (NF1) is a neurologic disease with a complex pathologic burden characterized by widespread tumor formation and various neurological manifestations. As a single gene loss-of-function disorder where more than 50% manifestations are caused by sporadic mutations, NF1 is amenable to gene therapy approaches aiming to restore function. The large size of the NF1 cDNA (~8.5 kb) is a considerable challenge for development of AAV-based gene therapies for this disease.

We have explored alternative gene therapy approaches that maintain NF1 regulation from its native promoter through trans-splicing to the endogenous pre-mRNA. We used a library approach to evaluate the efficacy of spliceosome mediated RNA trans-splicing (SMaRT) whereby a vector encoded mutation free mRNA, also known as a pre-trans-splicing molecule (PTM), hybridizes to the endogenous mutated pre-mRNA and provides splicing machinery with alternative splice signals to generate a chimeric mRNA encoding normal protein. Unfortunately, despite early success in splicing with artificial systems, next generation sequencing showed a lack of chimeric mRNA despite substantial expression of both the PTM and endogenous NF1 gene.

Recently a Cas13-based mRNA trans-splicing system was shown to work across a variety of genes using artificial constructs and endogenous sequences (Fiflis et al, 2024). We investigated this Cas13-based trans-splicing approach for NF1, but it is important to note that due to ineffectiveness of the CMV promoter in our target Neurofibroma cell lines, we transitioned the published CMV promoter to an EF1a promoter that was functional in our cells. Given the large size of the NF1 mRNA and limited packaging capacity of AAV vectors, we designed barcoded AAV gRNA libraries to identify candidates to replace the N- or C-terminus of NF1 CDS by targeting introns 22 and 38, respectively. We screened these libraries by transient transfection of HEK293T cells and identified several candidate gRNAs with significant enrichment between the plasmid library and chimeric trans-splicing derived NF1 mRNAs following RT-PCR and illumina NGS sequencing. We found that 0.7% of NF1 mRNAs were chimeric (carry gRNA-specific barcodes in exon 23) for the N-terminus replacement library but found no evidence of trans-splicing for the C-terminus library targeting intron 38. Enrichment calculations identified ~5 gRNAs for the N-terminus library with an enrichment greater than 10X (**Fig. 1**). We assessed the efficiency of each candidate gRNA and observed a maximum trans-splicing efficiency of 3.2%, ie the percentage of chimeric NF1 mRNAs in the cell.

We sought to analyze the therapeutic potential of trans-splicing in immortalized NF1 patient cell lines (ipN 02.3λ, ipNF 04.4, & ipNF95.6) by measuring changes in Ras pathway activity, cell growth and/or death. AAVD/J vectors encoding Cas13 and individual gRNA-N-terminus NF1 CDS were used to infect NF1 cell lines at 1E6 vg/cell and total RNA isolated to assess trans-splicing efficiency. Despite transducing 80-90% of cells (assessed by transduction with an AAVD/J-GFP vector), we found no evidence of trans-splicing despite our high vector dose. We are investigating different parameters that may influence the overall efficiency of Cas13-mediated trans-splicing, including intron 'targetability' (sequencing-specific accessibility in the pre-mRNA) expression levels of Cas13, gRNA-NF1 CDS, and endogenous NF1 mRNA levels.

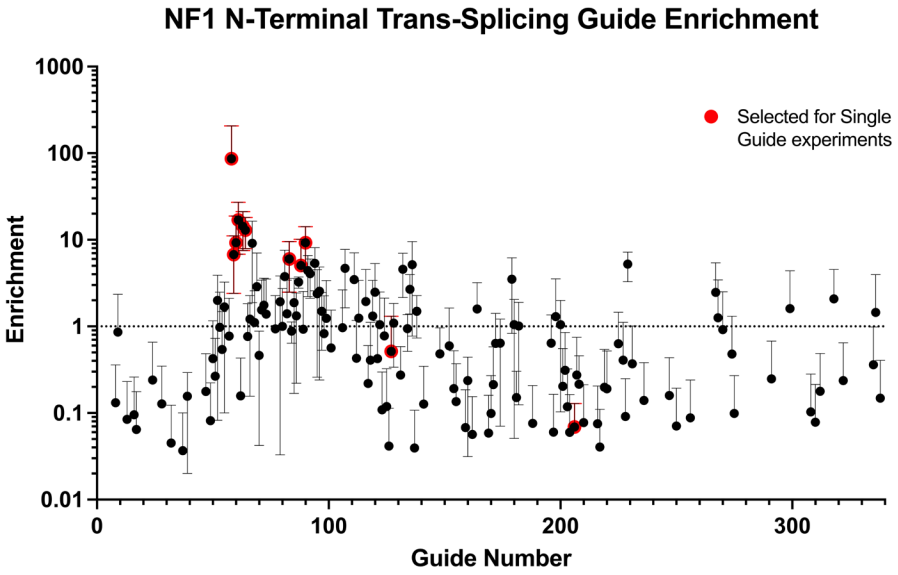

Figure 1. Guide Enrichment for NF1 N-terminal trans-splicing. Guides highlighted in red were cloned as single guides and tested for trans-splicing efficiency.

616 Discovery and Characterization of Novel Epigenome Editing Domains

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Although current epigenome editing technology is able to robustly activate or repress gene expression, only a small subset of mammalian epigenetic marks can be deposited with existing tools. In order to expand the capabilities of epigenome editing, we screened a library of ~2500 full-length human proteins recruited by dCas9 to several promoters for modulation of target gene expression. We found two proteins capable of facilitating target gene activation that have not previously been used to control expression of native genes.

One of these, CITED2, functions by recruiting the H3K27 acetyltransferase p300, and could provide additional specificity relative to overexpression of the p300 catalytic core domain, which can lead to target-independent acetylation. We also demonstrate that dCas9-CITED2 is a robust tool for high-throughput screening via gene activation, similar to the commonly used dCas9-VP64 activator.

This screen also revealed PHF15 as a novel activator. PHF15 functions by depositing H4K12 acetylation, a mark which has not previously been robustly programmable. PHF15 also mediates changes in H3K4 methylation. In cells from Friedreich's Ataxia patients, these marks are decreased at the *FXN* locus as a result of heterochromatin that forms in the first intron due to repeat expansion. This led us to hypothesize that dCas9-PHF15 might increase levels of these marks when targeted to *FXN*, leading to restored expression of its protein product, frataxin. We tested this hypothesis in Friedreich's Ataxia patient-derived iPSC lines and showed that PHF15 was indeed able to both

increase levels of these marks as well as increase expression of the frataxin gene. Ongoing work includes testing this approach in clinically relevant cell types and evaluating strategies for additional gene activation to wild type levels. This work expands the set of epigenetic states that can be robustly programmed and demonstrates the therapeutic potential of specifically reprogramming epigenetic marks.

617 Directed Evolution and Characterization of Cas Effectors in Mammalian Cells for Expanded Epigenome Editing Space

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CRISPR-mediated transcriptional and epigenetic regulation has broad potential applications in gene therapy and academic research. To improve the epigenome editing efficiency and achieve reduced cargo sizes suitable for delivery via single-AAV, we have previously engineered a compact RNP system with enhanced function (with dCas effectors under 500-amino acid in complex with gRNA scaffolds around 100-nucleotide long). However, the effective epigenome targeting range still desires further expansion from the canonical T-rich PAM recognition of our original dCas effector.

Here, we have established a directed evolution platform in mammalian cells to alter the PAM recognition profile of our dCas effectors towards a new PAM sequence with greater prevalence in the human genome and minimal initial recognition by our dCas effector. Using a CRISPRa synthetic reporter system to detect dCas recognition of the novel PAM, we were able to select and validate beneficial mutations from a site-saturation mutagenesis library of over one thousand variants. Combinatorial engineering of these variants significantly improved recognition of the novel PAM sequence, with top variants demonstrating over 400-fold improvement compared to the parent dCas effector. Recognition of the novel PAM was also observed in the context of CRISPRi when the variant dCas effectors were fused with a compact epigenetic suppressor and targeted to a synthetic reporter. To comprehensively characterize the PAM profile of these engineered dCas effectors in mammalian cells, we also developed a pooled CRISPRa assay which uses the activation of a synthetic target to sensitively query PAM recognition. This assay enabled us to detect the expanded or altered PAM profiles for our new dCas variants.

Overall, this work provides a novel mammalian cell-based platform for both directed evolution and characterization of altering the PAM recognition profile of dCas effectors. The resulting collection of novel dCas variants enables a broader or orthogonal epigenome targeting range when combined with the compact activation/suppression modulators we have developed in house. Increasing the epigenome editing range allows expanded applications in both basic research and therapeutics.

618 Beyond the Dot Blot: A Novel Method for dsRNA Detection for Gene Therapies

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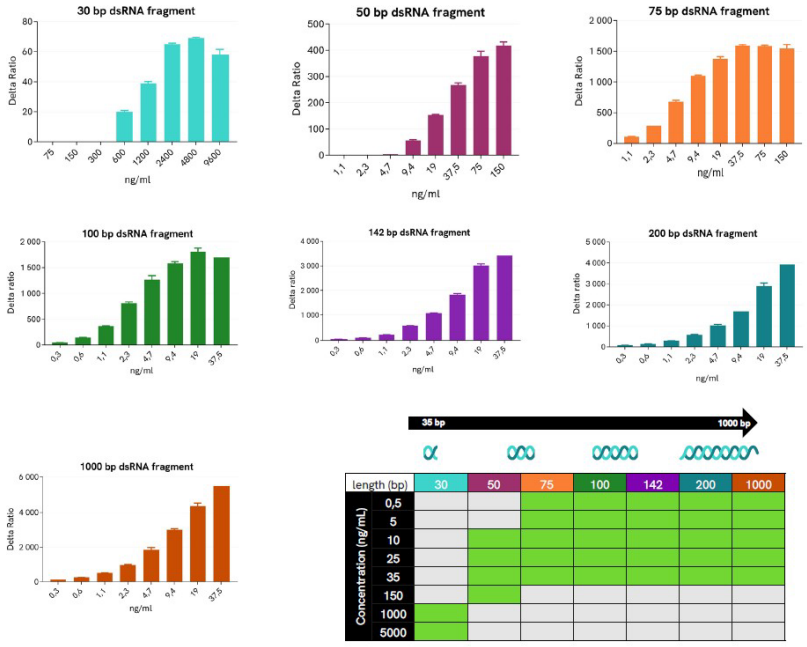
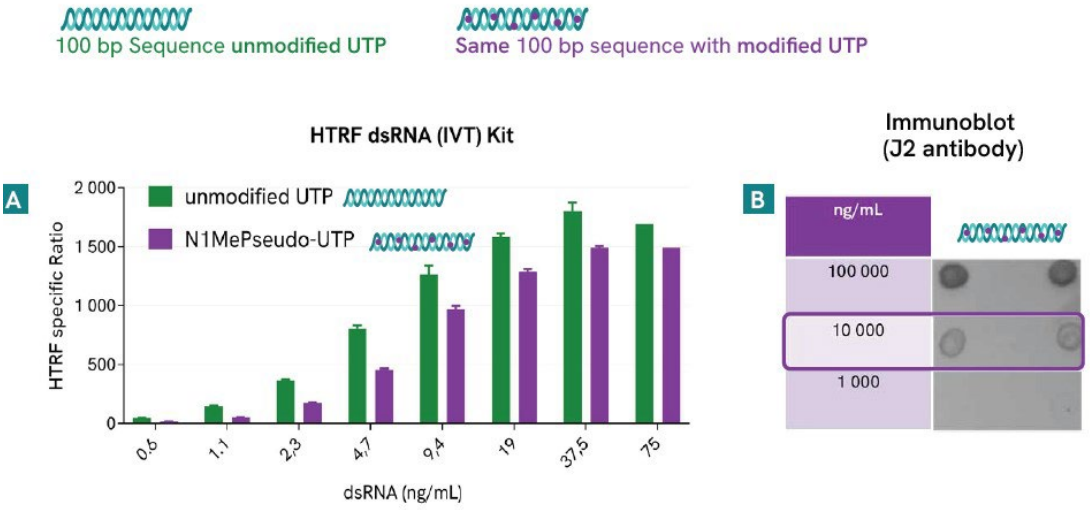
Introduction: In vitro transcription (IVT) is a go-to technique in molecular biology for creating RNA molecules from a DNA template. It is straightforward, precise, versatile, and scalable, making it essential for various research and biotech applications. IVT has been crucial in developing RNA-based therapeutics, like mRNA vaccines and gene therapy. The IVT process includes several steps: selecting and editing the template, setting up the transcription, purifying the RNA, and characterizing the final product. Detection of double-stranded RNA (dsRNA) contaminants, which can form due to secondary structures, self-annealing, or polymerase pausing, is performed using various immunoassay formats, such as ELISA, homogeneous immunoassays, and dot blot. Herein we describe the use of a no-wash homogeneous immunoassays that relies on FRET signaling to test for dsRNA **contaminants.**

Methods: All dsRNA samples were serially diluted in the kit diluent, and 10 µL of each dilution was plated in a 384sv white plate. After addition of 10 µL of HTRF dsRNA detection reagents mix, the HTRF signal was recorded following overnight incubation at room temperature. Two 100 bp dsRNA sequences were synthetized from the N-terminus portion of GFP protein. One sequence was generated without modification, while the other had a substitution of all uridines by N1mePseudo-UTP. The HTRF dsRNA assay was run following the protocol and compared to a dot blot assay using the J2 gold standard antibody.

Results: The novel method detected dsRNA for the whole range of short and mid-length sequences *(Figure 1)*. 50 bp sequences were detectable at low concentrations and detection of 30 bp sequences was possible at higher concentrations. Longer dsRNA sequences improved detection sensitivity, with successful detection of sequences up to 1000 bp at low concentrations. However, sequences of 20 bp and below were not detected due to their small size preventing antibody binding. HTRF signal and dot fluorescence intensity showed that both assays are capable of detecting N1mePseudo-UTP dsRNA *(Figure 2)*. HTRF assay sensitivity for modified dsRNA was in the same range as that of unmodified dRNA, with a similar detection limit ~1 ng/mL. For the immunoblot assay, however, the detection limit was much higher and around 10000 ng/mL. Using modified nucleoside triphosphates, like N1-methylpseudo-uridine, significantly advanced mRNA therapeutics by improving RNA stability and reducing immunogenicity. Comparative assays showed that both HTRF and immunoblot assays effectively detected uridine-modified dsRNA, with the HTRF assay demonstrating superior sensitivity. No detection signal was recorded using for any of the ssRNA, DNA Template, and T7 polymerase samples in comparison with positive controls of dsRNA. In these assay conditions, the kit shows detection specificity for dsRNA over other tested IVT reagents.

Conclusions: Developing robust dsRNA detection assays is crucial for advancing RNA therapeutics and ensuring the safety of biopharmaceutical products. The HTRF dsRNA assay provides a reliable and sensitive method for detecting dsRNA contaminants, supporting the rigorous quality control needed in IVT processes and gene therapy development.





619 Safety Assessment of on-/off-target aberrations in the generation of Universal CAR-T cells

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Allogeneic CAR-T cells have shown great clinical promise through the use of healthy donor-derived T-cells with reduced manufacturing times and consistent T-cell qualities. Advancements in gene editing tools such as CRISPR/Cas9 with improved guide design, and on-/off-target prediction tools have enabled the precise editing of genes, including B2M, PD-1 and TRAC, thereby successfully mitigating GvHD. Each Cas9-gRNA (RNP) induced gene editing event for the generation of allogeneic cells is associated with risks of potential off-target activity. The current therapeutic gene editing practices involve *in silico* guide design with off-target prediction, which can be confirmed by experimental validation. However, there is minimal guidance available for risk assessing off-target predictions and little known about variations between *in silico* and cell-based methods.

We aimed to compare *in silico* and experimental determination of on-/off-target editing events for clinically relevant targets in PBMC derived T-cells, to set out a methodical flow for guide selection and help set up clinically relevant thresholds for all editing events. The guide sequences targeting TRAC and B2M genes were categorised as high- and low-risk using the *in silico* tools. These *in silico* risk-assessments will be compared to DIGseq performed with gDNA and targeted NGS on enriched gene-edited populations from donor derived T-cells (n=3).

Analysis using web-based design tools of TRAC_guide1 and TRAC_guide3 targeting the TRAC locus, and B2M_guide1 and B2M_guide3 targeting the B2M gene for hindering the expression of TCR and MHC class I, respectively, have similar on-target editing scores (Table1). Although a variability in the number of predicted off-target sites was observed, a consensus was drawn as TRAC_guide3 and B2M_guide1 being ‘low-risk’ guides, while TRAC_guide1 and B2M_guide3 being ‘high-risk’ guides. However, all the selected guides have a potential for off-target edits in oncogenes (Table2). These guides will be tested further by experimental methods and compared to the *in silico* analysis to determine a methodical flow for performing a risk assessment of all the on-/off-target editing events.

While this work is in progress, we have shown that the *in silico* guide design tools offer a high throughput option for initial screening. However, the variations in predicted off-target sites warrants for the need of a methodical analysis of potentially dangerous off-target editing events.

Label	Chromosome	Locus location	IDT guide design checker scores		Total number of precited off-target sites (4, 3, 2 & 1 base mismatches)				
			On-Target (On-target score indicates higher editing efficiency at the intended site, higher value is better)	Off-Target (Higher value of off-target score indicates lower predicted off-target activity (scores are between 1-100))	IDT	Synthego	CHOPCHOP	Invitrogen TrueDesign	CRISPOR
TRAC_guide1	14	Exon 1	44	3	99	20	25	NA	4309
TRAC_guide3		Exon 3	46	84	99	20	1	NA	41
B2M_guide1	15	Exon 2	62	72	100	19	2	14	69
B2M_guide3		Exon 2	64	0	100	17	32	80	262

Oncogene	Chromosome	Guides with predicted off-target events			
EGFR	7	TRAC_guide1	TRAC_guide3	B2M_guide1	B2M_guide3
MYC	8	TRAC_guide1	TRAC_guide3		
KRAS	12	TRAC_guide1	TRAC_guide3	B2M_guide1	B2M_guide3
RB1	13	TRAC_guide1	TRAC_guide3	B2M_guide1	
BRCA2	13			B2M_guide1	
BRCA1	17		TRAC_guide3		

620 Leveraging gene delivery approaches for in vivo target validation and safety assessment

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At Calico, we aim to advance the understanding of aging or age-related diseases, and to develop therapeutics for these conditions. To understand whether age-related genes or pathways are valid therapeutic targets, we need precise tools to perturb them in live animals and to investigate the physiological consequences of such perturbations. Leveraging viral and non-viral gene therapy vectors, we optimized precise approaches to CRISPR-edit genes selectively in the liver or lung of mice, and applied these approaches to assess target safety or efficacy in early pipeline development. As the first example, we used lipid nanoparticles (LNPs) to perform a target safety assessment. We showed that a single dose of LNPs, through a systematic injection, can deliver genes selectively to >90% hepatocytes. Given the high selectivity and efficiency of liver delivery, we successfully deleted a target gene (X) in the liver by delivering LNP encapsulating gRNAs to Cas9 expressing mice, and confirmed that genetic perturbation of this gene did not induce liver toxicity. This approach allowed quick and robust liver toxicity assessment for pipeline targets without generating transgenic mouse models which is an extremely time consuming process. As the second example, we used adeno-associated viruses (AAVs) to validate the efficacy of a target gene for lung fibrosis. We found that, intranasal or intratracheal delivery of AAV6.2ff, an engineered capsid derived from AAV6, transduced >90% alveolar type 2 cells and >80% large airway epithelial cells. High targeting efficiency to lung epithelial cells made AAV6.2ff a promising tool for gene perturbation selectively in these cell types. Because our target gene (Y) is predominantly expressed by lung epithelial cells, we subsequently showed a successful deletion of gene (Y) in the lung by delivering AAV6.2ff carrying gRNAs to Cas9 expressing mice. This approach allows further investigation of target efficacy in mouse models of lung fibrosis without the need to generate tool compounds/antibodies or transgenic mouse models. Overall, we demonstrated that both viral and non-viral gene therapy vectors are valuable tools in early discovery research.

621 T-Cell Gene Editing via Integration-Free LNP-Mediated Delivery of gRNA and CRISPR Enzyme

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Background and Aims:
CRISPR technology has revolutionized genetic engineering by enabling precise genome editing. However, translating this potential into clinical applications faces a critical challenge: the efficient delivery of CRISPR components to primary T cells. While conventional viral vectors are effective, they are limited by immunogenicity, toxicity, and the risks associated with genomic integration. Lipid nanoparticle (LNP)-based systems have emerged

as a promising non-viral alternative, offering transient expression, scalability, and enhanced safety for delivering mRNA-encoded gene-editing tools.

Methodology:
This study introduces an optimized LNP platform for co-delivering guide RNA (gRNA), RNA-encoded Cas9 (via mRNA or circular RNA [circRNA]), and DNA donor templates to primary human T cells. By encapsulating the CRISPR machinery within a single formulation, the platform ensures synchronized delivery and temporal coordination of gene-editing components, improving editing precision while reducing off-target effects. To maximize efficacy, the LNP formulation was systematically engineered through rational lipid selection, RNA payload optimization, and refinement of the delivery protocol. Key parameters—including nanoparticle size, zeta potential, encapsulation efficiency, and biocompatibility—were characterized to enhance transfection performance. In vitro validation using primary T cells isolated from human peripheral blood demonstrated robust delivery and editing efficiency.

Results and conclusion:
LNP-mediated transient RNA expression reduced prolonged Cas9 activity, significantly lowering off-target risks and enhancing the safety profile of CRISPR editing. This approach enables precise genetic modifications without compromising cell viability, marking a critical advance for therapeutic applications. The platform holds transformative potential for developing next-generation T cell therapies, including customized CAR-T products with improved efficacy and safety. Future efforts will focus on adapting LNPs for in vivo delivery and exploring multiplexed gene-editing strategies to address complex disease models.

622 High-Fidelity Genome Editing with Cas-CLOVER: A Platform for Advancing Liver-Targeted Therapeutics

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Background
RNA-guided nucleases, such as CRISPR/Cas9, have greatly advanced genome editing by enabling precise genetic modifications, with the potential to address the root causes of genetic diseases. However, concerns about off-target edits, viral vector-related toxicities, and insertional mutagenesis remain significant barriers to clinical translation. To address these challenges, we developed a fully non-viral, liver-directed therapeutic platform using the high-fidelity Cas-CLOVER™ technology and proprietary liver-directed lipid nanoparticles (LNPs). Cas-CLOVER achieves its high fidelity by utilizing a dual gRNA strategy to narrowly restrict double strand breaks to the target site and reduce unwanted off-target edits.

Methods
Proprietary LNPs were engineered to co-encapsulate Cas-CLOVER mRNA and sgRNAs for targeted delivery to hepatocytes through an apoE/LDLR-dependent mechanism. Pharmacokinetic (PK) and safety profiles were evaluated in wild-type (WT) mice and non-human primates (NHPs). PK analysis included LC-MS and qPCR to assess serum clearance, tissue distribution, and persistence. Acute and chronic toxicity studies assessed transaminase levels, cytokines, hematology, coagulation markers, and histopathology. Efficacy was evaluated in humanized mice and NHPs through liver editing efficiency and biomarker reduction.

Results
PK studies in wild type mice dosed with 0.25-3 mg/kg of Cas-CLOVER LNP showed a rapid liver uptake and marked serum reduction of ionizable lipid and Cas-CLOVER mRNA, with minimal exposure of other major organs (80% liver exposure vs 15% in spleen, and 5% combined in lungs, heart, kidney, and testes), suggesting a favorable uptake and clearance profile. ──Safety studies (dose range 0.25-3 mg/kg) revealed transient serum transaminase (AST and ALT) and mild cytokine (IL6, MCP1, MIP-1β) elevations resolving within 48 hours, with no chronic toxicity. In humanized mice, a single administration achieved liver editing saturation (65%) and sustained plasma

biomarker reductions (>90%) for up to 180 days. In NHPs, a single administration achieved up to 45% liver editing and up to 65% reduction in plasma biomarker levels three weeks post-administration.

Conclusion
The Cas-CLOVER platform, delivered via proprietary LNPs, exhibits robust safety, durability, and efficacy in preclinical models. These results underscore its potential as a translatable, liver-directed therapeutic platform for treating rare and complex genetic diseases.

623 Design of Chemically Modified AsCas12a Guide RNAs for Increased Potency of LNP-Delivered Gene Editing Cargos

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In vivo therapeutic gene editing via delivery of CRISPR-Cas mRNA and guide RNA (gRNA) containing lipid nanoparticles (LNPs) is emerging as a powerful technology to treat genetic diseases. Establishing a favorable therapeutic index for this technology depends on engineering the gRNA and mRNA payloads for optimal potency. Optimizing the potency of the gRNA can require both sequence and chemical modifications to the gRNA, leading to a rapidly expanding number of gRNA variants to evaluate. Here we present the iterative incorporation of chemical modification motifs into AsCas12a gRNA that increases the potency of LNP gene editing cargos across multiple cell types and gRNA target sequences. We leverage biophysical measurements, including time-resolved DNA cleavage kinetics, gRNA binding affinity, and gRNA stability, coupled with *in cellulo* editing assessments, to inform the rational design of chemical modification patterns for improving gRNA potency. These measurements also provide mechanistic insight into the impact of CRISPR gRNA chemical modifications on specific steps along the pathway to productive gene editing. Identifying potency-enhancing chemical modification patterns for CRISPR gRNAs that translate across multiple cell types and target sequences represents a crucial step towards enabling a strategy for a gRNA target agnostic platform for ‘plug-n-play' *in vivo* gene editing pipeline development.

624 TALEN®- Mediated non-viral Transgene Insertion for the Advancement of Cellular and Gene Therapies.

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Cell and gene therapy approaches can rely on using gene-editing tools to modify disease-associated genes and introducing transgenes in the genome. These modifications can restore or create new cellular functions, thereby providing a potential therapeutic solution for a wide array of diseases. Despite clinical successes in the immuno-oncology and gene therapy fields, several challenges persist. The traditional application of lentiviral vectors for payload delivery results in random genomic integration, which may pose risks of genomic toxicity or cellular transformation. Advanced techniques use targeted transgene integration facilitated by DNA repair templates with specific homology arms, which can be delivered in AAV particles. These templates are introduced to the cell along with gene-specific double-strand breaks created by gene-editing tools. While more specific, this approach also faces challenges such as manufacturing constraints, potential AAV-associated toxicities or limited payload size. Increasing evidence suggests that non-viral methods, such as single-stranded DNA, to deliver transgenes have the potential to address current efficiency, safety and manufacturing challenges. Consequently, there is a need to integrate these methods into the next-generation of therapies to treat a broad range of diseases.

Here, we explore the combination of TALEN® mediated gene editing and the delivery of non-viral donor templates, particularly single-stranded DNA in its linear (LssDNA) and circular (CssDNA) formats, to mediate gene insertion in T-cells and hematopoietic stem and progenitor cells (HSPCs). We show that non-viral templates are amenable to be used to efficiently edit T-cells with CssDNA leading to higher integration levels. In HSPCs, we also observe an increase in transgene insertion frequency using CssDNA compared to LssDNA, alongside increased viability and insertion specificity. Additionally, we use CITE-seq coupled with long-term engraftment capacity assessment in NCG mice to compare CssDNA with traditional viral DNA repair templates (AAV). Our results demonstrate that CssDNA-mediated cell engineering favors stem-cell fitness and editing stability compared to traditional viral DNA repair templates (AAV). Our comprehensive dataset revealed critical transcriptomic and phenotypic differences regarding the efficacy of non-viral versus viral DNA donor template delivery methods.

Our findings highlight the potential of non-viral ssDNA delivery combined with TALEN® gene editing for gene insertion in long-term repopulating hematopoietic stem cells and primary T-cells. Implementing these advanced gene-editing techniques can pave the way for next-generation of therapies, aiming to provide alternative efficient, and safe therapeutic options for patients with cancer, autoimmune diseases, monogenic disorders, and other conditions.

625 Investigation of Double-Stranded DNA Donors and CRISPR-Cas9 RNP for Universal Correction of Cystic Fibrosis Causing Mutations in Human Airway Cells

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Introduction
Cystic Fibrosis (CF) is caused by disruptive mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene. *CFTR* encodes a chloride ion channel, essential for maintaining proper ion transport in the epithelial layers of the lungs and GI tract. Site-specific insertion of the *CFTR* cDNA into the endogenous locus in airway basal stem cells could prove curative for all disease-causing mutations of the *CFTR* coding region. Typically, approaches have relied on adeno-associated virus mediated delivery of a homology directed repair (HDR) donor, which limits therapies to *ex vivo* treatment. To overcome this limitation, we developed nonviral editing reagents that insert a *CFTR* cDNA cassette just downstream of the *CFTR* promoter with the eventual goal of delivery through lipid nanoparticles *in vivo*.

Methods
Editing reagents were all optimized in the T84 and 16HBE14o- cell lines. Different sgRNA were screened for their Cas9 directed on- and off-target editing profiles. Off-target double strand breaks were identified via GUIDE-seq analysis. Various naked dsDNA HDR donors were assessed for their effect on cell viability by MTT assay and their integration efficiency by droplet digital PCR. Following editing with the best HDR donor candidate in the CFTR-null CF mutant-cell line (16HBEgeG542X), CFTR protein expression was measured by Western Blot. Electrophysiological validation of protein restoration and function utilized the Ussing chamber assay to assess CFTR-dependent chloride ion current in bulk edited cell populations.

Results
An sgRNA that targeted the 5' untranslated region of *CFTR* with a cutting efficiency of 93% and minimal off-target activity was selected to move forward. We first screened potential nonviral HDR constructs that carried mCitrine and found that linear dsDNA had the lowest toxicity and highest rate of integration. To avoid including *CFTR*'s promoter in the homology arms of the HDR donor, we evaluated arm lengths of 500bp to 50bp and found no significant difference in integration. Furthermore, we evaluated the addition of 5' hexamines to our linear dsDNA donors to prevent free end recognition. The integration increased twofold with this modification. Still, with this optimized donor allelic integration was 7.0%, so we evaluated small molecule inhibitors of DNA-PKcs and Polθ,

two early mediators of the nonhomologous end joining and microhomology mediated end joining pathways, respectively. In combination, these inhibitors significantly increased the integration to 13.8% without significantly affecting cell survival. Editing with the full-length *CFTR* cDNA was performed in 16HBEgeG542X cells. Despite just 2% integration, expression of CFTR protein was detectable by Western Blot due to codon optimization. Additionally, bulk edited cell populations measured in Ussing chambers achieved >40% restoration of the chloride ion currents of the wildtype control.

Conclusions
We optimized a linear dsDNA HDR donor and developed a nonviral editing system for CF. Our approach demonstrated functional correction of the CFTR. Importantly, by not utilizing viral delivery the editing reagents can be packaged into lipid nanoparticles. This capability would potentially allow for *in vivo* delivery once targeting to airway basal stem cells is validated.

626 Achieving Therapeutic Endogenous FVIII Levels in Hemophilia A Mice Using Ultrasound-Mediated Gene Delivery of CRISPR/Cas9 Plasmids

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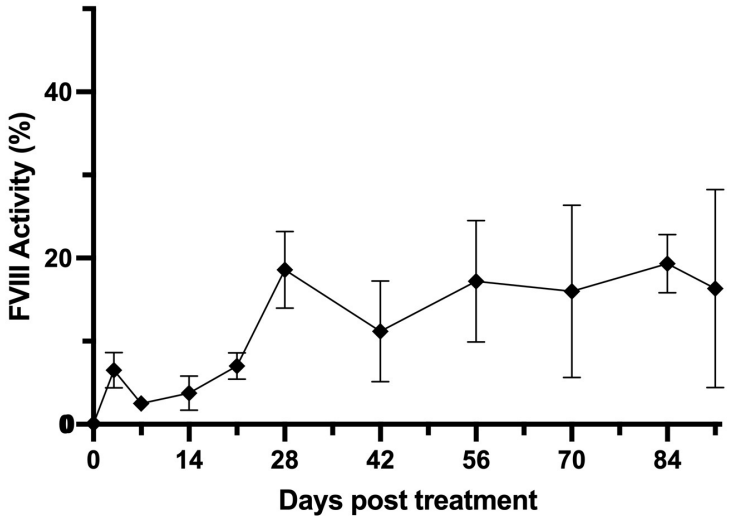
Hemophilia A (HA) is a genetic disorder characterized by the inability to produce functional clotting factor VIII (FVIII). Current treatment with FVIII replacement therapy need to be repeatedly administered that is both costly and inconvenient. Gene editing represents a promising alternative, offering the potential for correction of the underlying mutation. Ultrasound mediated gene delivery (UMGD) has emerged as a safe and effective non-viral method for gene transfer. This approach enables targeted delivery to specific cell types by altering ultrasound parameters and plasmid formulations. In this study, we evaluated the potential of UMGD to deliver CRISPR/Cas9 plasmids targeting liver sinusoidal endothelial cells (LSECs), the native site of FVIII production, to edit mutated FVIII gene and restore endogenous FVIII expression. Building on prior UMGD experiments, we used a therapeutic ultrasound protocol (1.1 MHz frequency, 14 Hz pulse repetition frequency, 50 W power, and 150 μs pulse duration), previously shown to preferentially transfect LSECs in mouse livers. The procedure involved injecting a mixture of MBs and a Cas9 plasmid carrying a single-guide RNA (sgRNA) targeting a 5-base pair deletion in exon 1 of the FVIII gene (NSGHAsgRNA) into the portal vein of NSG HA mice. Blood samples were collected over a 90-day follow-up period, and FVIII activity was assessed via the activated partial thromboplastin time (aPTT) assay. The livers were harvested post-experiment and hepatocytes and LSECs were isolated separately for DNA isolation and sequencing to evaluate gene-editing efficiency. FVIII activity in treated mice (n=5) reached an average of 20% of normal levels over the 90-day period (Fig 1). Next generation sequencing (NGS) analysis of liver DNA revealed an average in-frame editing efficiency of 5% in LSECs, highlighting the preferential targeting of this cell type under the applied ultrasound conditions.

This study demonstrates that UMGD can preferentially deliver CRISPR/Cas9 plasmids to LSECs in a mouse model of HA, enabling gene editing and partial restoration of FVIII activity. These findings provide a promising foundation for further optimization of UMGD as a non-viral approach to treat genetic disorders like HA. With advancements in ultrasound technology and gene-editing tools, UMGD could pave the way for safe, effective, and durable therapies for HA and other genetic diseases.



627 Highly Efficient and Easy-to-Operate HDR Gene Knock-in Editing Is Achieved by 'All-in-One' Delivery Vehicles (AiDVs)

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Introduction: CRISPR-Cas9 genome editing technology has revolutionized the landscape of gene therapy, immunotherapy, and regenerative medicine. Clinical trials using CRISPR-Cas9 for gene therapy are currently in progress or approved, such as in the treatment of patients with hemoglobinopathies. However, repairing a large defect or inserting a whole gene is not possible using CRISPR-Cas9, base editing, or prime editing. Hence, there is growing interest in homology-directed repair (HDR)-mediated precise gene knock-in to correct loss-of-function mutations and ultimately cure genetic diseases. However, the HDR efficiency is currently lower than indels (insertions and deletions), which has become a bottleneck for the application.

Methods: Here, we leverage advanced extracellular vesicle (EV) engineering methods to develop a novel modality for delivering Cas9-ribonucleoproteins (RNPs) as well as templates of a gene to integrate the correcting-cDNA at a specific locus by HDR simultaneously, all of which have been achieved by an 'All-in-One' Delivery Vehicle (AiDV).

Results: Our highly efficient nanoparticles can deliver the complete HDR machinery to multiple clinically relevant cell types, including T cells and hematopoietic stem cells (HSCs). With a simple addition to cell cultures, we achieve robust HDR editing rates (eg. 50% precise gene knock-in in Jurkat cells). Notably, in combination with the small molecule compound M3814, which inhibits non-homologous end-joining (NHEJ) repair, HDR is enhanced to over 90% at one endogenous locus.

Conclusions: This approach significantly improves HDR-mediated gene knock-in efficiency, making it applicable for clinical treatment. The straightforward operation and the possibility of in vivo application would make this treatment more economical than other options, paving the way for its development and improvement as an off-the-shelf treatment.

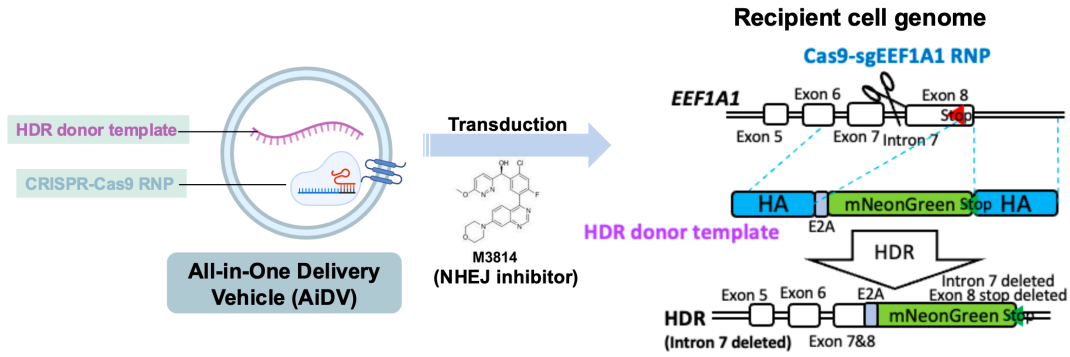

Fig.1 We leverage advanced extracellular vesicle (EV) engineering methods to develop a novel modality for delivering Cas9-ribonucleoproteins (RNPs) as well as templates of a gene to integrate the correcting-cDNA at a specific locus by HDR simultaneously, all of which have been achieved by an 'All-in-One' Delivery Vehicle (AiDV).

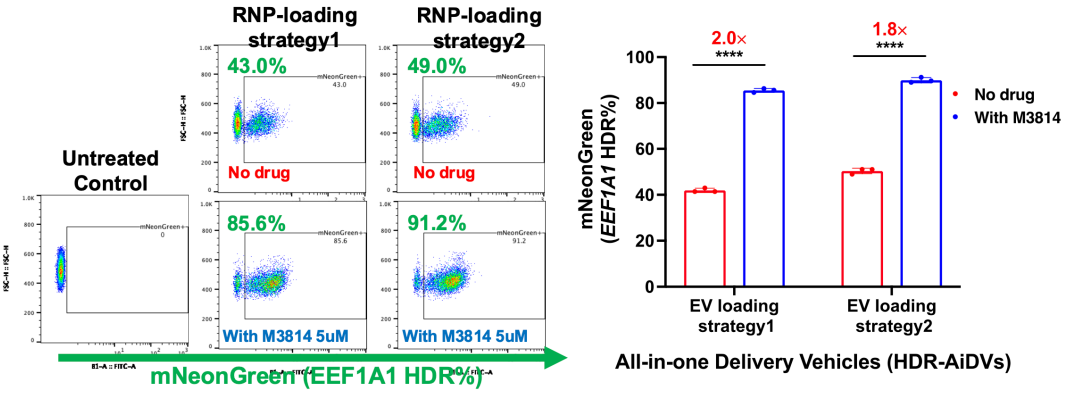

Fig.2 The modified AiDVs that delivered Cas9-RNPs and the HDR template simultaneously achieved efficient HDR in Jurkat cells at the EEF1A1 locus. Here the AiDVs were package with Cas9-RNPs and a mNeonGreen HDR template. The mNeonGreen positive cell percentage reflects HDR efficiency. HDR efficiency was further increased by M3814 (an effective inhibitor of the DNA-dependent protein kinase catalytic subunit, DNA-PKcs, involved in the phosphorylation of NHEJ-associated proteins), which is similar to previous data in Cas9-RNP electroporation and AAV6 delivery.

628 In Vivo Genome Editing with an Ultra-Compact Type V Nuclease for All-In-One AAV Delivery

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Adeno-associated viruses (AAV) are an established delivery technology with clinical validation in the field of gene therapy. The benefit of AAV delivery over other approaches, such as lipid nanoparticles, is tropism to organs and

tissues beyond the liver, including the central nervous system (CNS) and muscle. However, gene editing using AAV has been limited due to cargo size constraints, owing to the large size of many gene editing systems. Here, we describe an ultra-compact (488 amino acids) engineered type V nuclease capable of efficient genome editing across multiple cultured cell types and tissues in vivo when delivered using a single AAV vector. In vitro, this nuclease restored dystrophin protein levels by over 50% in Duchenne muscular dystrophy (DMD) model myocytes and achieved complete gene disruption at the SOD1 locus in K562 human cells. In vivo, AAV9-mediated delivery to the mouse central nervous system (CNS) via stereotactic injection resulted in up to 52% editing efficiency in olfactory bulb nuclei, and a significant reduction in target protein levels in the motor cortex. These results highlight the potential of this ultra-compact nuclease for AAV-based therapeutic genome editing beyond the liver, with implications for treating neuromuscular and neurodegenerative disorders.

629 AAV HITI for therapy of dominant Retinitis Pigmentosa

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Retinitis Pigmentosa (RP) affects 1 in 3,000 individuals worldwide, with 30-40% of cases having an autosomal dominant (AD) mode of inheritance. The rhodopsin gene (*RHO*) is most frequently mutated in ADRP (RP4), with the P23H mutation being the most common in the US. Traditional gene therapies are ineffective for this condition and eliminating the mutant *RHO* allele is critical to prevent its toxic effect. CRISPR-Cas9 holds promise by targeting the mutant allele while preserving the healthy one; however, its application is limited due to the need for guide RNAs specific for each *RHO* mutation.

We developed an adeno-associated viral vector-mediated homology-independent targeted integration (AAV-HITI) approach targeting the *RHO* locus irrespective of the mutation and demonstrated that HITI donor DNA design is crucial to achieve favourable knock-in events.

Moreover, comprehensive integration analysis at the on-target site revealed that HITI predominantly occurs in the desired direction of integration. Concurrently, we are employing unbiased long-read sequencing to gain deeper insights into the genomic outcomes of our platform. Finally, AAV-HITI tested in a humanized mouse model of RP4 resulted in significant improvements in retinal function and morphology, including visual acuity up to 1-year post-treatment. Overall, this human-centric approach represents a significant advancement toward clinical application of AAV-HITI.

630 A Compact and Potent Type II CRISPR System for CNS Gene Knockdown via AAV Delivery

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Adeno-associated viruses (AAVs) are important vehicles for therapeutic genome editing tools but have a restricted cargo capacity. Here we show that MG21-1, a novel type II nuclease that is small enough to be packaged together with its guide RNA in a single AAV, can produce high levels of protein knockdown in the central nervous system (CNS). Potent guides targeting the human ATXN2 gene were identified based on activity measured in K562 cells, with 97% genome editing efficiency measured by formation of insertions and deletions (InDels). These guides were

further validated in human neural progenitor cells (NPCs). Highly active human guides with the greatest similarity to the mouse genome were converted to the mouse sequence and then tested in mNeuro-2A cells, where editing in the range of 50-97% indels were detected. AAV cargo designs were evaluated with plasmid delivery to Neuro-2A cells, and the most active designs were subsequently packaged into AAV9 and CAP-B10 capsids. Stereotactic injections of AAV9 packaged with MG21-1 and guide into the mouse motor cortex showed up to 69% reduction in mean ATXN2 intensity in cells where MG21-1 was detected, as compared to a non-targeting guide control AAV9 delivered contralaterally. Systemic injections of the same editing cargo packaged into the blood-brain barrier-penetrant CAP-B10 capsid recapitulated the results of local injections, with an average 82% knockdown of ATXN2 protein detected in sagittal sections of the brain, validated across three animals by quantitative imaging. These results demonstrate the potential of a small CRISPR nuclease to efficiently knock-down a target in the CNS using a single AAV injected locally or systemically.

631 Improved Prediction and Activation of Homology-Directed Repair in Translational Ex-vivo Systems

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CRISPR-Cas enzymes can induce a double-strand break (DSB) at a specific genomic locus, which is subsequently repaired by various endogenous DNA repair pathways. Among these, homology-directed repair (HDR) is particularly crucial for introducing precise sequence alterations into the genome. However, enhancing the efficacy of this repair pathway to achieve therapeutically relevant frequencies is often a time-consuming and challenging process. In this study, we present a series of innovations aimed at optimizing HDR in therapeutically relevant systems, regardless of the format (ssODN, AAV, etc.) or size (small vs. large) of the donor template. Here, we demonstrate that the potential for HDR is dependent on the guide RNA (gRNA) and show predictive indications of non-homologous end joining (NHEJ) repair that are largely conserved to enable high-precision prediction of HDR potential across cell lines. We further demonstrate how HDR potential can be predictably enhanced by using different DNA repair modulation tools to achieve desired outcomes and are developing methods to detect undesired effects. Finally, we demonstrate that new protein-based DNA repair modulation can significantly enhance performance in translational settings without increasing off-target effects.

632 CRISPR Library Design Solutions for Efficient Gene Knockout Screening

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CRISPR-Cas systems are RNA-guided endonucleases that generate double-strand breaks (DSBs) at targeted loci, which can be repaired by pathways such as non-homologous end joining (NHEJ) to create indels. Leveraging this principle, guide RNA (gRNA) libraries can target large genomic regions for knockout, making them an essential strategy for gene knockouts and functional genomics. However, the lack of well-defined tools for flexible library generation with predictable performance has been a significant challenge. To address this need, we present a customizable gRNA library creation process featuring a customer-facing design interface, allowing parameters such as gRNA spacing and number to be tailored. We also demonstrate support for novel nucleases (e.g., Cas12 systems) and diverse genomes. We demonstrate the optimal number of gRNAs required for consistent, high-efficiency gene editing and identify design strategies that significantly improve frameshift and knockout frequency. To ensure the integrity of our synthesis platform, we synthesized a 96-well plate of select gRNAs (n=18 replicates/gRNA) and used multiplexed rhAmpSeq with UDIs to detect cross-contamination, finding none. Finally, we showcase an offering of advanced screening and analysis capabilities for CRISPR libraries through services available at IDT.

633 Excision of the *C9orf72* Hexanucleotide Repeat Expansion Using a Dual-ARCUS Gene Editing Approach Reduces Neurotoxic RNA Foci and Dipeptides in an *In Vivo* Model of ALS

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Amyotrophic Lateral Sclerosis (ALS) is a progressive, fatal neurodegenerative disease, characterized by the degeneration of upper and lower motor neurons leading to a loss of muscle control and eventual death. The most commonly known cause of familial ALS is a hexanucleotide repeat expansion (HRE) in the first intron of the chromosome 9 open reading frame 72 (*C9orf72*) gene. Bidirectional transcription of the HRE-containing allele generates toxic RNA foci and dipeptide repeats (DPRs), supporting a gain of function mechanism for *C9orf72* in ALS. To prevent production of these toxic species, two ARCUS nucleases were developed and optimized to excise the region of the gene that contains the HREs. Taking advantage of their small size, coding sequences for both ARCUS nucleases were packaged into a single AAV under the control of a neuron-specific promoter. To demonstrate proof of concept for this excision approach, induced pluripotent stem cells from an ALS patient with a *C9orf72* HRE were differentiated into motor neurons and transduced with the dual-ARCUS AAV. The target region of the *C9orf72* gene was excised from ~30% of alleles, which led to a ~30% reduction in toxic RNA foci. To confirm the efficacy of this approach *in vivo*, the dual-ARCUS AAV was administered via intracisternal magna injections to neonatal C9-500 mice, a transgenic mouse line that contains full-length human *C9orf72* with a HRE of 300-500 repeats. These mice display the hallmarks of *C9orf72* ALS, including detectable RNA foci throughout the central nervous system and DPRs in the brain. In both the motor cortex and spinal cord of ARCUS-treated mice, excision of the target region led to reductions in toxic products. These data provide proof of concept that this excision approach using a pair of ARCUS nucleases can remove the HRE region of *C9orf72* in neurons to eliminate the toxic gain of function products that drive degeneration in ALS.

634 Excision of the Expanded GAA Repeats from Fxn^{null}::$YG8s(GAA)_{>800}$Increases Frataxin Expression

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Introduction: Appropriate mouse models are essential in the processes of drug discovery and therapy development for Friedreich's ataxia (FRDA). The Fxn^{null}::$YG8s(GAA)_{>800}$ (Jax Stock No. 030395) humanized transgenic mouse is one of the best characterized mouse models of FRDA. It is also the only mouse model that expresses human frataxin encoded by the entire human *FXN* gene. It carries a large GAA repeat expansion of ~800 repeat and develops a mid to late onset progressive phenotype. It is the only FRDA mouse model that can be used for testing "human sequence-specific" therapies such as antisense oligonucleotides or CRISPR-Cas9 editing. Control mice (Y47R) were created by random integration of a human transgene containing the entire *FXN* gene with only 9 GAA repeats. The transgene is inserted in a different chromosomal location than that of the $YG8s(GAA)_{>800}$ model, resulting in very high frataxin expression. Progressive weight gain in these animals presents a concern for motor/behavioral analyses. Therefore, to address these issues and generate an appropriate control strain for $YG8s(GAA)_{>800}$ mice, we utilized CRISPR-Cas9 editing to excise the fragment of intron 1 containing 800 GAAs.

Methods: $YG8s(GAA)_{>800}$ embryos were electroporated with Cas9 protein and four gRNAs (two upstream and two downstream of the GAA repeats) to generate the YG8sD(GAA) strain. Embryos were transferred to pseudo-pregnant female mice.

Results: Genotyping and DNA sequencing of seven surviving pups demonstrated that all carried edited alleles. Two males were selected as founders of the YG8sD(GAA) colony and crossed with B6 wild type females to minimize potential off-target effects of genome editing. One of the seven pups was sacrificed, and RNA and protein analyses demonstrated significant upregulation of frataxin expression after GAA excision compared to the YG8s$(GAA)_{>800}$ strain.

Conclusions: Using CRISPR-Cas9, we generated a novel control mouse strain of critical importance to drug discovery and therapy development for FRDA

635 Single-Cell Genotyping and Transcriptomic Analysis of CRISPR/Cas9 edited hematopoietic stem and progenitor cells for Treating Sickle Cell Disease

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Sickle cell disease (SCD) is a severe inherited disorder caused by a single nucleotide substitution in the β-globin gene (*HBB*), resulting in the production of sickle hemoglobin (HbS) that leads to chronic complications, including hemolytic anemia, vaso-occlusion, and ineffective erythropoiesis. Gene-editing strategies such as that using CRISPR/Cas9 technology have demonstrated therapeutic potential by correcting the sickle mutation through homology-directed repair (HDR) with a donor template. However, this approach induces DNA double-stranded breaks, which can result in unintended editing outcome including small insertions and deletions (INDELs), and large gene modifications. These editing outcomes can have high genotypic diversity, which is largely overlooked by conventional colony-based genotyping methods due to the limited resolution, particularly at the single-cell level. To address these challenges, we developed a platform combining single-cell RNA sequencing (scRNA-seq) with the Genotyping of Transcriptomes (GoT) to analyze the diverse outcomes due to CRISPR/Cas9 gene editing. This platform was then applied to study the *HBB gene in edited and differentiated* hematopoietic stem and progenitor cells (HSPCs) from patients with SCD.

We first established a genotyping library preparation method to enrich for HBB cDNA and a pipeline to quantify editing-induced alleles and genotypes on *HBB.* This was integrated with standard scRNA-seq analysis to link genotype diversity with functional outcomes. Using this approach, we analyzed editing outcomes in SCD HSPCs edited with ribonucleoproteins (RNP) containing HiFi*Spy*Cas9 and either R-02 or R-66S guide RNA, with or without a corresponding single-strand oligodeoxynucleotide (ssODN) donor template for correcting the sickle mutation. Edited HSPCs were differentiated into the erythroid lineage and analyzed at days 3, 7, 10, and 13. We quantified 30,532 R-02 gRNA edited cells, 27,886 R-66S gRNA edited cells, and 14,688 untreated cells, revealing significant genotypic diversity. For example, on day 7, we identified 702 unique genotypes in 4,847 cells of the sample edited by the R-02 RNP plus ssODN and 1,230 unique genotypes in 4,163 cells of the sample edited by R-66S RNP. These genotypes are composed of unedited SCD mutation, HDR-mediated HBB correction, HBD-conversion, in-frame and out-of-frame INDELs as well as large deletions and insertions.

By linking genotypes to transcriptomic data, we found that while γ-globin expression partially compensates for β-globin deficiency in *HBB*-disruptive genotypes such as out-of-frame INDELs and large deletions, it does not fully restore the normal α/β globin ratio. This imbalance raises concerns about persistent functional deficit and globin dysregulation. We identified a distinct cell cluster in the edited samples that has significantly enriched *HBB*-disruptive genotypes. Cells in this cluster showed characteristics of ineffective erythropoiesis in late polychromatic erythroblasts, early and mid-orthochromatic erythroblasts (OrthoE). Further, they displayed significant upregulation of stress-related genes, severe α/β globin imbalances, and impaired progression into late-stage OrthoE. These findings underscore the importance of a comprehensive evaluation of the functional consequences of gene editing and highlight the urgent need to optimize the gene-editing strategies to improve the efficacy and safety of gene-editing therapies.

636 CasX gRNA Screening and Selection Enables Ex Vivo CD38 Knockout NK Cell Therapy

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Multiple myeloma is a malignant neoplasm originating from plasma cells expressing high levels of CD38. Although several treatments have been developed over the past decades, the 5-year relative survival rate for multiple myeloma is as low as 57%. Sarclisa® (isatuximab), a Sanofi anti-CD38 monoclonal antibody, is reported to increase the 60-month progression-free survival rate by 20% when used in combination with bortezomib, lenalidomide, and dexamethasone. Natural killer (NK) cell combination therapy has been proposed to increase the antigen-dependent cell-mediated cytotoxicity (ADCC) against malignant CD38+ plasma cells, potentially enhancing the efficacy of anti-CD38 antibodies. However, combining these two therapeutic strategies is challenging because NK cells express CD38 on their surface which results in fratricide upon exposure to anti-CD38 antibodies.

To minimize NK cell fratricide, we employed a CasX nuclease based genome editing strategy to eliminate CD38 protein on the surface of NK cells. We assessed the function of 52 CasX guide RNAs (gRNAs) in primary NK cells expanded from 6 unique CD3-depleted PBMC donors. In total, 6 CasX gRNAs achieved greater than 80% knockout (KO), with the top performing candidate reaching 95% KO. Importantly, screening of the lead gRNA was performed with CasX mRNA, obviating the need to produce CasX ribonucleoprotein. We defined gRNA potency across our top candidates by serially titrating CasX mRNA concentrations and found that the lead gRNA is more potent than other highly active gRNA. Surprisingly, while the lead gRNA maintained greater than 93% efficacy even with less than 10% of the amount of CasX mRNA used in the initial screen, other candidates exhibited a 10-35% reduction in efficacy with decreasing concentrations of CasX mRNA. Furthermore, insertions and deletions (indels) at the on-target cut sites for each gRNA correlated well with phenotypic protein KO.

We continue to develop a suite of gRNA off-target discovery and validation tools specifically for CasX using a surrogate gRNA. This gRNA exhibits similar on-target editing to other candidate gRNAs but has a higher *in silico* off-target signature. Using this surrogate gRNA, we detected off-target events in an *in vitro* biochemical off-target assay. One of the off-target sites that was identified in these assays, but not the *in silico* off-target algorithm, was validated to occur at 2% following editing in primary human NK cells using a targeted amplicon based sequencing method. In contrast, the lead gRNA has a more favorable *in silico* off-target signature and fewer off-target sites found in the *in vitro* biochemical off-target assay. Together, we present a robust CasX gRNA screening, gRNA off-target detection and gRNA off-target validation platform that can be used in combination to identify candidate gRNA used for therapeutics.

637 Machine Learning-Informed CRISPR Screening for Enhanced MSC Chondrogenesis

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Introduction: Traumatic and degenerative cartilage injuries are a leading source of pain worldwide, causing great financial burden and diminished quality of life. Cartilage has limited healing capacity, prompting the need for de novo cartilage regeneration through strategies such as the chondrogenic differentiation of primary mesenchymal stromal cells (MSCs). Yet, clinical translation is challenged by limited chondrogenic capacity in vivo and phenotype instability. CRISPR gene editing adds fine control of cell fate through precise manipulation of gene expression. However, editing a single gene can have a cascading effect throughout gene regulatory networks (GRNs) causing unpredictable phenotypic shifts. We have previously established CRISPR-GEM, a machine learning model that learns GRNs then evaluates CRISPR target genes for specific therapeutic applications. Our objective is to experimentally validate this model by screening CRISPR activation (CRISPRa) target genes to enhance MSC

chondrogenesis. We hypothesize that the identified genes will induce potent chondrogenesis as determined by PCR, high-throughput fluorescent assays and in vivo models.

Methods: mRNA encoding the nuclease null Cas9 fused to the tripartite transcriptional activator VPR (dCas9-VPR) and guide RNAs (gRNAs) were complexed at a 1:2 mRNA:gRNA molar ratio with the RALA peptide at a nitrogen:phosphorous (NP) ratio of 7. Quantitative PCR (qPCR) was performed using TaqMan Fast Advanced Master Mix for qPCR (Applied Biosystems) and specific TaqMan gene expression assays (Applied Biosystems) using the 2-ΔΔCt method with RPL13A as the housekeeping gene. SOX9 reporter knock-in was performed using Cas9 ribonucleic protein (RNP) targeting immediately before the SOX9 stop codon. A pDNA donor template (Genscript USA) containing GFP flanked by homology arms complementary to either side of the cut site was co-delivered with RNP using RALA at an NP ratio of 5 or a 100x RALA:RNP molar ratio, respectively. Finally, knock-in cells were sorted by flow assisted cell sorting (FACS).

Results: CRISPR-GEM identified PRG4, SERPINA3, SERPINA1, MGP, TNP1, and BMP2 as top scoring CRISPRa target genes for the chondrogenesis of MSCs. Multiple gRNA sequences were assessed for each gene using an mRNA/gRNA co-delivery system through the RALA cell penetrating peptide (Figure 1A). Significant activation of each gene was achieved relative to non-transfected controls and non-targeting controls with at least one gRNA for each gene (Figure 1B-G). To assess the functional impact of gene activations, we designed reporter MSCs to fluorescently trace SOX9 protein expression for high throughput analysis of target genes and gene combinations (Figure 2A). As a positive control, SOX9 activation was optimized resulting in a maximum fold change of 15.7 ± 5.5 (p=0.0005)(Figure 2B). Future work will use this reporter system to characterize the effect of CRISPR-GEM target gene activations on SOX9 expression to validate model findings. Optimized activation targets will then be assessed in vivo by embedding transfected reporter MSCs in alginate hydrogels then implanting them subcutaneously in immunodeficient mice and tracking the fluorescent intensity using an in vivo imaging system. In all, these results demonstrate efficient non-viral gene activation for the chondrogenesis of MSCs and presents a novel high-throughput strategy for CRISPR target gene assessment which can be applied to a range of therapeutic applications.

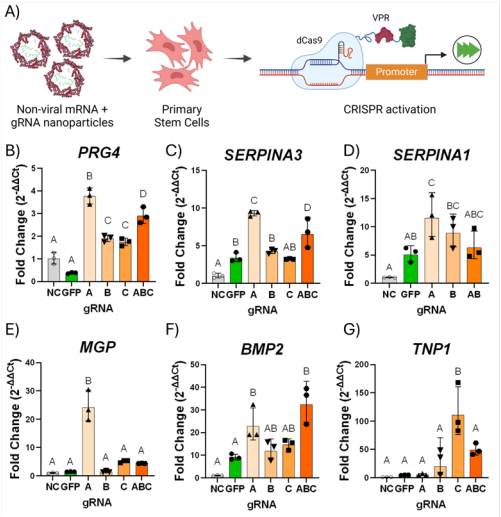

Figure 1. CRISPRa guide RNA (gRNA) Optimization. **A)** Schematic of gene activation strategy. mRNA encoding nuclease-null Cas9 (dCas9) fused to the tripartite transcriptional activator VPR (dCas9-VPR) was co-delivered with gRNA into primary MSCs using the non-viral RALA cell-penetrating peptide. This approach was used to optimize the gRNA sequences for each target gene by delivering three identified gRNA for each gene or two for *SERPINA1*. **B-G)** Using these methods, the top 6 genes were activated, and the fold change after 24 hours was determined via qPCR to identify the optimal gRNA sequences for future chondrogenesis studies. *PRG4, SERPINA1, SERPINA3, MGP, TNP1,* and *BMP2* activation levels corresponded to their basal expression but significant activation was achieved with at least one gRNA combination for each gene. The transfection of GFP was included as a control to determine the transcriptomic effects of the delivery vector. Groups labeled with the same letters within a graph are not statistically significant from each other (p<0.05) as determined by a one-way ANOVA.

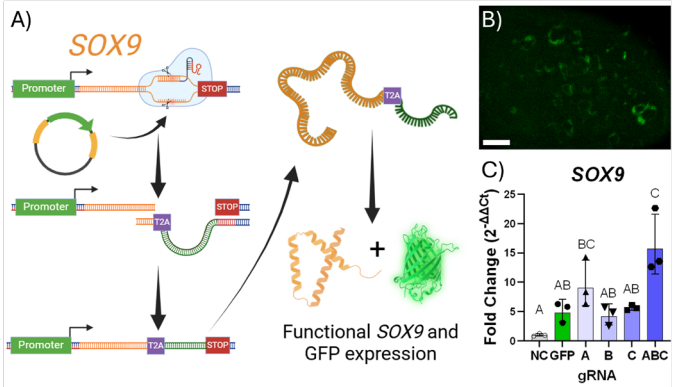

Figure 2. SOX9 reporter MSC experimental design. **A)** Schematic of experimental strategy. Cas9 ribonucleic protein targeting *SOX9* immediately before the stop codon will be delivered to MSCs to create a double stranded break (DSB). pDNA encoding GFP surrounded by homology arms complementary to the *SOX9* sequence was then delivered to align the GFP sequence over the DSB for copying into the *SOX9* transcript. The result is the expression of fully functional SOX9 and GFP allowing a quantification of SOX9 protein production following gene activations. **B)** Fluorescent images of knock-in cells following TGF-β3 treatment. Scale = 100 μm. **C)** *SOX9* activation was performed as a positive control in later studies. The transfection of GFP was included as a control to determine the transcriptomic effects of the delivery vector. Groups labeled with the same letters within a graph are not statistically significant from each other (p<0.05) as determined by a one-way ANOVA.

638 Rapid and Accurate Genotyping of 100 Gene-Edited Cells Using GeneAbacus

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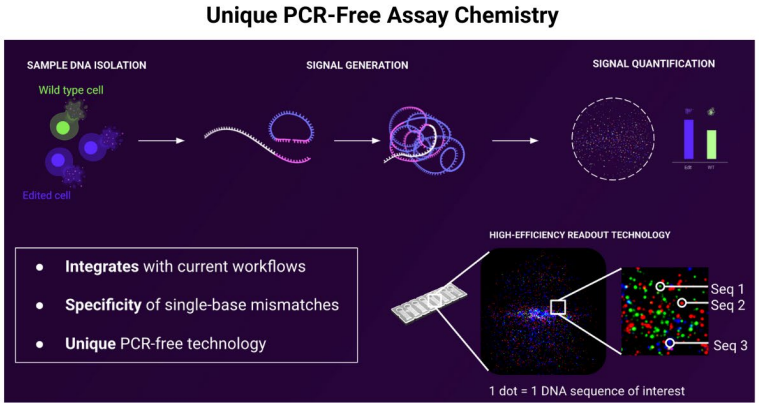
Introduction: Timely genotyping of gene editing outcomes remains a significant challenge, particularly when working with limited cell numbers or single-cell manipulation as long culturing times are needed for sufficient DNA amounts. Traditional PCR-based methods are powerful but prone to nonspecific amplification, especially in samples with low cell count. Here, we present a standardized PCR-free genotyping assay that delivers highly accurate results within a single day, starting from as few as 100 cells/sample.

Method: GeneAbacus leverages padlock probes and Rolling Circle Amplification (RCA) chemistry to convert genotypes into quantifiable signals that can be read out with a fluorescence microscope. The loss of wild type alleles and detection of specific edits are measured by digital quantification of signals obtained from edited and unedited cells. We have developed a sample preparation module for GeneAbacus to handle limited cell number samples without compromising accuracy. Comparative studies were conducted using human HCT 116 and human U-2 OS cell lines, analyzing knock-out efficiency in heterozygous pools and homozygous clones. Results were evaluated using next-generation sequencing (NGS) as a reference method.

Results: We demonstrated functionality of GeneAbacus from samples with a range of 100 to 1,000 cells. The assay achieved equal sensitivity for both cell lines used. Blind testing of knock-out efficiency revealed robust precision and reproducibility with high degree of concordance as confirmed by NGS data (R^2= 0.982) for tested gene editing efficiencies ranging from 20% to 100% edited alleles.

Conclusion and Outlook: With the extended capability of GeneAbacus to handle samples with limited cell numbers, the genotyping process of gene-edited cells can be streamlined.. This addresses not only bottlenecks in PCR-based workflows but can accelerate the gene editing validation step by for instance shortening the cell culture time, enabling cell engineering solutions on single cell level and on cell types with limited dividing capacity.





639 Advances in Cytogenetic Detection of Small Genomic Targets and Transgenes for Cell and Gene Therapy

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Cell and gene therapies often require complex editing systems that utilize nuclease-based editing in combination with delivery and/or insertion of one or more transgenes into a target genome. Some examples include in vivo gene therapy using liver-targeting AAV vectors, lentiviral particles to introduce Cas9 RNPs and CAR transgenes into human T-cells, and the use of DNA as a therapeutic agent to replace defective genes or kill tumor cells. While stable transgene integration can have considerable therapeutic benefits, any integration event into genomic DNA could disrupt host gene expression and potentially contribute to secondary malignancies via insertional mutagenesis. Factors such as vector choice (lentivirus versus gammaretrovirus), cell substrate (e.g., T-cells versus CD34+ cells), and even the individual donor can additionally influence this risk. The transgenic DNA sequences integrated into the genome are often smaller than what conventional FISH assays can detect. To address the need to characterize small-target integrations in a cellular-based assay, Kromatid has developed a platform, KROMASURE, with two fit-for-purpose solutions to provide single-cell cytogenetic assessment of transgene integrations. These are KROMASURE inSite and its counterpart, KROMASURE Pinpoint FISH, two advanced cytogenetic assays for identifying and measuring the copy number and true modal distribution of integration events on a single-cell basis. The number (and sometimes location) of the transgenic integration events in these complex edited products can be measured on average by NGS or dPCR methods. However, this averaging divorces structural variant and transgene data from any cellular context, making it impossible to observe co-localization of events or understand the true distribution in a batch of edited cells. For this study, we have combined our advanced DNA probe design strategies that avoid homology, repetitive sequence, and other sources of fluorescent signal noise, with intelligent image acquisition and image analysis technologies available at Nikon BioImaging Lab, to develop a scalable, robust imaging-based cytogenetics assay for small target detection. Briefly, Nikon developed an AI-enhanced “two-pass” approach to scan KROMASURE inSite slides, with the first pass being a low-magnification “large field of view” scan used to identify cells in interphase and metaphase states, and the second pass being a high-magnification Z-stack acquisition of detected interphase and metaphase cells. This imaging workflow was automated through a suite of Nikon NIS-Elements tools including JOBS (for automated imaging), General Analysis 3 (for object detection) and NIS.ai (for segmentation classifier training and noise reduction), to create a robust, scalable imaging solution with limited human intervention. Moreover, the Nikon microscope optics excelled at identifying small, fluorescently dim puncta, a critical requirement for using our DNA probe-based assay to accurately identify and score small genomic targets and transgene insertions. Using the Nikon BioImaging Lab workflow, we were able to obtain high-quality images of approximately 10,000 cells in about a week, a dramatic improvement over conventional, manual imaging workflows and one that enables us to search for and quantify rare genomic events. Using a transgene system as a model we demonstrate the use of KROMASURE inSite for understanding the true structural heterogenicity in batches of edited cells.

640 Personalized Molecular Engineering of CRISPR Systems to Improve Activity and Specificity

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The challenge of improving the activity and specificity of CRISPR in gene therapies lies in the facts that the human genome is large; that CRISPR effectors like Cas9 that increasingly enter clinical trials for therapeutic gene editing are tolerant to small differences between the intended target and other sites that might be present in an individual’s genome; and the existence of these “off-target” sites in a patient’s DNA may not even be known or detected *a priori.* However, developing CRISPR biotechnologies with extreme specificity would not only eliminate clinical risk for off-target mutation, but would also present new therapeutic opportunities of CRISPR, such as allele-specific gene editing.

Currently, two of the main approaches to reducing the potential for unwanted mutations are (i) computational optimization of the target site determined by a CRISPR effector's guide RNA (or gRNA) to minimize the predicted number of other sites that might be similar to the target of interest, and (ii) using engineered "high-fidelity" Cas9 enzymes with less activity at sites that diverge from the gRNA's target. These approaches suffer from 3 major limitations: (i) "high-fidelity" CRISPR effectors are "high-fidelity" because of their significantly reduced overall activity and effectiveness; (ii) the reduced flexibility and range of potential therapeutic targets that comes from computational optimization; and (iii) that computational optimization is not "personalized" but rather uses the "reference" human genome to predict "off-targets" and can catastrophically miss small but important variations between individuals.

Invisible line

My laboratory has developed an approach to experimentally screen 100's of thousands of gRNA variants known as extended gRNAs or x-gRNAs that we previously have shown can increase specificity in CRISPR activity at specific targets by orders of magnitude with known off-target sites and can significantly outperform CRISPR targeting even when compared to engineered "high-fidelity" Cas9 effectors. We have validated this approach using several well-characterized gRNAs with known-off-targets, including gRNAs for treating inherited metabolic disorders of the liver and others currently in clinical trial for treatment of sickle cell disease (SCD); we believe this approach would have great potential in genetic treatments for many diseases. More recently, we have developed "automated" microfluidic platforms for high-throughput gRNA characterization of both on- and off-target activity – simultaneously characterizing, for example, gRNAs for every possible CRISPR target within an exon of interest – and subsequently applied this approach toward the "hands-fee" discovery of gRNA variants with significantly enhanced activity profiles. Furthermore, we have taken this approach to the extreme: we are now ablet to readily generate gRNA variants capable of discriminating single-nucleotide variants (SNVs), even when the variants are located outside the protospacer adjacent motif (PAM) of the CRISPR effector – something otherwise extremely challenging – completely eliminating detectable activity at one SNV and with no loss of activity at the other SNV of interest; we have demonstrated this extreme level of specificity with two SNVs resulting from common pathogenic somatic mutations. We expect these contributions will not only improve the safety of CRISPR therapies but also enable new applications of CRISPR gene therapies.

641 A Robust Approach for CRISPR-Cas Therapeutic Guide Design and Selection Leveraging Variant-Aware Computational and Biochemical Approaches

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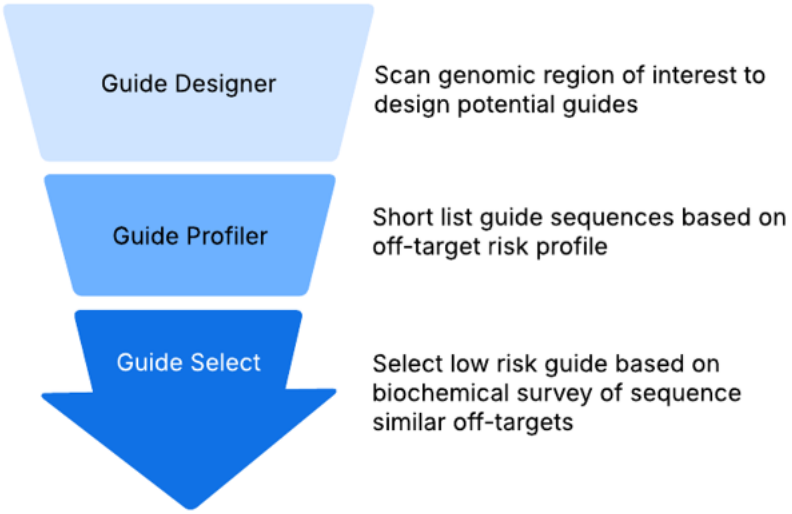
Guide RNA (gRNA) design for a CRISPR-Cas system routinely begins with computational tools to identify a small set of candidate guide sequences. While currently available computational tools that are designed for guide design take into account factors that impact on-target editing, they do not provide a comprehensive analysis of off-target risk or genetic variation present in human populations. Moreover, any off-target analysis is based on predictions from statistical models with no consideration for biological significance of putative off-target loci. These computational analyses are often followed by selection of a single guide based on experiments evaluating editing efficiency in a relevant model system without any biochemical assessment for off-target specificity. Motivated by experience in reducing off-target gene editing risk in therapeutic applications we have developed a more effective approach.

Here, we present an integrated, multi-step approach that utilizes a combination of computational tools (Guide Designer, Guide Profiler) and an in-vitro biochemical assay (Guide Select) to perform variant and off-target aware guide design, profiling, and selection.

The TRBC1 gene is a well-studied therapeutic target in immunotherapies. Using the Guide Designer software, we performed comprehensive design of candidate guides and profiled them based on several factors such as location within the transcript and previously published metrics of on-target and off-target performance. For the top 25 gRNA

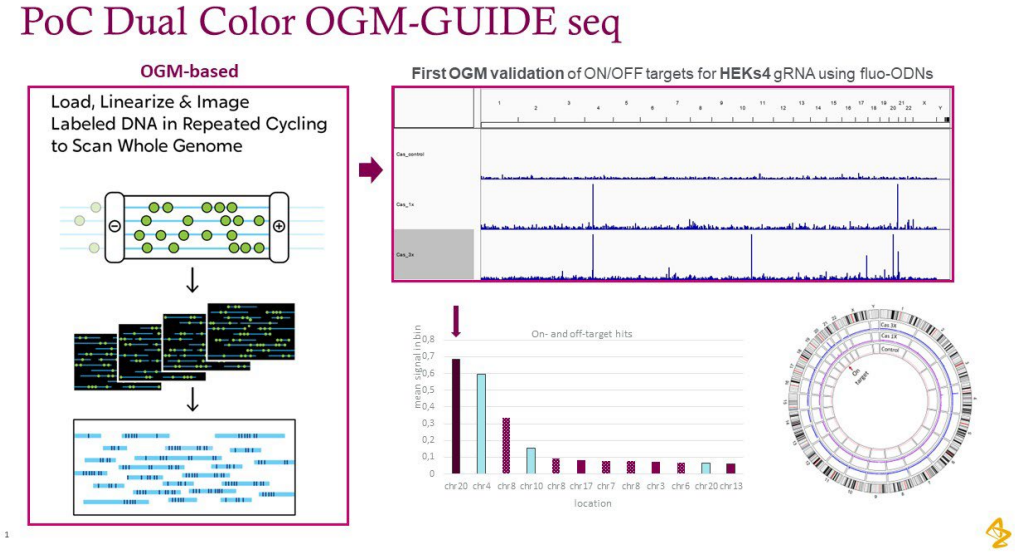
sequences, the Guide Profiler software was used to perform a comprehensive search for on-target variation and candidate off-target sites across 7 human superpopulations, annotation of putative off-target loci with biological features, and finally rank-ordering of their off-target risk profile. For the top 8 guide RNA sequences, this was then followed by empirically defining their biochemical editing specificity using the Guide Select assay. This assay is a multiplexed adaptation of the ONE-seq assay and is designed to biochemically identify off-target sequences with lower edit distance (<5 mismatches and indels) from the guide RNA sequence. Based on the assay read out, the guide RNA with lowest off-target risk is finally selected for downstream efficacy testing. Selection of the optimal candidate gRNA from this approach and comparing to a previously published guide, we present data from off-target characterization using the GUIDE-seq and SAFER-detection assays to underline the impact of this comprehensive guide RNA selection approach. In summary, improving the rigor around the design, profiling, and selection of guides earlier in the discovery process reduces the overall risk of a therapeutic program, prioritizes safety, and improves overall speed of a therapeutic program.



642 Dual-Color Optical Genome Mapping for Enhanced GUIDE-seq Analysis in CRISPR-Cas9 Editing

Roberto Nitsch, AstraZeneca, Mölndal, Vastra Gotalands lan, Sweden

CRISPR-Cas9 genome editing has revolutionized genetic engineering, yet ensuring its precision and safety remains a key challenge. Off-target effects and unintended structural variations, such as large deletions, duplications, and translocations, often evade detection by conventional sequencing-based methods, which rely heavily on small amplicon generation, and overly elaborated computational algorithms for variant calling. Here, we introduce a novel dual-color Optical Genome Mapping (OGM) approach that includes GUIDE-seq applications, providing a direct, PCR-free, bioinformatics-independent method for an all-in-one detection of CRISPR-induced genomic alterations. By leveraging two distinct fluorescent labels, this technique allows for simultaneous visualization of structural variants and the integration sites of GUIDE-seq oligonucleotides, enabling an unbiased, high-resolution assessment of double-strand break (DSB) repair outcomes across the genome. Unlike short-read or long-read sequencing, which can struggle with repetitive regions and complex rearrangements, dual-color OGM provides a single-molecule, high-throughput solution that captures large-scale genomic modifications with exceptional sensitivity and specificity. This approach offers a powerful alternative for evaluating Gene Editing safety, eliminating the need for bioinformatics pipelines and computational artifact corrections. By improving the accuracy of genome integrity assessments, dual-color OGM has strong potential for clinical and translational applications, particularly in the development of genome editing-based therapeutics.



643 Genome-Wide *In Silico* Analysis of Greater than Five Million CRISPR Guide RNAs Reveals Limited Predictive Power of Non-Human Primates for CRISPR Guide RNA Off-Target Activity in Humans

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Non-human primates (NHPs) such as rhesus and cynomolgus macaques are used for pre-clinical modeling of gene editing therapies, leveraging similarities to human anatomy and physiology as well as high sequence homology between NHP and human genomes. However, the functional relevance of NHP genomes in CRISPR guide RNA (gRNA) specificity analysis has yet to be determined. We describe a bioinformatic pipeline to annotate *in silico* genome-wide specificity analysis of over five million Cas9 and Cas12a CRISPR gRNAs with respective on-target sequence similarity across NHP species. We hypothesized that computational intersection of the annotated off-target profiles for each gRNA would reveal the functional relevance of multiple NHP species in modeling CRISPR off-target activity in humans.

CRISPR gRNA design tools were modified to enable prediction of human intragenic Cas9 and Cas12a spacer sequences genome-wide, and a bioinformatic pipeline was developed to annotate the panel of spacers with the names of the candidate human gene targets. Selection of NHP models for CRISPR efficacy and toxicity studies would require the selected NHP genome to exhibit a perfect on-target sequence match for the candidate gRNA, so another bioinformatic pipeline was developed to annotate the human-derived spacers against NHP genomes and characterize the strength of sequence match. Computational intersection of the species-specific spacer annotations produced a list of human spacers with perfect on-target sequence identity in common NHP genomes.

Next, an *in silico* CRISPR off-target analysis pipeline was modified and applied to the list of spacers with pan-species on-target sequence identity. For each spacer, the pipeline produced a list of potential off-target *loci* in each primate species' genome within defined mismatch criteria for both spacer and PAM sequences. Another bioinformatic pipeline was developed to annotate the off-target sites in each genome with the overlapping and nearest neighbor genes.

Comparison of the off-target profiles between species reveals the fraction of human off-targets also found in the off-target predictions in each NHP genome. Results were combined for all spacers stratified by Cas nuclease. The data, representing a robust and inclusive panel of guide RNAs across the human genome, demonstrate that NHP experimental models do not recapitulate a majority proportion of CRISPR guide RNA off-target activity in the human genome despite high overall genomic sequence similarity between primate species. These results highlight the importance of understanding limitations of NHPs for the study of CRISPR gRNA specificity and especially for contextualizing human risk informed by these studies.

644 Leveraging DNA Missing from the Human Genome to Generate CRISPR Targetable Sequences Without Off-Target Effects

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CRISPR off-target effects, including non-specific guide RNA mismatches, are a major hurdle for CRISPR therapy development. To overcome this limitation, we tested whether 15 nucleotide-long DNA sequences missing from human reference genomes and six model organism genomes (including mouse, rat, fly, worm, yeast, E. coli)—termed nullomers—are more precise protospacer targets for the CRISPR machinery. As a proof-of-concept for this approach, we used CRISPR activation (CRISPRa) and inactivation (CRISPRi) to test improvements in nullomer-protospacer targeting. We engineered thousands of previously characterized promoters and enhancers with nullomer-protospacer sequences. Using massively parallel reporter assays (MPRAs), we find that nullomer-protospacer sequences have a minor effect on endogenous regulatory activity and can be safely embedded in these elements. We are currently using these regulatory building blocks to downregulate (CRISPRi) or upregulate (CRISPRa) transgene activity with nullomer-targeting sgRNAs and analyzing them for off-target effects using a variety of genomic tools. In summary, our approach engineering nullomer-protospacer sequences into regulatory elements for CRISPRa/i systems provides a significant advancement in genome engineering by leveraging protospacer sequences absent from the human genome. Our work offers a promising strategy to improve the precision of genetic research tools and gene therapies by ensuring robust gene regulation with minimal off-target effects.

645 TOP-SECRETS Enables Cas9 Nucleases to Differentiate SNVs Outside of PAMs

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CRISPR-Cas9 has emerged over the past several years as a powerful biotechnological tool that holds tremendous therapeutic potential in treating genetic diseases, including several that have entered clinical trials. However, the possibility of unintended Cas9-induced mutational events raises significant concerns, especially in distinguishing single nucleotide variants (SNVs). To address this, we developed a novel high-throughput *E. coli*-based screening platform entitled, Selection of Extended CRISPR RNAs with Enhanced Targeting and Specificity (SECRETS), designed to quickly screen tens of thousands to hundreds of thousands of extended guide RNAs (x-gRNAs) and identify those that significantly enhance Cas9 specificity while maintaining robust on-target activity. Using an *in vitro* cleavage assay targeting several genes – *EMX1, FANCF, VEGFA,* and *HBB,* we consistently find that x-gRNAs selected through SECRETS outperform high-fidelity Cas9 variants by maintaining on-target activity levels while eliminating cleavage at off-target sequences differing by as few as two mismatches (Fig. 1). Additionally, these x-gRNAs do not generate new off-targets, as evidenced by CHANGE-seq analysis of Cas9 editing in A549 cells.

With further improvement of this system, we also developed Targeting Overlapping Protospacers with SECRETS (TOP-SECRETS) to discriminate between pathogenic and non-pathogenic alleles at SNVs outside the protospacer adjacent motif (PAM) – a capability that is currently unachievable with standard Cas9 approaches. We tested this by

targeting the KRAS G12D mutation, an undruggable oncogenic mutation frequently found in multiple cancers. We find that TOP-SECRETS can identify x-gRNAs that retain high on-target activity *in vitro* at the pathogenic allele with no detectable activity at the healthy allele (Fig. 2a). We also validated this approach with clinically relevant mutations in MED12, which are responsible for ~70% of uterine fibroids, and where mutations abolish a PAM site – the opposite of typical PAM-targeting approaches. Again, x-gRNAs identified via TOP-SECRETS maintain high on-target activity *in vitro* at the pathogenic allele while exhibiting no activity at the healthy allele (Fig. 2b).

These findings show that SECRETS and TOP-SECRETS can be used to robustly generate x-gRNAs in a design-free way that reduces the need for individualized optimization and is streamlined to test any identified on-target/off-target pair. This approach of designing gRNAs with extreme precision opens the door for allele-specific gene editing and has profound implications for personalized medicine, particularly in the treatment of monogenic disorders, cancer, and other genetic diseases where SNV-targeted correction is required, especially outside of a PAM.

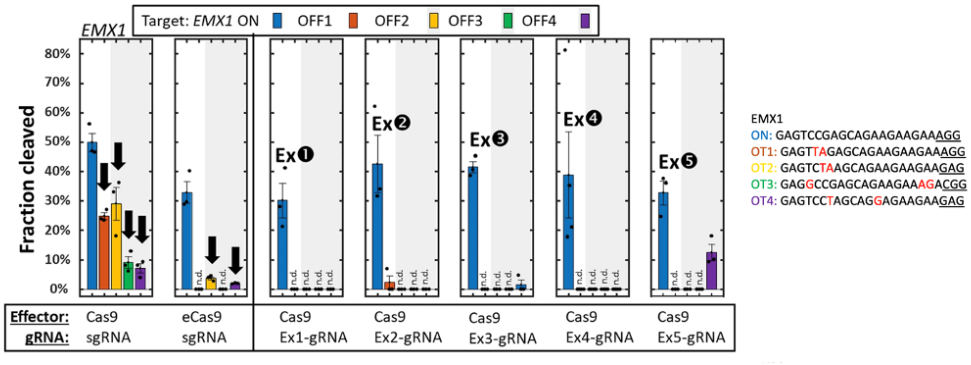

Figure 1. All five x-gRNAs exhibit remarkable on-target activity and greatly reduced off-target activities across four known off-target sites for the standard gRNA during *in vitro* cleavage assays using purified components. (n = 3 replicates each, dots are individual data points. Error bars = standard error of the mean. Black arrows highlight the off-target activity of Cas9 and eCas9 with the sgRNA that was blocked when using x-gRNAs).

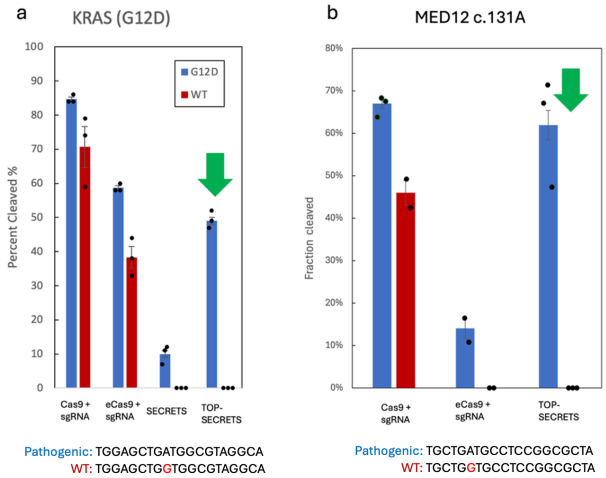

Figure 2. X-gRNAs identified via TOP-SECRETS exhibit increased specificity profiles compared to Cas9 and maintain robust activity as compared to eCas9. (n = 3 replicates each, dots are individual data points. Error bars = standard error of the mean. Green arrows highlight the activities of the x-gRNAs).

646 Reprogramming Large Serine Recombinases for Site-Specific Integration into Mammalian Genomes

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Accurate and efficient integration of large DNA sequences into specific genomic sites is essential for numerous medical, agricultural, and research applications. Current methods often rely on inducing double-stranded DNA breaks and relying on cellular DNA repair pathways such as NHEJ or HDR, which have their limitations. Serine integrases, such as Bxb1, offer a promising alternative as they can catalyze precise DNA recombination without generating free DNA ends. However, customizing these integrases for specific genomic targets remains challenging. We have employed directed evolution and rational engineering to generate Bxb1 variants capable of targeting defined genomic loci.

Through directed evolution of rationally designed libraries in bacteria, we successfully generated Bxb1 variants with activity at defined genomic sites in the human genome. Furthermore, we show that that the activity levels achieved in bacteria translate in reporter assays in human cells. Finally, we demonstrate and confirm target integration at the DNA level in the selected genomic locus using our evolved LSR variants.

Our study showcases the reprogramming of Bxb1 for integrating gene-sized DNA cargo into the human genome without requiring the prior integration of WT BxB1 binding sites (attB). Efficient integration was demonstrated for a target site that differs in 17 out of 38 nucleotides from the WT attB target site demonstrating that we do not require pre-existing pseudo recombination sites with high homology to attB. A genome wide analysis with our proprietary search motif revealed flexible targeting across the whole human genome. This novel approach highlights the versatility of Seamless Tx Recombinase Programming technology, offering efficient and precise genome editing capabilities for a wide range of applications.

647 CRISPR Engineered Humanized Albumin Mouse ("hAlb-CR") Achieves Robust Transgene Expression following Liver-Targeted Gene Insertion

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Humanized mouse models are crucial for preclinical drug development, particularly in the context of gene editing technologies. These models offer a unique advantage for *in vivo* evaluation of guide RNAs (gRNAs) that target human loci. We developed a CRISPR-engineered humanized mouse model ("hAlb-CR") where the human *albumin* exon 1 and intron 1 locus, a well-characterized safe harbor site, was integrated into the endogenous mouse *albumin* locus. This model allows for the direct evaluation of human-specific gRNAs within a phylogenetically relevant *in vivo* context.

We conducted systematic studies to validate the hAlb-CR mouse model. First, we compared hAlb-CR heterozygotes against age-matched Wild-Type control mice and an existing hAlb model ("hAlb-BAC") featuring a randomly inserted bacterial artificial chromosome containing the human albumin gene. These three models were treated with an AAV-reporter construct, packaged with a liver tropic capsid, and Lipid Nanoparticles (LNPs) containing SpCas9

and either human or mouse-specific gRNAs. This study showed 5-fold higher insertional reporter expression with hAlb-CR heterozygous mice compared to hAlb-BAC homozygous age-matched litter mate controls, while displaying nearly identical editing. The hAlb-CR model secreted similar levels of albumin as their age-matched wild-type littermates. This suggests that a nominal decrement in albumin expression following insertion at this locus is safe and will not adversely affect the maintenance of intravascular oncotic pressure. These models are relevant to evaluate the efficacy of potential clinical candidates utilizing the human albumin insertion approach, which is a predominant therapeutic locus of interest.

Next, we assessed the preclinical relevance of the homozygous hAlb-CR model by evaluating targeted insertion of a therapeutic protein at the human *albumin* locus. cDNA of a therapeutic protein was delivered via AAV, followed by LNP-mediated delivery of multiple nuclease variants and corresponding human gRNAs. All nuclease variants yielded robust therapeutic protein secretion. Notably, expression levels achieved through insertional editing were comparable to those observed with episomal AAV expression at similar doses. No significant changes in albumin levels were observed across treatment groups.

These findings demonstrate the utility of the hAlb-CR model for *in vivo* pre-clinical evaluation of nucleases, guides and therapeutic cassettes targeting the human *albumin* locus for relevant therapeutic applications.

648 A Protein-Guided Modular Integrase (MINT) Platform Enables Compact Therapeutic Payloads and Efficient Targeted Integration in T Cells

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Targeted integration (TI) of large DNA sequences into endogenous genomic loci has numerous potential applications in genomic medicine, agricultural trait development, synthetic biology, and basic research. Large serine integrases (LSIs) such as Bxb1 would represent ideal reagents to perform TI if they could be reprogrammed to integrate with high efficiencies at desired sites. LSIs recognize their targets using a protein-guided mechanism that allows for more compact and therefore more easily deliverable enzymes than the more common RNA-guided approaches. Notably, their covalent serine-DNA strand transfer mechanism is potentially safer than nuclease-based TI methods as it minimizes free DNA ends that can cause translocations and large deletions. However, high-efficiency direct reprogramming of LSIs has yet to be demonstrated.

With our Modular Integrase (MINT) platform we have identified three regions within Bxb1 that have a profound impact on the enzyme's specificity profile. Performing directed evolution upon 6-7 amino acid residues in each region can almost completely reprogram the targeting preferences for the corresponding portions of the target site. We build upon this work by combining our engineered Bxb1 variants with Bxb1 hyperactive mutations to dramatically increase integration efficiency. To enable therapeutic applications, we then explored alternative donor formats such as mini-circles and nanoplasmids.

With these improvements to our MINT platform, we demonstrate high-efficiency direct reprogramming of Bxb1 without relying on other gene editing tools to pre-install a natural Bxb1 recombination site. We further characterized our MINT platform by evaluating its genome-wide specificity profile and adding mini-circle and nanoplasmid donor delivery capabilities or our existing plasmid and AAV donor capabilities. This represents significant progress towards potential therapeutic applications such as integration into safe harbor loci and integration of a corrected coding sequence into introns of disease-causing genes.

649 PASTA: CRISPR Knock-In Paired with Integrase for Simple, Precise, and Efficient Large Transgene Insertion in T Cells

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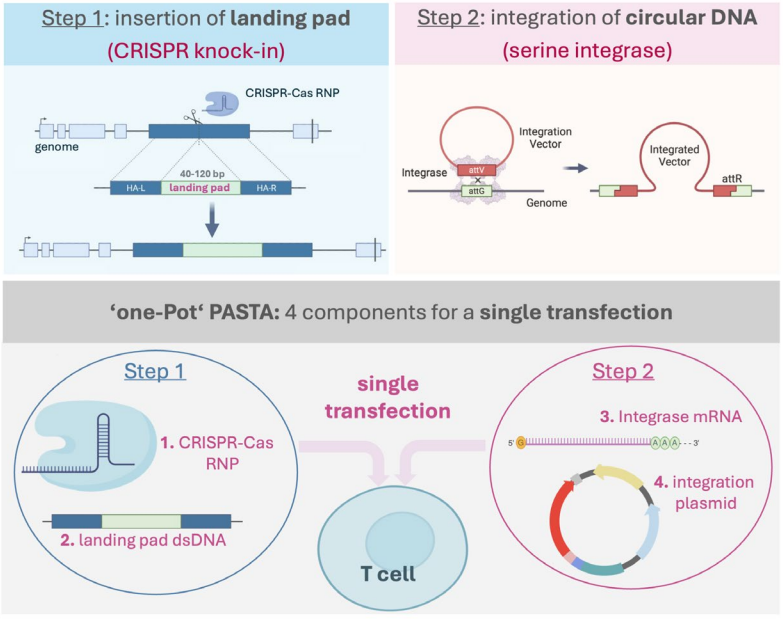
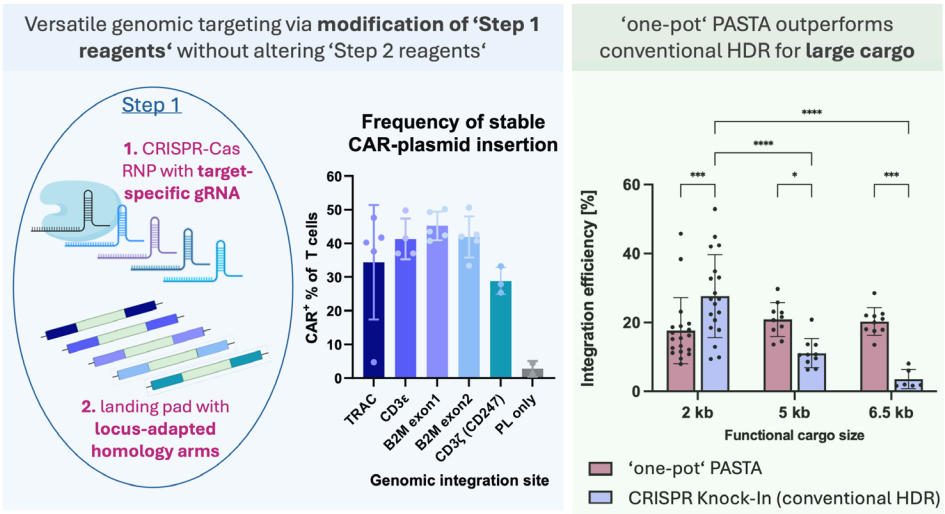
Adoptive cell therapy using gene-modified T cells expressing target-specific receptors has emerged as a powerful treatment for malignancies and autoimmune diseases. However, current gene transfer methods, such as lentiviral transduction and homology-directed repair (HDR), are limited in their ability to efficiently and precisely integrate large DNA sequences, constraining the scope of T-cell engineering.

To overcome these challenges, we developed PASTA (programmable and site-specific transgene addition), a two-phase approach enabling efficient, site-specific integration of large transgene cassettes. In Phase 1, a **small DNA landing pad** is introduced into the genome via CRISPR-Cas-mediated HDR. In Phase 2, a large serine integrase (e.g., Bxb1) precisely inserts a **circular DNA** vector into the landing pad. This streamlined "one-pot" process is initiated by a single transfection, simplifying the procedure and enhancing clinical applicability (**Figure 1**). We demonstrate that 'one-pot' PASTA enables efficient transgene insertion at key genomic loci for T-cell engineering, including TRAC, *B2M*, *CD3E*, *CD3Z (CD247)*, and *GAPDH* (**Figure 2, left**). Compared to conventional HDR, integrase-mediated insertion improves knock-in rates by 9-fold (range: 2.5–14-fold) when integrating a 6.5 kilobase (kb) therapeutic tri-cistronic transgene cassette. This results in targeted knock-in rates exceeding 20% after a single electroporation with an 8.5 kb plasmid (**Figure 2, right**). The transgene cassette contains two antigen receptors for dual-antigen targeting and a safety switch. The resulting dual-antigen targeting CAR T cells are currently being evaluated in mouse models for antigen escape in B-cell lymphoma, a common cause of relapse after mono-targeted CAR T-cell therapy.

Overall, 'one-pot' PASTA is a precise, versatile, and efficient non-viral gene transfer platform for large, multicistronic transgene cassettes. It simplifies T-cell engineering and provides a more flexible alternative to prime editing-based strategies like PASSIGE and PASTE.





650 Large DNA insertion at genomic safe harbors in human cells using a site-directed transposase

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Integrating large DNA sequences at specific genomic locations remains a challenge in gene therapy. Virus-based methods lack insertion site control, raising concerns about insertional mutagenesis. Non-integrating viral vectors like AAV are immunogenic, limiting repeat dosing. Sequence-specific strategies like CRISPR-Cas9 nucleases introduce unprotected double-strand breaks (DSBs), which can lead to deletions, chromosomal rearrangements, and chromothripsis via non-homologous end joining (NHEJ). While homology-directed repair (HDR) enables site-specific insertion, it is inefficient in non-dividing cells and impractical for large DNA sequences. Base editors and prime editors avoid DSBs but have limited edit sizes. CRISPR nucleases can also activate a p53-mediated DNA damage response, selecting for cells with impaired p53 function.

Transposons offer a promising alternative by integrating DNA without unprotected DSBs. Among transposable elements explored for gene therapy, piggyBac, Sleeping Beauty, and Myotis lucifugus transposase (MLT, or piggyBat) can integrate large DNA sequences (>100 kb) at TA or TTAA sites. These elements use a single transposase enzyme to excise and insert transposon DNA without generating unprotected DSBs.

A targeted transposase system could provide a powerful gene therapy tool. Previously, we demonstrated that fusing transcription activator-like effectors (TALEs) or dCas9 to piggyBac transposase enabled targeted DNA insertion, resulting in clonal isolated cells with single, site-specific integrations. However, this approach required extensive screening due to low targeting efficiency (~0.06%). Here, using structural predictions, we introduced mutations that disrupted MLT's native DNA-binding function while preserving its catalytic activity. By linking DNA-binding proteins to MLT, we aimed to restore targeted integration.

We systematically evaluated 10 factors influencing targeted integration: (1) spacing between the DNA-binding protein's target sequence and the target TTAA site, (2) amino acid linker composition (3) target sequence orientation relative to the TTAA site, (4) whether one or two DNA-binding protein target sequences flank the TTAA site, (5) transposase linkage site (N-terminus, C-terminus, or internally), (6) DNA-binding protein type (zinc fingers vs. TALEs), (7) sequence adjacent to the TTAA site, (8) genomic location of the binding protein's target sequence, (9)

whether the binding protein was covalently or non-covalently linked, and (10) mutations in the transposase DNA-binding domain to reduce off-target insertion while maintaining efficiency.

Using a plasmid-based assay, we identified strict targeting rules. Key factors included a non-covalent transposase–DNA-binding protein linkage and strict base-pair spacing between the binding protein's target sequence and TTAA site. Applying these rules to safe harbor sites, we achieved high-efficiency on-target insertions at multiple loci. Our most promising configuration resulted in over 50% of cells containing an on-target insertion. An off-target assay showed near-background activity, indicating DNA-binding domain mutations reduced unwanted insertions.

This programmable transposase system enables site-specific genomic integration without unprotected DSBs, minimizing off-target effects while allowing efficient insertion of large DNA sequences. These findings provide a framework for developing safer, more precise gene integration technologies.

651 Programmable Site-Specific Super piggyBac Transposase for Integration of Large DNA Payloads into Endogenous Genomic Sites

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[1]Poseida Therapeutics, San Diego, CA, [2]San Diego, CA, [3]Poseida Therapeutics, San Diego

The site-specific Super piggyBac (ssSPB) is a novel transposase system that combines the advantageous features of the Super piggyBac (SPB) transposase with the precision of targeted integration. The ability of SPB to insert large DNA payloads into mammalian genomes without introducing double-strand breaks (DSBs) in the genome (and thus not relying on endogenous DNA repair machinery) sets it apart from nuclease-based integration approaches using CRISPR-Cas9. The SPB cut-and-paste mechanism minimizes the chance of integrating truncated or mutated cargos. Additionally, SPB's propensity to integrate at TTAA sites in introns and intergenic regions is favorable from a genotoxicity perspective. Nevertheless, the inability to target integration to specific genomic locations can constrain its use in specific therapeutic applications.

Site-specificity expands the possibilities of transposase-based genome engineering by enabling gene replacement, promoter-less transposon designs, and expression uniformity among edited cells. By fusing a TAL effector DNA-binding domain (DBD) to the SPB transposase, we created a reprogrammable tool capable of integrating transgenes into specific genomic loci. Previous site-specific transposases suffered from low integration rates, making these systems inadequate for cell and gene therapy applications. In contrast, our structure-based design, informed by cryo-EM and AlphaFold simulations, precisely positions the DBD and transposase domains, resulting in efficient site-specific integration into mammalian genomes. Furthermore, re-engineering the transposase to function as a dimer rather than a tetramer, along with incorporation of additional thermostability and hyperactive substitutions resulted in a 2-log improvement in integration efficiency.

Given the DSB-free integration, the ssSPB platform is particularly well-suited for targeting repetitive loci, with observations that the integration efficiency scales with the number of target sites. By swapping its TAL DBD domain, ssSPB can be readily reprogrammed to such repetitive sites. Unlike nuclease-based methods that generate DSBs, ssSPB can target repetitive sequences like rDNA, without any genotoxicity. Targeting rDNA offers several advantages: high integration rates, safe harbor characteristics, species-to-species conservation, and the ability to enhance trafficking through fusion with nucleolar localizing proteins. In resting primary T-cells, 1-10 integrations per 100 haploid genomes were achieved using this enhanced trafficking strategy delivering a CAR expression cassette.

We next demonstrated the efficacy of the ssSPB system in vivo, utilizing a hybrid delivery strategy involving LNP-mediated mRNA delivery of the transposase targeting rDNA and AAV-mediated delivery of the transgene. This approach resulted in high editing efficiencies in juvenile mice of up to 5 integrations per 100 haploid genomes, quantified by ddPCR, with greater than 99% on-target integration fidelity as assessed by ligation-mediated PCR

(LM-PCR). Integration rates remained stable for the duration of the study (up to 56 days). We also demonstrate the feasibility of full non-viral delivery (utilizing nanoplasmid DNA-LNP transposon donors).

The ssSPB system offers a versatile and efficient platform for precise genome engineering holding great promise for the development of novel in vivo and ex vivo therapeutic strategies.

652 Site-Specific Integration of Therapeutic Transgenes with a Type V-K CAST System Engineered for Efficient and Targeted Human Genome Editing

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Gene editing technologies capable of efficient and specific integration of large transgenes into the human genome have the potential to bring curative treatments to diverse patient populations by providing a single approach that could cover the majority of disease-causing mutations. Recently, we and others demonstrated that CRISPR-associated transposases (CAST) discovered from microbes can be repurposed and programmed for site-specific, targeted integration of gene-sized DNA cargoes into the human genome. Here, we demonstrate that CAST protein engineering and delivery optimization enable efficient integration of diverse cargo designs across different cell types and target loci, including with therapeutically-relevant delivery approaches that are expected to be required for in vivo editing. Through structure and AI-guided protein design, we show that integration efficiency of a compact type V-K CAST system derived from uncultivated microbes can be improved by >50x compared with the natural system. Furthermore, an 'all-in-one' mRNA expressing multiple protein components can be used to deliver the system for efficient editing in primary hepatocytes. Combined with the fact that CAST integration uses a transposase to circumvent double-stranded DNA breaks, these advancements suggest important advantages over other gene editing approaches, especially those that are dependent on DNA repair machinery that may not be active in cell types targeted in vivo. Given their programmability and efficiency integrating large DNA cargoes, CAST systems have the potential to treat any genetic disease caused by a loss-of-function mutation, and contribute to other important advances in biotechnology and synthetic biology.

653 Proof of Concept for Automated Ntensify Manufacturing platform of mRNA: Productivity and Quality Assessment

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The field of mRNA therapeutics requires scalable, efficient, and Good Manufacturing Practice (GMP)-compliant production platforms to meet the increasing demands of both R&D and clinical applications. A proof-of-concept study was conducted to evaluate the first automated, standardized mRNA manufacturing platform, Quantoom's Ntensify™ system. Robust analytical assays were developed alongside production advancements to ensure the quality and regulatory compliance of mRNA therapeutics.

Key attributes like yield, purity, integrity, capping efficiency, poly(A) tail length, and residual contaminants are rapidly assessed using techniques such as High-Performance Liquid Chromatograph HPLC, (capillary) electrophoresis, and mass spectrometry. Integrating these GMP-compliant platforms with automated manufacturing has enhanced quality control and streamlines the transition from R&D to production.

Ntensify™ platform utilizes silica bead-based single-step purification and GMP-ready premixes, eliminating the need for custom process development while minimizing material variability. Robust scalability was demonstrated, with volumes ranging from 50 µL to 20 mL optimized for both R&D and GMP production. Linearized and purified plasmid DNA was used as a template, and the Ntensify™ in vitro transcription (IVT) process was first tested manually at small scale (125µL), achieving a total RNA productivity after one-step purification of 4.30 mg/mL with an RNA integrity of 89%. Scaling the manual process to a "Midi" scale (20ml) yielded 4.71 mg/mL with 91% RNA integrity. Applying the same process in the “Midi” automated system resulted in 4.77 mg/mL yield with 93% RNA integrity. Capping efficiency and poly(A) tail length were consistent between manual and automated processes, with capping efficiencies of 96% and 94%, and poly(A) tail lengths of 90% and 91%, respectively.

Quality assessments of the RNA drug substance produced in Midi automated system showed residual plasmid DNA levels of 0.18 ng/mg RNA and dsRNA content of <0.01%. Large-scale manufacturing is achieved through a scale-out strategy, bypassing the need for additional scale-up steps. These results highlight the advantageous of the automated Ntensify™ platform to streamline mRNA production, supporting a wide range of applications such as vaccinations, personalized cancer vaccines, and other therapeutics.

654 Development of a SMARTvector™ Multiplex shRNA Platform for Complex Cell Therapy Engineering with a Single Vector

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Chimeric antigen receptor (CAR) T cell therapy represents a new era of cell-based immunotherapies, allowing for targeted killing of cancer, often blood cancers, expressing a specific antigen. Despite the success of several CAR T cell therapies achieving FDA approval, these therapies often suffer from T cell exhaustion, which can present as reduced cytolytic activity, increased expression of inhibitory receptors, and reduced proliferative capacity. These outcomes render the treatment less potent, pointing towards a need to develop more advanced and tunable methods of modulating CAR T cells to be more robust and reliable. Gene editing approaches such as CRISPR-Cas9 have successfully been used for disruption of genes such as PDCD1, an inhibitory receptor involved in CAR T cell dysfunction, to enhance CAR T cell performance ex vivo. Despite the efficiency of CRISPR-Cas9, the number of targets that can be edited in a single sample is partially limited by increased cytotoxicity associated with DNA double-strand breaks (DSBs), thus limiting the therapeutic applicability. Additionally for some targets a knockdown approach might be favorable over a knockout approach. Here we present the SMARTvector lentiviral shRNA technology, a method for simultaneous introduction of a genetic payload, such as a CAR, with multiplex gene knockdown in immune cells from delivery of a single expression vector. We show that up to eight shRNAs and an anti-CD19 CAR can be expressed after a single lentiviral transduction, thus providing a safe alternative of targeting multiple genes while maximizing CAR T cell engineering.

We developed the SMARTvector multiplex shRNA technology with multiple repeats of a patented microRNA scaffold, flanking artificial DNA sequences that target mRNA transcript(s) of interest for efficient gene knockdown. The vector design includes a tunable promoter and optional selection markers for specific targeted development. Here, an array of multiple shRNAs was generated in a single expression vector with efficacy measured by RT-qPCR to assess relative mRNA transcript knockdown or flow cytometry for assessment of functional protein knockdown.

We have demonstrated that up to eight shRNAs can be expressed from a single expression vector, resulting in sustained expression and efficient gene knockdown in HEK cells, primary T cells, and iPSCs. In addition to achieving high knockdown efficiency, we have engineered a vector with optimal linker length between each microRNA-based shRNA encoding region and evaluated position related impacts of each shRNA on the multiplexed cassette. We demonstrate that the SMARTvector multiplex shRNA technology is highly modular and adaptable, making it primed for use in a wide range of cell therapy applications, such as generation of allogenic CAR T cell therapies.

655 CircVec: a powerful circular RNA expression platform to enhance viral and non-viral gene and cell therapies

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Circular RNA (circRNA) is a stable, endogenous class of non-coding transcripts found across most eukaryotes. Unlike mRNA, circRNA resists exonucleolytic RNA turnover, ensuring greater stability and persistence within cells. Advances in understanding circRNA biogenesis, functionality, and its engineering for efficient protein translation have established circRNA as a next-generation therapeutic RNA, with the potential to surpass conventional linear mRNA technologies.

Circio technology platform, circVec, is a novel powerful modular genetic cassette for potent and durable spliceosome-mediated intracellular circRNA expression from viral and non-viral DNA vectors. *In vitro* studies show that circVec achieves nearly 10x higher protein expression levels than conventional mRNA-based vectors one-week post-transfection.

In vivo, intramuscular injections of circVec DNA vectors have demonstrated up to 15x higher and substantially more durable protein expression compared to mRNA vector systems, with the most significant effects observed at low doses, reflecting the most clinically relevant conditions.

Bioinformatic modelling of RNA half-lives from long-term experimental *in vivo* data showed statistically inferred circRNA half-life of over 600 hours, compared to just 9 hours for mRNA, representing 70-fold improved RNA durability *in vivo*. This prolonged half-life results in a fundamentally distinct temporal expression profile, where the closed-loop circular structure enables a sustained accumulation and persistence of expression over a long period of time.

Continued optimization of the circVec genetic cassette has identified several critical genetic elements leading to significant enhancements in therapeutic protein expression compared to both mRNA expression vectors and earlier circVec generations These new genetic components impact the current understanding of gene expression biology in general and may be further modified to potentiate both viral and non-viral gene therapy vectors.

Systemic delivery of circVec has been tested in vivo using AAV9 viral constructs and LNP-packaged DNA vectors. Current results indicate tissue specific accumulation of circRNA expression particularly in extra-hepatic organs, such as muscle, heart and spleen. Ongoing experiments aim to further characterize and quantify the expression profile of the latest optimized circVec vector design and identify target tissues best suited for circRNA expression and accumulation. These data will support the identification of the most promising targets and tissues for development of future circVec-based therapeutic candidates.

With its ability to drive robust, durable circRNA biogenesis and sustained protein expression across a variety of vector systems and tissue types, the circVec platform offers transformative potential for next-generation gene and cell therapies. Circio is currently testing and optimizing circVec AAV and DNA vectors for gene and cell therapy applications, with the aim to advance one or more candidates towards clinical development.

656 Next-Gen Circular RNA: A Promising mRNA Alternative with Enhanced Stability and Therapeutic Applications

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Circular RNA (circRNA) is a type of single-stranded RNA with a covalently closed loop structure, which confers resistance to exonuclease-mediated degradation. This circular structure offers several advantages over linear

mRNAs, including a longer half-life, enhanced protein expression, functional stability, and reduced immunogenicity. To harness circRNA for therapeutic purpose, we have developed a novel method for producing circRNAs with efficient protein expression, utilizing ribozymes and RNA ligase. Our technology significantly improves circularization efficiency compared to traditional Group I permuted intron-exon (PIE) systems, especially for large genes. Using firefly luciferase as a model, we demonstrated that the activity of the protein by the circRNA was 80-fold and 7.6-fold higher compare to conventional mRNA *in vitro* and *in vivo*, respectively. Additionally, our circRNA technology has been successfully applied into CRISPR/Cas9 gene editing and Bio-PROTAC systems. Furthermore, we have developed and established a process for the mass production of GMP grade circRNAs. Taken together, the circRNAs generated by our technology hold great potential as a new class of therapeutics, offering a promising alternative to traditional mRNA-based therapies for a variety of diseases.

657 Advanced Capillary Electrophoresis Techniques for Monitoring mRNA-based Vaccine and Therapeutic Development: From Plasmid DNA to mRNA Encapsulation

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The flexibility and diversity of capillary electrophoresis (CE) have proven to be invaluable tools in the characterization of mRNA-based vaccines and therapeutics during the discovery and development process. Evaluations of key characteristics like purity, integrity, stability, and encapsulation efficiency play an important role in ensuring the safety and efficacy of the final product. This poster spotlights some of the CE-based analyses and kits used for this characterization, starting with the high-resolution separation of plasmid isoforms and linear DNA fragments for the analysis of plasmid DNA topology and purity. This is followed by the rapid assessment of mRNA poly(A) tail length and distribution. Additionally, stability is measured by monitoring the purity and integrity of mRNA molecules over time. Lastly, the encapsulation efficiency of mRNA-LNP is accurate determined utilizing size-based separations.

These studies can be performed using both the single-capillary PA 800 Plus system and the multi-capillary BioPhase 8800 system, providing valuable insights and comprehensive characterization of mRNA-based vaccines and therapeutics.

658 Repurposing CRISPR Knock Out Screens to Identify Cancer-Killing sgRNAs.

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Background: CRISPR/Cas9 is a prokaryotic adaptive immune system used widely for its programmable ability to induce double-strand DNA breaks (DSB) in the presence of a target sequence and protospacer adjacent motif (PAM). Our lab has previously shown inducing more than 12 DSBs simultaneously with CRISPR/Cas9 results in more than 95% cancer cell death. Hundreds of cancer-specific PAMs can be identified via alignment of a patient's normal whole genome sequencing (WGS) data to their tumor WGS data. How to best identify safe and therapeutically effective sgRNAs that induce a single DSB remains unknown.
Hypothesis: We hypothesize that CRISPR knock-out (CRISPRko) screens, where toxicity of a DSB-inducing sgRNA within cells is inferred from a decrease in abundance of the sgRNA over time, are sufficiently sensitive to identify top performing and safe sgRNAs for selective killing of cancer cells.
Methods: Cancer-specific PAMs were identified by aligning the patient's genome with their tumor's genome, identifying a set of mutations present in the patient's tumor and absent in their normal tissues. Mutations from the tumor genome were assessed for novel tumor-specific PAM formation, and novel PAMs were analyzed for potential off-target activity using Cas-OFFinder. Oligonucleotide libraries of cancer-specific sgRNAs were produced by Twist Biosciences and shotgun-cloned into a lentiGuide-puro (without Cas9) or lentiCRISPR-V2 (with Cas9) plasmid backbone. The patient's tumor-derived cancer cell line was transduced with the corresponding lentiviral sgRNA library and grown for 21 days. Genomic DNA was extracted from day 1 and day 21 samples of the transduced cell population, and PCR-amplified sgRNA loci were deep sequenced. A log-fold change (LFC) value was calculated based on the change in sgRNA abundance from day 1 to day 21. This procedure was repeated with

the same sgRNA library on a non-targeted cell line lacking the tumor-specific PAM to assess safety of the sgRNAs. *Results*: The Panc10.05 cancer-specific sgRNA library resulted in significant LFC of several sgRNAs when targeting Panc10.05 cells, however, most sgRNAs did not produce a significant LFC. The same sgRNA library was screened in TS0111, a cancer cell line lacking the Panc10.05-specific PAMs, and while most sgRNAs were non-toxic, a small number of sgRNAs did prove slightly toxic to TS0111 despite the lack of a corresponding PAM sequence. TS0111 cancer-specific sgRNAs were screened in a large library format as well as smaller cohort pools. These TS0111-specific libraries were cloned into a vector lacking Cas9 and screened in TS0111-Cas9^{+}, TS0111 without Cas9, and Panc10.05-Cas9^{+} cell lines. The libraries identified sgRNAs that were toxic in TS0111-Cas9^{+} but not the other two cell lines. Similar to the Panc10.05 sgRNA library, most TS0111-specific sgRNAs demonstrated no significant LFC.
Conclusions: Large library screens of cancer-specific sgRNAs were generally tolerated in cells, however, for both Panc10.05 and TS0111 cancer cells lines, a small number of cancer-specific sgRNAs were identified as both toxic to the target cell line and less toxic to the non-targeted cell line. Overall, CRISPRko screens were able to identify toxic sgRNAs that induce a single DSB, however, it remains unknown if identification of 5-10 slightly toxic sgRNAs is sufficient for a robust anti-cancer response.

659 Gene Expression Assays for PD Readout for siRNA Therapeutics: Validation Strategy to Support Clinical Studies

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Purpose: Quantitative reverse transcriptase-real time polymerase chain reaction (RT-qPCR) is a common approach to monitor the target gene expression modulation induced by a RNAi-based therapeutic. Despite the published guidelines for the qPCR analyses, the methodologies are not standardized across laboratories and there is an overall lack of harmonization on assay design, validation parameters and appropriate acceptance criteria for these assays. This case study presents the validation of a two-step RT-qPCR method including three duplex reactions for the quantification of a target gene mRNA expression relative to three housekeeping genes, in human liver biopsies.

Methods: Total RNA was isolated from naïve and diseased human liver samples and quantified by spectrophotometric measurement. RNA samples were then diluted with or without yeast carrier RNA and analyzed in parallel in three RT-qPCR duplex reactions. The comparative Ct method was used to normalize the target gene mRNA expression from each duplex to the corresponding housekeeping gene. The following parameters were assessed: range of response (linearity), intra- and inter-assay precision, reference range, multiplex interference, and RNA stability.

Results: The RT-qPCR assay performance was best in the presence of yeast carrier RNA when compared to nuclease-free water. Based on the linearity assessment and on the specificity evaluation (No RT controls), samples can be analyzed from 1000 to 1.40 ng per reverse transcription (RT) reaction, although a target concentration of 100 ng per RT reaction was selected for sample analysis, where possible. The intra-assay and inter-assay precision of the method was within 2.5% CV (coefficient of variation). A human liver RNA pool was also created and characterized (Ct range, Delta Ct range) to be used as quality control (QC) sample for sample analysis. No multiplex interference was observed for two out of the three duplex assays but differences between the single plex and duplex assay were seen in the third duplex assay. The Ct values for the target gene were lower in the duplex assay, compared to the single plex assay, and the maximum fold change observed between the single plex and the duplex assay was of 1.02 fold, indicating that the interference remained minimal and the sensitivity of the assay was not affected.

Conclusions: A two-step RT-qPCR assay combining three duplex assays for the quantification of a target gene mRNA expression relative to the expression of three housekeeping genes in human liver biopsies was successfully validated and used to support the PD assessment for a siRNA in a clinical study.

660 Harnessing Functional Genomics to Advance the Discovery and Development of Oligonucleotide-Based Therapies Targeting the Regulatory Genome

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Introduction: Chronic diseases are driven by complex pathophysiology, and the limited efficacy of single-gene targeting approaches has prompted a shift toward disease-modifying strategies that reprogram pathogenic cells back to a healthy state. Long non-coding RNAs (lncRNAs) are tissue- and cell type-specific regulators of disease, making them promising therapeutic targets within this emerging paradigm of more effective treatments achieved through cell state reprogramming. Unlike protein-coding mRNAs, lncRNAs do not encode proteins but modulate gene expression through epigenetic, transcriptional, and post-transcriptional mechanisms. While lncRNA functions are often conserved, their sequences can vary across species, challenging the use of a single antisense oligonucleotide (ASO) across preclinical stages. Thus, developing ASO-based therapeutics targeting lncRNAs requires a tailored approach. **Methods:** The development of ASO-based drugs targeting protein-coding mRNAs traditionally involves assessing ASO potency through dose-dependent target knockdown, with potency defined as the maximal target downregulation achieved at the lowest concentrations. However, this approach is not directly applicable to lncRNAs due to their broad regulatory functions and diverse mechanisms of action (MoAs). To design potent ASOs targeting lncRNAs, we developed a platform that leverages functional genomics across the entire drug development pipeline, from discovery to preclinical validation. Our platform combines genomic, epigenomic and transcriptomic profiling to comprehensively understand lncRNA MoAs and pinpoint optimal target regions to maximize ASO efficacy. This multi-omics approach allows the selection of ideal candidates from a vast in silico-generated ASO sequence pool by predicting disease-modifying target pathways and defines its downstream target engagement panel (TEP). As a result, it enables the selection of ideal candidates based on their ability to reprogram pathogenic cells back to a healthy state, while also incorporating off-target analysis and tolerability studies to optimize both therapeutic efficacy and safety. **Results.** Using our platform, we designed and selected ASO candidates targeting therapeutically relevant lncRNAs across multiple disease areas, including cardiac and pulmonary fibrosis. These ASOs exhibited potent on-target effects, efficient dose-dependent downregulation of TEP genes, and significant modulation of genes within predicted disease-modifying pathways, validating the efficacy of our platform. Through this approach, we identified a potent and safe ASO targeting WISPER, a cardiac fibroblast-specific lncRNA responsible for the fibrotic burden in cardiac diseases. The selected ASO demonstrated effective WISPER knockdown and dose-dependent downregulation of the WISPER TEP, leading to remarkable cell-state reprogramming of tissue-specific pathological myofibroblasts back into quiescent-like fibroblasts in the heart. IND-enabling activities are ongoing. **Conclusions.** Integrating multi-omics profiling and functional genomics assays provides a comprehensive approach to developing ASO-based therapeutics targeting lncRNAs. We have successfully designed ASOs targeting tissue-specific lncRNAs across multiple disease areas, demonstrating their therapeutic potential and safety, with promising prospects for advancing ASO-based disease-modifying treatments toward clinical applications.

661 Humanized INHBE Mice Provide a Robust *In Vivo* Platform for Evaluating RNAi Therapies.

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Introduction: Activin E, encoded by the INHBE gene, is a growth factor that belongs to the transforming growth factor-β (TGF-β) family. As part of the liver-adipose feedback loop, activin E is released from hepatocytes in response to elevated hepatic triglycerides and serum-free fatty acids. It then signals in adipose cells via ALK7 to suppress lipolysis, reducing the efflux of free fatty acids to the liver and preventing excessive hepatic lipid accumulation. Preclinical studies have shown that targeting INHBE with RNAi therapy can reduce fat mass and preserve lean muscle mass compared to currently approved obesity therapies. The drug with the fastest progress at present has initiated a Phase 1/2a clinical trial Biocytogen has developed a humanized INHBE mouse model, B-hINHBE mice, which exclusively express human INHBE but not mouse Inhbe. This model provides a valuable tool for preclinical studies of INHBE-related diseases.

Methods: To develop humanized B-hINHBE mice, exons 1-2 of the mouse *Inhbe* gene, which encode the entire molecule (from ATG start codon to STOP codon) including the 3'UTR, were replaced with their human counterparts. The promoter and 5'UTR regions of the mouse gene were retained. Human *INHBE* expression is driven by the endogenous mouse *Inhbe* promoter, while transcription and translation of the mouse *Inhbe* gene are disrupted. For mRNA expression analysis, liver RNA was isolated from wild-type C57BL/6 mice (+/+) and homozygous B-hINHBE mice (H/H). cDNA libraries were synthesized by reverse transcription, followed by PCR using mouse or human *INHBE* primers. For protein expression analysis, tissue lysates were collected from wild-type C57BL/6 mice (+/+) and homozygous B-hINHBE mice (H/H), and analyzed by western blot with anti-INHBE antibody. 40 µg total proteins were loaded for western blot analysis. To establish the diet-induced obesity (DIO) model, C57BL/6 and B-hINHBE mice (male, 7 weeks old) were fed a high-fat diet (60 kcal% fat) for 12 weeks to induce obesity. During the 9th week, mice were intraperitoneally injected with 15% glucose (1.5 g/kg) for glucose tolerance tests (IPGTT). During the 10th week, mice were intraperitoneally injected with 0.5 U/kg insulin for insulin tolerance Test (ITT). To evaluate the *in vivo* efficiency of RNAi therapy, DIO B-hINHBE mice were treated weekly with an siRNA drug targeting INHBE (provided by client; synthesized based on patent) at a dose of 9 mpk subcutaneously.

Results: RT-PCR results demonstrated that human *INHBE* mRNA was detectable only in homozygous B-hINHBE mice but not in wild-type mice. Western blot analysis showed that INHBE was present in the liver and kidney of wild-type and homozygous B-hINHBE mice, as the antibody cross-reacted with both human and mouse INHBE. The growth curve of DIO B-hINHBE mice closely resembled that of DIO C57BL/6 mice. No significant differences were observed in IPGTT and ITT between DIO B-hINHBE and DIO C57BL/6 mice. In the *in vivo* efficiency test, siRNA targeting INHBE inhibited body weight gain in DIO B-hINHBE mice compared to the saline-treated group. Treatment with siRNA targeting INHBE also resulted in fat mass loss while preserving lean mass.

Conclusions: B-hINHBE mice successfully express human INHBE, which functions normally. They serve as a powerful *in vivo* platform to evaluate RNAi therapy in obesity treatment and muscle preservation.

662 Developing Humanized APOC3 Mice As a Robust *In Vivo* Model for RNAi Therapy Assessment

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Introduction: APOC3 (Apolipoprotein C3) is primarily expressed by hepatocytes, with minor expression in intestinal epithelial cells. In the bloodstream, APOC3 is associated with triglyceride-rich lipoproteins (TRLs), which include chylomicrons (CM) produced in the intestine, very-low-density lipoproteins (VLDL) produced in the liver, and high-density lipoproteins (HDL). A small amount of APOC3 is also bound to low-density lipoproteins (LDL). APOC3 increases plasma triglyceride levels by inhibiting lipoprotein lipase (LPL) activity and raises triglyceride and cholesterol levels by interfering with the clearance of TRLs and their remnants through hepatic LDL receptors (LDLR) and low-density lipoprotein receptor-related protein 1 (LRP1). Reduced clearance of TRLs is strongly correlated with elevated plasma APOC3 levels, making APOC3 a promising therapeutic target for reducing the risk of dyslipidemia and cardiovascular disease. Biocytogen has developed a humanized APOC3 mouse model, known as B-hAPOC3 mice. These mice exclusively express human APOC3, with the mouse Apoc3 gene inactivated. This model provides a valuable tool for preclinical research on APOC3-associated diseases.

Methods: To develop humanized B-hAPOC3 mice, exons 2-4 of the mouse *Apoc3* gene, which encode the entire molecule (from ATG to STOP codon), including the 3'UTR, were replaced with their human counterparts. The promoter and 5'UTR regions of the mouse gene were retained. Human *APOC3* expression is driven by the endogenous mouse *Apoc3* promoter, while expression of the mouse *Apoc3* gene is disrupted. For mRNA expression analysis, liver RNA was extracted from C57BL/6 mice and B-hAPOC3 mice. cDNA libraries were synthesized by reverse transcription, and PCR was performed using primers specific to mouse or human APOC3. For protein expression analysis, serum from B-hAPOC3 mice was collected and analyzed using species-specific APOC3 ELISA kits. Lipid metabolism analysis: plasma levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured in B-hAPOC3 and C57BL/6

mice at 5 weeks of age. LDL-C, TC, and HDL-C levels in B-hAPOC3 mice were similar to wild-type. The efficacy of nucleic acid drugs in inhibiting human APOC3 was assessed in B-hAPOC3 mice. B-hAPOC3 mice were randomly divided into two groups. Each group received a subcutaneous injection of either 10 mg/kg of nucleic acid drugs targeting human APOC3 (synthesized as per the patent) or PBS. The nucleic acid drugs were delivered in a PBS solution.

Results: RT-PCR results indicated that human *APOC3* mRNA was detectable only in B-hAPOC3 mice but not in wild-type mice. Protein analysis showed that APOC3 was present in B-hAPOC3 mice. Lipid metabolism analysis showed that TG levels in male B-hAPOC3 mice were significantly higher than those in C57BL/6 mice. Compared to the control group, the treatment group showed a significant decrease in TG levels. Human *APOC3* mRNA expression in the treatment group was also significantly reduced, with an inhibition rate of 73.4%. These results show that B-hAPOC3 mice provide a powerful preclinical model for *in vivo* evaluation of human APOC3-targeted nucleic acid drugs.

Conclusions: B-hAPOC3 mice effectively express human APOC3 and maintain normal functions. These mice offer a robust in vivo model for assessing RNAi therapy in research on APOC3-associated lipid disorders.

663 Optimizing Purification Strategies for sgRNA and Therapeutic Oligonucleotides

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The development of CRISPR-based therapies and therapeutic oligonucleotides has opened new avenues for treating genetic disorders and rare diseases. Solid-phase synthesis is a key step in producing these molecules, but it generates impurities that necessitate rigorous purification. This study focuses on optimizing purification strategies for long single-guide RNA (sgRNA), particularly those over 90 nucleotides, to address these challenges. We employed a multi-step purification process, including DMT-on purification, detritylation, and a secondary purification step, to enhance the quality and scalability of these therapeutics. We compared different ion-pair reagents, such as triethylamine (TEA) and hexylamine (HA), for reverse-phase (RP) chromatography, as well as various buffer conditions, including chloride, bromide, and perchlorate, for anion-exchange (AEX) chromatography. Our results demonstrate significant improvements in purity and yield, with sgRNA purity exceeding 80%. Additionally, we are exploring enzymatic ligation as an alternative method for sgRNA production and testing oligo-antibody conjugates to expand the scope of therapeutic applications. The optimized process demonstrates robustness and reproducibility, making it suitable for both research and industrial applications. These advancements are particularly impactful for the rare disease community, where the demand for precise and effective therapies is growing. This work underscores the importance of innovative purification strategies in ensuring the safety and efficacy of next-generation genetic medicines.

664 Validated Impurity Test for Chemical Synthesized sgRNAs using NGS Detection Method

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Introduction: GMP (Good Manufacturing Practice)-grade single-guide RNA (sgRNA) is used for gene and cell therapy in clinical trials. In a seminar in 2024, the FDA has suggested that sgRNA for IND purposes should report sequence impurities with a frequency higher than 1%. Purity assessment and sequence confirmation for sgRNAs are typically performed with HPLC and Mass spectrometry. However, it is a difficult task for HPLC or Mass Spectrometry to distinguish sequence impurities of N+1 and N-1. Moreover, it is almost impossible to detect point mutations, especially when the mass difference between C and U is only 1 g/mol. Next-generation sequencing (NGS), on the other hand, is widely used for sequence detection. Discriminating between different nucleotide bases is a routine task for NGS. However, it has its own shortcomings where reads induced by PCR bias or sequencing bias can cause the quantification to be quite inaccurate.

In this study, we have developed a semi-quantitative NGS method to analyze sequence impurities in chemical synthesized sgRNAs.

Methods: 5'rApp and T4 RNA ligase are applied to ligate the sequencing adaptors to the single-stranded sgRNA molecule. The adaptors at both ends contain UMIs (Unique Molecular Identifiers) to reduce PCR bias during amplification. Reverse Transcriptase is then used to generate a reverse complement DNA chain. The library constructed through PCR amplification is then sequenced on an Illumina platform. The data analysis process is similar to the genome sequencing except that only UMIs with more than three reads are counted in our analysis pipeline. Tables and pie charts are drawn to display the sequence impurities and their distribution.

Results: Since there is only one reference sequence in the solution, the sequencing depth of the method can reach as high as 100,000X. To validate this method for quality control purposes, we have designed experiments to test the characteristics including specificity, linearity, accuracy, LOQ, and precision. The method was validated to detect N+1/N-1 or point mutations with an LOQ of 1%, which meets the FDA's requirement for IND sgRNA quality control. R^2 of the linear curve of variants with frequencies between 1% and 30% is higher than 0.99. N+1/N-1 in both ends of the sequence can also be detected with this method.

Conclusions: After neutralizing biases that occur in PCR amplification and sequencing, NGS is able to meet FDA requirements and serve as a QC method for synthesized IND sgRNA.

665 Optimizing VHH Ligand Density in T Cell-Targeted LNPs: An Inverted U-Shaped Relationship for Effective *In Vivo* CAR T Cell Generation

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Chimeric antigen receptor (CAR) T cell therapy has shown remarkable clinical success in treating B-cell malignancies. This approach also holds promise for autoimmune diseases, given its ability to efficiently target and profoundly eliminate pathogenic B cells expressing CD19. However, challenges such as cytokine release syndrome (CRS), neurotoxicity, and the complexity of the manufacturing process—resulting in high costs and extended production times—remain significant barriers. To address these issues, we developed T cell-targeted lipid nanoparticles (ctLNPs) loaded with CD19 CAR mRNA, enabling *in vivo* CAR T cell generation. By optimizing biodegradable ionizable lipids, we designed anti-human CD8 VHH-conjugated LNPs that efficiently and selectively deliver mRNA to CD8+ T cells. In this study, we emphasize the critical role of VHH ligand density on the LNP surface for enhancing mRNA delivery efficiency. The functional VHH ligands on the LNPs were visualized by their interaction with fluorescently labeled recombinant CD8 receptors (Figure 1A), and unbound receptors were excluded using size exclusion chromatography (SEC). Using a nano flow cytometer, we simultaneously measured particle size and fluorescence intensity at the single-particle level, allowing precise quantification of VHH ligand density (Figure 1B, 1C). We explored ligand density-activity relationships by preparing LNPs with varying VHH densities and assessing mCherry reporter mRNA delivery to activated T cells from human peripheral blood mononuclear cells (hPBMCs). The results revealed an inverted U-shaped relationship, with an optimal VHH density identified for maximum efficiency. At this optimal density, more than 80% of CD8+ T cells expressed mCherry at only 4 ng/mL of mRNA. This optimal VHH density was consistent across different VHH clones, lipid compositions, and LNP concentrations. To further validate the targeting efficiency of VHH-conjugated LNPs, *in vivo* studies in hPBMC-engrafted NSG mice were conducted. The results confirmed that the optimal VHH density for mRNA delivery to CD8+ T cells *in vivo* mirrored the findings from *in vitro* studies, with minimal off-target delivery on CD4+ T cells, Tregs, or B cells. The CD8+ T cell-targeted LNPs, loaded with CD19 CAR mRNA, led to dose-dependent CAR expression and B cell depletion. Moreover, intravenous administration of these ctLNPs induced dose-dependent efficacy (0.5–2.0 mg/kg) in Nalm6-Luc/GFP-bearing hPBMC-engrafted NSG mice and substantial depletion of splenic B cells in hPBMC-engrafted NSG mice (0.05–0.25 mg/kg). Notably, the ctLNPs containing our

proprietary biodegradable lipids did not affect mouse body weight at these doses, and we are planning non-human primate (NHP) studies and scale up production to advance this platform toward clinical proof of concept. We believe that these off-the-shelf ctLNPs with optimal VHH density have the potential to revolutionize autoimmune disease therapies.

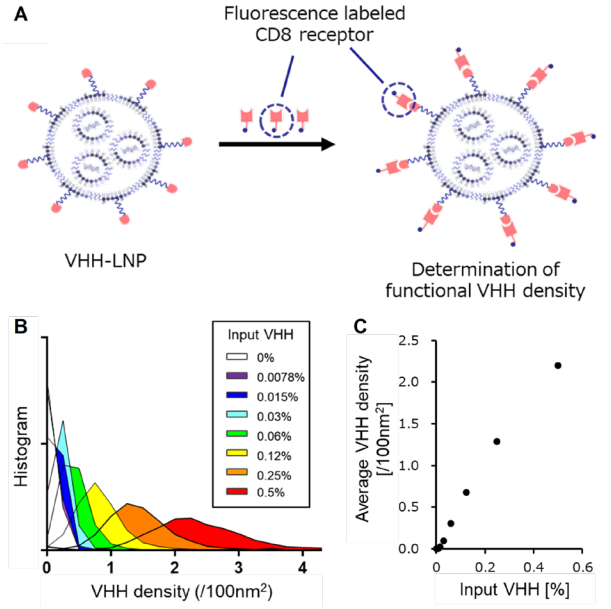

Figure 1. Quantification of the anti-human CD8 VHH Ligand Density of T Cell-Targeted LNP. A) Available VHH ligands on the LNP surface were visualized by interacting with Alexa Fluor 488 labelled recombinant CD8 receptors. B) The quantified VHH ligand density of each particle is represented as a histogram. C) The VHH density on the LNP surface increases with higher input VHH [mol%] relative to the total lipid.

666 T cell specific CAR-T mRNA Design for in situ T Cell Engineering

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Current CAR-T therapies are challenged with prohibitive cost, manufacturing difficulties and timelines, immune effector cell-associated neurotoxicity syndrome (ICANS). In addition, the malignant cells may lose their antigen that CAR-T cells are binding. Even with permanent genetic engineering CAR-T cells may get exhausted. Depending on the antigen, CAR-T cells may constantly attack healthy B cells. These limitations motivate versatile T cell engineering, that can be redosed if needed. Particularly for autoimmune disease therapy, temporary CAR-T treatment would be sufficient induce immune reset. At Kernal Bio, we have T cell targeted lipid nanoparticles that have higher than 90% transfection efficiency. To complement targeted delivery, we develop CAR-T expressing mRNAs that are translated specifically in T cells. With durable translation of mRNA temporary modification of T cells is sufficient to trigger malignant cell killing. Due to our tolerable ionizable lipids, it can be dosed multiple until the B-cells is fully cleared.

In order to eliminate ex vivo cell engineering, we need to constrain mRNA translation to target T cells upon intravenous administration of mRNA- LNPs, while sparing off-target cells. Using our proprietary computational and synthetic biology pipelines, Kernal Bio combs through large omics data sets to identify sequences that have the potential to shift activity away from off-target cell types into target cells. With this innovative approach of integrated omics analyses, we mechanism agnostically identify sequences of interest. Then the sequence hits are further altered for high throughput screening where the cells dictate what sequences are optimal. We can synthesize, purify, and

screen hundreds of putative cell-specific mRNA elements a week using this methodology and automated mRNA manufacturing. Using this approach, we identified RNA elements that boost translation up to 10-fold in T cells compared to monocytes. Omics driven mRNA design has the potential to time and dose the protein payload with synthetic mRNA. We use the data to fine-tune generative artifical intelligence models to extrapolate into untested combinations of sequences. Our iterative design strategy makes astronomic RNA sequence combinations tractable.

667 Improved T Cell Delivery and Expression of mRNA Using CD8-Targeted Lipid Nanoparticles with Athebody® Designed Ankyrin Repeat Proteins (DARPins)

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Lipid nanoparticles (LNPs) administered intravenously primarily accumulate in fenestrated tissues (e.g., liver, spleen, bone marrow) due to vascular permeability. In the liver, endogenous apolipoprotein E (apoE) binds to the LNP surface, mediating hepatocyte uptake and nucleic acid payload delivery via low-density lipoprotein receptors. While LNP delivery to the liver is well established, expanding delivery to extrahepatic tissues remains a challenge. One approach is "active" targeting, where ligand-conjugated LNPs facilitate direct uptake by target cells in the blood compartment. Here, we evaluate Athebody® designed ankyrin repeat proteins (DARPins), antibody mimetics generated through synthetic combinatorial libraries and *in vitro and in silico* selection methods, as high affinity and specificity ligands for targeted LNP delivery.

Using DiD-labeled LNP containing a fluorophore-expressing mRNA, we first evaluated binding and fluorescent protein expression in mouse splenocytes after IV injection of non-targeted LNPs. At 24 hours after administration, splenic macrophages (33%) and dendritic cells (29%) exhibited notable LNP uptake and payload mRNA expression, while granulocytes, B cells, and T cells showed minimal engagement (<2%). Similar trends were observed *in vitro* using human whole blood. To enable T cell targeting, we conjugated a murine CD8-targeting DARPin to LNPs, and injected these IV into mice to assess *in vivo* targeting efficiency. CD8 DARPin-targeted mRNA-LNP showed dose-dependent, target-specific binding (44%) and transgene expression (34%) in CD8 T cells, with no impact on viability (>90%). Using LNPs with extended circulation, *in vivo* targeting efficiency increased to 86% binding and 59% expression in CD8 T cells, with additional uptake in $CD8^+$ dendritic cells (88% binding, 61% expression). There was no significant increase in targeting to $CD8^-$ cells ($CD8^-$ DCs, CD4 T cells, B cells, or myeloid cells) compared to unconjugated LNPs. Translating this to human cells, a human CD8-targeting DARPin-LNP showed ~98% binding and 46–90% expression in CD8 T cells from healthy human donor whole blood. These findings highlight DARPin-conjugated LNPs as a promising strategy for highly specific mRNA delivery to hard-to-transfect cells accessible within the vascular compartment.

668 *In Vivo RNA Delivery to T Cells and Hematopoietic Stem Cells in Humanized Mice and Non-Human Primates using Targeting Lipid Nanoparticles*

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We believe that non-viral delivery of genetic medicines to cells and tissues *in vivo* could pave the way for new therapeutic possibilities and enhance patient access. However, achieving effective *in vivo* delivery requires development of safe, efficient delivery vehicles capable of transporting genetic medicines to their intended target tissues and cells. While lipid nanoparticles (LNPs) have emerged as a strong platform for delivering RNA

therapeutics to the liver, targeting extrahepatic tissues remains challenging. Tessera Therapeutics is advancing novel LNPs designed to deliver RNA-based gene editing payloads to T cells and hematopoietic stem cells (HSCs) *in vivo*.

Our approach to LNP design incorporates several strategies to enhance tissue-specific delivery, including the optimization of lipid chemistry and composition to skew LNP biodistribution. Our advancements are exemplified by our HSC-targeting LNPs. In B6 mice, after a single intravenous (IV) administered dose, our HSC-targeting LNPs reduced liver delivery by 11-fold compared to liver-tropic LNPs, as measured by transient green fluorescent protein (GFP) levels in various tissues. Addition of a targeting moiety to enhance cell-type specific delivery increased GFP expression in bone marrow by 55-fold, while minimizing liver delivery. In cynomolgus macaques, our LNPs also favored bone marrow, achieving 3-fold greater beta-2 microglobulin (B2M) gene editing in bone marrow versus the liver, and nearly 2-fold longer blood half-life compared to liver-tropic LNPs, enhancing exposure in difficult-to-reach tissues.

By combining our passive and active targeting strategies, we have previously presented efficient delivery to T cells and HSCs in humanized mice and non-human primates (NHPs). We have furthered these efforts by pairing Tessera's novel Gene Writing platform with our proprietary delivery system to enable *in vivo* gene editing. Tessera's Gene Writers facilitate a range of genetic edits, from transgene insertions to single nucleotide changes, using target-primed reverse transcription. Using HSC-targeting LNPs with a Gene Writer and a beta-2 microglobulin (B2M) template in an all-RNA format, we achieved an average of 74% and 76% B2M knockout in long-term HSCs (LT-HSCs, defined as Lin-CD34+CD38-CD90+CD45RA- cells) in humanized mice and cynomolgus macaques, respectively. Similarly, with T cell-targeting LNPs, we observed an average of 30% B2M knockout in splenic T cells of humanized mice.

Beyond successfully achieving reporter protein expression and editing in the B2M locus, we demonstrated robust editing of therapeutically relevant targets. For sickle cell disease, with a single IV administered dose of Gene Writer encapsulated in HSC-targeting LNPs, we achieved 62% HBB Makassar installation in LT-HSCs of humanized mice and an average of 24% in LT-HSCs of cynomolgus macaques. Separately, in the pursuit of developing an *in vivo* CAR-T therapy, a single dose of T cell-targeting LNPs with a Gene Writer and CD20 CAR template resulted in ~30% CAR-expressing T cells, and subsequent elimination of B cells in humanized mice. Together, we believe these results demonstrate our LNP delivery platform is capable of efficiently delivering RNA payloads to edit HSCs and T cells *in vivo*, providing a potential delivery platform for the application of genomic medicines in extra-hepatic tissues.

669 In vivo Targeting of Hematopoietic Stem Cell Receptor cKit by Lentivirus Particles Pseudotyped with chimeric VSV-G proteins

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Sickle cell disease (SCD) and beta-thalassemia (beta-thal) are high prevalence hemoglobinopathies resulting from abnormal or reduced production of beta globin, a component of adult hemoglobin. Persistent production of fetal hemoglobin (via continued postnatal gamma globin production) can strongly mitigate both diseases. Casgevy, recently approved by FDA, is an ex vivo hematopoietic stem cell (HSC) editing treatment that uses CRISPR/Cas9 to disrupt the erythroid-specific enhancer region of BCL11A, thereby restoring fetal hemoglobin production in adults with SCD or transfusion dependent thalassemia (TDT). To reduce the cost, complexity and toxicity of this highly effective "ex vivo" therapy we seek to develop virus like particles that can efficiently and specifically deliver BCL11A editing complexes directly to HSCs in vivo. To this end we engineered lentiviral vectors and lentivirus-derived virus-like particles (VLPs) to target the HSC receptor cKit. This was achieved by displaying stem cell factor, the natural ligand for cKit, as an N-terminal extension of the VSV-G protein with which our lentiviral particles were pseudotyped. Specific transduction of cKit positive cell lines and primary hematopoietic progenitor cells was demonstrated following their ex vivo exposure to the SCF-displaying cKit targeted vectors. Subsequently, cKit-targeted and control nontargeted, LDLR-blinded or EGFR-targeted vectors encoding the cre recombinase were

administered intravenously to Ai14 (loxP flanked STOP-tdTomato reporter) mice, following which tdTomato expression in PBMCs was monitored on days 17, 27, 57 and 80 post-infusion. Interestingly, tdTomato expression was clearly identified, initially in myeloid and later in lymphoid PBMCs (approximately 0.4% of circulating cells) from mice that had been infused with the cKit targeted vector, whereas no expression was detected in any of the control mice. Analysis of the tissue distribution of tdTomato positive cells was conducted in a smaller cohort of animals on day 7 post vector administration. Unexpectedly, and in striking contrast to the control vectors, sinusoidal endothelium in the bone marrow, spleen, and liver was transduced with high efficiency following administration of the cKit targeted vector. Further studies are underway to explore the HSC targeting potential of alternate ligands and combinations of ligands displayed on the lentiviral surface.

670 Efficient In Vivo Gene Editing of T Cells Utilizing Novel Targeted Lipid Nanoparticles

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Background: Immunotherapy in general and T cell therapy in particular have revolutionized the oncology field. Despite these successes, challenges such as cell manufacturing complexity, limited efficacy, and T cell dysfunction persist. In the study, we present results from a novel T cell-targeting lipid nanoparticle (tLNP) designed to deliver gene editing payloads to T cells in vivo, with potential applicability including next generation immunotherapies. These tLNPs are a product of our CellSeeker™ platform technology and comprise a novel LNP conjugated with a recombinant protein binder designed to deliver RNA payloads to specific cell types in vivo.

Methods: A gene editing payload, consisting of an mRNA encoding for Cas9 and a sgRNA targeting T-cell receptor alpha constant (TRAC), were encapsulated separately (single-payload) or co-encapsulated together (combined-payload). tLNPs were targeted to either pan-T cells via an anti-CD5 antibody, or a T cell subset via an anti-CD8 antibody. Single- and combined-payload tLNPs were administered to primary human T cells in vitro and intravenously to NSG mice engrafted with human T cells. T cell gene editing efficiencies were evaluated in vitro and in vivo.

Results: In vitro we observed robust gene editing of T cells with either single- or combined-payload tLNPs. Both CD5- and CD8-targeting combined-payload tLNPs demonstrated high targeting specificity and up to 55% editing efficiency. Interestingly, co-administration of CD5- and CD8-targeting combined-payload tLNPs resulted in an increase in gene editing efficiency (up to 71%) compared to CD5- or CD8-targeting alone at the same dosage. Delivering the Cas9 mRNA and TRAC sgRNA separately by single-payload tLNPs with either the same targeting antibody or different targeting antibodies, resulted in high editing rates in T cells and T cells subsets, reaching 93%. Single-payload tLNPs have the added benefit of easily titrating the mRNA/guide ratio to find the optimal dose. To test the translatability of this targeted gene editing to an in vivo setting, we intravenously administered combined-payload CD5- and/or CD8-targeting tLNPs to humanized NSG mice. After dosing, we observed robust gene editing consisting in target gene knockout in CD5+ and/or CD8+ T cells in vivo, dependent on targeting binder. Additionally, repeat dosing revealed enhanced editing efficacy in the targeted T cell population, highlighting the tolerability and redosability of the platform.

Conclusions: In this study, we demonstrate the applicability of our CellSeeker™ platform to genomic medicine. T cell targeted tLNPs could deliver gene editing payloads specifically and effectively to targeted cells, resulting in a robust genomic editing (target gene knock out) of T cells in vitro and in vivo providing a springboard for future development including gene knock in. These results demonstrate the flexibility of this platform, including customizability of multiple payload delivery, tunable payload ratio, adaptable dosing regimen, and potential for repeat dosing. These features position the platform as a possible solution for developing more accessible, effective and scalable in vivo cell and gene therapies.

671 Compositional Screening of Lipid Nanoparticles for Intracellular Delivery of Gene-Editing Proteins to T cells

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**Corresponding Author*

Statement of Purpose: Protein-based therapeutics targeting intracellular entities, such as the CRISPR-Cas9 system, have been employed in gene and immune therapies for diverse diseases. Despite considerable efforts, an efficient delivery system for the in vivo intracellular delivery of protein therapeutics remains elusive. Lipid nanoparticles have been engineered as carriers for mRNA-encoding antigens with validated biosafety profiles. However, their applications in protein delivery have been relatively umexplored. Previously, our lab has developed a multi-step screening method to optimize the type of helper lipid and component ratios in an LNP formulation for gene delivery. In this study, we aim to leverage this platform to optimize LNP formulations for the delivery of gene-editing proteins, including RNPs and Cre recombinase. Specifically, we will employ *in vitro* screening using the HEK293 Ai9 cell line and in vivo cluster-mode screening in Ai9 mice to identify formulations that effectively target the spleen for T-cell gene editing.

Methods: As shown in Figure 1a, we generated a 486-formulation LNP library with DLin-MC3-DMA as ionizable lipid, cholesterol, DMG-PEG2000, and one of six helper phospholipids that were previously used in FDA-approved or experimental LNP formulations. By varying the following parameters: (1) combined molar percentage of DLin-MC3-DMA and helper lipids; (2) weight ratio of cholesterol to DMG-PEG2000; (3) weight ratio of DLin-MC3-DMA to helper lipid; and (4) the weight ratio of total weights of lipid to protein, formulations were generated with RNP and Cre to screen in vitro on HEK293 Ai9 cell line. The top 50 formulations encapsulating RNP were then divided into 10 clusters and administered intravenously (*i.v.*) into Ai9 mice for in vivo cluster-mode screening. Analyzed by flow cytometry, the top three clusters with the highest gene editing efficiency in spleen immune cells were selected and tested individually on Ai9 mice. The most effective formulation F48 was then tested on C57BL/6 mice through i.v. injection to reduce CCR5 expression on T cells, and the results were validated through flow cytometry and western blot.

Results: To select LNP formulations that can deliver proteins with intracellular targets, we evaluated gene editing efficiency of the whole library treated with RNP with sgAi9 and measured the percentage of tdTom expression in the cells (Fig. 1b). The composition variation has generated different gene editing efficiencies under each helper lipid. Of 486 LNP formulations, the 50 top-performing LNPs are shown in Figure 1c. For in vivo systemic gene-editing evaluation, the most effective in vitro formulations were tested via i.v. injections in Ai9 mice with cluster-mode screening. In the following individual formulation assessment, the selected formulation F48 showed significant tdTom expression levels, specifically in T cells within spleens (Fig. 1d). We have also demonstrated the successful CCR5 reduction in T cells from blood 5- and 10-day post-injection, and from spleen 15 days post-injection shown in Figure 1e.

Conclusion: This LNP screening platform allows for the identification of the best-performing LNPs for intracellular delivery of protein for gene editing. In the study, we achieved promising gene editing in T cells in the spleen treated with the selected formulation. These findings highlight the potential of optimized LNP formulations for gene-editing proteins and their applications.

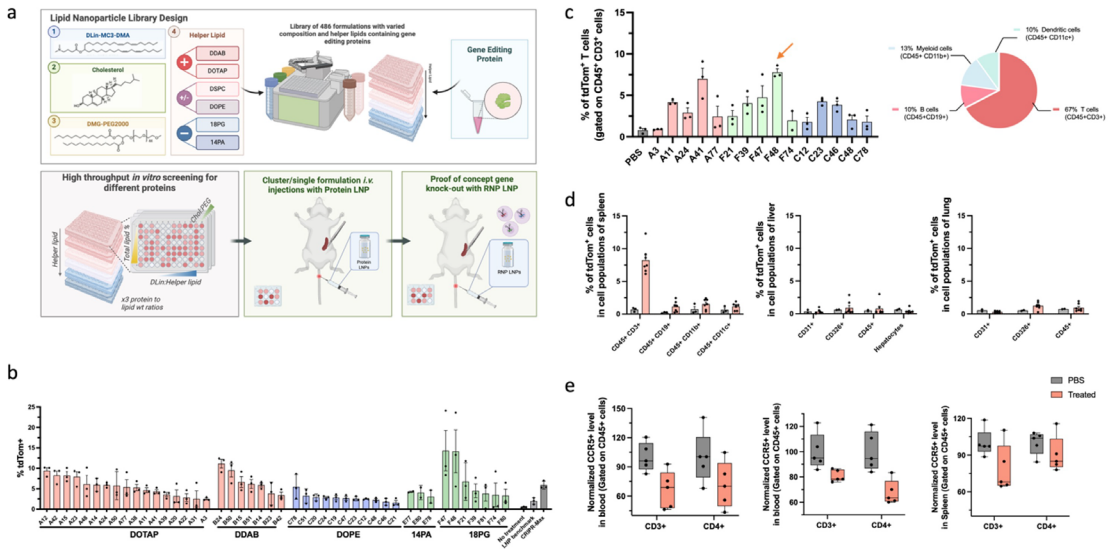

Figure 1. a) Schematic of multi-step screening for intracellular delivery of gene editing proteins **b)** Gene editing efficiency of RNP LNPs in HEK293 Ai9 cells via high-throughput screening platform ($n = 3$). Assessed with tdTom expression. **c)** The top 15 formulations were selected. **d)** FACS was used to quantify the percentage of tdTom$^+$ cells in spleen, liver and lung. **e)** CCR5 expression of CD3$^+$ and CD4$^+$ T cells were quantified 5-day and 10-day post-injection in blood, and 15-day post-injection in spleen.

672 Lipid nanoparticles- a game changer for primary B cell engineering mediated by mRNA-based Crispr/Cas9.

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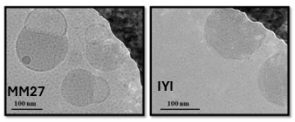
Delivery of mRNA to immune cells has emerged as a promising strategy for engineering and harnessing the therapeutical power of immune cells, especially T, natural killer and B cells. However, current methods, such as viral vectors or electroporation, have limitations in terms of safety, efficiency and scalability. Lipid-based nanoparticles evolved as an appealing alternative, offering a versatile platform for efficient and biocompatible mRNA delivery to these cells. Nevertheless, there is still a necessity for improving the performance and comprehending the underlying mechanisms of efficacy. Currently, there is a growing industrial and clinical interest in B cell engineering and subsequent adoptive B cell transfer, which already displayed good preclinical data. Efficacious, simple & scalable solutions to engineer B cells, particularly by utilization of CRISPR-Cas, remain to be uncovered and are a significant research priority. Thus, we aim to leverage the lipid nanoparticles (LNPs) for improved mRNA delivery to B lymphocytes and achieve therapeutic potency through CRISPR-Cas engineering. To achieve this, we assessed and further optimized our LNPs that we previously developed and described to have superior efficiency in transfecting NK cells. After validation that those LNPs achieved 100% GFP transfection efficiency without inducing toxicity in B cell lines, we exploited the Design Of Experiment approach by finely tuning production parameters to further improve the overall LNP potency. As a result, we obtained high and stable expression of GFP in the B cell lines. Then, we focused on the CRISPR-Cas system for B cell engineering with our model featuring GFP-expressing BL41 lymphoma cells. Cas9 mRNA and sgRNA targeted against the GFP gene were loaded into LNPs, resulting in more than 80% of GFP knock-out. Building on the promising results with cell lines, we next evaluated efficiency in primary B (pB) cells. Pre-activated cells treated with LNPs achieved over 90% transfection of pB cells that were positive for activation markers without notable mortality. This donor-independent efficiency was contrasted with the transfection failure of benchmark formulation, LNP SM102. Finally, as a proof-of-concept, we successfully delivered Cas9 mRNA and two sgRNAs encapsulated in LNPs to induce class switching recombination (CSR) in the antibody heavy chain locus of pB cells, showcasing the high potency of our approach.

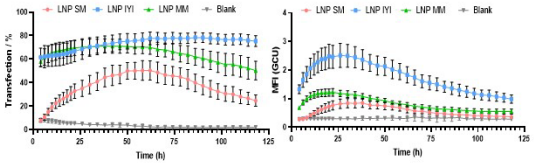
Overall, our LNPs provide a scalable platform for highly efficient transfection of B cells, enabling a versatile utilization of the CRISPR-Cas system - a key approach to fully harness the therapeutical potential of B cells.

Formulation & characterizations of LNPs



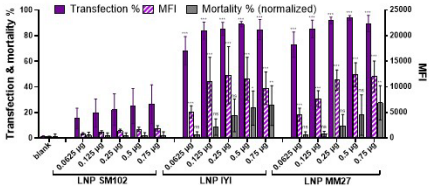
Formulation	Composition	N/P	RNAs	Size	PDI	EE%	RNA recovery / %	pKa
LNP SM	SM102-CHOL-DSPC-DMG PEG(2k)	6		83.2±8.6	0.061 ±0.049	95.6±2.4	74.9± 11.6	6.77
LNP IYI	IYI-Bsitosterol-DOPE-DMG PEG(2k)	10	eGFP, eGFP-Degron, Cas9+AAVS 1, Cas9-CSRs	153.5±23.9	0.053 ±0.029	92.5±4.0	73.8± 14.2	5.7
LNP MM	MM27-Bsitosterol-DOPE-DMG PEG(2k)	10		114.4±10.9	0.037 ±0.024	95.3±1.8	77.1± 10.9	6.4

Kinetic of GFP-Degron mRNA expression in primary B cells

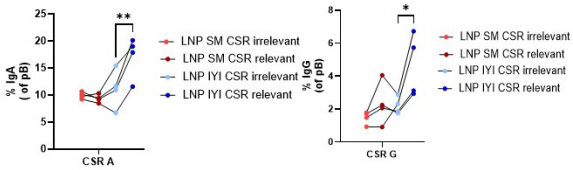


Class-switch recombination (CSR) by CRISPR-Cas9 system in human IgH locus of primary B cells after LNP delivery of Cas9 mRNA/sgRNAs

Potency of LNP formulations to transfect primary B cells



eGFP mRNA, Read-out: 24h



673 Invitrogen™ Vivofectamine™ Lipid Nanoparticles (lnps) for efficient nucleic acid delivery into immune cells ex vivo and in vivo

Yun Gong, Thermo Fisher Scientific, Waltham, MA

Neha N. Parayath, David Papp, Koshi Kunimoto, Vivek Chandra, Lindsay Bailey Steinitz, Maryam Mafreshi, Yun Gong, Jason Potter, Evgenia Verovskaya

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Lipid nanoparticles (LNPs) have proven themselves to be a potent delivery system for mRNA, demonstrating clinical efficacy and safety for several therapeutic applications, including vaccination for infectious diseases and genome editing for rare genetic disorders in the liver. Increasingly, ex vivo and in vivo delivery of nucleic acids to immune cells is highly attractive for the development of immunotherapies including CAR-T and novel cancer vaccines. Here, we have identified and characterized Invitrogen™ Vivofectamine™ LNPs suitable for high delivery efficiency to *ex vivo* human primary T-cells and NK cells as well as different immune cell populations *in vivo* without the need for active targeting.

Our leading LNP candidates were selected from **a library of over 6000 lipids that underwent iterative rounds of *in vitro* and *in vivo* screening and formulation optimization**. Different LNPs were evaluated for immune cell delivery using two desired use cases: 1) high levels of genome editing and protein expression in primary T cells and NK cells *ex vivo* 2) efficient delivery to immune cells after systemic administration *in vivo*.

To identify **LNPs for *ex vivo* mRNA delivery to T-cells**, efficiency of genome editing and protein expression was tested. We demonstrate >90% delivery of reporter eGFP mRNA at low doses of mRNA. Following a co-delivery of Cas9 mRNA and sgRNA to T cells, we observed >90% editing of the TRAC locus in primary human T cells, while maintaining higher cell viability compared to electroporation. Using CD19 CAR mRNA to generate CAR-T cells, we achieved >95% CAR+ expression and confirmed CAR-T functionality in cell killing assays. Additionally, **LNPs demonstrated ~60% transfection efficiency in primary NK cells**. To identify **LNPs for *ex vivo* DNA delivery to**

T-cells, we did multiple iterative screening to identify LNP formulation that can deliver DNA to human primary T-cells with ~60% transfection efficiency.

For identifying ***in vivo*** **immune cell targeting LNPs**, immune cell transfection efficiency was analyzed after systemic administration of selected LNPs to mice. Flow cytometry revealed that a single injection of 1 mg/kg dose of eGFP mRNA-LNPs can target up to 30% of CD11b+ myeloid cells and up to 28% CD11c+ MHCII+ dendritic cells in the spleen. Furthermore, when immune cell LNPs were administered to non-human primates (NHP), they showed efficient eGFP expression in CD11b+ myeloid cells (approx. 80%) and CD3+ T cells (approx. 40%) in peripheral blood. eGFP expression was durable being still detected in over 20% of myeloid and T cells after 2 weeks post-injection (collaborator generated data). Our data indicates that Vivofectamine™ LNPs are suitable for specific and efficient delivery of nucleic acids to a variety of immune cells in vivo and ex vivo for development of immunotherapeutic applications. **The immune cells specific delivery is driven by novel chemistry and LNP composition without utilizing active targeting** overcoming manufacturing and scale-up challenges for development of therapeutic applications.

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674 Rational Design and Preclinical Applications of Novel Unshielded Lipid Nanoparticles (uLNPs)

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Lipid nanoparticles (LNP) have revolutionized non-viral gene therapy and represent the most clinically advanced delivery modality. Onpattro, the first-ever approved RNAi therapeutic, is an siRNA-LNP against transthyretin and is indicated for the treatment of hereditary transthyretin amyloidosis. The LNP applied is a 4-component system composed of ionizable lipids, phosphatidylcholine, cholesterol and PEG-lipid (50/10/38.5/1.5 mol%, respectively). This composition is now the de facto gold-standard for intravenously administered nucleic acids for delivery to hepatocytes. The specificity arises from an endogenous targeting mechanism and lipid design that enables rapid unshielding of the LNP following i.v. administration. In particular, the PEG-lipid is designed in a manner so that it dissociates from the particle surface and allows for increased adsorption of Apolipoprotein E to the LNP surface thus promoting uptake into hepatocytes. During manufacturing and storage, PEG-lipid is required to promote LNP stability. Specifically, the amount of PEG-lipid within a formulation determines the particle size, and formulations lacking PEG-lipid rapidly flocculate post-production; the existing paradigm suggests that PEG-lipid or a relevant steric barrier is required to prevent particle aggregation. Comparatively, in vivo, this steric hinderance limits delivery. The hydrophilic coating of PEG-lipid has been reported to inhibit interactions with endosomal membrane resulting in prevention of endosomal escape and decreased formulation efficacy. It has been reported that PEGylated LNPs can induce hypersensitivity through complement activation and can cause undesirable immune responses in vivo against PEG. Additionally repeat dosing of PEGylated LNPs can results in anti-PEG antibodies that can clear subsequent PEG LNPs thereby reducing bioavailability. Therefore, there is a need for designing LNPs that have the desirable properties and stability for hepatic and extrahepatic delivery but do not suffer from the aforementioned issues associated with PEG-lipid.

He we report that we have rationally designed a novel set of LNP formulations termed unshielded LNPs (uLNPs) that comprise of 3 components namely ionizable lipids, phosphatidylcholine and cholesterol and eliminated the need for a fourth component. Our 3-component uLNPs lacking PEG-lipid in their compositions exhibit stable physicochemical properties and long term stability. We have demonstrated promising results with our uLNP technology for intramuscular vaccine applications, ex vivo immune cell applications and intravenous in vivo extrahepatic targeting. Firstly, several of our uLNP compositions resulted in similar or enhanced T and B cell responses against spike mRNA at 1 microgram dose in comparison to benchmark formulations. Secondly, we demonstrated enhanced mRNA delivery and efficient editing of ex vivo CD4+ and CD8+ cells and demonstrated 80% base editing of primary human hematopoietic stem cells (HSCs). Furthermore, we were also able to

demonstrate that uLNPs when administered intravenously, can deliver nucleic acid payloads to extra hepatic tissues of interest compared to LNPs with PEG. Our findings demonstrate that LNPs can be formulated without PEG that was previously thought to be essential LNP component. These novel 3-component uLNPs show excellent physicochemical properties and long-term stability making them an exciting platform to explore for range of other therapeutic applications.

675 *Mobilize*: A modular and versatile targeted LNP delivery platform for extra-hepatic RNA therapeutics

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Introduction: Lipid nanoparticles, or LNPs have demonstrated tremendous potential as a nonviral drug delivery system with successful clinical translation for both hepatic tissue and vaccine delivery. However, their utility for extrahepatic tissue delivery *in vivo* has been limited. Growing interest in *in vivo* chimeric antigen receptor (CAR) and genome editing technologies have led to a push for development of tissue-specific delivery methods with minimal off-target effects. Selective Organ Targeting (SORT) or CellSeeker™ technologies are prime examples that have demonstrated success in tissue-specific delivery *in vivo*. However, these approaches have specificity, scalability and translational challenges. We have developed *Mobilize* as a modular, versatile and scalable targeted LNP platform that solves the manufacturing challenges while retaining high efficacy.

Methods: *Mobilize* is a delivery system that enables the development of scalable, one-step assembly of a targeted LNP system. *Mobilize* produces high quality LNPs without typical limitations of downstream chemical functionalization, multistep production processes and variability in product quality. We have developed this platform with circular RNA (circRNA) encoding Green Fluorescent Protein (GFP) as payload, and three different targeting ligands for T cell specific delivery. The formulations are characterized by size, polydispersity index (PDI), encapsulation efficiency (EE) and stability using DLS and the Ribogreen assay. We further assess the impact of targeting ligand surface functionalization and fluorescence signal as a function of targeting ligand density *in vitro* in Jurkat cells, primary human T cells, and human PBMCs. *In vivo* efficacy of the *Mobilize* platform is confirmed in both a humanized mouse model and non-human primates (NHP), demonstrating the promise of the platform towards clinical translation.

Results: The *Mobilize* platform demonstrates physicochemical stability, dosing reproducibility and excellent efficacy in relevant *in vitro* and *in vivo* models. We confirm that our surface modification strategy provides the ability of LNPs to de-target from the passive LNP sinks in the liver and improve re-targeting to the cell of interest. As proof of concept, we validate the targeting ability of our platform using three independent ligands to target receptors on the surface of T cells with high efficiency. In humanized mouse models, we observe delivery to up to 55% of quiescent T cells at single and low doses. Finally, we validate our platform for successful delivery to quiescent NHP T cells with ~20% GFP positive population using a single dose of the *Mobilize* LNPs. Clinical observations, liver enzymes levels and extensive monitoring of cytokine/chemokine panels did not show any signs of overt toxicity in any of our mice or NHP study even at the highest tested dose.

Conclusions: Together, we demonstrate that the *Mobilize* LNP platform is versatile, modular and simple technology that is capable of efficient delivery of RNA payloads to extrahepatic tissues with high specificity. Our approach to developing this targeted delivery platform avoids several challenges and pitfalls faced by the industry to ensure product scalability, consistency, quality, efficacy and safety. Our data demonstrates that *Mobilize* provides a potential platform for *in vivo* delivery of RNA therapeutics to extrahepatic tissues.

676 Self-amplifying RNA-lipid nanoparticles for long-term protein expression in liver

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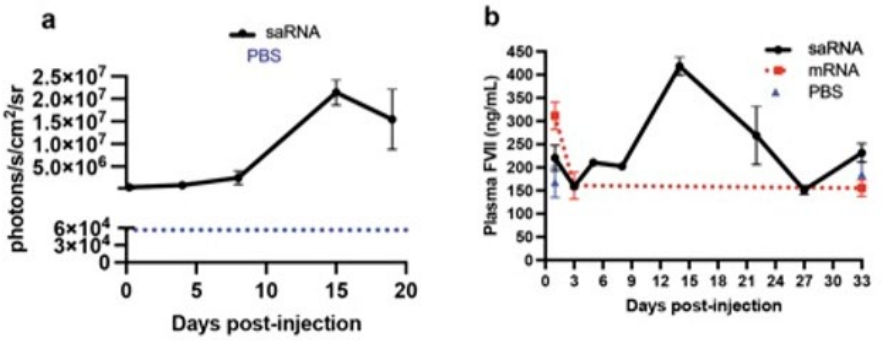
Background: Protein replacement therapies are used to treat over 50 disorders spanning a wide range of diseases including coagulation disorders, cancer, and rare diseases. Current protein replacement therapies have major limitations, including the need of continuous dosing due to the short half-life of proteins, and the risk of developing antibody inhibitors against the recombinant and natural protein. For example, in actively bleeding patients, including those with protein deficiencies, intravenous administration of recombinant activated coagulation factor VIIa (rFVIIa) decreases bleeding. rFVIIa has a short half-life of 2-3 hours and it increases risk of thrombosis due to the initial supra-physiological FVIIa concentration and because FVIIa is the enzymatically activate form of the zymogen protein FVII that normally circulates in blood. RNA therapies provide an opportunity to endogenously synthesize the missing protein from an exogenous RNA transcript, using the liver as a bioreactor following intravenous delivery of the RNA. mRNA therapies are an emerging technology that have potential to replace some protein replacement therapies, however, exogenous protein expression typically only lasts about one day and near-daily repeat injections is inconvenient and potentially dangerous. Self-amplifying RNA (saRNA) has the potential to produce exogenous proteins for up to a month, however its potential has been hindered by immunotoxicity concerns and lack of expression in the liver. saRNA uses a non-infectious, self-replicative viral replicase to enhance transcript lifespan of a therapeutic RNA.

Aims: Develop a protein replacement platform using saRNA encapsulated in lipid nanoparticles (LNP) for intravenous delivery and long-term protein expression in the liver.

Methods: We developed a novel immunosuppressant LNP formulation for the systemic delivery of saRNA and tested it by encapsulating an saRNA construct encoding for reporter protein, firefly luciferase (saFLuc). Protein expression was determined through in vivo imaging system (IVIS). Subsequently, we developed an mRNA (mFVII) and saRNA (saFVII) construct encoding for FVII and determined their therapeutic potential in vivo in mice. Plasma FVII levels were determined through an ELISA assay.

Results: saFLuc-LNP expressed exogenous protein in the liver for more than 20 days. mFVII increased circulating FVII by two-fold for up to 48 hours in mice and rats while saFVII-LNP increased circulating FVII for more than 32 days in mice. mFVII decreased blood loss in a bleeding injury model in both mice and rats. To determine therapeutic feasibly, swine were injected intravenously with mFVII encoding human FVII.

Conclusion: The novel saRNA-LNP platform may offer a more effective, longer-acting and safer treatment option for protein replacement therapies than standard mRNA. saFVII-LNP provides a more consistent replacement of FVII and an alternate treatment option for patients with rare bleeding disorders.



Hepatic delivery of saRNA using LNPs. A) Mice were injected intravenously with LNP containing saRNA expressing firefly luciferase, or PBS vehicle. Luminescence was measured by IVIS up to 20 days. **B)** C57BL/6J mice were treated with LNP containing saRNA encoding mouse FVII and euthanized 1, 3, 5, 8, 14, 22, 27, and 33-days post-injection. The plasma concentration of FVII was measured using ELISA.

677 Lipid Nanoparticle-Based Non-Viral Delivery of FVIII for Hemophilia A Gene Therapy

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Viral vector-based gene therapy has shown promise in treating hemophilia A (HA), but its application is limited by pre-existing neutralizing antibodies and the challenge of vector re-administration for maintaining therapeutic coagulation factor levels. Lipid nanoparticles (LNPs) represent a promising non-viral alternative for hemophilia gene therapy that can potentially overcome these limitations.

In this study, we evaluated various LNP formulations using either a biodegradable cationic lipid (LipidA) or FDA-approved MC3 lipid at different doses. Initial screening utilized a plasmid encoding secreted luciferase (pCAG-scLuc). For in vivo assessment of HA gene therapy efficacy, we treated both immunocompetent HA mice (mixed C57BL/6 and SV129 background) and immunodeficient HA mice (HA-NSG) with LNPs encapsulating FVIII-X10 plasmid (pCAG-FVIIIX10) at doses of 0.5 or 1 mg/kg. FVIII activity was measured using a one-stage clotting assay, while hepatotoxicity was evaluated through plasma alanine transaminase (ALT) levels and liver histology.
Mice receiving LipidA-LNPs encapsulating pCAG-scLuc demonstrated a 10-fold higher luciferase expression compared to MC3-LNP treated groups at both 0.25 and 0.5 mg/kg doses, with expression levels correlating with dosage. Luciferase activity persisted for approximately three months. Although LNP administration caused a transient elevation in ALT levels on day one, levels returned to baseline within one week, and liver histology at one-month post-treatment showed no evidence of sustained damage. Based on this superior transgene delivery efficiency, LipidA-LNPs were selected for subsequent HA gene therapy studies.

HA mice treated with LipidA-LNPs encapsulating FVIII-X10 variant at 0.5 mg/kg had therapeutic FVIII levels for approximately one month before declining due to inhibitor development. Given that inhibitor formation frequently occurs in HA mouse models but affects only about 30% of HA patients, we utilized immunodeficient HA-NSG mice to evaluate efficacy without inhibitor interference. HA mice produced FVIII activity above 10% for 6- and 12-weeks following administration at dosage of 0.5 and 1.0 mg/kg, respectively. Notably, subsequent re-administration achieved FVIII activity levels and duration comparable to the initial treatment. To extend the expression duration, we optimized the formulation by adjusting the molar ratio of lipid composition. Mice treated with optimized formulation had 60% FVIII activity in average for about a month. The expression level maintained above 20% for more than 8 months. Transiently elevated ALT level was observed one day post treatment but returned to baseline

level within one week. These findings demonstrate the potential of LNPs as a re-administrable, non-viral platform for HA gene therapy with minimal hepatotoxicity, offering a promising alternative HA gene therapy.

678 P-FVIII-101 is a Non-viral Genetic Medicine with the Potential for Controlled Modulation of FVIII Transgene Expression to Match Patient Needs

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The standard of care for patients with severe hemophilia A (HemA) has dramatically improved in recent decades with the advent of prophylactic bispecific antibodies such as emicizumab. However, patients still face the risk of breakthrough bleeds, chronic joint damage, and pain. Thus, a need exists for transformative therapies, such as genetic medicines, that may offer functional cures. Current AAV-based gene therapies for HemA are yet to consistently achieve such outcomes, with FVIII activity often failing to reach curative levels and/or declining over time. Moreover, pediatric patients and those with pre-existing AAV immunity are likely to be excluded from treatment.

To overcome these challenges, we deployed the Super piggyBac (SPB) transposon system for DNA insertion delivered via non-viral proprietary lipid nanoparticles (LNPs) to enable genomic integration of a human FVIII (hFVIII) transgene. This results in durable hFVIII expression in neonates, juveniles, and adults in which hFVIII expression is maintained for at least 13 months with full restoration of hemostasis in mouse models of HemA. In light of these promising data, we sought to further understand hepatocellular tolerability and explore potential titratability that may better tailor therapies to differing patient needs.

One benefit of transgene integration with SPB is the high level of hFVIII expression obtained from a relatively small number of modified cells. To understand the impact robust hFVIII expression and secretion may have on the health of targeted liver cells, we conducted experiments to assess ER stress in hepatocytes. Transcriptional responses in primary human hepatocytes and HepG2 cells showed no significant induction of the ER-stress pathway by hFVIII-X5, suggesting there is minimal hepatocellular burden to overexpressing and secreting hFVIII, confirming the feasibility of our low-VCN integration strategy.

To provide a more customizable approach to varying patient needs, we are pursuing technological innovations that enable either repeat dosing to titrate up to desired hFVIII levels, or the ability to step down expression through an inducible modulator switch.We previously demonstrated the feasibility and success of a hFVIII-LNP-SPB repeat dose approach in mice. Further exploration of translatability using rats, a larger representative species, revealed favorable tolerability and ~50% FVIII expression at low dose (0.5 mpk) and approaching 100% at 0.75 mpk after a single dose.Importantly, repeat administration 14 days after initial dose achieved over 100% hFVIII expression at 0.5 mpk and ~500% at 0.75 mpk with no added toxicity or tolerability concerns following the second administration.

To explore optionality to dial down FVIII in the event of supraphysiological levels, we incorporated an inducible modulator switch into the transposon. Delivery of small molecule rimiducid induces activation of iCasp9 in transduced cells triggering their death. In adult mice we show that circulating levels of hFVIII protein can be dose-responsively reduced or titrated down with no apparent impact to liver health. This innovation allows precise control over desired FVIII levels or, alternatively, the elimination of all transposed cells should an unwanted transformation event ever occur. Future preclinical studies will further explore the tunability of these systems to deliver tailored therapeutic levels that provide a durable solution for FVIII restoration in HemA patients.

679 Lipid Nanoparticle Delivery of Minicircle DNA for *LAMA2* Gene Replacement in Muscle

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Laminin-α2-related congenital muscular dystrophy (LAMA2-CMD) is a severe neuromuscular disorder caused by mutations in the *LAMA2* gene, leading to progressive muscle degeneration. Gene replacement therapy offers a promising strategy for restoring laminin-α2 expression; however, the large coding sequence (CDS) of *LAMA2* (~9 kb) presents a major delivery challenge. Here, we harness minicircle DNA (MC-DNA) technology in combination with lipid nanoparticles (LNPs) to enable efficient and sustained transgene expression in muscle tissues.

We engineered a human *LAMA2* minicircle construct designed for high expression and long-term stability. Optimized LNP formulations were developed to enhance transgene uptake and endosomal escape in muscle cells, ensuring robust delivery. We evaluated *hLAMA2* expression following intramuscular (IM) and hydrodynamic limb vein injection (HLVI) in murine models, comparing conditions with and without the inclusion of random insertion machinery. Insertion machinery components were delivered as RNA encapsulated in independent LNPs to facilitate genomic integration while potentially reducing genomic vandalism. Preliminary analyses suggest that this approach enables sustained transgene expression, with *LAMA2* detected for up to four weeks post-treatment in wild-type mice. Ongoing experiments aim to quantify and compare endogenous and transgenic *LAMA2* expression levels, as well as evaluate the therapeutic potential in an *LAMA2*-deficient mouse model.

Overall, these findings highlight the potential of MC-DNA with LNP-based delivery as a viable, non-viral alternative for *LAMA2* gene replacement therapy. Future efforts will focus on optimizing LNP formulations, refining dosing regimens, and assessing long-term efficacy and safety in preclinical models.

680 Protease enhanced gene therapy of squamous epithelial stem cells in the oral cavity and skin

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Squamous epithelium is an important tissue that covers the inner and outer surfaces of the body. Squamous cell epithelium is a stem cell driven tissue, characterized by rapid turnover. The stem cells express cytokeratin 14 (K14) and reside on a basement membrane. The progeny of the stem cells move upward from the basement membrane and become progressively differentiated and amitotic. The top layer cells are heavily keratinized and are continuously shed. Because of the rapid turnover, any correction of genetic diseases of the squamous cell epithelium needs to target the basement membrane resident K14+ stem cells. Unfortunately, current vectors and technologies have been unable to efficiently perform durable gene therapy in squamous epithelial stem cells (SESC) *in vivo*. To overcome this limitation, *ex vivo* gene therapy, followed by grafting of gene corrected skin is the current approach. Efficient delivery of genetic payloads to the epidermal stem cells *in vivo* would be much preferable. The situation is even more difficult for mucous membrane stem cells. There currently are no reports at all of gene therapy in that tissue, even in animal models, let alone humans.

Our laboratory has been exploring oral cavity gene therapy in the DNA repair disease Fanconi's anemia in an effort to prevent or reduce the incidence of head & neck malignancies in this disease. Initially, we were completely unsuccessful in targeting the SESC with any of the gene therapy vectors tried, including LNPs, lentivirus, AAV and DNA transposons. Vectors injected into the tongue targeted the musculature and cells below the basement membrane, but not the epithelium.

Reasoning that the basement membrane was the principal obstacle to targeting the stem cell compartment, we combined gene therapy with *in vivo* collagenase treatment. Importantly, collagenase injections are widely used in clinical medicine and considered safe. Using this approach, we achieved highly efficient (90%) stem cell targeting with lipid nanoparticle (LNP) mediated mRNA delivery and AAV vectors in the tongue after a single injection. Similarly, collagenase enabled highly efficient *in vivo* targeting of epidermal stem cells in the skin with LNPs.

We conclude that gentle proteolytic disruption of the basement membrane dramatically enhances the efficiency of gene transfer into squamous epithelial stem cells in vivo. This approach has clear clinical potential.

681 Peptide-Assisted Lipid Nanoparticles for DNA Delivery in Pulmonary Gene Therapy

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Purpose: Alveolar type 2 cells (AT2) in the lung epithelium play a crucial role in maintaining lung function, and their dysfunction contributes to diseases across all age groups. Lipid nanoparticles (LNPs) are the gold standard for non-viral nucleotide delivery. While DNA offers superior stability and sustained transgene expression, its therapeutic application is constrained by lower delivery efficiency. Additionally, conventional systemic injection presents challenges for targeting lung-specific gene therapy. This study aims to develop and optimize LNP-based delivery systems tailored for local lung delivery and enhanced DNA therapy efficiency. In collaboration with clinicians focusing on improving nanoparticle distribution using perfluorocarbon (PFC) liquid, we are optimizing DNA delivery to AT2 cells and expanding the application of DNA-LNP for lung-associated diseases.

Methods: A layer-by-layer design was employed to formulate LNPs consisting of a peptide-DNA complex core, lipid shell, and serum coating (Fig. 1A). A histone peptide sequence, inspired by histone tail signaling, was incorporated to facilitate DNA unpacking and nuclear uptake. LNPs were synthesized via microfluidic mixing, and various peptide-to-DNA charge ratios (NP) were tested to optimize encapsulation efficiency. LNP size, encapsulation efficiency, and stability were assessed using dynamic light scattering (DLS), gel electrophoresis, and a PicoGreen assay. Transfection efficiency and gene expression levels were analyzed in AT2 cell lines by delivering GFP pDNA. To enable local lung delivery in the absence of blood serum, serum pre-coating conditions were examined through size measurement and transfection efficiency. Off-target uptake and expression in a lung macrophage cell line were investigated by delivering Cy5-tagged DNA LNPs and measuring fluorescent intensity through flow cytometry. Lastly, the role of serum exosomes in facilitating peptide-enhanced LNP transfection was examined.

Results: Optimization of NP ratios revealed that an NP 0.5 formulation maintained DNA encapsulation with minimal size increase (Fig. 1B,C). Serum pre-coating remained stable after 16 hours and produced maximum reporter gene expression at 6% serum concentration (Fig. 2C). Transfection studies demonstrated that, despite no direct sequence-specific effects, the inclusion of the peptide in LNPs significantly enhanced overall gene expression. Peptide-modified LNPs achieved nearly 100% cell transfection efficiency, with a 17-fold increase in GFP expression compared to standard LNPs, while maintaining low cytotoxicity at 6% serum concentration (Fig. 2A,B). This improvement suggests a synergistic effect arising from serum pre-coating and peptide inclusion for lung cell delivery. Mechanistic investigations indicated that exosome interactions played a crucial role in enhancing gene expression (Fig. 2D). Macrophage studies confirmed minimal off-target expression.

Conclusion: The peptide-enhanced LNP system demonstrated significant improvements in DNA delivery efficiency to AT2 cells. Optimization of NP ratios and serum pre-coating conditions led to high transfection rates with minimal off-target gene expression. Exosome interactions were identified as a key factor in boosting gene expression. These findings provide a strong foundation for future in vivo testing and the development of targeted gene therapies for lung diseases.

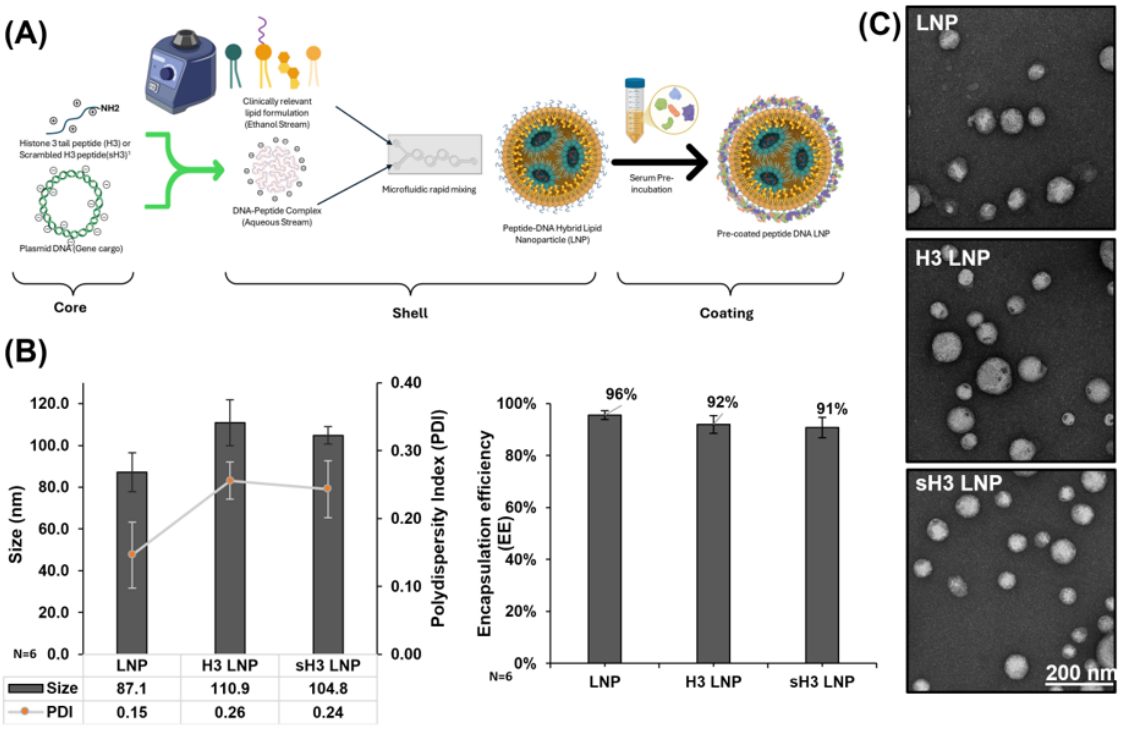

Figure 1: Layer by layer formulation of peptide inclusion LNP schematic and characterization. **A)** LNP is constructed by combining DNA and peptide through electrostatics interaction, then microfluidic mixed the DNA peptide complex with lipid mixture, and precoated with optimized serum protein for lung local delivery. **B)** LNP size and encapsulation measurement through DLS and picogreen assay. **C)** Representative TEM image of the LNPs.

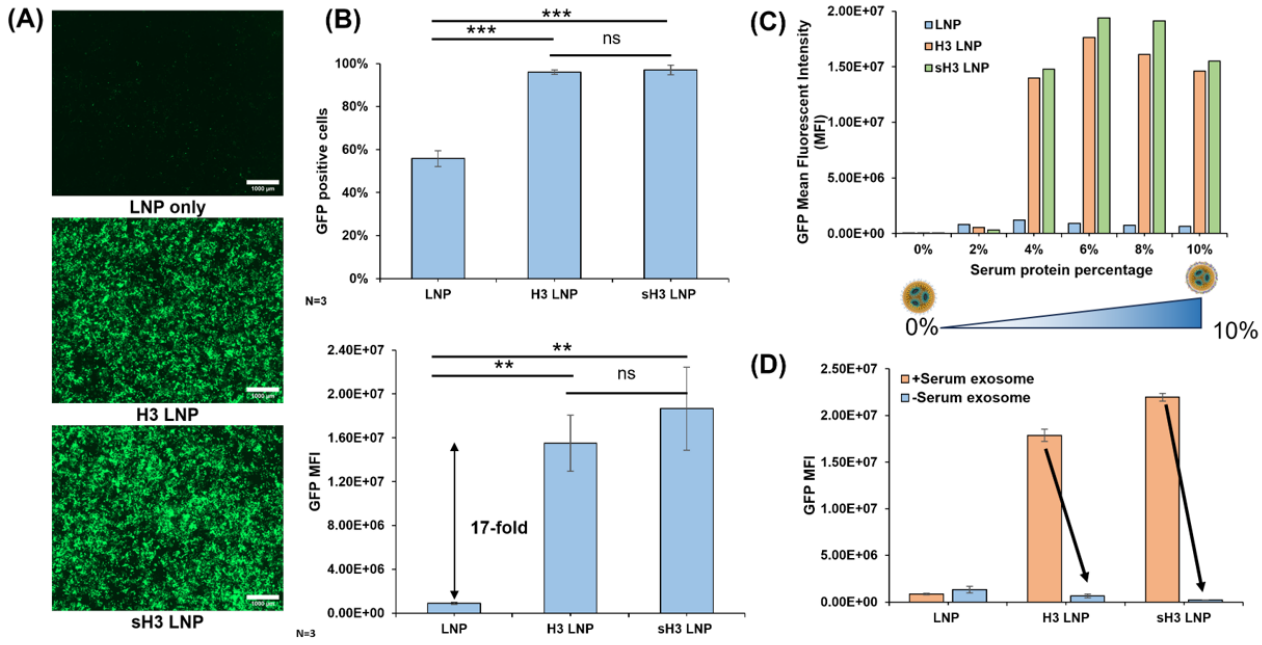

Figure 2: In vitro transfection investigation conducted using MLE-12 lung AT2 cells line. **A)** Representative fluorescent image of transfection study using the three DNA LNPs with GFP as reporter. **B)** Corresponding flow cytometry measurement on MLE-12 transfection efficiency at 6% and gene expression (N=3 for technical replication). **C)** Pre-coating condition testing varying pre-coating serum amount ranging from 0-10% FBS in culture media. **D)** Peptide LNP exosome interaction study using exosome depleted serum for pre-coating.

682 A Novel Polyvalent Ionizable Lipid Library for Nebulized Delivery of mRNA to Lung Cells

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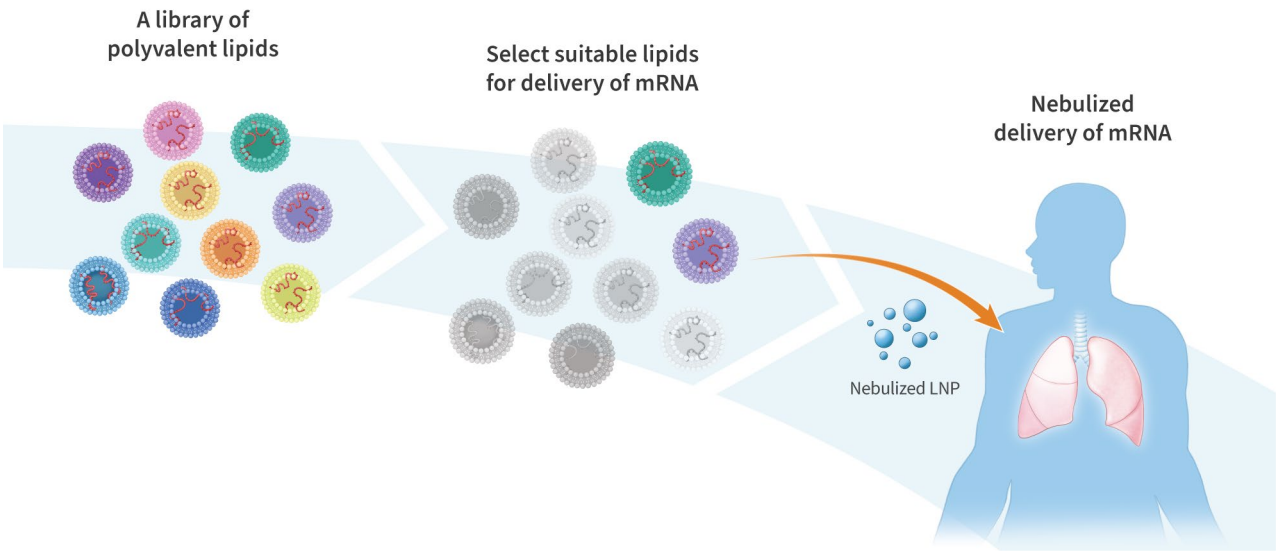
Nucleic acid delivery systems containing ionizable lipids enable non-viral gene expression and are being explored in numerous gene and cell therapy applications. These systems are typically administered intramuscularly (IM) or intravenously (IV), however these routes are not suitable to address diseases of the lung, such as cystic fibrosis (CF), idiopathic pulmonary fibrosis (IPF), or chronic obstructive pulmonary disease (COPD). Inhaled delivery of nebulized mRNA lipid nanoparticle (LNP) formulations is one of the most promising methods for lung cell targeting, however many nebulized mRNA LNP formulations are limited by nanoparticle disruption and aggregation induced by the nebulization process. Here we demonstrate novel ionizable lipids that enable efficient delivery of nebulized mRNA to lung cells.

From a previously synthesized library, we selected 10 lipids that exhibited high delivery efficiency and cell viability in vitro. LNPs were formulated by mixing 0.1 µg mRNA encoding green fluorescent protein (GFP) with 0.25 µg ionizable lipid, nebulized using a vibrating mesh nebulizer, and administered to 40,000 A549 lung epithelial cells. GFP expression and cell viability was assessed 24h after delivery by flow cytometry. 9 out of 10 lipid formulations outperformed a commercially-available lipid in both nebulized and non-nebulized experiments while maintaining cell viability above 85% in all cases. The top-performing lipids produced 93% GFP-positive cells without nebulization and 63% GFP-positive cells following nebulization, outperforming the commercial lipid, which produced 37% and 8% GFP-positive cells, respectively. Median GFP fluorescence intensity of GFP-positive cells in top-performing lipids was also up to 7 times higher than the commercial lipid without nebulization and up to 4 times higher following nebulization. Formulations maintained high delivery efficiency after being frozen at -80°C for 4 weeks. Lipids containing the dihydroxyspermine headgroup exhibited the strongest performance in nebulization experiments, possibly due to the enhanced capacity for intermolecular complexation with mRNA, increasing the resistance to nebulization induced disruptions.



683 Novel Physical Gene Delivery Method Enhances Expression in Skeletal Muscle

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Gene electrotransfer (GET) is an established physical method for gene delivery to various tissues and organs *in vivo*, including melanoma tumors, skin, cardiac muscle, and skeletal muscle. GET offers advantages such as nonimmunogenic and nonintegrative gene delivery to targeted tissues. However, limitations include low expression levels, short-lived/transient expression, and muscle contractions.

We investigated a novel physical method for gene delivery based on electrotransfer without applying external current to induce cell membrane permeabilization. We hypothesized that exposing tissues to a strong electric field—without external current—could enhance gene delivery while avoiding muscle stimulation.

Preliminary *in vitro* experiments involved exposing cells to an electric field without current in the presence of propidium iodide or Sytox Green dyes to assess whether electric fields alone could permeabilize mammalian cell membranes. Cells were then fluorescently imaged to determine permeabilization efficiency.

For *in vivo* studies, we developed a custom insulated surface electrode for monopolar gene transfer. In Sprague Dawley rats, skeletal muscle was exposed, and plasmid DNA encoding Firefly luciferase was injected, followed by pulsed electric fields. Muscle contractions were measured using an accelerometer, and pulse delivery was monitored with an oscilloscope. Negative control groups received only plasmid DNA injections, while traditional monopolar, monophasic pulses were used as positive controls for gene expression and muscle twitching. Bioluminescence imaging was performed periodically for over three months to quantify gene expression.

Our *in vitro* experiments demonstrated that cell permeabilization was possible without external current. *In vivo*, contactless electrotransfer significantly enhanced gene expression over injection alone—by more than 100-fold

($p<0.001$)—while completely eliminating muscle stimulation. The elimination of muscle stimulation could reduce pain responses and mitigate the risk of ventricular fibrillation.

This proof-of-concept study did not yet optimize pulsing parameters; therefore, future studies optimizing applied voltage, electrode design, and pulse number may further enhance gene delivery efficiency. Overall, our preliminary work introduces a novel physical method for *in vivo* gene delivery, opening the door to a new platform technology with potential applications in protein replacement therapies. While still in its early stages, this platform could also have broader applications in tissue ablation, as well as gene and drug delivery.

684 Continuous-flow electroporation platform for rapid optimization and seamless scalability for cellular therapy manufacturing

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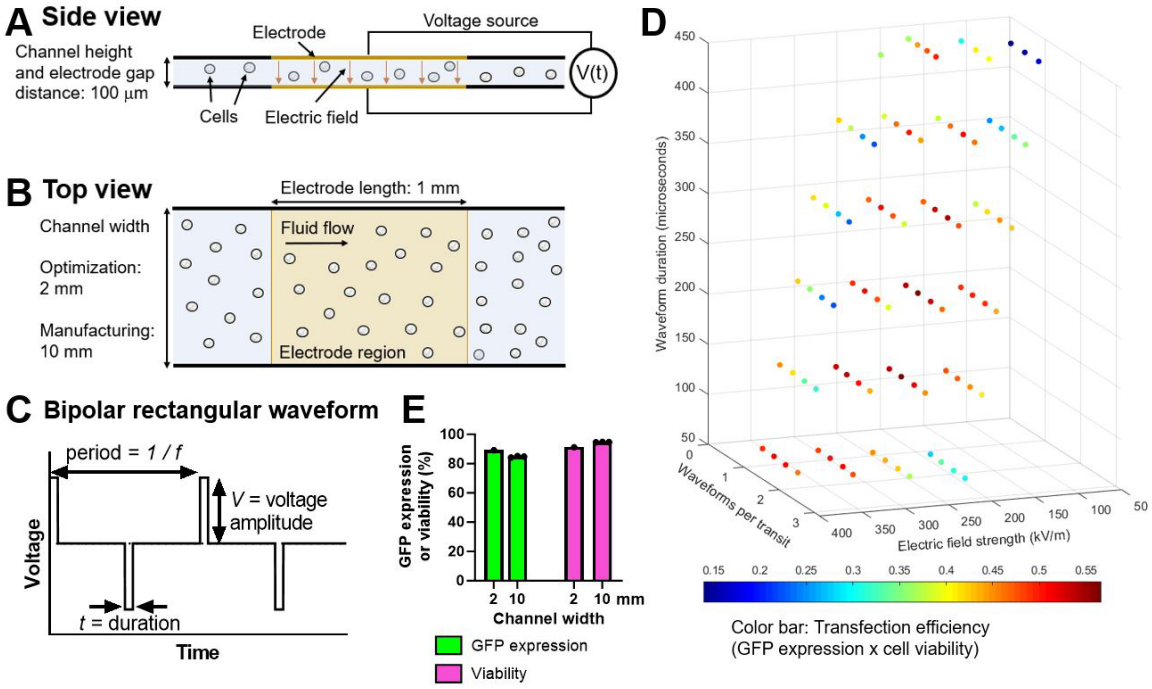
Electroporation has become an important tool to introduce genetic edits for cell therapies. However, current instrumentation is unable to address difficulties associated with electroporation such as limited throughput and arduous empirical process optimization. Here, we address these issues with our continuous-flow, microfluidic electroporation platform designed for rapid (3 seconds/well) process optimization in 96-well plates with low volumes (20 µL / well) of cell solution. Our platform combines automated liquid handling with computer-controlled electronics to enable rapid convergence on optimal parameters for electroporation such as cargo/cell concentration or voltage waveform. Once optimized, we can scale our volumetric throughput by swapping to a channel with a larger channel width without re-optimization because of the thin slab geometry of our microfluidic flow chip (Fig. 1A-B). Specifically, the thin flow channel height ensures each cell is subject to the same electric field for reproducibility whereas the width of the channel can be manufactured to match the desired volumetric throughput without changing the electric field strength experienced by the cells.

To demonstrate our optimization capabilities, we transfected Jurkat cells with plasmid DNA-GFP. We screened 93 different bipolar, rectangular waveforms with varying amplitude (V), duration (t), and frequency (f) over a single 7-minute experiment (Fig. 1C). The transfected cells were assigned a unifying efficiency value - calculated as the product of GFP expression and viability (GFP * Viability) - by measuring the GFP expression and viability 24-h post-transfection using flow cytometry. The optimized values were determined by plotting the electric field strength, the number of waveforms cells experience as they transit through the electrodes, waveform duration, and efficiency (Fig. 1D). Overall, we rapidly converged on the optimal waveform ($t = 225$ µs, $V = 18$V, $f = 63$ Hz) using small volumes of cells.

Scaling capabilities were demonstrated by transfecting Jurkat cells with nanoplasmid DNA-GFP using identical parameters in a flow chip with either 2- or 10-mm channel width. To keep the cells' linear velocity constant, we proportionally increased the volumetric flow rate with channel width. Fixing the cells' linear velocity and channel height creates identical electrical environments, providing identical transfection performance. Notably, we found that GFP expression and viability were less than 5% different between cells transfected in the 2 mm and 10 mm channel widths, showing our ability to scale without re-optimization (Fig. 1E).

These results demonstrate the capabilities of our electroporation platform for rapid optimization, which can be easily scaled to high-volume cell manufacturing without re-optimization. These capabilities bypass limitations of current instrumentation, making it a promising platform for non-viral cellular reprogramming for cell therapies.



685 Non-Viral Delivery of DNA Template and Gene Editing Components for Targeted Gene Insertion in Human Primary T-Cells: Electroporation vs. Lipid Nanoparticles

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Chimeric Antigen Receptor (CAR)-T cell therapy has revolutionized cancer and autoimmune disease treatment. Emerging non-viral delivery methods, such as electroporation (EP) and lipid nanoparticles (LNPs), enable precise nucleic acid delivery for targeted gene insertion. This study investigates the use of RNA-guided nuclease (RGN) mRNA, TRAC sgRNA, and synthetic DNA HDR templates (GFP and CD19-CAR) to achieve precise gene integration in human primary T-cells, comparing the efficiency of EP and LNPs for delivering these components.

Cryopreserved T-cells from healthy donors were activated and split into two transfection arms: EP and LNP delivery. Following transfection, cells were assessed for knockout and knock-in efficiency, viability, and phenotype. Electroporation achieved TRAC knockout efficiencies of 81–99% and HDR knock-in efficiencies of 19-39% for GFP and 10-31% for CD19-CAR. LNP-treated cells achieved TRAC knockout efficiencies of 48–87% and HDR knock-in efficiencies of 12–22% for GFP and 6–21% for CD19-CAR. HDR enhancers improved knock-in efficiencies in electroporated cells but had no effect on LNP-treated cells. Electroporated cells exhibited reduced viability (~60%) and slower growth (PDL = 3), while LNP-treated cells maintained higher viability (~89%) and faster growth (PDL = 5). CD4/CD8 ratios and memory T-cell phenotypes were consistent across EP and LNP conditions.

Both non-viral delivery methods demonstrated the feasibility of RGN-mediated gene editing in primary T-cells, with trade-offs in editing efficiency and viability. Electroporation achieved higher editing efficiencies under the conditions tested, while LNP delivery supported superior viability and expansion potential. These findings underscore the potential of novel non-viral approaches for CAR-T cell manufacturing and the need for further optimization to enhance efficiency and scalability.

686 Optimizing transposon-based gene delivery for cell therapy applications with the MaxCyte® ExPERT™ scalable electroporation platform

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Transposons are an efficient and proven method of gene transfer for cell engineering and have a wide range of applications from cell line engineering to generation of autologous cell therapies. Transposon systems, like piggyBac™, can support insertion of large DNA templates and achieve seamless editing with minimal disruptions to genomic integrity. Compared to gene delivery using viral vectors, transposons are more cost-effective and present fewer manufacturing complexities. Transposons are transfected into cells in the form of plasmids or linear dsDNA and the corresponding transposase enzyme is co-transfected as plasmid, mRNA or protein. Electroporation is a primary *ex vivo* method for delivering transposons due to its ability to load large cargoes with minimal impact on cell health and functionality. In this study we sought to optimize the delivery of the piggyBac™ (PB) transposon system for engineering of cell lines and primary T-cells using MaxCyte's® GMP-compliant ExPERT GTx™ platform with a specific focus on maximizing transposition efficiency, cell expansion, recovery and functionality while limiting vector copy number.

We first generated a PB transposon plasmid encoding a reporter gene (GFP) to study the relative effects of loading agent concentrations, electroporation parameters and other variables on transgene expression and integration efficiency. We initially electroporated K562 cells with a range of concentrations of PB GFP transposon plasmid and PB transposase mRNA to test different ratios and total quantities of transposon and transposase. Following expansion, a GFP expression of over 97% by flow cytometry was achieved at day 17 post-electroporation. Vector copy number (VCN), normalized to human albumin copies when analyzed by QIAcuity™ dPCR, varied in accordance with the relative amounts of transposon plasmid and transposase mRNA that were transfected. We also generated a PB Nanoplasmid™ transposon encoding a second generation anti-CD19 BB-z CAR and co-electroporated it with PB transposase mRNA into primary activated T-cells and we achieved over 50% CAR transposition efficiency by Day 5 post-EP. As with GFP, CAR expression and VCN correlated with the relative transposon and transposase concentrations in the electroporation reaction. These results support previously published data showing how MaxCyte® can enable efficient delivery of transposons with minimal impact on cell health for CAR-T cell manufacturing workflows.

687 Cell Engineering Solutions for T Cell-Based Immunotherapy Manufacturing: From Resting to Activated T Cell Editing

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Recent advancements in chimeric antigen receptor (CAR) T-based immunotherapies and the push for efficient, cost-effective cell therapies have increased the need for improved manufacturing workflows to enhance patient access while ensuring quality and performance. Traditional CAR T cell manufacturing involves T cell activation, payload delivery, and ex vivo expansion, which often leads to T cell differentiation, exhaustion, and resistance to therapy. Evidence from both murine and human studies indicates that T cell differentiation inversely impacts long-term anti-tumor efficacy, with early memory T cells providing sustained anti-tumor responses. A novel manufacturing approach that activates T cells post-Cas9 delivery has been shown to reduce chromosome loss in the final product. Using non-activated cells as the starting material also offers the benefits of shorter workflow times, crucial for point-of-care and decentralized therapy production, and reduced costs. We present a closed and modular system for producing CAR T cell products from both non-activated/resting and activated T cell populations.

We use an automated counterflow centrifugation method where PBMCs are isolated from fresh leukopaks using the CTS Rotea™ Counterflow Centrifugation system and then downstream T cell enrichment is performed. Electroporation (EP) reactions for payload delivery are set up and optimized at small scale using the Neon™ NxT™

electroporation platform and then scaled up using the CTS™ Xenon™ Electroporation System. Transfection efficiency and cell viability are measured by flow cytometry and resting T cell phenotype is monitored pre- and post-EP to monitor maintenance of stemness in comparison to the starting population. Electroporations are performed using the CTS™ Xenon™ Lower Conductivity Buffer that enables higher energy setting on the Xenon electroporation system for hard-to-transfect cell types thereby providing a wider design space for process development and commercial manufacturing. Cells are activated post-electroporation using CD3/CD28 CTS™ Dynabeads™ and expanded for downstream functional assays. For the activated T cell protocol, PBMCs are isolated from frozen leukopaks and activated prior to cell editing in the CTS™ Xenon™ Genome Editing Buffer. Post-expansion, electroporated cells are washed and concentrated using the CTS Rotea™ Counterflow Centrifugation system and analyzed for transgene expression and cryopreserved for downstream functional studies. Targeted Amplicon-seq validation assay (TAV-seq) confirmed no detectable off-targets in CAR T cells and functional cancer cell cytotoxic assays against CD19 presenting-Nalm-6 cells showed effective killing by anti CD19 CAR-T cells.

In summary, we established a closed modular workflow for CAR T cell generation using automated instrumentation for cell processing and gene delivery thereby minimizing human intervention in the cell therapy manufacturing process. The transfection reagents and gene editing payloads in combination with the closed automated workflows described here will help reduce labor-intensive open system hurdles and improve the engineering of hard-to-transfect primary cells used for the development of efficient adoptive immunotherapies.

688 Transfecting iPSCs Robustly and Safely Using Photothermal Electrospun Nanofibers

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Introduction: Photoporation is an emerging gentle transfection technology that combines laser stimulation with photothermal nanoparticles. It has been successfully used to deliver a wide range of payloads, including siRNA, mRNA, and proteins, into cells. To address concerns about potential toxic effects of nanoparticles on cells, we recently developed an alternative system in which the photothermal nanoparticles are embedded in biocompatible electrospun nanofibers (PEN). Cells can be cultured on the nanofiber substrate without direct contact with the embedded nanoparticles. At the same time, the nanoparticles are sufficiently close to the cell membrane to become permeabilized upon laser irradiation, allowing external payload molecules to enter and transfect the cells.

Having previously demonstrated that PEN photoporation can transfect cells with siRNA, we aimed to explore the possibility of delivering larger compounds into cells, including in iPSCs. First, we investigated how the physicochemical properties of the PEN substrate and laser fluence affect the delivery yield of FITC-dextran molecules of 10 kDa (FD10) and 500 kDa (FD500) in a model HeLa cell system. We then examined if repetitive laser activation of the PEN substrate increases the intracellular delivery efficiency. After that, we cultured induced pluripotent stem cells (iPSCs) on PEN nanofibers while maintaining high viability and the expression of pluripotency surface markers. Finally, we investigated the intracellular delivery of FITC-dextran molecules of 150 kDa and mRNA into iPSCs.

Methods: PEN nanofibers, composed of PCL and photothermal iron oxide nanoparticles (IONPs), were synthesized by electrospinning. A surface response methodology was used to simultaneously optimize the nanofiber diameter, IONP concentration, and fluence for optimal delivery of FD10 and FD500 into HeLa cells. The viability of iPSCs cultured on PEN substrates was assessed using a metabolic activity assay, while pluripotency markers were monitored via flow cytometry. The intracellular delivery of FD150 and the expression of eGFP after mRNA transfection were also investigated via flow cytometry.

Results: The delivery of FD10 and FD500 into HeLa cells demonstrated that laser fluence is the most significant factor influencing the delivery yield (57.3 ± 3.6% yield for FD10, and 35.3 ± 2.1% for FD500), while nanofiber diameter (400 – 600 nm) and IONP concentration (0.2-5%) had a minor effect. The delivery yield could be significantly improved by repetitively activating the PEN substrates, reaching 46.8 ± 7.2% for FD500). Next, PEN nanofibers were coated with Matrigel for iPSC culture. The concentration of Matrigel was titrated to optimize iPSC viability. The maintenance of pluripotency markers was confirmed with > 99% of cells expressing the TRA1-60, TRA1-81, and SSEA4 markers. Finally, PEN nanofibers were successfully used to deliver FD150 into iPSCs, achieving an efficiency of more than 80%. Additionally, iPSCs were transfected with eGFP mRNA, reaching a transfection efficiency of 25%.

Conclusion: PEN nanofibers are suitable for iPSC culture and enable the delivery of large payload molecules up to 150 kDa into cells without affecting pluripotency. Thus, they offer an attractive system for the safe engineering of iPSCs.

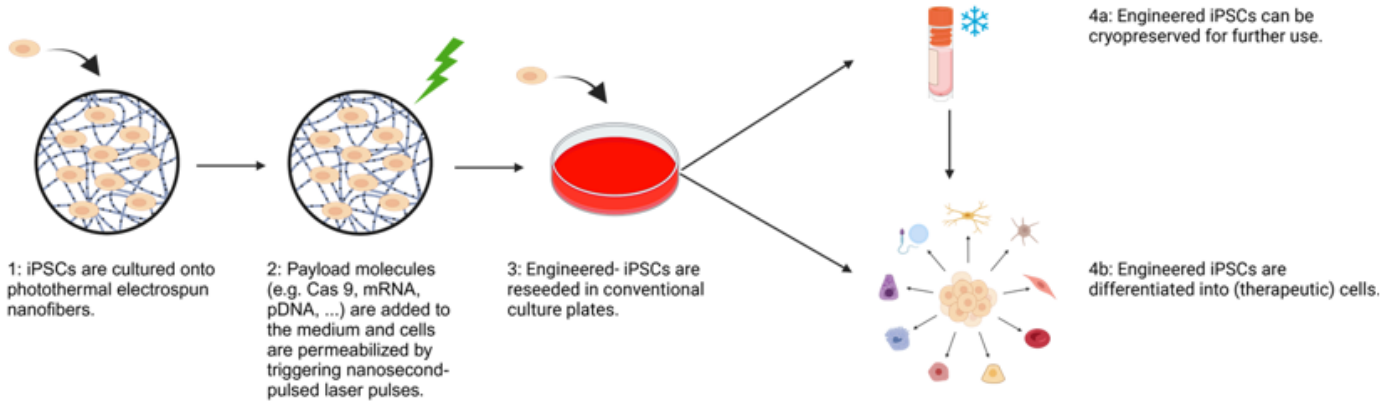

Figure 1: Schematic overview of iPSCs being transfected by photothermal electrospun nanofibers, and used as an off-the-shelf cell therapy product.

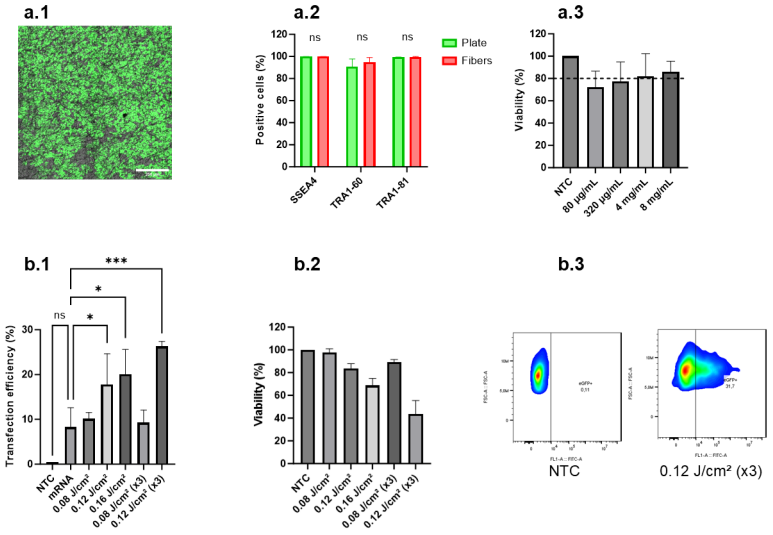

Figure 2: (a) iPSCs can be cultured onto PEN substrates while maintaining their viability and pluripotency. (a.1) Confocal microscopy image showing a monolayer of iPSCs labeled with calcein AM and cultured onto PEN nanofibers (transmission). (a.2) iPSCs cultured onto PEN substrates retain the expression of pluripotency markers similar to iPSCs cultured onto a conventional well plate. (a.3) iPSC viability when cultured onto PEN substrates coated with increasing concentrations of Matrigel. (b) PEN substrates enable the delivery of eGFP mRNA to iPSCs when irradited with a nanosecond pulsed laser (532 nm, 7 nanoseconds). (b.1) Percentage of cells expressing eGFP. (b.2) The cell viability, as determined by a metabolic assay 24 hours after transfection. (b.3) Exemplary flow cytometric data illustrating the expression of eGFP mRNA in iPSCs cultured onto PEN nanofibers.

689 Photoporation-Enhanced Drug Loading and Genetic Engineering of Mesenchymal Stem Cells for Glioblastoma Treatment

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**Corresponding Author*

Introduction: Glioblastoma is considered the most aggressive form of brain cancer with a poor prognosis. While various treatment options exist, the rapid disease progression requires more effective and targeted strategies. Currently, there is growing interest in using mesenchymal stem cell (MSC) therapy, because they can self-renew, differentiate into specific tissues, modulate the immune system and target tumor sites. MSCs offer an attractive option for delivering chemotherapeutics and other relevant effector molecules to the tumor site, thanks to their tumor-homing capacity. When using MSCs as drug delivery carriers to treat glioblastoma, there is a need for a technology that can efficiently and gently load MSCs with the relevant effector molecules before transferring them into the patient. Over the past decade, photoporation has emerged as an efficient and gentle cell transfection technology with translational potential for cell-based therapies. This technique uses cutting-edge laser and nanotechnology to permeabilize the plasma membrane with high precision and has been proven quite versatile in terms of cell type and cargo molecule (**Fig 1**). In this study, we aim to investigate whether photoporation can be used for drug loading of MSCs and to explore the potential of these drug loaded MSCs for the treatment of glioblastoma.

Methods: We first optimized photoporation (laser pulse fluence, AuNP concentration) for the delivery of payload molecules into human adipose-derived MSCs (hMSCs). For optimized conditions, we continued investigating the cell functionality after photoporation, e.g. by measuring cell proliferation.

Results: We optimized photoporation parameters using hMSCs from three different donors. FITC-dextran of 10 kDa (FD10) served as the model payload molecule. The percentage of FD10 positive cells was determined by flow cytometry, while the Cell Titre Glo assay was used to assess cell viability. The product of delivery efficiency and cell viability results in a yield value that expresses the overall percentage of viable positive cells. As the data in **Fig 2A** show, for a fixed laser fluence the delivery efficiency increases with higher AuNP concentration, at the expense of cell viability. A laser fluence of 0.53 J/cm^2 performed better than 0.21 J/cm^2, but there was no benefit using higher fluences. An optimal delivery yield of 62% was reached for 8×10^7 AuNPs/mL and a laser fluence of 0.53 J/cm^2. Next, we assessed cell proliferation after photoporation with four different AuNPs concentrations and a laser fluence of 0.53 J/cm^2. The cell proliferation rate was calculated as the fold change compared to day 1. As indicated in **Fig 2B**, there were no significant delays in cell proliferation 7 days after photoporation compared to untreated cells. In ongoing experiments, we are investigating important hMSC criteria (hMSC adhesion to plastic, expression of specific surface markers and multilineage differentiation potential) and hMSCs' immunomodulatory functions.

Conclusion: Our data so far indicate that photoporation can be successfully used for delivering macromolecules into hMSCs. Undelayed cell proliferation after photoporation suggests that it is a gentle approach, although further work is needed to assess its impact on hMSC functionality. Collectively, our results highlight the promise of using photoporation as an efficient technology for MSC engineering and drug loading.

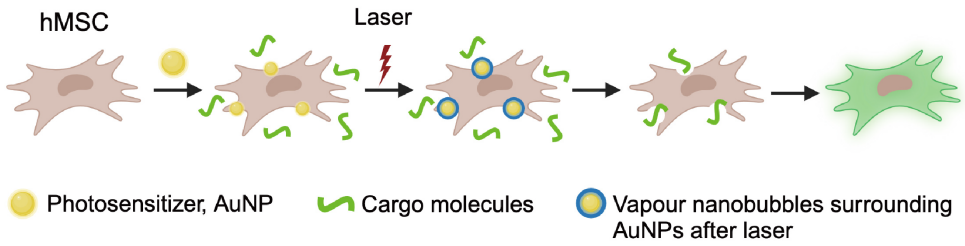

Figure 1. Illustration of the photoporation process. Photosensitizers, such as AuNPs, first bind to the cell membrane through electrostatic interactions. Applying nanosecond pulsed laser light leads to fast heating of the nanoparticles, resulting in heat and mechanical forces which locally disrupt the plasma cell membrane, allowing external payload molecules to enter the cytosol.

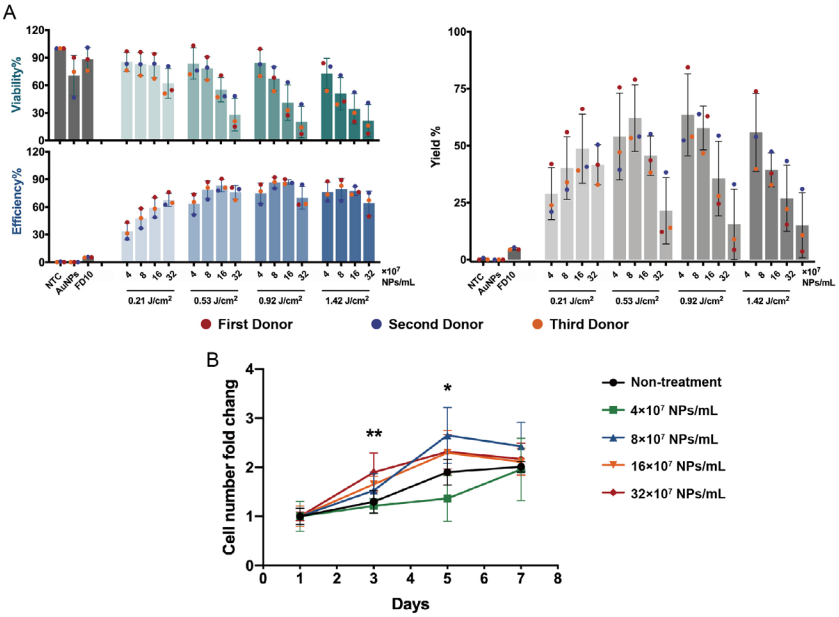

Figure 2. (A) The delivery of FD10 by photoporation into hMSCs from three different donors. Cell viability was measured by the Cell Titer Glo assay, delivery efficiency was measured using flow cytometry, yield was calculated by multiplying viability with efficiency. **(B)** The cell number fold change (relative to cell numbers on day 1) of hMSCs at different time points after photoporation with different AuNPs concentration under optimal laser fluence of 0.53 J/cm2. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test. (n = 3, *p < 0.05, **p < 0.01).

690 Scalable Non-Viral Gene Editing and Feeder-Free Production of CAR-NK Cells

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Engineered primary human natural killer (NK) cells demonstrate significant potential in immuno-oncology due to their proven safety and potent anti-tumor effects, particularly against solid tumors. Unlike primary T cells, NK cells do not rely on a matching human leukocyte antigen to function, reducing the risk of graft-versus-host diseases in allogeneic therapies. Gene delivery into NK cells is crucial for studying NK cell biology and developing NK cell-based immunotherapies. However, efficient, and safe non-viral gene delivery methods for NK cells remain limited. In this study, we describe non-viral based closed modular automated methods for CAR-NK cell engineering methods for clinical scale manufacturing.

We first optimized the electroporation parameters using the Neon™ NxT Electroporation System 8-channel, emphasizing gRNA, buffers and payload concentrations to enhance gene delivery efficiency and cell viability. We isolated PBMCs using CTS™ Rotea™, a closed counterflow centrifugation system. Isolated NK cells from PBMCs, were then expanded in feeder-free culture using CTS™ NK-Xpander™ Medium. The expended cells were washed, concentrated in genome editing buffer and subjected to cell engineering using CRISPR/Cas9 RNP and electroporation. First, we optimized conditions for small-scale cell editing using the Neon™ NxT, and successfully translated to a clinically relevant scale using the CTS™ Xenon™ Electroporation System. Our results showed that electroporation system efficiently achieved knockout (60-80%) and knock-in (15-25%) of different genes including site-specific CAR knock-in at AAVS1 locus. The edited NK cells maintained their viability and phenotypic characteristics and achieved clinically relevant levels of cell expansion. Further, we performed functional cytotoxicity assays of CAR-NK cells and showed enhanced cytotoxicity against target cells compared to unmodified NK cells.

In conclusion, this study presents closed, scalable, non-viral gene delivery methods for primary NK cell engineering that enables efficient transgene expression without compromising NK cell immunophenotyping or function. The ability to efficiently modify primary NK cells will facilitate further studies on NK cell biology and enable the development of NK cell-based immunotherapies.

691 Enhanced Gene Delivery with High-Frequency Asymmetric Biphasic Pulses: Balancing Efficiency and Reduced Muscle Contractions

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Introduction: Gene electrotransfer (GET) was first described in 1980s and since then emerged as a promising tool for non-viral gene delivery used for long-term cancer therapies and vaccine integration. One of the major obstacles hindering the transition of the GET and other electroporation approaches into the medical field is the pain and risk of ventricular fibrillation. One of the pulsing modalities shown to mitigate these effects is high-frequency biphasic waveforms, also known as H-FIRE pulses, used for tissue ablation. Due to bipolar cancellation, H-FIRE pulses are not well suited for gene delivery applications. In this study, we hypothesize that if an asymmetric, biphasic pulse sequence is applied to targeted tissue, then gene delivery will be enhanced while reducing skeletal muscle stimulation.

Methods: We studied microsecond-scale asymmetric biphasic waveforms with two opposite pulses (Fig. 1). *In vitro*, mouse myoblasts were exposed to luciferase reporter plasmid followed by immediate exposure to biphasic pulses. Gene expression was measured over the next two days. *In vivo*, Sprague-Dawley rats were subjected to intramuscular injections of luciferase plasmid followed by direct pulsing of skeletal muscles. Rats were then imaged for bioluminescence periodically over the next 3 months. Muscle contraction was measured with an accelerometer during pulsing. Additionally, gene delivery to the skin was assessed in guinea pigs, which received intradermal injections of luciferase encoding pDNA followed by surface application of pulses with either a monopolar platinum electrode or a four-plate electrode. All quantitative data were compared statistically with a two-way ANOVA and a multiple comparisons analysis, $p<0.05$ was considered statistically significant.

Results: Statistical analysis of in vitro results revealed that expression of luciferase in several biphasic pulse groups was significantly higher than the negative control of cells exposed to the DNA but not the pulses ($p<0.05$). Several biphasic groups had comparable expression levels with corresponding monophasic pulsing parameters ($p>0.05$). Similar results were demonstrated in muscle and skin where several biphasic groups showed a significant expression enhancement compared to negative control($p<0.05$). Biphasic pulsing parameters significantly reduced muscle contractions compared to monophasic pulses ($p<0.01$). The acceleration of hind and fore limb movement during several monophasic pulses exceeded acceleration under high-frequency biphasic conditions in both the maximum amplitude and the number of occasions at which maximum amplitude occurred.

Conclusion: This proof-of-concept study demonstrates the feasibility of using asymmetric biphasic pulses as an alternative to classic monophasic GET for gene delivery applications. More work remains on optimizing delivery parameters to reach gene expression levels within physiologically relevant ranges for specific therapeutic applications.

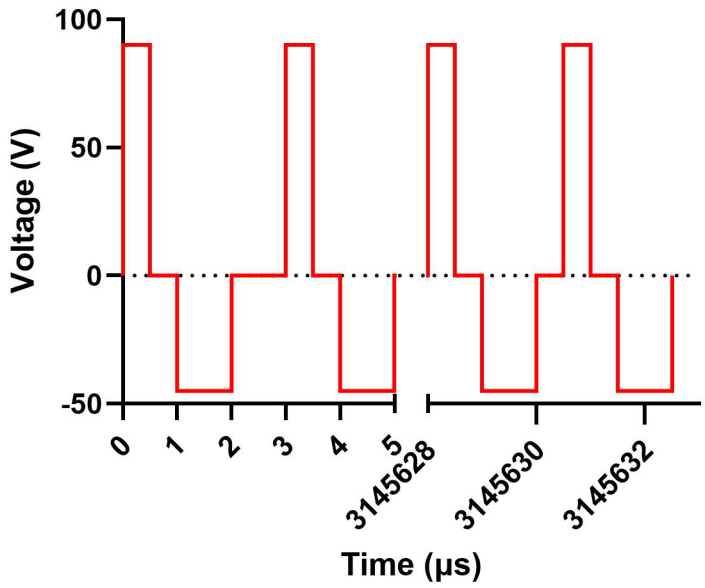

Figure 1: Asymmetric biphasic pulses were used to achieve luciferase plasmid transfection *in vivo* and *in vitro*.

692 Electroporation and LNP-mediated delivery of HPAI HA antigens supports robust anti-influenza immunity *in vivo*

Ebony Gary[1], Nicholas Tursi[2], Robert Vendramelli[3], Casey Hojecki[4], Yangcheng Gao[5], Elizabeth Parzych[1], Darwyn Kobasa[3], David Weiner[5]
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Highly pathogenic avian influenza (**HPAI**) viruses of the H5 subtype are responsible for an increasing number of zoonotic events, including an ongoing outbreak of clade 2.3.4.4b H5N1 influenza in North America. These viruses can infect a wide range of hosts, including humans, representing an ongoing threat to public health and agriculture. Synthetic DNA (**synDNA**) antigens are non-live, non-replicating antigens which are easily produced and do not require cold-chain transport. We designed plasmid-encoded H5 hemagglutinin (HA) antigens representing clades 2.3.2.1c (**pCamb**) and 2.3.4.4b (**pMich**), which have caused the most human zoonoses, and delivered them via electroporation (**EP**) or in optimized lipid nanoparticles (**DNA-LNP**). pCamb-immunized mice had high titers of clade 2.3.2.1c specific binding and pseudovirus neutralizing antibodies in their sera. We also detected significantly increased frequencies of clade 2.3.2.1c-specific IFNγ secreting T cells via ELISpot. Importantly, two immunizations with this plasmid via EP delivery supported complete protection from matched lethal challenge with clade 2.3.2.1c H5N1. Interestingly when mice were immunized with this construct alone, or co-immunized with pCamb and the mucosal adjuvant pCTACK, we observed intermediate protection from heterologous clade 2.3.4.4b H5N1 challenge. We designed a second construct expressing clade 2.3.4.4b HA (pMich) and delivered this plasmid as a single immunization formulated in lipid nanoparticles (**LNP**). A single immunization with pMich DNA-LNP resulted in significant increases in clade 2.3.2.1c and 2.3.4.4b specific serum binding and neutralizing antibodies and supported strong cellular responses 30 days post-immunization. These data suggest that DNA-delivered H5N1 HA antigens can have significant impact on HPAI challenge in vivo and support the continued translation of these antigens.

693 A FIH dose-escalation trial of the safety and pharmacokinetics of anti-SARS-CoV-2 DNA-encoded monoclonal antibodies (DMAb) formulated for delivery by CELLECTRA in healthy adults

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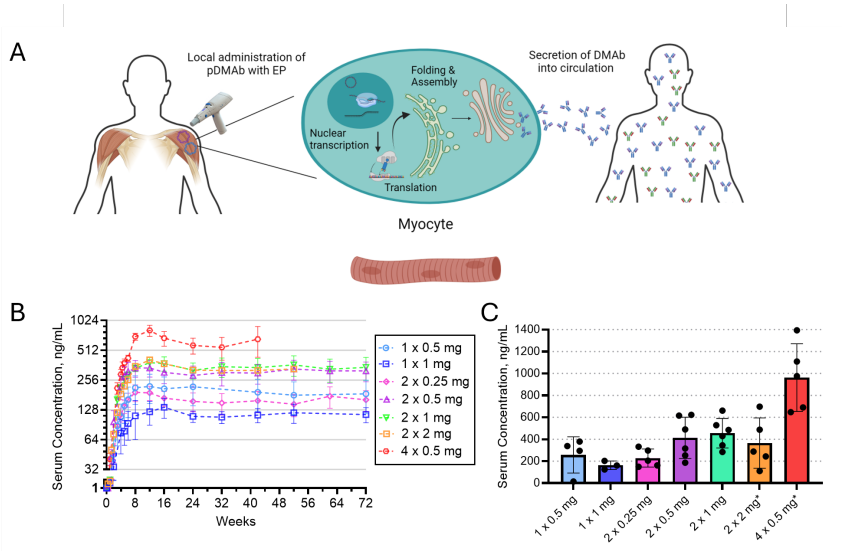
Background: Local intramuscular administration of synthetic plasmid DNA (pDNA) encoding monoclonal antibodies (mAbs) offers an alternative to recombinant protein technology. The dMAb platform has been developed preclinically in infectious disease and cancer immunotherapy models. However, no clinical trial data on this approach have been previously reported. This study advances a first-in-human (FIH) trial evaluating a two-dMAb formulation from preclinical small animal models to a non-human primate (NHP) pharmacokinetics (pK) and challenge study, ultimately leading to an Investigational New Drug (IND) application for assessing safety and pharmacokinetics in healthy individuals.

Methods: This multi-group Phase 1 dose-escalation study (NCT05293249) investigates the intramuscular (IM) administration of a pDNA cocktail encoding AZD5396 and AZD8076, modified for in vivo delivery and expression of the SARS-CoV-2 neutralizing mAb cocktail Evusheld. The vaccine is delivered using CELLECTRA™ electroporation technology. Participants received varying doses across multiple cohorts, and serum samples were analyzed for pharmacokinetics, durability, anti-drug antibody (ADA) responses, and SARS-CoV-2 neutralization activity. The study's primary endpoints include safety and pharmacokinetics of in vivo-expressed DMAbs.

Results: Muscle-expressed DMAbs were detected in the serum of all dosed participants (N=39), with concentrations reaching 1.11 µg/mL. In 24/24 participants who have completed 72 weeks of follow-up, DMAb levels remained stable. Functional assays confirmed SARS-CoV-2 receptor-binding domain (RBD) binding and neutralization activity in pseudovirus assays. No ADA responses were detected, suggesting sustained expression without immune-mediated clearance. Safety assessments revealed no serious adverse events, with mild injection site reactions being the most commonly reported.

Conclusion: This study provides the first clinical proof-of-concept for synthetic pDNA DMAb technology, demonstrating durable in vivo production of functional monoclonal antibodies. The findings underscore the importance of synthetic design, formulation, and delivery in achieving biologically relevant gene-encoded biologics expression. The DMAb platform may represent a valuable strategy for targeting a wide range of diseases treatable with monoclonal antibodies or their derivatives.



(A)Schema of DMab administration and process. (B) Longitudinal serum concentration of in vivo-expressed DMAbs AZD5396 and AZD8076. (C) Maximum DMAb serum concentration by group.

694 Enhanced Gene Therapy for Tay-Sachs Disease Using the PP6D5 Polymer and CRISPR/nCas9

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Tay-Sachs disease (TSD) is a fatal neurodegenerative disorder caused by a deficiency of β-hexosaminidase A (HexA), leading to GM2 ganglioside accumulation in the central nervous system. This study explores the use of the PP6D5 polymer as a non-viral vector for CRISPR/nCas9-mediated correction of HEXA mutations, assessing its transfection efficiency and therapeutic potential in TSD GM00515 fibroblasts.

Comparative analysis with Lipofectamine 3000 in healthy cell lines revealed that PP6D5-mediated transfection significantly enhanced HexA activity. In 3T3 cells, PP6D5 achieved a 1.5-fold increase in HexA activity compared to 1.16-fold with Lipofectamine after 30 days. Co-transfection with HEXA/HEXB cDNAs via PP6D5 nearly tripled HexA activity relative to HEXA alone. In U87MG cells, HEXA cDNA transfection using PP6D5 led to a fivefold increase in HexA activity after 25 days, surpassing Lipofectamine. Similarly, in SHSY5Y cells, PP6D5 induced a 2.3-fold enhancement after 15 days, with a striking 45-fold surge upon HEXA/HEXB co-transfection, stabilizing at 27.9-fold after 25 days.

In TSD fibroblasts, HEXA transfection improved HexA activity but did not fully restore normal levels. HEXA/HEXB transfection with Lipofectamine resulted in an eightfold increase after 15 days, whereas PP6D5-mediated transfection reached 3.5-fold after 30 days. Regarding lysosomal dynamics, Lipofectamine increased lysosomal mass intensity, while PP6D5 induced a 1.13-fold reduction. Both vectors effectively reduced ROS levels. Lipid compartmentalization exhibited contrasting patterns: neutral and polar lipid content decreased with Lipofectamine but increased with PP6D5. Additionally, autophagic flux analysis indicated partial recovery with Lipofectamine, while PP6D5 promoted autolysosome formation.

These findings underscore the potential of PP6D5 as a non-viral vector for gene therapy, demonstrating robust CRISPR/nCas9 delivery and sustained therapeutic efficacy across multiple cell models. The distinct cellular responses highlight PP6D5 as a promising, safer alternative to viral vectors for treating TSD.

695 High-Throughput Screening and Data-Driven Evolution of Polymeric Nanoparticles for Efficient Gene Delivery

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Polymeric nanoparticles (PNPs) are modular delivery systems that can be optimized for targeted delivery of therapeutics. The design and development of nanoparticles has been greatly accelerated by recent advancements in rational design approaches, such as high-throughput methods and computational techniques. These strategies move away from a trial-and-error model to a more structured, data-driven approach, improving the efficiency of preclinical discovery pipeline for nanomaterials. Here, we employ a high-throughput design-build-test-learn platform to enhance the delivery efficiency of PNPs for the treatment of Neurofibromatosis Type 1 (NF1), a genetic disorder caused by the loss of functional neurofibromin, a tumor suppressing protein. NF1 gene replacement therapy holds immense clinical potential but is hindered by challenges in delivering the large 8.5 kbp *NF1* gene using viral vectors.

To overcome this, we used cationic diblock PNPs for encapsulating and delivering the full-length human NF1 plasmid (EF1a-NF1-2A-EGFP, ~20 kbp) to human Schwann cells (SCs). However, designing the perfect PNP is difficult because of the vast chemical landscape, the complexity of biological systems, and the limitations of current modeling techniques. To address this, we used our High-Throughput Synthesis, Characterization, and Assessment of Nanoparticles (HIT SCAN™) platform to systematically test over 500 different PNPs and identify the most promising "hit" candidates. The polymers were synthesized using a reversible addition-fragmentation chain-transfer (RAFT) polymerization process, and self-assembled into PNPs. Our approach consisted of two rounds of iterations: in the first round, we screened a diverse library of ~450 first-generation (G1) PNPs, synthesized from six carefully selected monomers, to assess plasmid loading, transfection efficiency (TE), and cytotoxicity in immortalized human NF1-null SCs (hTERT NF1-/- ipNF97.4). *Select PNPs with specific combinations of three monomers emerged as highly effective in encapsulating the large plasmid and delivering it to SCs.* Twenty of these PNP hits were used to demonstrate partial neurofibromin restoration *in vitro* in SCs. Efficacy of the best performing PNP hit was confirmed with *in vivo* testing which showed gene expression in mouse and rat cortex. The overall TE of G1 PNPs was however not sufficient in fully restoring functional neurofibromin *in vitro.* In the second round, we leveraged a machine learning (ML) guided approach to refine the design space. The ML model, trained on successful G1 candidates, identified key structural and compositional features, which was used to generate a second-generation (G2) of 96 PNPs. *The resulting G2 PNPs exhibited a marked 5x increase in TE in SCs, from ~4% in G1 PNPs to ~20% in G2 PNPs.* Functional assays to assess biodistribution, delivery efficacy and restoration of neurofibromin function and downstream signaling pathways (e.g., pERK) both *in vitro* and *in vivo* are ongoing.

In conclusion, the combined use of our HITSCAN™ platform and a data directed evolution approach of PNPs led to the identification of a group of chemically distinct PNPs that were uniquely capable of carrying and delivering the large *NF1* gene to human SCs *in vitro*. The TE of second generation PNPs improved 5x over the first generation PNPs, highlighting the potential of high-throughput screening platforms for accelerating the development of novel nanomedicines for genetic diseases like NF1.

696 Improving Hematologic Gene Therapy by Mapping the Structure/Activity Relationship of Poly(Beta-amino ester) mRNA Nanoparticles

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Introduction: Disorders of the blood, including malignant conditions, such as leukemia, and non-malignant conditions, such as hemoglobinopathies, can be treated by blood cell (hematologic) gene therapy. Delivery of genes encoding chimeric antigen receptors to T-cells or gene editors to hematopoietic stem cells have offered new curative therapies to patients. However in most cases, delivery of these gene therapies to cells has relied upon viral vectors or electroporation. Viral vectors are difficult to scale up due to their high cost; electroporation toxicity reduces the recovery of modified patient cells. Non-viral, nanoparticle (NP)-based delivery is a promising alternative delivery method to hematologic cells. NP are chemically defined, easily scaled, and may have reduced toxicity. One such class of NP is polymeric NP made from poly-beta amino esters (PBAE). The plug-and-play chemistry used to synthesize PBAE allows a large, combinatorial design space. However, the intrinsic variability in the uncontrolled polymerization used to make PBAE polymers has limited wider adoption. The structure-activity relationship (SAR) between PBAE NP and delivery of mRNA to cells of the blood system is not well understood.

Methods: A new synthetic method for PBAE synthesis has been developed allowing highly reproducible polymers to be made. Using this methodology, three different length co-polymers of butanediol diacrylate and amino-butanol were made and then functionalized with three prototypical monomers (a diamine, a piperazine, or an amino-alcohol). Polymer-nucleic acid affinity was probed using fluorescent assays. mRNA transfection of human embryonic kidney (HEK/293) cell line, hematologic cell lines (K562 and HUDEP2) and primary human peripheral blood mononuclear cells (PBMC), and hematopoietic stem and progenitor cells (HSPC, CD34+) were investigated using luciferase and green fluorescent protein (GFP). Additionally, cas9 mRNA and gRNA PBAE NP-mediated gene editing of *B2m* was investigated with flow cytometry.

Results: Size characterization of PBAE polymers with gel permeation chromatography correlated with polymer size by NMR. Nucleic acid binding affinity correlated inversely with polymer length, and amino-alcohol functionalized polymers had lower binding affinity (Figure 1). From a library screen of PBAE-mRNA NP, the top performing candidate had 607-fold higher delivery of luciferase mRNA than a lipid nanoparticle based on a clinically used ionizable lipid. High activity of the best-performing PBAE polymer was conserved across HEK293 cells, HUDEP2, K562 (Figure 2A), PBMC, and CD34+ cells (Figure 2B). Delivery of cas9 mRNA and gRNA with PBAE NP is being studied in these cell types, as well as primary human T-cells.

Conclusions: PBAE NP are a promising non-viral delivery platform for delivery of mRNA to hematologic cells, including primary CD34+ cells. In this series, the profile of the best-performing polymer was conserved across cell types. Further studies of more diverse chemical structures are still needed to better understand the structure-activity relationship (SAR) between PBAE NPs and cell tropism. A better understanding of the SAR will allow the design of improved NP formulations for gene delivery, specifically to primary CD34+ cells and T-cells.

Figure 1

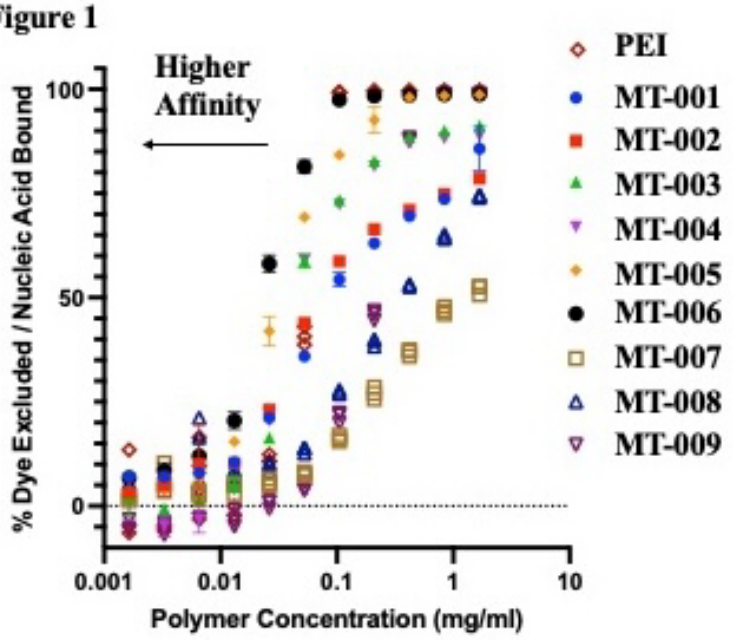

Figure 1. Nucleic acid affinity measurement by dye exclusion. Plasmid DNA was incubated with YOPRO-1 dye and PBAE polymer was added displacing the dye. Nine PBAE polymers were utilized with varying lengths (long, medium, and short) and each with three prototypical functionalizations (diamine, piperazine and aminoalcohol). Linear PEI 40 kDa is a positive control. N=3.

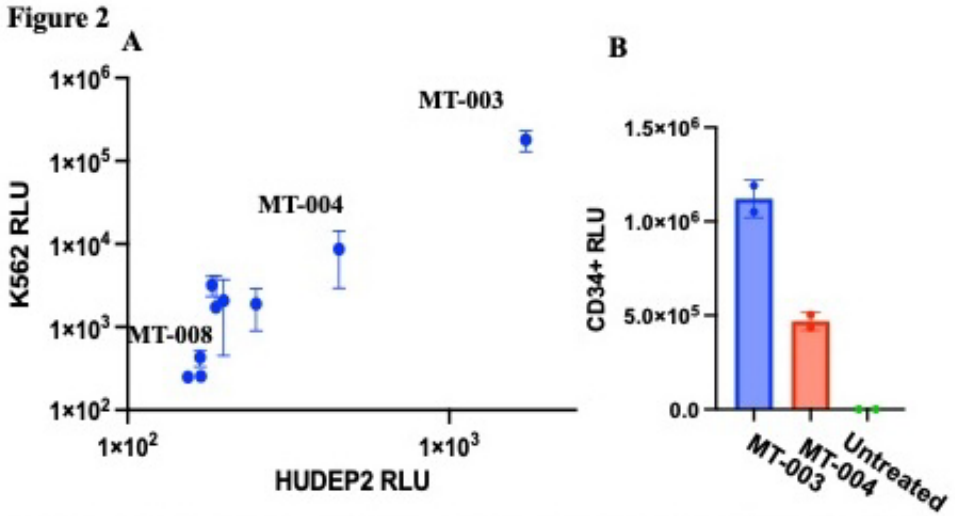

Figure 2. Correlation of mRNA transfection efficacy between K562 (myeloid leukemia, N=3) and HUDEP2 (erythroid progenitor, N=3) cell lines for a 3x3 PBAE polymer library of lengths and functional monomer. Transfection of 30,000 cells with 100 ng of mRNA at 24 hours and 90:1 PBAE to mRNA ratio. RLU: relative luciferase units (A). Efficacy of top-performing PBAE mRNA NP in primary human hematopoieitic stem and progenitor (CD34+) cells with similar conditions as in (A), N=2.

697 Optimizing Poly (Amine-Co-Ester) Polymeric Nanoparticles for Enhanced Nucleic Acid Delivery to Endothelial Cells

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Introduction: The intricate, multi-step process of non-viral gene delivery is highly sensitive to the alterations in the structure and properties of the vehicles (1). The interaction between vehicle material and drug delivery may be cell specific. Here, we explore the use of spherical poly-amine-co-ester (PACE) polymeric NPs to deliver therapeutic nucleic acids, including siRNA and mRNA (2), to endothelial cells, an understudied target for drug delivery. In this study, we modify the base cationic PACE polymer to affect the resulting NP surface chemistry and measure the impact on endothelial cellular uptake and total drug encapsulation, developing a correlation between surface chemistry and drug delivery in this cell type. We hypothesize that an increase in ionizable groups on the polymer backbone will result in enhanced nucleic acid loading, cell uptake, endosomal escape, due to stronger electrostatic interactions (3).

Methods: PACE polymers were modified from previously described polymers with (4) with various molecular groups such as carboxylic acid (COOH), polyethylene glycol (PEG), NH-NH2, NH2-OH and NH-OH (5). Fluorescent dyes and short-chain nucleic acid oligomers were encapsulated into NPs formed from these polymers. Dynamic light scattering and zeta potential measured the NP size and surface charge. An Optical Photothermal Infrared (O-PTIR) Spectroscopy characterized functional groups and the amino group density via mapping among different PACE NPs under the wavenumber set at 1173 (cm-1). A picogreen assay measured cargo encapsulation efficiency and TNS assay tested pKa values of PACE NPs. Flow cytometry was used for quantifying cellular uptake of different PACE NPs in Human Umbilical Vein Endothelial Cells (HUVECs).

Results: A library of NPs was formulated from an identical base polymer, each modified to exhibit different surface charges. Fig 1 illustrates PACE50 NPs with distinct end groups and surface charge variations. The amino group density on these NPs was characterized (Fig. 1B). PACENH-NH2 NPs contain more amines, making them more positively charged than base PACECOOH NPs, and enter endothelial cells more rapidly (Fig 2). Surface charge (Fig. 2B), pKa values (Fig. 2D) and nucleic acid loading (Fig. 2C) shift with amine incorporation. Increased charge density results in a greater nucleic acid loading (Fig. 2C) when additional amine groups are introduced. The differences in surface charges of PACE NPs also impact their uptake by endothelial cells (Fig. 2E). Thus, surface charge and pKa values corresponding to ionization of PACE NPs have significant correlation to the cellular uptake. A higher surface charge—excluding pegylated NPs owing to their hydrophilic properties—enhances the electrostatic interaction between the NP surface and the cell membrane, promoting faster cellular uptake and making them effective carriers for nucleic acid delivery.

Conclusions: The PACE NPs with various ratios of monomers can affect the surface charge values. The polymer can also be modified further by adding different end groups to affect the surface charge. The change of surface charge values and distribution of surface charge demonstrate the versatility of PACE NPs and their potential for tailored nucleic acid delivery to endothelial cells, offering promising applications in treating inflammatory conditions.

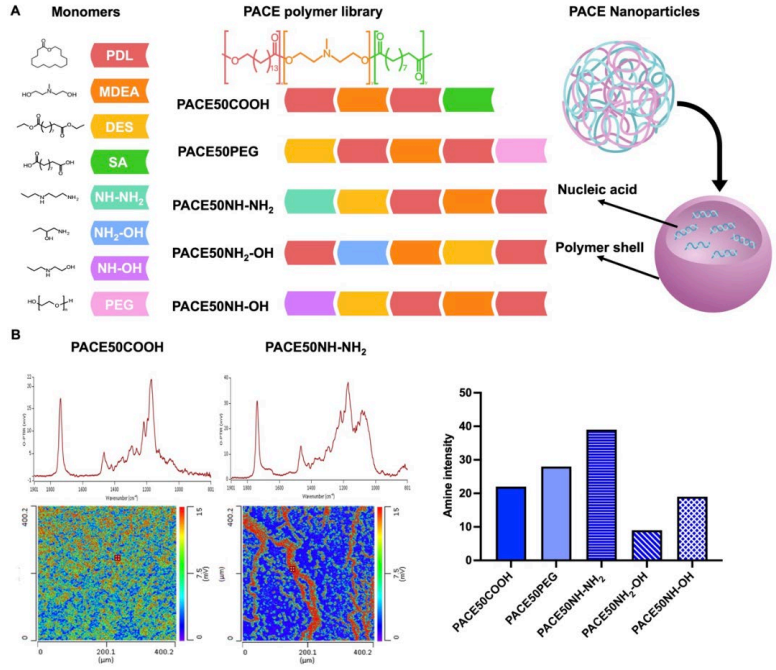

Figure1. PACE NP library formulation and characterization. (A). The structure of monomers and PACE NP formulation process. (B). Characterization of the functional groups of PACE NPs and amine group mapping at the wavenumber of 1173(cm^{-1}) of PACE NPs with different end group modification via O-PTIR.

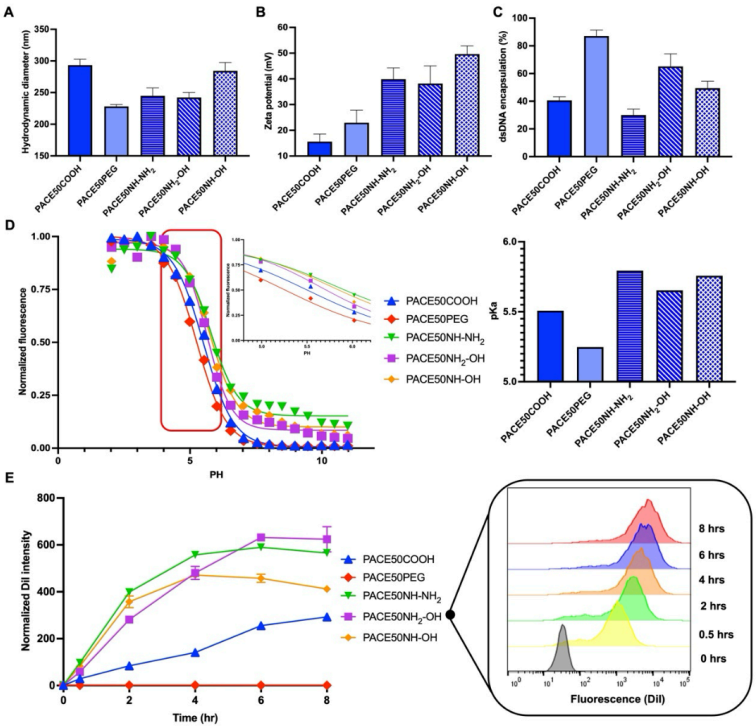

Figure2. Characterization and cellular behavior of different types of PACE NPs. (A). The average size of PACE NPs. (B). The average surface potential of PACE NPs. (C) Nucleic acid encapsulation percentage of PACE NPs via picogreen assay. (D). pKa values of PACE NPs via TNS assay. (E). HUVEC cellular uptake of PACE NPs.

698 Polymeric Nanoparticles Targeting Gut-Associated Lymphoid Tissue for Oral CRISPR/Cas9 and dCas9 Delivery

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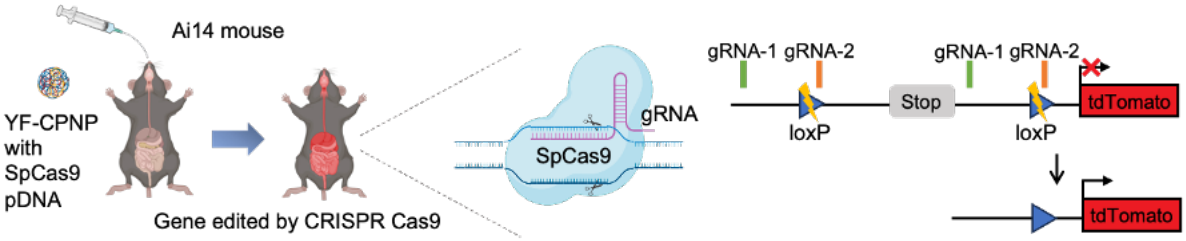
Effective delivery of CRISPR-Cas9/dCas9 elements is crucial to maximize therapeutic potential. Despite extensive efforts globally to improve the delivery of gene editing elements for somatic genome editing, achieving gene editing via oral delivery has remained an elusive challenge. Here, we present an innovative orally-administered, nonviral gene editing system that opens exciting new avenues for addressing the demands of both localized gene editing in the gastrointestinal (GI) tract and systemic gene editing in a patient-friendly, non-invasive, and repeatable manner. By incorporating yeast fragments into the polyplex, our oral polymeric nanoparticle (NP) platform YF-CPNP for gene editing plasmids can efficiently traverse tightly packed mucosal epithelium via intestinal microfold cells. Its subsequent endocytosis occurs in local and Peyer's patch macrophages, leading to a further accumulation in mesenteric lymph nodes (MLN). With these advantages, YF-CPNP can achieve gene editing in the macrophages, dendritic cells, T cells and B cells in the lymph nodes. This strategy not only prolongs gene editing within the local GI tract and gut-associated lymphoid tissues but also facilitates NP transport through the gut-associated lymphoid pathway to the systemic circulation, ultimately modulating the genome of distal organs such as the liver. This culminates in the manifestation of titin gene activation in the small intestine and liver through oral administration with YF-CPNP with dCas9 VPR plasmid. Our engineered delivery platform offers promise for oral gene editing and activation, representing a significant advancement with potential therapeutic applications.



699 In Vivo CAR Delivery as Supercoiled DNA with Lymphatic Tropism Polyasparagine Nanoparticles

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Viral vector and lipid nanoparticles had undoubtedly demonstrated enormous success as single dose gene therapy and annual vaccines, respectively. There remains a gap for a redosable, safe yet durable vector to address the need for in vivo immune reprogramming which has shown much promise to expand the use of CAR-T cell therapy to indications such as solid tumor and autoimmune disease. We produced and characterized a non-viral, biodegradable poly-asparagine nanoparticle for DNA delivery. When the GFP encoding DNA-nanoparticle was injected intravenously, the expression was found in thymus and surprisingly evaded the liver. T cells and lung resident macrophage were found to be GFP positive. We evaluated the delivery platform's utility for in vivo reprogramming using DNA encoding an anti-CD19 chimeric antigen receptor (CAR) in C57B/6 mice. The b cell population in blood and in spleen consistently reduced following tail vein injections. T cell expansion was modest and returned to near baseline within two weeks of the initial dosing. The mice exhibited no signs of distress under a biweekly dosing schedule, which was sufficient to result in significant b cell ablation. We deployed an in-silico binder optimization platform to design a single-chain antibody as the binder of a solid tumor targeting CAR in silico and deliver the CAR as a DNA-nanoparticle polyplex to evaluate the anti-cancer efficacy.

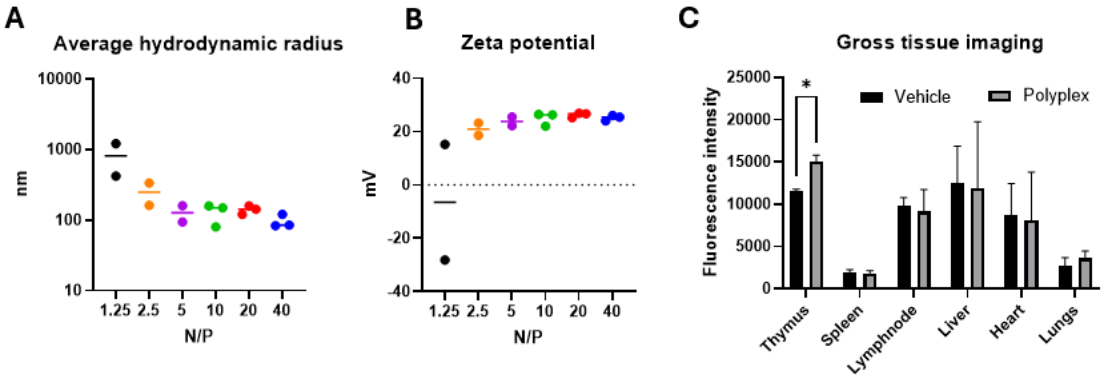

Figure 1, the physical properties and biodistribution of DNA polyplex. A) and B) are the hydrodynamic radius measured by dynamic light scattering and the zeta potential as the function of the asparagine to DNA ratio (N/P). The biodistribution of N/P 10 polyplex encoding GFP is shown in C).

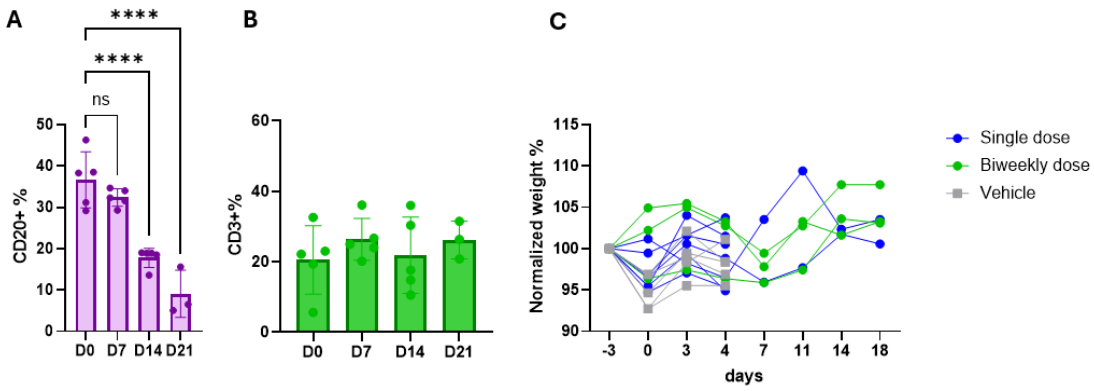

Figure 2, the b cell, t cell populations and weight changes observed in immune competent mice injected biweekly with DNA-nanoparticle encoding anti-CD19 chimeric antigen receptor. A) B cell population in PBMC steadily declined while B) T cell population undulates periodically coinciding the injection frequency. C) The normalized weight compared to baseline suggests no safety concerns.

700 In Vitro and In Vivo Delivery of Nucleic Acids using Poly(glycerol ester)

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Introduction: Poly(glycerol ester) (PGE) can be used to produce next-generation polyplex for cell and gene therapy and ocular delivery. PGE nanoparticles have performance based on (1) the selection of the diacid structure and the amine functionality used; (2) the type and molar ratio of the polyol; and (3) the complexation buffer and parameters. We investigated use of PGE polymers for delivery of mRNA and pDNA in serum-free, serum containing, and in vivo environments.

Method: PGE was synthesized through a polycondensation reaction followed by amine functionalization of hydroxyl groups using CDI coupling chemistry. Nanoparticle complexes were prepared using varied ratio of nucleic acid to polymer mass and particle size and zeta potential were measured (Zetasizer Ultra, Malvern). Transfection of HEK293T cells used a GFP mRNA or pDNA reporter (Aldevron). After 24 hours, cells were characterized using a flow cytometer (Cytoflex, Beckman Coulter) for viability, percentage of transfected cells (%TE), and expressed protein level (MFI). PGE formulations showing success in serum-free media were further tested in serum-containing media before being selected for testing in vivo in a mouse tail vein injection model to determine efficacy in vivo and localization of particles.

Results: PGE polyplex was able to successfully transfect HEK293T cells at a variety of PGE : nucleic acid ratios ranging from 20:1 to 40:1. Over 90% of cells were transfected with PGE in this range which was near the level seen with Lipofectamine 3000. Expression levels of the GFP reporter were high across the ranges tested and were able to exceed the expression levels seen with Lipofectamine 3000. A slight increase in expression occurred with the 20:1 group when serum was present, suggesting that serum had beneficial impact on the stability of the particle and its uptake by cells. PGE polyplex reagent was able to show notable pDNA and mRNA expression as early as 6 hours post tail vein injection in mouse models, with expression occurring primarily in the spleen of the animals.

Conclusions: Gene delivery using PGE polyplexes was demonstrated for the first time as having comparable in vitro performance to Lipofectamine 3000. PGE was able to maintain its performance in the presence of 10% FBS containing media. Furthermore, the PGE nanoparticle complex was able to transfect cells of the spleen in mouse model, indicating that they are able to protect mRNA from enzymatic degradation in vivo. These results show that the PGE nanoparticles have potential to provide an alternative to conventional delivery vehicles used for pDNA and mRNA both in vitro and in vivo.

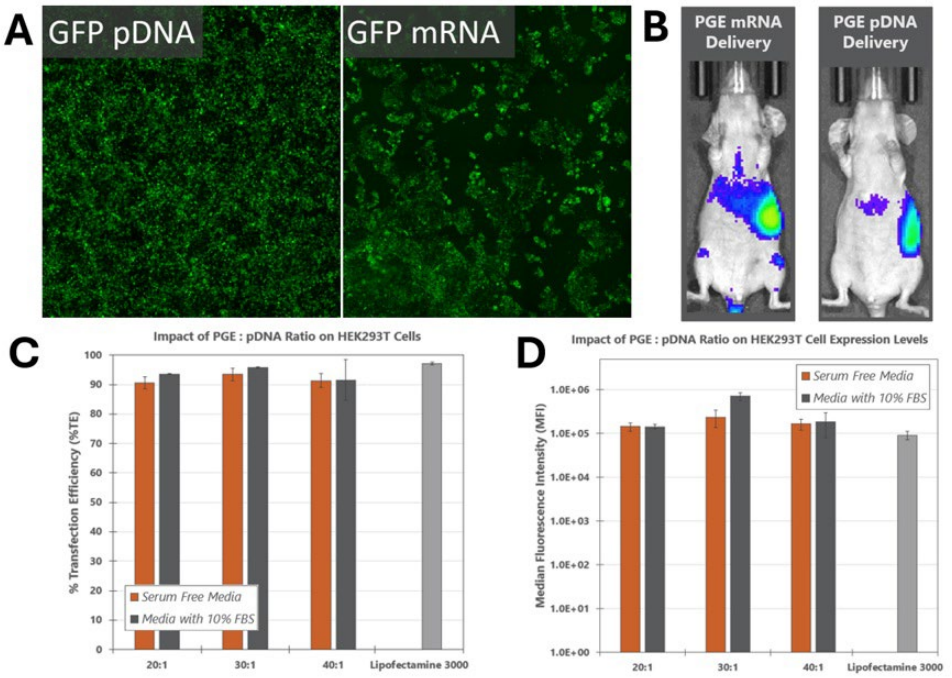

Figure 1: Poly(glycerol ester) (PGE) polyplex nanoparticles are able to deliver GFP reporter pDNA and mRNA to HEK293T cells (**A**). When delivered through a tail vein injection into mice, expression of either pDNA or mRNA is seen within 6 hours in the spleen (**B**). PGE is able to perform at or exceeding the performance of Lipofectamine 3000 across a range of PGE : pDNA ratios in both serum free and serum containing media (**C, D**).

701 High Level Persistent In Vivo Production of an anti-HIV Antibody via the Delivery of Plasmid DNA by a Polymer Nanoparticle

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While monoclonal antibodies are a prominent class of pharmaceutical products there remain significant issues of cost, complexity and especially delivery: concepts that overcome the need to infuse antibodies frequently are a desirable objective. An attractive approach is to deliver non-integrating DNA directly to tissue, allowing the patient to act as their own so-called "protein factory". Demonstrations of this concept have been made using LNPs and viral vectors, but these delivery methods face significant challenges, including extrahepatic delivery, cargo compatibility, safety, redosability and cost. Polymer nanoparticles (PNPs) offer a solution to these problems, but face their own challenges, such as a vast number of possible polymer structures, and polyplex formulation conditions. However, advances in machine learning, materials informatics, and high-throughput chemical synthesis techniques provide a foundation for addressing these challenges by efficiently exploring the polymer design space. Our SAYER platform utilizes a large computational dataset of pDNA polymer interactions to facilitate targeting agent discovery and refinement via deep learning, and to drive discovery of novel polymeric nanoparticles (PNPs) for a wide variety of target tissues. In this work, we demonstrate our ability to design PNPs that can deliver pDNA encoding for PGT121, a broadly neutralizing anti-HIV antibody that targets a V3 glycan-dependent epitope site on the HIV-1 envelope glycoprotein. SAYER-designed polymers formed small and stable PNPs with PGT121-encoding plasmids. Intravenous delivery demonstrated strong serum PGT121 protein levels at 1-day post-transfection and higher levels of protein expression when compared to other state-of-the-art DNA-delivery vehicles. More importantly, intramuscular delivery of Nanite PNPs showed greater than 1.0 ug/mL peak protein expression levels, with meaningful expression levels at > 56 days post-injection. Further, we showed that we can

increase antibody levels and durability by redosing. A general trend of lower dose and lower N/P ratio providing higher antibody levels is seen when PNPs were delivered intramuscularly. These parameters, separate from polymer structure, provide different mechanisms to optimize PNP in vivo delivery performance using machine learning techniques. Extension of the concept to the continuous production of other antibodies, proteins or enzymes are possible, suggesting the broad applicability of pDNA depoting via PNPs as a therapeutic modality. Finally, we emphasize that this strategy of delivering a DNA-encoded secreted proteins via a safe and effective PNP in vivo could be applicable to a wide range of other disease modalities.

702 Branched Polymers with a Crosslinked Core Offer Nanoparticle Size Retention and a Novel Method of Safe and Effective Non-Viral Gene Delivery

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Introduction: The delivery of nucleic acids remains a major hurdle in the development of innovative therapeutics. Adeno-associated viruses (AAVs) remain the gold standard, but they come with payload capacity limits and risks of myriad off-target effects. As such, non-viral delivery vehicles, such as polymeric nanoparticles (PNPs) and lipid nanoparticles (LNPs) are attractive alternatives for gene delivery solutions. PNPs represent a potential improvement beyond other non-viral vehicles such as LNPs, due to their increased compositional diversity, which confers the ability to fine tune physical properties beyond the traditional limits of LNPs. These physical properties include features such as size, charge, and shape, features which may be critical in biodistribution, cell uptake, payload release, reduced immunogenicity, and excretion.

Methods: We have developed the HIT SCAN^{TM} (High-Throughput Synthesis, Characterization, and Assessment of Nanoparticles) Platform, a design-build-test-learn PNP screening platform to guide the quick discovery of PNP delivery vehicles. It combines automated PNP library synthesis, in vitro and in vivo screening, and machine learning models to process generated data and direct new PNP synthesis. *Using the HIT SCAN platform, we have developed a family of novel polymer compositions that are designed to produce PNPs that are biodegradable, have a low elastic modulus, have a long half-life and have the potential to achieve a sustained release and/or slowed cellular uptake of their nucleic acid cargo.* The core of these PNPs is a copolymer of a cationic acrylic acid monomer containing tertiary amines, crosslinked with a dialkene functional monomer where the two vinyl groups are connected by an ethylene oxide oligomer linkage. Though most typical lipid nanoparticles and polymer micelles rely on self-assembly of amphiphilic molecules, to form nanoparticle structures, the cross-linked nanostructure of these branched PNPs may allow them to be smaller and maintain size under various formulation conditions. Moreover, the inclusion of the ester groups in the crosslinked core gives these nanoparticles the feature of biodegradability not present in most other polymer and lipid nanoparticles. Finally, the corona of the PNP may be tailored with various functional groups to tune the half-life of the nanoparticles, potentially resulting in sustained action nucleic acid drugs.

Results: We designed and synthesized a diverse of branched polymers, all of which were small (<50nm in diameter) when hydrated as unloaded PNPs in PBS. From this diverse library, a specific compositional family sharing a common functional group on the corona yielded PNPs that formed polyplexes with a small size (<50nm) upon loading with an eGFP circular RNA payload under conditions that often result in aggregated PNPs. In vitro transfection efficiency in HEK293T cells was assessed for the entire diverse library of branched polymer PNPs loaded with the same eGFP circular RNA payload above. Interestingly, there was an overlap between the compositional family of PNPs that formed small polyplexes with RNA, and those with high in vitro transfection efficiencies. We show that the crosslinked core polymers are useful for small polyplexes with nucleic acids that also demonstrate efficient transfection in cell culture. Future work will include evaluation of the in vivo delivery efficiency of this PNP family.

703 Programmable Polymer Nanoparticles Co-Package and Deliver Dual mRNA and pDNA Cargo to Cells

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Clinical success in the field of gene therapy underscores the importance of identifying affordable gene delivery options for broader public access to life-changing treatments. Lipid nanoparticles (LNPs) have emerged as the leading choice for mRNA-based therapies with the deployment of COVID vaccines, but are limited by their immunogenicity, inability to deliver pDNA and inadequate extra-hepatic delivery. In addition, viral vectors carrying DNA are particularly hindered by cargo capacity and inability to re-dose. Furthermore, virtually no nanocarrier system has shown potential for multi-cargo gene delivery. Polymer nanoparticles (PNPs) offer safe and resorbable capabilities for delivering mRNA or pDNA cargos in an economical way to achieve tissue targeted expression of transgenes. Nanite's proprietary AI-powered platform, SAYER™, rationally designs polymers across hundreds of thousands of collected datasets for effective mRNA or pDNA delivery to target tissues. Here we present optimally designed polymer candidates that can co-package mRNA as well as pDNA within a single nanoparticle, achieving >80% encapsulation efficiency. We also report successful transfection of dual cargo to human bronchial epithelial cell lines, and primary human basal cells with minimal cytotoxicity. The unique ability of Nanite's PNPs to co-deliver mRNA and pDNA into the same cell allows tunable and combinatorial gene expression control that is important across the gene and cell therapy landscape, including gene editing and gene silencing with simultaneous protein replacement, thereby offering competitive advantages over LNP and viral-based delivery systems.

704 Engineering Fusion Proteins to Enhance Efficacy of Adoptive Cell Therapy for Solid Tumors

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Adoptive cell therapy (ACT) is a "living drug" that provides the opportunity to genetically modify T cells to recognize and kill tumors. While this therapy has shown incredible curative potential in hematologic cancers, engineered immune cells can struggle to overcome immunosuppressive barriers in solid tumor microenvironments (TME), which has necessitated the development of novel engineering strategies. The TME presents obstacles that reduce T cell persistence and function, including hypoxia, upregulated inhibitory/death ligands, and immunosuppressive immune cells that promote tumor progression. Here, we describe the development of novel engineered fusion proteins called transform switch receptors (TSRs), that promote T cell persistence and antitumor efficacy against solid tumors by converting inhibitory signals to stimulatory signals to enhance T cell function. We developed Fas-TSRs that combine a negative receptor "decoy" ectodomain (Fas) with a toolbox of different costimulatory endodomains. FasL is a death receptor ligand highly expressed in many cancers that induces T cell apoptosis via FasL/Fas interaction and is a known limiting factor for ACT. We evaluated an array of novel Fas-TSRs in a murine model of pancreatic cancer and identified a leading Fas-TSR candidate that significantly outperformed all costimulatory domains tested – including the Fas-4-1BB that is currently in clinical trials. In preliminary studies, our leading Fas-TSR candidate significantly enhanced survival and improved tumor control *in vivo*. Fas-TSR T cells showed enhanced expansion in the blood, greater poly-functional cytokine expression, and reduced expression of markers associated with T cell exhaustion in the tumor. *In vitro*, Fas-TSR T cells showed enhanced proliferation and function under hypoxic conditions, demonstrating increased function in the immunosuppressive TME. Collectively, these data show that the Fas-TSR engineering strategy provides a safe and durable antitumor T cell response that significantly improves therapeutic efficacy against solid tumors. These data provide novel insights in ACT pre-clinical models to support the advancement of novel Fas-TSRs to clinical trials.

705 DNA delivery by lipid nanoparticles induces CD8$^+$ T cell-dependent antitumor responses and synergizes with anti-PDL1 antibodies in mouse models of hepatocellular carcinoma

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Background: The introduction of immune checkpoint inhibitors (ICIs) into frontline therapy has reshaped the treatment landscape of hepatocellular carcinoma (HCC). However, only a small subset of patients benefit from these therapies. Nucleic acid-based therapies, particularly those utilizing lipid nanoparticles (LNPs) as delivery vehicles, offer promising avenues for cancer treatment. In this study, we hypothesized that intracellular delivery of non-coding double-stranded DNA (dsDNA) via LNPs can activate innate immune signaling pathways to induce antitumor immune responses and potentiate ICI.

Methods: DNA-LNPs were formulated by combining promoterless plasmid and lipid components using a microfluidic device. Their antitumor efficacy, as monotherapy or in combination with anti-PD-L1 antibodies, was interrogated in two mouse models of HCC. The first involved genetic induction of HCC through hepatic overexpression of c-Met and ΔN90-β-catenin using the Sleeping Beauty transposon/transposase system. The second model involved chemical induction of HCC using diethylnitrosamine (DEN). Serum cytokine levels were measured 4 hours after DNA-LNPs intravenous administration. At study endpoints, livers were analyzed for macroscopic changes, immunohistochemistry staining, and gene expression. Survival outcomes were also assessed.

Results: Treatment with DNA-LNPs triggered a rapid, dose-dependent increase in serum cytokines including IFN-γ, TNF-α, IL-6 and IFN-β. This immune activation led to the recruitment of $CD8^+$ T cells to the tumor microenvironment, which mediated a significant antitumor effect as indicated by increased survival and reduced liver-to-body weight ratios in the genetically induced HCC model. The antitumor efficacy was abolished by $CD8^+$ T cell depletion. Transcriptomic analysis revealed downregulation of pathways associated with HCC progression in treated mice, alongside reduced expression of the oncogenes, β-cat and c-MET, and the proliferation marker Ki67, underscoring the therapeutic impact of DNA-LNPs. No cytokine elevation or antitumor effects were observed after administration of empty LNPs, suggesting that these responses were triggered by the activation of cytosolic DNA-sensing pathways. DNA-LNPs also demonstrated robust antitumor activity in the chemically induced HCC model, as evidenced by a significant reduction in tumor nodule count at the endpoint. The combination of DNA-LNPs with anti-PDL1 antibodies resulted in a synergistic effect, with 90% of treated mice remaining disease-free at the endpoint, compared to 20% and 50% with either treatment alone, respectively.

Conclusions: DNA-LNPs represent a promising therapeutic strategy for HCC, capable of eliciting potent antitumor immune responses. Their efficacy, particularly in combination with ICI, highlights their potential for clinical translation in the management of HCC.

706 Successful Treatment of Recurrent Respiratory Papillomatosis with INO-3107 is Irrespective of Papilloma Microenvironment and Molecular Subtype

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Introduction: Recurrent respiratory papillomatosis (RRP) is a chronic disease of the airway characterized by recurrent papilloma growth caused by Human Papillomavirus (HPV) infection. The current standard of care consists of multiple surgeries, which often lead to lasting damage to the airway and impaired vocal function. Treatments that minimize or eliminate the necessity for these surgeries while ideally targeting the known etiological agents of RRP (HPV-6 and/or HPV-11) are thus an area of need. Various molecular aspects of papilloma tissue are known to have immune suppressive/regulatory activity, inclusive of high viral load, cytokine/chemokine expression profiles and molecular subtype status. Such aspects may present hurdles to efficacy of an immune

therapy based on T cell activity. Here we report successful clinical treatment of RRP irrespective of these variables in a Phase 1/2 trial of INO-3107, a DNA therapy designed for the treatment of adults with RRP (NCT04398433).

Methods: INO-3107 was administered on study Weeks 0, 3, 6 and 9. Formalin-fixed paraffin-embedded papilloma tissue obtained prior to treatment was assessed by RNA sequencing. Data analyses were performed via R and Gene Set Enrichment Analysis (GSEA) platforms. Patients were classified as clinical responders if the frequency of required surgery during the 52-week trial was lower than the 52-week period prior to treatment.

Results: The overall clinical response for the trial was 81.3%. Pre-treatment papilloma tissue exhibited diverse viral loads in both HPV-6 and/or HPV-11 infected patients, and reductions in surgical interventions were observed irrespective of either HPV type or viral replication. GSEA driven assessment of pre-treatment papilloma tissue did not identify significant elevations of hallmark Interferon Alpha (IFNa) signaling or Inflammatory modules in patients who responded clinically as compared to those who did not. Likewise, individual assessment of cytokines such as IL-1b, IL-15 and IL-18 did not show elevations specific to patients who exhibited clinical responses, nor did expression of chemokines such as CXCL9 or CXCL10. Regression analyses of VEGFA and PD-L1 expression versus change in surgical intervention while on trial did not reveal a significant correlation. Additionally, categorization of pre-treatment papilloma tissue into Basal (immune permissive) or Differentiated (immune exclusionary) molecular subtypes did not trend with clinical benefit.

Conclusions: Reduction in surgical interventions for the management of RRP after immunotherapy were not contingent on low viral loads, a pre-inflamed papilloma status or reduced expression of modulators such as VEGFA or PD-L1. Presence of a Differentiated molecular papilloma subtype did not preclude clinical efficacy. Taken together these data indicate that INO-3107 was able to effectively reduce surgical burden in RRP patients irrespective of papilloma microenvironment and molecular subtype.

707 Platelet-hitchhiking Delivery of MSA-2 and Mn^{2+}-loaded Nanoparticles for Preventing Post-surgical Tumor Recurrence

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As a critical innate immune activation pathway in cancer immunotherapy, the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway enhances anti-tumor responses by promoting the release of type-I interferons (IFN) and other proinflammatory cytokines. MSA-2, an orally administered small molecule STING agonist, binds to human and mouse STING through dimer formation. However, systemic administration of MSA-2 requires high doses, which may cause off-target toxicity and limit its clinical application. Meanwhile, manganese ions (Mn^{2+}) have been shown to synergistically enhance STING activation when combined with STING agonists. Based on that, we propose to co-deliver Mn^{2+} and MSA-2 using spontaneously formed nanoparticles that could increase local drug concentration and amplify anti-tumor immune responses. Inspired by the wound-targeting ability of platelets, we hypothesized that platelet-hitchhiking delivery of MSA-2-loaded nanoparticles to the post-surgical tumor bed would improve drug accumulation at the tumor site, thereby preventing recurrence.

Here, we synthesized Mn^{2+}-coordinated tannic acid (TA) nanoparticles for MSA-2 delivery using a one-pot method. TAs serve as crosslinkers and anchoring sites for attaching nanoparticles to platelet membranes *via* polyphenol groups. We demonstrated that co-delivery of Mn^{2+} and MSA-2 through nanoparticles induced a stronger type-I IFN release compared to free-form drugs. Additionally, the TA-Mn-MSA-2 nanoparticles exhibited high drug-loading efficiency and pH-responsive disintegration, allowing controlled drug release. When administered intratumorally, these nanoparticles significantly enhanced dendritic cell (DC) maturation, T cell infiltration, and activation, leading to the efficient tumor eradication in the mouse CT26 tumor model. In an incomplete surgery model, particle-loaded platelets effectively targeted tumor residues and induced tumor regression. Our findings offer a promising alternative for systemic delivery of STING agonists to prevent post-surgical tumor recurrence.

708 Ex Vivo Modified Extracellular Vesicles as a Novel Immunotherapeutic Strategy in Glioblastoma

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Introduction: Despite significant advancements, the effectiveness of immunotherapy in treating glioblastoma remains unsatisfactory. Oncolytic virus (OV) therapies can kill tumor cells and stimulate the immune system. However, they face challenges posed by the tumor microenvironment. Our research investigates the use of extracellular vesicles (EVs) from glioblastoma stem-like cells (GSCs) following OV infection (EVOV) as vehicles to transport tumor antigens and activate immune signals.

Methods: We developed a spheroid model to assess EVOV's impact on immune responses by co-culturing GSCs with immune cells pre-treated with EVs or EVOVs. Patient-derived GSCs (n=6) were infected with OV (MOI 0.1), and 12 hours later, EVs/EVOVs were isolated and analyzed by Ultra Deep DIA-MS, then used to treat PBMCs. Immune responses and cytokine production were measured using multiparameter flow cytometry. Treated PBMCs were co-cultured with GSC spheroids for three days and analyzed for immune cell infiltration using Rarecyte™ technology and multiparameter flow cytometry. In a final in vivo study, humanized NSG mice (n=16) were injected with PBMCs and GSCs, with GSCs injected into the brain to create a glioblastoma disease model, and the ability of EVOV to activate immune cells circulating in the blood and recruit them to tumors was evaluated.

Results: Our findings revealed significant alterations in the EVOV proteome/peptidome profile upon OV infection. Bioinformatic analysis identified deregulated immunopeptides/proteins (n=879 and 2156; $p<0.05$, FC>1.5) linked to immune responses. Functional tests demonstrated that EVOV significantly enhanced cytokine production (IL-12p70, IFN-γ, MCP-1), activating NK cells, DCs, CD4+ and CD8+ T cells. EVOV-pre-treated PBMCs co-cultured with GSCs had increased activated immune cells (NKT, CD8+ T, and NK cells) and monocytes switching to DCs. The co-culture setup also demonstrated a higher percentage of GSC death when exposed to EVOV-pre-treated PBMCs, emphasizing the cytotoxic potential of this approach. UMAP analysis highlighted distinct activation states induced by EVOV. Using Rarecyte™, we observed significant infiltration of CD8+ T cells expressing granzyme B and TBX21 in tumor spheroids treated with EVOVs. Notably, these spheroids exhibited lower levels of PD-L1+ CD11C+ cells, indicating a reversal of the immunosuppressive microenvironment.

In a humanized NSG mouse model, EVOV treatment reduced CD8+ PD1+ CD25+ T cells and increased activated helper T cells in the blood, indicating enhanced immune responses and decreased T-cell exhaustion. The cytometric analysis also showed a more structured CD3+ T-cell population in the EVOV group, reflecting targeted activation in response to tumor antigens in EVOV.

Conclusions: Our findings suggest that EVOVs represent a promising platform for glioblastoma immunotherapy, potentially overcoming immune evasion mechanisms characteristic of glioblastoma and offering a new avenue for durable therapeutic responses. Thus, this approach can potentially establish similar therapies for other hard-to-treat cancers.

709 Safety and efficacy evaluation of retroviral vectors GEN-1018 and GEN-1013 toward clinical translation for *in vivo* immuno-gene therapy and combined suicide gene therapy

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Interleukin-12 (IL-12) is a potent anti-tumor cytokine which enhances tumor infiltration and cytotoxic activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTL), activates T_H-1-type immune responses [1-2], and exhibits anti-angiogenic properties [3]. However, systemic administration of IL-12 as a biologic therapy has never achieved approval due to life-threatening toxicities, primarily mediated by induction of IFN-γ overproduction [4-6]. IL-12 gene therapy represents an alternative therapeutic strategy which can be combined with prodrug-activated suicide gene therapy approaches. Our previous preclinical studies supported initiation of clinical trials for *in vivo* cancer gene therapy with GEN2, a retroviral vector for combined delivery of granulocyte-macrophage colony stimulating factor (GM-CSF) and an enhanced gain-of-function variant of the herpes simplex virus thymidine kinase suicide gene (HSV-eTK). Here, we performed a preliminary evaluation of GEN-1018, a retroviral vector encoding both p35 and p40 subunits of human IL-12 (hIL-12), and GEN-1013, encoding both HSV-eTK and IL-12, as well as corresponding surrogate vectors encoding murine IL-12 (mGEN-1018 and mGEN-1013, respectively).

Preliminary evaluation of therapeutic efficacy was performed by intratumoral (IT) or systemic (IV) administration of mGEN-1018 and mGEN-1013 to subcutaneous CT26 murine colorectal cancer models in syngeneic BALB/c mice, with mGEN-1013 experimental arms also treated with ganciclovir (GCV) prodrug. General health, body weight, and tumor growth were monitored over time, followed by immunophenotyping of peripheral blood, spleen, and tumor infiltrating immune cells. Additionally, preliminary safety evaluation of GEN-1013 was performed with pre-transduced A375 subcutaneous human melanoma models in immunodeficient athymic nude mice, enabling measurement of serum hIL-12 levels produced by a predetermined percentage of vector-transduced tumor cells.

Preliminary efficacy data showed that in the aggressive CT26 murine colorectal cancer model in immunocompetent mice, both IT and IV administration of mGEN-1018 and mGEN-1013 induce local and systemic antitumor immune responses by stimulating production of IFN-γ and Granzyme B from NK cells and CTL. Combined gene delivery of both mIL-12 and HSV-eTK by mGEN-1013 followed by GCV prodrug resulted in increased survival compared to control animals. Antitumor effects of systemic IL-12 gene therapy were not associated with visible signs of toxicity, as indicated by lack of significant body weight loss. In the preliminary safety study, increasing serum hIL-12 levels observed in the GEN-1013 pre-transduced A375 tumor model correlated with increasing tumor size over time. However, at all assessed time points, serum hIL-12 concentrations were found to be within the range previously reported to be well-tolerated in humans and non-human primates.

Overall, our results indicate the potential for in vivo gene therapy with retroviral vectors delivered by systemic or IT routes gene delivery to elicit a potent anti-tumor response through antigen release from cancer cells killed by HSV-eTK/GCV suicide gene therapy, leading to anti-tumor CTL responses that are further enhanced by IL-12-mediated activation of both CTL and NK cells. Based on these results, GEN-1013 is currently being further evaluated in definitive preclinical studies to support clinical translation and Investigational New Drug (IND) application.

References

1. Mazzolini G et al., Gene therapy of cancer with interleukin-12. Curr Pharm Des 2003; 9(24):1981-91.
2. Lasek, W et al., Interleukin 12: Still a promising candidate for tumor immunotherapy? Cancer Immunol Immunother. 2014; 63:419–435.
3. Kerbel RS and Hawley RG, Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors. J Natl Cancer Inst. 1995; 87:557-586.
4. Car, BD et al., The toxicology of interleukin-12: review. Toxicol Pathol. 1999; 27:58–63.
5. Atkins, MB et al., Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. Clin Cancer Res. 1997; 3:409–417.
6. Leonard, J et al., Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood. 1997; 90:2541–2548.

710 Generation of allogeneic CAR-NKT cells from hematopoietic stem and progenitor cells using a clinically guided culture method

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Cancer immunotherapy utilizing autologous chimeric antigen receptor (CAR) T cells encounters challenges in manufacturing and patient selection that could be circumvented by employing 'off-the-shelf' products, such as allogeneic CAR natural killer T (^{Allo}CAR-NKT) cells. Previously, we reported a system for differentiating human hematopoietic stem and progenitor cells into ^{Allo}CAR-NKT cells; however, the reliance on three-dimensional culture and xenogeneic feeders limited its clinical applicability. In this study, we describe a clinically guided method to differentiate and expand IL-15-enhanced ^{Allo}CAR-NKT cells, achieving high yield and purity. We generated ^{Allo}CAR-NKT cells targeting seven different cancers and demonstrated their antitumor efficacy, expansion, and persistence in a multiple myeloma model. These cells selectively depleted immunosuppressive cells within the tumor microenvironment and counteracted tumor immune evasion through triple targeting via CAR, TCR, and NK receptors. Additionally, they exhibited a stable hypoimmunogenic phenotype, which is associated with epigenetic and signaling regulation, and did not induce detectable graft-versus-host disease or cytokine release syndrome. These favorable characteristics of ^{Allo}CAR-NKT cells underscore their potential for clinical translation.

711 Allogeneic CD33-directed CAR-NKT Cells for the Treatment of Bone Marrow-resident Myeloid Malignancies

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Chimeric antigen receptor (CAR)-engineered T cell therapy holds promise for treating myeloid malignancies; however, challenges remain in bone marrow (BM) infiltration and targeting BM-resident malignant cells. Current autologous CAR-T therapies also face manufacturing and patient selection issues, underscoring the need for off-the-shelf products. In this study, we characterize primary patient samples and identify a unique therapeutic opportunity for CAR-engineered invariant natural killer T (CAR-NKT) cells. Using stem cell gene engineering and a clinically guided culture method, we generate allogeneic CD33-directed CAR-NKT cells with high yield, purity, and robustness. In preclinical mouse models, CAR-NKT cells exhibit strong BM homing and effectively target BM-resident malignant blast cells, including CD33-low/negative leukemia stem and progenitor cells. Furthermore, CAR-NKT cells synergize with hypomethylating agents, enhancing tumor-killing efficacy. These cells also show minimal off-tumor toxicity, reduced graft-versus-host disease and cytokine release syndrome risks, and resistance to allorejection, highlighting their substantial therapeutic potential for treating myeloid malignancies.

712 Reinvigoration of Exhausted T Cells Towards Improved Immunotherapy

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Cellular immunotherapies, including adoptive T cell transfer and genetically engineered T cell therapies, have transformed the treatment of hematologic malignancies and are showing increasing promise in solid tumors. However, the efficacy of these therapies is often compromised by **T cell exhaustion**, a dysfunctional state characterized by progressive loss of cytotoxicity, proliferation, and cytokine production. While immune checkpoint inhibitors (ICIs) can reinvigorate progenitor-exhausted T cells (T_{pex}) in some cancers, ICIs have shown little efficacy in hematologic malignancies despite their susceptibility to T cell immunity. Since **terminally exhausted** T cells are

thought to be refractory to checkpoint blockade, we sought to discover novel means of reversing deeply exhausted T cells.

To address this challenge, we developed a **CRISPR screening approach specifically designed to uncover genes that reverse T cell exhaustion**, rather than merely prevent its onset. A key innovation of this work is the ability to genetically modify **already-exhausted human T cells**, which required the development of a robust and tractable in vitro model of human T cell exhaustion. In this system, naïve human CD8+ T cells were redirected against a B cell acute lymphoblastic leukemia (B-ALL) cell line using a commercially available anti-CD19 × anti-CD3 bispecific T cell engager (BTE) and were subjected to repeated antigenic stimulation every 48 hours until they exhibited hallmark features of exhaustion, including loss of cytotoxicity, proliferation, and cytokine production (approximately 20 days). This model was then leveraged to perform functional genetic screening, employing both a pooled CRISPR sgRNA library targeting ~240 epigenetic modifiers via lentiviral transduction and a novel, non-viral cell-penetrating peptide (CPP)-mediated CRISPR/Cas9 system.

As a proof-of-concept, we first demonstrated efficient single-gene knockout in highly exhausted T cells. We then applied our CRISPR screen in "**exhaustion prevention" mode**, recovering known regulators of exhaustion such as components of the SWI/SNF chromatin remodeling complex, validating the fidelity of our model. More importantly, using our system in "**exhaustion reversal" mode**, we identified novel genetic hits, including genes known to enhance T cell persistence, as well as previously uncharacterized regulators of T cell function.

By identifying genes that may actively restore function in exhausted T cells, we hope to gain new insights into the molecular mechanisms of exhaustion reversal. Such findings could shift the focus from exhaustion prevention toward active rejuvenation of dysfunctional T cells, opening new therapeutic avenues to enhance the durability and efficacy of adoptive T cell therapies.

713 Vectorized Interferons Provide a Robust and Sustained Transcriptional Response Over Traditional Non-Vectorized Recombinant Cytokine Therapies in Human High-Grade Glioma Cells

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BACKGROUND: Interferons are pleiotropic immunomodulatory cytokines that drive anti-tumor functions and show promise as cancer therapies. However, their short half-lives limit their ability to mount sustained anti-tumor responses. Siren Biotechnology has developed an AAV immuno-gene therapy platform that delivers vectorized engineered interferons to overcome half-life challenges seen with traditional cytokine therapies. Our first program with this new modality targets high-grade gliomas (HGGs), aggressive brain cancers with no effective therapeutic options. We developed an *in vitro* model to measure cytokine durability and transcriptional responses in human HGG cells. Here, we compared the transcriptomic response of HGG cells when treated with a pulse of recombinant human interferon beta (hIFNβ) cytokines versus vectorized hIFNβ.

METHODS: Two human HGG cell lines (GBM6-FL and U251-FL) were treated with either an AAV carrying an engineered human IFNβ (AAV9-hIFNβ, 4e5-5e5 vg/cell), AAV9-GFP (4e5-5e5 vg/cell), recombinant hIFNβ protein (114-158 pg/mL), or media alone followed by a 24h washout period. Cell culture media and cells were collected 24, 48, 72, and 96h post-treatment for quantification of hIFNβ protein and mRNA purification, respectively. Bulk transcriptomic profiling was performed by RNA-sequencing on an Illumina NovoSeq 6000 with 150 bp paired-end reads. Reads were aligned to the human genome (GRCh38.p14) using STAR v2.7.11b and counted using subread v2.0.6. Differentially-expressed genes (DEGs) were identified relative to media alone and were considered significant if p-adj<0.01.

RESULTS: As expected, treatment with recombinant hIFNβ resulted in media hIFNβ levels below detectable limits (<23 pg/mL) within 24h due to progressive treatment washout. However, cells treated with vectorized hIFNβ induced hIFNβ protein as early as 15h, with expression above 2,000 pg/mL within 48h. Despite daily media

exchanges, hIFNβ levels in the transduced HGG cells were sustained for the 96h time course, demonstrating that vectorized delivery can induce durable supraphysiological levels of hIFNβ expression, directly overcoming half-life challenges of protein-based cytokine therapy. Importantly, measured media levels of hIFNβ protein treatments corresponded with time-matched transcriptional effects in the HGG cells. Cells treated with vectorized hIFNβ exhibited a robust and durable hIFNβ response, evidenced by sustained and increasing DEGs for the entire experiment (96h). Conversely, the response induced by recombinant hIFNβ cytokine was short-lived, with peak DEGs at 24-48h (2,600-5,000 DEGs, p-adj<0.01), followed by a 20-80X DEG reduction by 96h (30-260 DEGs, p-adj<0.01) for each cell line. Importantly, Type I IFN and TNFa signaling pathways were the most significantly enriched pathways in both cell lines treated with recombinant hIFNβ and vectorized hIFNβ when compared to their media alone treatments (p-adj<0.01), suggesting an immunostimulatory and anti-tumoral transcriptomic response specifically induced by the hIFNβ payload, which was absent in the AAV9-GFP control treated cells.

CONCLUSION: Taken together, our data suggests that vectorized hIFNβ induces sustained hIFNβ expression and an IFN-specific anti-tumoral transcriptomic response in HGG cells. Excitingly, this data supports cytokine vectorization as a powerful new modality class that overcomes the half-life challenges seen with traditional cytokine immunotherapy approaches.

714 Hemato-Chimeric Mice Hosting Human Liver-Resident Macrophages for Translational Studies Using In Vivo Gene Therapy of Liver Metastases

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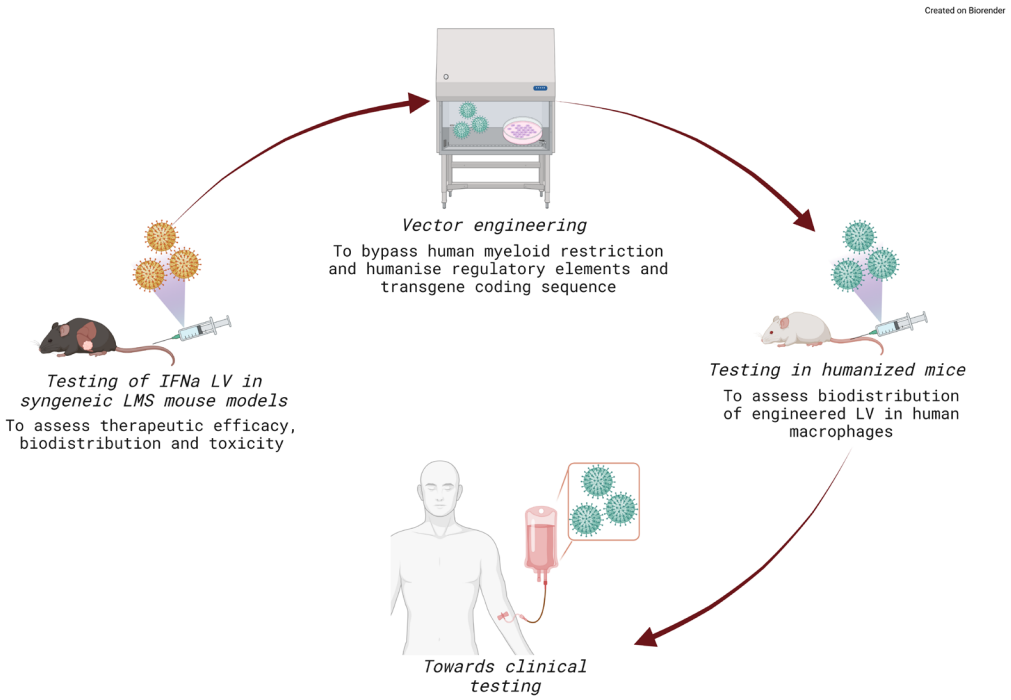
The liver hosts an immunosuppressive environment favoring occurrence of liver metastases (LMS), which render pharmacological treatments often ineffective, leading to poor patient prognosis. We have previously described an *in vivo* gene therapy platform, based on lentiviral vectors (LVs) to selectively engineer liver macrophages, including both liver-resident Kupffer cells (KCs) and tumor associated macrophages (TAMs), to secrete type I interferon (IFNα) within liver metastases. Selective transgene expression was achieved by a macrophage specific promoter and microRNA target sequences that, upon systemic delivery of the LV to mice, led to selective transgene expression in hepatic macrophages which was enhanced in areas surrounding LMS. Gene-based IFNα delivery caused reduced metastatic volume of both colorectal and pancreatic ductal adenocarcinoma liver metastases in mice. Therapeutic response was associated with macrophage immune activation, increased antigen presentation and increased CD8 T cell infiltration and cytotoxicity. In a subset of mice, we detected resistance to IFNα therapy, associated with increased IL-10 signaling, expansion of *Eomes* CD4+ T cells, a cell type displaying features of type I regulatory T (Tr1) cells, and CTLA-4. Combination of *in vivo* IFNα gene therapy with CTLA-4 immune checkpoint blockade expanded tumor-reactive T cells, attaining complete response in most mice. These findings support a promising therapeutic strategy with feasible translation to patients, however human-specific myeloid restriction to LV transduction poses a challenge. Among other mechanisms, SAMHD1-mediated interference with LV retrotranscription and integration in the human genome limits the potentiality of our platform. Here, we introduce a novel mouse model featuring human KCs, alongside LVs customized for human macrophage-specific transgene expression. We enabled the engraftment of human KCs in mice by administering *in vivo* LVs expressing human myeloid-specific growth factors, followed by transplantation of human hematopoietic stem cells. Compared at single cell-RNA level, human KCs predominated in the livers of hemato-chimeric mice and exhibited transcriptional profiles akin to patient-derived KCs. Additionally, we developed and characterized a human macrophage specific MRC1 promoter, which yielded significant transgene output, with heightened expression observed in M2-polarized macrophages. Engineered LVs, packaging the HIV-2 accessory protein VPX, bypassed SAMHD1 restriction enabling *in vivo* human KC engineering in humanized mice. The development of this humanized mouse model,

coupled with VPX-loaded LVs, represents a pivotal advancement for LV-based *in vivo* gene therapies. Humanised models not only enhance our understanding of LV-mediated transduction in human macrophages but also accelerate the translation of preclinical findings to clinical applications.



715 Recombinant Newcastle Disease Virus Expressing PTEN as a Novel Therapeutic Approach for Pancreatic Ductal Adenocarcinoma

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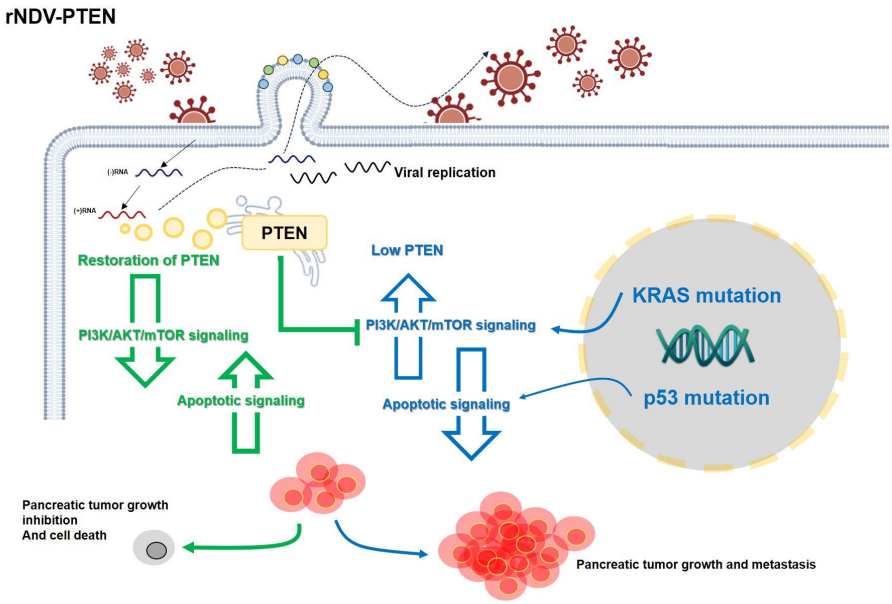
Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest and most treatment-resistant cancers, requiring innovative therapeutic approaches to improve outcomes. Genetic alterations such as KRAS mutations and reduced expression of the tumor suppressor PTEN are pivotal drivers of PDAC progression, leading to dysregulated PI3K/AKT/mTOR signaling and aggressive tumor behavior. To address these challenges, we developed a recombinant Newcastle disease virus engineered to express the PTEN gene (rNDV-PTEN). In vitro, rNDV-PTEN infection of KRAS-mutated PDAC cells significantly restored PTEN expression, suppressing PI3K/AKT/mTOR signaling, inducing apoptosis, and inhibiting cancer cell proliferation. *In vivo*, treatment with rNDV-PTEN effectively reduced tumor growth in orthotopic xenograft models, prolonged survival, and activated apoptotic pathways in PANC-1 cells. Importantly, rNDV-PTEN exhibited mild immune response without abnormal changes in blood glucose, triglycerides, or cholesterol levels. These findings highlight rNDV-PTEN as a dual-action oncolytic virotherapy and gene therapy, offering a novel, safe, and effective treatment option for PDAC patients with highly activated PI3K/AKT/mTOR signaling. This study provides a strong foundation for further clinical development of rNDV-PTEN in combating the devastating burden of pancreatic cancer.



716 More is Less: Cytotoxic Activity of Oncolytic Adenovirus Influences Therapeutic Efficacy in Disseminated Lung Cancer

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Oncolytic viruses have emerged as a promising therapeutic approach for cancer patients who fail to respond to existing treatments. We developed AVID-317, a novel engineered oncolytic adenovirus with targeted capsid mutations that significantly improved safety following systemic administration. In a preclinical model, systemic administration of AVID-317 (5×10^{10} v.p. per mouse) to NCr/Nude mice with disseminated lung adenocarcinoma significantly slowed tumor progression and extended survival compared to controls, with approximately 25% of mice achieving complete tumor rejection. However, dose escalation of AVID-317 did not yield additional therapeutic benefits. Instead, tumor-bearing mice treated with higher doses exhibited reduced median survival.

To investigate the mechanism underlying the reduction in median survival following dose escalation, we established an *in vitro* model using single-cell suspensions derived from the lungs of tumor-bearing mice. Cells were treated with escalating doses of AVID-317 and analyzed 48 hours post-infection. Flow cytometry revealed dose-dependent tumor cell elimination; however, pro-tumorigenic factor PD-L1 expression was upregulated in non-CD45+ stromal cells in a dose-dependent manner. Furthermore, inflammatory cytokines and tumor-promoting growth factors in the culture media increased with higher virus doses. These findings suggest that tumor cell killing by AVID-317 activates cellular components in the tumor microenvironment, which in turn produce pro-tumorigenic factors, leading to accelerated tumor growth.

To further dissect the mechanism of AVID-317-mediated tumor suppression and to design more effective therapies, we analyzed disseminated tumors from the lungs in NCr/Nude mice using single cell RNA-seq and Mass Cytometry (Cy-TOF) following virotherapy. scRNA-seq analysis revealed that in a subset of mice responding poorly to AVID-317 therapy, tumor-associated CSF1R-expressing myeloid cells expressed higher levels of MHC-II, Ctsb, and Arg1. Additionally, CD3e+ T cells from these mice expressed the exhaustion marker PD-1, a feature not observed in mice with favorable responses to virotherapy. Cy-TOF analysis using 37 metal isotope-labeled antibodies further confirmed increased expression of activation markers on myeloid cells following virotherapy. Based on these findings, we designed a combination therapy strategy to overcome immune suppression and enhance efficacy. In addition to AVID-317 administration, tumor-bearing mice were treated with anti-PD-1 antibody to block immune checkpoint signaling, anti-CSF1R antibody to inhibit CSF1R signaling, and anti-Asialo GM1 antibody to deplete antiviral natural killer (NK) cells.

Overall, our study uncovers complex mechanisms that limit the therapeutic efficacy of adenovirus vectors in treating disseminated cancer and demonstrates the potential of combining virotherapy with immunotherapy to improve outcomes.

Conflict of interest statement: DSH is a co-founder, shareholder, and officer of AdCure Bio, LLC, which develops adenovirus technologies for therapeutic use.

717 *In vitro* and *in vivo* efficacy of novel "armed" oncolytic viruses derived from adenovirus species B for breast cancer therapy

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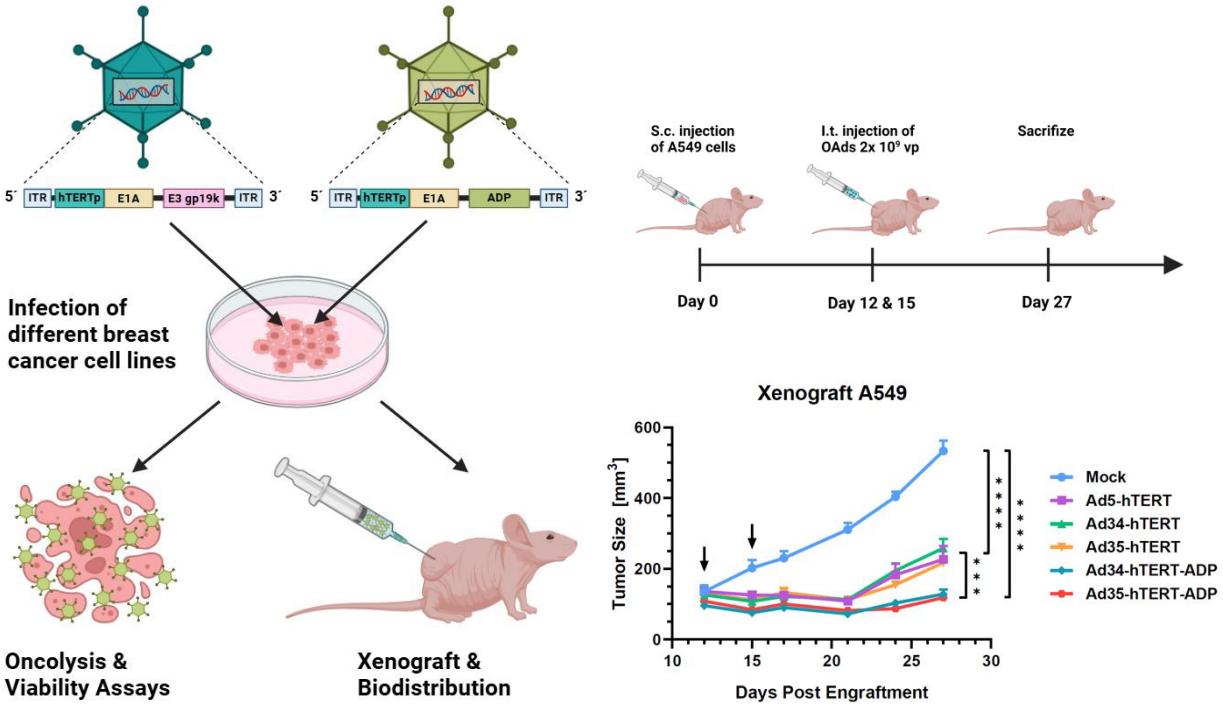
Oncolytic adenoviruses (OAds) are promising candidates for cancer therapy. Current studies focus on human adenovirus type 5 (Ad5), but robust therapeutic effects are hindered due to low expression of its main receptor, coxsackie and adenovirus receptor (CAR), on many tumor types and high seroprevalence among the population. Here, we explore species B Ads with low seroprevalence and different receptor usage. Based on our previous work, comparing several Ads regarding uptake and lysis of human breast cancer cell lines, two Ads were selected: Ad34 and Ad35, which utilize CD46 as receptor, and especially Ad34 with low seroprevalence and no binding to PF4. Here, the original Ad E1A replication unit promoter was replaced by the human telomerase reverse transcriptase promoter (hT). To obtain the strong lytic effect of Ad5 and to "arm" the non-Ad5 OAds, the adenovirus death protein (ADP) from Ad5 was inserted into the E3gp19k gene of Ad34 and Ad35 (hTA). As a control Ad5-hT was generated and all OAds were analyzed for infectivity, lytic effects and cell viability *in vitro* and explored *in vivo* in tumor-bearing mice.

The constructed OAds Ad5-hT, Ad34-hT, Ad35-hT, and Ad34-hTA and Ad35-hTA (ADP-OAds) were analyzed in four breast cancer cell lines (Hs578T and MDA-MB-231 (triple negative, TNBC), MCF-7 and SKBr3) and in A549 cells. To analyze replication of these novel OAds, TCID50 assays were performed. In SKBr3 and MCF-7 cells, ADP-OAds showed 50-fold higher plaque forming ability compared to Ad5-hT. In the TNBC cell lines, Ad5-hT and both ADP-OAds showed similar plaque forming units. For tumor cell viability, the ADP-OAds showed 30% and 75% reduced cell viability 3 days post-infection in SKBr3 and MCF-7 cells, respectively, compared to Ad5-hT which displayed no reduction in cell viability. For the A549 and TNBC cells, the ADP-OAds showed superior cell killing on day 5 compared to Ad5-hT. The results regarding lytic activity were confirmed by oncolysis assays. Next we performed neutralization assays with anti-human-Ad5 serum using 1/16,000 to 1/125 dilutions. Results revealed neutralization of Ad5 but not of Ad34- and Ad35-OAds, demonstrating that both Ad34 and Ad35 vectors are interesting candidates for *in vivo* applications. *In vivo* experiments in nu/nu NMR1/FoxN1 mice bearing subcutaneous A549 xenografts showed a significant decrease in tumor growth with the Ad34- and Ad35-based ADP-OAds compared to hTERT-only-OAds (Ad5-, Ad34-, Ad35-hT) 15 days after intratumoral application of 1x10e9 viral particles (vp) and 27 days post-engraftment. To examine if the OAds can reach the tumor site when injected intravenously, three additional OAds based on Ad5, Ad34 and Ad35 were generated encoding firefly luciferase (fLuc) driven by the SV40 promoter instead of E3gp19K transcription unit. Biodistribution experiments showed high replication activity in the tumors after intravenous injection of 1x10e9 vp as quantified by qPCR. Moreover, viral DNA for Ad34 and Ad35 was detected in lymph nodes in close proximity to the tumor, pancreas and spleen. To address the safety profile of the OAds and transduction properties in human blood cells, non-activated and activated T-cells from 9 donors were infected. 5 days post-infection no significant T-cell lysis was observed.

In summary, “armed” Ad34- and Ad35-hTERT-ADP OAds are promising candidates for breast cancer therapy that overcome Ad5 limitations by alternative cellular entry receptor usage and low seroprevalence.



718 Adenovirus Type 6 Expressing GM-CSF Exhibits Oncolytic Activity and Induces Antitumor Immunity in an Immunocompetent Syrian Hamster Model of Cholangiocarcinoma

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Cholangiocarcinoma (CCA), the second most common liver cancer, is highly resistant to chemotherapy and radiotherapy, leaving patients with unresectable tumors in urgent need of innovative therapies. Oncolytic adenoviruses (OAds) are a promising cancer treatment strategy due to their ability to induce direct oncolysis, activate the host's immune system, and their strong safety profile demonstrated in clinical trials. Adenovirus type 6 (Ad6), a species C human adenovirus, stands out for its low seroprevalence compared to a “standard” Adenovirus type 5 (Ad5) and its ability to evade Kupffer cells, enhancing its potential for liver cancer therapies such as CCA. To study oncolytic activity and antitumor immunity, an immunocompetent model permissive for OAd replication is essential. Syrian hamsters are one of the few such models, though limited by compatible cancer cell lines. Recently, a tumorigenic hamster CCA cell line, CCA-OF, was developed from tumors induced by Opisthorchis felineus infection. This cell line exhibits morphological characteristics and phenotypes similar to those of human carcinomas. In this study, we evaluated the efficacy of a novel Ad6-based vector, Ad6-d24-GM, against CCA in this immunocompetent, replication-permissive Syrian hamster model.

We have constructed Ad6-d24-GM to incorporate two critical modifications designed to enhance its therapeutic potential: 1) Expression of GM-CSF from the E3-6.7k/gp19k region, which stimulates both innate and adaptive

antitumor immune responses by recruiting and activating antigen-presenting cells; 2) A 24-bp deletion in the E1A gene, enabling tumor-selective replication and enhancing its specificity for cancer cells. We introduced a novel, efficient technology that leverages CRISPR-mediated genome digestion followed by in vitro recombination with a PCR product carrying the desired modifications.

Ad6-d24-GM exhibited dose-dependent cytotoxicity against the CCA-OF cell line, with reduced toxicity to normal human fibroblasts compared to wild-type Ad6 (Ad6wt). The secretion of GM-CSF was significantly higher in CCA-OF hamster cells infected with Ad6-d24-GM than in A549 cells at 24 and 48 hours post-infection. We further investigated in vivo antitumor effects of both Ad6-d24-GM and Ad6wt. Hamsters bearing CCA-OF subcutaneous tumors received 2 intratumoral injections of either Ad6-d24-GM, Ad6wt (3×109 TCID50 infection units), or PBS. The treatment effect was evaluated by comparing tumor growth rates using a linear mixed-effects model. Both viruses reduced tumor growth rate with only Ad6-d24-GM causing significant tumor growth inhibition. Furthermore, Ad6-d24-GM significantly increased the median survival to 34 days compared to 20 days in the PBS-treated group. Immunohistochemical quantification of immune markers revealed that both Ad6-d24-GM and wild-type Ad6 induced robust infiltration of CD4+ and CD8+ T cells and CD68+ macrophages in tumors, indicating activation of antitumor immunity 14 days after the first injection. Notably, the Ad6-d24-GM group exhibited a statistically significant increase in CD68+ macrophages compared to the Ad6wt, highlighting the immunomodulatory effect of the GM-CSF transgene.

These results demonstrate the oncolytic and immunostimulatory potential of Ad6-based vectors for CCA treatment and validate the Syrian hamster syngeneic CCA-OF model as a novel valuable platform for studying oncolytic adenovirus therapies.

719 Oncolytic adenovirus mediated antitumor immune response for the treatment of bladder cancer

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To overcome the limited therapeutic outcome of conventional treatments in non-muscle-invasive bladder cancer (NMIBC), oncolytic adenovirus (oAd) co-expressing interleukin 12 (IL12), granulocyte-macrophage colony-stimulating factor (GMCSF) and relaxin (RLX; oAd/IL12-GMCSF-RLX) with a clinical stage GSK3β inhibitor (9-ING-41; elraglusib; iGSK3β) was investigated. Our findings demonstrate that oAd/IL12-GMCSF-RLX and iGSK3β combination therapy (oAd/IL12-GMCSF-RLX+iGSK3β) exerted superior inhibition of tumor growth compared with respective monotherapy in a syngeneic NMIBC tumor model. oAd/IL12-GMCSF-RLX+iGSK3β induced superior degradation of tumor extracellular matrix (ECM) and antitumor immune response over the respective monotherapy. In detail, oAd/IL12-GMCSF-RLX+iGSK3β induced superior accumulation of intratumoral T and NK cells, prevention of immune cell exhaustion, and induction of tumor-specific adaptive immune response. Collectively, these results demonstrate that the combination of oAd/IL12-GMCSF-RLX and 9-ING-41 may be a promising approach to elicit a potent antitumor immune response against immunologically 'cold' bladder cancer.

720 Overcoming Barriers to Effective Cancer Therapy with Oncolytic Adenoviruses

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Oncolytic adenovirus vectors are an excellent platform for targeting cancer cells and enhancing the anti-tumor immune response. Adenoviral vectors have been used in clinical trials since the late 1990s. However, we still lack a universally effective oncolytic adenovirus to treat cancer. The answer lies in understanding the complex interplay of adenovirus and cancer biology, as well as immune system interactions.

To understand the molecular processes after adenovirus vector infusion, we used a nude mouse model with orthotopic disseminated xenografts in the lungs. This particular nude mouse line had low levels of T cell production, insufficient to mount an anti-tumor response, which allowed slow tumor growth, resulting in a complex cellular composition in the tumor sites. In this model, systemic intravenous treatment with oncolytic adenovirus AVID-317 cured 20% of treated mice, completely clearing cancer cells from the lungs. However, in 80% of tumor-bearing mice, tumor growth was largely unaffected, and the mice succumbed to cancer. Analysis did not show differences in tumor burden, nor were there differences in the amount of virus in the tumor-bearing lungs.

This led us to hypothesize that oncolytic virus-mediated cell death can cause a dysregulated response in tumor-associated cells, dictating treatment outcomes. If cancer cell death leads to an enhanced tissue repair response, cancer cells that escaped the virus proliferate, and the tumor progresses. However, if virus treatment repolarizes the tumor-associated cells to a non-tumor-supporting state, immune cells can clear the cancer cells.

To test this hypothesis, we compiled a signature list of genes known to enhance tumor progression as a "reparative call." The genes in the list included well-known mediators of cancer progression: Pdgfb, Vegfa, Igf1, Arg1, Tgfb1, Fgf, cytokines, chemokines and other genes. To understand which cellular populations were responsible for mounting this reparative call, we performed scRNA-seq on tumor-bearing lungs from mice treated with either buffer or AVID-317 oncolytic adenovirus. We selected mice exhibiting either a responder or non-responder phenotype at 2 weeks after virus infusion.

The scRNA-seq and flow cytometry analysis showed that the tumor cell composition in responder mice was similar to human lung tumor explants, with an abundance of myeloid, lymphoid, and stromal cells. In responder mice, expression of chemokines (Ccl3, Ccl5, Ccl6, Ccl9, Cxcl10) was lower, as was expression of Pdgfb, Vegfa, Tgfβ1, Fgf in stromal cells and macrophages. Wound healing markers like Arg1, Fn1, Chil3, Spp1, Retnla, and Psap were lower in various macrophage clusters. Moreover, lower expression of IL4, IL5, and IL13 in ILCs highlighted the role of these cytokines.

In conclusion, the major role of the reparative call response is played by the macrophage, and stromal compartments. In response to virus-mediated cancer cell death, these cells activate a reparative call that leads to tumor growth and disease progression. However, if these cells are silenced or absent, virus-mediated cancer cell death leads to complete clearing of tumor cells. Our study underscores the importance of the multi-lateral interactions of different immune and stromal cellular populations at the tumor site, highlighting the critical need to develop treatments targeting the myeloid and stromal involvement in the reparative call.

721 A Novel Method For Generating Unprecedented, Unbiased, Ultra-High-Sequence Diversity Libraries Of Plasmids And Recombinant Viruses (With Validation) For High-Throughput Screening, data for machine learning (AI) And Targeted Therapy.

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Background: A typical drug discovery pipeline needs good libraries of potential therapeutic candidates with high-sequence diversity. The probability of finding a good hit primarily depends on the quantity (diversity) and quality (distribution) of candidates in libraries used for screening. They include libraries of peptides, single-chain antibodies, nanobodies, CRISPRs, shRNAs, phage display, yeast display, Ribosome display, DARPins, BiKEs, TriKEs, CAR-T cells, and recombinant viruses (including adenoviruses, lentiviruses, AAVs, and other oncolytic viruses, etc.). Screening them for specific interaction with the target of interest to narrow down to fewer candidates, which can be further validated, are the basic steps in the generic discovery pipeline. Lately, artificial intelligence (AI)-based designing has also been used to design candidates for targeted interaction. However, the quality of such prediction depends on the data used in machine training. Since the current libraries are known to be biased, such libraries may not be as good as unbiased libraries.

Current limitations: Generating high-sequence diversity libraries is very challenging. Several published studies and industries claim to have very high diversity libraries ranging over 10^9 sequence diversity. However, most of those libraries lack validation by stringent experimental standards, and the diversity claimed is mainly based on sequencing a small number of candidates in the library and statistical extrapolations. Most of the time, we found that those who claim very high diversity (over 10^8) did not validate their libraries with stringent next-generation sequencing (NGS) based methods, and those who used stringent NGS methods for validation usually do not claim such big diversity. Therefore, there is an imminent need for a method to generate genuine high-sequence-diversity libraries of high quality.

Methods and results: We have developed a novel method to generate such libraries with very high sequence diversity and validated them with stringent NGS-based methods. Compared to other established methods, our libraries are at least 100 to 1000 times better in diversity (quantity) and have a more uniform and unbiased distribution (quality). Through benchmarking studies, we validated total diversity, duplication rate, sequence bias, and the ability to scale up. As a proof of concept, we generated libraries of plasmids (shuttle vectors to make recombinant adenoviruses) and recombinant adenoviruses with high sequence diversity in a structural protein (Fiber). We also used them for screening against a cancer-specific cell–surface protein.

Applications: Our methods can generate quantitatively and qualitatively better (100-1000 times) libraries. They can be used in high-throughput screening against targets of interest to narrow down to fewer candidates. Smaller libraries resulting after serial and multiple rounds of screening can serve as a dataset for machine training to aid AI-based design. Our unbiased, high-diversity libraries provide a high-quality dataset to improve Ai-based design criteria. We expect our libraries may help in avoiding AI-based design altogether in some cases.

722 Assessment of Oncolytic Properties of the Chimeric Poxvirus HOV-2 in Cholangiocarcinoma Models

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Introduction: Cholangiocarcinoma is a neoplasm of the biliary system and the second-most common primary hepatic malignancy. It has a highly unfavorable prognosis, regardless of type and location. Neoadjuvant chemotherapy has become mainstay, but five-year survival rates remain poor, prompting the need for more treatment options. Oncolytic viruses can selectively replicate in cancer cells and lyse them, representing a novel class of cancer therapeutics. We have developed a chimeric poxvirus that has enhanced oncolytic properties compared to other poxvirus strains. In this study, we evaluated the oncolytic properties of this chimeric poxvirus (HOV-2) in cholangiocarcinoma models.

Methods and Results: We infected four human cholangiocarcinoma cell lines (KMC-1, KMBC, KMCH and MzChA1) with the oncolytic virus HOV-2 at various multiplicities of infection and measured virus-mediated cytotoxicity at different timepoints. Dose- and time-dependent killing was observed in all four cell lines. Further analysis by flow cytometry revealed that the majority of cells died through apoptosis, as indicated by Annexin V staining. Next, we assessed the growth kinetics of the virus in these cell lines *in vitro*. While virus replication was observed in all cell lines, the levels of replication varied between them. The KMCH cell line supported the highest level of virus replication with 1e5-fold increase in virus titer 72 hours post-infection, while KMBC cells supported the lowest replication with only ~10-fold increase in virus titer over the same period. We tested the safety and therapeutic efficacy of this virus using murine models of cholangiocarcinoma. The KMBC and KMC-1 cell lines were used to generate two distinct xenografts models of cholangiocarcinoma in athymic nude mice. Bilateral subcutaneous tumors were generated by injecting the cells in either side of the lower flanks. Once the tumors reached 50-100 mm^3 in volume, mice were divided into four treatment groups (5 mice/group) such that each group had similar average tumor volume. Next, the mice were administered a single dose of virus (1e6 plaque forming units) either through intra-tumoral (I.T.) or intra-venous (I.V.) route. Tumor volume and weight of the mice were measured twice weekly. In the KMBC model, the I.V. treatment only resulted in mild delay in tumor growth whereas the I.T. treatment resulted in significant tumor regression. However, in the KMC-1 model, both I.T. and I.V. treatments were found to be equally efficient, resulting in almost complete tumor control. In a separate

experiment, mice treated as above, were euthanized on day seven post-infection and virus biodistribution was assessed using plaque assays. In all virus-treated groups, virus was detected in the tumors but was mostly undetectable in all normal organs.

Conclusions: The oncolytic virus HOV-2 can replicate within and kill human cholangiocarcinoma cells. In a human xenograft cholangiocarcinoma tumor model, HOV-2 causes significant tumor regression while remaining localized to the tumor microenvironment. These results signify that HOV-2 displays potential for treatment of patients with unresectable cholangiocarcinoma.

723 Using an Oncolytic Virus to Expand the use of GPC3-targeted Experimental Cancer Therapeutics

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Glypican-3 (GPC3) is a membrane-associated oncofetal protein that is overexpressed in hepatocellular carcinoma (HCC). In adult humans, GPC3 is not expressed in normal tissues making it an ideal target for HCC diagnosis and treatment. Several experimental therapeutics targeting GPC3 are currently under investigation, with some already in clinical trials. Among the most advanced GPC3-targeted therapies are GPC3-directed chimeric antigen receptor (CAR) T cells, bispecific T cell engagers (BiTEs), and the GPC3-directed monoclonal antibody, Condrituzumab. Although GPC3 expression is tumor-restricted, its presence within HCC tumors is heterogeneous, and many HCC patients are GPC3-negative. To address this, we have engineered an oncolytic virus capable of delivering a non-functional GPC3 to cancer cells. We hypothesize that this oncolytic virus could make GPC3-negative HCC tumors responsive to GPC3-targeted therapeutics. Additionally, this oncolytic virus could be applied to other solid tumors to render them susceptible to GPC3-targeted treatments. Our results show that the oncolytic virus effectively converts GPC3-negative tumor cells into GPC3-positive. The GPC3 expressed by the virus is specifically recognized by GPC3-targeted therapies, including GPC3 CAR-T cells, GPC3 BiTE, and condrituzumab. When GPC3 CAR-T cells were co-cultured with virus-infected cancer cells, they exhibited activation markers including cytokine secretion and demonstrated cytolytic activity.

Consequently, the combination of oncolytic virus and GPC3-CAR T cells significantly increased cancer cell killing compared to either treatment alone. Similarly, the bispecific T cell engager, composed of single-chain variable fragments (scFvs) targeting both GPC3 and CD3, activated T cells when co-cultured with virus-infected cancer cells, leading to potent tumor cell killing. Additionally, the monoclonal antibody Condrituzumab recognized the virus-encoded GPC3 on cancer cells and facilitated killing of virus-infected cancer cells by natural killer cells through antibody-dependent cellular cytotoxicity. More importantly, the combination of the oncolytic virus and GPC3-CAR T therapy improved therapeutic efficacy in murine tumor models. In conclusion, our findings indicate that GPC3 expression from an oncolytic virus can improve the effectiveness of GPC3-targeted therapeutics and broaden their potential applications.

724 A living CAR-T library combined with optofluidics and single cell sequencing for the rapid selection of functional CAR constructs.

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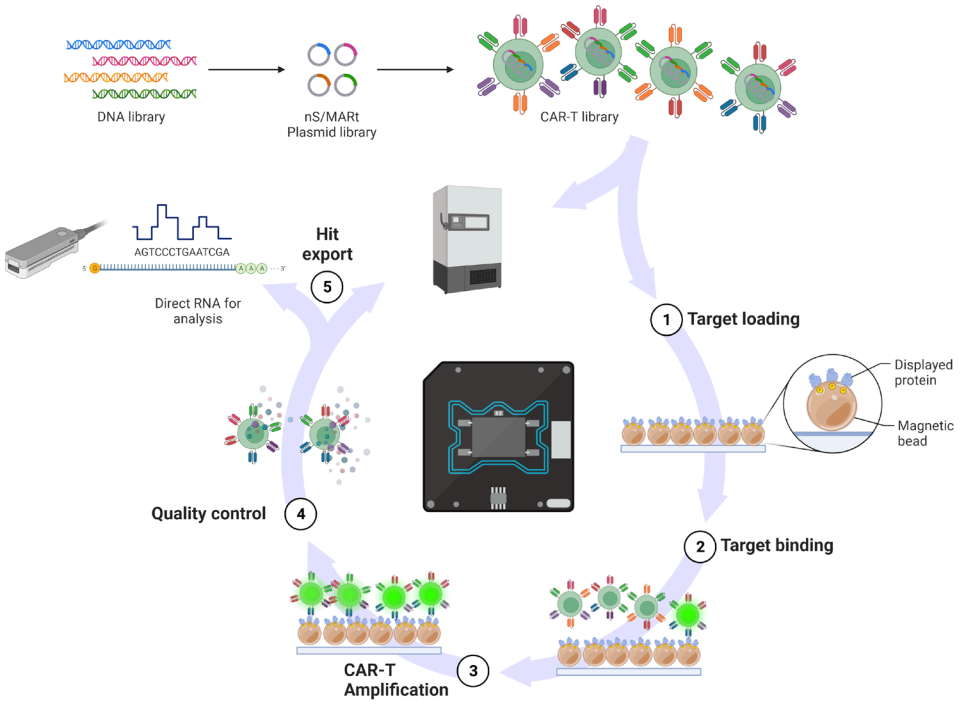
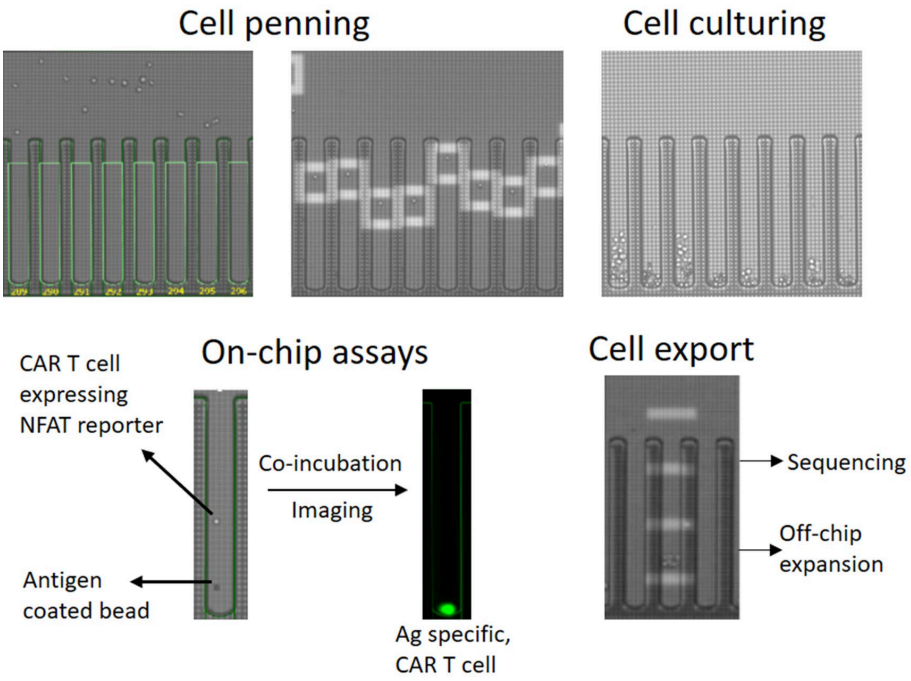
Chimeric antigen receptor (CAR)-T cell therapy has shown remarkable success in treating hematological malignancies, particularly B-cell leukemias and lymphomas, by addressing CD19 and BCMA as target. However, its application to solid tumors faces significant challenges that limit therapeutic efficacy under which antigen heterogeneity plays a prominent role. Recent advances in personalized medicine i.e. the exploitation of tumor neo-antigens as therapeutic target have opened an unprecedented window of opportunity to speed up and refine cancer treatments. Unfortunately, cellular therapies yet cannot hold pace with that development. Classically, CARs (i.e. the scFv binding domain) have been derived either from existing antibodies or are discovered via Phage-Display and subsequent CAR cloning, a process that can last up to several months.

Here, we propose an innovative platform, which can accelerate isolation of functional CAR constructs. In this approach, we generate a “living” fully human CAR library by engrafting a plasmid DNA library into a reporter cell line. Screening and selection of hits is performed on an optofluidic chip at single cell resolution using Bruker´s Lightning device. Oxford Nanopore sequencing of exported hits reveals the incorporation of less than 30 individual CAR constructs per single cell, which are accessible for validation. This novel method of quick identification and selection narrows the CAR discovery process down to maximum 14 days and therefore will allow the discovery of patient individual CAR-Ts for personalized treatment.





725 Enhancement of the Multimodal Cancer Therapeutic Efficacy in Non-Small Cell Lung Carcinoma by Hypoxia Reversal using Hemoglobin-Loaded Biomimetic Nanoparticles as Oxygen Delivery System

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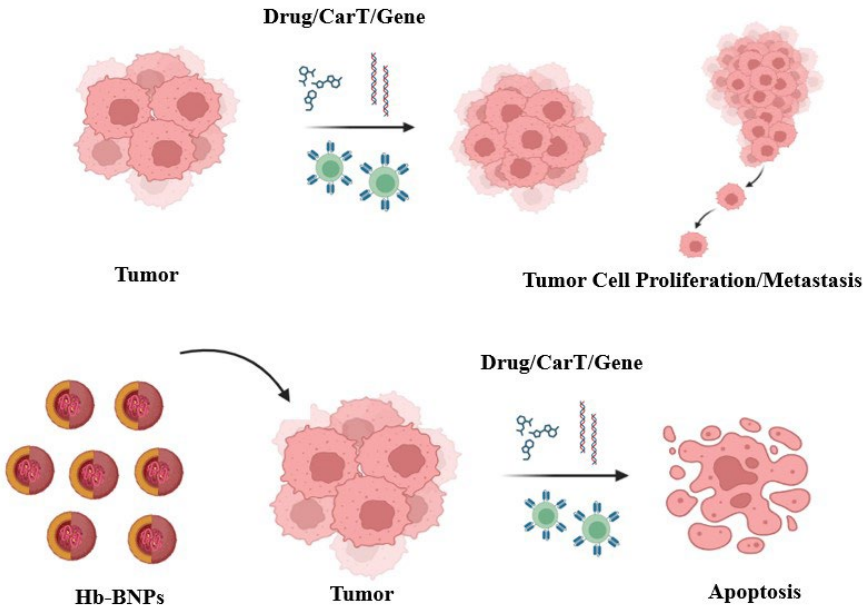
Cancer is a complex disease marked by uncontrolled cell growth that further leads to metastasis. Lung cancer results in most mortality globally, with non-small cell lung carcinoma (NSCLC) comprising about 85% of the cases. Tumor hypoxia generated by low oxygen levels in the tumor microenvironment poses a significant challenge, as it reduces the effectiveness of treatments like chemotherapy and immunotherapy. This further leads to the therapeutic resistance and poorer treatment outcomes. To address this challenge, we have developed cell membrane-camouflaged biomimetic nanoparticles loaded with natural oxygen carrier hemoglobin (Hb-BNPs) for controlled oxygen delivery to hypoxic tumor cores.

Our study demonstrated that the hypoxia-induced chemoresistance for the anticancer drug paclitaxel can be effectively reversed by delivering oxygen through Hb-BNPs, resulting in significantly improved therapeutic outcomes. Beyond chemoresistance, hypoxia in the tumor microenvironment also poses a major challenge for CAR-T cell therapy in solid tumors. Hypoxia impairs T-cell infiltration, persistence, metabolism, and effector function, all of which limit the therapeutic efficacy of these advanced immune therapies. To address this, we co-administered oxygen through Hb-BNPs alongside LunX CAR-T cells (an immunotherapy candidate for lung solid tumors). This strategy enhanced cytotoxicity under hypoxic conditions, effectively overcoming the functional limitations of immune therapies. Furthermore, hypoxia-induced reduced cellular uptake of gene and diminished transgene expression hinder therapeutic success of gene therapy. By delivering oxygen through Hb-BNPs, improved cellular uptake and transgene expression, can be achieved. Collectively, these findings underscore the broad potential of Hb-BNPs in overcoming hypoxia-driven limitations across multiple solid tumor therapies.

In addition to this, we have demonstrated and elucidated the role of KANK1, a gene associated with cytoskeleton organization in hypoxia. Our study revealed that KANK1 regulation can synergistically enhance the effectiveness of Hb-BNPs in chemotherapy and immunotherapy. By combining oxygen delivery through Hb-BNPs with KANK1

modulation, we are able to address both the oxygen deficit and the structural challenges within tumor cells. Hb-BNPs alleviate hypoxia, boosting oxygen-dependent therapies, while KANK1 modulation enhances cytoskeletal stability, increasing drug uptake and immune cell infiltration. Together, these mechanisms enhance therapeutic efficacy in hypoxic environments. This combined oxygen and KANK1 delivery approach holds the potential to reverse hypoxia-induced therapeutic resistance and enhance clinical outcomes in NSCLC patients.



726 Combining AI-Based Target Discovery with CRISPR/Cas9 Editing: Validating GBC-11004 as a Novel Target to Combat Osimertinib-Resistant NSCLC

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Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related mortality worldwide, accounting for approximately 85% of all lung cancer cases. Despite advances in early detection and treatment, the prognosis for advanced NSCLC remains poor due to metastasis and therapeutic resistance. To address this challenge, an advanced artificial intelligence (AI)-driven target discovery platform was employed to identify novel therapeutic targets from Osimertinib-resistant NSCLC patient-derived organoids (PDOs).

A novel kinase target, designated GBC-11004, was identified as one of the top-ranked target genes that were found to be overexpressed in PDOs resistant to Osimertinib. The selection of an appropriate delivery system for CRISPR/Cas9 cargo is a pivotal aspect in the advancement of gene editing for the treatment of diseases. Thus, four delivery systems were evaluated for their efficiency in introducing CRISPR/Cas9 into NSCLC PDOs: (1) plasmid-based transfection, (2) electroporation-based transfection, (3) lentiviral transduction, and (4) a newly developed delivery system. Following the optimization of the delivery system, CRISPR/Cas9-mediated gene editing was performed to assess the functional role of GBC-11004. Indel frequency was measured to confirm gene editing efficiency, and cell viability assays were conducted to determine the therapeutic impact of GBC-11004 inhibition.

Utilizing the most effective gene-editing platform identified, we successfully established PDOs with high efficiency, enabling precise functional validation of therapeutic targets. The present study has identified a new therapeutic target, GBC-11004, which has the potential to circumvent resistance to Osimertinib. In summary, the findings of this

study demonstrate the potential of integrating AI-driven target identification to advance therapeutic strategies in epidermal growth factor receptor-mutated NSCLC, providing a novel prospect for improved patient outcomes.

727 Lipid Nanoparticle Delivery of CRISPR/Cas9 Reagents for Disruption of Oncogenic Translocations in Alveolar Rhabdomyosarcoma

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Alveolar rhabdomyosarcoma (aRMS) is a rare and aggressive soft tissue cancer arising from skeletal muscle stem cells in children and young adults. This project leverages nanotechnology-based strategies to develop a lipid nanoparticle (LNP) platform designed to deliver CRISPR/Cas9 reagents configured to disrupt the PAX3-FOXO1 translocation that drives the most aggressive phenotypes of aRMS and mitigate its oncogenic effects. Here we present a two-pronged approach: first, designing and validating single guide RNA (sgRNA) sequences configured for the targeted knock out of PAX3-FOXO1 translocations; second, engineering LNPs for the efficient delivery of these gene-editing reagents for the direct therapeutic engineering of aRMS tumors. PAX3-FOXO1-targeting sgRNA sequences were first engineered by identifying conserved PAX3-FOXO1 fusion gene regions from ChimerDB's 4.0 fusion gene database and the UCSC Human Genome database. After targeting regions were identified, guides were then engineered via Benchling's guide design tool. Integrated DNA Technologies (IDT) guide design tool was then used to leverage on-target potential and off-target risk to maximize efficiency and minimize risk. Guide candidates were then tested in a representative PAX3-FOXO1 positive aRMS cell line (RH30) by electroporating Cas9 ribonucleoprotein complexes assembled with PAX3-FOXO1 sgRNA constructs and validated *via* qPCR analysis. Prior to packaging CRISPR/Cas9-based cargoes (*i.e.,* Cas9-mRNA and PAX3-FOXO1-targeting sgRNA), LNPs carrying green fluorescent protein (GFP)-encoding mRNA cargoes were applied to screen for nanoparticle formulations that maximize delivery to RH30 cells. Nanoparticles were prepared *via* microfluidic mixing with mean diameters <200 nm as measured by dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and cryo-transmission electron microscopy (cryo-TEM) with mRNA cargo encapsulation efficiencies >80% determined by Ribogreen fluorescence-based assay. Our best performing formulations achieved >90% GFP expression 24 h post treatment. In preliminary studies applying LNPs carrying PAX3-FOXO1 CRISPR/Cas9 cargoes, we observe a >50% reduction in PAX3-FOXO1 expression in targeted aRMS cells, as confirmed by qPCR. These findings highlight the potential for developing new CRISPR/Cas9 LNP-based cellular therapeutic strategies for directly augmenting high risk childhood malignancies through the manipulation of cancer driving mutations.

728 Tumoricidal Potential of CRISPR-Cas9 against Pancreatic Cancers

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Introduction: Pancreatic cancer (PC) is the third leading cause of cancer-related deaths in the U.S. with a dismal 5-year survival rate of 13%. Most patients present with advanced cancers at the time of diagnosis, and current treatment options are ineffective with most patients experiencing relapse or resistance. We have previously demonstrated the tumoricidal potential of CRISPR-Cas9 by targeting somatic mutations in PC cell lines. However, its ability to eliminate PC *in vivo* remains elusive. We hypothesize that CRISPR-Cas9 can eliminate PC *in vivo* by inducing multiple double strand breaks, provided that the cells receive the CRISPR-Cas9 therapeutic.

Methods: In a double-blind study, we transduced Panc10.05 Cas9-expressing cells with non-targeting sgRNA (NT), 715F(5) and 230F(12) sgRNAs (multi-target sgRNAs with 5 and 12 target sites in the human genome, respectively), ALU_112a sgRNA (positive control sgRNA targeting Alu repetitive elements), and a pool of 9 sgRNAs targeting 9 different noncoding mutations unique to Panc10.05 and absent in other cell lines (Panc10.05 pool, designed using a

novel protospacer adjacent motif-based, cancer-specific approach previously published[1]). After puromycin selection, we injected them into athymic, nude mice, and measured tumor growth and body weight for 6 weeks. Tumors were then surgically extracted and weighed. Genomic DNA extraction and next generation sequencing (NGS) were performed to detect CRISPR-Cas9 activity / mutations in tumors. Additionally, we developed an mNeonGreen-, Cas9-expressing Panc10.05 cell line, conditioned for *in vivo* growth, to screen lipid nanoparticles (LNPs) for *in vivo* transfection efficiency through intratumoral injection. We identified nine top LNP formulations from previous *in vitro* screening, and tested them in subcutaneous murine xenograft models using luciferase reporter assay. Tumors and organs (liver, spleen, and kidneys) were also harvested post-mortem to examine for off-target transfection. Flow cytometry was performed to identify PC cells in tumors.

Results: Tumor volumes decreased between NT and 230F(12) or Panc10.05 pool as early as week 4 (Dunn-Sidak tests, NT and 230F(12): $P=0.010$, NT and Panc10.05 pool: $P=0.043$, all N=10). By week 6, significant decreases in tumor volumes and tumor weights between NT and the remaining treatment groups were observed (all $P<0.0001$), but no differences in body weight were detected. While tumor growth after treatment with the 12-cutter sgRNA was significantly inhibited, we found that the mutation frequency of the two outlier 230F(12) tumors was extremely low (0.97% and 0.64%), suggesting that lack of CRISPR-Cas9 activity allowed tumor growth. In two separate LNP screening experiments, the LNP formulation used by Moderna mRNA-1273 (containing SM-102) consistently showed high *in vivo* transfection efficiency with some luciferase expression detected in liver. We were also able to identify PC cells from the tumors through mNeonGreen expression.

Conclusion: Our results show that CRISPR-Cas9 is tumoricidal *in vivo*. We have established a subcutaneous mouse model that allows for future optimization and delivery of CRISPR-Cas9 therapeutic to PC tumors.

[1]Teh and Bowland (2024). *NAR Cancer*. 6(2): zcae028.

729 CRISPR/Cas9 knockout of *c-Met* to evaluate its effects on reducing drug resistance against gemcitabine in pancreatic cancer

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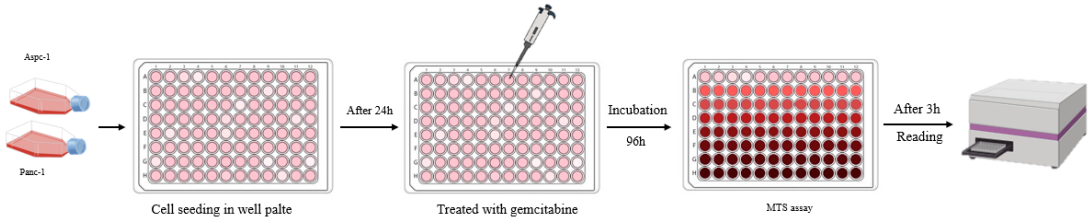
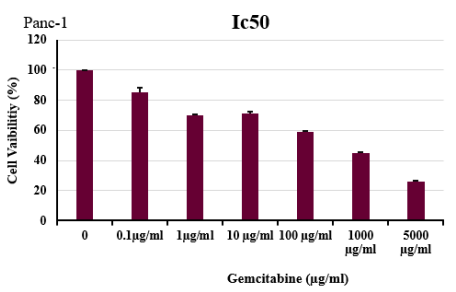
Background: Pancreatic cancer is one of the most lethal malignancies, with drug resistance to gemcitabine posing a significant therapeutic challenge. The c-Met receptor tyrosine kinase has been implicated in tumor progression and drug resistance. This study aimed to knock out the c-Met gene in Panc-1 cells using the CRISPR-Cas9 system and evaluate its effect on reducing drug resistance to gemcitabine in pancreatic cancer.

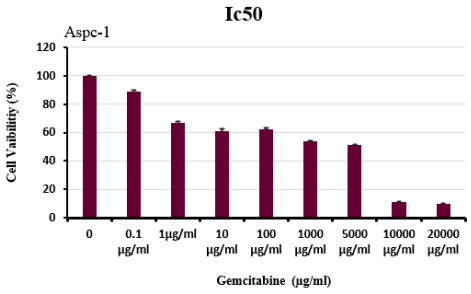
Methods: Initially, c-Met expression levels were measured in Panc-1 and Aspc-1 cell lines by RT-qPCR. Subsequently, the IC50 values of gemcitabine were determined in these cell lines using the MTS assay with varying concentrations of the drug (Fig. 1)Additionally, a vector encoding two designed gRNAs and the spCas9 nuclease was constructed, and transfected into the cell line with a high c-Met expression. The transfected cells were then treated with puromycin to select genetically modified clones, followed by single clonal selection and evaluation using genomic PCR and Sanger sequencing (Fig.2).

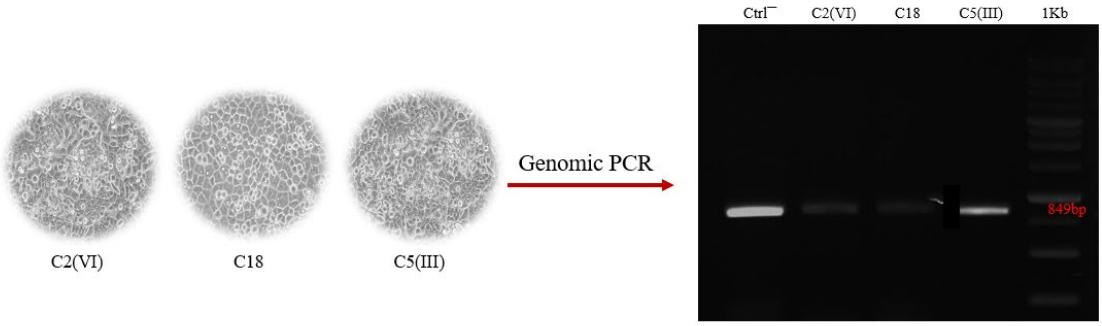
Results: After evaluating the expression level of c-MET using real-time PCR, the PANC-1 cell line was selected for the continuation of the resserch. Following the confirmation of the successful knockout of the c-MET gene through genomic PCR and Sanger sequencing, a notable decrease in cell growth and proliferation was observed. The gemcitabine IC50 values in the c-MET knockout clones were also significantly reduced.

Conclusions: Knockout of the c-Met gene in Panc-1 cells using the CRISPR/Cas9 system significantly decreased drug resistance to gemcitabine. This research underscores the potential therapeutic advantage of targeting c-Met to overcome chemoresistance in pancreatic cancer, offering a hopeful path for enhancing gemcitabine treatment results.









730 mRNA Aggregate Analysis using SEC-MALS

Yinan Zhang[1], Linting Li[1], **Minxin Wang[1]**, Konstantin Musiychuk[1]
[1]*Sepax Technologies, Newark, DE*

The recent developments in mRNA vaccines, therapeutics and CRISPR-Cas9 genome editing technologies call for a robust, accurate and reliable analytical quantification and characterization methods for critical quality attributes of mRNA to ensure the quality of mRNA-based therapeutics and vaccines.

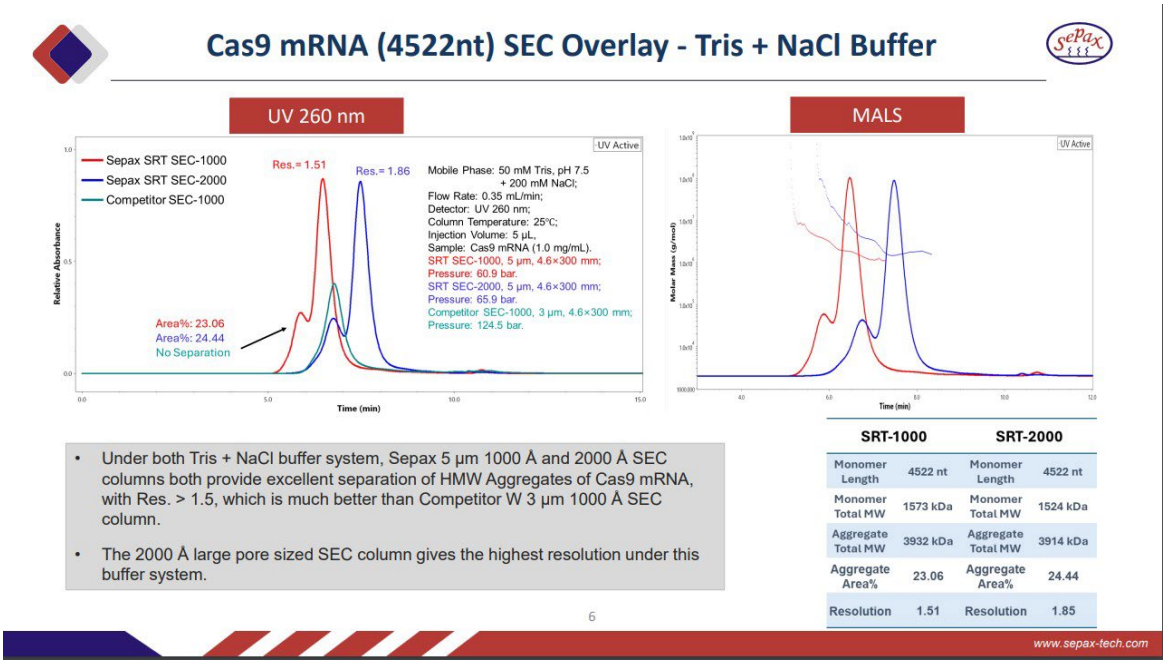
Size Exclusion Chromatography (SEC) along with MALS is one of the methods which can quickly quantify amount of mRNA, its size and amount of high molecular weight aggregates and evaluate its purity.

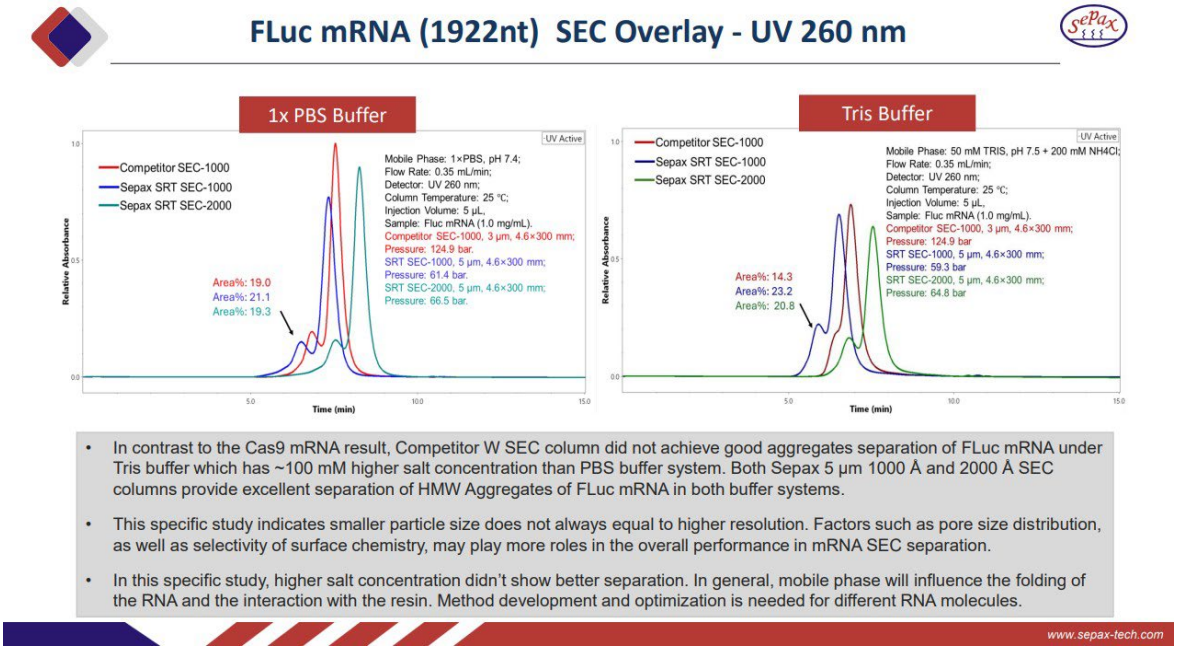
Large size of the modern RNA based therapeutics requires adequate pore size resins with suitable surface chemistry for selectivity and hydrophilicity to achieve high resolution and efficiency separation. It is important to choose adequate column for separation of mRNA – size and sample dependent. Smaller particle size of the resin may not always equal to higher resolution. Factors such as pore size distribution, as well as selectivity of surface chemistry, may also play an important role in the overall performance in RNA SEC separation.

In this study, we present SEC HPLC and MALS data to analyze the effect of pore size, surface chemistry, type of buffer system, salt concentration and temperature on the monomer and aggregate separation of Cas9 mRNA (4500

nt) with estimated molecule weight of 1449 kDa and FLuc mRNA (1922nt) with estimated molecular weight of 616 kDa.

Sepax SRT SEC-1000Å and SEC-2000Å, 5µm particles columns outperform one 3 µm SEC-1000Å column from other branded vendor under all buffer systems with significantly higher resolution. The 2000Å pore sized SEC column provides the highest resolution among the three columns being screened. Conditions such as buffer system, ionic strength and temperature can affect the folding of the mRNA species, and its interaction with the resin separation. Method development and optimization are needed for different RNA molecules. Sepax SRT SEC unique surface chemistry provides more universal buffer compatibility, which is important for developing a robust, accurate and reliable analytical method.





731 Improved ApoptiCIDe™ Safety Switch Provides a Novel, Controllable Therapeutic Platform for Regulated Klotho Anti-Aging Gene Therapy

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Background: Just as the increasing complexities of cell therapies has demonstrated the need for a rapid, safe and effective safety switch, novel gene therapies that extend past gene replacement for rare pediatric diseases would benefit from the same level of controllability. It remains difficult to control systemic levels of novel proteins produced by newer types of gene therapies simply by modifying gene therapy dose. Therefore, a regulatable gene therapy platform that could be used for such products would be advantageous. Currently, the rimiducid/inducible Caspase-9 (ApoptiCIDe™) system remains the most clinically validated attenuator for cell and gene therapy; however, it's broadest adoption is limited by the burden of a 2-hour infusion of the rim activating agent, along with infusion reactions that can occur as a result of the current excipient, Solutol hydroxy stearate (HS) 15. To improve efficacy and broaden potential applications of the ApoptiCIDe system, we reformulated rimiducid that can be administered quickly and easily *via* the subcutaneous or intramuscular route. Moreover, we demonstrated its broad utility *in vivo* by regulated attenuation of both reporter genes and therapeutically relevant anti-aging candidate therapeutics, Klotho and Follistatin-344.

Results: We screened a large number of combinations of excipient combinations and chose one formulation compatible with subcutaneous and intramuscular injection, with bioavailability above 30%, named PTC-1202, for further clinical development. Formal stability testing revealed 99.8% stability under accelerated conditions (40°C, 75% relative humidity) over 3 months. To demonstrate *in vivo* utility, bioluminescent (BLI) reporter, N-Luc, was co-expressed with ApoptiCIDe under the constitutive CMV promoter/enhancer and delivered to mice using AAV2 and AAV9 vectors via IV, IM, IP and SQ routes. In each case, administration of as little as 10 mg/kg rimiducid (0.3 mg total) led to ~ 90% elimination of BLI within days.

To demonstrate utility with a therapeutically useful gene target, we replaced N-Luc with human secreted Klotho (sKL1), associated with longevity or Follistatin (FST) 344, associated with maintaining muscle mass and delaying sarcopenia or overcoming muscular deficiencies. In both cases, levels of human proteins were easily detectable in the serum at physiological levels and elimination of protein(s) of up to 100% was achievable with one or more rimiducid administration. After a second injection, 60% of mice had undetectable human Klotho and the remaining animals revealed >90% decreased peak protein levels. Ongoing experiments are evaluating the use of lower PTC-1202 doses as a "rheostat" to enable turning down rather than turning off the production of the therapeutic protein.

Discussion: We demonstrated that our improved formulation of rimiducid, PTC-1202, the activating agent for our ApoptiCIDe cell elimination technology, could rapidly and effectively turn off production and reduce systemic levels of Klotho using our controllable gene therapy platform. PTC-1202 should not only provide a new controllable gene therapy platform to improve the safety for patients treated with novel agents, but it should also enhance the ease of use of CaspaCIDe in cell therapies as a safety switch.

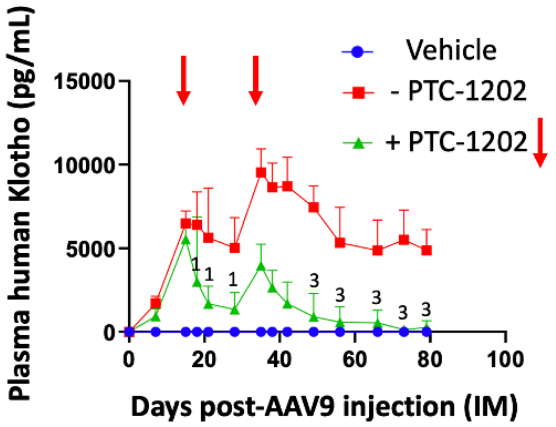
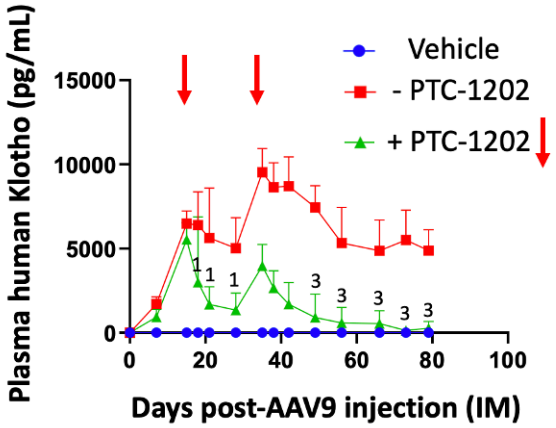


Figure Legend: On day 0, cohorts of mice (n = 5) received vehicle alone (circle), or a single IM injection of AAV9 encoding a bicistronic vector encoding CMV-driven ApoptiCIDe (rimiducid inducible Caspase-9) and secreted human Klotho (square and triangle). Vector-injected mice received reformulated rimiducid on days 19 and 39 (triangle) or control excipient (square) and serum Klotho was analyzed by ELISA at the indicated timepoints. *Note:* Number shown represents the number of animals (of 5) with serum Klotho below the lower limit of quantification (LLoQ).



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732 Gene Studies About Development of Renal Cell Carcinoma

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Background: Renal cell carcinoma (RCC) is a widespread and dangerouos disease. The clear-cell renal cell carcinoma (ccRCC) is the most prevalent type of RCC and is highly malignant. The ccRCC is associated with the loss of the von Hippel-Lindau tumor suppressor gene VHL. The VHL protein (pVHL) binds and stabilzes the renal suppressor protein Jade-1 and plays important growth suppressive roles through Jade-1. Previous studies found that many solid tumors, such as ccRCC, exhibit chromosomal instability and hence have defects in DNA double-strand breaks (DSBs) repairs, we sought to investigate on Jade-1 and pVHL to illustrate mechanisms of cancer development.

Methods: Primary clear cell adenocarcinoma 786-O was the renal cancer cell model. 786-O HAVHL and 786-O HAV0 were transfected by vectors with and without VHL gene respectively. The human embryonic kidney cell line 293T was the healthy renal cell model. Gamma irradiation, doxorubicin, or neocarzinostatin was applied to cause DSBs in renal cells, which were labeled by γH2AX antibodies. The overlapping between Jade-1 and DSBs under the impact of pVHL was visulized in immunofluorescence microscope. The impact of pVHL on Jade-1 expression and transcription levels was innvestigated by immunoblotting and qPCR.

Result: Irradiation and doxorubicin treatments accumulated Jade-1 inside nucleus. DSBs increased Jade-1 protein level in cells without VHL gene, but caused no changes in cells with VHL gene. The colocalization coefficient value between Jade-1 and DSBs did not increase in cells with VHL gene.

Conclusion: Jade-1 participated in DSBs process, and pVHL did not enhance the overlapping between Jade-1 and DSBs. This implies that Jade-1 may not require pVHL in DNA repair process. DSBs upregulated Jade-1 protein level, and pVHL inhibited this elevation. A possible explanation is that pVHL stabilizes the amount of Jade-1 diffusing in cells. More experiments are needed to consolidate these results.

733 Drug-responsive Domains Coupled with Cognate Small-molecule Ligands Drive Synthetic Gene-ON or -OFF Circuits to Regulate Expression of Therapeutic Proteins

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Development of increasingly powerful biological drugs, such as cytokine-armored cell therapies, requires tight control of in vivo protein expression to avoid severe side effects and thereby expand the therapeutic window. We have previously shown that the activity of different classes of therapeutic proteins can be regulated via fusion to drug-responsive domains (DRDs) that can be stabilized with FDA-approved small molecules (Inniss et. al. *Commun Biol* 2025). Currently, this approach is showing promising activity in a Ph1 clinical trial (NCT05470283) in patients with immune checkpoint inhibitor-resistant melanoma treated with engineered tumor-infiltrating lymphocytes expressing regulatable membrane-bound interleukin 15 (OBX-115; Amaria ASCO 2024). While direct fusion of a DRD to proteins of interest can yield effective functional regulation in many cases, in other cases a DRD fusion might affect protein function by interfering with protein binding or post-translational processing. Further, when regulating a secreted protein therapeutic it might be desirable to secrete the native protein rather than a DRD-fused protein. Here, we show that by using a DRD to regulate an artificial transcription factor consisting of a fusion of the chimeric DNA binding domain, ZFHD1, and the transcription activation domain of p65 (Fig 1a) we can construct genetic circuits to regulate expression of either mRNA or short inhibitory RNA. By stabilizing the DRD with ligand, we increase the abundance of the artificial transcription factor, which recruits to the cognate DNA binding sites placed upstream of a minimal promoter. By placing a transgene-encoding protein downstream of the minimal promoter (Fig 1b), mRNA will be transcribed in the ligand-ON state, thereby expressing the protein of interest (ligand-ON/gene-ON circuit). If a short inhibitory RNA is placed downstream of the minimal promoter (Fig 1c), in the ligand-ON state, we can potentially downregulate the expression of any gene in the genome by altering the specificity of the inhibitory RNA (ligand-ON/gene-OFF circuit). The capabilities of the ligand-ON/gene-ON circuit were demonstrated with the in vitro cellular regulation of protein in the cytoplasm (GFP), mitochondria (frataxin) and a secreted protein (interleukin 12 [IL12]). IL12 secretion shows a ligand dose-dependent rheostat-like regulation, with proportional increases in IL12 levels with increases in ligand dose (Fig 1d). Differential basal secretion states for IL12 in the absence of ligand were enforced by altering the composition of the artificial transcription factor (v1-v4, Fig 1d), whilst using fully human components. The capability of the ligand-ON/gene-OFF circuit was demonstrated by dampening the expression of tool proteins like GFP, neuronal proteins such as ApoE (Fig 1e), HTT and members of the TCR signaling pathway, ZAP70 and LAT in a drug-dependent manner. The gene-ON and gene-OFF circuits have the potential to be important tools for gene and cell therapies where precise and variable control of delivered gene product is needed to manage disease and repeat dosing of biologics or anti-sense oligonucleotides is challenging.

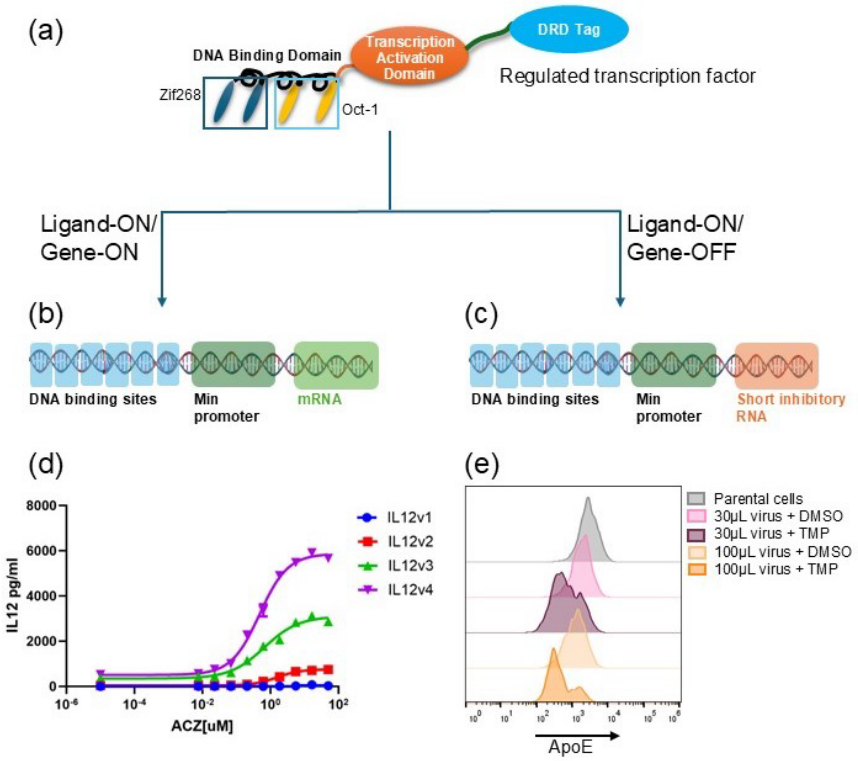

Fig 1. DRD-regulated chimeric transcription factor **(a)** can enable a ligand-ON/gene-ON functionality if combined with an mRNA under the control of a minimal promoter **(b)**. The ligand-ON/gene-OFF switch functionality is enabled when the DRD regulated chimeric transcription is combined with a modified short inhibitory RNA under the control of the minimal promoter **(c)**. Ligand dose-response curves for secreted IL12 using the ligand-ON/gene-ON circuit using trimethoprim (TMP) as the ligand **(d)**. Downregulation of ApoE using the ligand-ON/gene-OFF circuit in a TMP-dependent manner in transduced cells **(e)**.

734 Alectinib Enhances Anti-tumor Efficacy of Disialoganglioside 2 Chimeric Antigen Receptor T Cells in ALK-Mutated Neuroblastoma by Suppressing Programmed Death-Ligand 1

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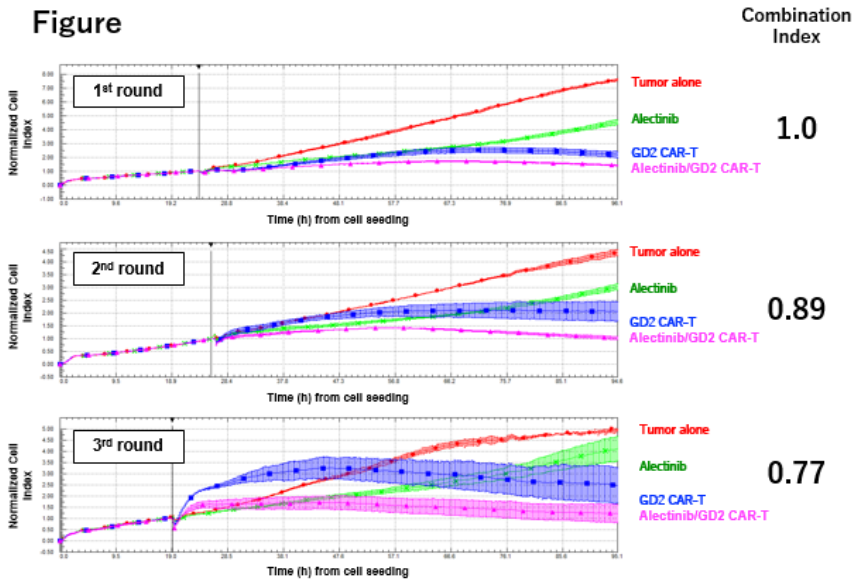
Background: High-risk ALK-mutant neuroblastoma (NB) presents significant therapeutic challenges due to drug resistance and an immunosuppressive tumor microenvironment (TME). Chimeric antigen receptor T (CAR-T) cell therapy, particularly targeting disialoganglioside 2 (GD2), has shown potential against NB, but its efficacy is limited in solid tumors by immune evasion mechanisms, such as the upregulation of programmed death-ligand 1 (PD-L1). Aberrant ALK signaling is implicated in the regulation of PD-L1 expression, contributing to immune escape. Alectinib, a second-generation ALK inhibitor, has demonstrated efficacy in ALK-driven malignancies. This study investigates the synergistic potential of Alectinib with GD2 CAR-T cells to enhance anti-tumor activity in ALK-mutant NB. **Methods:** GD2 CAR-T cells were generated by retroviral transduction using a second-generation CAR construct. Their phenotype and function were assessed using flow cytometry and real-time impedance-based cytotoxicity assays. ALK-mutant and wild-type NB cell lines were stimulated with interferon-gamma (IFN-γ) to induce PD-L1 expression, which was measured via flow cytometry and Western blotting. The effects of Alectinib on ALK downstream signaling pathways (STAT3, Akt, Erk1/2) were evaluated by Western blotting. Sequential co-

culture assays were performed to assess the synergistic effects of Alectinib and GD2 CAR-T cells over multiple tumor rechallenges. In vivo efficacy was evaluated using an ALK-mutant NB xenograft model, with tumor growth and survival analyzed across treatment groups. PD-L1 expression in tumor tissues was assessed by immunohistochemistry.

Results: Flow cytometry confirmed that 95.9% of GD2 CAR-T cells expressed GD2, with 35.6% displaying a naïve/stem cell memory phenotype and 44.6% expressing PD-1, indicative of early T-cell exhaustion. In ALK-mutant NB cell lines, IFN-γ stimulation robustly induced PD-L1 expression, significantly suppressed by Alectinib pretreatment. Alectinib inhibited phosphorylation of key ALK downstream signaling molecules, including STAT3, Akt, and Erk1/2, disrupting PD-L1 upstream signaling. Sequential co-culture assays demonstrated enhanced anti-tumor efficacy of GD2 CAR-T cells in the presence of Alectinib, with combination indices improving from 1.0 to 0.77 over three rounds (Figure). Notably, Alectinib did not compromise CAR-T cell phenotype or function. In vivo, the combination therapy of Alectinib and GD2 CAR-T cells significantly suppressed tumor growth and extended survival in ALK-mutant NB xenografts compared to monotherapies ($p < 0.01$). Immunohistochemical analysis revealed reduced PD-L1 expression in tumor tissues from the combination group, suggesting that Alectinib mitigated immune evasion by disrupting the PD-1/PD-L1 axis.

Conclusion: Alectinib synergistically enhanced the anti-tumor efficacy of GD2 CAR-T cells against ALK-mutant NB by suppressing PD-L1-mediated immune evasion and preserving CAR-T cell function. This combination therapy represents a promising strategy to improve outcomes in refractory ALK-mutant NB, warranting further clinical investigation.

Figure



735 Developing Novel Chimeric Antigen Receptor Therapies for Glioma

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High-grade gliomas remain one of the most formidable challenges in oncology, characterised by aggressive progression and dismal survival outcomes. Despite remarkable advancements in cancer therapies over the past two decades, treatment options for brain cancers, such as glioblastoma (GBM) and diffuse midline glioma (DMG), have stagnated. Current standards, including surgery, chemotherapy, and radiotherapy, offer limited efficacy; GBM patients face a stark 5% five-year survival rate, while DMG, tragically, leaves no survivors, particularly in paediatric cases.

Our research addresses the urgent need for innovative therapeutic strategies. Leveraging the transformative potential of immunotherapy, our program focuses on designing personalised Chimeric Antigen Receptors (CARs) to enhance T cell immunotherapy for brain cancer. Through cutting-edge cell-surface proteomics, we have identified novel brain cancer antigens and established a robust discovery pipeline for therapeutic targets.

We have evaluated the efficacy of CAR T cells in preclinical models, demonstrating significant therapeutic activity both in vitro and in vivo. Recent findings from our group highlight the successful targeting of EphA3 and HER2 in DMG models. In both adult and paediatric models of high-grade glioma, we show that not only do EphA3-targeted CAR T cells eliminate brain tumours, but also persist and form a memory, that can be successfully recalled and clear subsequent tumours.

In humans, recognising the limitations of single-target approaches, we are advancing EphA3-targeted CAR T in combination therapies by engineering CAR T cells to target multiple antigens simultaneously, including GD2, EGFR, HER2, EphA3, CD276, and CD63. Furthermore, we have integrated CAR T cell therapy with immune-modulating strategies, such as checkpoint inhibitors and co-stimulatory agonists, to enhance therapeutic outcomes. We show that systemic administration of anti-41BB in PDX models improves the clearance of syngeneic orthotopic brain tumours.

We have developed innovative methods for tracking CAR T cell dynamics in vivo to address tumour heterogeneity and improve precision. These efforts reflect our commitment to delivering novel therapeutic options for patients with high-grade gliomas, offering hope to individuals and families facing these devastating diseases.

736 GITRL Coexpression in Anti-GD2 CAR-T Cells Results in Persistent Antitumor Activity and Exhaustion Resistance in a Glioblastoma Model

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Background: CAR-T cell therapy for solid tumors is impaired by the complex tumor microenvironment, which limits cell infiltration and sustained antitumor activity. Thus, enhancing persistance and resistance to suppressive cells will be crucial for improving the efficacy of CAR-T cells against solid neoplasms. Full activation of T cells requires the engagement of costimulatory molecules, as CD28, expressed constitutively, and GITR, expressed right after the initial activation of T cells. GITR engagement with its ligand GITRL promotes enhanced effector functions, as a consequence of T cell proliferation, cytokine production, and antiapoptotic signals. Besides that, GITR activation on Treg cells was found to repress their immunosuppressive functions, which may help to sustain the antitumor activity of CAR-T cells in situ. Thus, our hypothesis is that CAR-T cells coexpressing GITRL will display enhanced antitumor effects.

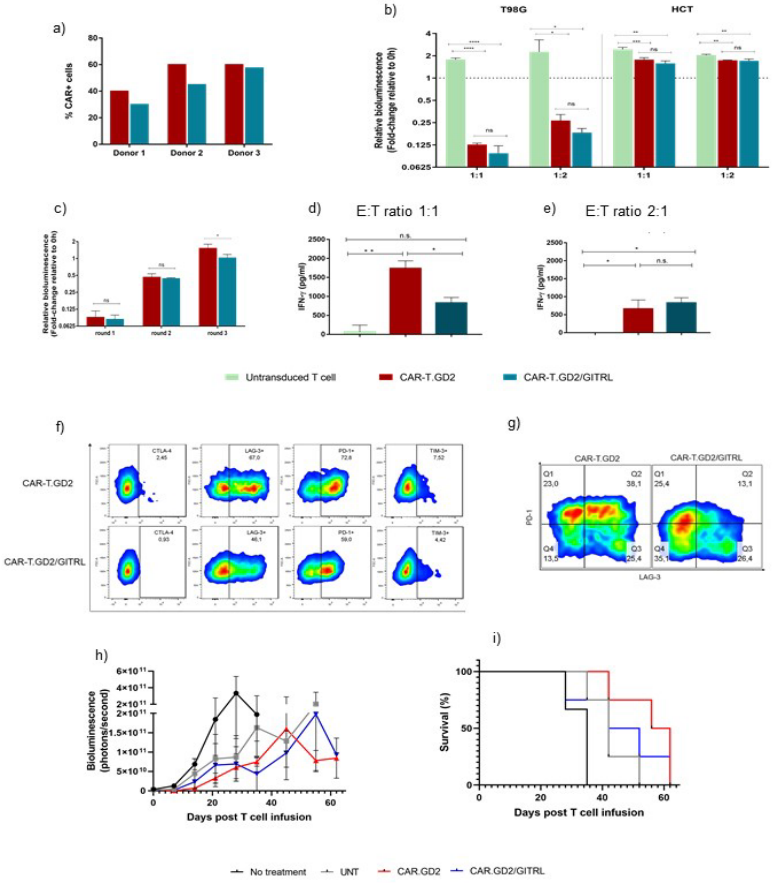
Aim: The aim of this study was to evaluate in vitro and in vivo the antitumor efficiency of anti-GD2 CAR-T cells coexpressing GITRL in a glioblastoma model.

Methods: To test our hypothesis, we generated human anti-GD2 CAR-T cells coexpressing GITRL (CAR.GD2/GITRL) or the CAR molecule alone (CAR.GD2). In order to investigate the functional activity in vitro of the CAR-T cells, we performed a coculture assay with glioblastoma cells T98G-luciferase (GD2+) and the HCT-166-luciferase (GD2-) using two effector-to-target cell (E:T) ratios. Also, the levels of secreted IFNy were measured by ELISA. Also, T cells recovered from coculture were stained with antibodies against four exhaustion markers (PD-1, CTLA-4, TIM-3, LAG-3) and analyzed by flow cytometry. The persistence of lysis capacity was evaluated by additional rechallenges of coculture. Finally, to access the antineoplastic effect of CAR-T cells in vivo we

induced tumors in NSG mice using T98G-luciferase cells and treated them with both CAR-T cells, untransduced T cell and vehicle.

Results: The lentiviral particles were able to efficiently transduced primary T cells resulting in 30-60% of CAR+ cells. In coculture assays, both CAR-T cells demonstrated a potent cytotoxic activity, with around 90% of T98G cell lysis, while sparing HCT-116 cancer cells, demonstrating GD2-restricted cytotoxic activity. Additionally, CAR.GD2 cells showed higher secretion in coculture in 1:1 ratio, but not in the 2:1 ratio, demonstrating that IFNy secretion by CAR.GD2/GITRL cells was less susceptible to the higher proportion of tumor cells compared to CAR.GD2 cells.The analysis of exhaustion markers revealed an upregulation in all four markers observed in CAR-T.GD2 cells. When comparing the double-positive cell populations for the most expressed markers (PD-1 and LAG-3), we identified a higher frequency of CAR-T.GD2 cells expressing both markers simultaneously (38%) than CAR-T.GD2/GITRL (13%). Also, after three rounds of coculture with T98G tumor cells, CAR-T.GD2/GITRL cells displayed a higher cytotoxic activity than CAR.GD2 cells. In the NSG xenograft model, we observed that both CAR-T cells demonstrated similar in vivo tumor regression and increased survival.

Conclusion: These data suggest that the coexpression of GITRL in anti-GD2 CAR-T cells might enhance T cell resistance to exhaustion and contribute to functional activity. These findings could also be applied to enhance the cytotoxic activity and persistence for other CAR constructs.



a) Transduction efficiency of lentiviral particles in primary T cells from three different donors. **b)** Quantification of relative bioluminescence after 48h of coculture assay using two effector:target (E:T) ratios 1:1 and 1:2 against tumor cells T98G-luc (GD2+) and HCT-luc (GD2-). Statistical analysis: One-way ANOVA (ns = not significant; * = p<0,0189; ** = p<0,0010; *** = p<0,0001) n=3 **c)** Relative bioluminescence after three sequential rounds of coculture against T98G-luc 1:1 E:T ratio. **d)** INF-g secretion during coculture assay in 1:1 and **e)** 1:2 E:T ratio. **f)** Flow cytometry of CAR.GD2 and CAR.GD2/GITRL lymphocytes labeled with antibodies against the exhaustion markers CTLA-4, LAG-3, PD-1, and TIM-3 after 48 hours of coculture against T98G cells. **g)** Flow cytometry of CAR-T lymphocytes labeled with anti-PD-1 and LAG-3 antibodies. **h)** Bioluminescence quantification of the NSG mices after T cell infusion (black line represent no treatment group (n=3), grey line representes UNT group (n=4), red line represents CAR-T.GD2 (n=4) and blue line represents CAR-T.GD2/GITRL (n=4). **i)** Survival analysis (percentage) of the NSG mices after treatment

737 Enhanced anti-GD2 CAR-T efficacy due to the addition of encoded armored elements of persistence, expansion and shielding modules

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Neuroblastoma is the most common malignant (cancerous) extracranial (outside the cranium) solid tumor of childhood that accounts for 15% of cancer deaths in children, and is the primary cause of death from pediatric cancer in children ages 1–5. Disalogangliosid (GD2) is a sialic acid-containing glycosphingolipid uniformly expressed in neuroblastomas. Common challenges of CAR-T cell therapies in solid tumors, such as the limited survival due to the activation induced cell death (AICD), the decreased expansion by their development of a depletion stage and the repressed activation in the immunosuppressive tumor microenvironment (TME). To overcome these challenges, we developed an anti-GD2 Turbo CAR-T which is an enhanced anti-GD2 CAR-T cells with the addition of encoded armored elements of persistence, expansion and shielding modules. The anti-GD2 Turbo CAR-T cells shows enhanced cytotoxicity and increased cytokine release than regular GD2 CAR only when co-cultured with tumor target cells *in vitro*. In addition, the anti-GD2 Turbo CAR-T cells shows enhanced persistence to activation induced T cell apoptosis, increased survival without further stimulation and exogenous cytokines, and potent shielding of anti-tumor activity and cytokine release after TGFβ1 treatment. Evaluation of anti-GD2 Turbo CAR-T in the subcutaneous SY5Y (GD2+ human neuroblastoma cell line) xenograft model demonstrate the significant increase anti-tumor efficacy. The anti-GD2 Turbo CAR-T is more effective at low dose (1e6 or 3e5) to remove tumor due to the addition of encoded armored elements. Compared with regular sc-Fv anti-GD2 CAR-T, the turbo CAR-T also increases the median survival by one week. In summary, these results demonstrate that anti-GD2 Turbo CAR-T as a promising approach for cancer immunotherapy could overcome multiple suppressive mechanisms in the solid tumor microenvironment.

738 Orthogonal IL-2/IL-2Rβ Armoring is Superior to TGF-β Receptor Blockade in Overcoming TGF-β-Driven Inhibition of GPC3 CAR T Cells in Hepatocellular Carcinoma

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CAR T therapy has demonstrated success in treating certain hematologic malignancies, achieving deep and durable responses. However, efficacy of CAR T cells is limited, particularly in solid tumors, due to factors contributing to an inhospitable tumor microenvironment. The immunosuppressive cytokine Transforming Growth Factor-beta (TGF-β) accumulates in many cancers. TGF-β inhibits T cell activity by direct inhibition of T cell proliferation, inhibition of Interleukin 2 secretion by T cells, and the inhibition of the cytolytic activity of effector T cells. Strategies to overcome the intratumoral inhibition of T cells by TGF-β are therefore essential to achieving the full potential of CAR T therapy.

IL-2 promotes the proliferation and activity of T cells and has been shown to counteract the inhibitory effects of TGF-β on T cell function. In addition, IL-2 induces IFNγ, which directly inhibits TGF-β signaling. However, therapeutic use of IL-2 is limited by systemic toxicity due to its non-specific activation of lymphocytes. To overcome this challenge, we previously developed a human orthogonal IL-2/IL-2R system consisting of a pegylated, IL-2 mutein (STK- 009) that does not significantly signal through the wild-type IL-2 receptors and a mutated IL-2 Receptor Beta (oRb) that does not significantly respond to wild-type IL-2. This system provides a private IL-2 signal for engineered oRb-expressing cells while avoiding IL-2 signaling in bystander immune cells and IL-2 related toxicities. We have demonstrated STK-009/oRb's ability to selectively expand oRb-expressing CAR T cells (SYNCAR T cells) targeting antigens in both hematological (SYNCAR-001, CD19) and hepatocellular (HCC) malignancies (SYNCAR-002, GPC3) . STK-009 significantly improved anti-tumor efficacy, yielding lasting responses in lymphoma and hepatocellular carcinoma models. In non-human primates, STK-009 did not activate wild-type T or NK cells and showed no drug-related clinical symptoms. Currently, STK-009 + SYNCAR-001 is in Phase I trials (Palomba et al., ASH 2024; NCT05665062, and NCT06544330).

Here, we evaluated the inhibition of CAR T mediated tumor efficacy by intratumoral TGF-β using human anti-GPC3 CAR T cells and GPC3$^+$ TGF-β expressing HCC xenograft models. We compared CAR T cells armored with the orthogonal IL-2/IL-2R system with CAR T cells, whereby TGF-β signaling was abrogated by the expression of a dominant negative TGFBRII (DNTGFBRII). To assess TGF-β accelerated CAR T dysfunction CAR T cells were subjected to continuous antigen exposure of GPC3$^+$ HCC cells engineered to overexpress TGF-β (HEPG2-TGFβ). While DNTGFBRII armoring improved effector cytokine secretion, proliferation, and suppressed exhaustion during chronic CAR stimulation, STK-009 treated SYNCAR-002 T cells demonstrated a greater and more durable enhancement. Both armoring strategies were then tested in HCC xenograft mouse models expressing significant amounts of TGF-β. STK-009/SYNCAR-002 treatment demonstrated superior tumor control, cell proliferation, and better cell fitness compared to the DNTGFBRII armored CAR T cells.

These findings support the advantage of an orthogonal IL-2/IL-2R system that selectively drives and sustains potent T cell effector function even in an adverse tumor microenvironment saturated with TGF-β.

739 JW004: An Armored anti-GPC3 CAR T Therapy for Hepatocellular Carcinoma Enhanced through Multiple Modules

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Introduction: Hepatocellular carcinoma (HCC) is an aggressive malignancy, resulting as the third cause of death by cancer each year. Advanced therapeutics have dramatically changed the systemic treatment landscape of advanced HCC. CAR T therapies have become the current focus of treatment investigation in advanced HCC, and show remarkable therapeutic potential. Glypican-3 (GPC3) is a glycosylphospatidyl inositol-anchored membrane protein, is overexpressed in HCC tissues but not in the healthy adult liver. Thus, GPC3 is becoming a promising candidate for HCC diagnosis and immunotherapy. However, the effectiveness of CAR T cells is limited due to the complex tumor microenvironment (TME) and immune-suppressed. To overcome these challenges, we explored JW004, the multiple armored modules to enhance CAR T's function in TME including resistance activation-induced cell death (AICD) and enhance T cell persistence and proliferation.

Methods: Enriched primary CD4$^+$ and CD8$^+$ T cells were used for the generation of CAR-T cells by lentiviral transduction. In vitro, flow cytometry was used to assess the expression level of anti-GPC3 CAR and armored modules' overexpression. The xCELLigence RTCA was used to measure CAR cytotoxic activity post co-culture with target cells from Day0 to Day6, CAR-T cell proliferation was calculated by cell counter. For the function verification of armors were validated by cytotoxic activity and T cell proliferation under non-activation cytokine cell culture medium and soluble TGFb1 treatment which is simulating the TME. In vivo JW004's efficacy was evaluated in NCG xenograft models HCC (via HepG2 and Hu7 two human HCC tumor cell line inoculation), tumor elimination was calculated by tumor volume post CAR T cell dosing. The CAR T cell number and percentage were assessed from mice blood leukocyte by flow cytometry post CAR T cell dosing.

Results: JW004 showed higher potent of cytolysis, T cell proliferation and persistence than anti-GPC3 CAR only when co-cultured with GPC3 expression (low and high level) HepG2 cells in vitro. Furthermore, JW004 improved tumor clearance, also enhanced T cell persistence and proliferation compared to anti-GPC3 CAR only in HepG2 (low GPC3 expression) NCG mice model.

Conclusion: These results demonstrate that armored modules enhance GPC3 CAR T cells' potency and proliferation to support the continuous expansion in vitro and in vivo. JW004 demonstrated more antitumor immunity, potent CAR targeting, resilience to attenuation by TGFβ, reduced activation induce T cell apoptosis, and favorable preclinical efficacy and safety characteristics compared to anti-GPC3 CAR only. Supported by these data, JW004 has potential to address GPC3+ advanced HCC.

740 Armoring iPSC-derived allogeneic therapies with a TGF-b neutralizing synthetic receptor that enhances solid tumor elimination in TME-mimicking conditions in vitro.

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Allogeneic immunotherapy holds immense promise for solid tumor treatment with significant challenges remaining in overcoming the barriers imposed by the solid tumor microenvironment (TME). Extensive preclinical research has led to the pharmacological targeting of the Transforming Growth Factor Beta (TGF-β) pathway, which plays a key role regulating TME. TGF-β drives immunosuppression, stromal remodeling, and angiogenesis to support tumor progression. Here we describe solid-tumor-targeted induced pluripotent stem cells (CAR-iPSC) armored with a synthetic dominant negative receptor (DNR) that neutralizes TGF-β in the environment, thereby potentially inhibiting tumor growth and enhancing immune responses against cancer. We established compatibility of TGF-β DNR engineering with iPSC differentiation and successfully generated TGF-β DNR CAR-iT cells. We confirmed that TGF-β DNR-engineered CAR-iPSCs have reduced SMAD signaling in the presence of soluble TGF-β compared to non-engineered CAR-iPSC by flow cytometry. We evaluated CAR-iT (+/- TGF-β DNR) function in the presence of inhibitory TGF- β (5ng/mL) signals in a repeat-killing assay across multiple Nectin-4 positive target cells. We observed significant advantages of TGF-β DNR CAR-iT in maintaining prolonged cytotoxic activity against labeled target cells by Incucyte in the presence of TME-mimicking conditions. Killing capacity was observed to be 4 times higher with expression of the TGF-β DNR (1.05x10e10 for CAR-iT and 2.5x10e9 CAR-iT with TGF-β DNR). Moreover, we observed a similar effect in a spheroid tumor model, indicating the ability of TGF-β DNR to sustain cytotoxic activity in a 3D culture system. Additionally, we've observed improved function in armored Peripheral blood mononuclear cell-derived iPSC-derived CAR-iNK with TGF-β DNR. In summary, we can improve cytotoxic activity of targeted CAR-iT and CAR-iNK engineered with TGF-β DNR against solid tumor cell lines in the presence of recombinant human TGF-β. These findings highlight a modality that can confer improved anti-tumor activity of future iPSC-derived immunotherapies designed to withstand the immunosuppressive nature of the TME.

741 Preclinical Development of CTSR-Armored B7H3 CAR-T Cells for Pediatric Solid Tumors

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Background: TGF-β-targeted CAR-T cells that seek to overcome TME's immunosuppression remain a major opportunity to improve current CAR-T cell therapies. B7-H3, a pan-cancer antigen found to be highly expressed in many pediatric solid tumors, is currently being explored in several trials. Interim reports suggest that the treatment was tolerated with mediocre responses. We proposed to arm these B7-H3 CAR-T cells with a novel chimeric TGF-β switch receptor (CTSR) to convert the immunosuppressive TGF-β signal to a Toll-like receptor (TLR)-mimicking response. We expected that TLR activation in these CAR-T cells would boost their efficiency in TGF-β-rich tumors such as Neuroblastoma (NB), Ewing's sarcoma (EWS), and osteosarcoma (OS).

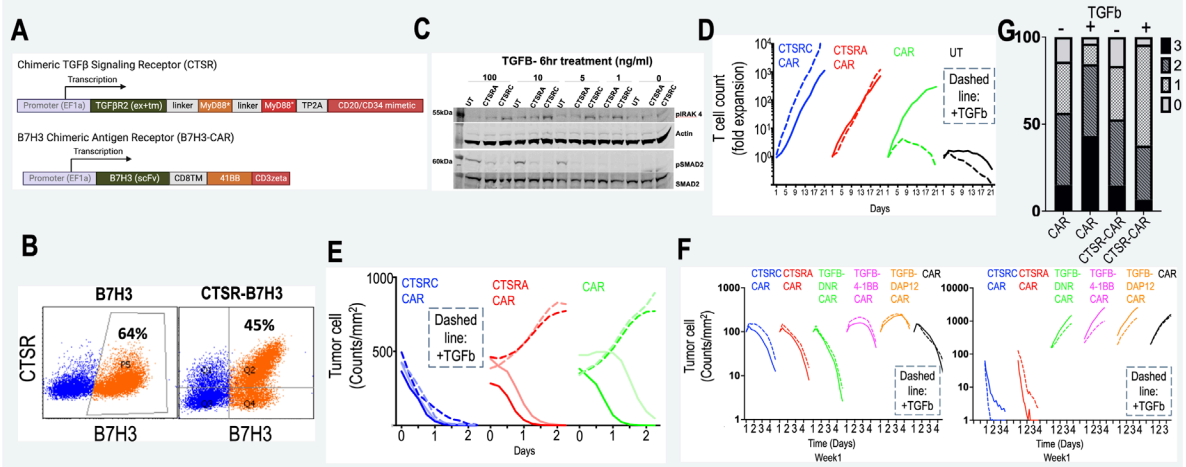
Methods: We engineered the CTSR by fusing the extracellular TGFBR2 domain with the activating domains of MYD88. Human T cells were engineered to express a second-generation CAR against B7-H3 (B7H3-4-1BB-CD3ζ), with or without CTSR.

Results: First, we successfully engineered variations of CTSR and demonstrated a dose-dependent suppression of TGF-β signaling (lower SMAD2/3) and activation of the TLR response (higher IRAK4) upon exposure to TGF-β. We then showed that CTSR reversed the inhibitory effect of TGF-β on CTSR B7-H3 CAR-T cells. While B7-H3 CAR-T cells alone failed to control NB in vitro when exposed to physiological doses of TGF-β, CTSR B7-H3 CAR-

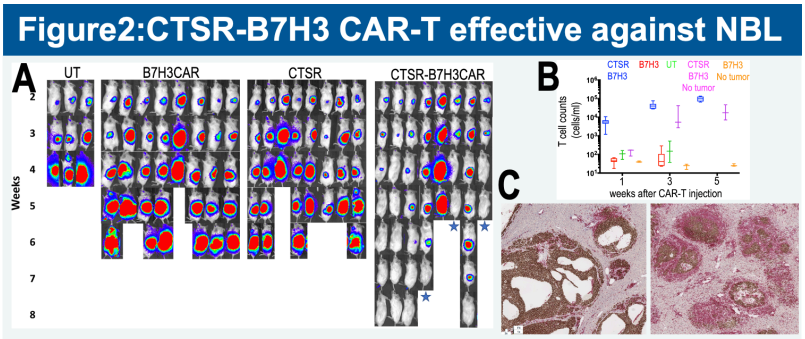
T cells maintained effective tumor control despite increasing doses of TGF-β. More importantly, we observed that CTSR B7-H3 CAR-T cells retained superior anti-tumor efficacy in repetitive tumor exposure, where B7-H3 CAR-T cells alone or armed with alternative switch receptors such as TGFβR2-4-1BB, TGFβR2-DAP12, or TGFB-DNR eventually failed. CTSR B7-H3 CAR-T cells subjected to repetitive tumor exposure showed augmented expansion profiles, enhanced CD4 populations, and longer-lasting T cell memory phenotypes. In an orthotopic disease model of NB, CTSR B7-H3 CAR-T cells demonstrated impressive tumor penetration and disease control, whereas B7-H3 CAR-T cells alone universally failed. The extent of disease control correlated with higher levels of human inflammatory cytokines and several-fold higher T cell counts in the peripheral blood of the mice receiving CTSR CAR-T cells. These findings were similarly replicated in orthotopic models of pediatric EWS and OS.

Conclusions: CTSR armored B7-H3 CAR-T cells have demonstrated superior efficacy against TGF-β-rich pediatric solid tumor disease models and are poised to overcome the immunosuppressive TME barrier, a critical roadblock to the success of CAR-T cells in solid tumors. Our preliminary data provides the rationale for a first-in-human phase I trial of this promising product.

Figure1:CTSR switches TGF-B signaling and reverses its inhibitory effect on CAR-T cells



A-B, Construct design, and CAR expression profile. **C,** CTSR CAR-T cells activate the TLR pathway (via IRAK4) and downregulate the TGF-β signaling pathway (via SMAD2/3). **D,** CTSR-CAR variants demonstrate sustained expansion even under TGF-β treatment. **E,** CTSRC-CARs effectively control tumor cells, overcoming the suppressive effects of high-dose TGF-β. **F,** CTSR-CARs maintain robust, long-term tumor killing, while other CAR variants fail within one week under physiological TGF-β conditions. **G,** CTSR-CAR-T cells exhibit lower cumulative expression of exhaustion markers PD-1, LAG-3, and TIM-3 in response to TGFB exposure.



A, BLI images reveal that CTSR-B7H3 CAR-T cells successfully treat aggressive orthotopic NBL, whereas B7H3 CAR-T cells fail to achieve this outcome. **B,** CTSR CAR-T cells show significantly greater expansion in the peripheral blood of treated mice. **C,** IHC images (brown indicating NBL, red indicating T cells) illustrate strong tumor infiltration and potent anti-tumor effects of CTSR-B7H3 CAR-T cells (right), as compared to the limited efficacy of B7H3 CAR-T cells in the orthotopic model.

742 Nrf2 Deficiency Improves CAR-T Cell Survival and Function in Breast Cancer Models

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$CD8^+$ cytotoxic T lymphocytes play a pivotal role in tumor rejection but frequently face dysfunction and exhaustion within the immunosuppressive tumor microenvironment (TME). Elevated levels of reactive oxygen species (ROS) in the TME exacerbate T cell impairment, yet the precise molecular mechanisms underlying this phenomenon remain unclear. Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor activated by ROS, is associated with tumor progression. We hypothesized that ROS might influence the anti-tumor activity of $CD8^+$ T cells via Nrf2-mediated pathways. Our study revealed that tumor growth was significantly reduced and reversed in Nrf2-deficient mice upon T cell depletion. We observed increased Nrf2 expression in tumor-infiltrating (TI) T cells, suggesting a role in regulating their function. Importantly, Nrf2 deficiency in $CD8^+$ T cells enhanced their effector functions and resistance to ROS in a murine solid tumor model, enabling sustained activity in a ROS-rich TME. Furthermore, Nrf2 knockdown in human chimeric antigen receptor (CAR)-T cells improved their survival and functional activity in a humanized breast cancer model, resulting in effective tumor control. These findings uncover a novel mechanism linking oxidative stress to immune suppression in cancer and identify Nrf2 as a promising therapeutic target for enhancing T cell-based immunotherapies in ROS-rich solid tumors.

743 Metabolic Reprogramming of HER2 CAR-T Cells Under Hypoxic Conditions: Insights from a multi-omics approach.

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Background: Chimeric Antigen Receptor (CAR) T-cell therapy has revolutionized hematological cancer treatment, yet its efficacy in solid tumors remains constrained by the challenging tumor microenvironment. Solid tumors characteristically exhibit oxygen levels ranging from 0.5% to 5%, dramatically different from normoxic conditions. This hypoxic environment profoundly impacts immune cell function, particularly T cell metabolism and persistence. Previous research has demonstrated that T cells undergo metabolic reprogramming in low-oxygen conditions,

transitioning from oxidative phosphorylation to glycolytic metabolism. However, the precise metabolic adaptations of CAR-T cells in hypoxic environments and their consequent anti-tumor efficacy remain incompletely understood.

Methods: Our comprehensive investigation utilized an integrated multi-omics approach to characterize HER2 CAR-T cell responses under varying oxygen conditions. Human T cells were isolated from healthy donors, transduced with a HER2-specific CAR construct, and cultured under normoxic (21% O2), moderate hypoxic (5% O2), and severe hypoxic (1% O2) conditions for 24 and 72 hours. We performed 10x Genomics single-cell RNA sequencing to capture transcriptional dynamics, Seahorse metabolic flux analysis to assess cellular energetics, and mass spectrometry to quantify intracellular metabolite profiles. Restimulation assays with HER2-expressing K562s cells evaluated functional capabilities, while advanced computational approaches enabled integrative analysis of the multi-dimensional datasets.

Results: Our analysis revealed nuanced transcriptional and metabolic variations across different hypoxic conditions. Significant differences emerged in gene expression patterns, metabolic pathway utilization, and functional capacities between normoxic and hypoxic environments. These variations were particularly pronounced when comparing moderate and severe hypoxic states, suggesting complex cellular adaptation mechanisms. Metabolic pathway analysis highlighted dynamic shifts in cellular energetics, with notable focus in glycolytic and mitochondrial metabolic nodes.

Conclusion: By integrating single-cell sequencing, metabolomics, and functional data, we have uncovered critical metabolic adaptation mechanisms of CAR-T cells in hypoxic conditions. Our findings provide a foundational framework for developing synthetic biology approaches to engineer more resilient and effective CAR-T cell therapies. Future research will focus on translating these insights into targeted metabolic modifications that enhance CAR-T cell performance in challenging solid tumor microenvironments.

744 EGFR-Targeting Ligand-Based CAR-T Cells Demonstrate Favorable Antitumor Activity Against Gynecologic Malignancies.

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Objective. In recent years, the introduction of PARP and immune checkpoint inhibitors has improved the treatment outcomes of maintenance therapy and recurrent cases of gynecological malignancies. However, there is still no new treatment for cases that are resistant to these treatments. Epidermal growth factor receptor (EGFR) is reported to be overexpressed in gynecologic malignancies. However, the clinical efficacy of existing molecular EGFR-targeted therapies against gynecological malignancies has not been demonstrated. This study investigated the antitumor effects of ligand-based EGFR chimeric antigen receptor (CAR)-T cells on gynecological malignancies.

Methods. EGFR expression in patient surgical samples was evaluated using immunohistochemistry. Ligand-based EGFR CAR-T cells were generated via piggyBac-mediated gene transfer. EGFR CAR-T cells were co-cultured with six EGFR-positive gynecologic malignancy cell lines, and their antitumor effects were assessed via flow cytometry. In addition, to check the serial killing activity of CAR-T cells, a long-term culture was performed by repeatedly adding malignancy cells. In our in vivo study, tumor-bearing mice implanted with gynecological malignancy cell lines expressing firefly luciferase in their intraperitoneal cavity were administered EGFR CAR-T cells, CD19 CAR-T cells, or phosphate-buffered saline (PBS) intraperitoneally. Similarly, a subcutaneous transplantation model was created, and CAR-T or PBS was injected into the tail vein. Tumor growth was monitored using an IVIS® Lumina II in vivo imaging system.

Results. EGFR positivity was observed in 41%, 82%, and 79% of ovarian, endometrial, and cervical malignancies in patient samples, respectively. EGFR CAR-T cells were successfully generated with high CAR positivity and a high proportion of naïve/stem cell memory-like T cells. On the other hand, there was almost no expression of the PD-L1 exhaustion marker. In the co-culture experiment, the growth of all six gynecologic malignancy cell lines was significantly suppressed by EGFR CAR-T cells compared to mock T cells. In long-term culture, CAR-T cells also showed favorable cytotoxic activity. In intraperitoneal gynecological malignancies mouse models, mice treated with EGFR CAR-T cells displayed significantly decreased tumor burden compared to those treated with either CD19 CAR-T cells or PBS. Tail vein intravenous injection of CAR-T cells proved effective against cervical malignancies in a subcutaneous transplantation model.

Conclusions. Ligand-based EGFR CAR-T cells may be a promising therapy for various gynecological malignancies.

745 Development of GMP-Compliant Processes for Novel Mesothelin Targeting hYP218 CAR Lentiviral Vector and T Cell Production

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Development of GMP-Compliant Processes for Novel Mesothelin Targeting hYP218 CAR Lentiviral Vector and T Cell Production

Hui Dong[1], Lianghua Bin[1], Cynthia Ng[1], Xiaoyi Yang[1], Warren Rowe[1], Tanusree Sen[1], Chinatsu Mukai[1], Brian Browser[1], John Roach[1], Ray Harris[2], Jason Yovandich[2], Mitchell Ho[3], Raffit Hassan[4], Vinay Vyas[1]

Mesothelin is a cell-surface antigen overexpressed in a variety of solid malignant tumors, making it an attractive target for chimeric antigen receptor (CAR) T cell therapy. Here, we report the development and optimization of a novel CAR T cell therapy process based on a humanized hYP218 antibody targeting the membrane-proximal epitope of mesothelin[i]. This study focused on production of replication-defective lentiviral vectors encoding hYP218 CAR and the subsequent engineering and expansion of CAR T cells in both small-scale and Good Manufacturing Practice (GMP)-scale setups.

Lentiviral vectors were produced using a third-generation lentiviral platform, involving four-plasmid co-transfection, tangential flow filtration (TFF), chromatography, and sterile filtration. These vectors demonstrated a physical p24 titer of 6.72 $x10^6$ pg/mL and an infectious titer as 1.79 $x10^8$ TU/mL. This lentivirus process was further scaled up and GMP grade lentiviral vectors were produced in a GMP facility.

CD4+/CD8+ enriched T cells from peripheral blood apheresis were transduced using purified hYP218 CAR lentiviral vectors and further expanded. T cell process was developed in a small-scale laboratory setup starting at $1x10^6$ cells and scaled up to starting at 55 $x10^6$ cells in an automated device CliniMACS Prodigy (Miltenyi Biotec). The resulting hYP218 CAR T cells achieved CAR expression levels ranging from 47.64% to 57.58% across donors, with a multiplicity of infection (MOI) of 2.06 and a vector copy number (VCN) of 2.1-2.7 copies/transduced cell. The CAR T process produced 2.17-2.19 $x10^9$ hYP218 CAR T cells starting from 55 $x10^6$ CD4+/CD8+ enriched T cell. These CAR T cells demonstrated robust dose-dependent killing potency against mesothelin-expressing cancer cells in cytotoxicity assays.

In summary, we successfully developed and scaled up the production of high-quality hYP218 CAR lentiviral vectors and CAR T cells under GMP conditions, demonstrating high-quality outputs suitable for clinical use. These advancements establish a robust GMP-compliant manufacturing platform for hYP218 CAR T cell therapy into clinical trials for mesothelin-positive solid tumors.

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746 Drug-Inducible Transcriptional Control of CAR-T Cells for Enhanced Safety and Efficacy

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Chimeric Antigen Receptor (CAR) T cell therapy has demonstrated remarkable clinical success for the treatment of hematological malignancies and holds great promise for expanding its application to other types of cancer and autoimmune disease. However, extending CAR T cell therapy to solid tumors presents significant challenges including limited efficacy, poor expansion and persistence of the engineered cells and potential on-target off-tumor toxicity. Furthermore, the severity of the adverse effects may be enhanced by strategies to improve the efficacy by e.g. co-expression of cytokines. In order to address both, safety and efficacy questions, a tight control of the engineered cells would be favorable. We have developed a drug-inducible ON-switch system that allows transcriptional regulation of either the CAR or a booster factor in presence of an orally available small molecule.

Our novel drug-inducible switch system consists of a split synthetic transcription factor (sTF) that can be functionally reconstituted in presence of caffeine. To generate the split sTF, we made use of an anti-caffeine nanobody heterodimer pair and fused a zinc finger DNA binding domain to one of the nanobodies and a transcriptional activator to the other. Transcription of the gene of interest is regulated via an engineered inducible promoter specifically targeted by the sTF.

We demonstrate the versatility of the system, being functional with a wide range of different DNA binding domains and activation domains used in different sTFs, leading to different expression levels of the regulated gene. We also show that response of the inducible promoter can be modulated by modifying the number of sTF binding sites, expanding the design features available to tune our transcriptional switch. All of these, together with the drug dose, highlight the potential to tailor our system to meet specific requirements of the therapy in terms of the expression level of the gene of interest (e.g. CAR or booster molecule).

Using primary human T cells *in vitro,* we demonstrate caffeine dose-dependent expression and cytolytic activity of inducible anti-CD20 CAR and, importantly, no background expression in the absence of the inducer. Furthermore, we investigated the ON-OFF kinetics of the transcriptional switch to prove that the expression of the anti-CD20 CAR can be re-induced after complete shutdown of the system. In contrast to suicide switches, this allows to resume the therapy in case of e.g. relapse. Moreover, we explored the drug-inducible control of additional factors with the potential to enhance efficacy, expansion and persistence of engineered cells constitutively expressing a CAR. Our results reveal improved persistence and efficacy *in vitro* of anti-CD20 CAR cells expressing an active mutant of STAT5B in a caffeine-regulated manner. Importantly, this effect was strictly dependent on the presence of inducer and the strength of the response correlated with the caffeine dose.

We expect that our novel drug-inducible transcriptional switch will enable the development of more potent CAR T cell therapies by regulating the expression of booster proteins while preserving safety and mitigating side effects.

Additionally, when applied to control the expression of the CAR, it allows for a tight regulation of CAR T cell activity to finetune response and enhance safety.

747 Computationally Guided Point Mutations for Screening of Enhanced CAR T cells

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Chimeric antigen receptor (CAR)-T cell therapy has transformed cancer treatment, showing substantial promise against various malignancies. However, the optimization of CAR constructs remains an ongoing challenge. While most clinically approved CAR-T cell therapies rely on combinations of CD3ζ, CD28 and 4-1BB, there is growing interest in refining CAR design through more targeted engineering approaches. Traditional methods, such as domain shuffling, have been used to generate CAR diversity, but we propose a more precise method involving point mutations to enhance CAR function. In this study, we computationally selected mutation sites and amino acid substitutions based on their evolutionary conservation, aiming to introduce beneficial changes without disrupting the receptor's structural integrity. This strategy allows for the targeted optimization of specific regions within the CAR, such as the hinge and co-stimulatory domains, for improved signaling and overall CAR-T cell performance. We constructed a library of ~10^8 CAR variants, all featuring second-generation CD28-based receptors targeting CD19. Point mutations were introduced into the hinge and co-stimulatory domains to enhance CAR-T cell activity. From this library, 500 unique CAR variants were selected, transfected into human T cells and tested for their anti-tumor activity against CD19+ tumor cells. Several CAR-T cell clones expressing mutated receptors demonstrated significantly improved anti-tumor responses compared to control CAR-T cells expressing the parental CD28-based construct. These findings highlight the potential of using point mutations as a precise tool for optimizing CAR-T cell therapies and advancing cancer immunotherapy.

748 ICANS and CRS Following Lymphodepletion in CAR-T Therapy: A Real-World Data Analysis

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Lymphodepletion (LD) chemotherapy is an essential preparatory regimen employed prior to CAR-T cell infusion to enhance the body's ability to accept and proliferate infused CAR-T cells. While overall response rates (ORR) for both LD regimens are reported to be similar, varying rates of Immune effector Cell-Associated Neurotoxicity Syndrome (ICANS) and Cytokine Release Syndrome (CRS) have been reported. This study investigates the relative risk of ICANS and CRS post LD with these regimens as represented in Real-World Data (RWD) from 2018 through 2024.

RWD analysis utilizing the TriNetX database including data from over 229 million patients worldwide was employed to analyze the relative incidence of ICANS and CRS in patients undergoing LD via either Benda or Flu/Cy within 10 days prior to CAR-T infusion with any FDA-approved CAR including group matching and propensity analysis.

Results from 3,730 patients (3240 Flu/Cy and 490 Benda) with identical median follow-up times of 180 days indicate no significant difference in rates of ICANS or CRS between LD regimens. ICANS risk was slightly higher in the Flu/Cy group (16.7%) compared to Benda (14.3%) but this difference was not statistically significant ($p = 0.184$). Survival analysis showed similar results with no significant difference in survival probabilities (83.39% vs 86.32%, $p = 0.118$) or hazard ratio (1.227, $p = 0.551$). CRS risk was similar between Flu/Cy (37.0%) and Benda (38.8%) with no statistically significant difference ($p = 0.458$). Survival analysis showed no significant differences with similar survival probabilities (62.71% vs 61.76%, $p = 0.492$) and hazard ratio (0.947, $p = 0.075$). The number of instances of CRS was also not significantly different.

Propensity score matching was employed to balance demographic characteristics. The matching process reduced both cohorts to 480 patients each effectively balancing most demographics with minor residual differences in some racial categories. Follow-up times were similar between cohorts with a median of 180 days for both before and after matching. For ICANS Flu/Cy showed a slightly higher risk (18.8% vs 14.6%) and lower survival probability (82.00% vs 86.27%) but these differences were not statistically significant. CRS risks were identical (39.6%) with very similar survival probabilities but again not statistically significant.

This study found no significant differences between the rates of ICANS and CRS post-CAR-T with Benda vs Flu/Cy LD per RWD, even after propensity matching . This RWD analysis re-confirms earlier findings although additional confound analysis is needed to clarify the comparative safety profiles of these LD regimens.

749 Epidemiology and Impact of Acute Gastrointestinal Hemorrhage on Outcomes of Chimeric Antigen Receptor T-Cell Therapy in Patients With Hematologic Malignancies: Insights From a Nationwide Database

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Introduction: Patients with hematologic malignancies are predisposed to both bleeding and thrombotic events due to a range of risk factors. Following chimeric antigen receptor T-cell (CAR-T) therapy, these risks may be amplified by hematologic abnormalities such as impaired hematopoietic recovery, coagulation defects, and increased thrombotic tendencies. Additionally, cytokine release syndrome (CRS), a common CAR-T-related complication, can exacerbate coagulopathy. Despite these risks, limited data are available regarding the epidemiology of gastrointestinal hemorrhage (GIH) and its influence on CAR-T therapy outcomes. This study evaluates the impact of GIH on clinical outcomes in CAR-T recipients using data from the National Inpatient Sample (NIS) database.

Methods: We analyzed data from the NIS to identify adults diagnosed with non-Hodgkin's lymphoma (NHL), B-cell acute lymphoblastic leukemia (ALL), and multiple myeloma (MM) who underwent CAR-T therapy between 2018 and 2021, as identified by ICD-10 codes. Patients were stratified based on the presence or absence of GIH. To ensure national representativeness, complex sampling weights were applied. Sociodemographic characteristics and comorbidities were compared across groups. The primary outcome was all-cause in-hospital mortality, with secondary outcomes including various complications. Multivariate regression models were used to assess outcome disparities, with statistical significance defined as $p < 0.05$.

Results: Out of 4,120 adults who received CAR-T therapy, 82.5% had NHL, 12.6% had MM, and 4.9% had ALL. GIH occurred in 1.46% of these patients. Baseline demographic characteristics were similar between the GIH and non-GIH groups; however, atrial fibrillation was significantly more prevalent in the GIH group (41.67% vs. 11.33%, $p < 0.001$). While the overall in-hospital mortality rate was 3.0%, patients with GIH had markedly higher mortality (16.7%). After adjusting for confounders, GIH was associated with significantly increased odds of all-cause mortality (adjusted odds ratio [aOR] 5.04, 95% CI 1.01–29.01) and higher risks of complications, including acute kidney injury (aOR 4.41, 95% CI 1.07–18.21), respiratory failure (aOR 9.17, 95% CI 1.88–44.79), and shock (aOR 5.29, 95% CI 1.19–23.58). No significant differences were observed between groups regarding the incidence of immune effector cell-associated neurotoxicity syndrome, deep venous thrombosis, pulmonary embolism, intracranial hemorrhage, acute blood loss anemia, acute heart failure, or acute pulmonary edema. Additionally, there were no significant differences in length of hospital stay (LOS) or mean hospitalization costs, which were 16.96 days and $1,196,413, respectively, across the entire CAR-T cohort.

Conclusion: Among patients with hematologic malignancies undergoing CAR-T therapy, the development of GIH was associated with significantly higher in-hospital mortality and increased rates of severe complications. These findings highlight the detrimental impact of GIH on CAR-T therapy outcomes and emphasize the need for targeted management strategies in this high-risk population.

750 A United States Population-Based Study on the Epidemiology and Impact of Hemophagocytic Lymphohistiocytosis on In-Hospital Outcomes in Patients Receiving Chimeric Antigen Receptor T-Cell Therapy

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Introduction: Hemophagocytic Lymphohistiocytosis (HLH) is a life-threatening hyperinflammatory state characterized by uncontrolled immune cell activation, leading to a cytokine storm and hemophagocytosis. This cascade results in cytopenia, coagulopathy, progressive multi-organ failure, and potentially death. In patients undergoing chimeric antigen receptor T-cell therapy (CAR-T), HLH has emerged as a recognized complication and is termed immune effector cell-associated HLH-like syndrome (IEC-HS). While efforts have been made to define its characteristics, pathogenesis, and treatment options, studies examining its epidemiology and population-level impact on CAR-T outcomes remain limited. In this study, we investigate the epidemiology of HLH and its impact on in-hospital outcomes in CAR-T patients using United States population data.

Methods: We used the National Inpatient Sample to identify adults with non-Hodgkin's lymphoma (NHL), B-cell acute lymphoblastic leukemia (ALL), and multiple myeloma (MM) who underwent CAR-T from 2018 to 2021 using ICD-10 codes. The cohort was stratified by HLH status, and complex sampling weights ensured national representation. We compared sociodemographic characteristics and comorbidities between groups, with all-cause mortality as the primary outcome and various secondary outcomes also examined. Multivariate regression assessed outcome disparities, with statistical significance set at $p < 0.05$.

Results: We identified 4,120 adults who received CAR-T (82.5% NHL, 12.6% MM, 4.9% ALL), of whom 50 (1.2%) had a concurrent diagnosis of HLH. The HLH group had a higher proportion of female patients (80.0% vs. 39.7%, $p < 0.05$); otherwise, there were no significant differences in demographic characteristics or comorbidities between the groups. Overall mortality was 3.0%, but 20.0% in HLH patients. HLH was associated with higher odds of all-cause mortality (aOR 7.1, 95% CI 1.1–44.2). Moreover, patients with HLH had higher odds of experiencing shock (aOR 6.3, 95% CI 1.5–25.3), disseminated intravascular coagulation (aOR 21.6, 95% CI 4.2–110.5), intracerebral hemorrhage (aOR 51.3, 95% CI 3.8–685.3), and tumor lysis syndrome (aOR 10.0, 95% CI 2.7–37.3). They also had higher odds of requiring mechanical ventilation (aOR 18.2, 95% CI 4.5–73.0) and vasopressor support (aOR 24.2, 95% CI 6.0–97.9). There were no increased odds of respiratory failure, acute kidney injury, gastrointestinal hemorrhage, acute liver failure, or immune effector cell-associated neurotoxicity in patients with HLH compared to those without HLH. Patients with HLH had longer hospital stays (32.4 vs. 16.8 days, $p < 0.05$; adjusted incidence rate ratio [aIRR] 1.9, 95% CI 1.3–2.6). The mean hospitalization charges for CAR-T patients in general were $1,196,413, with no significant difference between the groups.

Conclusion: HLH in CAR-T patients is linked to higher odds of all-cause mortality, adverse outcomes, a greater need for intensive care, and extended hospital stays. Based on the findings of this study, we stress the importance of early identification and prompt management of HLH in CAR-T patients to reduce its negative impact on outcomes and alleviate the associated healthcare burden.

751 Does the Occurrence of ICANS After CAR-T Therapy Increase the Risk of Late Neurological Complications: A Real-World Data Analysis

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A common and challenging adverse event (AE) associated with CAR-T cell therapy is immune cell-associated neurotoxicity syndrome (ICANS) occurring in 20–60% of patients. ICANS typically occurs within hours to weeks of CAR-T therapy presenting with a wide range of neurological symptoms ranging from mild confusion to severe encephalopathy and potentially life-threatening cerebral edema. While the acute management of ICANS has improved concerns persist about potential long-term neurological sequelae. This study aims to evaluate whether ICANS after CAR-T therapy is associated with the development of Parkinson's disease (PD), in the months and years following treatment.

The TriNetX Real-World (RW) global dataset comprising electronic health records (EHR) of 229 million patients from diverse healthcare organizations worldwide was utilized for this analysis. A total of 4,700 patients in this dataset received one of the US FDA-approved CAR-T therapies, including axicabtagene ciloleucel, tisagenlecleucel, lisocabtagene maraleucel, brexucabtagene autoleucel, and idecabtagene vicleucel. Among these, 820 patients developed ICANS (Cohort 1), while 3,880 patients did not develop ICANS (Cohort 2). Lifetime follow-up data from this RW dataset as of January 10, 2025 revealed that 10 patients (1.22%) in Cohort 1 developed Parkinson's disease (PD) any time after three months of CAR-T therapy whereas 30 patients (0.77%) in Cohort 2 developed PD in the same timeframe. Although the incidence of PD was higher in Cohort 1, the difference was not statistically significant (p-value = 0.2095; Fisher’s Exact Test).

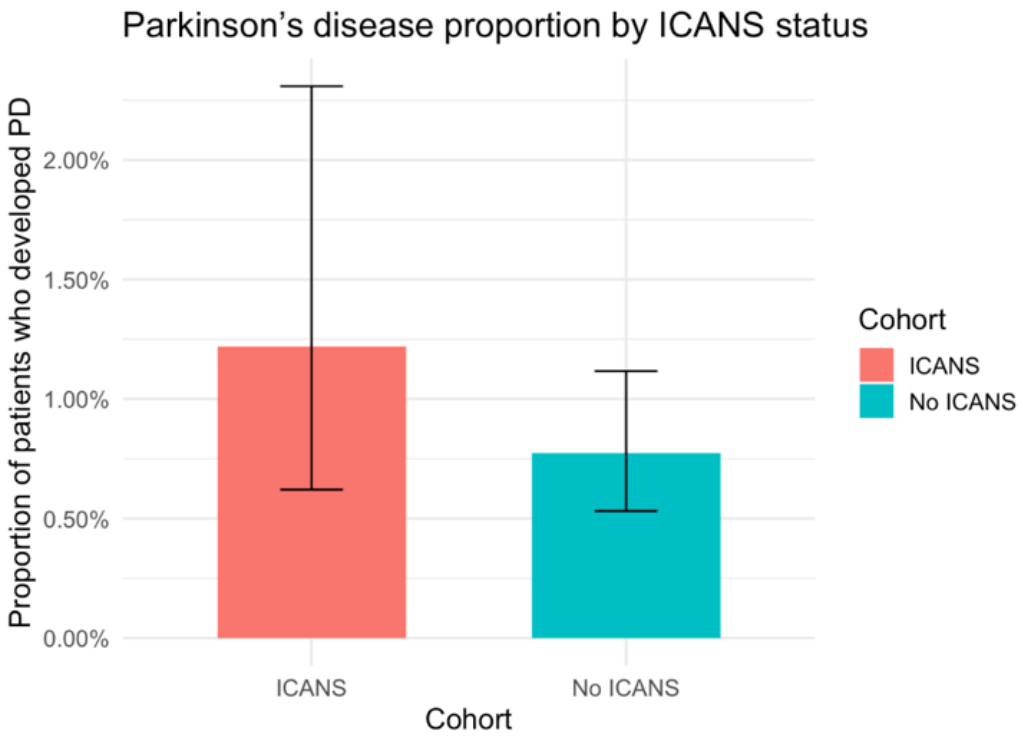
This real-world data analysis revealed that there is no significant association between the development of PD and ICANS post-CAR-T therapy. While the analysis of this large RW dataset reconfirms the findings from earlier studies the slightly higher incidence of PD in the ICANS group while not statistically significant warrants further investigation with longer follow-up- periods and larger patient cohorts. Future studies should consider potential confounding factors including age, prior cancer treatment, and underlying cancer type which could impact the risk of developing neurodegenerative disorders after CAR-T.

Table- 1

Patient Cohorts

Cohort	Cohort Description	Sample size (# of patients)	# of patients developed Parkinson Disease (anytime after 3 months of CART therapy)	Percentage of patients
Cohort 1	Patients who developed ICANS within 3 months following CART	820	10	1.22
Cohort 2	Patients who did not develop ICANS within 3 months of CART	3880	30	0.77

Graph -1



752 Social Determinants Hindering CART Access in Civilian and Military Life

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Chimeric Antigen Receptor T-cell (CART) therapy represents a promising advancement in the treatment of certain hematologic malignancies. Underserved patient populations, including veterans, face unique challenges linked to service-related disabilities, mental health conditions, patient financial barriers and reliance on limited healthcare availability, limiting access to CART. Analysis of real-world data (RWD) on Social Determinants Health (SDOH) was conducted to identify factors impacting access to CART.

Review of RWD from the TriNetx claims database covering 4,990 patients across 35 Health Care Organizations (HCOs) from 2019-2025 was conducted together with analysis of clinical trial data on Veteran Affairs (VA) centers from Citeline.

Results of this analysis indicate significant impact due to lack of CART availability in community-based treatment facilities with zero of the 88 United States facilities providing CART at community-based centers. Across the United States, there are 300 academic medical centers, but a small portion—13%—offer CAR-T therapy to patients. Citeline data shows that oncology clinical trials involve only 18 VA centers. Within the extensive network of the Veterans Health Administration (VHA), compromising of 170 medical centers and 1,193 outpatient clinics, the capability to provide CAR-T therapy is limited to a single facility. The VA serves 9.1 million veterans each year with approximately 56,000 veterans in the VHA system being diagnosed with cancer annually. TriNetx data brought to light that of the 4,990 patients undergoing CART treatment 75.75% of those were white with 8.0% black or African American and 12.21% Asian, Other or Unknown. Ethnicity analysis reveals 74.14% not of Hispanic or of Latino descent 7.61% Hispanic or Latino and 18.43% of unknown ethnicity. Differences in gender appear but may be explained by disease state.

For the general population challenges to CART access are multifaceted. The high cost of CART therapy which can exceed $500,000 per treatment creates significant barriers. High insurance deductibles, out-of-pocket expenses along with limited insurance coverage present challenges to patients with health insurance while the uninsured are often excluded from CART access outside of clinical trials. Geographic disparities also play a critical role, as CART therapy is only available at a limited number of certified treatment centers primarily located in urban or academic medical centers. Patients in rural or underserved areas often face significant travel burdens, logistical challenges, and delays in receiving care. The availability of only one VHA facility equipped to administer CART exacerbates the problem. While CART has been proven to extend the life of many patients access to this life-saving treatment must be adapted to meet the needs of underserved populations as RWD demonstrates that the impact of SDOH disproportionately effects vulnerable populations. This analysis highlights the urgent need to integrate SDOH considerations into the deployment of novel therapies to bridge gaps in healthcare delivery for underserved populations.

753 CD45 as a Universal Target for Adjuvant TCR-T Cell Therapy Following Allogeneic Hematopoietic Cell Transplantation

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Engineered cell therapies have transformed the treatment of B-cell malignancies, but myeloid malignancies like AML, MDS, and some forms of ALL remain a high unmet need. To date, allogeneic hematopoietic cell transplantation (HCT) remains the only curative option for patients with these malignancies, but up to 40% of patients relapse post-HCT, with >80% mortality within 2 years of relapse. One way to prevent relapse is to administer donor-derived TCR-engineered T cells (TCR-T cells) immediately following transplantation that target antigens presented on patient-derived, but not donor-derived, hematopoietic cells. This provides a way to eliminate residual malignant and pre-malignant hematopoietic cells while sparing normal, donor-derived cells. Early data from the ALLOHA trial suggest that TCR-T cells targeting HA-1 or HA-2, antigens presented on HLA-A*02:01, reduce relapse rates in patients with AML, MDS, and ALL undergoing allogeneic HCT with reduced intensity conditioning (Al Malki, ASH 2024). To expand this trial to include TCR-Ts targeting additional HLA types, we report a novel strategy in which TCR-Ts are developed that target antigens derived from the universal heme-restricted protein CD45 (PTPRC). We demonstrate preclinical proof-of-concept studies using a lead, HLA-A*03:01-restricted, CD45-targeted TCR identified using novel in vitro stimulation and screening platforms. We show that the TCR-T candidate is target-specific, with good safety and efficacy profiles, sparing HLA-A*03:01-negative hematopoietic cells and HLA-A*03:01-positive non-hematopoietic cells, while eradicating HLA-A*03:01-positive, CD45-positive hematopoietic cells of normal and transformed origin in vitro and in vivo. This TCR-T candidate has now been advanced to IND-enabling studies and will be incorporated into the ongoing ALLOHA Phase 1 study.

754 Impact of Protein-Energy Malnutrition on Outcomes of Chimeric Antigen Receptor T-Cell Therapy in Patients with Hematologic Malignancies: Insights from a National Database

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Introduction: Protein-energy malnutrition (PEM) is common in cancer patients, including those with hematologic malignancies, and can affect multiple organ systems, including cardiovascular, immune, and cognitive functions.

PEM is linked to poorer outcomes and longer hospital stays across various cancer types. In patients with hematologic malignancies receiving chimeric antigen receptor T-cell (CAR-T) therapy, PEM is associated with prolonged hospitalizations. However, limited research exists on how PEM influences CAR-T outcomes and related complications. This study aims to assess the impact of PEM on CAR-T complications and outcomes using United States population data.

Methods: We used the National Inpatient Sample to identify adults with non-Hodgkin's lymphoma (NHL), B-cell acute lymphoblastic leukemia (ALL), and multiple myeloma (MM) who underwent CAR T-cell therapy between 2018 and 2021, based on relevant ICD-10 codes. The cohort was stratified by the presence or absence of PEM, and complex sampling weights ensured national representation. We compared sociodemographic characteristics and comorbidities between PEM and non-PEM patients. The primary outcome was all-cause in-hospital mortality, with secondary outcomes including complications also examined. Multivariate regression models assessed outcome disparities between the groups, with statistical significance set at $p < 0.05$.

Results: We identified 4,120 adults who received CAR-T therapy (82.5% with non-Hodgkin's lymphoma, 12.6% with multiple myeloma, and 4.9% with acute lymphoblastic leukemia), of whom 790 (19.2%) had concurrent PEM. The PEM group had a higher proportion of patients with anemia (92.4% vs. 76.7%, $p < 0.001$); otherwise, there were no significant differences in demographic characteristics or comorbidities between the groups. Overall mortality was 3.0%, but 10.8% in the PEM group. PEM was associated with higher odds of all-cause mortality (adjusted odds ratio [aOR] 9.4, 95% confidence interval [CI] 3.7–23.8). Additionally, PEM patients had higher odds of acute kidney injury (aOR 2.1, 95% CI 1.3–3.4), respiratory failure (aOR 4.3, 95% CI 2.3–8.0), shock (aOR 2.8, 95% CI 1.4–5.7), immune effector cell-associated neurotoxicity syndrome (aOR 2.7, 95% CI 1.7–4.2), disseminated intravascular coagulation (aOR 10.1, 95% CI 4.0–25.4), and gastrointestinal hemorrhage (aOR 6.5, 95% CI 1.7–24.8). PEM was also associated with higher odds of renal replacement therapy (aOR 5.1, 95% CI 1.5–16.6), mechanical ventilation (aOR 4.7, 95% CI 2.3–9.7), and vasopressor use (aOR 3.4, 95% CI 1.7–7.0). Furthermore, PEM patients had longer hospital stays (24.7 vs. 15.1 days, $p < 0.001$; adjusted incidence rate ratio [aIRR] 1.6, 95% CI 1.4–1.8) and higher mean hospitalization charges ($1,638,050 vs. $1,092,026, $p < 0.001$; aIRR 1.4, 95% CI 1.2–1.6).

Conclusion: Our study found that patients with hematologic malignancies and concurrent PEM who received CAR-T therapy had significantly higher odds of all-cause mortality, adverse outcomes, increased need for intensive care, longer hospital stays, and higher hospitalization charges. These findings emphasize the critical need to address the nutritional needs of this population to improve in-hospital outcomes and reduce healthcare burdens.

755 Comprehensive Immune Reconstitution Profiles Following Chimeric Antigen Receptor T-Cell Therapy

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Introduction: Chimeric Antigen Receptor T (CAR-T) cell therapy has revolutionized the treatment of hematologic malignancies. Significant challenges persist regarding disease relapse and infection-related morbidity, impacted by delayed immune reconstitution (IR). Detailed characterization of CAR-T therapy IR remains limited.

Methods: We conducted a single-center retrospective study analyzing IR patterns in CAR-T patients treated between 1/2016 and 9/2024. Comprehensive flow cytometric analysis assessed immune cell subsets, including myeloid cells, monocytes, dendritic cells, T cells (CD4+, CD8+, Treg, αβ, γδ, naïve, memory, activated, and exhausted), B cells, NK cells, and NK-T cells. Planned measurements were prior to lymphodepletion, at CAR-T infusion, and at defined intervals (days 30, 90, 180, year 1, month 18, and year 2) post-infusion. Clinical data were

collected to analyze IR differences. Response rates and survival analyses with landmark analysis at days 30, 90, and 180 post-CAR-T infusion were performed to evaluate patient outcomes with differing IR profiles.

Results: A total of 319 patients were enrolled, including 220 B-cell lymphoma (BCL) and 39 acute B lymphoblastic leukemia (B-ALL) patients who received CD19-CAR-T cells, and 60 multiple myeloma (MM) patients receiving BCMA CAR-T cells. Early recovery (day 30) was observed in monocytes, dendritic, CD8+ T, NK-T, and T-REG cell subsets, while CD4+ T and B cells showed delayed recovery influenced by underlying disease (Fig 1). Median CD4+ T cell count reached 200 cells/μL by day 180 in BCL and day 365 in MM patients, while B-ALL patients failed to achieve this threshold by day 365. B cell recovery patterns varied by disease type. MM patients showed earliest recovery at day 90, followed by BCL patients at day 180, while B-ALL patients showed minimal B cell recovery even at day 365. In BCL patients, CD4+ T cells <100 cells/μL correlated with significantly reduced progression-free survival (PFS) and overall survival (OS) across multiple timepoints, with day-30 landmark analysis hazard ratios (HR) of 3.35 for OS (95% CI 1.73-6.37, p<0.001) and 1.88 for PFS (95% CI 1.14-3.08, p=0.013) (Fig 2). T-cell activation peaked at day 30, coinciding with maximum exhaustion marker expression. At day 30, patients exhibited variable T cell expansion patterns, ranging from high T cell expansion, particularly in γδ T cells, while others lacked T cell recovery (<300 cells/μL). Patients without T cell reconstitution demonstrated significantly lower OS (30-day landmark analysis HR 2.24, 95% CI 1.28-3.93, p=0.005). Patients receiving bendamustine lymphodepletion demonstrated lower depth of immune suppression at CAR-T infusion in all immune subsets vs fludarabine/cyclophosphamide. However, B cells were low at baseline, presumably from prior therapy. Despite less suppression, all immune subsets were further suppressed, except dendritic cells, which remained unsuppressed in some patients receiving bendamustine. MM patients with unsuppressed myeloid dendritic cells at infusion demonstrated reduced PFS (HR 2.29, 95% CI 1.02-5.14, p=0.045).

Conclusions: This study provides the first comprehensive characterization of CAR-T IR through a detailed analysis of multiple immune cell subsets and identifies key immune subset thresholds that may predict survival outcomes. These findings could inform personalized post-CAR-T monitoring strategies and guide interventions to improve clinical outcomes.

Immune subset	Reference ranges(Cells/uL)	Median at LD(Cells/uL)	Median at D0(Cells/uL)	Median at D30(Cells/uL)	Median at D90(Cells/uL)	Median at D180(Cells/uL)	Median at D365(Cells/uL)
MYELOID CELLS	1,800 - 7,700	2568 (1677-3704)	1860 (1143-3382)	1878 (955-2678)	2479.5 (1725-4033)	2758 (1777-3251)	2287 (1294-6699)
MONOCYTES	100 - 900	314 (145-578)	94 (24-227)	218 (126.5-309.5)	271.5 (200-356)	333 (201-426)	146 (130-377)
MDCS	4-32	13.5 (6-19)	4 (1-8)	10 (4-17)	15 (9-20)	18 (11-29)	10 (10-17)
PDCS	1-20	2 (1-4)	0 (0-1)	1 (0-3)	3 (1-4)	3 (2-7)	2 (1-3)
T-CELLS	411 - 2,061	552 (324.5-906.5)	49 (20-144)	391 (207-842)	481 (227.5-793)	489 (302-1024)	583 (431-762)
HELPER T-CELLS	212 - 1,391	245 (157-409)	31 (14-78)	138 (84-237)	179 (100-267)	203 (139-357)	219.5 (142.5-302)
CYTOTOXIC T-CELLS	59 - 699	228 (119.5-552)	14 (4-40)	211 (80-483)	298 (111.5-479.5)	222 (99-500)	292 (227-393)
DNT-CELLS		11.5 (6-18)	1 (0-3)	7 (3-17)	6 (3-18)	10 (3-18)	28 (7-89)
B-CELLS	32 - 341	13 (13-13)	0 (0-0)	0 (0-0)	0 (0-0)	13 (0-13)	13 (13-77)
NK-CELLS	71 - 499	103 (60-183.5)	5 (1-12)	66 (36-123)	100 (60.5-149.5)	131 (94-171)	132 (107-180)
T-NK CELLS		22 (5-39)	2 (1-6)	15 (7-44)	16 (5-34)	18 (9-39)	56 (18-101)
T-REG		18.5 (7-37)	4 (2.5-9.5)	15 (9-27)	17 (11-25)	17 (10-26)	14 (10-32)

Figure 1. Temporal dynamics of immune cell subset recovery in B-cell lymphoma patients during first year post-CAR-T cell therapy

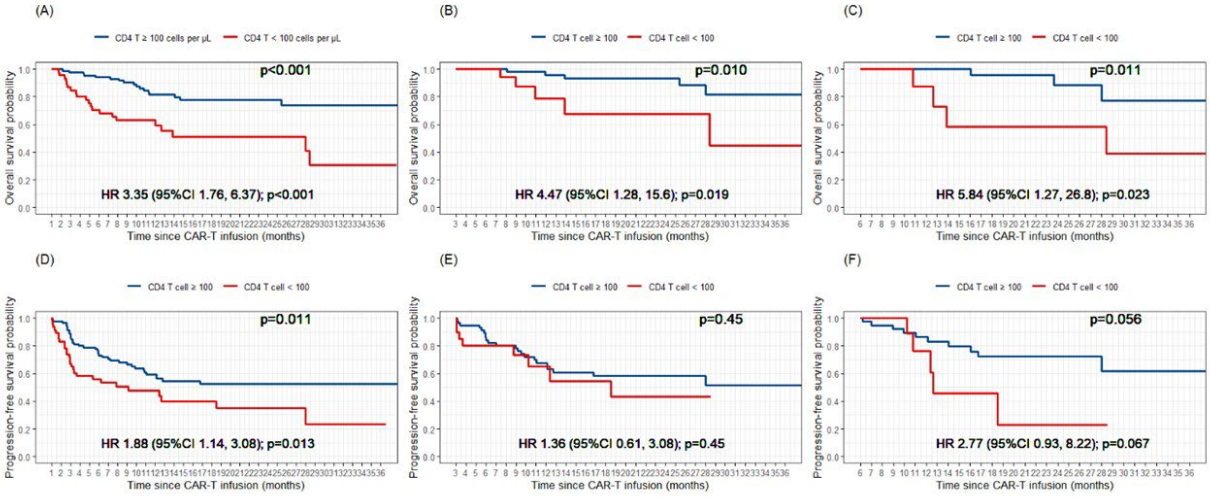

Figure 2. Impact of CD4+ T cell recovery on overall survival and progression-free survival in B-cell lymphoma patients: threshold analysis at days 30, 90, and 180 post-CAR-T therapy. (A) 30-day landmark analysis of overall survival, (B) 90-day landmark analysis of overall survival, (C) 180-day landmark analysis of overall survival, (D) 30-day landmark analysis of progression-free survival, (E) 90-day landmark analysis of progression-free survival, (F) 180-day landmark analysis of progression-free survival.

756 Stage-specific Expression and Signaling of a CAR in TRAC$^{-/-}$ iPSC-ATOs Rescues Positive Selection and Produces More Naïve and Cytotoxic CAR T Cells

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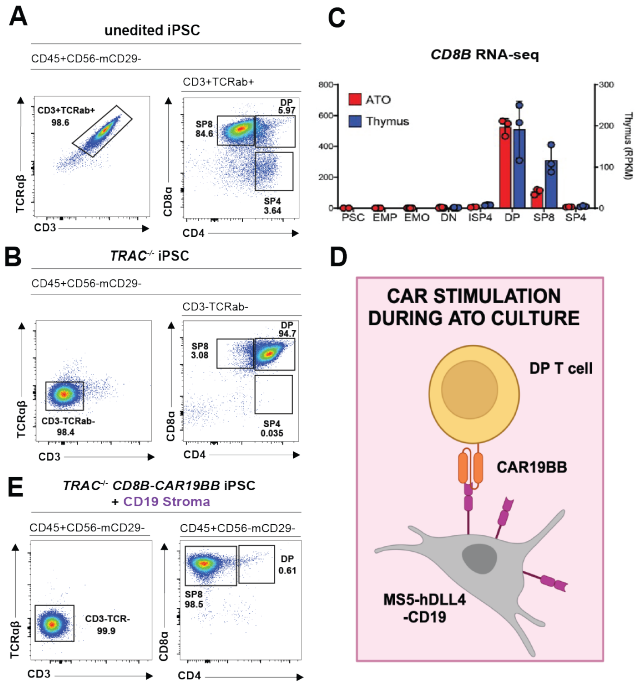
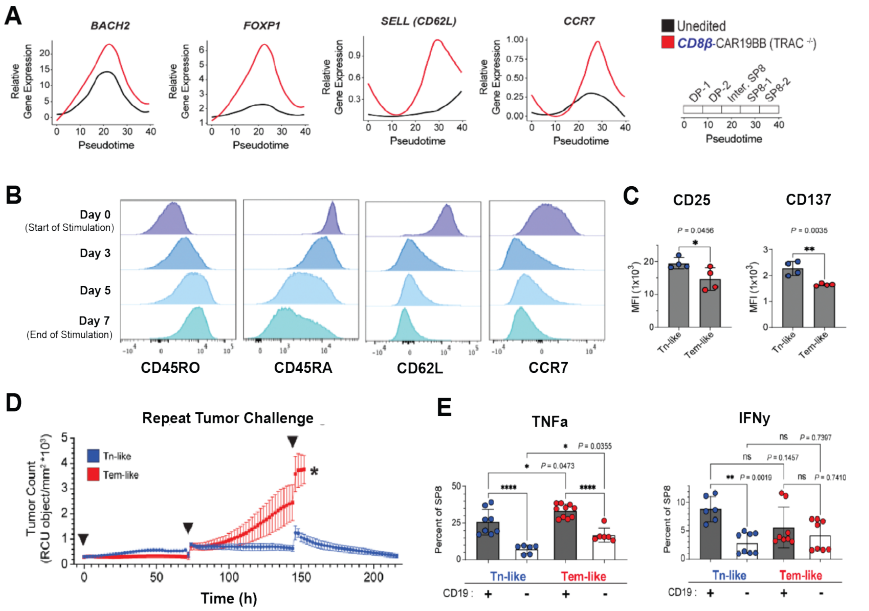
A primary barrier to Chimeric Antigen Receptor (CAR) adoptive T cell therapies is the ongoing reliance on autologous T cell sources and the resulting and unpredictable response rates due to inconsistent T cell potency and *in vivo* exhaustion of cancer patient-derived T cells. Thus, an important goal for the field is to develop strategies to enhance T cell effector function. Induced pluripotent cells (iPSCs) serve as a unique tool in this effort as they can undergo complex multi-gene editing and unlimited clonal expansion. Genetic alterations can be introduced in iPSC to create a universal non-alloreactive transgenic CAR T cell source that is functionally optimized and readily available. However, genetic modifications performed in iPSC may negatively impact subsequent T cell development. Most critically, the elimination of cell surface expression of endogenous T cell receptor (TCR) to prevent graft versus host disease also blocks positive selection, the critical developmental process required to generate mature T cells. During positive selection double positive (DP) T cells mature into single positive CD4+ (SP4s) and CD8+ (SP8s) after a productive interaction between the TCR and a major histocompatibility complex (MHC)-peptide. Our group has developed a method to overcome the block in positive selection through gene editing strategies that produce stage-specific expression of an exogenous TCR or CAR in iPSCs. After differentiation of these engineered iPSC lines in the Artificial Thymic Organoid (ATO) platform, it was observed that mature SP8 T cells that underwent CAR-mediated positive selection were phenotypically and transcriptionally uniformly like naïve T cells (Tn), whereas TCR-mediated positive selection induced mostly effector T cells (Teff). Further, naïve-like CAR T cells produced from the iPSC-ATO when compared to effector memory (Tem) like CAR T cells showed higher *in vitro* tumor killing capacity. In future studies we aim to further define CAR signaling during positive selection and to interrogate the impact of specific CAR signaling on development of mature SP8 phenotype and function.

Figure 1: Stage-specific expression & signaling of a CAR rescues positive selection and T cell development of SP8s.



(A) At 6 weeks of unedited iPSC-ATO development, TCR/CD3 is expressed and primarily single positive CD8+ (SP8) T cells are seen. **(B)** T cells from *TRAC*$^{-/-}$ iPSCs do not express the TCR/CD3 and cannot undergo positive selection. **(C)** RNA-seq data of normal Thymus and ATO differentiation was used to identify candidate genes that were developmentally regulated. *CD8B* was expressed at the DP and SP8 stages of T cell development. Using a CRISPR/-Cas9 editing strategy, a CAR targeting CD19 (CAR19BB) was introduced into the 3' UTR of the *CD8B* gene in the iPSCs, introducing CAR expression during the same expression window as the endogenous *CD8B* gene. **(D)** The CAR was stimulated throughout positive selection by CD19 antigen provided in the stroma within the ATO. **(E)** Delayed expression and stimulation of CAR19BB at the developmental stage of *CD8B* expression completely rescued positive selection in *CD8B-CAR19BB TRAC*$^{-/-}$ iPSCs.

Figure 2: SP8 CAR T cell transcriptional phenotype and impact of culture and stimulation on T cell phenotype and function.



(A) Multiomic analysis of week 3 iPSC-ATOs identified five major clusters as T cells developed from DP stages to mature SP8s. Comparison of gene expression within the defined clusters identified common naïve transcription factors and genes were differentially upregulated in the *CD8B-CAR19BB TRAC*$^{-/-}$ (red) T cells versus unedited T cells (black). **(B)** SP8s from the ATO were stimulated by CD19 expressing K562s (CD19-K562) across 7 days of co-culture. By day 7, naïve markers were largely downregulated, mimicking a more effector memory-like T cell (Tem) phenotype. **(C)** Activation phenotypes of Tem-like *CD8B-CAR19BB TRAC*$^{-/-}$ T cells (red) were compared to the naïve-like *CD8B-CAR19BB TRAC*$^{-/-}$ T cells (Tn) (blue) produced directly from the ATO culture. **(E)** After a 6h co-culture with either CD19-K562s (black bar) or antigen negative K562s (white bar), quantification of T cell effector cytokines was assessed in both Tem-like and Tn-like SP8s. **(D)** *In vitro* cytotoxicity was assessed using an Incucyte repeated tumor challenge assay of CD19-K562 growth during co-cultures with Tn-like or Tem-like SP8 T cells at a Target:T cell ratio of 1:1.

757 Targeting CCL2 to Improve Efficacy and Reduce Toxicity of CAR T-cell Therapy

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Chimeric antigen receptor (CAR) T-cell immunotherapy has emerged as a transformative treatment for haematological cancers. However, it is not effective in all patients, and it has still not found use in solid tumours, largely because of their immunosuppressive microenvironments. Furthermore, its clinical application may be limited by severe toxicities, most notably cytokine release syndrome (CRS). A unifying axis regulating these processes is that mediated by the chemokine, CCL2, also known as monocyte chemoattractant protein-1 (MCP-1). CCL2 activates its target cells by binding its receptor, CCR2. CCR2 is expressed on regulatory T-cells (Tregs), a key driver of immunosuppression in the tumour microenvironment. It is also expressed on monocytes, a central player in the pathophysiology of CRS; where CCL2 binding leads to chemotaxis to the tumour site, interleukin-6 secretion, and recruitment and activation of surrounding immune cells. CCL2 is thus a major driver of CRS. Indeed, it is found at elevated levels in the serum of patients receiving CAR T-cell therapy and it is a reliable predictor of high-grade CRS. Targeting CCL2 therefore represents a promising therapeutic strategy, enhancing efficacy by inhibiting Tregs at the tumour site, and reducing toxicity by preventing excessive monocyte recruitment. However, it is important to demonstrate that this strategy does not impair the activity of the CAR T-cells themselves.

To explore this approach, we developed fourth-generation, CD19-directed, armoured CAR T-cells that secrete proteins to modulate the effects of CCL2 in the tumour microenvironment. These proteins include both single-chain variable fragments (scFvs) and nanobodies directed against CCL2, plus eotaxin-3, a natural antagonist of CCL2.

These proteins neutralize CCL2 and block the CCR2 receptor on monocytes and Tregs. To overcome the challenge of poor transduction efficiency in primary T-cells, we employed an antisense vector in which the CAR is under a constitutively active CMV promoter, while therapeutic protein secretion is regulated by a T-cell activation-inducible NFAT promoter. Each gene in the vector is separated by polyadenylation sites to minimize transcriptional interference. We demonstrated that this optimized vector could be efficiently transduced into primary T-cells, at a level similar to the parental, unarmoured vector. To ensure that armouring these CAR T-cells did not impair their cytotoxicity, we co-cultured them with CD19+ Ramos B-cells and assessed target cell killing via flow cytometry. Indeed, the armoured CAR T-cells demonstrated equivalent cytotoxicity to control, parental, non-armoured second-generation CAR T-cells. Importantly, we confirmed that these CAR T-cells effectively secreted scFv/nanobody proteins against CCL2 and that these armouring proteins had binding and functional blocking activity. Further studies are ongoing to confirm inhibition of monocyte migration.

In conclusion, this study presents a novel, unified approach to improving CAR T-cell therapy by enhancing efficacy, whilst targeting one of the major axes of toxicity.

758 Alloimmune Defense Receptor Combined with Genetic Ablation of Adhesion Ligand CD58 is a Comprehensive Approach to Promote Functional Persistence of Allogeneic Cell Therapies without Conditioning Chemotherapy

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Clinical administration of chimeric antigen receptor (CAR) T-cell therapy relies on conditioning chemotherapy (CCT) to deplete host immune cells and maximize homeostatic cytokines to promote CAR T cell expansion and functional persistence. However, CCT also requires hospitalization and costly administration of therapy in tertiary healthcare settings. Providing off-the-shelf CAR T cell therapy without requiring CCT has the potential to significantly reduce treatment-related toxicities, cost of care, and extend patient reach. To this end, we established Sword and Shield Technology, a unique approach combining a novel synthetic alloimmune defense receptor (ADR), which targets 4-1BB and evades alloreactive immune cell response, and knock-out of CD58 ($CD58^{KO}$) to disrupt the ability of alloreactive immune cells to form a functional synapse. Both ADR and $CD58^{KO}$ were engineered into a clonal master iPSC line that can be used for consistent and uniform production of off-the-shelf multiplexed-edited CAR T cells. $ADR^{+}CD58^{KO}$ CAR iT cells demonstrated resistance to allo-rejection and uniquely expanded in cell number over the duration of an MLR assay with unmatched donor PBMCs, whereas control CAR iT cells (ADR^{null} $CD58^{wt}$) were eliminated. Notably, $ADR^{+}CD58^{KO}$ CAR iT cells exhibited similar levels of host T cell evasion observed with HLA I/II deficient controls. In addition, $ADR^{+}CD58^{KO}$ CAR iT cells avoided NK cell-mediated rejection, an observation not seen with controls. The $ADR^{+}CD58^{KO}$ CAR iT cells proved capable of persisting even in supraphysiologic conditions, co-cultures with primed alloreactive T cells (pAllo-T) generated from an unmatched donor, where in these very stringent conditions, control CAR iT cells were depleted in a few days. Upregulation of 4-1BB on pAllo-T was observed in co-cultures with control CAR iT cells but not in co-cultures with $ADR^{+}CD58^{KO}$ CAR iT cells, indicating that $ADR^{+}CD58^{KO}$ CAR iT cells are hypoimmunogenic to highly reactive pAllo-T. To confirm $ADR^{+}CD58^{KO}$ CAR iT cells retain potent anti-tumor activity under alloreactive pressure, we used a serially challenged tri-culture assay combining the pAllo-T MLR with daily-dosing of tumor cells. Control CAR iT cells showed rapid loss of tumor control and expansion of $4\text{-}1BB^{+}$ pAllo-T. In contrast, $ADR^{+}CD58^{KO}$ CAR iT cells prevented expansion of $4\text{-}1BB^{+}$ pAllo-T (not detected vs 47% $4\text{-}1BB^{+}$ in control) and maintained potent anti-tumor activity and durable persistence. Similar results were observed in a xenograft tumor model where $ADR^{+}CD58^{KO}$ CAR iT showed enhanced tumor control compared to controls in vivo in the presence of pAllo-T ($p < .05$). To demonstrate that $ADR^{+}CD58^{KO}$ CAR iT cells do not inhibit the host immune response against tumor cells, a tri-culture set up was investigated consisting of Nalm6 tumor lacking CAR antigen, host anti-tumor T cells, and $ADR^{+}CD58^{KO}$ CAR iT cells. Anti-tumor T cells co-cultured with Nalm6 tumor reduced tumor burden by 5-fold and in tri-cultures using $ADR^{+}CD58^{KO}$ CAR iT cells, we observed no inhibition of the anti-tumor T cell activity or

associated 4-1BB expression. Importantly, these results show that ADR^{+}CD58^{KO} CAR iT cells do not aberrantly target or disrupt the anti-tumor activity of non-alloreactive T cells. In conclusion, the potential of ADR^{+}CD58^{KO} CAR iT cells to elicit robust cytotoxic responses against diseased cells in immune competent settings shows promise for the use of cell-based immunotherapies without administration of CCT to patients.

759 Single Cell RNA-seq Detection of AAV in Circulating Leukocytes via Antibody-Derived Tags

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Introduction: The presence of anti-AAV antibodies pose a potential obstacle to AAV-mediated gene therapy. Recent reports have highlighted the innate immune compartment's role in response to recombinant AAV vectors – often facilitated by anti-AAV antibody opsonization and Fc receptor-mediated uptake. These reports were proteomic-based, namely conventional[1] or high-dimensional[2] flow cytometry with a capsid reporter for single cell AAV detection. While impactful in identifying the reactive populations of the immune system and demonstrating intervention strategies, these methods were limited by the number of cellular biomarkers characterizing the immune response. Single cell RNA-sequencing (scRNA-seq) provides unparalleled phenotyping of individual cells with potential to generate molecular and proteomic data through antibody-derived tags (ADTs). Herein we leveraged this multimodal platform with the first of its kind viral detection using anti-AAV ADTs to identify reactive leukocytes via *sequencing of extracellular protein targets from AAV* (SEPTA) to address fundamental questions in AAV immunology.

Methods: Three AAV8 seropositive individuals were recalled from a healthy donor pool screened in the Philadelphia area. Whole blood draws were stimulated *ex vivo* for 24 hours with AAV8 empty capsid or AAV8 expressing nano-luciferase paired with unstimulated control. Post hemolysis, cells were stained with a panel of antibody-derived tags for surface protein quantification. An additional anti-AAV monoclonal antibody, ADK8, was conjugated with a molecular barcode and spiked in during surface staining to label extracellular AAV+ cells. Cells were then fixed and permeabilized, followed by intracellular stain with ADK8 conjugated to a second unique molecular barcode to identify AAV+ phagocytes.

Results: The characterization of neutrophils is technically challenging due to rapid cellular degradation and paucity of gene expression. Using SEPTA, we captured ~50,000 cells and defined >12 distinct neutrophil states acutely post *ex vivo* stimulation, with >600 molecular and protein markers per leukocyte. Molecular signature-based computation clustering resolved neutrophil heterogeneity ranging from immunosuppressive to interferon-inducible viral responsive, degranulating, or neutrophil extracellular trap (NET) priming. Consistent with previous reports, capsid uptake was observed in neutrophils and monocytes. These frontline populations exhibit upregulation of toll-like receptor (TLR), cytosolic DNA sensing, NOD-like receptor, and chemokine signaling pathways in AAV-stimulated conditions. We observed variation in antiviral response between agonists and donors categorized as tolerant or moderate-to-highly immunogenic. Interestingly, a greater magnitude and proportion of leukocytes expressed activation markers in the AAV8 nano-luciferase stimulation compared to donor-paired AAV8 empty capsid.

Conclusion: The complexity and heterogeneity of human response to AAV warrants the use of the most sophisticated tools at our disposal. Though ephemeral in nature and understudied in the context of AAV, neutrophil states may correlate to several clinical outcomes including disease severity and thrombotic microangiopathy (TMA). Here we demonstrate the utility of our multimodal scRNA-seq method with viral detection, SEPTA, in resolving the initial immunological populations and pathways ensuing from AAV administration in seropositive individuals.

1. Smith CJ, et al. Front. Immunol. 2022 Sep;13:999021.

2. Rust BJ, et al. Molecular Therapy. 2024 Volume 32, Issue 4, –Page 194

760 Characterization of Antigen-specific Memory B Cell Repertoire in Human Tissues by Single-Cell Tools (LIBRA-Seq)

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Memory B cells (Bmem) play a critical role in generating antibodies upon repeated pathogen exposure. Research on human B memory cells has predominantly focused on their circulating pool, which poorly reflects the true B memory repertoires in other immunological sites. There remains a significant knowledge gap regarding tissue-resident Bmem, which constitute over 90% of the total Bmem pool. To address this gap with a closer focus on antigen (AG)-specific B cells, we investigated AG-specific Bmem in multiple tissues of an organ donor seropositive for SARS-CoV-2 and OC43 antibodies. We leveraged the LIBRA-seq tool, which integrates single-cell RNA sequencing (scRNA-seq), V(D)J sequencing, and barcode-enabled antigen mapping, to evaluate the tissue residence and transcriptome profile of OC43 and SARS2-reactive Bmem from human spleen, mediastinal, and mesenteric lymph nodes.

Methods: Lymphocytes were isolated from the spleen, mediastinal, and mesenteric lymph nodes of an organ donor. The cells were stained with an antibody panel, including barcoded OC43 and SARS-CoV-2 Spike tetramers. CD19+/IgD− AG++ cells were bulk sorted for single-cell analysis. 10,000 cells were submitted for 10X libraries GEX, VDJ, and FB. Libraries were sequenced on an Illumina platform, and the final reads were processed using the Cell Ranger multi pipeline, followed by various single-cell tools to calculate LIBRA-scores, analyze gene expression and molecular characteristics.

Results: Our findings reveal that subsets of AG-specific Bmem exhibit distinct transcriptional profiles driven partly by antigen specificity. Significant differences in repertoire characteristics (clonality, abundance, and diversity), gene usage, VH-VL pairing, CDR3 length, and frequency of somatic hypermutations (SHM) were observed between the OC43- and SARS-CoV-2-reactive subsets. The OC43 subset demonstrated highly biased V gene usage, predominated by IGHV1-69**01, IGHD6-19**01, and IGHJ4*02 gene combinations, VH:VL gene pairing, longer CDR3 lengths (up to 30 amino acids), and a higher frequency of SHM. In contrast, the SARS-CoV-2 reactive receptors exhibited greater clonal diversity exhibiting broader (unbiased) VH gene utilization with lower mutation frequencies. The OC43 repertoire displayed features of a well-developed adaptive response, with broad clonal expansion and affinity maturation, while the SARS2 repertoire likely reflects the response to a new infection, characterized by minimal clonal expansion, shorter CDR3 regions, higher diversity, and lower mutation frequency.

Conclusions: Our high-throughput single-cell analysis enables the study of the diversity and distinctive characteristics of OC43 and SARS-CoV-2 -specific B memory cells within human lymphoid tissues. This study provides detailed insights into the molecular characteristics of OC43- and SARS-CoV-2 -reactive antibody repertoires, including clonal structure, antibody specificity, and the functional potential of memory B cell subsets. These insights are crucial for understanding the immune response to coronaviruses in general. For instance, the available pool of OC43-reactive Bmem can now be evaluated for cross-reactivity to SARS-CoV-2, suggesting the potential for recalling pre-existing OC43 Brem to combat a new coronavirus infection. Additionally, our study provides a wealth of paired sequences of human antibody genes, with potential applications in therapeutic or diagnostic fields, particularly in gene therapy.

761 *Pre-Existing Immunity to AAV - A Qualitative Comparison Between Different Neutralization Assay Methods*

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Adeno-associated virus (AAV)-based gene therapy continues to offer transformative potential for treating a wide array of genetic disorders. However, natural exposure to AAV has resulted in the development of antibodies in a

significant portion of the population. These pre-existing antibodies can pose challenges by neutralizing AAV vectors or eliciting immune responses, which may compromise the efficacy and safety of gene delivery. Thus, screening patients for AAV-specific antibodies prior to treatment is crucial. To evaluate whether patients possess pre-existing neutralizing antibodies (NAbs), neutralization assays are performed. These assays determine the ability of antibodies to inhibit AAV genetic payload delivery into target cells. Complementary to neutralization assays, total antibody (TAb) assays—such as TAb ELISAs—offer a broad assessment of AAV antibodies. Positive results from TAb assays may warrant further investigation using specific NAb assays, which are more selective but usually also more cost-intensive.

In this study, data from three distinct NAb assays with distinct read-outs were compared for AAV2 and AAV8. The assays include a system using AAV vectors encoding Renilla luciferase (Luc), a system employing AAV-NanoLuc® and a solution utilizing the iLite® AAV Nab platform. With each method the neutralization potential of three monoclonal antibodies, that recognize intact AAV particles, have been evaluated by determining their half-maximal effective concentration (EC_{50}) values. The EC_{50} represents the antibody concentration at which 50% of viral infectivity is neutralized, serving as a quantitative measure of neutralizing activity. Additionally, ten human serum samples were tested using the before mentioned systems. The results were qualitatively compared among the three NAb assays and further evaluated against data from a total antibody (TAb) ELISA, which detects general AAV antibody presence.

The assays exhibited variations in their evaluation methodologies, driven by the distinct technical requirements inherent to each system. Nevertheless, the resulting order of magnitude for the EC_{50} values obtained from the tested monoclonal AAV antibodies demonstrated qualitatively comparable outcomes across all three NAb assays. Similarly, the results generated for the tested human serum samples were qualitatively consistent among the three assay systems and showed strong alignment with the outcomes derived from the TAb ELISA. These findings demonstrate the overall methodological comparability of the tested systems.

762 Characterization of the Immune Landscape of the AML Bone Marrow Microenvironment by Cellular Indexing of Transcriptomes and Epitopes (CITE-Seq)

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Single-cell technologies have enabled important advances in acute myeloid leukemia (AML) by characterizing the heterogeneity of tumor cell populations; however, the immunological landscape of the bone marrow microenvironment (BMME) in AML has not been as extensively explored at the single cell level. In these studies, flow cytometric and high-dimensional single-cell technologies were applied to characterize immune states in the AML BMME. Preliminary analysis of the AML BMME was initially performed using multiparametric flow cytometry of bone marrow (BM) samples from 17 AML patients at diagnosis (Dx) and following remission (Rm) induction (total 34 samples). The frequency of 11 BM resident immune cell subsets was determined. Hierarchical clustering of this data revealed 3 major patterns of immune configurations and included, 1) a group exhibiting a high frequency of monocytes and myeloid derivatives, 2) a group characterized by high frequency of plasma cells, and 3) a group composed predominantly of $CD4^+$ and $CD8^+$ T lymphocytes. These immune cell patterns remained consistent in Dx and Rm stages, indicating the conservation of certain immune profiles. To enable a more detailed mapping of the immune profiles in the BMME, single-cell level proteomic and RNA analysis was performed using Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq). This approach, combined with unsupervised clustering, enables an unbiased characterization of both individual cells and the overall immune landscape of the BMME. CITE-seq enabled us not only to move beyond traditional immune cell types, but also to identify 20 distinct transcriptomic cell states—both immune and non-immune. Among these, we observed several $CD8^+$ T cell and NK cell subtypes that have been previously associated with anti-tumor functions.

To identify the primary axis of cell composition variation, we applied principal component analysis (PCA) to uncover recurrent immune patterns in the BMME across Dx and Rm samples using CITE-seq data. Each group was

analyzed independently, and in both cases, three principal components captured a significant portion of the heterogeneity—accounting for approximately 50% of the total variation. Specifically, we observed four distinct immune patterns in the Dx samples, whereas the Rm samples appeared largely homogeneous. In both groups, the variation was primarily driven by monocytes (MO), B cells, and plasmacytoid dendritic cells (pDCs). This suggests that while the immune environment is initially heterogeneous at Dx, various chemotherapy regimens may reset the BMME to a more homogeneous state.

In light of these findings, we investigated whether the trajectory of immune composition changes between Dx and Rm could serve as a robust classifier for AML patients. Using PCA on paired biopsies, we identified four primary classes of immune compositional shifts following chemotherapy. Notably, $CD8^+$ T cell subtypes emerged as a key feature, independent of treatment type or AML subtype. These results suggest that the immune compositional shifts between Dx and Rm biopsies define distinct immune archetypes, driven by the transcriptomic profile of bone marrow-associated $CD8^+$ T cells, and may serve as an orthogonal disease classifier. We anticipate that this BMME-based classifier could enhance the prediction of disease trajectories and therapeutic responses to AML.

763 Understanding and characterizing the immune response to adeno-associated virus (AAV) in the central nervous system (CNS)

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Evaluating immune responses to adeno-associated virus (AAV) is critical for safe and durable gene therapy. AAV is a leading viral vector for the delivery of therapeutic cargo in part because pre-clinical data has demonstrated low immunogenicity compared to other viral vectors. However, immune responses have been observed in clinical trials for AAV gene therapies including capsid-specific T cell responses that led to clearance of transduced cells. Striking differences in immune responses to AAV gene therapies from different administration routes have been observed. Intravenous (IV) delivery of Zolgensma provoked elevated liver enzymes, acute liver failure, and cardiac dysfunction, whereas intraparenchymal (IP) delivery of Upstaza elicited little to no adverse immune responses, suggesting that direct brain injections of AAV may have a safer immune profile for central nervous system (CNS) indications. The clinical data further highlighted that the mechanisms underlying the differential immune response to routes of delivery, capsid and transgene are not fully understood.

To investigate this further, we injected 6–12-week-old mice bilaterally in the thalamus (IP) or IV with either PBS, a low immunogenic capsid, AAV9, or a highly immunogenic capsid, AAVrh32.33. These capsids further delivered either an immunologically inert transgene, secreted embryonic alkaline phosphatase (SEAP), or a highly immunogenic transgene, Ovalbumin (OVA). At 2-weeks post IP injection, serum IFNγ and CXCL10 spiked in animals in the rh32.33-OVA group, indicating potential recruitment of immune cells to the brain. 6-weeks post injection, animals injected with AAVrh32.33, but not AAV9, exhibited an increase in the percentage of CD3+ T cells in the brain. Interestingly, CD4+CD25+FoxP3+ regulatory T cells were observed in the brain of all animals treated IP and not IV, indicating stimulation of an immunoprotective response. IFNγ ELISPOTs performed on splenocytes isolated at 6-weeks demonstrate capsid-specific T cells in IP-delivered rh32.33-SEAP treated animals, but not those treated with rh32.33-OVA. However, 25% of the animals in the rh32.33-OVA group reacted to the immunogenic OVA peptide, SIINFEKL. In the brain, 25% of rh32.33-SEAP animals clearly demonstrated capsid-specific T cells as measured by capsid dextramer staining. IV-injected animals in the rh32.33-SEAP group however, all had circulating capsid-specific T cells at 6-weeks post-injection. Surprisingly, all animals in the IV-injected AAV9-SEAP group also had capsid-specific T cells. Serum expression of rh32.33-OVA was higher in animals injected IP compared to animals that received rh32.33-OVA IV, where there were no detectable transgene levels, indicating that Treg recruitment to the brain may allow for transgene expression. In IP injected animals, vector genomes at 6-weeks are higher in the brain than in the liver and are similar across all capsid- and transgene-groups. IV injected animals, however, have no detectable vector genomes in the brain and low-levels in the liver.

Overall, we found an interplay of route of delivery, transgene and capsid elicited surprising T cell responses in AAV treated mice. Future work will include testing the role of Tregs in transgene expression through Treg depletion and further elucidating the mechanism of T cell recruitment to the brain for the creation of safer and more effective AAV gene therapies.

764 Elucidating the efficacy and immune response to retinal gene therapy in non-human primates using spatial transcriptomic and single-cell approaches

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Adeno-associated viral (AAV) vector-mediated gene therapies to treat inherited retinal degenerations have gained momentum since the approval of *voretigene neparvovec* (Luxturna), a subretinally-administered AAV2 vector encoding human *RPE65*. A wide range of AAV-based gene therapies are in advanced stages of development for treating both rare and common retinal diseases. While the eye is considered relatively immune-privileged due to the blood-retinal barrier, chronic intraocular inflammation has been reported in both preclinical and clinical studies following AAV gene therapy, which has become a major clinical concern for the broader application of this treatment modality.

To better understand the primate ocular immune response to AAV gene therapy, we treated two wildtype non-human primates (NHPs) with subretinal gene therapy: NHP1 received the clinical dose of *voretigene neparvovec* (AAV2-CAG-*RPE65*) in both eyes; while NHP2 received an engineering grade AAV8 vector encoding a human codon-optimised retinitis pigmentosa GTPase regulator (*coRPGR*) transgene under the control of a photoreceptor-specific promoter (*GRK1*), alongside an equivalent vector encoding *mScarlet* reporter transgene, in separate subretinal blebs of both eyes.

Retinal structure and function were monitored for 3 months by *in vivo* retinal imaging and electroretinograms. At three months, retinal punches from treated and untreated areas were harvested for histological, spatial transcriptomic and single-cell transcriptomic analyses. The results revealed the subretinal gene therapies to be clinically well-tolerated with minimal perturbation of retinal structure and function. Robust expression of human *RPE65* and *coRGPR* transgenes were identified in the treated retina. Single-cell RNA-seq indicates that AAV8 vectors transduced ~80% of cones and rods within the bleb area, while AAV2 transduced 30% of rods and RPE cells. No correlation was found between the expression levels of the viral transgene and apoptotic marker. Single cell analysis also identified retinal infiltration by significant populations of macrophages, T cells and B cells at 3 months post-gene therapy, indicating a subclinical but prolonged type 1 cell-mediated response. Moreover, spatial transcriptomic data revealed migration of retinal microglia to the subretinal space following gene therapy. This, when combined with marked upregulation of pro-inflammatory pathway genes (including TNFα, IL1b, CXCL8, CCL2 and CCL5), implicates resident and infiltrating monocytic phagocytes as key orchestraters of retinal inflammation.

Our findings provide mechanistic understanding of clinical observations of gene therapy-associated uveitis (GTAU) and reveal molecular targets for controlling these deleterious immune responses.

765 Transient Treg Expansion Mitigates Cas9-Specific Immune Responses to Enhance Durability of AAV Gene Editing Therapy in Duchenne Muscular Dystrophy

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Adeno-associated virus (AAV)-mediated CRISPR editing offers transformative potential for Duchenne muscular dystrophy (DMD), but immunotoxicity directed against AAV and the bacterial-derived Cas9 nuclease remains a critical barrier. To reach therapeutic efficiency, high therapeutic doses are used which could exacerbate the humoral and cytotoxic T lymphocyte (CTL) responses. Particularly, the Cas9-induced immunotoxicity leads to elimination of edited muscle fibers and promotes muscle inflammation, reducing the long-term efficacy and safety of AAV-mediated gene editing therapy for DMD. Here we explored an immunomodulatory strategy using F5111-IC, a single-chain human IL-2/antibody fusion protein, to selectively expand regulatory T cells (Tregs) while minimizing CD8+ T cell and NK cell activation—key drivers of AAV/transgene-directed immunity.

Male *mdx*^{4cv} mice were primed with four consecutive intraperitoneal doses of F5111-IC followed by intravenous administration of MyoAAV4A-CMV-SpCas9-ABE (1E+14vg/kg) one day after priming. At the time of AAV administration, F5111-IC selectively expanded peripheral blood FOXP3 CD25 Tregs to ~60% in CD4 while suppressing CD8+ and NK cell populations. This immunosuppressive state persisted for ~7 days, after which Tregs levels returned to physiological baseline at 10%. IFN-γ ELISpot analysis of splenocytes at 5 weeks post-AAV injection revealed a 54% reduction in Cas9-specific T cell responses compared to untreated AAV controls, though residual immunity remained detectable.

To further improve the regime, we include rapamycin that synergizes with IL-2 on Treg expansion and immune suppression. Treatment was extended to twice weekly for 4 weeks post-AAV administration. Preliminary data showed sustained Treg expansion and suppression of CD8+, NK, and CD19+ populations in peripheral blood. Additionally, the treatment group exhibited an 83% reduction in serum AAV-specific IgG levels compared to untreated AAV-only controls at Day 5 post-AAV treatment, highlighting its strong immune suppression potential. Our ongoing experiments are investigating whether this combinatorial regimen can yield stronger or even full suppression of Cas9-directed CTL activity, thereby improving the durability and safety of AAV-CRISPR therapy in DMD.

We expect that this study will provide critical insights into the potential of transient, targeted Treg modulation to overcome immune toxicity in AAV-mediated gene editing and to enhance long-term therapeutic outcomes in DMD.

766 AAV cross reactivity elicits a serotype specific response and complement activation in type I SMA patients post Zolgensma treatment

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Gene therapy offers lasting benefits but also faces hurdles in the form of seropositivity to AAV vectors from prior exposure. This includes generation of neutralising antibodies that can reduce efficacy and complement activation that can trigger adverse immune reactions. In this study, we have developed and applied a fully quantitative, multiplexed targeted proteomic method to measure and profile anti-AAV antibody responses and complement binding. In a pilot study of 20 spinal muscular atrophy patients who received Zolgensma, an AAV9 based gene therapy, we examined the cross-reactivity of antibody/complement responses against AAV2, AAV8, and AAV9.

When assessing antibody response longitudinally against AAV9, we observe shifts from an IgM dominant response towards a complement activating IgG1 profile from 2 months post dose. We detected cross-reactivity of IgG1, IgG2, IgG3, IgG4, IgM, and IgA1 against all three serotypes, despite no known exposure to AAV2 or AAV8. Notably, AAV8 exhibited significantly lower complement deposition than AAV9, with reduced levels of C1Qc ($p=0.0049$), C4b ($p<0.0007$), and C9 ($p=0.0012$). Additionally, immunocomplex responses were assessed in 3 patients pre-

treatment, demonstrating serotype specific evolved immune responses. In post-treatment samples, we observed a complement response to AAV2 from 0.5 months. This indicates a non-IgG associated response either driven instead by IgM through the classical pathway or with also a notable absence/reduction of C1q could indicate activation of the lectin pathway against AAV2 in AAV9 exposed individuals.

By applying a novel targeted proteomic based methodology to profile the immune complex to AAV vectors we can achieve a significant advancement in assessing a patient's immune response to AAV compared to traditional methods. We demonstrate here that not only can we determine cross-seropositivity against a range of capsids, but also determine whether these antibody responses are likely to stimulate complement deposition. This allows for a more informative, fully quantitative analysis of individual patients immunocomplex raised against varying capsids. This could potentially open the door towards a personalised medicine approach and re-dosing.

767 Classical Complement Ablation Leads to Divergent Lymphocyte Activation by Antigen Presenting Cells in Response to AAV

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Systemically delivered adeno-associated viral vectors (AAV) activate classical and alternative complement pathways. Excessive complement activation leads to thrombotic microangiopathy (TMA). While drug interventions can dampen TMA, the split products that arise from complement activation cannot be completely eliminated. These split products act as anaphylatoxins that modulate downstream immune responses that limit the safety and efficacy of AAV-based therapies. Prior work demonstrated that dual dosing AAV in mice, with a 4-week interval, can recapitulate some immune responses observed in humans dosed with AAV such as anti-AAV antibody formation, chemokine signaling, and classical complement activation (Emami, et al, 2023). To mechanistically understand how split products of classical complement influence downstream immune responses to AAV, we used C1qa knockout mice (C1q KO) (*B6(Cg)C1qatm1d*(EUCOMM)Wtsi/TennJ) lacking the first component of classical complement. Homozygous C1q KO knockout mice and C57Bl6/J wildtype mice were given an initial dose of AAV9 empty vector to prime adaptive immunity and four weeks later administered a second dose of AAV9_CMV_mCherry (1.16 E14 vg/kg). Splenocytes were isolated from singly dosed mice after 1 and 2 weeks as well as from dual dosed mice. Splenocytes were subjected to single cell RNA sequencing. B-cell, CD4 T-cell and CD8-cell frequencies were delayed after a single dose of AAV in C1q KO mice at 1 week compared to wildtype splenocytes. However, T follicular helper cell populations increased two weeks post dosing and presented signs of enhanced activation at 2 weeks post dose and after dual dosing. C1q KO monocytes upregulated activation markers at 1- and 2-weeks post dosing. Following a second dose of AAV, the difference in cellular frequencies observed between the two genotypes previously was reduced. However, select immune activation markers persisted including those on monocytes, T follicular helpers and B cells. Chemokine protein levels were significantly elevated for Cxcl10, Ccl4, and Ccl2 in both genotypes at the 5-hour post dose 2 timepoint and were significantly elevated in C1q KO mice compared to wildtype at this same time point. Blood samples were collected from DMD patients treated with Elevidys (AAV Rh74-microdystrophin) prior to dosing and on days 4, 7, and 14 post dosing, revealing significantly elevated levels of CXCL10 and CCL2 from plasma. The chemokines detected are suggestive of macrophage activation, therefore we examined how antibodies and complement C3 influence THP-1 macrophage activation in vitro after exposure to AAV-immune complexes. These studies showed that AAV-antibody complexes increased both viral entry and activation, in an Fc receptor dependent manner, while complement C3b or iC3b did not influence uptake or activation. We tested whether increased chemokines observed in C1q KO mice was due to higher antibody levels but found equivalent anti-AAV IgG levels between genotypes. However, we observed evidence of continued complement activation in the mice, demonstrated by enhanced iC3b generation in C1q KO mice 5 hours post dose 2, suggesting activation of the alternative pathway. These results suggest that inhibition of classical complement pathway could lead to increased activation of compensatory immune mechanisms that worsen downstream immune responses. Studies are needed to determine if acute inhibition of classical complement results in similar responses.

768 AAV2 Delivery of the saCas9 Gene Results in MHC I Presentation of an HLA-A*02:01 Restricted T cell Epitope Potent to Induce T Cell Cytotoxicity.

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In vivo genome editing with CRISPR-Cas9 systems is generating worldwide attention and enthusiasm for the possible treatment of genetic disorders. However, the consequences of potential immunogenicity of the bacterial Cas9 protein and the AAV capsid have been the subject of considerable debate. Here we model the antigen presentation in cells after in vivo gene editing by in vitro transduction of a human cell line with an AAV2 vector that delivers the Staphylococcus aureus Cas9 transgene. Through HLA class I enrichment, peptide elution and highly sensitive LC-MS interrogation, we identified a highly conserved saCas9-derived T cell epitope that is restricted to HLA-A*02:01 and induces CD8+ T cell activation and killing. We conclude that AAV delivery of Cas9 protein results in presentation of a T cell epitope that can activate CD8+ cells and induces killing of the transduced cell, with important ramifications for in vivo genome editing strategies.

769 All-in-one AAV Vectors Delivering Compact *Dbp*-CasX, *Plm*-CasX, *Un1*Cas12f and *As*Cas12f Nucleases as Antiviral Gene Editing Therapeutics for the Treatment of Hepatitis B Virus

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In recent years much research effort has gone towards the development of curative therapies for highly prevalent but uncurable chronic infectious diseases that place a high burden on public health systems globally. Pathogens including malaria, tuberculosis, human immunodeficiency virus (HIV), herpes simplex virus (HSV) and hepatitis B virus (HBV) are now being targeted for 'cure'. To this end, we and others are developing targeted nucleases including CRISPR family nucleases as antiviral therapeutics, and we are using AAV vectors to deliver these nucleases to sites of persistent viral infection, a process made challenging by the large size of the commonly used *Sp*Cas9 nuclease. Previously we showed that administration of an HBV-targeted AAV-*Sa*Cas9 vector containing a 3345 bp *Sa*Cas9 ORF expressed from a short EFS promoter resulted in gene editing of viral genomes in chronically HBV infected liver-humanized FRG mice. However, the gene editing efficiency was low, which was likely due to low nuclease expression levels from a suboptimal AAV-*Sa*Cas9 vector in HBV infected human hepatocytes. Here we describe the development of compact all-in-one AAV vectors expressing engineered Cas family nucleases derived from *Dbp*-CasX (*Dbp*-CasX-R3), *Plm*-CasX (*Plm*-CasX-R1), *Un1*Cas12f (CasMINI_v3.1) and *As*Cas12f (en*As*Cas12f-HKRA), plus their respective sgRNAs. Compact all-in-one AAV vectors contain nuclease ORFs of 3078, 3063, 1737 and 1329 bp respectively so can accommodate increasingly larger promoters and additional regulatory elements to facilitate improved nuclease expression in human hepatocytes and subsequently HBV gene editing. Strong ubiquitous or liver-specific promoters, as well as post-transcriptional regulatory elements or microRNA binding motifs can now be accommodated to optimize nuclease expression. Compact all-in-one AAV vectors express a sgRNA from the U6 promoter containing a 3' 20-23bp protospacer that can recognize target sequences with adjacent 5'TTTR, 5'NTTR, 5'TTCN and 5'TTCN PAM sequences respectively. We generated compact all-in-one vectors expressing either *Dbp*-CasX-R3, *Plm*-CasX-R1, CasMINI_v3.1 or en*As*Cas12f-HKRA from the strong ubiquitous CBH promoter and complementary sgRNAs targeting 9 conserved HBV genome sequences within essential HBV ORFs, plus previously validated sgRNAs targeting either the human VEGF or β2-microglobulin genes as internal controls (40 total targets). CpG-motif deleted non-coding GAPDH intron 2 stuffer DNA was included in CasMINI_v3.1 and en*As*Cas12f-HKRA all-in-one vectors to create AAV vector genomes of comparable length to *Dbp*-CasX-R3 and *Plm*-CasX-R1 all-in-one vectors (4002-4631). We then tested the ability of each compact nuclease to edit a HBV genotype D genome in HEK293 and HepG2 cells. Efficient gene editing by compact nucleases was detected at HBV target sites via TIDE analysis and NGS sequencing of target site PCR amplicons at levels comparable or greater than our previously used AAV-*Sa*Cas9 vector. HBV-specific compact all-in-one AAV vectors packaged into the human hepatocyte-tropic AAV.LK03 capsid are currently being tested for their antiviral efficacy in HBV infected primary human hepatocytes and in HBV infected liver-humanized NSG-PiZ

mice. Preliminary results suggest all-in-one compact nuclease AAV vectors can be viable alternatives to previously used AAV-*Sa*Cas9 vectors as antiviral therapeutics targeting replicating and persistent HBV.

770 In Vivo Generation of CAR-T Cells for the Treatment of B-Cell Mediated Autoimmune Diseases

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Chimeric antigen receptor T cell (CAR-T) therapy has demonstrated remarkable success in treating hematological malignancies [1]. Building on the achievements of anti-CD19 CAR constructs in oncology, there is increasing interest in adapting these approaches for autoimmune disorders driven by autoreactive B cells, such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, to restore immune tolerance. Notably, ex vivo–manufactured CD19 CAR-T cells have shown robust B cell depletion, inducing complete remission in patients with treatment-refractory systemic lupus erythematosus and dermatomyositis [2].

Despite these impressive outcomes, ex vivo CAR-T cell therapies face significant challenges, including the need for lymphodepletion, a complex production process, and limited control over peak CAR-T cell levels due to uncontrolled expansion of cells in vivo, which can lead to adverse events such as cytokine release syndrome [3]. To address these limitations and harness the full potential of CAR-T therapy for autoimmune diseases, we are developing an in vivo CAR-T cell engineering platform. This system employs an antibody-based ligand targeting strategy to direct lipid nanoparticles (LNPs) carrying mRNA encoding CD19 CAR to specific T cell populations in vivo, enabling controlled, transient CAR expression without the need for ex vivo manipulation or lymphodepletion. Our approach demonstrated dose-dependent mRNA delivery to resting T cells from both human and non-human primate (NHP) sources in vitro, resulting in the generation of highly functional, antigen-specific CAR-T cells. These CAR-T cells exhibited strong cytotoxic activity, antigen-induced proliferation, and effective B cell depletion in peripheral blood mononuclear cells from multiple donors. In vivo, in a $CD34^+$ humanized mouse model, single and multiple doses of targeted LNPs delivering CD19 CAR mRNA produced significant, dose-dependent B cell depletion. Additionally, the LNP platform demonstrated robust cargo mRNA expression in non-human primates, highlighting its potential for clinical translation.

In summary, our in vivo CAR-T engineering platform offers a simpler, scalable, and potentially safer alternative to traditional ex vivo CAR-T therapies, with broad therapeutic implications for treating autoimmune diseases and beyond.

References:
1. Maude SL, et al. N Engl J Med 2014;371:1507-1517
2. Müller F, et al. N Engl J Med 2024;390:687-700
3. Brudno JN, Kochenderfer JN. Blood 2016;127:3321-30

771 Chemokine-Engineered CAR-T Cells for Autoimmunity: Multiplexed mRNA Technology for Improved Mode of Action and Safety

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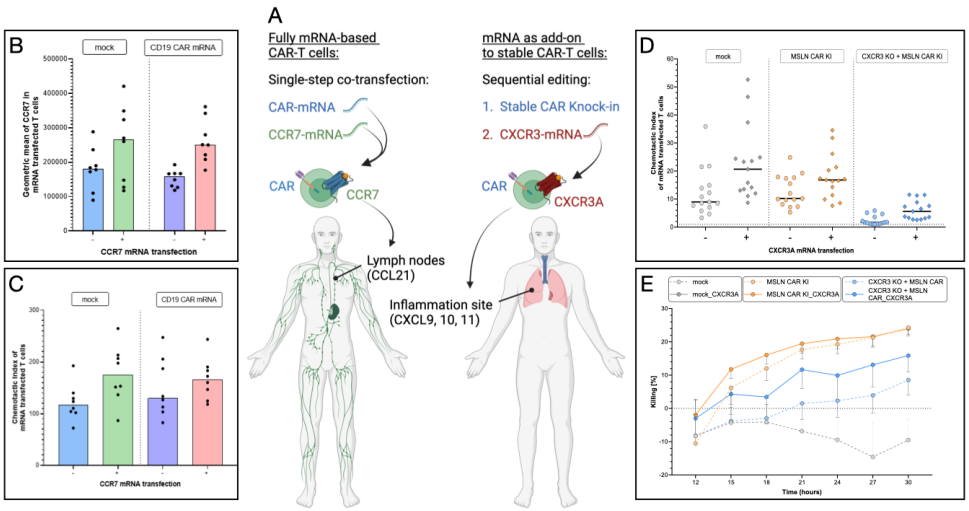
Chimeric antigen receptor (CAR)-T cells have recently emerged as a potential cure for systemic autoimmune diseases [1] but their widespread application is limited by manufacturing cost, lymphodepletion and poor control over biodistribution and pharmacodynamics.
As an immune reset in autoimmunity does not appear to require long-term CAR-T engraftment, mRNA technology bears the promise for transient CAR-T cells with an improved safety and treatment controllability [2].
We aim to design such mRNA-CAR-T cells for deeper B cell depletion. We optimize mRNA delivery to human T cells and co-express mRNA-based CD19-CAR or stable CRISPR/Cas9 CAR knock-in (KI) alongside mRNA-encoded chemokine receptors for targeting lymphatic tissues (CCR7) and inflammation (CXCR3A; Figure 1A) [3].

mRNA was transcribed in vitro [4], LNPs were formulated using microfluidic mixing. Primary human T cells were activated and transfected by LNPs vs electroporation with CAR-mRNA and CCR7- or CXCR3-mRNA. For stable CAR-T cells, CAR was knocked into the T cell receptor alpha chain locus using CRISPR-Cas9 and CXCR3 mRNA was transfected 6 days later.
Transfection efficacy and cell viability, mRNA expression kinetics (flow cytometry) and changes in cytokine production (ELISA) were quantified. CAR-T cell functions were compared between migration-engineered CAR-T cells, concentional CAR-T and untreated T cells: killing of B cells, CAR-T cell migration toward target cytokines (CXCL11; CCL21), and a combination of migration and target elimination were quantified in cell-based human in vitro assays.

Both mRNA transfection methods resulted in effective transfection (>80%) and high T cell viability (>90%). In CRISPR/Cas9-engineered CAR-T cells, CAR-KI rate was >20% and cells could be re-transfected with mRNA 6 days post-KI without significant viability loss. In "mRNA-only" CAR-T cells, co-expression of CAR and chemokine receptor mRNAs could be achieved in 80% of cells upon single treatment and lasted ~3d, whereafter cells assumed their original phenotype.
Transfection with CCR7-mRNA increased receptor expression (Figure 1B) and strongly boosted the migration of (CAR-)T cells toward the CCL21 chemokine, a marker of secondary lymphoid organs (Figure 1C), while CXCR3A-mRNA increased migration toward the inflammatory mediator CXCL11 (Figure 1D).
Chemokine-engineered CAR-T cells functionally outperformed T cells transfected with "CAR only" in a combined killing and migration assay (Figure 1E).

We demonstrate mRNA-based CAR-T cells with a transient phenotype and no transfection-related toxicity. Their elimination of tissue-resident B cells may be enhanced by the co-expression of tissue-targeting chemokine receptors. Such products promise improved controllability and safety through re-dosing and a mode of action tailored to deep B cell depletion in autoimmunity.
Validation in relevant disease models and transition to GMP-compatible manufacturing are ongoing.

1. Mackensen, A. Anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus. Nat Med, 2022
2. Baker, D.J. CAR T therapy beyond cancer: the evolution of a living drug. Nature, 2023
3. Vollmer, T. The intratumoral CXCR3 chemokine system is predictive of chemotherapy response in human bladder cancer. Sci Transl Med, 2021
4. Drzeniek, N.M. In Vitro Transcribed mRNA Immunogenicity Induces Chemokine-Mediated Lymphocyte Recruitment and Can Be Gradually Tailored by Uridine Modification. Adv Sci, 2024



772 Innovative Lipid-Based Delivery Solution: Unlocking New Opportunities in Manufacturing Genetically Modified Immune Cell Therapies

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Gene-modified cell therapies, such as engineered T cells or Natural Killer (NK) cells expressing chimeric antigen receptors (CARs), are among the most promising treatments for cancer and autoimmune diseases. Traditionally, these therapies rely on lentiviral vectors to transfer the gene of interest; however, this approach is associated with lengthy manufacturing timelines, high costs, and potential safety risks. To overcome these limitations, our research focuses on lipid-based solutions as a promising alternative for producing CAR-T and CAR-NK cells.

773 Cell Targeted Lipid Nanoparticle (ctLNP)-based CAR-T Therapy: A Novel Strategy for B cell Malignancies and Autoimmune Diseases

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Chimeric antigen receptor (CAR)-T cell therapy, which can be engineered to target pathogenic B cells, has demonstrated clinical success in treating B cell malignancies and is now being explored for B cell–driven autoimmune diseases. However, current genome-integrated *ex vivo* CAR-T therapies face multiple limitations, including the risk of cytokine release syndrome (CRS) and neurotoxicity, as well as complex manufacturing and high costs that restrict broader clinical implementation. Nitto has pioneered targeted lipid nanoparticle (LNP) technology (Nat Biotechnol, 2008, PMID: 18376398; Hepatology, 2022, PMID: 34605045) and developed a cell-targeted LNP (ctLNP) platform to enable the *in vivo* generation of mRNA-based CAR-T cells. Because CAR expression is driven by ctLNP-delivered mRNA, the dosing can be flexibly adjusted and repeated, allowing efficient regimens that minimize the risk of CRS while maximizing therapeutic efficacy. Furthermore, this off-the-shelf formulation shortens production timelines, lowers costs, and improves accessibility by circumventing the need for labor-intensive *ex vivo* procedures. Our ctLNPs comprise three principal components: (i) a VHH (the variable heavy domain of the heavy chain) that specifically binds CD8 T cells, for which we precisely optimized its surface density on the LNP to maximize delivery efficiency; (ii) Nitto's biodegradable lipids, formulated to support safe and

efficient *in vivo* delivery; and (iii) an mRNA payload encoding a CD19-specific CAR. To evaluate this system, we activated human peripheral blood mononuclear cells (hPBMCs) with CD3/CD28 beads and IL-2, then transfected them at varying ctLNP doses. We observed robust, dose-dependent mRNA expression in CD8 T cells, which yielded efficient CAR-T cell generation. These CAR-T cells exhibited potent cytotoxicity against primary B cells across ctLNP concentrations of 0.003–3 µg/mL. To further characterize CAR-mediated activity, we co-cultured ctLNP-transfected T cells (0.3 or 3 µg/mL) with the CD19-expressing Nalm6 leukemia cell line at effector-to-target (E:T) ratios of 1:1 to 1:9 for 72 hours. The results demonstrated clear dose-dependent target cell elimination and corresponding IFNγ production, underscoring the efficacy and specificity of this mRNA-based CAR-T platform. In *in vivo* studies, intravenous ctLNP administration (0.05–0.5 mg/kg) in humanized NSG mice (bearing hPBMCs) achieved complete B cell depletion. In a separate Nalm6 tumor model, ctLNP treatment (0.5–2 mg/kg) yielded dose-dependent tumor suppression (Fig. 1) without evident toxicity such as weight loss. Collectively, these findings demonstrate that ctLNP-based *in vivo* CAR-T therapy is a promising new modality. We are now preparing for non-human primate studies and GMP Production to advance this platform toward clinical proof of concept, ultimately broadening therapeutic options for patients with B cell malignancies or B cell–driven autoimmune disorders.

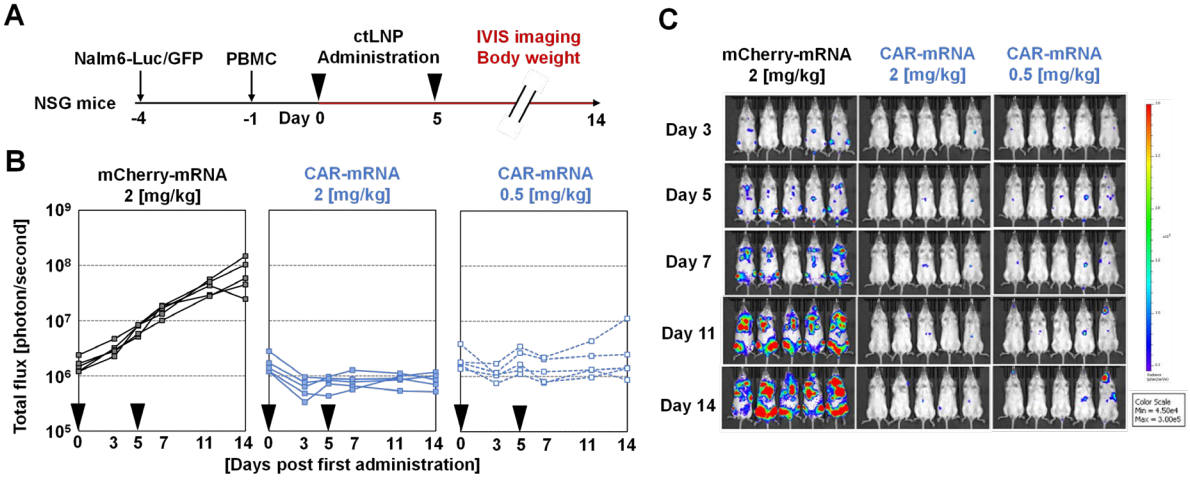

Fig 1. ctLNP treatment suppresses leukemia growth in humanized mice
(A) Timeline of schedule. Luciferase-labeled Nalm6 (5x10^{5}cells) and hPBMC (1x10^{7}cells) were injected intravenously into NSG mice and after randomization, ctLNP was administered twice. (B) Total flux value of Nalm6 burden at indicated timepoints quantified by *In vivo* imaging system. (C) Representative images of the luciferase signal in each mouse.

774 *In vivo* Transient Programming of T cells with SAIL's Targeted Nanoparticles Encapsulating eRNA™-encoded hCD19 CAR Achieves Deep Depletion of B cells in blood and lymphoid tissues in a B-cell Repopulating Humanized Mouse Model.

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Background: Early clinical data with autologous lentiviral chimeric antigen receptor (CAR) T cell therapy in autoimmune disease (AID) has been paradigm shifting with potential to transform patient care. However, traditional

CAR-T has limitations on scalability, access, procedure complexity, and safety associated with conditioning that may limit its potential reach. SAIL is developing a targeted LNP-eRNA platform to enable *in vivo* transient programming of a patient's immune cells with an off-the-shelf IV injectable product.

In vivo CAR-T platform: SAIL's in vivo CAR-T platform is composed of three key components: (1) circular, Endless RNA™ (eRNA™) designed for enhanced stability and durable expression of CD19 CAR in T cells; (2) optimized nanoparticle (NP) compositions to maximize endosomal escape and expression of eRNA in T cells; and (3) surface functionalization of NPs with binding moieties to increase the efficiency and selectivity of T cell-targeted LNPs.

Results: Delivery of a GFP reporter-encoded payload resulted in efficient and selective expression in 60% to 80% of CD4 and CD8 T cells in blood and lymphoid tissues across C57BL/6 WT mice, hPBMC and hCD34+-humanized mice, and NHPs. *In vivo*-engineered T cells with eRNA-encoded hCD19 CAR expressed ~1,000-7,000 CAR molecules per cell; a level of expression above the threshold to effectively mediate target cell killing *in vitro*. Accordingly, single administration of SAIL's targeted LNP-eRNA at a dose as low as 0.01mg/kg led to deep depletion of naïve, memory B cells and plasmablasts in PBMC-humanized mice. In a more translational B-cell repopulating CD34-humanized model, a short cycle of injections achieved deep depletion of B cells in both blood and lymphoid tissues; the latter of which is known to be critical to achieve long-lasting immune reset in autoimmune disease (AID) patients.

Conclusion: SAIL's targeted LNP-eRNA technology platform enables *in vivo* transient programming of T cells with a superior dose efficiency profile, with the potential to replace a complex, costly, and genome-integrative cell therapy product by an off-the-shelf and non-integrative IV injectable product that does not carry the safety risks associated with lymphoablative conditioning. Plans are in place to rapidly advance a first-generation *in vivo* eRNA-encoded CAR product to human clinical studies for autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, idiopathic inflammatory myositis, and systemic sclerosis.

775 Next-generation synthetic signal peptides Cell and Gene Therapies

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For secreted or cell-surface proteins, signal peptides are short sequences (15-30 amino acids) that target the nascent polypeptide into the secretory pathway. They are cleaved during co-translational translocation into the endoplasmic reticulum at specific recognition sites. Signal peptides are inherently diverse and protein-specific (~3,700 sequences/genome). Critically, at the intersection of key cellular processes – mRNA translation and protein-folding - signal peptide mediated translocation can significantly impact the rate at which proteins are transited through the secretory pathway. Importantly, signal peptides are highly context specific: alternation of a signal peptide sequence may significantly increase cellular production of one target protein whilst reducing the production of another.

In cell and gene therapy, signal peptides used often face limitations such as inefficient targeting, degradation, immunogenicity, context-dependent activity, and potential interference with cellular functions. Whilst most therapies utilise endogenous sequences, directed engineering of signal peptide sequence can substantially improve the efficiency of protein production from an introduced recombinant DNA or mRNA sequence. Moreover, relevant to the quality of the encoded therapy, correct cleavage of a signal peptide to yield the predicted protein product cannot be assured in the therapeutic context, even when using endogenous sequences.

We have developed a proprietary computational platform that integrates large-scale 'omics data with advanced feature analysis, which enables protein product-specific design and engineering of novel signal peptides. Based on in-house comparative empirical analysis of a large library of natural signal peptides, we designed novel, patentable, synthetic compositions with enhanced functionality. Our approach confers expression control, with validated peptide cleavage, and provides a flexible, efficient solution for various therapeutic needs. To exemplify application of our platform, for both Factor VIII (FVIII; Hemophilia A) and a CD19 CAR-T (B-cell malignancies), we used proprietary algorithms to curate and select libraries of 200 signal peptides in each case. Protein expression was measured in HepG2 and Jurkat T-cells, respectively, with reference to the homologous FVIII and CD28 CAR-

T/GM-CSFRα signal peptide controls. Demonstrating the effect of signal peptide sequence, across all signal peptides tested, ~10-fold range of expression was observed for FVIII and >100-fold range for CD19 CAR-T. Based on these data, key sequence features were computationally identified and utilised to derive novel synthetic signal peptides, which exhibited 2.5-fold increases in both FVIII expression in HepG2 cells and CD19 CAR-T expression in Jurkat cells. For CD19 CAR-T, signal peptide cleavage was measured by mass spectrometry. Whilst control signal peptides were correctly cleaved with 60% efficiency, synthetic signal peptides were cleaved at up to 90% efficiency.

In summary, we demonstrate that computational design can be successfully deployed to yield novel protein-specific synthetic signal peptides that can significantly improve cellular protein production and processing. For gene and cell therapies, this is a powerful tool to increase the cellular efficiency of therapeutic protein production.

776 Leveraging Immuno-STAT stimulation via TCR and IL-2 signaling to specifically control CAR T cell expansion and persistence *in vivo*

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***Introduction*:** CAR-T cell therapy (CAR-T) has remarkable success in hematologic malignancies but limited persistence remains a challenge contributing to disease relapse. IL-2 signaling through a CAR-independent circuit uniquely eradicated solid tumor models [1], but the clinical utility of non-specific cytokine CAR-T support (e.g. IL2; aldesleukin) is limited by toxicity. Immuno-STATs^{TM} are designed to activate and expand antigen-specific CD8^{+} T-cells via co-delivery of peptide/HLA-based TCR stimulation and an IL-2 mutein (*Fig. 1A*; [2]). The first clinical-stage Immuno-STAT, CUE-101, selectively activates and expands HPV-specific CD8^{+} T-cells and is associated with durable anti-tumor responses in HPV+ recurrent/metastatic head and neck cancers, with the objective response rate more than doubled in combination with pembrolizumab vs historic control of pembrolizumab alone (NCT03978689, [3]). While IL-2 signaling pathway remains a major component of T-cell persistence, combining natural TCR signaling could lead to optimal expansion and persistence of CAR-T cells. Here we report pre-clinical proof-of-concept for genetic engineering of combined exogenous-TCR co-expression plus a CAR (*E*xogenous-*T*CR; E.T. or E.T.CAR) paired with TCR and IL-2 signaling from an Immuno-STAT to enable direct regulation of E.T.CAR expansion and persistence in vivo (*Fig. 1A*).

***Methods*:** Human CD8^{+} T cells were transduced with an HPV-E.T., spiked into autologous PBMCs and cultured in the presence of increasing concentrations of CUE-101 or irrelevantly targeted Immuno-STAT. For in vivo studies, multi-cistronic viral vectors were engineered to encode E.T.CAR (TCRα/β + CAR) and nano-luciferase. HPV-E.T.CAR T-cells or irrelevantly-targeted CMV-E.T.CAR T-cells were transferred into immunocompromised mice, followed by 3 weekly-doses of CUE-101. T-cell expansion/persistence was measured by bioluminescence imaging (BLI) and flow cytometry.

***Results*:** *In vitro*, in contrast to an irrelevant-peptide/HLA Immuno-STAT, CUE-101 treatment resulted in selective and dose-dependent expansion of HPV-E.T.-transduced T-cells (enrichment from 3% to ~30% E.T.+ of CD8+ T cells; *Fig. 1B–C*). In a tumor-free (to assess CUE-101/E.T. contribution, without confounding by CAR signaling) *in vivo* model, CUE-101 treatment induced dramatic and selective expansion of HPV-E.T.CAR T-cells compared to irrelevant control CMV-E.T.CAR T-cells (>80-fold E.T.CAR+ increase at day 21 post transfer; *Fig. 2*).

***Conclusion*:** We demonstrate that Immuno-STAT treatment together with engineering co-expression of cognate exogenous TCR can specifically control the expansion and persistence of CAR-T cells. Leveraging the clinical experience with CUE-101, we anticipate future evaluation of this approach to determine whether Immuno-STAT-controlled expansion of E.T.CAR-T can enhance and control expansion/persistence of adoptive cellular therapies in patients.

References:
1. GM. Allen *et al.*, *Science* 378, 6625 (2022). doi: 10.1126/science.aba1624. Epub 2022 Dec 16. PMID: 36520915.
2. RD. Seidel *et al.*, *Sci Rep.* 11(1):19220 (2021). doi: 10.1038/s41598-021-98716-z. PMID: 34584159.
3. CH. Chung *et al.*, *ImmunoTherapy of Cancer* 12 (2024). doi: 10.1136/jitc-2024-SITC2024.0649.

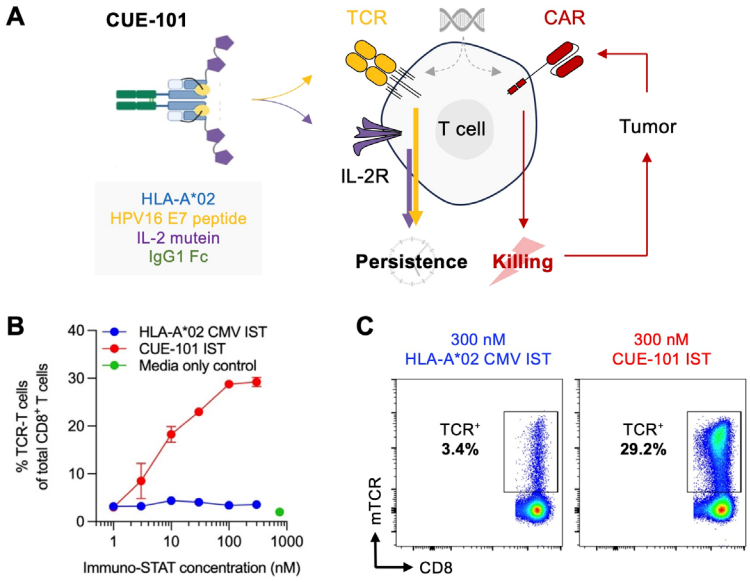

Figure 1. Principle and in-vitro proof-of-concept of CUE-101-induced T cell expansion through TCR and IL-2R. (**A**) CUE-101, an Immuno-STAT consisting of an HPV16 E7 peptide bound to an HLA-A*02 complex and an IL-2 mutein, is used in this study for promoting persistence of T cells engineered to co-express an exogenous HPV-specific TCR and a CAR (E.T.CAR T cells) while maintaining CAR expression for tumor antigen targeting. (**B–C**) In vitro CUE-101 stimulation leads to selective expansion of exogenous HPV TCR$^+$ CD8$^+$ T cells. Transduced CD8$^+$ T cells expressing the HPV-E.T. were spiked into autologous PBMCs and treated with increasing concentrations of on-target (CUE-101) or off-target (CMV) Immuno-STATs (IST). After one week in culture, the percentage of TCR$^+$ cells among live CD8$^+$ T cells was quantified by Flow cytometry (staining of murine TCR-β constant region). **(B)** Quantification and **(C)** representative flow cytometry analysis showing HPV-E.T. cell enrichment following CUE-101 treatment.

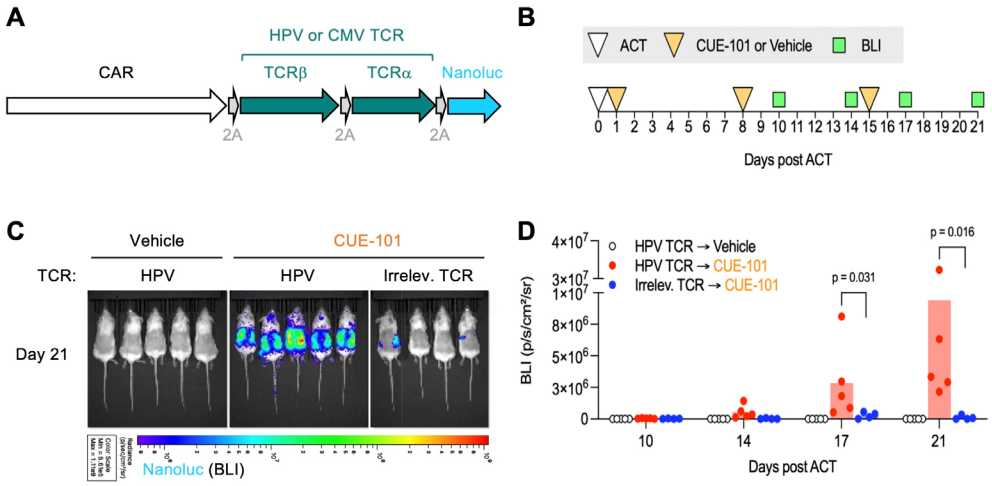

Figure 2. In vivo CUE-101 stimulation leads to substantial and selective E.T.CAR T cell expansion in tumor-free mice. (A) Schematic of multi-cistronic viral vectors for CAR, exogenous TCR (E.T.) and NanoLuc expression. **(B)** Timeline of adoptive cellular therapy (ACT; single dose), CUE-101 treatment (3 weekly doses), and bioluminescence imaging (BLI). **(C)** Representative BLI images at day 21 showing expansion of transduced T cells. **(D)** Quantification of BLI signal over time.

777 Exploration of Allogeneic Shielding Strategies by Precise CRISPR/Cas9 Genome Engineering of Primary Human B Cells to Enable Off-the-shelf B Cell Medicines for Sustained Delivery of *in vivo* Biologics

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B cells can be precisely genetically engineered to produce therapeutic proteins and further differentiated into plasma cells (PCs) that secrete the target proteins (referred to as a B Cell Medicine, BCM). BCMs have the potential for longevity, capacity for high protein secretion, the ability to engraft without preconditioning and to be re-dosed[1], making them an attractive platform for the sustained supply of biologics. To expand BCM accessibility, we explored allogeneic models to test the potential of BCMs as an allogeneic therapy, and ways to enhance hypoimmunogenicity by multiplexed CRISPR/Cas9 genome engineering.

Engineered BCMs are currently being clinically evaluated as an autologous cell therapy for hemophilia B[2]. The natural hypoimmunogenicity of plasma cells has been observed in the clinic, including detection of patient-derived PCs 9-month post allogeneic bone marrow transplant[3], suggesting the potential of BCMs as an allogeneic cell therapy. Herein we first present data using both *in vitro* and *in vivo* assays to determine the potential of BCMs as an allogeneic off-the-shelf cell therapy without additional engineering. We determined changes to cell surface markers relevant to allogeneic rejection during the process of B cell to PC conversion and observed major histocompatibility complex (MHC) downregulation and modulation of other immunologically relevant molecules. We used mixed lymphocyte reactions (MLR) to compare BCM recognition and, relative to activated B cells, noted reduced CD4 (>80%) and CD8 (40-90%) T cell activation. To evaluate allogeneic resistance *in vivo*, we used immunodeficient mice that express human IL-15 (NOG/NSG-hIL15) which were further humanized with either NK or CD34 cells. Human cell chimerism was >3% for NK and > 12% for T cells and both human T and NK cells were demonstrated to be functional in the CD34 humanized mice.

We used CRISPR/Cas9-based B cell engineering to introduce potential allogeneic shielding strategies. Three categories of shielding prototypes were evaluated: 1) “Don’t-eat-me” signals; 2) MHC modulation; and 3) Immune synapse modulation. With engineering optimization, we demonstrated multiplexed gene knock-out (>85% at 3 loci)

and multiplexed transgene knock-in (up to 25% dual positive cells without selection). *In vitro* all prototypes showed complete CD4 T cell resistance and increased CD8 T cell resistance compared to unedited BCMs in the MLR assay, with the MHC modulation category facilitating the most NK resistance.

Top prototypes in each category, along with control BCMs with luciferase as a surrogate transgene, were evaluated *in vivo*. In the NOG-hIL15 NK model, the Don't-eat-me and MHC modulation categories showed complete NK resistance as demonstrated by equivalent bioluminescence with or without NK cells at week 7. Both T and NK resistance was evaluated in the hCD34-NSG-hIL15 model. All prototypes were well tolerated, and persistence was observed at week 7 for all prototypes except for the MHC knockout approach. Consistent with human clinical observations, unedited BCMs shared many of the survival advantages observed with the allo-engineered cell prototypes.

In summary, we demonstrated BCMs are immunologically stealthy and can be further engineered to evade host immune responses, opening the possibility of exploring them as off-the-shelf cellular therapies without HLA matching to serve broader patient populations.

[1]Young 2024 *Mol Ther*
[2]NCT06611436
[3]Griffith 2005 *BJHaem*

778 A Differentiated and Durable Allogeneic T cell Strategy by Leveraging Orthogonal Cas9 Enzymes and an AAV & LNP Delivery Platform

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Autologous chimeric antigen receptor (CAR) T cell therapies have shown great success for treating several types of hematological malignancies, but their widespread use has been hindered by several challenges, including manufacturing failures and the high cost of individualized products. This has sparked a strong desire for "off-the-shelf" allogeneic products. A key safety concern with allogeneic T cells, the risk of graft-versus-host disease (GvHD), has largely been addressed by removing the endogenous T cell receptor (TCR) using gene editing technologies. However, key challenges remain for generating persistent allogeneic T cells due to the recognition of mismatched human leukocyte antigen (HLA) alleles by host T cells or the recognition of cells lacking HLA class I (e.g., when removed via beta-2-microglobulin (*B2M)* knockout) by NK cells, both of which can lead to rejection of such allogeneic T cells by the host immune system. Other approaches involving deep or prolonged lymphodepletion of the host can improve allogeneic T cell persistence but come with an increased risk of infections. To address these challenges, we developed an allogeneic T cell strategy by leveraging a dual CRISPR/Cas9 cleavase/base editor and lipid nanoparticle (LNP) delivery platforms to perform one site-specific insertion for a targeting CAR or TCR and four knockouts, removing the endogenous TCR (via *TRAC*) to prevent GvHD, and disrupting *HLA-A*, *HLA-B*, and the class II transactivator (*CIITA*) to prevent T and NK cell rejection. Edited donor T cells did not induce GvHD in an immunocompromised mouse model over a 90-day evaluation period. Cells knocked out for *HLA-A*, *HLA-B*, and *CIITA* were protected from rejection by alloreactive T cells and NK cells when the residual HLA-C allele was matched to the hosts. The matching approach is further simplified using donors homozygous for *HLA-C*, achieving HLA matching across a broad patient population with a minimal number of donors. Using our proprietary T cell engineering process, we successfully generated allogeneic T cells incorporating four gene knockouts plus site-specific insertion of a tumor-specific TCR or CAR, with high yields and efficiency. Importantly, these allogeneic T cells had comparable functional activity to the control cells engineered without the *HLA-A*, *-B*, and *CIITA* edits in *in vitro* and *in vivo* assays, suggesting no unfavorable functional impact from these gene edits. In summary, we have successfully developed a differentiated "off-the-shelf" approach for CAR-T and TCR-T therapies, which is expected to be cost-effective to produce and durable without requiring intense lymphodepletion. This promising strategy is being applied to our cell therapy pipeline, and its implementation in other cell types is being investigated.

779 Electroporation-free delivery of CRISPR enzymes for efficient multiplex genome editing of primary human immune cells and repair of patient mutations in the rare disease hemophagocytic lymphohistiocytosis (HLH)

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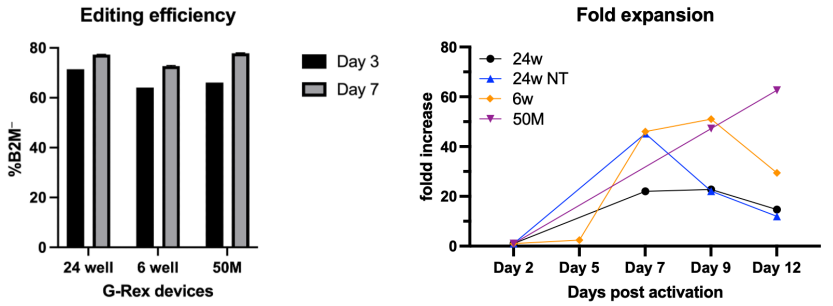
Peptide enabled ribonucleoprotein delivery of CRISPR enzymes (PERC) is a new approach for delivery of genome editing enzymes to primary human immune cells, an important cell type in the field of cell-based genetic therapies. Currently, the predominant mode of CRISPR enzyme delivery is electroporation, which has several disadvantages including reliance on dedicated hardware, incompatibility with repeat dosing, and negative impacts to cell phenotype and viability. In contrast, PERC relies on an amphiphilic peptide that is combined with CRISPR enzymes in their GMP-friendly ribonucleoprotein (RNP) format and applied on to cells in a cultured (ex vivo) setting to mediate high-efficiency editing. PERC has been shown to be compatible with Cas9 and Cas12a as well as adenine base editors and could potentially work well with any CRISPR enzyme. When paired with an AAV carrying a homology directed repair template (HDRT) DNA, PERC can produce high efficiency knock-in of desired transgenes.

Cells edited using PERC have improved viability, resulting in significantly higher edited cell yields, which can be critical in a clinical setting. PERC preserves naïve phenotype, minimally impacting the transcriptome as compared to electroporation. It does not require dedicated hardware and is gentle on cells, which allows for sequential multiplex editing, reducing the risk of chromosomal translocations. PERC is especially appealing because it is "lentivirus"-like in practice: PERC reagents are simply applied to the cells. This is in stark contrast to the cumbersome electroporation approach to CRISPR delivery that has been used out of necessity in recent years. PERC is compatible with the transformational G-Rex technology, which enables culturing and expansion of T cells with minimal handling. Cell health and editing efficiencies are well maintained as we scale from 24 well to 6 well to larger G-Rex vessels. These findings suggest a clear path towards manufacture of precisely edited CRISPR-based cell therapies, potentially involving a "closed loop" approach, requiring minimal handling while maintaining sterility and quality of manufacturable chimeric antigen receptor (CAR)-T cells.

Mutations in the gene *PRF1*, which encodes for the protein perforin, is among the leading causes for familial Hemophagocytic lymphohistiocytosis (HLH), a rare immunological disease. Gene variants may have an unknown effect on its function, and therefore on the disease. To study the effects of these variants of unknown significance (VUS), we introduce a patient mutation in healthy donor cytotoxic T cells by electroporation of CRISPR RNPs and homologous-directed repair templates (HDRTs).This leads to a reduction of perforin levels, which is associated with decreased efficiency in a target cell killing assay. Because PERC can easily be paired with other methods of delivery where repeated sequential editing would not be possible, here we utilize PERC to deliver base editors and revert the simulated patient mutation in cells that were previously electroporated.

In conclusion, we show PERC as a versatile delivery method that can be paired with G-Rex technology for multiplex genome editing and scalable manufacture of CAR-T cells. PERC can also be paired with other delivery technologies such as electroporation, and thus enable the study and correction of disease-causing mutations.

PERC can be readily scaled up in G-Rex® to edit T cells



PERC editing of primary human T cells can be readily scaled in different G-Rex® devices starting with initial cell counts of $1x10^6$ (24 well G-Rex®), $5x10^6$ (6 well G-Rex®), and $25x10^6$ cells (50M G-Rex®) resulting in efficient editing rates and ~50-fold expansion in 9 days. This technology can be applied to multiplex editing of T cells to efficiently manufacture high quality CAR-T cells with minimal handling.

780 Adapter-targeted lentiviral vectors for gene transfer *in vitro* and *in vivo*

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Selective gene delivery to the desired cell type using targeted lentiviral vectors (LV) is an effective and safe strategy for immunotherapeutic applications such as chimeric antigen receptor (CAR) T cell therapy. Current CAR-T cell production processes lasts up to two weeks and include several *ex vivo* cell modification steps. By shortening the manufacturing process or directly generating CAR-T cells *in vivo*, the field aims to eliminate this laborious process while improving product quality. Furthermore, T cell subsets and phenotypes have been shown to have a significant impact on CAR T cell anti-tumor efficacy, therefore, understanding the functional differences between T cell subsets may promote shorter production processes and *in vivo* approaches.

Previously, we described the development of a modular gene delivery system called Adapter-LV, which is based on universally targeted LVs, as directed by adapter specificity. Additionally, a single chain variable fragment (scFv) specific for an artificial tag on the adapter molecule (e.g. an antibody or a fragment thereof) is fused to the viral attachment protein (H) of the LV. In general, transduction of a specific cell type expressing a certain target antigen is dependent on the concentration and specificity of tagged adapter molecules. We optimized Adapter-LV by employing engineered envelope proteins from the canine distemper virus (CDV) to overcome low viral vector yield and limited functionality targeting non-activated cells, as with the previously used measles virus (MV) pseudotype. With the CDV based Adapter-LV, the productivity is improved while the selectivity and flexibility of the system are maintained.

We successfully generated CAR-T cells with varying CD4:CD8 ratio *in vitro* and confirmed CAR-T cell function by specific lysis of tumor cells with all generated CAR-T cell products. Next, we evaluated the functionality *in vivo* by injecting a single dose of CDV-Adapter-LV into PBMC-transplanted NSG mice to generate CD8+ CAR-T cells using a CD8-specific adapter. CD8+ T cells were selectively transduced, while B cells were depleted, confirming the generation of functional CAR-T cells *in vivo*. Furthermore, we investigated adapter-mediated gene transfer to generate CD4+ CAR-T cells or a mix of CD4+ and CD8+ CAR-T cells *in vivo*. In the presence of CD4 and CD8 specific adapter molecules, CAR-T cells of both T cell subsets were successfully generated.

In conclusion, we can confirm that our improved targeted gene delivery system is suitable for both, *in vitro* and *in vivo* use, by selectively generating for example CD4+ and CD8+ CAR-T cells using the Adapter-LV platform. This system can also be used to analyze and optimize effective CD4:CD8 T cell combinations, as well as to better understand their individual and bystander importance for CAR-T cell and non-CAR-T cell therapy applications.

781 Long-acting Circular RNA CAR Drives Potent and Durable Tumor Elimination In-vivo

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In vivo engineered mRNA-based cell therapies can overcome many of the limitations of current stably integrated cell therapies such as Chimeric antigen receptor (CAR) T cell therapy. Broader patient access, ease of manufacture, significantly lower cost, immediate availability of drug product, ease of repeat dosing and improved safety profile with the absence of stable integration and debilitating lymphodepletion regimens are some of the key advantages of an in vivo engineered mRNA cell therapy approach. However, due to the short-lived nature of RNA expression, levels of CAR protein expression and duration must be improved to match the effectiveness observed with stable integration-based cell therapy.

We previously demonstrated the development of circular RNA (circRNA) IRES sequences that enable 10x higher levels than the current state of the art IRES and up to 25 days of CAR expression when transfected into non-activated primary human T cells. These circRNA CAR-T cells drove multiple rounds of robust repeat tumor cell killing *in vitro* over 15 days in culture. When adoptively transferred into a tumor bearing mouse the circRNA CAR-T cells effectively controlled tumor burden up to 7 days post transfer.

Building on this work, we show that our circRNAs can transfect T cells with up to 80% efficiency within the mononuclear cell (MNC) population in fresh leukopaks using lipid nanoparticle (LNP) based delivery. These transfected cells when adoptively transferred into tumor bearing mice showed complete tumor elimination, comparable to lenti-virus transduced T cells, with sustained tumor control lasting 21 days post transfer. We also demonstrate here, the ability of miRNA-based genetic circuits within circRNA to be highly effective in de-targeting expression in non-target cells while preserving expression in target cells. By integrating Strand's proprietary circuit platform for highly specific payload expression, advanced circRNA production processes, and enhancements in expression duration and levels, we are developing an in vivo engineered circRNA CAR platform for potent and long-lasting CAR therapy.

782 Development and Evaluation of Two BCMA-Targeted CAR-T Cell Products for Multiple Myeloma Treatment: A 2nd vs. 4th Generation Approach with IL-15 Production in Brazil

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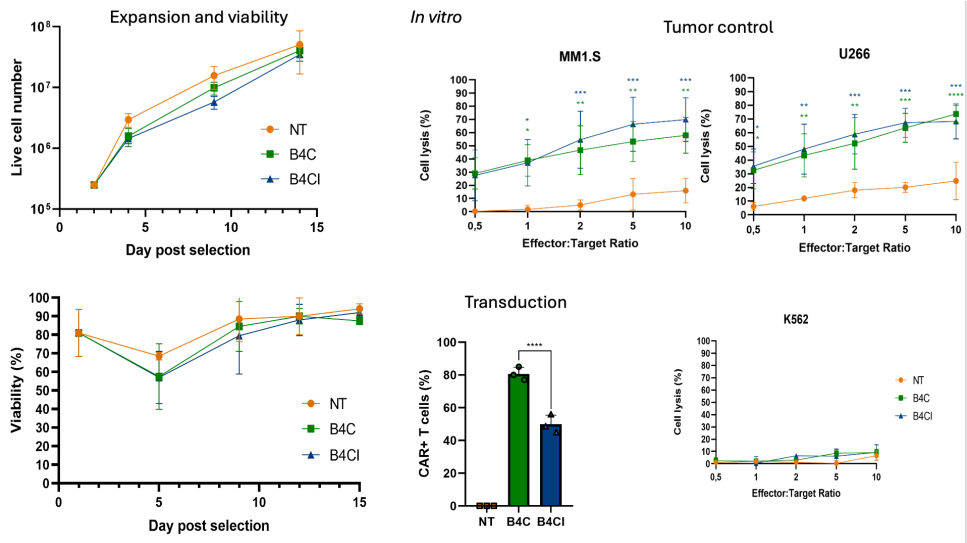
Introduction: B cell maturation antigen (BCMA)-targeted chimeric antigen receptor (CAR)-T cell therapy has emerged as a breakthrough treatment for multiple myeloma. However, disease relapse remains a significant challenge following therapy. In this context, fourth-generation CAR-T cells, which include cytokine production, have emerged as a potential strategy to enhance both tumor cell eradication and CAR-T cell persistence. In Brazil,

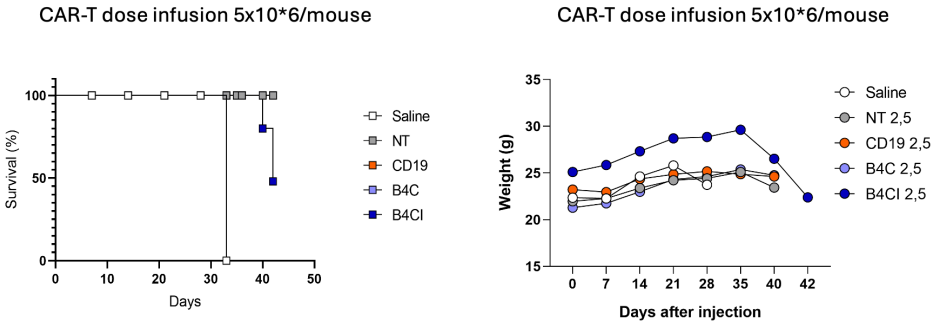
however, access to CAR-T cell therapy is constrained by prohibitively high costs and lengthy production timelines. This study aimed to develop two BCMA-targeted CAR-T cell products: a 2nd generation and a 4th generation CAR with sIL-15 production, for the treatment of multiple myeloma patients.

Methods: We developed two BCMA-targeted CAR constructs: one 2nd generation CAR (BCMA-4-1BB-CD3ζ) and one 4th generation CAR (BCMA-4-1BB-CD3ζ-IL-15). Lentiviral particles containing the CAR sequences were generated and used to transduce primary T cells. The functionality of the CAR-T cells in vitro was assessed through cytotoxicity and cytokine production assays against BCMA-positive (MM1.S, U266) and BCMA-negative (K562) cell lines. Additionally, T cell memory and exhaustion profiles were evaluated. For in vivo efficacy, luciferase-labeled BCMA+ tumor cells (MM1.S) were intravenously injected into immunodeficient mice, followed by CAR-T cell infusion. Control groups included saline, non-transduced T cells, and CD19-targeted CAR-T cells. Tumor progression was monitored through weight tracking, bioluminescence imaging, and survival analysis.

Results: Both 2nd and 4th generation BCMA-targeted CAR-T cells achieved high transduction rates (40-85% CAR+ cells) in primary T cells derived from healthy donors. In vitro assays revealed BCMA-specific cytotoxicity, with both CAR-T products effectively killing BCMA+ target cell lines (MM1.S, U266) and producing TNFα and IFNγ. No cytokine production or cytotoxicity was observed against the BCMA-negative K562 cell line. Notably, no significant increase in T cell exhaustion markers (PD-1, TIM-3, LAG-3) was observed on CAR-T cells compared to non-transduced T cells. Moreover, IL-15-producing CAR-T cells exhibited a higher proportion of memory T cell populations. In vivo, both CAR-T products exhibited strong anti-tumor activity compared to the control groups. The 4th generation CAR-T cells provided superior tumor control relative to the 2nd generation CAR-T cells. However, mice treated with the 4th generation CAR-T cells experienced significant toxicity, leading to mortality within 35 days, despite no tumor progression. In contrast, mice treated with the 2nd generation CAR-T cells remain alive to this day, demonstrating nearly 3 months of survival, while all control animals did not survive.

Conclusion: We developed two novel BCMA-targeted CAR-T cell products: a 2nd generation CAR and a 4th generation CAR with sIL-15 production. Both showed strong BCMA-specific cytotoxicity and cytokine production, with favorable memory and exhaustion profiles in vitro. In vivo, both CAR-T products controlled tumor growth, but the 4th generation demonstrated superior efficacy at the cost of significant toxicity. Further research is needed to address these toxic effects. Notably, CAR-T production and testing were completed in just 10 days, marking a key advancement in reducing production timelines and improving access to CAR-T therapy for multiple myeloma patients in Brazil.





783 Development of a Method for the Gene Editing of T-cells in a Closed Single-Use System

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The genetic modification of T-cells involves viral transduction for the delivery of the Chimeric Antigen Receptor (CAR), which is often inefficient. In this study, we present a method to ensure a robust T-cell transduction rate in a closed single-use system, the VueLife® cell culture bags from Saint-Gobain. VueLife® bags are highly permeable to oxygen and carbon dioxide, while being impermeable to water. VueLife® C Series bags are designed for culture and expansion of suspension cells, including T-cells. The VueLife® AC Series are composed of a treated film. This proprietary surface treatment results in the VueLife® AC bag having a higher surface energy and is intended to promote better cell and protein adhesion to the bag surface, a feature than can be leveraged for transduction applications.

VueLife® C and AC bags were used to perform the lentiviral transduction using a viral concentration of 10^9-10^{10} TU/ml and an MOI of 1, 5, 10 and 20. To identify the optimal transduction conditions, we tested different coating combinations in VueLife® C and AC bags prior to transduction. We evaluated coating with a range of RetroNectin® concentrations, both in the presence or absence of the blocking agent BSA. T-cells were incubated in the coated bags along with the GFP-labelled lentiviral vector for an additional two days. We obtained 20%, 70%, 80% and 85% GFP positive cells with an MOI of 1, 5, 10 and 20, respectively, in the presence of 20ug/cm^2 RetroNectin® for T-cells transduced in both VueLife® C and AC bags. The effect of BSA coating, in the presence or absence of RetroNectin®, did not have a significant impact on transduction rates.

Using a lentiviral vector with an MOI of 10, we found that, in the absence of RetroNectin®, the T- cell transduction rate in VueLife® C bags was 35%, while it was 50% in VueLife® AC bags, two days post-transduction. Following a ten-day expansion, transduced T-cells were collected and analyzed. The GFP levels were found to range between 60 and 70%. We also performed cell surface staining, to assess the distribution of CD4 and CD8 cells, as well as the distribution of effector, effector memory and central memory T-cells, which were the dominant population in all conditions tested. VueLife® C bags require RetroNectin® coating to achieve high transduction rates (>50%), while VueLife® AC bags can be used without coating. In summary, these results demonstrate the capabilities of the VueLife® bags series for efficient T-cell transduction.

784 Improved Genetic Engineering of Primary Human Hematopoietic Stem and Progenitor Cells Using SMAR DNA Nanovectors

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Transplanting hematopoietic stem and progenitor cells (HSPCs) that have been electroporated with cytotoxic drug-resistance genes could enable robust anticancer therapy without compromising adequate hematopoiesis. Achieving sustained genetic modification in HSPCs is critical for this approach. While recombinant integrating viruses are commonly used, their propensity for random genomic integration raises concerns about insertional mutagenesis. In addition, inherent viral immunogenicity complicates clinical implementation.

To address these challenges, we developed a non-viral DNA vector platform incorporating Scaffold/Matrix-Associated Regions (S/MAR). Unlike viral or CRISPR/Cas9-based systems, these SMAR DNA nanovectors (nSMAR) remain episomal and avoid the risk of integration-mediated genomic damage. Their large cargo capacity also facilitates the delivery of complex therapeutic transgenes, making them suitable for advanced gene therapy applications. Notably, the mitotic stability of nSMAR-based constructs provides the potential to lower manufacturing costs for genetically engineered HSPCs. Here, we assessed the efficiency and longevity of reporter gene expression mediated by nSMAR constructs in the K562 cell line and in primary HSPCs. We also generated nSMAR vectors carrying a mutant human dihydrofolate reductase (hDHFR) gene to confer resistance to methotrexate (MTX). All electroporations were performed with the MaxCyte ExPERT® platform, and transfection efficiency along with cell viability were evaluated via flow cytometry.

We observed persistent reporter transgene expression in K562 and primary HSPCs. In primary HSPCs from three donors, transfection efficiencies reached up to 82% with acceptable viability (75–93%) three days post-electroporation. Cells maintained reporter expression for at least 21 days in culture. Furthermore, in K562 cells, the nSMAR vector containing double-mutant *hDHFR* effectively conferred MTX resistance. Ongoing work aims to adapt these nSMAR vectors for primary HSPC modification and to investigate engraftment potential and MTX resistance in more detail. These findings highlight nSMAR vectors as a promising strategy for stable and safe gene modification of HSPCs. This platform may serve as a powerful tool for hematopoietic cell transplantation, potentially benefiting patients with malignancies or inherited hematological disorders.

785 Epitope Editing of CD90 Protects Hematopoietic Stem Cells from Immunotherapy and Enables Targeted Enrichment for Gene Therapy

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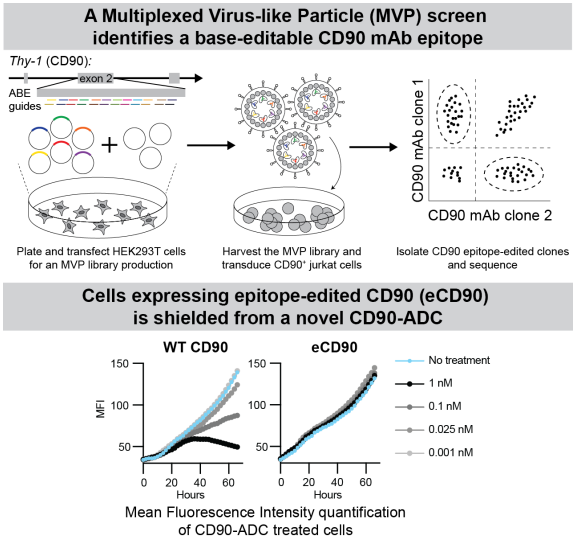
Introduction: Hematopoietic stem cell (HSC) gene therapy holds immense potential to cure blood and immune disorders. Its success and long-term efficacy depend on the treated HSCs' ability to efficiently repopulate the hematopoietic system. However, current cytotoxic conditioning regimens used to deplete host HSCs and enable engraftment of ex vivo gene-modified HSCs are associated with significant toxicities. To address this issue, non-genotoxic approaches utilizing cell type-specific antibody-drug conjugates (ADCs) have been developed to selectively target specific cell populations. Building on this concept, gene editing of epitope sites can prevent ADC binding, protecting edited cells from ADC-mediated depletion and enabling selective enrichment of desired cells to reach the therapeutic threshold. Here, we present an epitope editing strategy targeting the long-term HSC (LT-HSC) marker CD90. This approach shields edited cells from a novel CD90-ADC, allowing for selective elimination of unedited CD90-expressing LT-HSCs from the bone marrow niche and supporting improved HSC gene therapy outcome.

Methods: To identify epitopes for a CD90 monoclonal antibody (mAb), a guide RNA library targeting all adenine base-editable sites in exon 2 of the *Thy1* gene (encoding CD90) was packaged into virus-like particles containing adenine base editors. The resulting multiplexed virus-like particle (MVP) guide library was used to transduce CD90-dependent Jurkat human lymphoblastic cells. Viable clones with reduced mAb binding were isolated and sequenced to identify epitope-edited CD90 variants (eCD90). HSCs and cell lines expressing eCD90 were evaluated for proliferation, differentiation potential, and resistance to a novel CD90-ADC.

Results: The MVP screen identified an eCD90 variant with reduced binding to the CD90 mAb while retaining recognition by a different antibody, indicating preserved CD90 protein structure. Cryo-electron microscopy localized the epitope to the mAb interaction site. Human HSCs expressing eCD90 demonstrated normal engraftment, proliferation, and multilineage potential in an in vivo immunocompromised mouse model. Upon exposure to a novel CD90-ADC, eCD90-expressing cells were protected and proliferated normally, whereas wild-type CD90-expressing cells were effectively eliminated.

Conclusions: The eCD90 variant provides complete protection from a CD90-ADC without disrupting CD90 protein structure or functionality in HSC engraftment and proliferation. This approach offers a robust strategy to enrich gene-corrected LT-HSCs by removing uncorrected HSCs and creating niche space for corrected cells to engraft, ultimately enhancing the efficacy of HSC gene therapy. Further investigations are underway to assess eCD90 functionality, its long-term effects in vivo, and eCD90-mediated HSC enrichment using the CD90-ADC and chimeric antigen receptors.



786 NK cells derived from expanded cord blood HSCs provide an optimal source for allogeneic cell therapy

Ece Canan Sayitoglu[1], Everardo Hegewisch-Solloa[1], Grace Eppolito[1], **Rui Tostoes**, Miguel Maduro[1], Nina Horowitz[1]
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Natural Killer (NKs) cells are a promising allogeneic immunotherapy for several malignancies. Their natural anti-tumor activity is orchestrated by a diverse array of activating and inhibitory receptors, enabling potent effector function against heterogeneous tumors while maintaining a favorable safety profile. However, there have been several roadblocks in their production as cell therapies: (1) lack of scalable cell sources platforms for clinical-scale manufacturing, (2) significant donor-to-donor variability, and (3) need for robust and predictive preclinical assays to validate NK efficacy. We developed an integrated screening and manufacturing platform that leverages a proprietary small molecule, IBR403, to achieve 1E3-1E6 fold expansion of cord blood unit (CBU)-derived hematopoietic stem cells (xHSCs). This platform enables use of CBU segments, containing less than 300 µL of

blood, while leaving the 25 mL CBU cryopreserved and intact for selective manufacturing. xHSCs from segments are differentiated into NK cells (xHSC-NKs) and characterized using flow cytometry, xCELLigence, and advanced assays (e.g. serial rechallenge, organoid co-culture, and in vivo models). xHSC-NKs from optimal donors express high levels of canonical activating receptors including NKG2D and DNAM-1, and degranulate and produce inflammatory cytokines upon stimulation with target cells similarly to peripheral blood (pb) NK cells. xHSC-NKs exert cytotoxicity against diverse tumor cell lines, allowing us to identify optimal donors for downstream clinical applications. Serial rechallenge assays show NKs exhibit significant donor-derived variability in their responses to repeated tumor exposures. xHSC-NKs also eradicate 3D patient-derived cancer organoids comparably to pbNKs. Finally, xHSC-NKs acquire a mature phenotype (KIR^+CD16^+) and exhibit long-term persistence and high killing capacity after 10 weeks in vivo. Overall, xHSC-NK cells demonstrate high efficacy against tumor cells and clear therapeutic potential, with our platform enabling scalable, cost-effective manufacturing and rigorous donor prescreening to produce thousands of potent allogeneic NK doses from a single CBU - providing access to durable therapies and improving patient outcomes.

787 Enhanced Ex Vivo Expansion of Hematopoietic Stem Cells by a Novel Compound Cocktail

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A major challenge in utilizing hematopoietic stem cells (HSCs) for cell and gene therapies in blood and immune disorders is the loss of self-renewal and potency during in vitro culture. Developing optimized cell culture conditions that preserve and expand functional HSCs is essential to overcome this limitation. To address this, we tested the effects of more than 40 compounds either individually or in combination for their capacity to expand and maintain long-term hematopoietic stem cells during in vitro culture. We identified a novel combination of compounds (CMs) that improved the expansion and maintenance of HSCs derived from human cord blood (CB), bone marrow (BM), and mobilized peripheral blood (mPB). CB-derived CD34+ cells or CD34+CD38-CD45RA-CD90+ cells (an HSC enriched population) were cultured in StemSpan™ SFEM II, a serum-free medium containing SCF, FLT3L, TPO, IL-3, and IL-6, with or without CMs (control). After 7 days of culture, cells were counted and analyzed for expression of CD34, CD45RA, CD90, CD133, and EPCR by flow cytometry. The results demonstrate that supplementation with CMs significantly increased the frequency of CD34+ cells relative to the control (75.6% ± 10.8% vs. 59.2% ± 9.9%, respectively; n=24, $p < 0.0001$). More importantly, the HSC population, as defined by CD34+CD45RA-CD90+CD133+EPCR+ marker expression, showed significantly higher fold expansion (4.6 ± 1.92 vs 0.12 ± 0.07, n=24, $p<0.0001$) and frequency (10.5% ± 3.7% vs 0.19% ± 0.11%, n=24, $p< 0.0001$) compared to control. The effects of CMs on HSC frequency and expansion were similar in cultures initiated with mPB and BM-derived CD34+ cells. When CMs were added to cultures initiated with CD34+CD38-CD45RA-CD90+ cells, it resulted in 18-fold increase in expansion and 64-fold higher frequency of HSCs compared to cultures without CMs after 12 days of culture. Finally, to determine whether CMs preserve in vivo engraftment potential of HSCs, 2,500 uncultured CB CD34+ cells or the progeny of 2,500 CB CD34+ cells expanded for 7 days with CMs were transplanted into immunodeficient NSG mice. Bone marrow analysis at 20 weeks post-transplantation demonstrated higher levels of human leukocyte engraftment in mice transplanted with CM-expanded cells compared to those receiving uncultured CD34+ cells (19.2% ± 15.14% vs 11.23% ± 8.76%; n=8 mice/group). Together, these findings demonstrate that supplementation with this novel combination of compounds improves HSC expansion, preserves self-renewal capacity and enhances their in vivo repopulation potential. This novel approach provides a promising solution for preserving functional HSCs during in vitro culture, facilitating their application in cell and gene therapies for hematological and immune disorders.

788 Transcription Factor-Wide Screens to Identify Regulators of Tissue Resident Memory T cell States

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Memory T cells exhibit distinct functional characteristics depending on their location within the body. Recent advancements have unveiled a significant population of memory T cells residing in non-lymphoid tissues. These resident memory T cells are critical for combating infections and their abundance has a positive correlation with improved survival rates across many solid tumor types. Motivated by this observation, we aimed to harness the potential of resident memory T cells for adoptive cell therapy. However, isolating these cells from donor-derived tissues is impractical. Therefore, we focused on epigenetic reprogramming of blood-derived T cells towards a tissue resident state. We conducted two cDNA overexpression screens, using open reading frames (ORFs) encoding all known transcription factors, in primary human CD8 T cells to identify genetic regulators of *CD103* (a residency marker) and *IL7R* (a memory marker). The intersection of these screens identified the transcription factor *TGIF2LX*, capable of upregulating both markers. Interestingly, this transcription factor is not expressed in human T cells. As opposed to the reported effects of TGIF family members in other cell types, we show that *TGIF2LX* overexpression potentiates the effect of TGF-b and retinoic acid signaling in T cells. Varying levels of these signaling pathways have been demonstrated to control the generation of tissue resident memory T cells in the skin, small intestine, and liver. Bulk RNA-sequencing revealed that *TGIF2LX* overexpression in T cells leads to widespread transcriptional reprogramming, including activation of genes associated with increased tissue residency programs (e.g. *ITGAE*, *CD69*, *CXCR6*) and decreases in genes associated with circulation programs (e.g. *KLF2*, *S1PR1*). We further characterized *TGIF2LX* overexpression in T cells using a multimodal single cell readout, pairing scRNA-seq with a CITE-seq library of 134 unique cell surface markers. This demonstrated a distinct cell state with *TGIFL2X*-overexpressing T cells existing in clusters unique from those containing unedited cells. Of note, these cells also clustered separately from T cells overexpressing *RUNX3*, a transcription factor previously identified to enhance tissue residency and improve tumor control, suggesting a novel axis for controlling tissue residency.

789 Targeting UPAR With Multiplexed-Engineered iPSC-Derived CAR T Cells to Reverse Age- and Insult-Related Fibrotic Disease

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Urokinase plasminogen activator receptor (UPAR) is a component of the plasminogen system, contributing to diverse biological processes including fibrinolysis, cell adhesion, and cell migration. Increased UPAR expression has been implicated in cancer and inflammatory disease. Recent work has demonstrated that treatment with anti-UPAR CAR T cells can eliminate fibrosis and restore liver function in models of metabolic dysfunction-associated steatohepatitis (MASH). Here, we present the development of novel anti-UPAR CAR constructs, characterization of UPAR as a target in MASH and radiation induced pulmonary fibrosis (RIPF), and development of iPSC-derived CAR T cells for treatment of fibrotic diseases.

To select an optimal binder sequence for targeting UPAR in human disease, we profiled a set of candidate binders across several parameters. The UPAR protein is comprised of three domains and is highly susceptible to cleavage between domains 1 and 2. To assess the binding location of our binders, we generated target lines overexpressing either full-length or cleaved UPAR for use in cytotoxicity assays. Two of our candidate binders showed activity only against full-length UPAR, while two other binders effectively targeted both full-length and cleaved UPAR. In addition to mapping domain specificity, we generated a series of target cell lines expressing specific amounts of UPAR to compare the relative binding affinity of each of our candidate CAR binders. From these experiments we selected the two binders that recognized the post-cleavage UPAR domains, with two distinct binding affinities, for further development. To characterize UPAR expression in human disease, we analyzed patient samples of MASH, hepatocellular carcinoma-related cirrhosis, and pulmonary fibrosis, as well as normal adjacent tissue for comparison. UPAR expression was elevated in diseased tissues, with the highest expression found in fibrotic regions. Staining with cell identity markers revealed that UPAR+ cells express markers of either macrophages (CD68) or activated fibroblasts (αSMA), cell types known to drive fibrotic disease progression. Profiling UPAR across a panel of 30 normal tissues revealed consistently low levels of expression, demonstrating the potential of UPAR as a potent CAR target to treat fibrotic disease with low risk of cross-reactivity.

In addition to developing CAR T cells targeting human UPAR, we have developed a new mouse model for targeting UPAR in RIPF. In humans, RIPF is a common side effect of radiation therapy with minimal treatment options. In preclinical studies, after a single dose of radiation to the left lung, we observed significant fibrosis in the irradiated tissue within two weeks (Fig. 1). As in models of liver fibrosis, UPAR expression was upregulated in the fibrotic region following irradiation, evidence that targeting UPAR+ cells with CAR T-cell therapy will be widely applicable to a range of fibrotic diseases with few current therapeutic options. Moreover, targeting fibrotic diseases such as MASH and RIPF with iPSC-derived CAR T cells will avoid drawbacks of traditional CAR T-cell therapy including manufacturing challenges overcome by our off-the-shelf approach and the need for conditioning chemotherapy mitigated by our sword and shield technology. We are now investigating iPSC-derived anti-UPAR CAR T cells in various preclinical models, with the goal of developing an off-the-shelf CAR T-cell therapy for fibrotic disease and cancer.

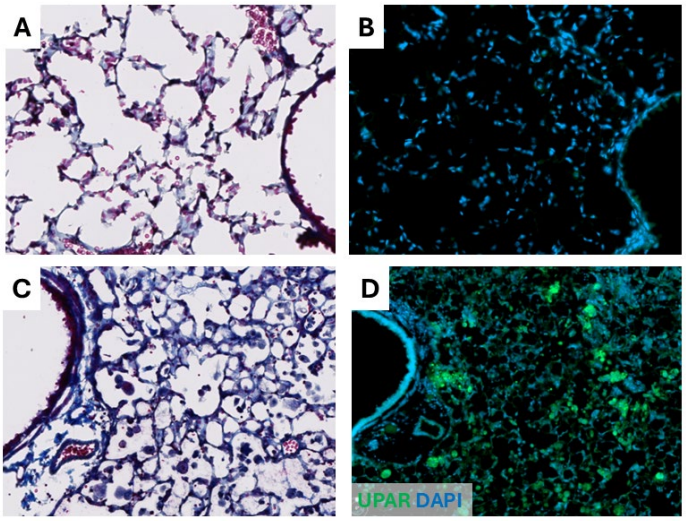

Figure 1. UPAR is upregulated in fibrotic regions in a mouse model of RIPF. Two weeks after irradiation of the left lung (90 Gy), fibrosis was observed by trichrome staining of collagen (**C**). UPAR expression was increased in the fibrotic regions of irradiated lungs (**D**) compared to naïve lung (**A, B**)

790 Role of integrins in engineered plasma cell localization and function

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Introduction: Plasma Cells (PCs) play an integral role in long-term humoral protection. After antigen exposure, PCs are formed in the peripheral organs, migrate into the bone marrow (BM) and secrete large amounts of antibodies (Abs) for several decades. Due to their longevity and high secretion capacity, engineered PCs are being developed as cell-based protein-secreting therapeutics. Engraftment of PCs in the BM depends on the interactions between the donor PCs and the host BM niche. It is therefore important to assess the surface proteome of PCs to characterize potential interactions that enhance or inhibit engraftment into the BM.

Methods: Here, we employed mass spectrometry-based proteomics to define the proteins expressed on the surface of engineered PCs and undifferentiated B cells. To determine whether identified proteins were required for PC biology, we used CRISPR-Cas9-based gene editing to knock out specific proteins and studied PC functions *in vitro*. To study the *in vivo* functions of edited PCs, we utilized luciferase tracking in a murine model to determine their localization and engraftment in various immune compartments, and ELISA to quantify human Abs secretion in the murine serum. Finally, we characterized the phenotype of the engrafted PCs upon sacrifice via flow cytometry.

Results: With the surface proteomics approach, we identified several adhesion proteins that were expressed on the membrane of PCs, some of which were not found in undifferentiated B cells. We found that adhesion proteins belonging to the Integrin and SLAMF family were predominantly expressed on PCs. Specifically, PCs highly expressed integrin proteins ITGA4 and ITGB1, which form a heterodimer known as Very Late Antigen-4 (VLA-4), previously shown to be necessary for PC retention in the BM. In *in vitro* studies, upon CRISPR-Cas9 based knock out of ITGB1, we discovered that surface expression of ITGB7 is upregulated, due to pairing with ITGA4, which would otherwise be complexed with ITGB1. Assessment of the engrafted PCs demonstrated that, in contrast to published literature, neither ITGB1 nor ITGB7 are necessary for engraftment or function of PCs in the BM niche. These findings suggest that integrin proteins either play a redundant role or are dispensable for driving and retaining edited PCs into the BM niche.

Conclusions: Contrary to existing literature, VLA-4 is dispensable for migration to or retention of PCs in the BM niche. This challenges existing paradigms and requires a reevaluation of PC-BM interactions. The ITGA4 and ITGB7 heterodimer is believed to be a homing marker that drives immune cells to gut-associated mucosal sites. Future experiments will compare the differential localization of ITGA4 and ITGB7 co-expressing PCs to that of ITGA4 and ITGB1 co-expressing PCs in the BM and gut-associated lymphoid tissues.

791 SR-02, a Novel Islet Replacement Therapy for Insulin-Requiring Diabetes

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Islet replacement therapy is the best option to improve long-term health outcomes for patients of type 1 diabetes (Rickels 2019). Allogeneic cadaveric islets infused to the portal hepatic circulation with concurrent immune suppression (Lantidra) and an experimental, embryonic stem cell-derived product (Zimislecel), also infused to the portal circulation with immune suppression, have both resulted in insulin independence in patients of type 1 diabetes.

Regardless of their source, pancreatic cell clusters infused to the portal vein can cause microvascular obstruction injury that stimulates a local inflammatory reaction harmful to the infused cells. This uncertainty complicates the delivery of a calculated therapeutic dose and likely reduces the potency of the cell product. Furthermore, the surviving cell clusters are dispersed throughout the liver parenchyma, making their tracking, or removal (for safety reasons) impossible.

SR-02, a novel stem-cell derived Islet replacement therapy have advantages that are described here.

- The cells were reprogrammed from mature islet tissue of a pancreas from a highly compatible, healthy organ donor. Administration of this cell product will not be limited to patients of certain ABO blood types.
- The SR-02 cells differ from pluripotent cells (including embryonic stem cells and induced pluripotent stem cells) in that they retain pancreatic character that restricts their fate potential, yielding a purer endocrine pancreatic product.
- SR-02 is administered into an omental pouch in lieu of the portal vein. This allows a precise application of a calculated dose, which can be visualized radiographically and retrieved without causing long-term complication to the patient. SR-02 is manufactured to meet 21 CFR part 210 and 211 requirements and is currently being evaluated in a phase 1/2 clinical study (NCT06651515).

A required feature of omental administration is the ability to remodel the implant site to form a niche, including microvascularization, capable of supporting a functional extra-pancreatic endocrine pancreas. Nonclinical studies have shown that the implant coordinates with host omental tissue to create a graft with structural features of a native pancreas. In particular, the bolus of implanted endocrine cell clusters become the organized endocrine cell clusters and ribbons separated by host stroma reminiscent of the distribution of islets in the pancreas. This implies coordination between host and implant to remodel the combined graft.

The retrievability of the graft without immune suppression withdrawal enables the implant of clusters to patients on immune suppression therapy due to an existing organ transplant and enables the implant of clusters that have been modified to reduce or eliminate immune-targeted rejection. SR-03, a version of the Seraxis stem cell line has also been modified and is in nonclinical development. Omental administration and elimination of de novo systemic immune suppression therapy will make islet replacement therapy widely accessible to millions of patients in need.

792 MxV Glycoproteins: A Novel Fusogen for In Vivo CAR-T Generation

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Chimeric antigen receptor (CAR) T-cell therapies have revolutionized cancer treatment, yet their widespread use is hindered by complex manufacturing processes and high costs. To overcome these challenges, we developed a novel lentiviral vector pseudotyped with MxV glycoprotein (MxV-G), demonstrating promising efficacy for in vivo CAR-T generation. First, we observed that MxV-G significantly outperformed the traditional VSV-G pseudotyped vectors, achieving 2-5 fold higher viral titers, both for reporter gene construct GFP and CAR constructs. Additionally, the MxV-G pseudotyped vector exhibited superior CAR-T transduction efficiency at equivalent multiplicities of infection (MOI). We next utilized this vector, incorporating a T-cell targeting module, to generate CAR-T cells in vivo. In a MHCI/II knockout NSG mouse model bearing Nalm6-Luc tumors, intraperitoneal injection of the vector one day after peripheral blood mononuclear cell (PBMC) infusion resulted in complete tumor eradication. Notably, high levels of CAR-T cells were detected post-virus administration. To abolish natural tropism and enhance specificity of MxV-G, we designed a number of mutants with single and multiple amino acid mutation based on an AI-Driven protein model and a Large Language Model. Our results showed that a mutant with multiple AAs mutation diminished infectivity in non-T cells, while the T-cell targeting module restored comparable transduction efficiency on T cells, similar to the wild-type MxV-G vector. These findings collectively highlight mutated MxV-G as a novel fusogen for in vivo CAR-T generation, offering a potentially more efficient and cost-effective approach for CAR-T therapy.

793 ENaBL, a Novel Lentiviral Vector Platform for In Vivo Engineering of Therapeutic Anti-BCMA CAR T Cells, Shows Effective T Cell Transduction and Tumor Control in a Humanized Murine Model

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Chimeric antigen receptor (CAR) T-cell therapies have transformed treatment for B-cell malignancies, though their availability is restricted by manufacturing limitations, logistical demands, long waiting time and high cost of treatment. To address these challenges, we developed ENaBL, a nanobody-targeted, immune-shielded lentiviral vector platform for *in vivo* cell engineering. The ENaBL-T vector, specifically designed to transduce T cells, is a third-generation, self-inactivating lentiviral vector encoding a BCMA-targeted CAR under the control of a T cell-specific synthetic promoter to minimize off-target expression. The envelope of ENaBL-T is MHCI-depleted and enriched with human CD47 to reduce complement-mediated inactivation and vector phagocytosis (Milani et al., 2017 & 2019). T-cell-specific transduction is achieved through a llama-derived, membrane-anchored nanobody (VHH) targeting the TCRαβ receptor, combined with a targeting deficient VSV-G protein for envelope stability and efficient membrane fusion. *In vitro*, ENaBL-T effectively transduced both activated and resting CD4+ and CD8+ T cells from healthy donors and multiple myeloma patients. Exposure of resting T cells to ENaBL-T induced T-cell activation, expansion, and enrichment of transduced cells. In human CD34+ hematopoietic stem cell humanized

NCG mice, a single intravenous administration of ENaBL-T led to selective T-cell transduction, achieving clinically-relevant levels of circulating BCMA CAR-T cells, complete tumor clearance in a systemic multiple myeloma model, and no observed toxicity. Biodistribution of ENaBL-T transduced cells was consistent with the intravenous route of administration, with integrated vector copies detected in liver, spleen, and bone marrow, while CAR expression remained specific to human T cells, confirming the specificity of the T cell synthetic promoter. These results demonstrate that ENaBL-T can support safe and effective *in vivo* generation of functional CAR-T cells, potentially expanding patient access to cost-effective, off-the-shelf CAR-T therapies without the need for apheresis, lymphodepletion or *ex vivo* cell manufacturing. We have successfully manufactured ENaBL-T encoding a clinically validated BCMA CAR construct (referred to as ESO-T01) at clinical scale using adherent fixed bed bioreactor and first in human testing was initiated in 4Q2024 (NCT06691685).

794 A Vivo CAR T Producer (HN2301) for Autoimmune Disease Enables B cell Depletion in NHP Tissues and Performs Therapeutic Effect in an SLE Mouse Model

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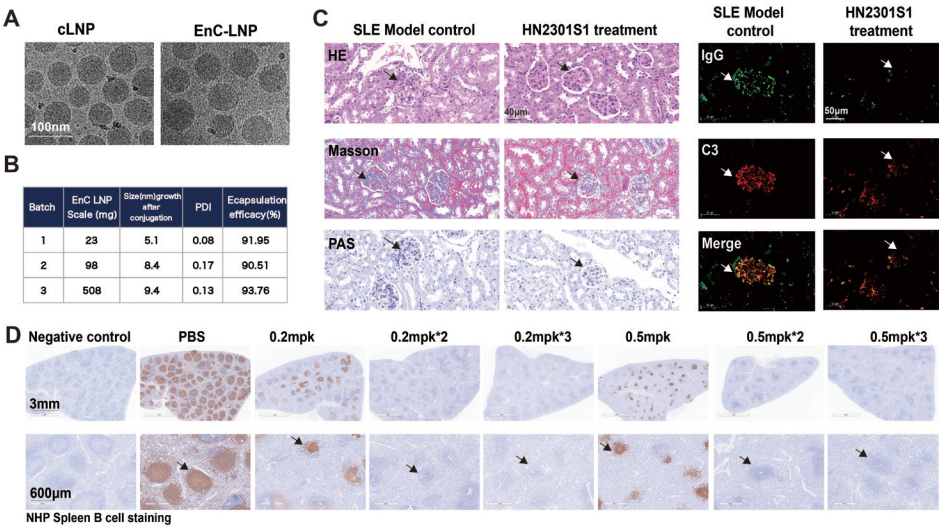
Background: Autologous chimeric antigen receptor (CAR) T cell therapies demonstrated clinical efficacy in various autoimmune diseases through the “B cell reset”. But the challenges of CAR T therapy involved in manufacturing costs, including time and economy, expandability, risk of the integrating viral vectors, and the process of lymphodepleting chemotherapy, highlight the necessity for an “off the shelf” substitution CAR T therapeutic. To this aim, we developed an in vivo CD19 CAR T producer, HN2301. It is an engineered CD8 T cell lipid nanoparticle (EnC-LNP) encapsulated with CD19 CAR mRNA. HN2301 reprogrammed CD19 CAR T cells in vivo and depleted the B cells, inducing a “B cell reset” to cure the B cell-involved autoimmune diseases. We tested the activity of HN2301 to eradicate B cells from systemic lupus erythematosus (SLE) patients and healthy adults. Then we explored the murine cross-reactive CD19 CAR surrogate 1 (HN2301S1) in a SLE murine model to test the therapeutic effect of HN2301 on lupus and evaluated the “B cell reset” by a NHP cross-reactive CD20 CAR surrogate 2 (HN2301S2) in non-human primates (NHPs).

Methods: To test the activity of HN2301, peripheral blood mononuclear cells (PBMC) from patients with SLE and healthy control adults (HC) were treated with HN2301, and CAR T cell reprogram efficiency and B cell depletion efficiency were evaluated for the function of HN2301 invitro. Therapeutic effects were testified in a SLE murine model that utilized HN2301S1. HN2301S2 was used for the CAR T cell reprogramming, “B cell reset” verification, and safety evaluation in Cynomolgus macaques.

Results: HN2301 holds the regular morphology under an electron microscope and has almost the same characteristics as conventional LNP. The production of HN2301 is controllable and repeatable, and the scalable production of HN2301 reached 500 mg for now. HN2301 efficiently and specifically engineered the CAR T cells in vitro and eliminated the autologous B cells of the HC and SLE patients. HN2301S1 is a surrogate of human CD8 EnC-LNP that carried anti-mouse CD19 CAR mRNA, successfully reprogrammed the CAR T cells and induced “B cell reset” in CD8 humanized SLE model mice, and significantly decreased the level of anti-dsDNA antibody, ameliorating the lupus nephritis manifestations of the SLE model mice. HN2301S2 efficiently enabled the CD20 CAR T cells reprogrammed in cynomolgus macaques in a dose-dependent manner (0.05-1 mpk), inducing profound B cell depletion in peripheral blood (PB). In addition, a low dosage of 0.2 mpk*3 doses induced B cell elimination in the spleen, bone marrow, and lymph nodes of NHP. B cells recovered in PB within 10 days after the HN2301S2 administration, and the repopulated B cells were predominantly naïve (CD27-), demonstrating the “B cells reset” after the administration of HN2301. In addition, HN2301S2 demonstrated excellent safety and tolerability in NHPs.

Conclusion: The vivo CAR T producer HN2301 is a safe "off-the-shelf" product that reprogrammed the CAR T cells in vivo, eliminated the B cells quickly in different tissues, induced the “B cell reset” in NHPs, and performed excellent therapeutic efficacy on SLE mice. HN2301 holds the controllable, repeatable, and scalable production. HN2301 brings limitless potential for autoimmune diseases involved by the autoantibody produced by auto-reactive B cells and enables the autoimmune diseases patients to achieve a durable, drug-free remission.



Batch	EnC LNP Scale (mg)	Size(nm)growth after conjugation	PDI	Ecapsulation efficacy(%)
1	23	5.1	0.08	91.95
2	98	8.4	0.17	90.51
3	508	9.4	0.13	93.76

Figure. A. EnC-LNP morphology under electron microscope. **B.** Scalable production of HN2301 in different batch. **C.** HE, Masson, and periodic acid-Schiff (PAS) staining and the deposition of IgG and C3 in renal sections from SLE mice after the HN2301S1 treatment. **D.** B cell staining of the spleen from the NHP after the different dosages of HN2301S2.

795 Preclinical Development of UB-VV400, a Surface Engineered Lentiviral Vector for the In Vivo Generation of CD22-Directed CAR T Cells

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BACKGROUND: Autologous CD19 CAR T therapies have transformed treatment paradigms for B-cell malignancies (BCMs); however, many challenges remain that limit patient access to these therapies, including operational and logistical complexity, and high manufacturing and distribution costs. Furthermore, there is a need for alternative treatment strategies for patients who are refractory or relapse following CD19 CAR T therapy. CD22 is a B-cell specific antigen expressed on most BCMs and autoreactive B cells and is a validated target for CD22-directed CAR T products. Umoja is developing UB-VV400, an off-the-shelf, multidomain fusion (MDF) protein surface engineered lentiviral vector (VivoVec^{TM}) designed to generate fully human anti-CD22 CAR T cells *in vivo* without the need for lymphodepletion. In addition to the anti-CD22 CAR, UB-VV400 encodes the rapamycin-activated cytokine receptor (RACR^{TM}) system as part of the payload delivered to T cells. The RACR system enables rapamycin-activated enrichment and expansion of CAR T cells *in vivo* through the delivery of IL-2/IL-15-like signaling to transduced cells. Here, we describe preclinical studies demonstrating that UB-VV400 ± rapamycin is well-tolerated and generates anti-CD22 CAR T cells *in vivo* that deplete CD22-expressing B cells and tumor cells.

METHODS: UB-VV400 lots were manufactured in a clinical scale suspension process at Umoja's manufacturing facility (Louisville, CO). In vitro studies with UB-VV400 used PBMCs from healthy donors and patients with hematologic malignancies or autoimmune disease. Functional assays were performed by co-culturing UB-VV400-generated CAR T cells with target cell lines. In vivo studies were performed in PBMC-humanized NSG MHC-I/II DKO mice bearing systemic tumor and in non-tumor bearing CD34-humanized NSG mice, which contain CD22+ human B cells.

RESULTS: In vitro culture of PBMCs with UB-VV400 demonstrated MDF surface engineering mediated selective binding and activation of T cells and subsequent transduction of T cells resulting in expression of RACR and the anti-CD22 CAR. UB-VV400 generated CAR T cells mediated antigen-specific tumor cell killing and cytokine secretion in response to CD22^{hi} (Raji) and CD22^{lo} (Nalm6) cells, but not CD22-negative cells (K562, Raji-CD22^{KO}). In PBMC-humanized NSG MHC-I/II DKO mice bearing systemic tumor, UB-VV400 generated CAR T cells that mediated anti-tumor activity. In non-tumor bearing CD34-humanized NSG mice, UB-VV400 generated CAR T cells that resulted in B-cell depletion in the circulation, spleen, and bone marrow. Combination treatment of UB-VV400

with rapamycin further enriched and expanded CAR T cells *in vivo* in both humanized mouse models and corresponded with complete target cell eradication and prolonged survival in tumor-bearing mice. UB-VV400 ± rapamycin was well tolerated in both mouse models.

CONCLUSIONS: UB-VV400 demonstrated specific and efficient transduction of T cells, which corresponded with the generation of CD22-directed CAR T cells capable of killing both $CD22^{hi}$ and $CD22^{lo}$ tumor cells *in vitro* and *in vivo*. Moreover, the administration of rapamycin promoted RACR-mediated enrichment and expansion of CAR T cells *in vivo* resulting in complete tumor clearance and B-cell depletion. Overall, nonclinical studies of UB-VV400 ± rapamycin demonstrated a favorable safety profile, with no associated adverse events. These data support active, ongoing clinical studies in R/R LBCL and autoimmune disease.

796 Development of In Vivo CAR T Cell Therapy Using Nanoparticle and RNA-Based Gene Delivery Systems

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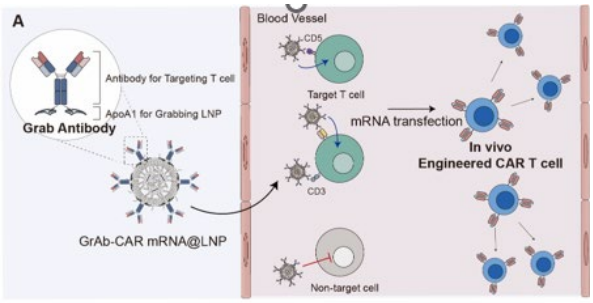
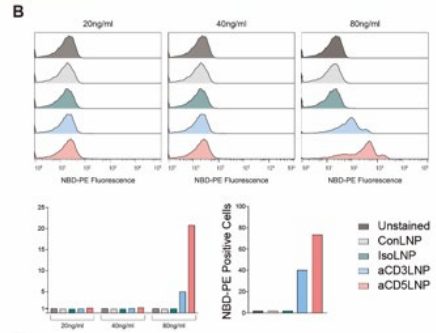
Introduction: CAR-T cell therapy has demonstrated groundbreaking efficacy in relapsed or refractory malignant hematologic tumors, such as leukemia and lymphoma. Currently, most CAR-T cell therapies require a complex ex vivo manufacturing process, involving T cell extraction, activation, genetic modification, expansion, and purification before administration to the patient. This process is time-consuming and costly, limiting its feasibility for rapid, patient-specific treatment and broad clinical application. To overcome these challenges, an alternative strategy is to directly deliver CAR genes into immune cells, eliminating the need for ex vivo manipulation. Among various gene delivery systems, mRNA@LNP has emerged as a promising alternative due to its advantages, including ease of large-scale production, high delivery efficiency and flexibility, excellent safety. However, since LNPs can be absorbed by various cell types indiscriminately, non-specific mRNA delivery has been reported to cause significant side effects. Although antibody-mediated active targeting can address this issue, existing chemical conjugation strategies typically involve multi-step reactions, toxic reagents, and PEG detachment problems, highlighting the need for simpler and more stable alternatives.
To this end, we developed a chemical-free platform by fusing apolipoprotein A1 (ApoA1) directly to an antibody, termed “Grab Antibody” (GrAb). Due to the strong lipid-binding affinity of ApoA1, GrAb spontaneously assembles into a stable protein corona on the LNP surface, ensuring proper antibody orientation while maintaining stability. In this study, we applied the GrAb approach by fusing ApoA1 to the Fc domain of a CD3 antibody (Foralumab) or a CD5 antibody (5D7). CD3 and CD5 are highly expressed on T cells, enabling receptor-mediated delivery of mRNA to this cell population. We demonstrate that CD3- or CD5-targeted GrAb-LNPs efficiently deliver CAR mRNA to T cells, leading to a strong antitumor effect.

Results: We generated modified nucleoside-containing mRNA encoding a CAR targeting CD19 (a marker of B cells) and encapsulated it in CD3- or CD5-targeted GrAb-LNPs (hereafter referred to as aCD3LNP or aCD5LNP, collectively T-LNP) (Fig. 1A). As a proof-of-concept experiment, we incubated NBD-PE-labeled T-LNPs with freshly isolated human Peripheral Blood Mononuclear Cells (hPBMCs) in vitro. T-LNPs exhibited selective binding to lymphocytes, resulting in an increased NBD-PE fluorescence signal in this population. In contrast, LNPs without antibody coating (ConLNP) and isotype control antibody-coated LNPs (IsoLNP, non-targeting LNPs) did not show this increase (Fig. 1B). Next, we incubated T-LNPs containing modified mRNA encoding Green Fluorescent Protein (GFP) with hPBMCs in vitro. T-LNPs successfully delivered mRNA to most lymphocytes, with 50.2% of cells expressing GFP following aCD3LNP-GFP treatment and 21.9% following aCD5LNP-GFP treatment (Fig. 1C). In in vitro experiments, GFP protein expression peaked at 12 hours and declined over 24 and 48 hours. Furthermore, GFP expression increased in a dose-dependent manner (Fig. 1D, E). Finally, we generated T-LNPs encapsulating CAR-encoding modified mRNA. After co-culturing PBMCs with luciferase-expressing NALM-6 cells (a CD19-expressing cancer cell line), we delivered aCD3LNP-CAR or aCD5LNP-CAR. These LNP-generated CAR-T cells effectively eliminated CD19-expressing target cells in vitro (Fig. 1F).


A
Blood Vessel
CD5
Antibody for Targeting T cell
ApoA1 for Grabbing LNP
Grab Antibody
Target T cell
mRNA transfection
In vivo
Engineered CAR T cell
CD3
GrAb-CAR mRNA@LNP
Non-target cell


B
20ng/ml
40ng/ml
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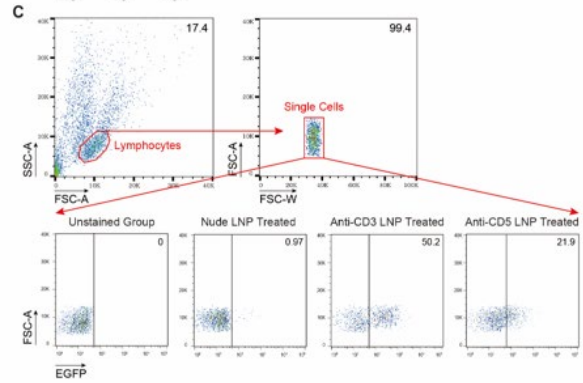
C
17.4
99.4
Single Cells
Lymphocytes
SSC-A
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FSC-W
Unstained Group
Nude LNP Treated
Anti-CD3 LNP Treated
Anti-CD5 LNP Treated
0
0.97
50.2
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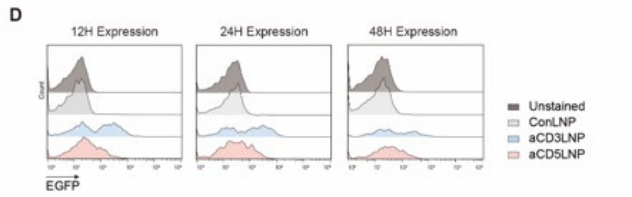
EGFP


D
12H Expression
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ConLNP
aCD3LNP
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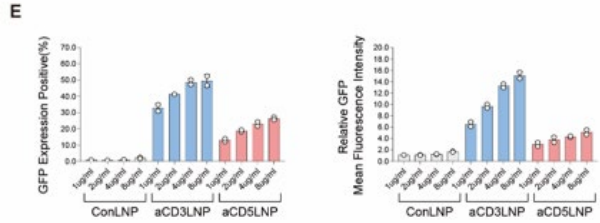
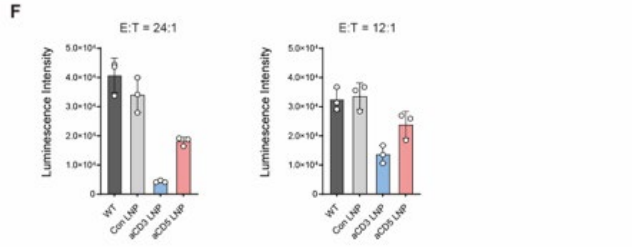

E
GFP Expression Positive(%)
Relative GFP
Mean Fluorescence Intensity
1ug/ml
2ug/ml
4ug/ml
8ug/ml
ConLNP
aCD3LNP
aCD5LNP


F
E:T = 24:1
E:T = 12:1
Luminescence Intensity
WT
Con LNP
aCD3 LNP
aCD5 LNP

797 Efficient and Effective *in Vivo* CAR T Generation Using Recombinase-Based, Precise, High-Capacity, Off-the-Shelf, LNP-Based *In Vivo* Genomic Engineering

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Engineered cell therapies are revolutionizing medicine. However, problems of high cost and high complexity associated with *ex vivo* manufacturing are restricting access by patients and threatening the commercial viability of these life-saving therapies. *In vivo* cell engineering with off-the-shelf medicines offers the solution.

Herein, we describe an elegant, efficient, and low-cost approach to *in vivo* CAR T therapy using recombinase-mediated, site-specific genomic insertion of high-capacity payloads by non-viral, targeted lipid nanoparticle (LNP) delivery. This strategy avoids fundamental obstacles to potency, safety and accessibility including viral vector immunogenicity, random genomic insertion, payload limitations and transient expression.

CAR T cell engineering using a built-for-purpose large serine recombinase targeted to a safe harbor in the human genome was first analyzed *in vitro*. Using transfection of a mRNA-encoded recombinase and a CAR-encoding DNA donor, we observed precise, efficient, and reproducible payload integration. Cell-surface analysis of engineered T cells revealed uniform and high CAR expression - consistent with the recombinase's defined insertion profile. Functional analysis of recombinase-engineered CAR T cells *in vitro* and *in vivo* demonstrated potent anti-tumor cytotoxicity, polyfunctional cytokine production, and stable long-term CAR expression.

Recombinase-mediated *in vivo* CAR-T generation using targeted-LNP and subsequent anti-tumor activity was next evaluated in a NSG mouse model engrafted with Nalm6-luciferase tumor cells. Following transfer of human PBMCs, mice were treated with a single intravenous dose of CD3-targeted LNP co-encapsulating an mRNA-encoded recombinase and a DNA donor encoding a CD19-specific CAR (or appropriate LNP controls). Subsequent analysis of peripheral blood revealed cell surface CAR-expression (FACS) on T cells and genomic integration (ddPCR) of the CAR construct indicating successful *in vivo* CAR T generation. IVIS-based tumor analysis demonstrated potent and sustained anti-tumor responses consistent with efficient *in vivo* CAR T generation and stable long-term CAR expression.

In summary, we have developed a recombinase-based platform capable of precise, polygenic, off-the-shelf, LNP-based *in vivo* cell engineering. We demonstrate efficient and effective *in vivo* generation of CAR T cells using this approach. This work lays the foundation for genomic medicines that are differentiated, effective, safe and broadly accessible.

798 Development of a Scalable HEK293/HEK293T Suspension Cell Line Platform for Efficient Viral Vector Production

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Introduction: HEK293/HEK293T cell lines are widely used in viral vector production processes. However, their tendency to form aggregates during scale-up or high-density culture poses challenges in suspension process development compared to CHO cell lines. This study presents a scalable, regulatory-compliant cell line platform for viral vector production using the MatiMax HEK293T suspension cell line. It addresses the challenges through experimental screening by DOE and statistical analysis, without any genetic modifications or engineering of the cells.

Methods: The cell line was generated through serum-free, suspension adaptation from an adherent cell line. The effects of throw diameter, agitation speed, media composition, media supplements, and different flask types were tested to identify the optimal condition for HEK293T suspension cell line. The effects of different throw diameters for agitation were evaluated and compared in terms of P/V ratio. Cell density, viability, cell size distribution, cell aggregates, and cell cluster formation were assessed using an automatic cell counter (Vi-cell, Nucleocounter), flow cytometry, and microscopy. Productivity was evaluated through the lentiviral vector production process.

Results: Agitation conditions were found to play a critical role in HEK293T cell aggregate formation and aggregate size during culture in Erlenmeyer flasks. The experimental results demonstrate that conditions which reduced the aggregate formation and allow only smaller-sized aggregates not only enhanced viability but also led to more stable and robust process data. Approaches involving supplementation or physical modifications, such as changes in flask type, were effective in reducing aggregate formation. However, due to potential risks on productivity and viability, these conditions were not incorporated into the final setup.

Conclusions: This study presents a cell line platform that can be used to develop processes for viral vector production, which is essential for gene therapy product manufacturing. We successfully identified the optimal high-density culture conditions for cell expansion in Erlenmeyer flasks that minimize aggregate formation. This platform not only provides valuable insights into overcoming challenges associated with HEK293T cell culture in viral vector production but also offers potential as a host cell line for advanced technologies, such as stable packaging cell lines or stable producer cell lines.

799 Breaking the Bottleneck in Lentiviral Production: Achieving Ultra-Low Infectivity-to-Particle Ratios and Cost Efficiency for Transformative Gene Therapies

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Lentiviral vectors (LV) are increasingly favored for both *in vivo* and *ex vivo* gene therapy due to their distinct advantages over other viral vectors such as adeno-associated virus (AAV). These advantages include a larger genetic cargo capacity, efficient transduction of both dividing and non-dividing cells, stable integration into the host genome for long-term expression, and relatively low immunogenicity. As a result, lentiviruses have emerged as a powerful platform for delivering therapeutic genes across various clinical applications.

We employed a Design of Experiments (DoE) approach, integrated with advanced high-throughput process technologies and state-of-the-art analytical characterization tools, to optimize the upstream production of lentiviral vectors. This multi-pronged strategy enabled us to systematically assess critical process parameters and refine manufacturing conditions for enhanced LV yield and quality.

In this work, we present an optimized approach to produce LV encoding the CD19 gene of interest, benchmarked against a green fluorescent protein (GFP) control. Our process achieved high upstream LV titers while consistently maintaining an infectivity-to-particle ratio below 1:1000. Moreover, we observed a notable reduction in manufacturing costs without compromising infectivity, highlighting the potential of this platform to streamline LV production for both clinical and research applications. This optimized approach for producing lentiviral vectors encoding the CD19 gene of interest demonstrates a key advance in establishing robust, scalable, and cost-effective manufacturing processes. By consistently achieving high upstream LV titers with infectivity-to-particle ratios below 1:1000, our method addresses one of the critical barriers in lentiviral vector production—ensuring product potency while controlling costs. These findings underscore the promise of this platform for accelerating clinical and research applications, and the potential to extend these optimization strategies to other gene targets and therapeutic indications.

800 Systematic Assessment and Scalable Bioreactor Process Development of Lentiviral and Gamma-Retroviral Vectors for CAR-T and TIL Therapies

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[1]CTMC, Houston, TX, [2]CTMC, Houston

Genetically modified immune cell therapies such as CAR-T and TIL have shown great promise in treatment of hematological and solid tumors. Lentiviral vectors (LVV) and gamma-retroviral vectors (RVV) are mainstay platforms for genetic modification in CAR-T and TIL manufacturing processes, however, cost and timeline associated with viral vector manufacturing often presents a significant hurdle to new therapies during early and late stages of development.

CTMC, a joint venture between National Resilience and MD Anderson Cancer Center, is uniquely positioned to address the unmet need of viral vector supply chain in CAR-T and TIL product development. CTMC has taken the two-pronged approach of 1) from a technical perspective, identifying and prioritizing the development of viral vector platforms most suitable for CAR-T and TIL processes with the greatest benefit in cost and scalability dependent on the phase of clinical development; and 2) from an organizational perspective, reducing white space in the product development timeline through robust platform building and vertical integration of development activities.

Here we present a systematic assessment of LVV and RVV performance attributes in T cell processes, and evaluation of RVV manufacturing in multiple bioreactor platforms. Using in-house representative constructs, we generate like-for-like LVV and RVV with multiple envelopes and compare them in T cell transduction. Vector- and envelope-specific differences are observed in transduction efficiency and expression pattern, highlighting differences in performance attributes and informing selection of suitable vector system for specific applications. Furthermore, we evaluate a stable cell line-based RVV process in manual and bioreactor production platforms including hollow fiber and fixed-bed bioreactors and show that such a process is highly scalable with maintenance of productivity over multiple harvests. We compare attributes of different bioreactor platforms tested and show that a manual RVV process can readily transition to a suitable bioreactor platform with comparable or superior unit and total batch yield. Bioreactor platforms drastically reduce hands-on time and operational complexity, enabling the scaling of stable cell line-based RVV processes through product development stages. Through this work, we aim to reduce the viral vector supply chain bottleneck, accelerate development timeline, and expand accessibility of innovative cell therapies.

801 From bench to dose: building a robust and scalable cGMP manufacturing process for non-replicative Herpes Simplex Virus 1 (nrHSV-1) vector as a powerful gene therapy platform

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Viral vectors have become one of the leading delivery systems for genetic medicine in humans. The specific combination of their key properties offers several advantages tailored to a broad spectrum of gene therapies for both acute and chronic diseases (K. Lundstrom, 2023). nrHSV-1 vectors are attractive for gene therapy mainly due to the large HSV genome (152 kb) which can accommodate therapeutic transgenes larger than 30 kb in size. Moreover, they are particularly useful in nervous system (NS) gene therapy applications due to their innate neural tropism and ability to establish latent infections with prolonged transgene expression (D. R. Knop and H. Harrel, 2007). Many of these indications address large patient populations necessitating efficient, large-scale production to reach scale and lower COGS per dose. Thus, developing a highly productive, scalable and completely closed production process represents a critical requirement to manufacture the vector under cGMP conditions to cover the clinical demands. This study describes the development of a fully scalable production process for a nrHSV-1 viral vector using the Fixed-bed Scale-X technology from the bench up to the Scale X Carbo 30 m^2, using a derivative clone from an adherent Vero cell line. The upstream and downstream process parameters were initially investigated by small-scale studies using a Cell Stack system, then the optimal parameters were adapted and transferred to the Scale-X Hydro

2.4m^2. A number of optimization runs were executed to increase productivity and maximize the yield and clearance of the final process. By infecting the cells at a density of >1.2E+05 cell/cm^2, a productivity comparable to the values obtained in Cell Stack systems was achieved. In terms of downstream, the release of the virus from infected cells was coupled to an endonuclease digestion in recirculation mode inside the bioreactor, ensuring an efficient system to recover the intracellular virus, while reducing the host cell DNA released from the cells. Furthermore, a 0.45 µm clarification step was introduced to reduce cell debris and aggregated impurities, while the TFF with hollow fiber was carried out to formulate the final DS. Our modular purification platform ensured optimal overall yield (≥50% in terms of PFU/mL) and good impurities clearance (up to 785-fold HCP reduction and HCDNA amount ≤10 ng/dose) in final product. To prove the full scalability of the process, additional runs were executed on both Scale-X Carbo 10m^2 and Carbo 30m^2 bioreactors, as demonstration and engineering runs respectively, to mimic the final process implemented during GMP manufacturing. The process parameters were linearly scaled-up based on the higher fixed bed surface, while the same seeding and infection cell densities were applied. Although a higher fixed bed scale was used, both vector-specific productivity (pfu/cm^2) and process yield (~ 50%) were achieved. The manufacturing of the clinical lot was successfully executed, proving the development of a robust and scalable platform fulfilling clinical dose expectations and satisfying regulatory requirements for the release of the drug product.

802 Implementation of an Institutional Gene Therapy Safety Committee at the University of Utah

Julie Porter[1], Sarah Moldt[2], Brian Shayota[2], Russell Butterfield[2]
[1]University of Utah Salt Lake City, UT, Salt Lake City, UT, [2]University of Utah, Salt Lake City, UT

Gene therapy has rapidly expanded with hundreds of active clinical trials and 14 FDA-approved non-oncologic therapies. Safety events, including death, are a significant consideration with gene therapy products and without proper prevention and management can limit their use and benefit. Management of systemically delivered AAV gene therapies, including management of adverse events, crosses established medical disciplines. Many of these adverse events are immunologically mediated and a better understanding of immunology, the effects on various organ systems, and appropriate interventions is critical for providers administering gene therapy. Thus, a multidisciplinary framework is needed to review, monitor, and mitigate risks associated with gene therapy treatments.

At the University of Utah, we recently initiated an institutional Gene Therapy Safety Committee (GTSC) to (1) build a base of knowledge and experience among providers regarding care for patients and research subjects receiving gene therapy, (2) create an established forum for individuals to discuss gene therapy, including in-depth review of adverse events , and (3) develop a set of institutional guidelines and practices for implementation of systemically delivered gene therapies including standards for pre-treatment evaluation, treatment, and post-treatment surveillance and management. The GTSC integrates expertise from diverse medical specialties including Neurology, Genetics, Gastroenterology/ Hepatology, Nephrology, Hematology/ Oncology, Cardiology, Dermatology, and Ophthalmology. Clinical research coordinators and nurses are invited to participate, ensuring a comprehensive evaluation of complex patient cases. The GTSC meets bi-monthly to discuss internal cases as well as those from the literature. Previous and planned meeting topics typically relate to a particular adverse event type, such as acute liver injury, thrombotic microangiopathy, or acute cardiac injury; or management strategies such as immune modulation beyond steroids. Additional resources of include a listserv, which members can use to ask questions or get advice outside of regular meetings, and a literature archive containing relevant articles relating to gene therapy safety. The GTSC is also available to help review potential clinical trial opportunities as our institutional confidentiality agreements allow. Early outcomes demonstrate the GTSC's effectiveness in reducing adverse event severity, improving interdisciplinary communication, and enhancing the ethical and scientific rigor of gene therapy treatment. We likewise anticipate that further development of the Gene Therapy Safety Committee will allow for expanded implementation of both FDA- approved and clinical trial gene therapies as physicians and primary investigators feel more empowered and supported in managing potential safety concerns, ultimately leading to more opportunities for patients.

803 Case Study: Resilience's SnapLVV™ Program from Bench Scale to GMP (USP Process and Lessons Learned)

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Lentiviral vector production is a highly sensitive gene delivery system. The execution of bench-scale bioreactors, scale-up runs, and the technology transfer for GMP manufacturing of a 4-plasmid transient transfection system can be difficult without proper procedures and templates in place. This case study exemplifies the lentiviral vector platform process performance from 2L to 50L, while also including the at-scale technology transfer to a GMP manufacturing facility. In six months, we utilized scale-up methods and templates for all process parameters, including but not limited to, gassing strategy, agitation, PID tuning, media warming procedure, and cell counting. Executing the process at a smaller scale allows for identifying potential issues before the commitment to large-scale manufacturing. With the benefit of lesser consumption of process material, there is also faster process optimization, and higher confidence in at-scale operations, all while maintaining viral vector yield and titer. Following successful process evaluation, an at-scale demonstration run was executed while the technology transfer was ongoing between the development team and the manufacturing counterparts. The result was the maintenance or increase in the recovery yield across scales. We will highlight lessons learned from this program, upstream processes, execution, and templates from thaw to harvest.

804 Development of Cell-Based Functional Assays to Predict CAR-T Potency and Persistence

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Introduction: Cytokine secretion assays are routinely deployed to predict the effectiveness of adoptive cell therapy products. Cytokine secretion occurs when effector cells bind to target tumor cells, with secreted factors such as IFNg, TNFa, and IL-2 assessed to determine the 'potency' of manufactured CAR-T cells. However, secretion assays lack direct functional information on the status of the target cell (cytolysis, viability), relying solely on the activation of status of the effector cells to determine its effectiveness. Furthermore, secretion assays are limited in their ability to predict the 'persistence' of the adoptive cell therapy product, a critical factor for determining long-term in vivo effectiveness in patients. To overcome these limitations, we have developed a set of functional and phenotypic assays that can be deployed in all phases of development to accurately predict the potency and persistence of both autologous and allogenic adoptive cell therapy products.

Methods: Utilizing the AVATAR platform (Xcellbio), effector and target cell populations (CD19 CAR-T, Nalm6) were screened under hypoxic and hyperbaric culture conditions to mimic the in vivo tumor microenvironment. Oxygen and pressure levels of 1% O2 and 2.0 PSI were used for screening conditions, along with effector to target ratios (E:T) that ranged from 1:4 to 1:32. Serial tumor-restimulation was performed up to 5 challenges to measure the persistent killing capacity of CD19 CAR-T cells. Functional assays were paired with immunophenotyping to determine populations of central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-), naïve/stem cell memory (CD45RA+CCR7+) and terminally differentiated effector memory cells (CD45RA+CCR7-).

Results: CD19 CAR-T cells manufactured with the AVATAR platform exhibited improved tumor killing potency compared to conventionally manufactured cells (CO2 incubator, control), particularly at E:T ratios below 1:4. AVATAR expanded CD19 CAR-T cells also maintained high levels of killing at fourth and fifth tumor challenges, whereas conventionally expanded cells exhibited consecutive 50% decreases in tumor killing capacity across the same set of tumor challenges. Further analysis of the AVATAR expanded CD19 CAR-T cells showed two-fold higher percentages of naïve/stem cell memory and terminally differentiated effector memory cells, whereas both central memory and effector memory were decreased when compared to conventionally manufactured cells.

Conclusions: In this study, we have shown that cell-based functional assays can be used to measure the killing persistence of an adoptive cell therapy product and can be complemented by immunophenotyping efforts to improve manufacturing strategies to produce cell therapies with long-term persistence in patients.

805 Point of Care Automated Manufacturing Enables Rapid Translation of Both Novel TCR-T Cell Therapies as well as Conventional CAR T Cell Therapies into the Clinic

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[1]Miltenyi Biotec BV & Co KG, Köln, Nordrhein-Westfalen, Germany, [2]Leiden University Medical Center, Leiden, Netherlands, [3]Miltenyi Biotec BV & Co KG, GERMANY, [4]Bergisch-Gladbach, Germany, [5]Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Nordrhein-Westfalen, Germany

Although cell and gene therapies are showing impressive efficacy, a number of serious barriers to widespread patient access remain, including manufacturing logistics, manufacturing upscaling and the cost of the therapies themselves. One possible solution to reduce costs and improve patient access is to manufacture using a local point of care (PoC) model.

Using closed, GMP-compliant, automated manufacturing systems, it is possible to standardize ATMP manufacturing procedures to greatly accelerate and simplify the translation of novel therapies into the clinic. To illustrate this, 2 different manufacturing protocols for generating TCR-modified T cells were established on the CliniMACS Prodigy® at a PoC clinical manufacturing site in the Netherlands. The first of these was a T cell Transduction large-scale (TCT-LS) 8-day protocol to generate T cells modified with a lentiviral vector encoding an HLA-A*02:01 restricted recombinant ΔNPM1 neoantigen-specific TCR to be used in a clinical trial targeting relapsed or refractory AML. The second was a standard scale (TCT) 6-day shortened protocol to enable expression of an HLA-B*07:02 restricted recombinant BOB1-specific TCR, which will be used in a trial for treatment of relapsed or refractory B-cell malignancies. BOB1 is a B-cell lineage-specific transcription coactivator shown in studies to be essential for survival of certain B cell malignancies and therefore provides an attractive novel target.

Method transfer and process establishment was simplified due to a high level of automation. During process validation runs using healthy donor material, a suitable yield of TCR-modified CD8+ T cells for clinical application was reached: 5.6E9 ± 1E9 ΔNPM1.TCR+ CD8+ T cells (n=4) and 9.4E8 ± 3.1E8 BOB1.TCR+CD8+ T cells (n=3). As all processes are performed at the clinical site and a fresh product is infused into the patient, a short vein-to-vein time is readily achievable. The ΔNPM1-TCR trial is currently recruiting patients in the Netherlands (NCT06424340) while the BOB1-TCR trial is in preparation.

A complementary strategy will be discussed which is focused less on novel therapies but on more established approaches using CAR T cells. A concept has recently been implemented to supply “blueprints” for manufacturing and quality control along with GMP-quality reagents, consumables, GMP lentiviral vector encoding a selection of clinically-relevant CAR constructs as well as regulatory documentation packages to academic clinical centers. This approach has enabled rapid establishment of PoC manufacturing either for early-phase CAR T cell clinical trials or for treatment of patients under hospital exemption. First clinical results have been reported by investigators in several countries including India and Thailand. These examples demonstrate how the use of functionally-closed, automated technologies along with robust manufacturing and quality control procedures can greatly simplify the establishment of manufacturing ATMPs in a PoC setting. It has the potential to foster innovation, improve patient access to life-saving therapies in areas where treatment is not available while giving the treating physician more control of the patient treatment and therapeutic scheduling.

806 Expansion and Characterization of Natural Killer Cells in a Closed Single-Use System

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Nature Killer (NK) cells are innate immune cells that belong to the same family as T and B cells. They account for 5-10% of peripheral blood mononuclear cells (PBMCs). Their role is to control tumor growth as well as microbial infections. NK cells are cytotoxic and do not require prior antigen exposure to mediate their anti-tumor effects, making them a cell of choice for both autologous and allogeneic immunotherapies. NK cell therapies require a large number of cells per infusion (10^7-10^8 cells). The establishment of a robust, reproducible protocol for *in vitro* expansion of NK cells from PBMCs, without the need for feeder cells or serum supplementation, will have significant implications for the number of cancer patients who will potentially benefit from this therapeutic option.

In this study, we present a method to ensure robust NK cell expansion in a closed single-use system, the VueLife® cell culture bags from Saint-Gobain. VueLife® bags are highly permeable to oxygen and carbon dioxide, while being impermeable to water. VueLife® C Series bags are designed for culture and expansion of suspension cells, including NK cells. Cells were cultured in serum-containing and serum-free media, following isolation from PBMCs. There were no significant differences between the two growth conditions in terms of fold increase over a 14-day expansion (70-80-fold). However, cells expanded in serum-free conditions had a lower overall viability (75% vs. 90% for serum-containing media). In addition to expansion from freshly isolated cells, we also performed a successful NK cell expansion from a cryopreserved stock, with similar fold increase and viability to cells freshly isolated from PBMCs.

Finally, we performed an extensive flow cytometry analysis of cell surface markers. In all examined conditions, cytotoxic NK cells were expanded ($CD56^{bright}$ $CD16^{+}$). The activation marker NKG2D was consistently expressed, with the largest population recorded in the frozen NK cells. Exhaustion markers (PD-1 and TIGIT) remained at low levels in all conditions. Functional markers Granzyme B and Perforin showed the highest level of variability, with the largest population recorded in the NK cells expanded from the cryopreserved stocks. Using the current protocol for a 14-day expansion, this set of experiments show that VueLife C bags can support the ex-vivo expansion of functional NK cells with little to no effect on their viability and exhaustion markers.

807 G-Rex (Gas Permeable Rapid Expansion) Technology used for T regulatory Cell Culture

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Introduction: Finding robust methods to culture T regulatory (Treg) cells can be challenging when looking to develop a clinical scale cell and gene therapy process. Examples of popular methods used for GMP manufacturing are T-Flasks, gas permeable culture bags, and WAVE bioreactors. These methods allow for gas exchange and nutrients via diffusion. The G-Rex unit tested in our studies shows that the technology of nutrient and gas exchange via convection with the G-Rex technology produces similar or higher expansion fold for Treg cells compared with conventional methods mentioned above.

Methods: In our study, we tested seeding densities ranging from 0.5 x 10^6 – 4 x 10^6 cells /cm^2 with Treg cells across 4 donors. The G-Rex 24 well units were used where each well is 2cm^2. Cells were cultured for 5 days with X-VIVO™ 15 Chemically Defined Medium with gentamicin supplemented with 10% heat inactivated Human male AB serum and 10% L-glutamine, 10% Sodium Pyruvate and 10% non-essential amino acids (NEAA) in the presence of recombinant human IL-2 (500 IU/ml), and stimulated using Dynabeads® Human T-Activator CD3/CD28 at 1:1 Cell:Bead ratio. Cell counts were completed/performed with Nexcelom's Cellometer Auto 2000.

***Results*:** Culture seeded at 0.5 x 10^6 cells /cm^2 had fold expansion of 1.79 with cell viability dropping from 94.6% to 88.6%. Cultures seeded at 1.0 x 10^6 cells /cm^2 had fold expansion ranging from 2.63 to 7.9 while maintaining viability between 93.5% to 98%. Cultures seeded at 2.0 x 10^6 cells /cm^2 had fold expansion ranging from 2.6 to 6.3 while maintaining viability from 93.9% to 95%. Cultures seeded at 4.0 x 10^6 cells /cm^2 had fold expansion of 3.78

while maintaining viability at 92%. When comparing to tissue culture plastic, fold expansion of Tregs is similar ranging from 2.47 to 3.47 with viability ranging from 93.2% to 94.3%.

***Conclusion*:** Across 4 seeding densities that were cultured in the G-Rex 24 Well plate we were able to conclude that it is feasible to scale up T regulatory cell culture using the G-Rex culture technology with a seeding density ranging from 1 x 10^6 cells /cm^2 - 4 x 10^6 cells /cm^2.

808 Development and Translation of PPL-001, an autologous, gene-edited $CD34^+$ HSPC Therapy for Friedreich's Ataxia

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Friedreich's ataxia (FRDA) is a multi-systemic, autosomal recessive, neurodegenerative disorder caused by homozygous GAA repeat expansion mutation in intron 1 of frataxin (*FXN*) gene.This leads to reduced FXN expression, a mitochondrial protein essential for cellular homeostasis. FRDA is characterized by ataxia, areflexia, sensory loss, muscle weakness, and cardiomyopathy, which is the leading cause of mortality.Currently, there are no effective treatments for FRDA. We previously showed complete rescue of neurologic, muscular, and cardiac complications in YG8R FRDA mouse model with a single, systemic infusion of wild-type hematopoietic stem and progenitor cells (HSPCs).This rescue was mediated by FXN transfer from engrafted HSPC-derived microglia/macrophages to neurons/myocytes.We next developed an autologous transplantation therapy using an ex-vivo CRISPR/Cas9 gene-editing strategy that excises GAA expansion in CD34+ HSPCs from patients (PPL-001).Here, we present IND-enabling studies evaluating pharmacology, manufacturing, and safety of PPL-001, aimed at initiating first-in-human HSPC and genome editing clinical trial for FRDA.To evaluate therapeutic efficacy, we generated murine analogous, bone-marrow derived Sca1+ HSPCs (mPPL-001) from YG8s(GAA)>800 mice (YG8JR) carrying human FXN with >800 GAA repeats, using the same gene editing components as PPL-001.Two-month-old myeloablated YG8JR mice were transplanted with either mPPL-001 or mock HSPCs and analyzed 6-7 months post-transplant.With a mean gene editing efficiency of 18.5% in mPPL-001, hematopoietic tissues maintained a substantial proportion of mPPL-001 derived cells: ~7.24% in bone marrow cells, ~8.8% editing in blood, 25.05% in spleen and 16.68% in thymus. mPPL-001-derived cells were also present in FRDA-affected organs, such as the heart(2.78%), spinal cord(1.49%), cerebellum(0.85%), and skeletal muscle(3.33%).Mice transplanted with mPPL-001 exhibited significant preservation of cerebellar architecture and granular cell layer neurons, compared to untreated or mock-transplanted mice. Additionally, cardiac mitochondrial ultrastructure was significantly preserved in mPPL-001 transplanted mice compared to mock.These findings demonstrate that single infusion of mPPL-001 engraft in the bone marrow niche to become a reservoir of healthy cells that can engraft into injured organs for their long-term preservation.We next optimized a robust and reproducible manufacturing process for PPL-001 using G-CSF/Plerixafor mobilized apheresis products from two healthy adult donors (<36 GAA), Cas9 protein, two single guideRNAs (sgRNA) flanking the mutation (UP4, DN4), an electroporation facilitator and Xenon electroporator. CD34+ HSPCs were isolated using the CliniMACS Plus system with >80% viability and >98.75% purity, pre- and post-electroporation.7 days post-electroporation, gene editing efficiency of PPL-001 from the two donors were 19.31% and 22.35%.To evaluate potential off-target effects, comprehensive ONE-Seq analysis of the two sgRNAs identified <5 Tier 1 sites (>1% rate of on-target with functional annotation) and <71 Tier 2 sites (>1% rate of on-target with low functional annotation), confirming low number of potential off-target sites. The nominated off-target sites will be validated using rhAMP-Seq in PPL-001 cells. In summary, these promising results of scale-up feasibility, efficacy and safety support our efforts in clinical translation of CRISPR/Cas9 gene corrected HSPCs for FRDA.

809 Expansion of human T cells with greater quantity and quality via small molecule (CMT-01)

Ling Wu, ChemT Biotechnology, Singapore, Singapore

T cell therapy products require ex vivo modification and expansion to reach the necessary cell numbers and quality before being administered to patients. Traditionally, this process has relied heavily on serum and cytokine-based culture systems, which present challenges such as variability, batch-to-batch inconsistencies, and high costs. As the field transitions toward large-scale allogeneic T cell manufacturing to meet growing demand, selecting the appropriate culture reagents is increasingly critical to achieving robust and reproducible yields. Small molecules have emerged as a promising alternative, offering distinct advantages over traditional reagents, including enhanced stability, scalability, and cost-effectiveness. Despite their potential, small molecules remain underexplored in the context of T cell production, creating an opportunity to innovate and optimize this critical step in T cell therapy development.

Our discovery, CMT-01, exemplifies this potential by small molecules to significantly improve T cell culture outcomes. In preclinical studies, CMT-01 has consistently demonstrated a remarkable 5-10x increase in T cell expansion compared to conventional methods, while preserving key functional characteristics such as a naïve memory phenotype. This is crucial for maintaining long-term efficacy, as it enhances T cell persistence and activity in vivo. Moreover, CMT-01 has been shown to augment the functional capabilities of expanded T cells, resulting in improved therapeutic performance in vivo. These findings position CMT-01 as a transformative solution for next-generation T cell therapy manufacturing, addressing industry challenges and paving the way for more efficient, scalable, and cost-effective production processes.

810 FAST-STEM: A Synthetic Biology Landing-Pad Toolkit for Next Generation Stem Cell Engineering and Regenerative Medicine

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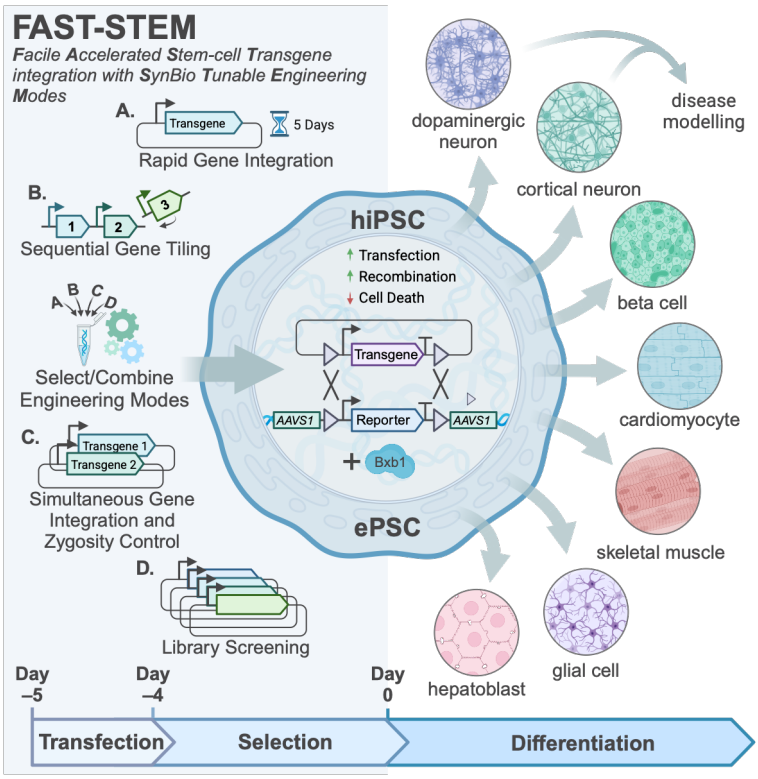
Very recent clinical advancements in human stem cell (hPSC) based tissue replacement have opened a new frontier of possibility: *therapeutically augmented human cell* engraftment. Therapeutic augmentation requires the design of synthetic gene circuits that are compatible with hPSCs. Such design is highly challenging due to: (a) poor translation of circuits developed in immortalized cell lines; (b) absence of effective tools for synthetic human stem cell development. To address this, we developed FAST-STEM (*Facile Accelerated Stem-cell Transgene integration with SynBio Tunable Engineering Modes*), a landing-pad platform that enables *design–build–test–learn* style hPSC engineering. FAST-STEM facilitates fast iterative synthetic cell evolution, zygosity manipulation, and highly controlled library screening. While previous site-specific methods involve laborious multi-step engineering protocols which take weeks to complete prior to expansion, FAST-STEM enables generation of *differentiation ready* engineered stem cells in 5 days. We demonstrate the potential for FAST-STEM to accelerate stem cell synthetic biology by creation of engineered human liver, glial-cells, muscle, cardiomyocytes, pancreatic beta-cells, and cortical neurons in addition to dopaminergic neuron Parkinson's disease models.



811 Feeder-Free Expansion of iPSC-derived NK Cells to Clinical Scale

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Background & Aim: Natural killer (NK) cells play a critical role in immune surveillance, targeting and eliminating transformed cells before they develop into life-threatening cancers. Unlike T cells, NK cells can be used as allogeneic therapies without the risk of graft-versus-host disease. This enables large-scale manufacturing from a single donor for off-the-shelf therapies, potentially offering improved safety compared to T-cell-based treatments. Traditional donor-derived NK cell manufacturing faces challenges, including donor-to-donor variability and reliance on bulk gene delivery methods such as viral vectors, leading to inconsistent production. iPSC-derived NK cells offer a consistent and practically unlimited source material, requiring only a single precise gene-editing step at the master cell bank level. However, current methods using iPSCs often depend on embryoid body-based differentiation and cancerous feeder cells, creating additional complexities for clinical scalability.

Methods: An iPSC-derived NK cell platform was developed to address these challenges by enabling feeder-free expansion at clinical scale. This process uses defined media to expand and bank NK precursor cells at small scale before transitioning to cost-effective media for large-scale expansion. The approach eliminates the need for feeder cells and uses GMP-compatible protocols. NK cell purity and functionality were assessed through the expression of KIR receptors, NK activation markers, and cytotoxic efficacy against K562 cells.

Results: The developed protocol successfully expanded up to several billion NK cells per cm² of seeded iPSCs in a feeder-free process. The resulting NK cells exhibited high purity, robust KIR receptor and activation marker expression, and potent cytotoxic activity against target cells. To our knowledge, this is the first method to achieve the expansion of iPSC-derived NK cells without feeder cells, using a GMP-compatible process, and at scales sufficient for clinical applications.

Conclusions: This feeder-free iPSC-derived NK cell platform offers a consistent, scalable, and GMP-compatible solution to produce NK cells for clinical use. By overcoming the limitations of traditional donor-derived methods and current iPSC-based approaches, the platform supports the development of off-the-shelf NK cell therapies with enhanced safety, reliability, and manufacturing efficiency.

812 Generation of Functional BCMA CAR-iNKT Cells from Clinical-Grade iPSCs via a GMP-Compliant Manufacturing Process with Capacity for Linear Scale-Up

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Induced pluripotent stem cells (iPSCs) have emerged as a consistent cell source for 'off-the-shelf' CAR-T or CAR-NK cell therapies. This study presents the first successful establishment of an iPSC line engineered with a BCMA-targeting CAR, derived from invariant natural killer T (iNKT), and subsequently differentiated into functional iNKT cells suitable for clinical application. We also demonstrate, for the first time, the efficient production of functional CAR-iNKT cells from an iPSC platform with the capacity for linear scale-up to clinical-scale production.

iNKT cells are a rare subset of T lymphocytes with an invariant Vα24-Jα18 TCR (Vα24 TCR) that recognizes glycolipids presented by the monomorphic MHC-like molecule CD1d, which enables allogenic use without inducing graft-versus-host disease. The uniqueness of iNKT cells as a cell therapy platform lies in their dual anti-tumor functionality: direct cytotoxicity via TCR and NK receptors, and indirect tumor suppression through the activation of tumor antigen-specific T cells. The induced endogenous host immune cells are expected to persist within the patient even after the administered allogeneic cells are eventually cleared. We previously reported that iPSC-derived iNKT cells retained these functional properties of native iNKT cells, irrespective of CAR gene editing.

To establish a parental iPSC source for further engineering, we established iNKT cell-derived iPSC banks. iNKT cells were enriched from leukapheresis products obtained from qualified healthy donors and subsequently reprogrammed into iPSCs. The resulting colonies were screened and evaluated based on the pluripotency markers, TCR gene rearrangement, differentiation potential, and genetic integrity. These iPSC lines successfully differentiated into CD34+ hematopoietic progenitor cells (HPCs) and subsequently into iPSC-derived iNKT (ipsNKT) cells, achieving robust proliferation with an expansion capacity of up to 6,000-fold. Successively, we developed a BCMA-targeting CAR-iPSC line. Using CRISPR-Cas12a-based technology, iPSCs were engineered to knock out beta-2-microglobulin (ß2M) and knock in BCMA CAR along with human IL-15. This modification resulted in BCMA CAR expression, IL-15 secretion, and the loss of MHC Class I expression. The edited cells were cloned, screened by PCR, and evaluated for genetic characteristics and differentiation potential. The gene-edited iPSCs retained robust differentiation capability, and the resulting BCMA CAR-ipsNKT cells exhibited high purity (>95% CD3+/Vα24-Jα18 TCR+ cells) with CAR expression reaching up to 99.5%. This established manufacturing process utilized feeder-free culture conditions, with all reagents being xeno-free and GMP-compliant.

BCMA CAR-ipsNKT cells demonstrated potent in vitro cytotoxicity against multiple myeloma cell lines. In a xenograft model, mice treated with BCMA CAR-ipsNKT cells showed significantly prolonged survival compared to those treated with controls or non-CAR-engineered ipsNKT cells.

In summary, we successfully established clinically compliant iNKT cell-derived iPSC lines and a GMP-compliant, scalable manufacturing process for gene editing and large-scale production of CAR-ipsNKT cells. This study highlights the potential of iPSC-derived CAR-iNKT cells as a versatile and scalable platform for allogeneic cell therapies.

813 Formulating recombinant proteins with chemically defined ingredients to replace human-derived proteins for mesenchymal stem cell culture

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[1]Evonik, Singapore, Singapore, Singapore, [2]Evonik Corporation, Singapore, Singapore, [3]Evonik, Singapore, Singapore

Introduction: Xeno-free (XF) media containing human blood-derived proteins is state-of-the-art for manufacturing mesenchymal stem cells (MSC). However, a recent Food and Drug Administration (FDA)'s 2024 draft guidance highlighted the risks of disease transmission and ingredient impurities arising from the use of human derived materials, there is increasing regulatory scrutiny of raw ingredients used in cell therapy manufacturing. To address the unmet need for alternatives to human derived proteins with lower risk profile, we screened, optimized, and evaluated different formulations comprising of recombinant proteins and other chemically defined ingredients.

Methods: An in-house xeno-free (XF) MSC medium formulation was used as a basis to evaluate our recombinant protein formulations. In internal tests, this XF formulation, containing human proteins, performed favorably in comparison to commercial xeno-free media in terms of cell growth and stemness. In a first step, the human proteins were removed from formulation, and combinations of recombinant proteins with other chemically defined ingredients were screened for their ability to re-create the effect of human-derived proetins. The most promising combinations were then used to prepare chemically defined version of the XF media to be tested in small scale experiments. The best performing media were identified and further evaluated using MSC derived from different donors and tissue sources for three passages. The critical quality attributes of cell growth, stemness and senescence were assessed.

Results: In order to understand how to formulate recombinant proteins as alternatives for human proteins in cell media, we first tried to directly change from human-derived proteins with recombinant proteins. This proved inadequate for MSC cell survival and growth (Figure 1). Moving ahead, combinations of recombinant proteins and chemically defined ingredients were designed and tested. Out of the tested formulations, four demonstrated the ability to support MSC growth over four passages in small-scale experiments. The best-performing formulation was subsequently validated in T25 flasks across multiple donors. This recombinant protein based formulation exhibited superior performance in cell growth and stemness maintenance across two donors over four passages, indicating stable efficacy (Figure 2). This formulation was also added to umbilical cord MSCs cultured in commercial low-serum media. This supplementation supported stem cell expansion in later passages (>P6) and significantly increased the production of cellular antioxidant glutathione while reducing the percentage of senescent cells.

Conclusions: The incorporation of additional chemically defined ingredients alongside recombinant proteins successfully replaces human proteins in MSC media. This combination may also be applicable to other cell types as an alternative of human-derived proteins, significantly aiding in addressing regulatory challenges associated with the use of human-derived materials in cell therapy production.

Figure 1. Directly replace human derived protein with recombinant proteins provide inadequate for Mesenchymal cell survival and growth

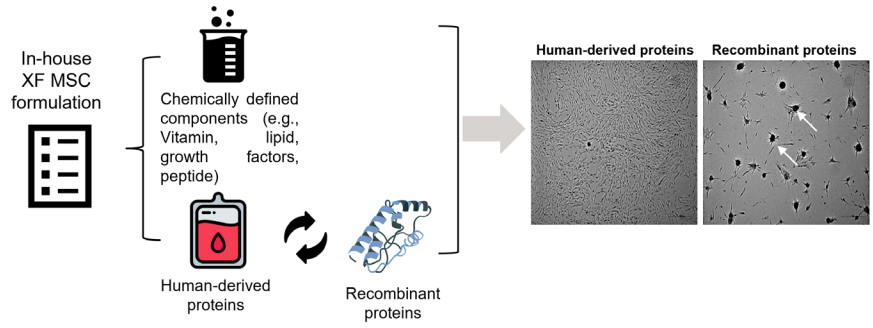
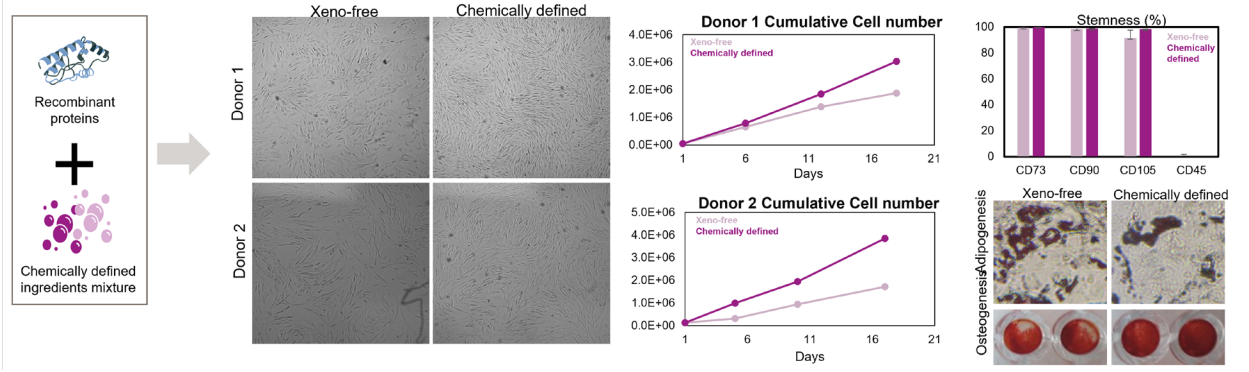


Figure 2. Recombinant protein-based formulation with chemically defined ingredients successfully re-create the effect of human derived proteins and exhibited superior performance in cell growth and stemness maintenance.



814 Development of Tools to Support Coating-Free Adherent Culture of Human Mesenchymal Stromal Cells Under Reduced-Serum and Serum-Free Conditions

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Human mesenchymal stromal cells (hMSCs) have found applications in cell therapies for a wide variety of diseases such as bone and cartilage disorders, diabetes, neurological disorders, and more due to their capabilities in differentiation, immune regulation, tissue repair, and cell signaling. As with all cell therapies, high quantities of hMSCs are required to prepare therapeutic doses. Therefore, the process of expanding hMSCs needs to be efficient, reliable, and consistent to provide adequate quantities of cells for both research purposes and therapeutic development. Adherent hMSCs cultures generally require extracellular matrix (ECM) coatings and/or media containing animal-derived serum. These factors reduce the efficiency and reproducibility of the hMSC expansion process by introducing variable culture conditions from animal-origin components, requiring additional time for coating, and adding extra costs.

To support progress towards more efficient and less animal-dependent processes for expanding hMSCs, we have developed an animal-origin free (AOF) version of Insulin-Transferrin-Selenium (ITS) supplement. Through multiple experiments, we demonstrated that this supplement supports the culture of adipose-derived hMSCs (as well as other primary cell types) under reduced-serum conditions on coating-free surfaces over multiple passages.

To support complete serum-free expansion of adherent hMSCs under coating-free conditions, we developed the Nunclon™ Supra cell culture surface and applied it to a wide variety of cell culture vessels ranging from 96-well plates to T175 flasks. Using hMSCs derived from adipose tissue, bone marrow, and Warton's jelly, we demonstrated that serum-free, coating-free culture on the Nunclon™ Supra cell culture surface allows for similar cell culture metrics (including viability, doubling time, and morphology) as compared with serum-free, ECM-coated culture on a standard cell culture surface. In addition, we also demonstrated that hMSCs expanded under serum-free, coating-free conditions on the Nunclon™ Supra surface maintain equivalent expression of multipotency markers and demonstrate comparable differentiation into chondrocytes, osteocytes, and adipocytes when compared to hMSCs cultured under serum-free, ECM-coated conditions. Taken together, these results indicate that the enhanced Nunclon™ Supra cell culture surface can play an important role in reducing hMSC culture workflow demands and easing the expansion of hMSCs into large volume cultures.

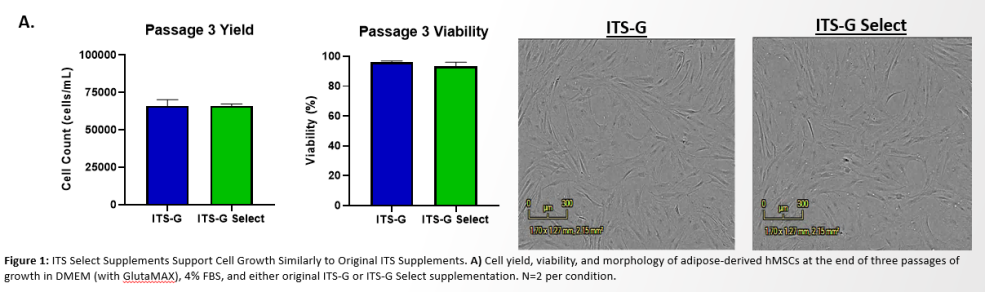

Figure 1: ITS Select Supplements Support Cell Growth Similarly to Original ITS Supplements. **A)** Cell yield, viability, and morphology of adipose-derived hMSCs at the end of three passages of growth in DMEM (with GlutaMAX), 4% FBS, and either original ITS-G or ITS-G Select supplementation. N=2 per condition.

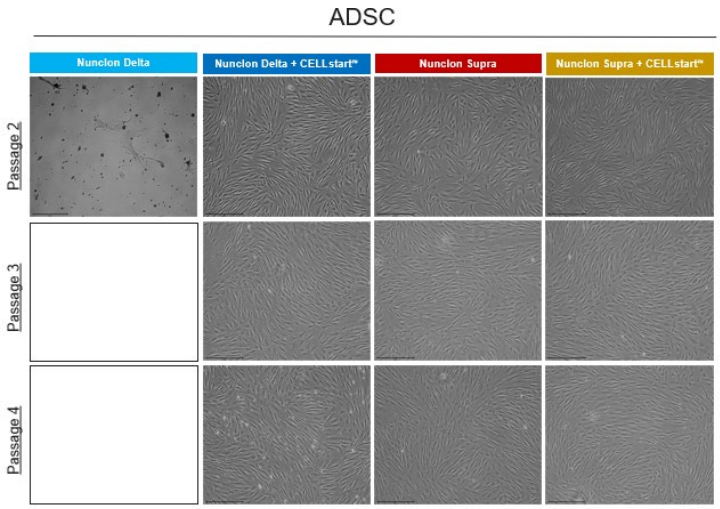

Figure 2. Morphology of hMSCs derived from adipose tissue (ADSC) over multiple passages when cultured without serum on the Nunclon™ Delta or Nunclon™ Supra surface with or without CELLstart™ coating. hMSCs cultured serum-free on the Supra surface without coating are viable unlike hMSCs cultured serum-free and coating-free on the Delta surface. This experiment was repeated with similar results for bone marrow-derived and Wharton's jelly-derived hMSCs.

815 Bioinspired Microbeads for Boosting Feeder-free Manufacturing of Immune Cells

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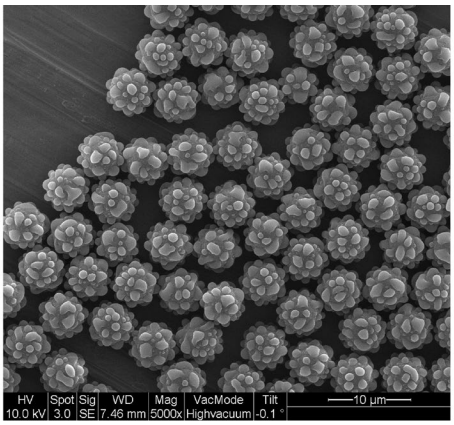
Introduction: Cell-based immunotherapy carries significant potential for treating cancer and other diseases; however, it faces substantial challenges in immune cell manufacturing. The intricate processes, especially for T and NK cells, demand careful selection of raw materials to ensure safety and quality. To address these challenges, we are thrilled to introduce iKNOBeads—a magnetic microbead with a unique bioinspired design engineered explicitly for immune cell activation.

Methods: We meticulously fabricated iKNOBeads, focusing on size and shape to ensure optimal design. Their bumpy surface, which enhances the contact area for cell interactions, significantly improves activation efficiency while minimizing the required bead amount. To validate the effectiveness of our new technology, we rigorously compared the iKNOBeads-based platform with commercially available products for manufacturing CAR-T and NK cells, implementing our optimized processes and carefully selected reagents.

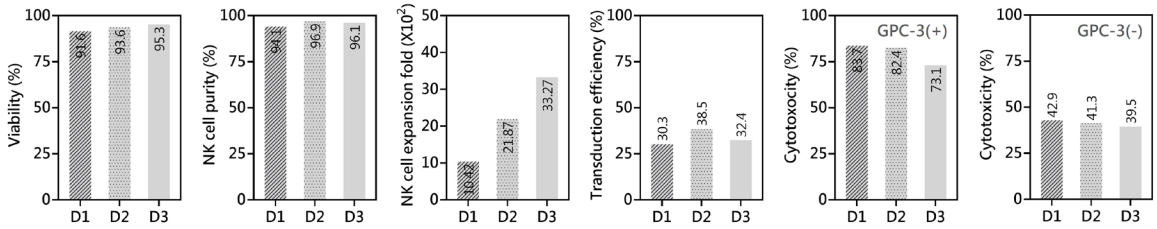
Results: Our compelling results reveal that the iKNOBeads platform substantially elevates CAR-T and NK cell products' quality, quantity, and functionality. When scaled up using a 500 mL G-rex bioreactor, we achieved an

impressive milestone: successfully producing up to 2 billion functional NK cells in just two weeks. Moreover, we have validated the efficacy of our innovative feeder-free platform in producing CAR-NK cells in vitro.

Conclusion: iKNOBeads represents a transformative leap in immune cell manufacturing efficiency. Its unique bioinspired design and superior performance promise more effective and affordable cell-based immunotherapies. With iKNOBeads set for commercial release this year, we are excited to continue our research and explore its potential in preclinical and clinical applications.



Scanning electronic microscopy image of the core of iKNOBeads



Manufacturing of GPC-3 CAR-NK cells in 14 days from 3 healthy donors using the iKNOBeads-based platform

816 Serum- and Feeder-Free Generation of Multipotent Hematopoietic Progenitors from Pluripotent Stem Cells in a 3D Culture System

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[1]STEMCELL Technologies, Inc., Vancouver, BC, [2]STEMCELL Technologies, Inc., Vancouver, [3]STEMCELL Technologies Inc, Vancouver, BC, [4]STEMCELL Technologies, Vancouver

Hematopoietic stem and/or progenitor cells (HSPCs) and their derivatives, including natural killer (NK) cells, T cells, and B cells, show immense potential for the treatment of various malignancies and diseases. However, generating therapeutically relevant yields of HSPCs remains a major challenge.The derivation of HSPCs from human pluripotent stem cells (hPSCs) provides an alternate source of unlimited cells to overcome this bottleneck and enable their use in diverse cellular therapies.

We have developed a scaleable, serum- and feeder-free 3D culture system capable of generating large numbers of functional, multipotent HSPCs. hPSC aggregates (5 independent cell lines) were seeded into either 6-well non-treated tissue culture plates on an orbital shaker or into a 100 mL PBS-MINI MagDrive Bioreactor containing STEMdiff™ Hematopoietic - EB Medium to form embryoid bodies (EBs) in 3D suspension cultures. After 12 days, both cultures were filtered with 37 µm reversible filters to remove the EBs and harvest the hPSC-derived HSPCs

remaining in the supernatant as single cells. HSPCs were assessed for yield and frequency of CD34+ cells by flow cytometry. The differentiation potential of these hPSC-derived HSPCs was assessed by colony-forming unit (CFU) assay and by lineage-specific differentiation to myeloid cells, erythroid cells, and lymphoid cells.

The 6-well 3D suspension cultures yielded 1.3 x 10^6 ± 1.6 x 10^5 CD34+ cells per 1 mL of culture vessel volume, with a CD34+ frequency of 73 ± 2% (mean ± SEM; n = 36). The scaled-up 100 mL bioreactor cultures performed comparably, producing 1.6 x 10^7 - 1.1 x 10^8 CD34+ cells (n = 4). In the CFU assay, hPSC-derived HSPCs generated 116 ± 14 CFU per 5,000 cells seeded, with 101 ± 16 granulocyte/macrophage colonies and 9 ± 4 erythroid colonies (n = 8) counted.

Lineage-specific differentiation confirmed that these hPSC-derived HSPCs were capable of differentiating to myeloid cells (macrophages, megakaryocytes, and microglia), erythroid cells, and importantly, lymphoid cells (B, NK, and T cells), indicating that these cells are definitive multipotent HSPCs. Lymphoid-specific differentiation resulted in 27 ± 14% CD19+ B cells (8 ± 4 per input hPSC, n = 6), 79 ± 2% CD56+ NK cells (1504 ± 394 per input hPSC, n = 25), and 54 ± 7% CD4+CD8+ double positive T cells (701 ± 293 per input hPSC, n = 9). Of note, 33 ± 5% of the CD4+CD8+ double positive T cells were also CD3+TCRαß+ indicative of a more differentiated phenotype.

These results demonstrate that our 3D suspension culture system can generate functional and multipotent HSPCs from hPSCs in both 6-well plate and larger-scale bioreactor formats to support the manufacturing of clinically relevant yields of HSPCs. This system is a valuable tool for researchers to aid in the study and development of cellular therapies.

817 Metabolic Profiling to Predict In-Vitro T-cell Fitness

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Adoptive T-cell therapies rely on the expansion of functional and metabolically fit T-cells to ensure therapeutic efficacy. Comprehensive evaluation of T-cell fitness in vitro is critical for optimizing these approaches, yet predictive metabolic metrics are not fully established. In this study, we employed luminescent plate-based assays to measure key metabolic and functional parameters in human T-cells as a model for cell expansion in CAR-T or other cell therapy applications.

Cellular reducing capacity, NAD(H)/NAD(P)(H) concentration, ATP levels, dehydrogenase activity, and markers of differentiation were measured at multiple timepoints in the activation and expansion process. IFN-γ/TNF-α cytokine secretion and T-cell-dependent cell killing served as functional metrics of cell fitness.

Correlations between these metrics and differing cell culture conditions revealed distinct metabolic signatures associated with T-cell functional fitness. Increased reducing capacity highlighted stronger activation when using one distinct culture medium, while increased proliferation in another medium correlated with higher ATP levels and increased dehydrogenase activity. Differentiation markers, analyzed alongside metabolic profiles, showed expected increases in glycolysis during T-cell activation and differentiation, and provided insights into the relationship between metabolic states and lineage commitment (e.g., effector vs. memory-like cells).

Collectively, these metabolic parameters serve as predictive biomarkers for T-cell fitness, demonstrating their potential utility in screening T-cell products for CAR-T and other therapeutic applications, and highlighting the importance of metabolic profiling in the optimization of T-cell expansion protocols.

818 Microfluidic Magnetic Bead Capture of Target Cells for In-Process Phenotyping of CAR T Cells

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Chimeric antigen receptor (CAR)-T cell therapies have in the past decade revolutionized the treatment of haematological cancers with impressive long-term remission rates achieved in clinical trials resulting in seven US Food and Drug Administration (FDA) approvals as of November 2024. However, the establishment of CAR T cell therapy as a standard of care is limited by complex logistics and manufacturing protocols, which contribute to their high prices. Producing a drug of consistent quality is critical in reducing the overall costs, which requires tight control of process parameters. To monitor them robust technologies are necessary to inform of critical quality attributes (CQAs), highlighting the importance of quality control (QC) in manufacturing of CAR T cell therapies.

Flow cytometry, a great research tool widely used for in-process CAR T cell phenotyping, does have limitations when implemented in Good Manufacturing Practice (GMP) environment. It is typically a highly manual procedure in both assay execution and data analysis and so is prone to human error and therefore not reliable. Some attempts have been made to automate flow cytometry assays, using robotic arms or microfluidic cartridges. These, however, do not offer online, inline or at-line testing, nor are they affordable. This highlights the lack of Process Analytical Technology (PAT) for determination of cell phenotype. To truly introduce PAT into CAR T cell manufacturing, there is a need for novel approaches that not only accurately measure cell marker expression levels but also integrate with cell processing equipment.

Here, we evaluate effective approaches to incorporate magnetic bead-based cell detection in microfluidic devices. Preliminary results show efficient cell capture on antibody-functionalised magnetic beads. The fabrication of microfluidic devices using a hard polymer allows not only for rapid prototyping but also aligns with the needs of low-cost mass manufacture. Efficient CNC machine milling of the chips enables screening of a range of designs to best fit the proposed analytical assay. The use of a clear, hard polymer allows for stable tubing connection of the chip device with syringe pumps for automation of fluid flow as well as on-chip cell visualisation. Small footprint of the chip paves way for its integration with CAR T cell manufacturing equipment. All these aspects strive to challenge the status-quo of analytical testing in advanced therapy processing by creating a viable, low-cost alternative.

819 Recirculated Tumor Chip Platform to Study How Microenvironmental Factors Affect Kinetics of Immune Cells and Immunotherapeutics

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Microenvironmental factors, including mechanical, chemical, immune, and stromal components, control tumor development. However, there is yet little understanding of how these factors affect the kinetics of cancer therapeutics and the downstream drug response, although there have been multiple models reported to study tumor microenvironment and to screen new therapeutics. Patient-derived xenograft (PDX) and high-throughput 2D screening are the most representative in vivo and in vitro models used for cancer drug development, respectively. Despite their popularity in cancer drug screening, PDX mouse models only offer murine physiological microenvironment to transplanted human tumors, while conventional 2D culture models provide very limited insights as 2D culture lacks many components seen in tumor. In light of these limitations, we develop a tumor chip platform that enables control and measurement of certain microenvironmental variables, including physiological flow, endothelial barrier and stroma compositions, along with live-cell imaging to understand the dynamics between tumor-microenvironment-cancer therapeutics and to evaluate how these components respond to the treatment. This multi-layered tumor chip reconstructs the tumor architecture and microvessel. Recirculation in this platform is

controlled by the recirculating circuit unit we created, consist of four one-way valves to generate the unidirectional flow. Through our optimization, our platform could recapitulate the tumor-microvascular interface and physiologically relevant interstitial flow in the reconstructed triple-negative breast cancer (TNBC) tumor compartment. To understand the role of tumor microenvironment in affecting the dynamics of immune cells and the efficacy of immunotherapeutics, we created different stromal compositions on chip with recirculation of CD8+ cytotoxic T cells. Tumor endothelium was found to be the key barrier for T cell infiltration, and cancer-associated fibroblasts in the tumor stroma further impeded this cell infiltration. In addition, affected by the cancer-associated fibroblasts in tumor stroma, tumor microvasculature reconstructed on chip showed a different expression pattern of endothelin B receptor (ET_BR) and intercellular adhesion molecule-1 (ICAM-1), thereby inhibiting T cell infiltration. Treatment of anti-PD-L1 antibody therapeutics could overcome these barriers and enhanced T cell's cytotoxicity against the TNBC cancer cells, while additional treatment of the ET_BR antagonist (BQ788) could tackle the negative dynamics between ET_BR and ICAM-1 in tumor microvasculature. Taken together, our results confirm that our chip recapitulates the characteristics of tumor-microvascular interface as well as tumor stoma. This platform could potentially serve as an ex vivo toolkit to optimize immunotherapeutics for cancer treatment.

820 Development of Potency Assays for Recombinant AAV vectors Independent of both the Promoter and Transgene as well as for AAV Encoded Transgenes Regulated by Photoreceptor and Retina Specific Promoters

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Current methods for quantifying the potency of recombinant AAV vectors are laborious, imprecise, and time consuming. A universal AAV potency assay based on the i*Lite*® reporter-gene technology has been developed that is rapid, precise, and independent of both the transgene and its promoter. Data will be presented illustrating the utility of this assay. An essential safety requirement for the successful development of therapies based on AAV-encoded transgenes is that the transgene is expressed exclusively in the target tissue. This can be accomplished by the use of tissue or even cell-type specific promoters. This poses significant challenges, however, for the development of appropriate potency assays that accurately reflect tissue or cell-type specific expression of the transgene.

The i*Lite*® reporter-gene technology has been used to develop potency assays for AAV encoded transgenes regulated by both photoreceptor-specific promoters and retina-specific promoters that reflect to a high degree the mechanism of action of the AAV-encoded transgene. The development of one such assay for the quantification of the potency of the retina-specific guanylate cyclase GUCY2D regulated by the hGRK1 promoter derived from the rhodopsin kinase G-protein coupled receptor kinase will be described. The assay is based on the stable co-transfection of ARPE-19-HPV-16 cells, derived from the retina pigmented epithelium, with a cGMP responsive reporter-gene construct and guanylyl cyclase-activating protein-1 (GCAP-1) to obtain maximal GUCY2D activity. The resulting cell line was also stably transfected with the Renilla luciferase (RL) reporter-gene under the control of a constitutive promoter that allows both FL and RL activity to be quantified sequentially in the same well of a microtiter plate. FL activity can thus be normalized with respect to RL expression rendering the assay independent of cell number and allowing non-specific effects to be detected.

The resulting i*Lite*® cell line has been used to quantify the potency of an AAV5 encoded GUCY2D transgene under the control of the hGRK1 promoter. A dynamic range of some 750-fold was obtained in a 4PL potency assay and a relative potency assay without the need to add live Adeno virus or other external stimuli. The cells have been converted into a frozen ready-to-use format that confers both ease of use and low coefficients of variation. Data will be presented illustrating the utility of this assay.

821 A Novel and Reliable *In Vitro* Copper-Excretion Potency Assay with Engineered Huh-7-CuEP Cells for Evaluating Gene Therapy Candidates in Wilson Disease

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Wilson disease (WD) is a rare autosomal recessive disorder caused by mutations in the *ATP7B* gene, leading to impaired copper excretion by the dysfunctional ATP7B protein and resulting in systemic copper accumulation, primarily affecting the liver, brain, and kidneys. LY-M003, an investigational adeno-associated virus serotype 8 (AAV8)-based gene therapy, was designed to produce a modified miniATP7B^{LY} protein in the liver, thereby reducing copper accumulation in WD. The development of a biological potency assay that accurately mimics the endogenous mechanism of action of the target gene/protein is essential for advancing gene therapy products. In this study, we developed a robust in vitro potency assay utilizing a Cu^{2+}-dependent luciferase reporter system in an engineered Huh-7-CuEP^{TM} cell line, providing a sensitive tool to evaluate the copper-excretion capacity of therapeutic candidates for WD.

In this Huh-7-CuEP^{TM} cell line, a metal responsible luciferase reporter cassette was stable integrated into Huh-7 cell line, with the endogenous *ATP7B* gene being inactivated. Upon the addition of excess CuSO4 to the culture medium, cellular copper levels increased significantly and the luciferase expression was markedly increase under the activation of the copper responsible cassette. The luciferase signal showed a correlation with excess $CuSO_4$ concentration, ranging from 10 μM to 100 μM (4-PL, $R^2 = 0.98$). Following infection with LY-M003, cellular copper concentration decreased as ATP7B expression was restored, which was reflected by a substantial reduction in luciferase activity. At specific concentrations of $CuSO_4$ in the medium, the inhibition of excess copper response can be used to quantify the biological potency of the gene therapy products. The inhibition curve demonstrated a strong correlation with LY-M003 multiplicity of infection (MOI), from 1.0×10^4 to 3.0×10^6 (4-PL, $R^2 = 0.97$), simulating different levels of functional product. This assay has been established and validated for specificity, linearity and range, precision and robustness in accordance with GLP guidelines. Specificity testing confirmed that the luciferase signal was exclusively induced by excess Cu^{2+} and inhibited by LY-M003. The quantitative range was found to be between MOI 1.2×10^4 and 2.7×10^6. When performed by two analysts on separate days, the coefficient of variation (CV) for six replicates per analyst and twelve replicates across both analysts was less than 20%, indicating high precision.

In conclusion, the Huh-7-CuEP-based copper-excretion potency assay has been successfully established and validated. It is a suitable tool for release testing and stability evaluation of Wilson disease products, particularly gene therapy candidates.

822 Functional Potency-Based Bioactivity Characterization of Viral Vectors with Supportive Physical and Genomic Titer Analyses

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Abstract: The field of Cell and Gene Therapy (C>) has generated a variety of novel solutions to the treatment of certain diseases such as cancer and genetic disorders. In this space, viral vectors are employed as the drug product itself, or a critical reagent to generate the therapeutic cell. These viral vectors require a variety of analytics to be performed for the evaluation of their critical quality attributes. Among these attributes are genomic titer, particle titer, and vector potency. While the analytics for genomic and particle titer, often PCR and ELISA-based, respectively, vary modestly among viral vectors, potency analytics are more varied. As each viral vector is unique in its intended bioactivity, no one-size-fits all potency assay is in place and therefore each program must develop product specific potency assays.

Despite the commonality of the physical and biochemical techniques applied to evaluate genomic and particle titer, there are some differences that necessitate one or more augments to these methods. For instance, while qPCR and ddPCR can be utilized to titer DNA-carrying vectors, RNA-containing ones require the addition of a reverse transcriptase step. Moreover, to evaluate particle titer, due to the variation in particle size and composition of the base vector, ELISA, HPLC, microscopy, and light scattering techniques are employed. Each of these methods require some variation in their execution to accommodate for these vector differences.

Potency assays, however, require the greatest variation among vectors to characterize their bioactivity. Vector potency refers to the construct's ability to exert its intended therapeutic effect upon the target cell type. As such, the potency assay must be developed to directly evaluate the therapeutic effect of the vector whether this effect is oncolytic for the treatment of cancer cells, or transducing, resulting in short, or long, term expression of a specific gene product. Incompatible, or inefficient, methods to determine potency can lead to program complications and regulatory delays.

At Matica, we have developed a variety of potency assays to support our clients' programs. These include one of multiple different viral vectors generated using Lentiviruses, AAV's, Adenoviruses, and others. Each vector exhibits its own characteristics which determine the analytical techniques that are applicable. For instance, Adenoviruses, often employed as oncolytic vectors, allow for a more direct characterization through traditional plaque assays, as these vectors are cytopathic. Whereas AAV's, due to their nonpathogenic nature, traditional infectivity assays are not applicable. Instead, to evaluate an AAV vector's infectivity, a TCID50 can be employed, where the readout is by qPCR or ddPCR after a short infection period.

We show here multiple methods developed and executed at Matica, on different representative vectors systems, to demonstrate the variety of techniques available to interrogate viral vector potency along with data showing characterization of genomic and particle titer.

823 Functional cell-based models for gene therapies that utilize cell-specific promoters are enabled through CRISPR-activation

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As the field of AAV gene therapy evolves, payload design strategies have begun to focus on minimizing potential off-target toxicity that may be associated with systemic transgene expression. One approach to address this issue is the use of cell type-specific promoters that restrict payload expression to therapeutically relevant cell types, as exemplified by use of the synapsin promoter[1] to minimize non-neuronal expression in brain-targeted AAV gene replacement.

While this specificity is desirable for maximizing the therapeutic index of a gene therapeutic, it introduces challenges in designing functional cell-based assays principally due to the lack of readily-culturable cell lines that both express the specific cell type promoter and are tropic to AAV. To address this issue, we used CRISPR-activation[2], coupled with promoter-specific guides to enable transgene expression in HEK293T cells. In this approach, a panel of guides spanning the human promoter sequence are transiently co-transfected into HEK293T along with CRISPR-activation components (dCAS9-VP64, gRNA-MS2, MS2-p65HS1) in 96-well plates. AAV harboring a promoter-GFP payload is introduced into the wells, followed by outgrowth and imaging. The guide yielding the highest and most consistent GFP expression is then used to make a monoclonal derived stable cell line suitable for gene therapy expression, potency and functional assays.

In the representative system described here, we focused on an appropriate cell line that can express transgenes tied to a proprietary ON bipolar mGluR6 (metabotropic glutamate receptor 6) promoter. After identifying an active mGluR6 promoter guide and establishing a clonal CRISPR-activator cell line, we demonstrate the use of this cell line in an optogenetic potency assay suitable for GMP lot release. This allows potency assays previously developed for the universal CAG promoter to be seamlessly repurposed for the mGluR6 promoter.

This strategy should be applicable to any promoter whose activation in HEK293T is tolerated and potentially enhanced via guides that span artificial junctions in synthetic promoters, overcoming one of the practical roadblocks hindering the implementation of next-generation AAV gene therapies.

Citations
[1]Kügler, S., Kilic, E. & Bähr, M. Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene Ther* **10**, 337–347 (2003)
[2]Heidersbach, A.J., Dorighi, K.M., Gomez, J.A. *et al.* A versatile, high-efficiency platform for CRISPR-based gene activation. *Nat Commun* **14**, 902 (2023)

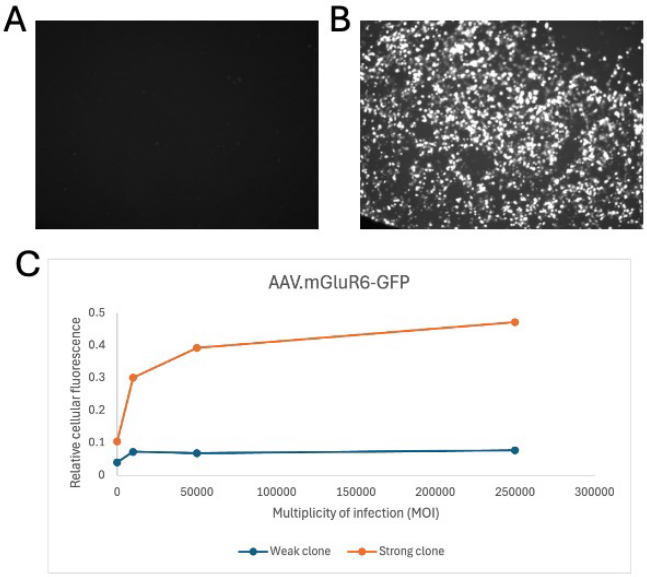

Figure 1: (A) Wild type HEK293T transduced with AAV.mGluR6-GFP show poor GFP expression. (B) HEK293T transiently transfected with CRISPRa components and an mGluR6 promoter-targeted guide express high levels of GFP upon transduction with AAV.mGluR6-GFP. (C) Clonal selection of HEK293T/CRISPRa-mGluR6 yields stable cell lines, including those with stably high expression levels.

824 BGT-DTDS vector *in vivo* potency analysis in mouse model of Dopamine Transporter Deficiency Syndrome

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BGT-DTDS and BGT-PD (AAV2-SLC6A3) are first-in-class gene therapies expressing dopamine transporter (DAT) evaluated for different indications. DAT is a key regulator of dopamine homeostasis, and perturbations have

been associated in genetic and idiopathic Parkinson's disease (PD). Dopamine transporter deficiency syndrome (DTDS) is caused by biallelic loss-of-function mutations in *SLC6A3* gene encoding DAT, and is a form of childhood parkinsonism disorder similar to AADC deficiency. We have previously shown preclinical efficacy of BGT-DTDS in a DTDS mouse model, and efficacy in managing debilitating levodopa-induced dyskinesia (LID) in PD with complete LID suppression in a rat model. We aim to progress with these promising preclinical data to clinical application.

One of the challenges in translation is AAV scale-up manufacturing to current good manufacturing practice (cGMP). In this study, we successfully scaled up BGT-DTDS/BGT-PD production to a 2L feasibility batch with Viralgen, and evaluated potency *in vivo*. Pre-cGMP batch had an improved empty:full capsid ratio than research grade vector, in absence of aggregates. *In vivo* potency assay was performed in neonatal DTDS mice by bilateral intracerebroventricular injections at two-log dose range ($1.7x10^9$-$1.7x10^{11}$vg/pup), and research grade at $1.7x10^{10}$vg/pup (n=7-9/group). Compared to dose-matched research grade vector, pre-cGMP vector had increased potency showing significant improvements in open field parameters at 4 and 12 weeks. No in-life adverse effects were observed in both pre-cGMP and research vectors, and at all doses evaluated. Overall, our data demonstrates pre-cGMP vector superiority in purity and *in vivo* potency, supporting successful scale-up manufacturing towards cGMP applications.

825 Optimized Automated DNA Extraction: A Pathway to Improved Patient Monitoring in CAR-T Trials

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Chimeric Antigen Receptor T- cell (CAR-T) therapies are key gene therapy for both oncology and autoimmune diseases. CAR T-cells as well as TCR-T therapies are produced by stable integration of the CAR/TCR transgene constructs in the genome predominantly by lentiviral /retroviral vector transduction. The use of viral vectors and transgene integration in the genome need monitoring of patients treated with CAR -T therapies for replication competent lentivirus/retrovirus (RCL/R) and vector copy numbers (VCN).

CAR therapies can be autologous or allogeneic cell therapies. In both cases, patients must undergo lymphodepletion before being treated with CAR-T therapies. As a result of this lymphodepletion, patients have little to no nucleated cells within the first few days' post infusion of therapy. RCL/R and VCN are monitored at specific time points before and after CAR administration. In particular, VCN is done from the first few days to 4 weeks of CAR therapy. In this window, only a limited amount of DNA is available before recovery of the lymphocytes including the CAR-T cells in the subject. To ensure sufficient DNA is available for CAR monitoring during the first 2 weeks, DNA should be extracted using as much sample input volume as possible. This poses challenges to any automated extraction procedure as well as for efficiency of downstream molecular assays.

We optimized our standard automated DNA extraction procedure with sample input volumes <0.5 mL and managed to increase sample input volumes above 1 ml and elute DNA in volumes sufficient to enable efficient RCL/R and VCN detection directly (n=5 different donor samples). The RCL/R results showed a CV <20% for all copies tested within the quantification range, while the VCN data showed a CV <20% for >50 copies/PCR and a CV< 40% for copies <50 copies/PCR. The results from both our standard and optimized workflows are comparable and demonstrate no impact on molecular assay performance. This provides us with a scalable workflow that can handle different volumes of clinical samples, both above and below 1 ml. The workflow is being tested for other sample matrices and being validated for clinical sample testing.

826 Assessment of an Automated, Multi-dimensional Digital PCR Workflow for the Rapid Evaluation of Recombinant AAV Genome Integrity.

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Digital PCR (dPCR) is the gold standard for quantifying recombinant adeno-associated virus (rAAV) vector following production because of its ability to deliver absolute and precise measurements of viral genome titers without a need for external standards. Its multiplexing capability enables the simultaneous detection of multiple genomic regions, providing critical insights into rAAV genome integrity through co-localization analysis. However, dPCR has a narrow dynamic range primarily due to the fundamental principles of its operation and the statistical constraints of its partitioning-based quantification method, which necessitates testing multiple dilutions of each sample to avoid errors from subsampling or saturation.

After capsid lysis, the released single-strand rAAV genome is fragile and prone to degradation. It is hypothesized that the measured integrity by multi-dimensional dPCR underestimates the actual rAAV integrity due to sample treatment during and after capsid lysis. Manual preparation of dPCR samples is further complicated by the need for custom multiplexed primer/probe sets for different rAAV genomes and for varying dilution strategies for both crude and purified samples across different production stages. This complexity makes manual workflows labor-intensive, error-prone, and difficult to scale.

To overcome these challenges and obtain consistent results, variations in sample preparations must be minimized, highlighting the need for robotic systems. We have developed an automated workflow using a cost-effective benchtop robotic system to streamline dPCR sample preparation for rAAV quantification. The system automates DNase digestion of the cellular DNA, capsid lysis, serial dilutions, and the preparation of dilution-optimized samples with the appropriate dPCR mix, ready for analysis. By eliminating manual preparation challenges, this method improves efficiency, consistency, and accuracy while enabling the simultaneous processing of diverse rAAV sample types on a single dPCR plate. This approach, together with a custom downstream data analytics software, makes dPCR workflows more accessible and scalable in the context of rAAV production analytics.

This automated workflow for multi-dimensional PCR is used alongside long-read sequencing technologies for the quality assessment of AAV-based gene therapy product candidates.

827 High-Coverage Whole-Genome Sequencing Reveals Adverse CRISPR Editing Events in Human HSPCs Targeted by Therapeutic gRNAs

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CRISPR-Cas genome-editing technology is rapidly advancing into diverse clinical applications for the treatment of various diseases. However, its therapeutic use requires a thorough assessment of potential adverse editing outcomes at both on-target and off-target sites. These outcomes include insertions and deletions at target and non-target sites, as well as structural variations (SVs) such as long deletions, inversions, translocations, and copy number variation (CNV). Such events can result in harmful genomic rearrangements, which, if they confer a selective advantage, may promote clonal expansion and drive tumorigenesis. Therefore, precise and robust detection methods are essential to evaluate the safety and efficacy of CRISPR-based genome-editing therapeutics.

The gold-standard approach for quantifying adverse genomic effects relies on targeted amplification followed by deep sequencing of pre-selected sites. However, this method requires an extensive preliminary nomination of potential off-target sites, is limited to analyzing only a few hundred regions, and demands the design and optimization of custom primers for each target. Furthermore, targeted sequencing is inherently constrained in its

ability to detect all types of large-scale genomic rearrangements, such as inversions and chromosome loss. Fish-and-bait methods enable broader detection but are restricted to pre-designed bait sequences. Amplification-based long-read sequencing (LRS) can identify large-scale SVs but remains limited to selected targets and is subjected to amplification bias. While amplification-free LRS eliminates this bias, it suffers from low read yield and is still target-specific, further limiting its utility for unbiased, genome-wide analysis.

A promising solution to the challenges of detecting adverse genomic effects lies in the application of whole-genome sequencing (WGS). This unbiased, genome-wide approach is versatile, applicable to all cell types, and capable of detecting a comprehensive range of CRISPR-editing events. However, traditional WGS methods have been constrained by high costs and insufficient coverage, limiting their utility for identifying low-frequency events. We have advanced WGS capabilities by utilizing Ultima, a sequencing technology that delivers dramatically reduced costs, enabling ultra-high coverage (up to 1000×) as the new standard for evaluating and understanding Cas9 on-target and off-target effects.

Leveraging this technology, we conducted high-coverage WGS on CRISPR-edited human hematopoietic stem and progenitor cells (HSPCs) modified with CRISPR-ribonucleoprotein (RNP) complexes, with gRNAs used in clinical studies, including the Casgevy gRNA—the first FDA-approved CRISPR-based therapy. This analysis revealed a diverse spectrum of genomic aberrations, including various genomic rearrangements at the cut-sites, that are challenging to identify using targeted approaches. This study highlights the wide range of potential genomic alterations caused by CRISPR editing and emphasizes the vital role of unbiased, high-coverage approaches in their detection. By enabling a streamlined, one-step, and comprehensive assessment of genomic integrity, WGS-based approach represents a major advancement in ensuring the safety of CRISPR-based cell therapeutics.

828 Simultaneous measurement of viral vector titer and genome integrity with long-read amplicons enabled by Countable PCR

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We introduce Countable PCR, a novel platform that enables genome integrity and viral titer characterization, including long-read amplicons, within a single assay, with high sensitivity and low variability. Current methodologies in the field present notable drawbacks for providing reliable viral titer measurements: qPCR is too variable; and dPCR suffers from a narrow dynamic range, high Poisson errors, and arbitrary thresholding. Further, it is impossible to conduct both titer and genome integrity assessments in a single assay, as dPCR has partitioning limitations. Countable PCR overcomes these challenges by directly counting single molecules.

Here, we demonstrate the value of direct molecule counting by designing a novel multiplex assay that interrogates different regions of the gene of interest (GOI) to accurately quantify the ratio of partial vs. complete transgene while simultaneously measuring titer in the same sample. Unlike dPCR, this single-molecule approach prevents false co-occupancy of fragmented, unlinked targets, ensuring accurate genome integrity data. Full genome integration is further validated with the ability to analyze long-read amplicons, which we demonstrate here up to 2000 bp. Together, this work demonstrates how Countable PCR makes gene therapy viral characterization workflows more versatile and approachable compared to legacy methods.

829 The Good, the Bad, & the Chimeric: Using Nanopore Sequencing to De-Risk rAAV Genome Packaging

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Adeno-associated virus (AAV) vectors are widely used in gene therapy due to their efficacy and safety. The vast majority of AAV capsids contain the correct vector transgene ("the good"), but a small fraction harbors unwanted DNA sequences. These packaged DNA impurities ("the bad") are derived from the biological starting materials used to manufacture AAV, e.g. plasmids used in transient manufacturing platforms, or viruses used for infection-based manufacturing and from the manufacturing cells. Fortunately, their safety implications are mitigated to some extent by the absence of inverted terminal repeats (ITR) which prevent episomal persistence in the transduced cells, reduce the risk of double-strand formation of DNA impurities and, therefore, limit the risk of (persistent) expression of impurities in patient´s cells.

However, chimeric reads, i.e. hybrid DNA sequences with ITRs and contaminants arising from vector recombination or bidirectional ITR packaging, could be rapidly transcribed into translationally active double-stranded DNA molecules that may persist in transduced cells for years raising safety concerns with respect to immunogenicity and cell transformation when comprising oncogenic sequences. To this end, a deeper understanding of their origins and implications is needed. Here, we report a thorough characterization of more common DNA impurities derived from plasmid backbone and host cell DNA sequence packaging, and of very rare sequences such as HCD-ITR and rep-ITR fusion sequences in a rAAV9 vector batch.

Using Illumina and nanopore deep sequencing we examined the identity, origin, and abundance of various classes of chimeric reads using proprietary analysis pipelines. We speculate on their biological relevance and potential to impact gene therapy. Finally, we present strategies demonstrating how comprehensive analytical approaches can mitigate risks associated with packaged DNA impurities.

830 A Novel Reference Standard for Assessing AAV Titer and Genome Integrity

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Introduction: Viral vectors hold great promise for treating a broad array of genetic conditions by enabling the delivery of therapeutic genes into target cells. We developed a reference standard for evaluating adeno-associated virus (AAV) titer and genome integrity, incorporating polymerase chain reaction (PCR) target sites, including the Bovine Growth Hormone Polyadenylation Signal (bGHpA), Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE), Cytomegalovirus (CMV) Enhancer and Promoter, Green Fluorescent Protein (GFP) reporter gene, Simian Virus 40 Polyadenylation Signal (SV40pA) and Promoter, and Human Growth Hormone Polyadenylation Signal (hGHpA). These targets, evenly distributed across the AAV genome, allow for comprehensive titer and genome integrity assessment (GIA). The reference standard also incorporates the Green Fluorescent Protein (GFP) reporter gene.

Material and Methods: A cross-site evaluation of genome titer and GIA of the rAAV8 lot produced in AE1-BHK cell line containing the reference standard as the genome was performed. Titer and genome integrity were assessed with the use of QIAcuity Cell and Gene Therapy (CGT) dPCR Assays, with use of QIAcuity Digital PCR System. Qiagen CGT Viral Vector Lysis Kit was used to perform capsid lysis, and no restriction digestion was performed. Titer was measured in duplex reactions with the following target sets (Figure 1): Duplex 1 (CMV Promoter, GFP, fragment length ~1.5kbp), Duplex 3 (WPRE, SV40 Promoter, fragment length ~2.6kbp), and Duplex 4 (bGHpA, hGHpA, fragment length ~3.5kbp).

Results: The mean AAV genome titer based on all targets was determined to be 1.13×10^{13} vg/mL ± SD 2.98×10^{12}. We compared results of our standard analytics that accompany a certificate of analysis (COA) and partnered with Qiagen as an outside vendor to validate in parallel. These data showed reproducibility between both groups and the percentage differences in titer obtained from duplex reactions for Duplex 1, 3, and 4 between Agathos Biologics and Qiagen laboratories ranged from ~2% to ~15%. dPCR targets located at the distal ends of the AAV genome (bGHpA, hGHpA, near the ITR regions) were lower than those determined for regions positioned closer to the center of the AAV genome. Genome integrity analysis showed similar percentages of AAV genome intactness, with cross-site precision (%CV) of 11%, 4%, and 53% for Duplex 1, 3, and 4, respectively. Intra-assay precision (%CV) was below 20% for both Agathos and Qiagen data. The results indicated the highest percentage of intact

genomes for shorter fragments (fragments 1.5 kbp and 1.9 kbp (Agathos Biologics data only)) near the position of the gene of interest (GFP), with lower percentages of genome intactness for targets located near the ITR region.

Conclusions: Cross-site evaluation of the rAAV8 sample with the reference standard showed similar titer and genome intactness results. The lower percent genome intactness at distal regions may indicate PCR inhibition from ITR secondary structures, as reported previously, and/or AAV genome truncation. Further optimization and standardization of genome integrity protocols are needed to reduce inter-laboratory variation.

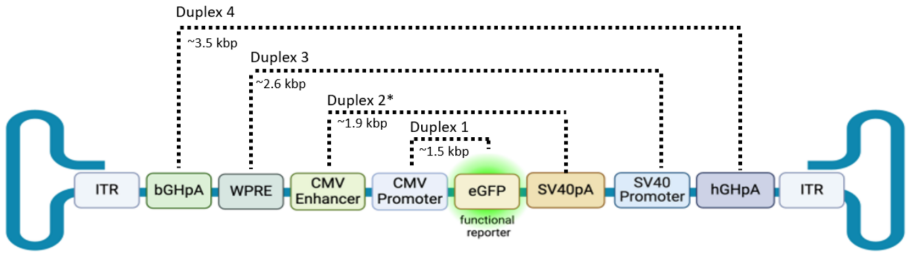

Figure 1. Graphical representation of the rAAV standard and dPCR assays used. The figure illustrates the types of duplex assays employed to evaluate titer (vg/mL) and genome integrity of the rAAV standard in a cross-site evaluation (*Duplex 2 not included in the cross-site evaluation). The duplex assays include Duplex 1: CMV Promoter and GFP (fragment length ~1.5 kbp); Duplex 2: CMV Enhancer and SV40pA (fragment length ~1.9 kbp); Duplex 3: WPRE and SV40 Promoter (fragment length ~2.6 kbp); Duplex 4: bGHpA and hGHpA (fragment length ~3.5 kbp). Image not drawn to scale.

831 Nanopore Sequencing and Screening of AAV Genomes for Optimal Production and Function

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Adeno-associated virus (AAV) has become a popular tool for gene therapy due to its ability to safely and efficiently deliver genetic elements to multiple tissue types in vivo. Genetic elements with a total size within ~4.7 kilobases (kb) can be assembled between two ITRs, packaged into AAV particles, and be used to deliver expression cassettes to animal and human tissues.

Production of AAV particles can be challenging and result in a heterogeneous mixture of capsids with complete and incomplete genomes, which can increase the amount of virus needed to achieve the desired expression in vivo. Indeed, sequence elements have been shown to impact packaging of complete AAV genomes, though a thorough characterization of how sequence affects AAV genome packaging is not available. Here we sought to develop a system to 1) identify truncated AAV genomes and the underlying sequence elements responsible for inefficient AAV genome packaging, and 2) evaluate how these sequence elements impact AAV manufacturing and transduction using a high-throughput assay system. To this end, we have established a pipeline where AAV genomes are evaluated by gel electrophoresis and sequencing using the Oxford Nanopore Technology (ONT) platform, and pooled libraries of AAV plasmid constructs are evaluated for manufacturing and transduction potential using bulk and single-cell sequencing. This pipeline identified a promoter sequence element that was detrimental to AAV manufacturing and transduction. The approach presented herein can be used to select AAV constructs with properties needed for safe and effective gene therapies destined for the clinic.

832 Improved Quantification of Adeno-Associated Virus (AAV) Viral Genome Using Nanoneedle Technology for Enhanced Molecular Specificity

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Introduction: Recombinant adeno-associated virus (rAAV) exhibits significant heterogeneity throughout the manufacturing process. Accurate quantification of capsid and genome titers, particularly the integrity of the viral genome, is crucial for ensuring the efficacy and safety of gene therapies. Current methodologies (analytical ultracentrifugation [AUC], electrophoresis, and molecular assays, such as PCR and ELISA), often fall short in distinguishing between full-length viral genomes and truncated or partial genome fragments, which impacts the interpretation of AAV vector quality.

Methods: We developed a novel viral genome assay utilizing a nanoneedle technology on the Tessie platform. Tessie enables accurate and highly reproducible quantification of full-length transgenes and offers molecular specificity to distinguish those from long partial genomes of similar sizes. In our study, Tessie's performance was compared to established methods, including ELISA for capsid titer, droplet digital PCR for genome titer, and AUC and electrophoresis for assessing viral genome integrity. A reference AAV sample, potentially containing a high level of full-length genomes along with a certain level of contamination from long partial genomes, was analyzed using all platforms. This comparison aimed to evaluate Tessie's ability and accuracy in identifying and quantifying distinct viral species within heterogeneous AAV preparations.

Results: The AUC characterization displayed a single peak (**Fig. 1a**), suggesting that the predominant species accounts for 70% of the total population. In contrast, electrophoresis revealed multiple side bands, highlighting the heterogeneous nature of the sample. When quantifying the area under the curve within the 3-5 kb size range, electrophoresis indicated that less than 11% of the total population corresponds to the full-length viral genome size **(Fig. 1b**). We hypothesize that the discrepancy between AUC and electrophoresis results arises from the presence of a significant population of long partial genomes that are similar in size to the full-length viral genome. On the Tessie platform, we developed two assays: the full-length transgene assay, which specifically quantifies the titer of the full-length viral genome (4.3 kb), and the long partial transgene assay, which quantifies left-truncated partial transgenes (3.9 kb). Additionally, we quantified the capsid titer on the same platform, enabling us to calculate the empty/full/partial ratio. When accounting for the long partial genome populations, the empty/full ratio aligns well with the AUC characterization. Furthermore, the pure full-length population corresponds more closely with the electrophoresis results.

Conclusions: Our results demonstrate that traditional methods, such as AUC, can misclassify long partial genome subpopulations as full-length viral particles due to their limited ability to distinguish between full-length genomes and truncated transgene variants. In contrast, Tessie's quantification method exhibited a high degree of correlation with molecular methods, including ELISA and ddPCR, and effectively reconciled the discrepancies observed between different biophysical methods, such as AUC and electrophoresis. The new methodology enables accurate quantification of viral genomes, distinguishing full from partial genomes, thus enhancing the development and production of AAV-based gene therapies.

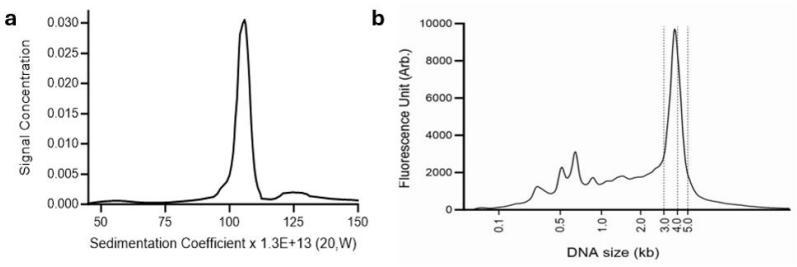

Fig. 1: **a**. AAV9 samples were analyzed using a sediment velocity analytical ultracentrifuge at 20,000 rpm, equilibrated at 20°C for 1 hour. Absorbance at 230 nm was then measured. **b**. AAV9 sample was DNase I treated, lysed by heat treatment, purified by AMPure XP beads, and analyzed on the Tapestation with a denatured double-stranded DNA size ladder. The sizes of the dsDNA ladder are shown and should serve as a rough approximation of fragment sizes. The dotted lines specify the region overlapping the peak.

833 Comprehensive Integrity Assessment of rAAV Batches Through an Integrated Workflow Coupling Long Read Sequencing with Multiplex dPCR

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While recombinant adeno-associated viral vectors (rAAV) are one of the leading platforms for gene therapy, the manufacturing process remains complex. The assessment of potential incomplete or truncated genomes and their effects on the safety and efficacy of the therapy is crucial. Several methods have emerged to support rAAV integrity analysis. Digital polymerase chain reaction (dPCR) allows for absolute quantification of AAV genomes and assessment of integrity by multiplexing up to six targets to cover the whole genome. While faster and cheaper, dPCR requires an upfront setup to establish primer sets and can only be used for quantification of known and expected targets. Long-read sequencing technologies extensively characterize the AAV genome, including sequence identity and identification of all packaged species, without the need for known targets. However, these methods only provide a relative quantification of full-length genomes. Here, we present a study where we evaluate the integrity of the AAV genomes packaged within a research-grade AAV9 control batch, with an expected length of 2,996bp.

We sequenced the rAAV batch on an Oxford Nanopore MinION. The vector capsids were sequentially digested with a combination of DNases and Proteinase K to release only the packaged DNA. The size distribution was assessed using automated electrophoresis before and after the material was converted into double stranded form. Results showed a reliable and consistent conversion, without alteration of the size distribution when looking at the single-stranded profile versus the double stranded profile. Next, the library was prepared for long read sequencing following the sequencing kit instructions for the V14 chemistry, with several optimizations for the expected size and type of molecule. A total of >2,5 million reads were obtained and further analyzed using an in-house pipeline. Most reads mapped to the genome (>85%), and most were at full length (2,900-2,996 bp). The second most frequent length size identified was at 100-200bp pointing to a possible presence of additional ITRs or truncation.

In parallel to the Nanopore efforts, to leverage dPCR multiplexing, we designed five primer sets spanning the whole AAV genome (ITR-UTR junction, 5' region of the promoter, promoter-transgene junction, transgene-polyA junction, and polyA-ITR junction). After establishment, these primers were used in a multiplex dPCR assay with part of the material extracted for the previous method, which was serially diluted down to 1E+03 cp, and added to a QIAcuity Nanoplate 8.5k 96-well plate. dPCR results were used to confirm the findings from the Nanopore data. Efforts to refine the accuracy of the quantification are currently ongoing, focusing on the de-multiplexing of occupancy data.

In summary, we have shown that coupling the long-read sequencing and dPCR enables an extended characterization of the vector particles and the genomes packaged, as well as accurate quantitation of full-length vectors.

834 Leveraging Next-Generation Sequencing for CMC of Gene and Cell Therapy Products

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In the rapidly evolving field of gene and cell therapies (CGTs), Chemistry, Manufacturing, and Controls (CMC) is instrumental. The development and production of these therapeutics present significant challenges, including shorter shelf life, Critical Quality Attributes (CQAs) assessment, manufacturing complexity, complex logistics and higher costs, and stringent regulatory compliance requirements. By harnessing the power of NGS technology, biopharmaceutical companies can produce high-quality CGTs that meet good manufacturing practice (GMP) requirements and clinical standards, ensuring that CMC concerns are addressed. Biopharma and biotech organizations can significantly reduce development timelines and improve product quality by using the NGS multi-attribute method (MAM) to monitor several CQAs of CGTs simultaneously for e.g., adventitious agent detection, vector integration, and genome integrity assessment.

Having a validated in-house environment is vital for the successful implementation of overarching NGS platforms. However, to successfully validate such platforms, teams need to consider variability in methods, instruments, and protocols, as well as robust data handling, and adherence to regulatory compliance. Strategic partnerships are crucial for success.

In this poster, we present three use cases from leading biopharmaceutical companies showing best practices for validation of NGS platforms in-house for CGT development.

835 Advancing rAAV Manufacturing with Long-Read Sequencing

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Recombinant adeno-associated virus (rAAV) vectors require robust quality control to ensure patient safety and therapeutic efficacy. Conventional assays, such as restriction enzyme digests, denaturing alkaline gels, and PCR-based quantification, while valuable, often fall short in fully characterizing genome integrity, confirming sequence identity, and detecting contaminants. Many current sequencing-based approaches, particularly those based on Sanger and Illumina sequencing, rely on targeted or amplification-based methods, which may introduce biases or fail to capture the full complexity of vector populations. Next-Generation Sequencing (NGS), particularly long-read sequencing, offers a more comprehensive analytical approach to enhance detection of partial genomes, structural abnormalities, and unexpected contaminants throughout the rAAV production lifecycle.

Long-read sequencing using Nanopore technology serves as a key component of our rAAV characterization strategy throughout the manufacturing process, from research-grade plasmid screening to final cGMP drug product testing. This approach enables full-length genome characterization, structural variant detection, and contaminant analysis. By avoiding amplification biases, native DNA integrity is preserved, ensuring a more accurate representation of vector composition. Long-read sequencing offers detailed insights into plasmid integrity and rAAV vector composition, identifying partial genomes, heterogeneous populations, and unexpected structural variations.

For GMP-grade rAAV drug product testing, Illumina sequencing serves as an orthogonal method to confirm sequence identity and improve confidence in variant detection. Its lower per-base error rate allows for the precise identification of small sequence variants, including single-nucleotide polymorphisms (SNPs) and small insertions or deletions (indels).

Optimized DNA extraction and library preparation protocols maximize data accuracy and reproducibility within sequencing workflows. The data analysis workflow is performed using Forge's proprietary bioinformatics pipeline, which integrates read alignment, variant calling, and contaminant screening to provide a comprehensive assessment of genome integrity, purity, and sequence identity. These methodologies support both research applications and regulatory compliance, ensuring the reliability of rAAV vector characterization from early development through commercial manufacturing.

This approach enables comprehensive rAAV characterization throughout production, supporting confirmatory release testing and early detection of manufacturing inconsistencies before cGMP batch production. The ability to assess genome integrity, sequence identity, and contaminants provides valuable insights for refining vector production, guiding risk assessments, and ensuring regulatory compliance

836 Full Length Transgene Quantification Utilizing NanoMosaic Tessie Technology

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Product characterization is a critical aspect of adeno-associated virus (AAV)-based gene therapy development, where accurate quantification of full-length transgenes and capsid titers is essential for maintaining consistent quality, safety, and efficacy. Currently the AAV field utilizes several approaches to quantify product integrity, including both molecular and biophysical methods. However, none of the currently available methods allow for genome integrity and capsid titer measurements on the same sample utilizing the same device. Combining data obtained from multiple systems compounds assay variability resulting in decreased accuracy, thereby lessening the value of the obtained results.

Traditional methods, such as qPCR and ddPCR, are limited in their ability to distinguish full-length genomes from truncated or fragmented sequences, and when combined with ELISA for capsid titer, suffer from compounded variability. Similarly, current methodologies for analyzing the empty/full capsid ratio, such as analytical ultracentrifugation (AUC), are not just labor-intensive, with a low assay throughput, they cannot identify what is being packaged. While these methods provide value at specific stages in the AAV production process, newer technologies are needed that can provide a more accurate quantification of true full-length vector genome. This abstract discusses advantages of using NanoMosaic's Tessie technology to identify full-length transgene and capsid titers in one platform.

The Tessie nanoneedle platform enables measurement of AAV transgenes and AAV capsids in one platform. The Tessie nano-plate nanoneedles are densely integrated on a silicon chip and are configured into 96 or 384 well format for high throughput workflows.

We report the development of a viral genome assay utilizing two affinity-tagged oligonucleotide probes to selectively bind specific regions of the transgene. Through limited primer extension, these probes incorporate into a double-stranded DNA product, enabling differentiation between full-length and truncated transgenes. Full-length genomes were detected by probes positioned at opposite ends of the therapeutic region, while truncated variants were identified using probes targeting suspected breakpoints. The resulting labeled DNA was quantified with high sensitivity using Tessie nanoneedle sensors, having more than 20,000 nanoneedles in each well, resulting in an accurate and scalable method for assessing AAV genome integrity.

Improvements to both the transgene and capsid assays for use on our custom capsid platform were made over traditional methods with the goal of providing an accurate and fast measurement of the empty/full ratio of our AAV products. For example, for the transgene assay, DNA probe sequences and locations along the transgene were enhanced to allow for quantification of full-length transgene; and capsid titer assay developed ligand/antibody identification, and standard curve. A combination of assays capable of characterizing the titer, genome integrity, capsid titer and empty/full ratio of AAV products compatible with samples from various process stages was developed.

837 Impact of Sample Collection Container Material, Hold Time, and Storage Temperature on Adeno-Associated Virus Endotoxin Testing

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Endotoxins are components of the outer membrane of gram-negative bacteria, and they are released upon cell lysis of the intact bacteria. The Inspection Technical Guide with the title "Pyrogens, Still a Danger" issued by U.S. Food and Drug Administration (FDA) in 1979, described the severe impacts of this pyrogen when injected in sufficient amount. Since endotoxin is a common contaminate found in biopharmaceutical product and that the amount of endotoxin in the product dose is critical, it is crucial to test the drug product samples to ensure the endotoxin level in parental solution is within the FDA's guideline of acceptance level. The questions we are trying to answer for the produced adeno-associated virus (AAV) material are: what would be the ideal sample collection container material, sample hold time, and sample storage temperature to safeguard the endotoxin data as consistently and accurately reflecting the endotoxin concentration in drug material? Also, do AAV serotypes play a role in endotoxin recovery in solution? Endotoxin recovery means the amount of endotoxin measured under different conditions versus the starting endotoxin measurement. There is limited literature on these topics, hence this study was conducted to better understand the best endotoxin testing practice for AAV.

Two common AAV serotypes were selected to verify any differences in endotoxin recovery between them. AAV samples were spiked in with an appropriate amount of Control Standard Endotoxin (CSE) to achieve comparable endotoxin concentration at time 0 of this study. A negative and a positive buffer control were included, where the positive control had similar starting endotoxin level as the spiked-in AAV samples. All these samples were tested in polypropylene and polystyrene collection containers because these two plastic materials are two of the commonly used sample collection container types. The samples were stored at three different temperatures: room temperature, 2-8ºC, and -80ºC. Then, they were measured at different time points in replicates. After all data was collected, statistical analyses were applied to check for outliers, significant differences between the data, and if there was a trend for the endotoxin recovery under different parameters.

Data indicates there is minimal difference for endotoxin recovery between polystyrene and polypropylene at 2-8ºC and -80ºC. However, at room temperature, endotoxin recovery in polypropylene sample collection containers is impacted more prominently than in polystyrene material. Data suggests the presence of AAV reduces the impact of storage temperature on endotoxin measurements for both plastic materials. This is particularly noticeable at -80ºC. Finally, regardless of which type of plastic material was used for the collection tube, or storage temperature, the endotoxin values reduced with time for both serotypes.

In summary, endotoxin measurements can be affected by sample container material, time of exposure to the container material, and storage temperature. The recovery differences between serotypes are not evident. Polystyrene is the preferred sample storage container, and testing time plays a role on the endotoxin levels measured. This study helped to determine the parameters to better test AAV samples that will yield results to more accurately reflect the endotoxin content in drug material.

838 Ultra-Cold Storage of Lentiviral and Adenoviral Vectors in Cyclic Olefin Polymer Containers

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Adenoviral (AdV) and Lentiviral (LV) vectors are major platforms for developing vaccines and cell and gene therapies. Ultra-cold storage of the adenoviral viral vectors can be helpful to ensure uninterrupted supply of vaccines in critical times such as a pandemic and in oncologic and advanced therapies. Lentiviral vectors are commonly used in cell therapy, in particular CAR-T cell generation. Thus, well-controlled ultra-cold storage of viral vector plays an important role in ensuring the quality of cell products.

Polypropylene (PP) vials and tubes have been commonly used as the primary container in research, but they may be not suitable for clinical manufacturing of drug products, due to concerns related to container closure integrity of the snap cap- or screw cap-based systems and high particle generation from the material. Cyclic olefin polymer (COP) vials may be helpful to overcome these challenges and maintain better drug safety, purity and efficacy.

2 mL Daikyo Crystal Zenith® (CZ) cyclic olefin polymer (COP) vials with bromobutyl serum stoppers and aluminum seals were compared to 1.8 mL PP screw cap vials and evaluated for the ultra-cold storage of Ad5-CMV-eGFP and Ad5.F35-CMV-eGFP viral vectors for 1 to 14 days. In a separate experiment, the vials were compared for the functional recovery of Lenti-GFP viral vectors, imitating a short-term storage condition and addressing potential concerns related to pH excursions upon exposure to and after removal from a dry ice-based shipping condition.

STUDY FINDINGS

- AdV physical recovery was higher from COP vials than PP vials particularly upon shorter terms of ultra-cold storage. In either vial types, functional recovery was comparable to fresh sample after 1 day but declined before 7 days.
- LV functional recovery was comparable between COP and PP vials, in ultra-low freezer or dry ice. There was significant functional titer loss after filling and immediately dispensing from the PP vials, whereas there was no significant loss from COP vials.
- COP vials can be a suitable alternative to PP vials for the cold chain containment of the growing generation of viral vector-based therapeutics.

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839 Recombinant Animal Origin Free Albumin in Downstream Wash and Cryopreservation for CAR-T Cell Therapy Manufacturing

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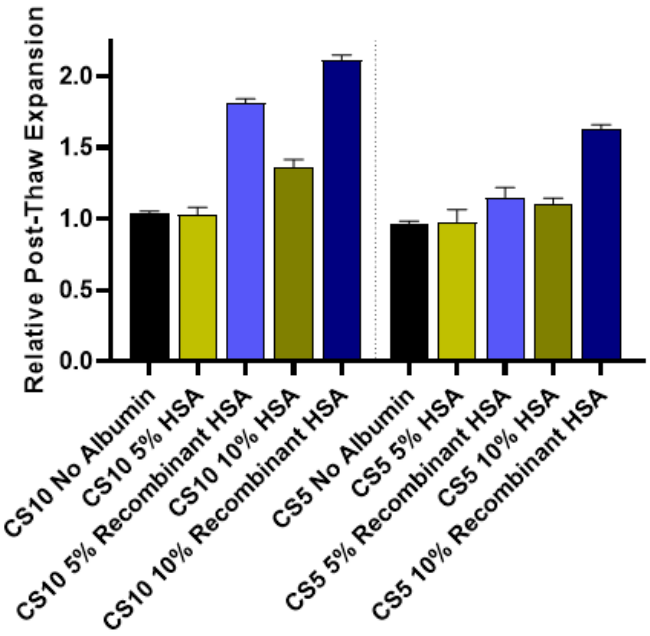
Background and Aims: Cryopreservation and cell washing are critical steps in T cell therapy manufacturing, where preserving T cell viability and key phenotypes is essential for therapeutic success. Traditional cryopreservation media and washing buffers often use clinical blood-derived human serum albumin (HSA), which presents safety, regulatory, and supply chain challenges. Recombinant human serum albumin (rHSA) produced in an animal free expression system, offers a safer, more consistent alternative. This study aimed to evaluate the efficacy of rHSA in enhancing T cell cryopreservation and improving post-wash T cell retention while maintaining CD4/CD8 ratio, memory phenotype, and proliferative capacity.

Methodology: Primary human T cells from two healthy donors were cryopreserved in commonly used formulations containing 5 or 10% DMSO with varying concentrations of rHSA (0%, 5%, or 10%) or clinical HSA. Post-thaw, T cell viability, proliferation, and memory phenotypes (Tscm, Tcm, Tem, Temra) were assessed over 72 hours using live-cell imaging and flow cytometry. For cell washing, T cells were thawed, activated, and washed three times with 1% or 5% rHSA or clinical blood-derived HSA solutions in PBS. The CD4/CD8 ratio, memory phenotypes, cell retention, and post-wash proliferation were evaluated immediately after washing and after three or six days of culture.

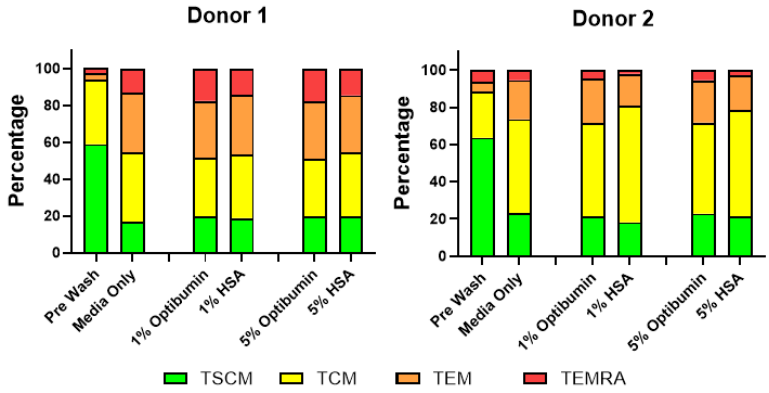
Results: In cryopreservation, rHSA significantly improved T cell recovery and proliferation compared to controls. Animal origin free rHSA also reduced final DMSO concentrations, improving the safety profile. The rHSA preserved early memory phenotypes (Tscm and Tcm) comparably to clinical HSA and helped maintain a balanced CD4/CD8 ratio by mitigating CD8+ T cell loss. In the washing process, rHSA significantly improved cell retention,

preserved the CD4/CD8 ratio, and maintained early memory phenotypes (Tscm and Tcm). These cell washing experiment results were also comparable to clinical HSA.

Conclusion: Animal origin free rHSA significantly improved T cell cryopreservation outcomes by enhancing viability, proliferation, and preserving phenotype, while reducing DMSO concentrations. It also improved cell retention during washing and preserved T cell phenotype, supporting critical early memory phenotypes essential for CAR-T therapies. As an animal origin free alternative, rHSA addresses regulatory and safety concerns, providing consistent performance for both cryopreservation and washing steps in cell therapy manufacturing.



Normalized T cell expansion after 72 hours post-thaw, comparing cryopreservation conditions inCS10 (10% DMSO) and CS5 (5% DMSO) with no albumin, 5% or 10% rHSA (recombinant human serum albumin), or a clinically used blood-derived HSA. rHSA enhanced post-thaw expansion in a dose-dependent manner.



Proportions of stem cell memory (Tscm, green), central memory (Tcm, yellow), effector memory (Tem, orange) and terminal effector memory (Temra, red) cells in T cells from two different donors. Flow cytometry analysis performed before the wash step (*Pre-Wash*) and on Day 3 post-wash using 1% and 5% concentrations of either *Optibumin* or Clinical HSA.

840 Development of In Vitro Potency Assay for AAV-mediated Gene Therapy for Muscular Dystrophy

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Adeno-associated virus (AAV)- mediated gene therapy has accelerated the therapy development for muscle disorders in recent years. AAVs with multiple modalities including gene replacement, knockdown, CRISPR and exon skipping, are at the forefront of delivering these therapeutics for patients with Muscular Dystrophy. With many of these programs gearing up for the in-human clinical trials, potency determination of the clinical grade AAV therapeutic remains one of the crucial aspects of the vector release as well as continued stability testing. For most of the current AAV serotypes, the potency assessment is currently performed in vivo in rodent models of the disease, which is extremely time consuming, labor intensive and expensive. Thus, there is critical need for the development of a robust and quantitative in vitro cell based assay for potency determination. Unfortunately, the current vectors in use (especially AAV9) do not readily transduce the cells in vitro. In the current study, we have developed a method to determine the potency of AAV9 vector in vitro using patients-derived fibroMyoD (FM) cell line from healthy donors and patients with Duchenne Muscular Dystrophy (DMD), as well commercially available primary myoblasts. We show that this method can be utilized to determine the dose dependent increase of expression of the therapeutic protein or exon skipping upon transduction of AAV9 vector at increasing multiplicity of infections (MOIs). This novel method is suitable not only for initial determination of efficacy of the vectors, but also for the potency determination of clinical grade vectors for release testing, lot-to-lot variability testing and stability.

841 pART Platform Process for Plasmid Manufacturing: Scalable, Robust, and Built for Tomorrow's Therapies

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The demand for plasmid DNA (pDNA) in different qualities has grown substantially in recent years, as it plays a critical role in the rapidly expanding sector of next generation vaccines and therapies. It acts as an active pharmaceutical ingredient (API) in DNA vaccines and cell and gene therapies (C>), as a critical raw material in the production of viral vectors, and as a template for therapeutic mRNA. Meeting the growing demand for pDNA and securing a reliable supply chain require the development and validation of robust platform manufacturing processes. These processes must deliver consistent quality across various scales, while complying with stringent regulations and remaining cost effective.

Manufacturing processes based on the fermentation of *E. coli* as a host have proven to be the most reliable method for industrial pDNA production, offering flexibility, robustness and scalability to bioreactors of several thousand liters. *E. coli* grows rapidly in inexpensive media and produces large quantities of pDNA within short timeframes. A typical manufacturing process consists of three main stages: the fermentation of *E. coli* in a bioreactor (USP – Upstream processing), cell lysis and clarification of the pDNA containing supernatant (MSP – midstream processing), and the use of advanced purification techniques (DSP – downstream processing) for the recovery of highly purified supercoiled (SC) pDNA, which is widely recognized as the therapeutically most relevant isoform.

The development of a robust and cost-effective platform process involves addressing several challenges in a systematic manner. While growth medium optimization and the use of sophisticated feeding strategies can be used to increase output and volumetric productivity during the USP, the produced pDNA molecules are large and sensitive to mechanical stress, which can ultimately lead to changes in their topological form. This, coupled with the increased viscosity of high cell density lysates, makes standard clarification methods, such as those using depth filters or hollow fibers, particularly difficult. Additionally, the target SC pDNA must be purified from a complex mixture of host cell proteins (HCP), genomic DNA (gDNA), endotoxins and unwanted pDNA isoforms. Some of these contaminants share similar physicochemical properties, comparable sizes, or a net negative surface charge. The pART process was developed to overcome all these issues in a ready-to-use approach, offering tailored solutions for pDNA supply that ensure consistent quality from small to large scales. These results have been demonstrated for plasmid sizes ranging between 3 to 19 kb.

842 Risk Assessment for Cross-Contamination of Plasmids Used for AAV Production

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Plasmid transient transfection is a fast and flexible method to produce adeno-associated virus (AAV) used as a gene therapy vector. Recently, health authorities have highlighted cross-contamination between different plasmids produced in multi-product plasmid facilities as a potential safety risk for gene therapy products. This study examines factors influencing the safety risk of plasmid cross-contamination between AAV products.

There are three general types of plasmids used to produce AAV products through triple transfection: 1) gene-of-interest (GOI) plasmids encoding a transgene flanked by inverted terminal repeats, 2) plasmids encoding AAV *rep* and *cap* genes, and 3) plasmids encoding adenovirus helper genes. From a patient safety perspective, the highest risk scenario is cross-contamination of an AAV GOI plasmid into another plasmid used for production of a different AAV product. Such a contamination event results in production of an AAV capsid capable of delivering a contaminant transgene to patients. Within the AAV process, plasmid ratios and relative titer of contaminant and contaminated plasmid both impact the concentration of contaminated AAV product found in an AAV drug substance. The risk to the patient is further influenced by the toxicity of the contaminant transgene and the dose of the contaminated product.

The risk from cross-contamination in multi-product plasmid production facilities can be mitigated by employing cleaning solutions which degrade plasmid DNA. While it is a common practice to use NaOH to clean equipment in plasmid facilities, we provide data demonstrating that NaOH does not robustly degrade plasmid DNA. To address this gap, we will show data on an alternative cleaning solution which degrades plasmid DNA more effectively.

Finally, we consider all these factors in a case study using the GOI plasmid pGOIx as a model contaminant. pGOIx is used to produce the AAV gene therapy product GOIx, and clinical data from the GOIx program was used to perform the safety assessment. The outcome of the pGOIx case study is that the risk to patients from plasmid cross-contamination of pGOIx into other AAV plasmids is low, as long as appropriate cleaning and changeover procedures are used in the plasmid production facility. This safety assessment requires product toxicity data and production process parameters which may not yet be available in other AAV products prior to plasmid production. Thus, it should not be expected that a safety assessment such as this will be performed to justify routine plasmid changeover; rather, this case study is intended to aid plasmid and AAV manufacturers in reducing the risk of plasmid cross-contamination by highlighting relevant risk factors.

843 Advanced Plasmid Linearization Using Type I and Type II Restriction Enzymes: A Scalable, High-Purity Platform for Cell and Gene Therapy Applications

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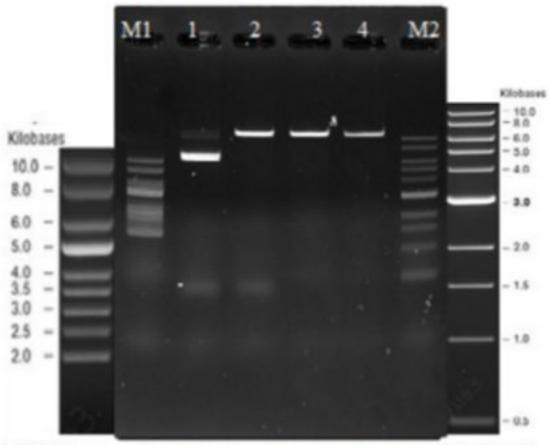
Plasmid DNA serves as a critical starting material for cell and gene therapies due to its ease of design, low production costs and high stability for long term storage.1 Today, plasmids are used to manufacture several advanced therapies which include DNA vaccines, viral vectors (AAV and LVV), mRNA therapies and vaccines, transient protein therapies (mAbs) and gene modified therapies, such as CAR-T.2 For some processes, plasmids are linearized as this can increase transfection efficacy, improve accessibility for transcription and allow for enhanced gene expression for non-viral delivery therapies.1 ProBio has developed a 3-step platform plasmid purification process for the purification of supercoiled plasmid DNA with capabilities of generating greater than 90% supercoiled plasmid with endotoxin levels less than 10 EU/mg. When followed by plasmid linearization, the entire process has a quick turnaround time of 4 weeks.

The linearization process is done by using restriction enzymes to cut circular plasmid DNA at specific sites, which transforms it into a linear plasmid.3 Restriction enzymes are categorized into three classes, Type I, Type II and Type III. In this poster, we demonstrate our plasmid linearization capabilities using Type I and Type II enzymes for three plasmids with our platform process. Type I enzymes were used to linearize LVV plasmid NF04 and pHelper plasmid JGIA, respectively. Type II enzymes were used to linearize a polyA plasmid, referred to as, Plasmid A. The plasmids were incubated with the enzymes for 16-20 hours until digest was completed and purified using mixed modal Capto Core 700 resin. Figure 1 shows Agarose Gel Electrophoresis results for undigested and linearized JGIA respectively. Our Analytical data summarized in Table 1 confirms that the purified supercoiled and linearized plasmids (NF-04, JGIA, Plasmid A) have >95% purity which meets current FDA standards. Further, the current platform plasmid linearization process has a recovery rate of >40%, host cell protein impurities less than 1% and endotoxin level less than 10 EU/mg. In all cases, we were able to remove undigested non linearized plasmid using mixed modal chromatography resin and able to achieve 100% linear plasmid as the final product.



M1: Supercoiled DNA Ladder Marker (NEB:N0472s)
M2:1kb DNA Ladder (NEB:N0550S)

Lane#	Sample ID
1	Undigested JGIA
2	Digested JGIA
3	CORE700 Elu
4	Final JGIA

Table 1: Analytical Data for ProBio Linearized Plasmids

Analytical Data	NF-04	JGIA	Plasmid A (Poly A)
% Plasmid Recovery	41.67	49.06	51.08
% Linearization in final plasmid	100	100	100
% HCP	<1	<1	<1
Endotoxin Conc. in Linearized Plasmid	<10 EU/mg	<10 EU/mg	<10 EU/mg

844 Revolutionizing Plasmid DNA Production: A Chromatography-Free, High-Yield, High-Quality Platform

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Background: Plasmids are indispensable tools in the cell and gene therapy (CGT) field, serving as critical raw materials for advanced therapies such as viral vector production and enabling the delivery of therapeutic genes. Their ability to carry and express genetic information has revolutionized treatments for genetic disorders, cancers, and infectious diseases. Despite significant advances in plasmid design and delivery systems, downstream processing remains a major bottleneck in plasmid biomanufacturing. Traditional reliance on multiple chromatographic steps often leads to low yields, high costs, and operational complexity, underscoring the need for innovative solutions to streamline production while maintaining high-quality standards.

Methods: In this study, we developed and validated a novel, chromatography-free plasmid purification platform designed to produce therapeutic-grade plasmid DNA with reduced processing complexity and manufacturing costs. The platform was optimized through three key innovations: (1) enhanced alkaline lysis conditions informed by a

mechanistic understanding of cellular disruption and precipitation dynamics, (2) controlled solid-liquid separation to preserve precipitate integrity, and (3) development of control strategies to minimize supercoiled to open circular plasmid conversion and (4) novel approach for selective removal of open circular plasmid during purification.

Results: The optimized platform technology consistently preserved high supercoiled plasmid integrity while efficiently removing host cell-derived contaminants and controlling product-related impurities, such as open circular and linear plasmid DNA forms. The final product met stringent quality specifications, achieving >90% supercoiled purity, endotoxin levels <10 EU/mg, residual host cell protein <0.1%, host cell DNA <0.1%, and RNA content <0.1%. The chromatography-free process achieved a recovery rate of >50%, outperforming traditional chromatography-based methods in both efficiency and cost-effectiveness. Furthermore, the process inherently reduces cross-contamination risks, making it well-suited for large-scale GMP manufacturing and ensuring robust regulatory compliance.

Conclusion: This innovative chromatography-free platform represents a transformative advancement in plasmid DNA manufacturing. By eliminating chromatographic steps, it offers a scalable, cost-effective, and operationally simplified solution for commercial-scale production. Its ability to consistently meet therapeutic-grade quality standards positions it as a powerful tool for accelerating the development of plasmid-based therapeutics, vaccines, and personalized medicine, addressing critical challenges in the rapidly growing CGT field.

845 Animal Origin-free Yeast and Soytone-based Fed batch Fermentation as a Versatile Platform for High-Yield Plasmid Production

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The production of plasmids, crucial for gene therapy applications, vaccine development, and biotechnology, often faces yield limitations during the upstream fermentation. Fermentation media design and nutrients screening have been a point of focus in improving overall yield. This study investigates the use of Gibco™ Animal Origin Free (AOF) yeast and soytone to enhance cell growth and plasmid production across various cell lines and plasmid constructs including plasmids destined for viral vector and *in-vitro* transcription applications. The impact of yeast and soytone-based feed approach on plasmid yield was tested under controlled and identical media formulation (CD media), fermentation temperatures, pH, Agitation, and dissolved oxygen conditions across *E. Coli* strains and constructs. The process demonstrated a consistent improvement in both cell growth and plasmid yield, particularly in conditions where traditional media fall short. The findings suggest that Gibco™ AOF yeast and soytone feed can be an effective supplement to standard fermentation media, offering a cost-effective solution to optimize plasmid production and increase yields up to 1 g/L of culture. Data showed that under optimal fermentation conditions the use of yeast and soytone minimizes the accumulation of byproducts such as acetate, regardless of the *E.Coli* strain.

846 Scalable and Efficient Production of Plasmid using Yeast and Soytone Fed batch: Optimizing Single Use Technology Fermentation and Purification Processes for cGMP production

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With advances in the biotechnology industry, the demand for plasmid DNA (pDNA) to support viral vectors, mRNA/DNA-based biotherapies and vaccines has significantly increased. As several plasmid-based products

advance towards FDA approval, there is a growing need for cost-effective and safe processes capable of producing plasmids at both gram and kilogram scales. Although specialized production facilities have been developed to meet the increasing demand, the expansion of capacity brings challenges such as large-volume handling and the risk of cross-contamination between production campaigns. These challenges highlight the need for a highly productive fermentation process that utilizes fully disposable product-contact materials to mitigate these risks. Culture metabolic profiles and pDNA production trends were used to design and optimize a Gibco™ yeast and soytone-based feed to supplement Gibco™ CD Media. This process consistently showed high performance across a range of plasmid constructs. The developed process was scaled up using our process transfer strategy from the 0.25L Ambr250M to 5L scale during process development, followed by scaling up to cGMP 50L and 200L single use Fermenters. Results showed scalability in the fermentation process and consistency in cell growth and plasmid productivity. Moreover, downstream purification using a 2-column purification process on our single-use platform demonstrated consistent yields from development through cGMP production. Quality control test results for cGMP grade final formulated plasmids meets release specifications for 5L, 50L and 200L scale batches

847 Advances in Gene Therapy for Profound Hearing Loss: An Update on the CHORD Phase 1/2 Trial

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Introduction: Otoferlin plays a critical role in inner hair cell (IHC) signal transmission to auditory nerve fibers. Biallelic pathogenic variants of the *otoferlin* (*OTOF*) gene typically cause severe-to-profound deafness. Preclinical data for DB-OTO, an adeno-associated virus gene therapy carrying the human *OTOF* cDNA (complementary DNA), showed IHC-selective otoferlin expression and auditory brainstem response (ABR) recovery in mutant mice that may translate into high-quality physiological hearing in humans. The ongoing, first-in-human, multicenter phase 1/2 open-label CHORD clinical trial (NCT05788536) is evaluating the safety, tolerability, and efficacy of DB-OTO in pediatric patients with profound *OTOF*-related deafness.

Methods: In this phase 1/2 trial, participants <18 years of age with biallelic pathogenic or likely pathogenic variants in *OTOF*, with profound hearing loss by behavioral pure tone audiometry and no detectable response on ABR testing at maximum stimulus levels, are being enrolled. DB-OTO, at a dose of 7.2×10^{12} vector genomes, was administered as a unilateral or bilateral intracochlear injection using a typical facial recess approach via the round window. Safety and efficacy measurements were performed at multiple timepoints before and after treatment.

Results: Previously, early safety and efficacy data were presented. Surgical delivery has been uneventful in all participants, no dose-limiting toxicities or DB-OTO–related adverse events have been reported, and no persistent vestibular manifestations have been observed to date. Longer-term efficacy and durability data from the first participant, and efficacy data from the first 11 patients enrolled in the trial including initial data from bilaterally dosed patients will be presented.

Conclusion: Gene therapy has recently emerged as a promising approach to treat congenital hearing loss. In this first-in human gene therapy trial, DB-OTO was well tolerated overall. These encouraging data suggest that DB-OTO gene therapy has the potential to enable physiological hearing in children with *OTOF*-related deafness.

848 Initial Results from Gene Therapy for X-linked Retinoschisis *via* Subretinal Delivery of scAAV8-hRS1 in Pediatric Patients

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Introduction: X-linked retinoschisis (XLRS) is an X-linked recessive retinal degenerative disease, resulting from mutations in *RS1* gene. It is the leading cause of macular degeneration in male children. We report the safety and efficacy of subretinal delivery of scAAV8-hRS1 in pediatric patients with XLRS.

Methods: This single-arm, single-center trial enrolled pediatric patients (aged 5–18 years) with confirmed *RS1* mutations. All participants received a single unilateral subretinal delivery of scAAV8-hRS1. The primary outcome was the incidence of treatment-emergent adverse events (TEAEs), while secondary outcomes were best-corrected visual acuity (BCVA) and swept-source optical coherence tomography (SS-OCT). Initial data through the 26-week study period is reported. This trial is registered at the Chinese Clinical Trial Registry, ChiCTR2300076682.

Results: Nine patients completed the 26 weeks of follow-up. They were divided into two dose cohorts: cohort 1 (n=6) and cohort 2 (n=3). A total of 38 TEAEs were reported, with no grade 3 or higher TEAEs. No TEAE was related to the study drug. No ocular inflammation was observed. The treated eyes showed a mean BCVA improvement of 9·3 letters (SD 3·7) in cohort 1 and of 10·0 letters (SD 3·6) in cohort 2. SS-OCT revealed that by week 13, macular schisis cavities completely closed in all patients with no recurrences till the 26-week follow-up. The mean central retinal thickness in treated eyes decreased by 479·3 µm (SD 150·3) in cohort 1 and by 432·7 µm (SD 150·1) in cohort 2. Seven out of nine patients demonstrated restoration of the continuity of outer retinal layers, including the external limiting membrane and ellipsoid zone. Both retinal structure and function were restored in the treated eyes of all 9 participants.

Conclusion: In the 26-week study, the treatment successfully restored both retinal structure and function in the in pediatric patients with XLRS, with no drug-related severe adverse events. These promising results supported the further development of scAAV8-hRS1 gene therapy for patients with XLRS.

849 Safety and Efficacy of ZM-02 Optogenetic Gene Therapy Clinical Study for Advanced Retinitis Pigmentosa

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Introduction: Retinitis pigmentosa (RP) is the most common inherited retinal disease, affecting over 200 million people globally, with no approved treatment. Advanced RP causes significant decreases in visual acuity, visual field, and quality of life. ZM-02 is an optogenetic gene therapy using an adeno-associated virus to deliver a novel photosensitive protein gene, PsCatCh2.0, via intravitreal injection. Thus, we conducted a clinical study, MOON trial (NCT06292650) to evaluate a single injection of ZM-02 in advanced RP patients with various causative genes.

Methods: MOON trial is a randomized, single-blinded, dose-escalation, first-in-human study investigating the safety, tolerability, and efficacy of ZM-02 (AAV-PsCatCh2.0) in RP patients. Eligible participants had best-corrected visual acuity (BCVA) no better than finger counting (≥ logMAR 2.0), loss of photoreceptor layer, and retained ganglion and bipolar cell layers. Participants were randomly assigned (3:1) to intervention (cohort 1-high dose, Cohort 2-low dose) or control group (Sham injection). Participants were assessed before and after treatment at designated follow-ups for 1 year. The primary endpoints were incidence of adverse events (AEs), serious adverse events (SAEs), and dose-limiting toxicities (DLTs). Secondary endpoints were BCVA, and multi-luminance tests (MLTs).

Results: Eight RP participants (mean 36.25 ± 9.25 years old) were enrolled: Cohort 1 (n=4), 2 (n=2), and 3 (n=2). All participants in Cohort 1 and 3, and one in Cohort 2, completed at least 9 months' follow-up; the other in Cohort 2 completed 6 months. Most AEs were mild (≤ CTCAE Grade 2), resolved with topical treatment, and were attributed to the surgical procedure. No DLTs or SAEs were reported (Figure 1). At the most recent visit, 83.3% of the intervention group (Cohorts 1 and 2) showed clinically significant BCVA improvement (0.54 ± 0.30 logMAR), and 100% had improvement in at least two out of three MLTs. Notably, the intervention group showed progressive improvement in color vision (Figure 2).

Conclusions: This trial indicated that ZM-02 has a notable safety and tolerability profile with no drug-related AEs. Efficacy results showed significant BCVA improvement, supported by MLTs, with an unexpected bonus in color perception. These findings suggest that ZM-02 holds promise in advanced RP treatment.

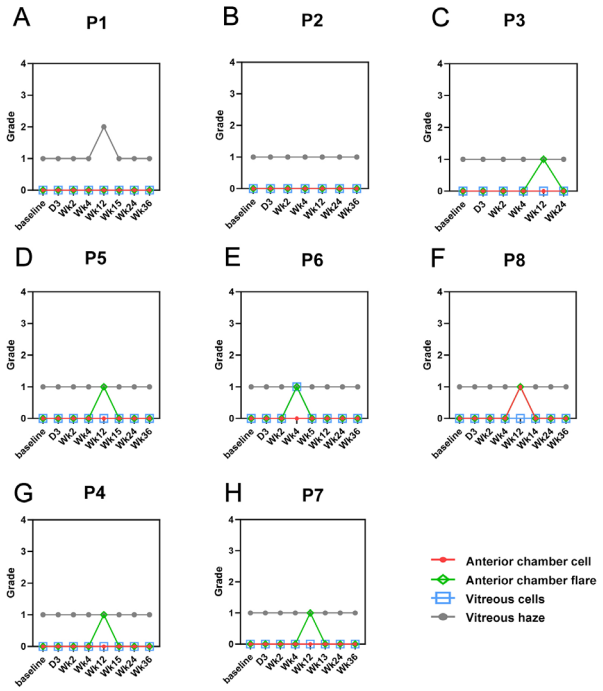

Figure 1. Adverse Events of Participant 1-8 according to SUN (Standardization of Uveitis Nomenclature). (A-F) Inflammation Grade according to SUN from baseline to the most recent visit of intervention Group (Cohort 1 and 2, Participants 1, 2, 3, 5, 6, 8). (G-H) Inflammation Grade according to SUN from baseline to the most recent visit of intervention Group (Cohort 3, Participants 4,7).

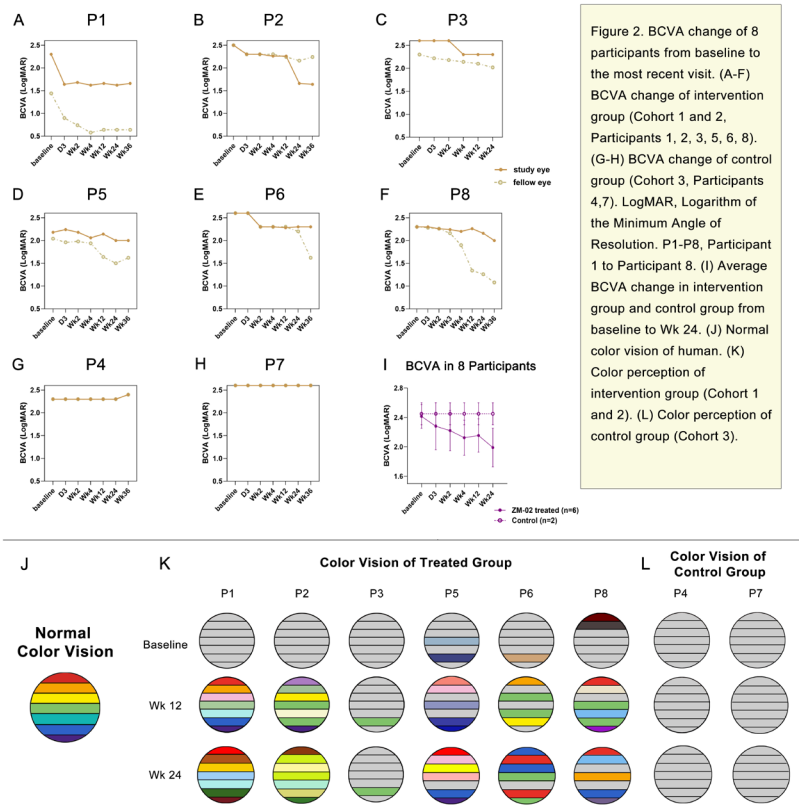

Figure 2. BCVA change of 8 participants from baseline to the most recent visit. (A-F) BCVA change of intervention group (Cohort 1 and 2, Participants 1, 2, 3, 5, 6, 8). (G-H) BCVA change of control group (Cohort 3, Participants 4,7). LogMAR, Logarithm of the Minimum Angle of Resolution. P1-P8, Participant 1 to Participant 8. (I) Average BCVA change in intervention group and control group from baseline to Wk 24. (J) Normal color vision of human. (K) Color perception of intervention group (Cohort 1 and 2). (L) Color perception of control group (Cohort 3).

850 Gene Therapy Clinical Trials Targeting Optic Neuropathies and Other Ocular Diseases until 2024

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Introduction: Gene therapy is a promising approach that has the potential to treat incurable conditions such as optic neuropathies. We have compiled and analyzed all gene therapy clinical trials on optic neuropathies and other ocular diseases until 2024.

Methods: The ocular gene therapy clinical trials were searched from the following websites: US National Institute of Health ClinicalTrials.gov, International Standard Randomised Controlled Trial Number Register, EU Clinical Trials Register, Gene Therapy Net, Australia New Zealand Clinical Trials Registry (ANZCTR), EU Deliberate release database on clinical trials (JRC), Chinese Clinical Trial Registry (ChiCTR), US National Cancer Institute Cancer Trials, Belgian Biosafety Server Clinical Trials Database, World Health Organization International Clinical Trials Registry Platform, German Clinical Trials Register, Japan Registry of Clinical Trials (JRCT), UMIN Clinical Trials Registry (UMIN-CTR), Wiley database on Gene Therapy Trials Worldwide, and UK Gene Therapy Research.

Results: There were a total of 260 ocular gene therapy clinical trials from 1998 to 2024. Among these, 30 trials (12%) targeted optic neuropathies, with Leber's hereditary optic neuropathy (LHON) being the most studied condition, comprising 16 trials. Other optic neuropathy trials included 3 for autosomal dominant optic atrophy, 2 for nonarteritic anterior ischemic optic neuropathy (NAION), and 1 for ischemic optic neuropathy. 8 trials targeted systemic disorders affecting the optic nerve such as neuromyelitis optica spectrum disorder (NMOSD) and multiple sclerosis. Of the remaining ocular trials, 221 focused on retinal diseases, 4 on glaucoma, 3 on corneal diseases, and 2 on uveitis.

In terms of therapeutic approach, 174 trials (67%) used gene augmentation, 31 trials used RNA therapy, 9 trials utilized systemic gene therapy, 7 trials used gene therapy targeting pathogenic viruses, and 3 trials used oncolytic viruses. 18 trials were observational extensions of interventional studies. 26 trials used cell-based gene therapy; of these 18 trials used encapsulated cell technology, 7 trials used CAR T cell therapy and 1 trial used another approach. Of the gene augmentation trials, 159 (61%) used adeno-associated virus (AAV) as a vector, 8 used lentiviral (LV) vector, 2 used simian immuno-deficiency virus (SIMV), and 1 used adenoviral vector (AV). Of all the years, 2023 had the maximum number of trials at 53, followed by 2024 with 32 trials. 61 trials (23%) were academic, and 199 trials (77%) were sponsored by industry.

In terms of the trial phase, 65 trials were phase 1, 81 trials were phase 1/2, 43 trials were phase 2, 12 trials were phase 2/3, 25 trials were phase 3, and 1 trial was phase 4, 30 trials did not specify the phase.

Conclusion: Our analysis shows that of the gene therapy clinical trials targeting optic neuropathies, the majority targeted LHON, followed by systemic conditions affecting the optic nerve. Overall, clinical trials on gene therapy for ocular diseases have been increasing vigorously as the years progress with a peak of 53 trials in 2023. This growth highlights the expanding interest and advancements in gene therapy approaches for addressing ocular conditions.

851 Efficacy and Safety of ART002, a Single-dose Gene Editing Therapy, in Patients with Severe Heterozygous Familial Hypercholesterolemia

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Background and Aims: Heterozygous familial hypercholesterolemia (HeFH) is one of the most common hereditary metabolic disorders. Severe HeFH (LDL-C>5 mmol/L or 190 mg/dL with other risk factors) confers significant increased risk for atherosclerotic cardiovascular disease (ASCVD) but only a small proportion of patients have achieved the LDL-C goal. Statins are the primary treatment but it is not enough to effectively reduce LDL-C, and a significant proportion of patients are unable to tolerate high-intensity statins. PCSK9 inhibitors as an add-on therapy to statins can further reduce LDL-C levels but the real-world reported adherence to PCSK9 antibody therapy is suboptimal. While siRNA has improved drug convenience and adherence, the average LDL-C reduction of Inclisiran over 4 years is only 44% even with maximally tolerated statins. ART002 is an investigational CRISPR/Cas9-based *in vivo* gene editing therapy delivered via lipid nanoparticles (LNP), designed as a single-course treatment to permanently inactivate the liver *PCSK9* gene. We aim to evaluate safety and efficacy for ART002 in patients with severe HeFH.

Methods and Results: In this open-label, single-arm clinical trial (ChiCTR2400093099) in China, 6 adults with HeFH (diagnosed by LDLR or APOB pathogenic mutants) were subjected to single intravenous infusion of ART002 at doses of 0.3, 0.75 or 1 mg/kg, with N=1, 3 or 2, respectively. At study baseline, 4 of 6 patients did not receive statins and mean LDL-C was very high (>6.2 mmol/L or 240 mg/dL). For those without baseline statin use, low- or moderate-intensity statin was initiated as a combination therapy three weeks or more after ART002 treatment. The primary endpoint was safety and tolerability of ART002 in HeFH patients. Secondary endpoints included the assessment of pharmacodynamics, specifically changes in plasma PCSK9 and serum LDL-C levels at week 24. The data cutoff for the analysis was 17-Jan-2025. ART002 demonstrated a favorable safety profile with no serious adverse events (SAEs) or significant immune toxicities reported. No elevations in liver enzymes beyond CTCAE Grade 2 was observed. Adverse events were limited to mild, self-limiting and mostly have resolved without any intervention. The administration of ART002 achieved remarkable reductions in plasma PCSK9 levels reaching up to 91% with mean reductions of 73%, 86% and 86% in the 0.3, 0.75 and 1 mg/kg groups, respectively. Similarly, LDL-C levels were reduced by up to 68%, with mean reductions of 62%, 57% and 56% across the same dose groups at the latest follow-up (8–24 weeks post-infusion). Notably, a patient with a challenging heterozygous LDLR-negative mutation demonstrated a substantial 81% reduction in PCSK9 and 43% in LDL-C. Patients with heterozygous

LDLR or APOB-defective mutations in the 0.75 and 1 mg/kg groups achieved noteworthy reductions of 87% in PCSK9 and 59% in LDL-C. Overall, an impressive 83% of patients successfully reached the target of ≥50% LDL-C reduction, underscoring the efficacy and potential impact of ART002 in effectively lowering LDL-C levels across diverse patient populations.

Conclusions: ART002 is the first single-course gene-editing therapy targeting PCSK9 that has been clinically proven to be safe and effective for the treatment of severe HeFH across all treated patients to date. This innovative therapy may potentially revolutionize the treatment landscape for hypercholesterolemia and ASCVD.

852 Initial clinical results from OTC-HOPE, the first *in vivo*, liver directed, AAV-mediated gene insertion study in neonatal OTC deficiency: complete clinical response observed in first male infant to receive ECUR-506 and complete 24-week Phase 1/2 study

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Background: Ornithine transcarbamylase deficiency (OTCD) results in impaired ureagenesis and hyperammonemia due to congenital deficiency of the urea cycle enzyme, ornithine transcarbamylase (OTC). Severe neonatal onset OTCD patients have marked reductions in OTC activity resulting in severe hyperammonemia and low blood urea nitrogen (BUN). Males with severe neonatal onset OTCD typically become hyperammonemic within the first 48-72 hours of life and are commonly misdiagnosed. Current standard of care includes limiting protein intake and administration of nitrogen scavenger drugs. While this aids in lowering plasma ammonia, impairment of ureagenesis and risk for hyperammonemia persist. Despite aggressive medical management, repeat hyperammonemic events are common and mortality rates are high thereby necessitating liver transplantation for some patients. ECUR-506 is a liver-directed, investigational gene editing product being developed for the treatment of neonatal onset OTCD. ECUR-506 is a mix of two AAVrh79 vectors, one encoding a meganuclease, M2PCSK9, for targeted editing of the human PCSK9 gene, and the other, a codon-optimized human OTC donor gene co-administered intravenously in a 1:3 ratio, respectively. Dual vector administration is designed to guide OTC transgene integration into the hepatocyte genome which may result in the durable production of functional OTC enzyme.

Materials and Methods: The OTC-HOPE clinical trial (NCT06255782) is a 24-week, first in human, single arm, Phase 1/2, open-label, global, multi-center trial designed to assess safety and efficacy of ECUR-506 in males with neonatal onset OTCD who are <9 months of age and 3.5 kg to 10 kg at the time of dosing. Planned dose levels were informed by non-clinical studies. Study participants will be included in a 14.5-year follow-up study. Herein, initial clinical and biomarker data of the first study participant to complete the 6-month clinical trial are described.

Results: The participant experienced 2 hyperammonemic events by 3.5 months of age and underwent ECUR-506 (1.3×10^{13} GC/kg) infusion at 6.5 months of age. Asymptomatic transaminitis was observed between weeks 4-8 post-infusion, and a liver biopsy confirmed an acute T-cell inflammatory response, which resolved with immunosuppression. Normal ammonia and reduced glutamine levels prompted nitrogen scavenger medication discontinuation and liberalization of protein intake by weeks 12 and 15, respectively. Subsequently, mean ammonia levels were normal, no hyperammonemic events were observed, mean BUN levels and urinary nitrogen were increased relative to pre-treatment levels, and normal infant growth trajectory were observed over the remainder of the 6-month study.

Conclusion: These are data from the first infant to undergo in-vivo, liver directed, AAV-mediated gene insertion and to complete the OTC-HOPE clinical trial. The observed increase in BUN and urinary nitrogen along with normal mean ammonia levels and increased protein intake following ECUR-506 administration in conjunction with scavenger medication discontinuation are suggestive of increased nitrogen flux through the urea cycle and restoration of at least partial functional hepatic OTC enzyme activity. The complete clinical response, as defined in the OTC-HOPE clinical protocol, achieved in this patient support the continued evaluation of low dose ECUR-506 (1.3×10^{13} GC/kg) in the clinical trial.

853 Durable Efficacy and Safety of DTX301: Long-term Follow Up (LTFU) of a Phase 1/2 Trial in Adults with Ornithine Transcarbamylase Deficiency (OTCD

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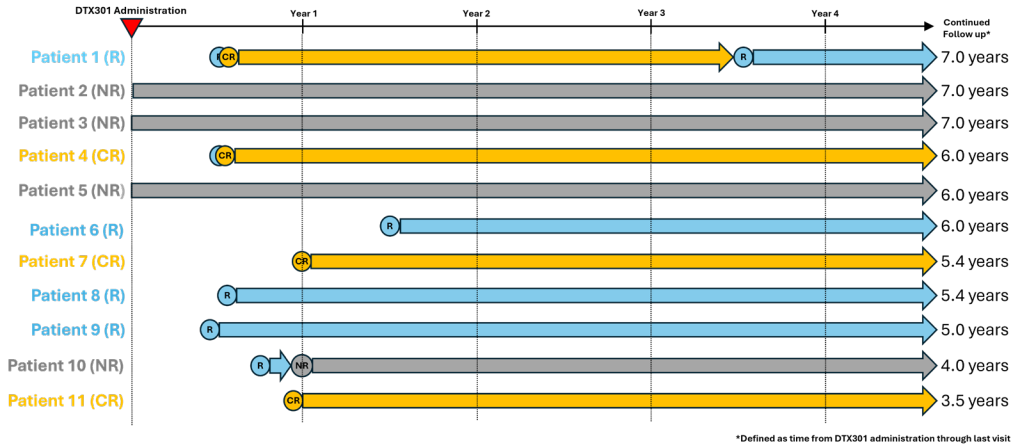
Introduction: OTCD is an X-linked urea cycle disorder resulting in a life-long risk of episodic hyperammonemia that can lead to cumulative brain damage, coma, or death. DTX301 is an AAV8 vector containing the OTC transgene being investigated for treatment of OTCD. We report the LTFU of 11 adults with late-onset OTCD who received a single IV infusion of DTX301 in the Phase 1/2 trial (NCT02991144).

Methods: The initial study was a global, multicenter, open-label phase 1/2 dose-finding study that lasted 52 weeks with an ongoing long-term extension for an additional 8 years. Patients received one IV infusion of DTX301. Relative to baseline in the Phase 1/2 trial, Complete Responders discontinued all ammonia-scavenger medications and protein-restricted diet, and Responders had ≥50% reduction in medications and dietary protein restriction.

Results: Eleven patients were enrolled in the Phase 1/2 trial. All have continued in the extension study, with up to 7 years of follow up to date. No treatment-related serious AEs, dose-limiting toxicities, or infusion-related events have been reported. During the primary study, six patients experienced transient changes in liver function due to AAV8 vector administration. All resolved following corticosteroid administration. During LTFU, three patients experienced mild ALT elevations and two experienced hyperammonemia or hyperammonemic crises, all considered unrelated to DTX301. Corticosteroids were not associated with increased ammonia, hyperammonemia, or hyperammonemic crisis. Five patients had corticosteroid-related AEs. Most were mild (grade 1), though one patient had grade 3 AEs of bone infarction, osteonecrosis, and subcortical collapse that led to knee replacement. As of 09 December 2024, 10 of 11 patients have remained clinically stable and 7 have shown a meaningful clinical response to DTX301. 3 patients were Complete Responders, 4 were Responders, and 4 were Nonresponders at last follow up (**Figure 1**). The overall mean rate of ureagenesis increased as early as Week 6 after DTX301 administration and increases were maintained. The rate of ureagenesis increased from baseline to last visit for 10 patients (91%); only 1 Nonresponder showed no increase over this time. From Baseline to last visit, mean 24-hour plasma ammonia area under the curve (AUC) values decreased in 8 patients (73%), 2/3 Complete Responders, 3/4 Responders, and 3/4 Nonresponders. One patient was reclassified from Responder to Nonresponder at W52 due to the need to decrease protein intake. This patient continues to experience recurrent hyperammonemia and HACs not considered related to DTX301. For the remaining 3 Nonresponders, no changes were made to ammonia scavenging drugs or dietary protein restrictions. Another patient was reclassified from Complete Responder to Responder after restarting an ammonia-scavenging drug at Week 182 due to personal preference despite being clinically stable and experienced two serious AEs at Week 300 and Week 333 with hyperammonemia although no elevated ammonia levels of clinical significance were reported. All other patients have maintained their Complete Responder or Responder status (**Figure 1**).

Conclusions: DTX301 continues to show manageable safety and durable improvements in clinical responses. The Phase 3 trial (NCT05345171) is currently evaluating DTX301 in adults and children ≥12 years of age.

Figure 1. Clinical response to DTX301 over time



854 First patient treated in the first phase 1–2 trial of AAVLK03hOTC gene therapy for ornithine transcarbamylase deficiency in children

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Ornithine transcarbamylase deficiency (OTCD) is a rare X-linked urea cycle disorder with recurrent metabolic decompensations characterised by hyperammonaemia, high glutamine levels, liver dysfunction and encephalopathy causing long-term neurological damage. Conventional treatments have limited efficacy and whilst liver transplantation is effective, it has associated morbidity and mortality, requires lifelong immunosuppression and is challenged with a shortage of organ donors. We developed the adeno-associated virus (AAV)-based gene therapy AAVLK03hOTC encoding a human codon-optimised OTC gene and pseudoserotyped with highly human hepatotropic LK03 capsid to target the liver and restore functional expression of OTC enzyme. Preclinical in vitro and in vivo studies demonstrated restoration of functional OTC activity in OTC deficient hepatocytes following treatment with AAVLK03hOTC. A 26-week GLP toxicity and biodistribution study in naïve Cynomolgus monkeys demonstrated elevated liver OTC activity 26 weeks after administration of AAVLK03hOTC in the absence of notable toxicological alterations. AAVLK03hOTC was expressed in almost all fluids and organs analysed, highest levels were in the liver. "Halting Ornithine transcarbamylase deficiency with Recombinant AAV in ChildrEn" (HORACE) is a UK based phase I/II clinical trial with a single administration of AAVLK03hOTC to paediatric OTCD patients with severe phenotype which aims to achieve normalisation of liver metabolism, thus reducing acute risk of morbidity and mortality from hyperammonaemic decompensations. Three doses (6x10^11vg/kg, 2x10^12vg/kg and 6x10^12vg/kg) are proposed in the dose escalation part of the trial. For dose-escalation cohorts, all patients will be 6-16 years to ensure homogeneity between cohorts. Based upon predicate data from other AAV gene therapy trials, there is a risk of asymptomatic transaminitis likely caused by T-cell mediated immune response to AAV, readily controlled by immunosuppression. Following escalation, recruited patients will be adaptively allocated to dose according to model-based simulation. After the initial 12 month-follow

up period participants will enter a 4-year follow up. The first patient treated with low dose (6x10^11vg/kg) AAVLK03hOTC gene therapy was a 10-year-old male with late-onset OTCD. Previous history included recurrent episodes of hyperammonaemia treated with dietary protein restriction and daily ammonia scavengers. The patient tolerated the gene therapy administration remarkably well. Two weeks after administration he presented with hyperammonaemia and encephalopathy caused by mycoplasma pneumonia encephalitis unrelated to gene therapy, followed by excellent recovery. Significant reduction in ammonia and glutamine levels were noted 6 and 8 weeks after gene therapy administration respectively, which allowed initiation of ammonia scavenger withdrawal. 7 months after gene therapy administration the patient developed raised liver transaminases >2 times above upper limit of normal. He remained asymptomatic but treated with prednisolone and tacrolimus which achieved normalisation of the transaminases within 2 weeks. 12 months post infusion glutamine and ammonia levels remain below the lower limit of normal and withdrawal of ammonia scavengers with normalisation of dietary protein intake was achieved whilst maintaining metabolic stability.

855 rAAV Gene Therapy for Classic Fabry Disease: ZS805 Results from An Ongoing Investigator-Initiated Clinical Trial

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Introduction: Fabry disease is a lysosomal storage disorder, caused by the pathogenic mutations in the *GLA* gene located on the X chromosome, which results in the progressive accumulation of GL3 and Lyso-GL3 in multiple cells, tissues and organs. ZS805 is a rAAV genetic drug carrying the *GLA* gene with an engineered signal peptide driven by a liver specific promoter, which was packaged in the AAV5 capsid (ChiCTR2300073886). Patients with Anti-α-Gal and Anti-AAV5 were enrolled, and the efficacy with or without prophylactic treatment of immunosuppressant was compared.

Study Design: Dose escalation was performed at two doses: 1.0×10^{13} vg/kg (low dose) and 2.0×10^{13} vg/kg (high dose). Eleven participants were enrolled into dose escalation and dose expansion cohorts (Figure 1). Ten patients were given a prednisone prophylactic treatment one week prior to injection while one patient had no prednisone prophylactic treatment. Data from nine participants with relatively long treatments were presented. The key patient eligibility criteria included male classic participants aged ≥18 years without ERT (enzyme replacement therapy) within 45 days prior to enrollment. The primary objectives was safety and tolerability, and the secondary objectives included the assessment of PK/PD (α-Gal A activity and vector shedding), immunogenicity (α-Gal A antibody, T cell response, anti-AAV5 antibody) and efficacy (GL-3 and Lyso-GL3 level, renal function, BPI and QOL scores).

Results: **Plasma α-Gal A Activity**
At low dose, plasma α-Gal A activity levels increased to 2.2 to 14 times the normal median level (8.1 nmol/h/mL) one week after administration, remaining stable with peak levels reaching 4.8 to 29.5 times the normal. After one week of high-dose administration, plasma α-Gal A activity levels increased to 4.4 to 16.1 times the normal median level, and remained stable, achieving peak levels that were 5.5 to 62.6 times the normal. All four participants who had either anti-α-Gal A antibody or anti-AAV5 antibody showed the elevated plasma α-Gal A activity.

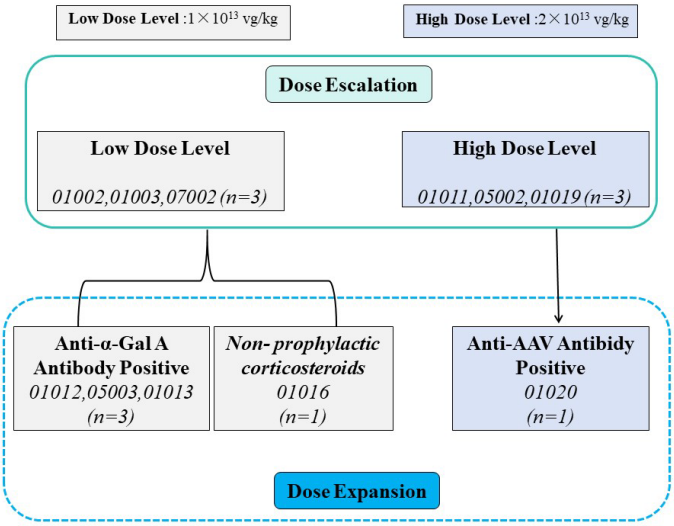
Substrates Lyso-GL3 and GL-3
Plasma Lyso-GL3 in all participants showed a significant reduction, with the **maximum decrease ranging from 20.1% to 66.6% in the low dose group and 9.2% to 67.6% in the high dose group.** The changes in plasma GL-3 levels exhibited a similar trend to that of Lyso-GL-3.

Safety and Tolerability
ZS805 was well-tolerated with no dose-limiting toxicity (DLT) among the eleven participants enrolled, which indicated a favorable safety profile. Two participants experienced three adverse events (AEs), but of all of them recovered without any lasting effects.

Vector shedding
Vector DNA levels in the blood peaked within 10 minutes, rapidly declined by the end of week 1, and were

completely cleared by about 6 months. Full clearance periods of the vector were as follows: week 1-3 for urine, week 3-7 for saliva, week 3-12 for semen.

Conclusions:
ZS805 was well tolerated at both dosage levels.
The α-Gal A activity remained stable and sustained in two participants for more than 52 weeks.
The level of plasma Lyso-GL-3 significantly decreased.



856 Title: Patient Centered Work on Considerations for Outcomes in Cerebral Creatine Deficiency Syndromes Trial Design

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This poster will present findings and ongoing work from two related Patient Centered Outcomes Research Institute (PCORI) grants led by the Association for Creatine Deficiencies. We established a Core Outcome Set for patients with Cerebral Creatine Deficiency Syndromes (phase 1), and work is now underway on considerations for how to choose optimal outcome measures for clinical trials in these conditions (phase 2). In this second phase, we also focus on identification of Clinically Meaningful Differences.

This process is important for our patients and families because oftentimes individuals with genetic conditions associated with neurodevelopmental disorders score at the floor of the standardized tests used or in some cases no tests have been validated for the population. The FDA is interested in meaningful differences in areas relating to functioning and feeling, but when parents observe small improvements and repeat testing, the metrics utilized may not be sensitive to these changes. This issue is exacerbated when the "symptom" under evaluation is neurodevelopment itself, which is critically important since many neurodevelopmental domains (e.g. expressive communication, cognitive functioning) were prioritized in the core outcome set established by the first phase of this work. We aim to promote disease understanding and prevent the use of inadequate endpoints in clinical trials by working ahead of potential upcoming trials to establish the considerations necessary for selecting appropriate measurement tools. Another complexity that we consider in this work is that very different neurodevelopmental phenotypes are observed within the same disease (for example, early versus later diagnoses in guanidinoacetate methyltransferase deficiency, and female versus male phenotypes in X-linked creatine transporter deficiency).

Understanding differences based on heterogeneity in presentation and clinical meaningfulness will be important to consider when selecting outcome measures.

857 Initial Safety Results from the PROPEL Trial Evaluating SBT101 as a Potential Gene Therapy for Spinal Cord Disease in Adult Males with X-linked Adrenoleukodystrophy

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Introduction: X-linked adrenoleukodystrophy (ALD) is a progressive neurodegenerative disorder caused pathogenic variants in the *ABCD1* gene, which is localized to the X chromosome and encodes ABCD1 protein that transports very long chain fatty acids (VLCFA)-CoA esters into the peroxisome for degradation. Pathogenic variants in *ABCD1* result in accumulation of VLCFA in all tissues. Patients with ALD can develop a leukodystrophy, adrenal failure, progressive peripheral neuropathy and spinal cord disease with or without cerebral involvement. When cerebral involvement has not been documented, this phenotype has historically been referred to as adrenomyeloneuropathy (AMN). There are currently no treatments for AMN, leaving patients with a progressive disease that leads to lifelong physical disability. SBT101 is a recombinant AAV9 vector encoding the functional *ABCD1* gene. Preclinical studies demonstrated dose-dependent ABCD1 protein expression, reduction of VLCFA levels, and functional improvements as measured by grip strength following administration of SBT101 in models of AMN. SBT101 has progressed to the clinic and has the potential to address the primary cause of AMN by restoring expression of the ABCD1 protein in spinal cord cells, potentially halting progression of disease and/or leading to improvements in the neurological pathology.

Methods: PROPEL is a Phase 1/2 dose-finding study in adult males with ALD and no cerebral involvement (historical AMN phenotype) to evaluate the safety and efficacy of a single intrathecal administration of SBT101 in two dose cohorts: low dose (LD: 1.0E14 vg) and high dose (HD: 3.0E14 vg). All patients receive prophylactic immune management (IM). The primary endpoint is safety. Secondary endpoints include multiple clinical assessments of mobility, bladder/bowel impairment, imaging, patient reported outcomes, and biomarkers of response. A natural history study, CYGNET is also ongoing with 65 patients enrolled and duration of follow up 3-36 months.

Results: Eight patients have been dosed to date (4 LD, 4 HD). Patient age ranged from 19-36 years, with disease duration at time of treatment of 2-14 years. Duration of follow up after dosing was 1 week-20 months. All patients tolerated the infusion well with 2 receiving a blood patch after the procedure. The most common adverse drug reaction (ADR) reported more than once was positive ELISPOT test. To date, no serious adverse events (SAEs) deemed related to SBT101, the procedure, or IM have occurred. One patient in the LD cohort died during the trial due to underlying disease, which was deemed unrelated to study product, study procedures, or IM. Evidence of transduction from droplet digital PCR will be presented along with early read out of markers of axonal damage in plasma and cerebral spinal fluid (CSF) (ie., neurofilament light chain; NfL).

Conclusion: Initial results from the PROPEL trial in adult, male ALD patients, show SBT101 has been well tolerated to date at both low and high doses, with cellular evidence of transduction in the relevant tissues and encouraging biomarkers of response. Patient outcomes will be analyzed and compared to natural history databases including those of the ongoing CYGNET study. The current data support the development of SBT101, a one-time, first-in-class investigational gene therapy to treat the high unmet medical need in individuals with AMN.

858 AlloNK® Cell Therapy ± Rituximab in Patients with Relapsed or Refractory Non-Hodgkin Lymphoma

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Introduction: AlloNK is a non-genetically modified, allogeneic, off-the-shelf, cryopreserved natural killer (NK) cell therapy candidate in development for the treatment of cancer and autoimmune diseases. A highly scaled manufacturing process enables production of thousands of 1 billion cell doses from a single donor cord blood unit (CBU). AlloNK has been optimized for combination with monoclonal antibodies (mAbs) to enhance antibody-dependent cellular cytotoxicity (ADCC) and anti-tumor responses through selection of CBUs with the natural high-affinity variant of CD16 (158V/V polymorphism) and a killer cell immunoglobulin-like receptor (KIR)-B haplotype.

Methods: The objectives of this Phase 1/2 clinical trial (NCT04673617) were to assess the safety and anti-tumor activity of AlloNK alone and in combination with rituximab in patients with CD20 positive relapsed or refractory (R/R) non-Hodgkin Lymphoma (NHL) of B-cell origin. Patients received a standard lymphodepleting (LD) regimen and were administered up to 4 cycles (4 weekly doses per cycle) of AlloNK at doses of $1x10^9$or $4x10^9$ cells/dose in an outpatient setting. Efficacy was evaluated using the Lugano response assessment criteria (2014).

Results: Sixteen patients received AlloNK across 3 different monotherapy cohorts, and 29 patients received AlloNK + rituximab across 4 different combination cohorts. Most patients were white (82%), male (62%), with a median age of 68 years, had aggressive NHL subtypes (84%), and had received a median of 4 prior lines of systemic therapy (range: 1-9). In line with the usage of LD regimens, the most common treatment-emergent adverse events (TEAEs) were hematologic, including neutropenia (84%), leukopenia (82%), and lymphopenia (71%). Infusion related- reactions (IRRs) and febrile neutropenia (n=3; 7% each) were the only related serious TEAEs reported in more than 1 patient. TEAEs of cytokine release syndrome (CRS) were reported in 4 patients (9%; 3 Grade 1 cases and 1 Grade 2 case) and were attributed to IRR as they happened within 24 hours of cell infusion, resolved without specialized treatment, and no cytokine elevations were noted. Other cell therapy-associated toxicities such as immune effector cell associated neurotoxicity syndrome (ICANS) and graft-versus-host disease (GVHD) were not observed. There have been no deaths related to AlloNK, and no patients have discontinued from the study due to AEs related to AlloNK. The objective response rate (ORR) in the monotherapy treatment group was 37.5%, including 4 (25.0%) complete responses (CRs) and 2 (12.5%) partial responses (PRs). In the combination treatment group, the ORR was 55.2%, including 13 CRs (44.8%) and 3 PRs (11.1%). In the 14 patients who were naïve to prior CAR-T therapies, 64% had CRs, with a median duration of response of 19.3 months.

Conclusions: AlloNK cell therapy is a safe and effective cellular therapy for B cell lymphomas. Given its alternative mechanism of action, AlloNK has the potential to enhance the ADCC response, thereby increasing anti-tumor activity without the safety challenges associated with T cell approaches, including high rates of CRS and ICANS. The combination of AlloNK and rituximab has demonstrated promising safety and efficacy in a heavily pretreated R/R NHL patient population, including older patients. The low risk of acute toxicities and preliminary efficacy results support the administration of AlloNK in the outpatient setting.

859 Trial in progress: Phase I study of non-viral gene-modified CAR-T cell therapy for malignant solid tumors with expression of EPHB4 receptor（CARTiEr）

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Background: EPHB4 has been reported to be overexpressed on the surface of tumor cells in various tumors, including malignant bone and soft tissue tumors. AP8901 is a CAR-T cell that can specifically recognize and kill EPHB4 receptor-expressing malignant tumor cells by modifying the ephrin-B2 protein, the natural ligand for the EPHB4 receptor. AP8901 is being developed using a genetic manipulation technique called the "piggyback transposon method" and the "genetically modified feeder cell method", which enables the stable expression of CAR proteins in T cells and prevents T cell exhaustion. A preclinical study demonstrated the therapeutic efficacy and tolerability of AP8901 in mice transplanted with a rhabdomyosarcoma cell line. We planned Phase I study evaluating safety and efficacy of AP8901 for metastatic solid tumors.

Methods This is a single-center, single-arm Phase I study of a single intravenous dose of AP8901 to evaluate the safety, tolerability, pharmacokinetics/pharmacodynamics and preliminary antitumor activity in patients with Ewing's sarcoma or solid tumors expressing the EPHB4 receptor. This study is a dose escalation study with a 3+3 design. Doses are calculated on the basis of body weight at the time of peripheral blood mononuclear cell collection. Key inclusion criteria include: (1) Subjects must have histologically diagnosed Ewing's sarcoma or solid tumor with confirmed metastasis or recurrence/no standard treatment for metastasis or recurrence or refractory or intolerant to standard treatment. (2) Subjects have a most recent biopsy or surgical resection specimen with a pre-screening immunohistochemical stain positive for EPHB4 in at least 1% of tumor cells. (3) ECOG performance status of 0 or 1. Major exclusion criteria include (1) Subjects being treated with systemic corticosteroids or systemic immunosuppressive drugs. (2) Subjects with symptomatic or uncontrolled brain metastases requiring concurrent treatment, impaired major organ function, active autoimmune disease, or active infection. (3) Subjects with a history of allogeneic hematopoietic stem cell transplantation. Primary endpoint is percentage of dose-limiting toxicities from the start of lymphocyte depletion therapy to 28 days after AP8901 administration. The maximum number of subjects to be enrolled is 18. This study is being conducted at the National Cancer Center Hospital East, Japan. (jRCT2033230299)

860 Phase I Open-label Single-arm Study to Evaluate the Safety, Tolerability and Preliminary Efficacy of CHT102, an Allogeneic Mesothelin-targeted CAR-T Cell, in Patients with Advanced Solid Tumors (CHAPTER)

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Background: CHT102, an allogeneic CAR-T therapy targeting mesothelin (MSLN), enhanced by proprietary ROBUS-T technology, demonstrates remarkable preclinical specificity, cytotoxic efficacy and persistence. This report presents preliminary results from a Phase I, open-label, single-arm trial evaluating the safety and efficacy of CHT102 in patients (pts) with advanced solid tumors. **Methods:** The primary objective was to assess safety and tolerability, with secondary objectives of evaluating efficacy and pharmacokinetics (PK) via flow cytometry and qPCR. Eligible pts included those with advanced/metastatic MSLN-expressing tumors, progressive disease despite systemic therapy, and ECOG status ≤1. Pts underwent lymphodepletion with cyclophosphamide and fludarabine,

with/without nab-paclitaxel, followed by CHT102 infusions on Days 1, 5 and 9. Dose escalation employed accelerated titration and a 3+3 design across four cohorts (3×10^8, 6×10^8, 12×10^8 and 18×10^8 CAR+ T cells). **Results:** Between August 9, 2024, and January 27, 2025, seven pts (4 pancreatic cancers,1 gastric cancer, 2 ovarian cancers) were enrolled. Six pts completed CHT102 infusions and were evaluated for safety and efficacy. One pancreatic cancer pt in cohort 2 received a second infusion on D43. The 7th pt is awaiting lymphodepletion, and cohort 4 enrollment is set for February 2025. The six infused pts had a median age of 66 years (range: 57-68) with a median of two prior systemic therapies (range: 2-5). No dose-limiting toxicities were observed. Common treatment-emergent adverse events (TEAEs) were hematologic: leukocytopenia (100%, Grade 3/4 83.3%), neutropenia (100%, G3/4 83.3%), lymphopenia (100%, G3/4 100%), anemia (66.7%, G3 50%), and thrombocytopenia (66.7%, G3/4 33.3%). One pt in cohort 3 experienced Grade 2 CRS on D1, resolved with tocilizumab and steroids. No neurologic toxicities or GVHD occurred. A Grade 3 cerebral infarction was reported in a cohort 2 pt on D35, attributed to pre-existing hypercoagulability. Non-hematologic TEAEs, including nausea (100%) and fever (50%), were Grade 1-2. At a median follow-up of 56 days (range: 11-99), no objective response rate (ORR) was observed. However, five pts (83.3%) showed 5-27% target lesion reduction, achieving a disease control rate (DCR) of 83.3%. Pancreatic cancer pts had a DCR of 75%. PK analysis demonstrated robust CHT102 expansion, with a median peak of 10,909 cells/ml (range: 1,040–15,819) and a median time to peak of 10 days (range: 7-13). **Conclusions:** The CHAPTER study indicates CHT102 has a favourable safety profile and encouraging efficacy in advanced solid tumors. Most AEs were transient and manageable. With an 83.3% DCR, CHT102 shows promise as a therapeutic option, particularly in pancreatic cancer pts, who demonstrate a DCR of 75%.

861 Improved HDAd Vector Design for Multiplex Editing and Gene Addition

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We are using capsid-modified, helper-dependent adenovirus (HDAd) vectors for *in vivo* transduction of hematopoietic stem cells (HSCs) with the goal to treat hemoglobinopathies, neurodegenerative diseases, or HIV. HDAds have an insert capacity of 35kb and are produced in HEK293 cells expressing Cre recombinase (116 cells). During HDAd amplification, massive amounts of transgene products are produced in 116 cells, which can affect their viability, greatly reduce the yield of produced HDAds, and/or cause HDAd genome rearrangements that mostly involve the deletion of the transgene. This problem is more acute for HDAd vector expressing genome editors (CRISPR Cas9, base editors, and prime editors), specifically for multiplex editing HDAd vectors containing more than one sgRNA or sg/peg RNAs. We speculate that this is due to enhanced cytotoxicity, associated with off-target editing together with high-level expression of the editors containing dCas9. Our goal was therefore to suppress editor expression in 116 cells, while allowing it in target HSCs. In addition to microRNA regulation (PMID: 2605252), we use here two approaches: *i)* We expressed a natural anti-CRIPR peptide (Acr) from the helper vector, from 116 cells, and also used an Acr-expressing plasmid for transfection together with the pHDAd plasmid in the beginning of the rescue process. *ii)* To completely suppress base or prime editor expression in 116 cells, we employed proteasomal degradation of the anti-CRISPR peptide AcrIIA4 using the chimeric degron ZFP91-IKZF3 (superdegron) and pomalidomide. Addition of pomalidomide restores the function of the dCas9 domain within the base or prime editors. After addition of pomalidomide base or prime editor editing rates in HSCs increased in a pomalidomide concentration-dependent manner. The plateau of editing is reached on day 2 after exposure to pomalidomide. The editor is active for 3 days after removal of pomalidomide. These improvements allowed us to produce HDAds vector with two prime editors (to truncate tEpoR for erythroid cells auto-expansion and to simultaneously correct the sickle mutation in the b-globin gene) or with two Tad CBE base editors (for tEpoR truncation and reactivation of g-globin). Furthermore, we improved HDAd vectors for gene addition (GFP, g-globin) by using a novel hyperactive Sleeping Beauty transposase (SB200x) that allowed for a higher integration rate than its predecessor SB100x. We are currently testing whether this will allow us to reduce the intensity of *in vivo* selection of stably transduced HSCs with O^6BG/BCNU. Overall, these improvements make HDAd vector more suitable for *in vivo* HSC gene therapy.

862 Cross-Species Enhancer AAV Platform for Targeting Spinal Motor Neurons and Cardiomyocytes

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The spinal cord contains a diverse array of cell types that work together to coordinate motor and sensory functions by relaying information between the brain and the rest of the body. Understanding and targeting these specific cell types is critical for advancing knowledge and developing therapeutics for movement disorders such as Amyotrophic Lateral Sclerosis (ALS). These conditions, characterized by motor neuron (MN) degeneration, currently lack effective cures. Recent advances in single-cell transcriptomics have provided deeper insights into the cellular diversity, however selective targeting of these cell types remains a significant challenge. To address this gap, we aimed to create an adeno-associated virus (AAV) toolkit for targeting spinal motor neurons. We expanded our single-cell epigenetic datasets to include mouse and macaque spinal cords to identify putative cell-type-specific enhancer elements compact enough to fit into an AAV backbone while providing ample room for therapeutic cargos. From these datasets, we identified novel enhancer elements that target broad motor neuron populations as well as specific spinal cord motor neuron (SMN) subtypes (α and γ). Each enhancer was cloned into an AAV backbone and validated using fluorescence reporter expression *in vivo*. We further tested the top pan-MN enhancers in rats and macaques through various routes of administration, confirming the conservation of enhancer activity across species. Additionally, we combined enhancers targeting both upper motor neurons (UMNs) and SMNs into a single AAV vector for simultaneous labeling of the corticospinal target. This approach demonstrates the capability to target multiple cell types within a single viral vector and could be directly applied to diseases like ALS. Beyond the central nervous system, we developed enhancer AAVs for other targets. Specifically, we identified putative cardiomyocyte-specific enhancers derived from human epigenetic data and validated enhancer AAVs in mouse hearts. Overall, our enhancer AAV platform enables precise, cell-type-specific targeting, which can drive therapeutic cargos for a range of diseases affecting the CNS and other organ systems.

863 A New Generation of Inducible All-In-One Tet-On-ORF/shRNA Vectors

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Tet-On is a powerful inducible system and also a classical tool to regulate gene expression in mammalian cells. It has also been applied to regulate Pol III-driven transcription, such as shRNA or gRNA driven by U6 or H1 promoter. However, there are unresolved technical issues that limit its applications. 1). The Tet-On inducible system is composed of Tet regulatory protein and inducible gene expression cassettes, however co-transfection of two vectors will cause the imbalance of the two functional groups in transfected cells. 2). Due to the limited insert size and the unique structure of the lentivector, it poses a great challenge to incorporate all functional cassettes into a single lentiviral transfer plasmid. Our All-in-one new system is built upon the tetracycline activator protein, Tet-On 3G, combined with a new plasmid structure, which could tightly regulate the downstream transcription of gRNA or shRNA. The responsiveness of our system to Doxycycline regulation is also dramatically improved compared with the current versions. The new Tet-On system is further optimized into a compact structure to be compatible with the Lenti-virus package (All-In-One Lenti-Tet-On system), which still keeps the leaky expression at an undetectable

level. Combined with all these features, the new generation of the system imposes a wide application in the genomic editing fields.

864 AAV-Based Cell Type-Specific Targeting (ACTStar) System: A Novel Method for Brain Cell Access

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Determining cell types and their function in the brain enables understanding of the complex structure of the brain, developing treatments, and illumination of cellular mechanisms. Current methods depend on transcriptomic methods, compact promoter isolation, the Cre-loxP system, and germline modifications and are usually of low efficiency and high cost. Thus new methods are needed. The aim of this study is to design a method, AAV-based Cell Type-Specific Targeting system (ACTStar), to overcome the limitations of the current methods. Specifically, the system should access brain cells of multiple species without the need of germline modification and promoter isolation. ACTStar enables brain cell access via CRISPR/Cas9-mediated targeted integration of recombinase DNA into the 3'UTR of marker genes for brain cells. The system consists of three AAV vectors: a gene-specific recombinase driver, a Cas9-expressing vector, and a reporter vector, the latter two vectors are shared between all genes and specifies. The gene-specific recombinase driver vector expresses a sgRNA targeting the 3'UTR of the marker gene and a sgRNA to target both ends of an IRES-recombinase DNA template on the vector for integration into the marker gene. The gene-specific recombinase driver and the Cas9-expressing vector work together to integrate IRES-recombinase into the 3'UTR of the marker gene to enable marker gene-specific expression of the recombinase, which will then activate reporter expression from the reporter vector. We tested targeting IRES-Cre to the 3'UTR region of mouse *Gad1* and *Slc17a7* in mouse N2a neuroblastoma cells, and observed Cas9-dependent integration of IRES-Cre in the expected loci. Off-target analysis and in vivo validation of the method are under way.

865 GRAd as Vaccine Platform for COVID-19 and HIV

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The GRAd platform, based on a low-seroprevalent gorilla-derived adenoviral vector, represents a transformative approach for the rapid generation of vaccines against a variety of pathogens, proving especially suitable for outbreaks and experimental medicine. ReiThera's pioneering efforts in the development of simian adenoviral (SAd) vector vaccines have led to the isolation of several SAds from chimpanzees and gorillas, belonging to different genetic groups. Among these, Group C adenoviral vectors stand out as the most potent inducers of T cell responses, owed to their lower induction of innate immunity (type 1 IFNs) and higher, prolonged antigen expression.

GRAd-COV2, a GRAd-based COVID-19 vaccine, has been the first product based on this platform technology, tested in humans and demonstrated to induce a potent and broad CD8 and Th1-skewed CD4 response, associated to a good safety profile and elevated productivity.

Building on the success of GRAd-COV2, we are now leveraging the GRAd vector technology to develop a potent T-cell inducing HIV vaccine, relying on an innovative T cell immunogen design based on mutationally constrained CD8 epitopes. These epitopes are selected from several clade C and B HIV proteins by structure-based network analysis, are commonly targeted by highly functional CD8 T cells in HIV elite controllers and provide broad coverage of prominent HLA class I alleles.

Preclinical immunogenicity in inbred and HLA-A2.1 knock in mice have confirmed the successful induction of high magnitude, broad and functional CD8+ T cell responses, and the GRAdHIVNE1 vaccine manufacturing and release for the IAVI C-114 phase I clinical trial scheduled for Q1 2025 in Southern Africa are underway. This innovative approach underscores GRAd's potential as a versatile and potent vaccine platform for addressing global health challenges.

866 Differential expression from reconstituted AAV hybrid dual vector systems with splicing enhancers is dependent on tissue specific SRSF protein expression

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The limited packaging capacity of recombinant AAV presents a major challenge, which is effectively addressed by the hybrid dual (HD) vector system. However, splice signals flanking the overlapping sequence play a crucial role in intron removal, directly impacting final protein expression levels. To overcome this bottleneck, we developed a novel 90bp recombinogenic splice enhancer intron (SEI) to improve splicing efficiency. When compared to the previously reported 297bp AP sequence (OAP), the SEI dual vector demonstrated significantly higher reporter gene expression *in vitro.* We observed a higher percentage of LacZ positive cells with SEI (13.51% ± 3.28) compared to that of OAP (7.29 % ± 0.56) upon cytological staining. Similarly, the β-galactosidase activity obtained from the SEI vectors (0.0082 ± 0.0009 U/µg protein) were significantly higher ($p < 0.0001$) when compared to levels from the OAP vectors (0.0034 ± 0.0006 U/µg protein).

Molecular analysis of splicing *in vitro* revealed that the SEI dual vectors achieved (23.83% ± 4.68) a significantly higher ($P \leq 0.05$) percentage of spliced to unspliced mRNA ratio compared to OAP (6% ± 1.21). To validate that the spliceosome factors are indeed contributing to the higher expression from SEI, we used splicing inhibitors during transfections with pDNA containing SEI or OAP in cis, flanked by the 5' and 3' halves of the *LacZ* reporter gene on either side. The effect of pre-mRNA splicing inhibitors was partially mitigated by SEI, yielding a higher percentage of spliced/unspliced product ratio (7.245% ± 1.892) compared to OAP (3.898% ± 0.9235) upon quantitative PCR analysis, confirming the interaction with splicing factors.

The SEI and OAP dual vectors were tested for stable gene expression *in vivo* in C57BL/6 mice. Percentage of LacZ positive TA muscle fibres were significantly ($P \leq 0.01$) higher with SEI vectors (86.09% ±3.41) compared to OAP (42.63% ±8.94). In retina, LacZ expression were significantly ($P = 0.03$) higher with SEI (45.75 ±0.84) vs. OAP (40.53 ±1.39). We further validated the efficiency of the SEI vectors in retinal and muscle disease mouse models. AAV dual vectors administered *Abca4*$^{-/-}$ retina demonstrated reduced lipofuscin deposits (~60-70%) and significantly recovered retinal function on ERG (n=6/group, $P < 0.05$) with the SEI vectors. In the D2.mdx mice, the SEI vectors yielded 65.19 ± 4.984 dystrophin positive fibers and the OAP vectors yielded 49.52 ± 3.818 fibers.

Interestingly, in all experiments the expression efficacy from the SEI vectors was significantly higher in the muscle compared to the retina. We observed higher expression levels of the spliceosome factors such as SRSF1 and SRSF2 in muscle compared to the retina. The SEI contains enriched binding sites for these factors. These SR proteins bound to SEI (acting as an Exonic Splice Enhancer) stabilize U2AF-65kDa recruitment to the polypyrimidine tract via U2AF-35kDa and aid interactions across intron and the U1 snRNP-specific 70kDa, further promoting efficient intron removal. Gene expression driven by this enhanced splicing mechanism is likely independent of reconstitution efficiencies from the ITR and overlap-mediated concatemerization.

In conclusion, our results in Stargardt disease and Duchenne muscular dystrophy models further validate the application of this novel dual vector system for gene expression across various tissues.

867 *Purified Anelloviral Capsid Proteins can Bind and Encapsidate Different Nucleic Acids Payloads into Anellovectors for Gene Delivery*

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Human anelloviruses are an abundant and highly divergent class of commensal viruses with no known disease association. In addition, their diversity and abundance in all tissues presents a potential for tissue tropism. These features position them as ideal candidates for potentially safe and efficient gene delivery of a diverse range of therapeutic nucleic acid payloads for various disease indications. We have developed a cell-free, in vitro assembled genetic medicines platform which is composed principally of two components: recombinant anellovirus capsid protein and a nucleic acid payload (e.g., DNA, RNA), offering payload diversity while also simplifying production to conventional and readily scalable systems at dramatically lower cost.

Here we've tested assembly of various sizes and types of nucleic acids with recombinant anellovirus capsid protein for Anellovector formation. Using electrophoretic mobility shift assays followed by electron microscopy, we evaluated the binding and encapsidation of different nucleic acid structures with purified capsid proteins. The tested payloads included single and double stranded DNA and RNA with different structures and sizes. Successful formation of an anellovirus vector (ANV) was achieved with DNA and RNA payloads as confirmed by electron microscopy. Several of these novel ANVs were tested in in vitro transductions of HEK293s and found to be functionally active.

This work has allowed us to better understand the permissiveness of ANVs to potentially deliver an unprecedented diversity of nucleic acid payloads. Such a uniquely versatile genetic medicines platform opens the possibility to more finely tailor the viral delivery product to the gene-delivery need.

868 Development of Novel T Cell-Specific Synthetic Promoters for Safer and More Effective In Vivo CAR-T Cell Therapies

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In vivo reprogramming of Chimeric Antigen Receptor (CAR) T cells holds transformative potential for cell therapy. A major challenge of this approach is the risk of off-target toxicity due to CAR transgene expression in non-T cell populations. To address this, EsoBiotec has developed ENaBL-T, a T cell-targeted lentiviral vector that induces CAR transgene expression under the control of a T cell-specific promoter derived from endogenous human gene regulatory elements. This synthetic promoter is engineered to achieve functional expression of the CAR transgene exclusively in T cells while maintaining a compact size compatible with lentiviral vector packaging constraints.

Core promoters and enhancers from genes specifically expressed in T lymphocytes were identified from functional genomics datasets using proprietary bioinformatics pipelines developed at Chromatin Bioscience. The selected regulatory sequences were assembled into synthetic promoters with a size below 1kb and initially tested in a dual reporter expression assay on a panel of cell lines and primary cells from different tissues. Synthetic promoters were selected based on specificity and potency of expression compared to the constitutive promoter EF1a and subsequently tested for functional expression of a CAR construct in primary T cells and for impact on lentiviral titer during production.

Mining of functional datasets for T cell specific regulatory sequences yielded multiple proximal-core promoters and enhancers sequences which were assembled in 35 synthetic promoters each containing one core and 2 enhancer sequences. Promoters including regulatory sequences from CD3δ and TCRab induced expression of reporters significantly above those of EF1a in lymphocyte cell lines as well as in primary T cells, while minimizing expression in non-target cells. In repeated co-culture of primary CAR-T cells expressing a BCMA CAR under the control of selected synthetic promoters (SYN), cytotoxicity and cell expansion of SYN BCMA CAR-T cells were higher compared to EF1α BCMA CAR-T cells when exposed to target tumor cells. In addition, infectivity and production yield of ENaBL-T SYN BCMA CAR vectors were significantly higher compared to ENaBL-T EF1α BCMA CAR and correlated with a reduced level of BCMA CAR on the surface of lentiviral producer cells.

These findings demonstrate that ENaBL-T synthetic promoters drive potent and selective CAR expression in T cells, outperforming constitutive promoters, while also enhancing vector production yield and infectivity. As a key component of the ENaBL-T vector, the synthetic promoter plays a critical role in building a platform for cost-effective, safe, and efficacious in vivo CAR-T cell therapies.

869 Optimization of Plasmid Cloning Methodology to Support Continued Development of the Pinnacle PCL™ Gene Therapy Platform

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As Ultragenyx continues to advance its portfolio of gene therapy programs, there is continual investment in iterative improvement of plasmids supporting rAAV generation. Here, we report advancements in the Triple-Play Plasmid (pTP), which contains AAV *Rep* and *Cap* genes, and the therapeutic vector construct, and is stably integrated into the Pinnacle Producer Cell Line (PCL) system. The Pinnacle PCL system is capable of supporting scalable 2000L clinical grade rAAV production and has been used to generate material for the ongoing UX701 clinical trial for Wilson Disease. Cloning techniques to facilitate faster, more efficient cloning with a higher probability of technical success have been optimized. This effort has culminated in “pTemP 2.0”, a method for scarless cloning of an insert into a standardized pTP template backbone in a single step, bypassing subcloning and generation of intermediate plasmids, thereby saving time and resources. We have also focused on improvement of supplying helper virus functions; however, the traditional, widely used pAD helper plasmid has proven challenging to modify due to its large size (~15-20 kb) and lack of available restriction sites. We have successfully employed the use of Gateway Cloning technology to overcome historic challenges observed with pAD Helper cloning workflows. This work has facilitated ongoing efforts to improve rAAV productivity and quality via higher throughput plasmid cloning.

870 High-Quality Engineered Plasmid Vectors for Advancing Therapeutics Development

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High-quality plasmid vectors are critical for biologic manufacturing, next-generation cell and gene therapies, and vaccine development. Vector purity and the integrity of inverted terminal repeats (ITRs) are essential for genome replication, packaging, and regulatory compliance in therapeutic applications.

Our engineered ssAAV and scAAV vectors with customizable features, including two distinct backbone options, three ITR sequence variations, and multiple restriction sites on both sides of the ITRs enable scientists to meet diverse research and clinical needs. These vectors ensure superior ITR stability and plasmid yield, outperforming traditional systems to deliver consistent and reliable results.

For mRNA research, our optimized plasmid vectors that outperform conventional systems such as pUC57 and pVAX1, offering improved cloning efficiency, enhanced poly(A) tail stability, and higher plasmid yields. With two unique backbones and four poly(A) sequence variations, these new state-of-the-art vectors incorporate dual

restriction enzyme sites, providing exceptional flexibility and precision for diverse and complex mRNA applications.

Combining innovative design with robust performance, these plasmid solutions empower researchers to achieve scalable, high-quality viral vector and mRNA-based therapeutic development. In this poster, we show how these innovations enhance the transition from discovery to commercialization and ultimately advance the field of precision medicine.

871 Enhancing Gene Transfer Efficiency in Viral and Non-Viral Vectors for Therapeutic Delivery

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Maximizing the efficacy of therapeutic delivery while minimizing risks is key to improving therapeutic development. Viral vectors, such as lentivirus and adeno-associated virus (AAV), are noted for their high efficiency and stable integration, but pose risks such as immunogenicity and insertional mutagenesis. Non-viral methods, including lipid-based nanoparticles (LNPs) and CRISPR/Cas9-based delivery offer safer alternatives with lower immunogenic risks; however, the typical payload can have limited bioavailability. Each of these methods has a critical component of either the delivery vehicle or payload which governs experimental success. Here we describe optimized workflows to minimize screening time while maximizing successful research results across the top three delivery vectors of choice: lentivirus, AAV, and mRNA.

Lentiviral vectors are powerful cell engineering tools used to infect target cells followed by integration of the gene of interest into target cell genome. For difficult-to-infect target cells including hematopoietic stem cells and neuronal cells, high viral titer is critical for efficient transduction. We describe an efficient lentiviral packaging method with robust packaging to produce high-titer lentiviral vectors, tailored for difficult-to-transduce cells and high-throughput packaging in 96-well plates suitable for CRISPR screening.

The ITR regions in AAV plasmids are notoriously unstable, prone to full or partial deletions during propagation in bacteria. As a result, a significant portion of clones after transformation may contain mutated ITRs, and plasmid DNA preparations from a liquid culture may contain a mixed population. Our results show that proprietary plasmid synthesis and preparation techniques maintain ITR integrity through AAV packaging and improve gene delivery.

Lastly, sequence accuracy of the DNA template and integrity of transcribed mRNA are crucial factors in determining the success of downstream experiments for mRNA therapies. Here we illustrate a streamlined gene-to-mRNA workflow to overcome these limitations in mRNA production. This includes rapid synthesis with optimization of long polyA regions in mRNA for enhanced LNP encapsulation and efficient delivery, in vitro transcription, and novel sequencing of mRNA constructs for complete characterization of critical components such as the polyA tail.

872 Gene Delivery Vector Comparison Study Using the Celigo™ Image Cytometer

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Recombinant adeno-associated virus (rAAV) is one of the preferred gene delivery vectors due to its safety and efficacy attributes for cell-based therapies. To develop effective gene delivery vectors, scientists need to consider multiple elements, such as the promoter, insertion location, genetic target, and target gene to name a few. Green fluorescent protein (GFP), or other detection markers are often used to measure vector transduction and gene expression efficiency. Using fluorescent microscopy and flow cytometry, cell samples can be analyzed, however, both methods can be quite time consuming, labor intensive, and lack high-throughput capabilities to quickly monitor gene expression over the time course of the experiment. In this work, we developed and employed the Celigo™

cytometer to screen different gene delivery vectors including, plasmid, mRNA, AAV with or without gene integration element, to determine the GFP gene delivery efficiency in SupT1 cells. GFP signal intensity was monitored 24-, 48-, 72-, and 96-hours following transduction and the viability was tested at 96 hours. Using the Celigo™ image cytometer we were able to monitor transient and integrated GFP expression amongst multiple gene delivery vectors. SB-P-AA-101-07 displayed stronger expression levels at 24- and 48-hours as transient gene delivery reagent. With stable gene expression, both plasmid and rAAV with gene integration element mRNA exhibited over ~90%+ GFP transduction efficiency at 96-hours. The end-point viability assay used a duo fluorescent staining method with Hoechst 33342 and Drag 7™ dyes to display various results ~60-95% amongst gene delivery vectors used, while untransduced samples displayed high viability 95%. Based off this study, the Celigo™ cytometer may prove useful for other researchers developing and screening gene delivery vector efficiency and viability in a high throughput manner.

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873 New gene therapy vehicle for the expression of multiple transgenes with distinct kinetics of expression

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During the last decade, viral delivery systems for gene therapy have undergone significant improvements, enabling the introduction, replacement, or counteraction of genes to treat a variety of human diseases. However, current systems are not adapted for the expression of multiple transgenes with distinct expression kinetics.

Herpes simplex virus type 1 (HSV-1) is a non-integrative virus that establishes a life-long, latent infection within sensory neurons. Its 152kb double-stranded DNA genome is packed into chromatin and organized in two distinct epigenetic programs: Heterochromatin marks repress all the viral lytic genes, while euchromatin marks allow expression within a specific genomic region called the Latency-Associated Transcript (LAT).

We developed a single, non-replicative HSV-1 (nrHSV-1) vector bearing two transgenes inserted in two different epigenetic regions of the HSV-1 genome. *In vitro* studies using glutamatergic cortical neurons derived from iPS cells showed that this unique vector allows the two transgenes to be expressed with different durations. (Figure 1) The transgene inserted in the heterochromatin region (Fig. 1B) displays shorter expression while the one inserted in the euchromatin counterpart (Fig. 1A) is stably expressed over the tested time points. The expression pattern of these transgenes from this single nrHSV-1 vector is currently being assessed *in vivo* in mouse DRG sensory neurons after footpad injection.

This innovative dual-payload nrHSV-1 vehicle represents a promising approach for next-generation gene therapy products, enabling the delivery of multiple transgenes with distinct expression dynamic within the same cell. This research paves the way to *in vivo* gene deletion and replacement by a long-term expression of the corrected gene, as a therapeutic tool for autosomal dominant diseases.

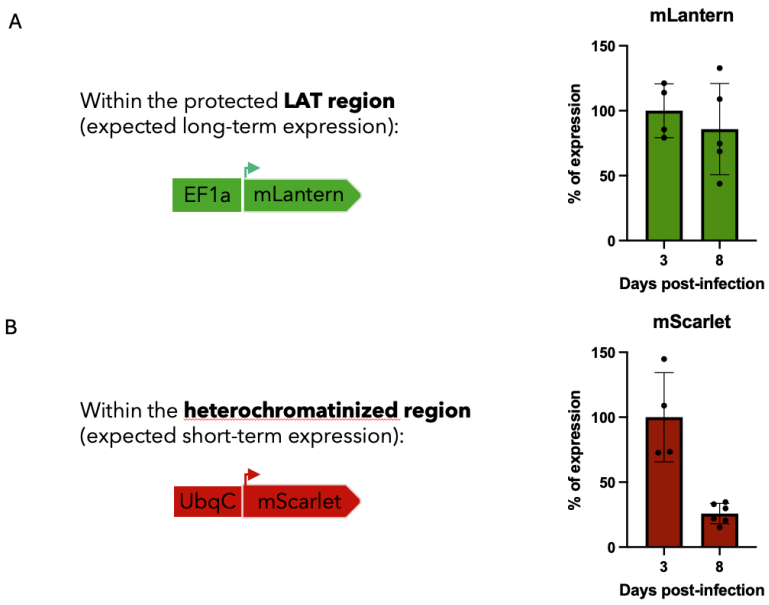

Figure 1: Distinct kinetics of transgene expression in iPS-derived cortical glutamatergic neurons infected with nrHSV1 dual-payload vectors. **A.** RT-PCR quantification of the mGreen Lantern reporter gene inserted within the protected LAT region, showed a stable expression level overtime. **B.** RT-PCR quantification of the mScarlet reporter gene inserted in the heterochromatin region of HSV1 genome presented a strong decrease of expression overtime.

874 Comparison of dual AAV vector strategies for lung gene therapy

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Introduction: Cystic Fibrosis and other monogenic lung diseases have long been a pertinent target for AAV-vectored gene therapies. However, the limited coding capacity of AAV has been a serious barrier to delivering larger transgenes. To overcome this issue, two AAVs can be co-administered, each carrying one half of the gene, effectively doubling AAV's coding capacity. Many dual vector strategies have been developed to achieve recombination of the full-length transgene. The overlap method uses homologous recombination, while the hybrid method uses this in addition to splicing. The novel mRNA transplicing (MTS) method involves mRNA transcript binding and splicing.

Methods and Results: Here, we engineered dual AAV vectors employing either the overlap or novel MTS strategy to deliver the Cre-recombinase gene, with eGFP in the 5' AAV and mCherry in the 3' AAV as reporter genes. These AAV vectors were delivered to mouse-derived precision-cut lung slices (PCLS) as a lung modelling system, and fluorescence was monitored via fluorescence microscopy. Both eGFP and mCherry expression in single cells were observed after co-transduction, suggesting both AAVs had transduced single lung cells (Figure 1). PCLS generated from the Rosa-LacZ strain will be used to evaluate successful Cre expression via LacZ staining and quantification. These vectors will then be co-administered intranasally to Rosa-LacZ mice to evaluate their efficiency *in vivo* in the lungs. The lungs will be harvested, stained, and LacZ quantitative assays will be run. Additionally, we developed dual AAV vectors using the overlap and hybrid method to deliver the clinically relevant CFTR gene (tagged with eGFP). Mouse PCLS were transduced with these vectors and GFP expression monitored. Both the overlap and hybrid vectors resulted in GFP-CFTR expression; however, the overlap method outperformed the hybrid method (Figure 2). These CFTR dual vectors have been administered to Balb/c mice intranasally. At 3 weeks post-AAV administration, the lungs will be harvested, and GFP-CFTR expression will be quantified by flow cytometry.

Conclusion/Relevance: Dual vectors are vital to expanding AAV's coding capacity. This study suggests that the overlap method may be an effective option for delivering the oversized CFTR gene to lungs.

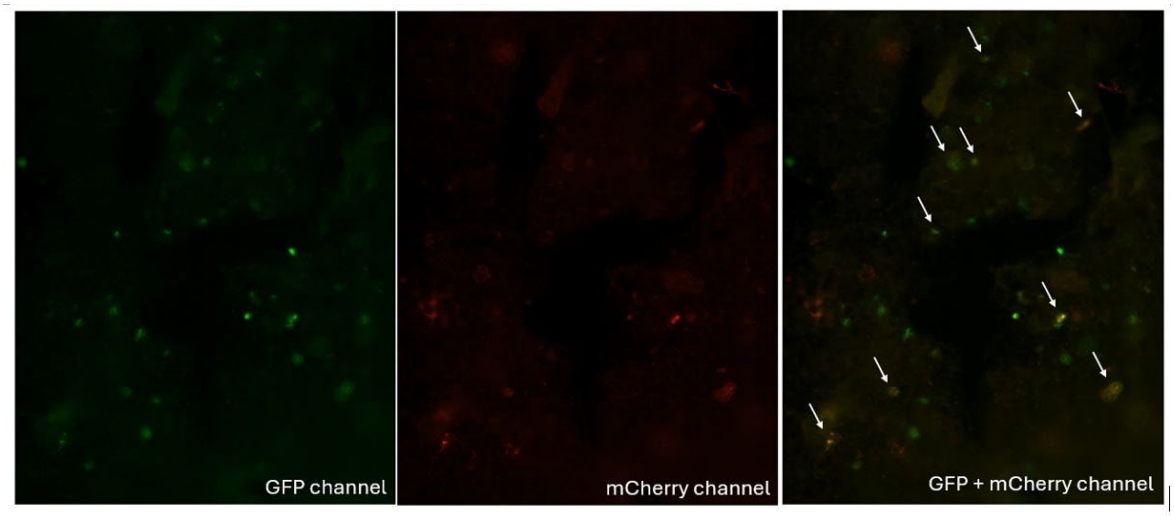

Figure 1. Mouse-derived PCLS were transduced with dual AAV Cre mRNA transplicing vectors. Five days post-transduction, slices were imaged using fluorescence microscopy to detect GFP and mCherry, and images were merged to identify co-transduced cells (white arrows).

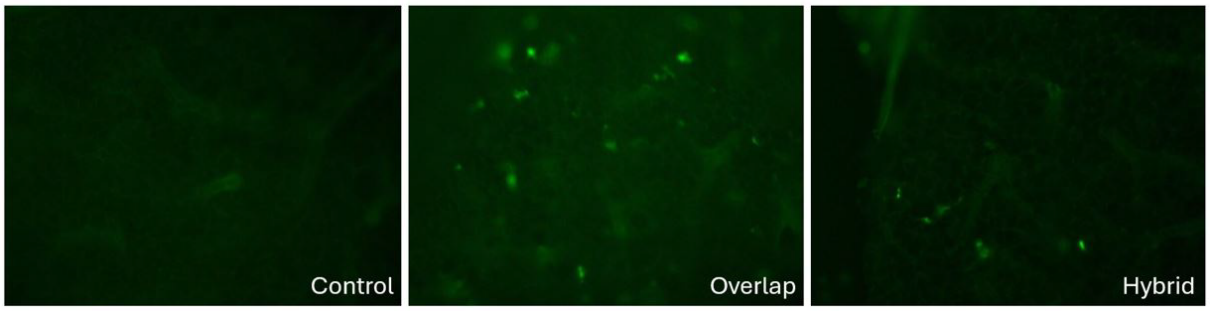

Figure 2. Mouse-derived PCLS were transduced with dual AAV vectors expressing eGFP-tagged CFTR using either the overlap or hybrid method. PBS-administered slices were used as a negative control. Five days post-transduction, slices were imaged under fluorescent microscopy to detect GFP.

875 AAV Vectors in Focus: Overcoming Challenges in Gene Delivery Systems

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Adeno-associated virus (AAV) vectors have become a cornerstone of gene therapy due to their safety, versatility, and ability to achieve long-term gene expression. However, challenges such as limited cargo capacity, pre-existing immunity, and off-target effects continue to hinder their full potential. This abstract examines recent advancements aimed at overcoming these obstacles to enhance the efficiency and applicability of AAV-based gene delivery systems. Innovations in capsid engineering have resulted in vectors with improved tissue specificity and reduced immunogenicity. Novel strategies, including the development of hybrid AAV systems, enhanced manufacturing

processes, and immune modulation techniques, are enabling more effective delivery of therapeutic genes. These breakthroughs have significant implications for treating a wide range of genetic disorders and chronic diseases, underscoring the potential of AAV vectors to transform the future of gene therapy.

876 Biomechanical Assessment of the Impact of Adenoviral Delivery of Three VEGF Isoforms on Rotator Cuff Tendon-to-Bone Healing in Mice

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Tendon healing is the main limiting factor of recovery after rotator cuff repair. Outcomes from surgery may be enhanced by faster healing and a reduced rate of repeated injury. This study assesses the local administration of an adenovirus vector (Ad) expressing multiple isoforms of vascular endothelial growth factor (VEGF) to promote recovery from rotator cuff repair in a mouse model. We used Ad serotype 5 to express a hybrid cDNA/genomic VEGF gene coding for the 3 major VEGF isoforms: VEGF121, 165, and 189. *In vitro* and *in vivo* studies established that the highest VEGF expression was achieved with Ad5VEGF(3i), $E1^{-}E3^{-}$ Ad5 with an expression cassette in the 3' to 5' configuration driven by the CAG promoter in the deleted E1 region. This vector expresses VEGF 189>165>121. The C57Bl/6 mice (n=10, per time point, per treatment condition) underwent complete transection of the supraspinatus tendon, followed by immediate surgical repair using a modified Kessler suture pattern with a 6-0 prolene suture. The potential utility of Ad5VEGF(3i) was assessed by its administration into the deltoid muscle at a total dose of 10^8 or 10^9 particle units at the time of rotator cuff repair. To assess the impact on recovery, biomechanical testing of the tendon attachment site was assessed at 1, 2, 4 and 8 weeks post-surgery. Rotator cuff injury and subsequent repair had a profound impact on many biomechanical parameters, with a >2.5-fold decrease in stiffness at all time points. However, the tendons of the animals treated with Ad5VEGF(3i) showed a faster recovery compared to untreated surgical mice with a higher load to failure ($p<0.05$), higher maximum stress ($p<0.03$) and higher energy absorbed by the tendon ($p<0.02$) 2 weeks following surgery. The biomechanical properties of the tendon were maintained at 4 weeks after surgery. At 8 weeks, the high dose treated tendons also showed higher energy absorbed ($p<0.01$). We conclude that the delivery of Ad5VEGF(3i) at the time of surgery has the potential to accelerate recovery following rotator cuff repair and maintain a better biomechanical phenotype.

877 A novel miniaturized filamentous phagemid as a gene delivery vehicle to target mammalian cells

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The filamentous phage M13 is a single-stranded DNA phage with several attractive characteristics for gene delivery, including a capsid amenable to the display of foreign peptides and a simple well-characterized genome that is easy to genetically modify. Previously, we constructed a targetable DNA minivector production system based on M13 (a miniphagemid), called *'intelligent Phage-Assembled Gene Expression'* (iPhAGE) which minimized the inflammatory bacterial and phage DNA content in the vector. In general, DNA minivectors, devoid of their prokaryotic components have shown improved gene transfer and safety. We examined the miniphagemid system's capacity for *in vitro* transgene delivery to target cells through phage display of epidermal growth factor (EGF) to target its cognate receptor. The absence of the prokaryotic backbone and minimized vector size conferred dramatically improved transgene expression of both the targeted miniphagemid virion particles as well as purified single-stranded phagemid miniDNA compared to their 'full' counterparts. We further optimized this system to improve the packaging efficiency of DNA minivectors by deleting the packaging signal and introducing multiple novel mutations to the helper plasmid used for miniphagemid production. These enhancements resulted in higher yields and exceptional purity of the miniphagemids. In all produced lysates, contamination of prokaryotic backbone DNA in iPhAGE lysates was $< 2.0 \times 10^{-8}$ and contamination from helper phage/plasmid was $< 1.0 \times 10^{-10}$. Another notable improvement was the yield of miniphagemids and hence retrievable miniDNA, which was between 1.0–10 mg/L/OD_{600} depending on the packaged sequence. The improved system further enhanced phage-mediated gene

expression in mammalian cells by >100-fold as assessed *in vitro*. In summary, we present a highly pure, scalable, and efficient vector production system that combines cell-targeting ligand display with high capacity, minimized DNA vector capabilities.

878 An *E. coli*-based platform for the production and assembly of anellovirus vectors in *vitro* for gene therapy platform

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Gene therapy (GT) offers immense potential for treating various acquired and inherited diseases, but the safety and efficacy of this treatment modality largely depends on the vector. Generally, viral vectors show high transduction efficiency along with transgene expression, but their use is limited by challenges such as insertional mutagenesis, immune responses, and high manufacturing costs. While non-viral vectors may offer the possibility of improved control and redosability, and often easier to produce, they tend to exhibit lower transgene delivery efficiency. These limitations underscore the need for novel and more effective delivery systems that are safe, efficacious, and provide greater transgene expression control via vector redosability and titratability.

Anelloviruses are ssDNA orphan viruses with the ability to transfect a wide range of human cells and tissues. As they naturally contribute to the human virome, they offer a safer and potentially redosable/controllable alternative to more conventional viral vectors. The application of these viruses as gene therapy vectors has been limited by the dearth of efficient propagation systems that permit the exploitation of anelloviruses as suitable gene therapy vectors.

Here, we describe a novel prokaryotic system for a two-step *in vitro* of TTV19 anellosomes in *E. coli*. This work successfully demonstrates the expression and assembly of anellovirus capsid proteins, leading to the production of ssDNA viral gene delivery vectors *in vitro*. Self-assembled particles were capable of efficiently transducing mammalian cells with a 100- to 1000-fold improvement in gene expression compared to the molar equivalent of dsDNA and ssDNA in HEK 293T cells respectively. This two-step anellovirus vector production was further enhanced into a single-step production system enabling fully formed anellosomes in *E. coli*. The proposed *E. coli*-based anellovirus system represents a novel and highly scalable/cost-effective platform for the development of anellovirus-based viral vectors for gene therapy applications.

879 Engineering T Cell-Specific Lentiviral Vectors for Safe and Effective In Vivo CAR-T Therapy

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Ex vivo Chimeric Antigen Receptor (CAR)-T cell therapy is constrained by a complex manufacturing process and a high cost of goods, significantly limiting patient accessibility. In vivo generation of CAR-T cells offers a cost-effective, “off-the-shelf” approach by reprogramming a patient’s own cells directly in situ, addressing the challenges of both autologous and allogeneic ex vivo CAR-T therapies. However, translating this approach into the clinic requires overcoming key hurdles, such as ensuring precise T cell transduction to minimize off-target toxicity and effectively reprogramming a sufficient number of T cells to achieve meaningful clinical outcomes. To address these challenges, EsoBiotec has developed a 3rd generation self-inactivating Engineered NanoBody T cell-specific Lentiviral vector (ENaBL-T). ENaBL-T features an engineered viral envelope incorporating a T cell-specific nanobody binder (VHH) that enables precise targeting of T cells and induces their activation and expansion. Efficient internalization of the vector into T cells is achieved through a newly designed targeting-deficient mutant of VSV-G.

VSV-G mutant envelope proteins were created via site-directed mutagenesis and candidates were screened to identify mutations that abrogate the natural tropism of VSV-G for the LDL receptor while retaining its membrane fusion activity. Selected VSV-G mutants were paired with T cell-specific nanobody binders (VHH) including CD8 and TCRαβ and tested on panels of cell lines and primary cells. The optimized vector, combining anti-TCRαβ VHH and VSV-G^{I331E} targeting deficient mutant, demonstrated a high level of T cell specific transduction with minimal off-target transduction in TCR-negative cells. VSV-GI^{331E} TCRαβ VHH ENaBL-T efficiently transduced both resting and activated human primary T cells, inducing significant T cell activation and expansion compared to broad tropism VSV-G^{WT} lentiviral vectors. BCMA CAR-T cells generated using VSV-G^{I331E} TCRαβ VHH ENaBL-T (referred to as ESO-T01) exhibited potent antitumor activity *in vitro* against target tumor cells.

These findings demonstrate that the ENaBL-T vector, incorporating the VSV-G^{I331E} targeting deficient mutant and a VHH targeting TCRαβ, achieves precise T cell targeting while also inducing T activation and expansion through the stimulatory activity of the TCRαβ VHH, resulting in high levels of T cell transduction. This engineered lentiviral vector enables the generation of potent CAR-T cells with robust antitumor activity. ENaBL-T represents a promising step toward the clinical translation of cost-effective, scalable, and safe in vivo CAR-T cell therapies.

880 Infectivity, Expression and Redosing in mice of an Anellovirus vector produced by in vitro assembly.

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Recent advances in gene therapy have led to FDA approvals of several treatments for a wide range of genetic disorders. Despite these successes, significant challenges remain, including issues with immunogenicity, redosing, serious adverse events, and high manufacturing costs. To address these limitations, Ring Therapeutics is developing a novel class of gene delivery vectors (ANVs) derived from Anelloviruses. Anelloviruses are a diverse family of commensal, single-stranded DNA (ssDNA) viruses that constitute >97% of the human virome. Anelloviruses are widely distributed across vertebrate species, are immune evasive, and exhibit broad tissue tropism in humans, making them promising candidates for therapeutic gene delivery. Here we show that ANVs are amenable to production via assembly in vitro, which has the potential to dramatically reduce manufacturing costs for genetic medicines.

We recently demonstrated that these engineered vectors can be produced at research scale by assembly in vitro combining purified capsid proteins and nucleic acid payloads in a highly controlled encapsidation reaction. Further, ANVs have been shown to be functionally active exhibiting transduction in cell cultures using a marker gene, green fluorescent protein (GFP). Here, we demonstrate, for the first time in vivo (in mice), the activity of an ANV assembled in vitro containing ssDNA payloads encoding GFP under the control of a CMV promoter (ANV.CMV.GFP). Local injections in eye and muscle of mice were performed to evaluate the expression of the ANV.CMV.GFP vector. Initial repeat dosing experiments were also conducted. For the ocular study, C57BL/6 mice received bilateral subretinal injections of ANV.CMV.GFP, AAV2.CMV.GFP, or vehicle control on Day 0. A repeat unilateral intravitreal injection was administered on Day 21. Retinas and posterior eyecups were harvested on Days 21 and 42 for analysis of DNA, mRNA, and protein. For the muscle studies, Balb/c mice were injected intramuscularly with ANV.CMV.GFP, AAV2.CMV.GFP, or vehicle control on Day 0, followed by a repeat intramuscular injection on Day 21. Caudal muscles were harvested on Days 21 and 42 for analysis.

GFP DNA, mRNA, and protein was detected, and redosing was well tolerated in the mice. This study represents the first demonstration in vivo of the infectivity, expression, and repeat-dosing potential of ANV assembled in vitro and data are consistent with our transduction results in cell culture. These findings support further studies in vivo of ANV assembled in vitro and highlight the potential of ANVs to offer a more versatile therapeutic, with substantially less cost than traditional viral therapeutics, possibly overcoming key limitations of current gene therapy.

881 Detyrosinated Microtubules Limits AAV2 Trafficking and Regulates Gene Transduction Efficiency

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Introduction: Adeno-associated virus (AAV) is a widely utilized vector for gene delivery due to its safety and efficacy. However, its transduction limitations lie in its interaction with host cell machinery, particularly its trafficking through the endosomal compartments and routing toward degradation pathways. This study investigates the role of microtubule detyrosination—a microtubule post-translational modification (Mt-PTM)—in regulating AAV2 trafficking, and its transduction efficiency.

Methods: By using super-resolution Structured Illumination Microscopy (SIM) and correlative live-cell and STochastic Optical Reconstruction Microscopy (STORM), we studied the interaction between AAV2 and detyrosinated microtubules and the impact of microtubule detyrosination on AAV2 motility. Microtubule tyrosination was enhanced by overexpressing tubulin-tyrosine ligase (TTL) and the detyrosinated tubulin was modulated pharmacologically by using parthenolide. Transduction efficiency was quantified, and preclinical models of hemophilia B were used to evaluate the therapeutic potential of dimethylamino parthenolide (DMAPT) in enhancing AAV2-mediated factor IX expression.

Results: AAV2 infection led to an increase in microtubule detyrosination, which hindered the cytoplasmic motility of the virus and delayed its nuclear entry. Either overexpression of tubulin-tyrosine ligase (TTL) or the pharmacological inhibition of microtubule detyrosination using parthenolide, improved the AAV2 motility by two-fold and its perinuclear accumulation. These modulations facilitated the early escape of the virus, resulting in a three-fold improvement in transduction efficiency. DMAPT enhanced AAV2-mediated factor IX expression in hemophilia B preclinical models, demonstrating its potential as a co-therapeutic agent.

Conclusion: These findings underscore the critical role of detyrosinated microtubules in managing AAV2 trafficking and transduction efficiency. The pharmacological targeting of microtubule detyrosination with modulators like DMAPT offers a promising strategy for improving the effectiveness of AAV-based gene therapies. This study establishes the foundation for novel therapeutic interventions that enhance AAV transduction by modulating host-cell microtubule dynamics.

882 The influence of VP1 N-termini on heat-induced genome release of adeno-associated virus serotype 8

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Recombinant adeno-associated virus (rAAV) is used as a gene delivery vehicle for gene therapy. rAAV encapsidates the designed genome flanked by the two inverted terminal repeats in the wild-type genome, that contains a promoter and gene of interest to treat diseases. rAAV capsid consists of 60-mers of three major viral proteins (VPs), VP1, VP2, and VP3. VP1 contains the VP1 unique (VP1u) region at the N-terminus that is not present in other VPs and possesses phospholipase A2 activity for endosomal escape. The VP2 N-terminus, which is shared with VP1, is called the VP1/VP2 common region and contains a nuclear localization signal. Although the stoichiometric ratio of VPs composing the capsid has been considered to be VP1:VP2:VP3 = 5:5:50, recent studies have shown that rAAV is a mixture of heterogeneous particles with various VP stoichiometric ratios, and that the incorporation of VP1 or VP1/VP2 into total VPs is highly correlated with rAAV transduction efficiency.

Here, we focused on the VP stoichiometric ratio of rAAV serotype 8 (rAAV8) and investigated the heat-induced genome release and the capsid thermal stability using several biophysical methods, mass photometry, nano differential scanning fluorimetry, hydrogen/deuterium exchange mass spectrometry, and sedimentation velocity analytical ultracentrifugation. We prepared three rAAV8 samples with different VP stoichiometric ratios, including particles with a high VP1/VP2 to VP3 ratio (FP_{LD}), a low VP1/VP2 to VP3 ratio (FP_{HD}), and only VP3 ($FP_{VP3only}$). All the samples were extensively purified to remove empty particles (EPs) with no encapsidated genome inside the capsid using two cycles of CsCl density gradient ultracentrifugation. As a result, upon heating to the point of capsid disassembly, most rAAV8 particles remain as monomers in three states: capsids encapsidating their ssDNA, capsids that fully eject the genome while tethering the genome on the capsid surface, and empty capsids that have fully released their encapsidated genome. The ratio of VP1 and/or VP2 to total VPs barely altered the extent of genome release, whereas the possession of VP1 and/or VP2 influenced on the extent of genome release. Importantly, thermal unfolding of the VP1u and VP1/VP2 common region, i.e., VP1 N-terminus was closely monitored at about 60°C, lower than the VP3 denaturation initiation temperature of 71-72°C. Interestingly, $FP_{VP3only}$ had a greater proportion of empty particles (EPs) than did FP_{LD} and FP_{HD} after incubation at 45–55°C, whereas when incubated at 65°C, the FP_{LD} and FP_{HD} had a significantly greater proportion of EPs than did $FP_{VP3only}$. This result indicates that the unfolding VP1 N-terminus related to the genome release upon the heating; folded VP1 N-termini prevented the genome release at temperatures below 60°C, whereas the unfolding of the globular structure in VP1 N-termini facilitated the genome release.

This study provide us the new insights into heat-induced genome release with a focus on VPs and contribute to a better understanding of the rAAV particles at a molecular level.

883 A Single Conserved F-to-Y Mutation at the 5-Fold Interface Is Exclusively Required for an AAP-Dependent-to-Independent Capsid Assembly Switch

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Assembly activating protein (AAP) facilitates a multitude of functions resulting in AAV capsid assembly. However, the process through which AAP assists VP subunit assembly resulting in the formation of AAV capsids is not completely understood. Data from our group and others have shown that certain AAV serotypes (AAP-independent serotypes; AAPi serotypes: AAV4, 5, 11, 12 and rh32.33) can assemble capsids independently of AAP's involvement while the rest are AAP-dependent serotypes (AAPd serotypes) that include but are not limited to AAV2,3,6,7,8,9, and 10. To uncover amino acids underlying AAP requirement, we had previously focused on AAV2. Our iterative evolution study on an AAV2 mutagenesis library identified AAV2F370Y as a mutant capable of assembling capsids without AAP. However, this observation does not exclude the possibility that there are alternative pathways that contribute to the transition from AAPd to AAP-independent AAPd serotype capsid mutants because directed evolution approaches do not necessarily capture all potential pathways leading to the desired phenotype. Here, by extending our directed evolution approach to AAV9, we reveal that the F-to-Y mutation at the position corresponding to AAV2F370 is an exclusive requirement for the phenotype switch from AAV9, an AAPd serotype, to an AAP-independent AAV9 capsid mutant. To uncover the amino acids underlying AAP requirement in AAV9, we generated an AAP knockout of AAV9 utilizing an ITR2-Rep2-AAV9CapAAP9KO-ITR2 plasmid. The plasmid was subjected to error-prone PCR to generate an AAV9 mutagenesis library. The library was selectively enriched for AAP independent mutants by cycling through HEK293 over three rounds of selection in the absence of AAP9. Similar to the finding with AAV2, arbitrarily chosen mutants after third round showed a common AAV9F372Y mutation (corresponding to AAV2F370Y) and were capable of assembling capsids in the absence of AAP. The AAV2F370Y and AAV9F372Y mutants exhibited transduction efficiency in HEK293 cells comparable to their respective wild-type capsids, indicating that the F-to-Y mutation does not impair function. This suggests that the mutation is neutral, apart from its role in determining AAP dependence in capsid assembly. As this F-to-Y mutation lies on the 5-fold axis interface, we propose that VP subunit assembly at this axis requires conformational changes promoted by AAP, while the F-to-Y mutation alone induces these changes even in the absence of AAP. Importantly, our finding that the same F-to-Y mutation emerged as the sole mutation capable of conferring AAP independence in two independent directed evolution studies conducted on two separate serotypes (AAV2 and

AAV9) strongly indicates that, within the AAPd serotypes, this F-Y mutation is the only viable pathway to AAP independence and no alternative pathways can confer AAP independence. This F residue is fully conserved across the AAPd and AAPi serotypes while the F-Y mutation appears to have a neutral effect on infectivity, indicating that there are evolutionary constraints that strongly disfavor reversion to AAP independence once capsid assembly becomes reliant on AAP. Further investigation of AAP-independent mutants derived from AAPd serotypes will help better understand how AAP participates in AAV capsid assembly and why AAP has become an indispensable factor in this process.

884 Functional Orthogonality of Parvoviral Phospholipase A_2 (PLA_2) Domains in Recombinant AAV Vector Transduction

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Dependoparvoviruses are a genera of Parvoviridae which are of interest in viral vector development for gene therapy applications. Engineering approaches that modify the AAV capsid surface have been employed to retarget vectors and enhance transduction efficiency in desired tissues. Separately, buried capsid protein domains that are not surface exposed, such as the viral protein 1 unique (VP1u) region or nuclear localization signals (NLS), have been shown to play a critical role in viral infectivity and post-entry trafficking. However, functional exploration and engineering of these domains with the goal of improving vector potency has lagged that of the capsid surface. Within this framework, the conserved group XIII phospholipase A_2 domain (PLA_2) within the VP1u of parvoviruses, which must be externalized during infection is particularly noteworthy. Here, we describe a library-based approach to evaluate a diverse panel of both rationally engineered and natural parvoviral PLA_2 domains by replacing the adeno-associated virus serotype 9 (AAV9) enzymatic domain. Key findings highlight which parvoviral genera contain PLA_2 domains that exist in sequence space compatible with the VP1u region of AAV9 capsid proteins. Notably, a selection of Avian dependoparvovirus/AAV9 chimeras deemed high performers based on infectious library cycling *in vitro* demonstrate improved transduction efficiency compared to both single-stranded (ss) and self-complementary (sc) AAV9 vectors. The best performing chimeric isolate is equivalent to AAV9 with regard to cell surface binding but shows improved uptake into the cytoplasm as well as nuclear entry as evidenced by sub-cellular fractionation and confocal microscopy. The lead identified avian PLA_2 domain is also functional in the context of serologically distinct AAV5 vectors, demonstrating broader compatibility within the genera. Overall, this study underscores the functional orthogonality of antigenically distinct PLA_2 core enzyme domains derived from Parvoviridae in commonly utilized AAV capsids with prospects for improving post-entry trafficking and AAV vector potency in gene therapy.

885 Identification of key amino acids near the N-terminus of AAV AAP essential for driving its degron function

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Assembly activating protein (AAP) interacts with AAV VP subunits and plays a critical role in AAV capsid assembly. It has been shown that AAP binds to VP through the domain near its N-terminus. This AAP-VP interaction, however, cannot remain persistent as prolonged binding may hinder VP oligomerization. Studies have shown a decline in AAP steady-state levels over time during capsid assembly, and capsid structural and biochemical analyses have revealed that AAP is absent from the capsid. Together, these findings strongly indicate that AAP indeed dissociates and is ultimately degraded. To understand the mechanism by which AAP degrades, our group has

conceived an AAP degron model wherein AAP undergoes facilitated degradation triggered through its degron motif. We have previously shown that the Hydrophobic Region (HR) for AAP1, 2, 3, 5, 6, 7, 8, 9 and 12 and the Conserved Core (CC) for AAP 4 and 11, both of which are present near the N-terminus, contain a degron. Here we further extend our observations and uncover key amino acids within the HR or CC region that are critical for AAP's degron function. To pinpoint the key amino acids within each of HR and CC critical for AAP's degron function, we utilized two approaches, with each tailored to the respective region. For the HR region, we scanned overlapping motifs across its entirety, and for the CC region, we manually selected a residue based on the AAP sequence alignment. For the HR region, we chose AAP9 as the representative serotype and divided HR into 7 subregions (R1 to R7) wherein each subregion had 5 overlapping residues with the adjacent region(s). To assess the degron function of these subregions, each subregion was fused to FLAG-GFP, and GFP expression from each subregion construct was compared against controls. Interestingly, both R5 and R6 showed substantial decrease in GFP expression comparable to that from the full-length HR, whereas the other subregions showed GFP expression comparable to the FLAG-GFP control. The overlapping 5 residues between R5 and R6 was found to be QWLQV, indicating its critical role in HR degron function. For the CC region, sequence alignment of AAPs revealed that most residues are conserved exhibiting similar biochemical attributes except at position 135(AAP4 numbering), where AAV4 and11 have V while all other serotypes have E. Accordingly, we generated AAP4CCV135E-FLAG-GFP and assessed its GFP expression against controls. True to our hypothesis, we found AAP4CCV135E retained GFP expression albeit at a level lower than the FLAG-GFP control, indicating a critical role of this residue for the degron function in CC. These findings confirm that AAPs are inherently prone to degradation, while previous studies including ours have consistently shown that AAP can stabilize in the presence of VP, indirectly indicating that AAP-VP complex formation prevents AAP degradation. Taken altogether, we postulate a hypothetical model where the formation of AAP-VP complex masks the AAP degron motif thereby suppressing its function and supporting AAP's function for assisting VP oligomerization. Once VP oligomerization begins, it induces a conformational loosening of the AAP-VP interaction, leading to degron exposure. This, in turn, facilitates AAP degradation, promotes AAP-VP dissociation and ultimately VP oligomerization into a fully assembled capsid.

886 Understanding DNA host cell factors as potential restriction factors to efficient AAV gene transfer in skeletal muscle cell

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Adeno-Associated Viral (AAV) vector mediated gene therapies are at the forefront of novel treatments for muscular dystrophies. In recent years, the number of clinical trials and approved AAV-based treatments have increased. Nonetheless, certain roadblocks still arise during clinical trials, such as immune responses due to the high doses administered. While efforts to reduce the dose by capsid engineering are being made, investigating cellular to responses to AAV transduction would enable us to define new strategies to improve gene therapies. Studies have been conducted to help understand the cells innate viral responses, such as DNA damage response pathways. However, most of these studies have been performed in dividing cells where these pathways are more abundant and might not reflect the environment in post-mitotic skeletal muscle fibers. Using next generation sequencing technologies such as RNA-Seq and single-nuclei RNA-Seq, we aimed to investigate changes in the transcriptional landscape of myogenic cells following rAAV transduction. The aim was to identify known and potential new host cell restriction factors which might restrict efficient AAV gene transfer in skeletal muscle fibers. A preliminary study was performed in immortalized differentiated human myotubes transduced with LICA1 or AAV9 encoding a GFP-Luciferase gene at MOI 5,000. Cells were harvested at 24h post-transduction and analyzed by bulk RNA-Seq comparing differential gene expressions to control vehicle infected cells. In the LICA1 transduced cells, but not the AAV9 cells, we observed an increase in DNA damage response genes linked to p53 class mediator resulting in cell cycle arrest. These findings are being further investigated by single-nuclei RNA-Seq in immortalized human myotubes to assess for potential limitations due to heterogeneous transduction of myonuclei. Additionally, co-localization studies by confocal imaging allow to validate the interaction between AAV transduction and the observed molecular changes. These results allow us to further understand the interactions between AAV genome and host cell factors, leading to an improved knowledge of factors influencing efficient AAV gene transfer in

muscle fibers. Additionally, they will pave the way to develop novel therapeutic strategies to increase gene transfer and potentially lower doses in muscular dystrophies gene therapy.

887 EGFR Inhibition Reduces AAV8 Capsid Entry to Hepatocytes, Posing a Potential Liver De-Targeting Strategy

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Adeno-associated virus (AAV) based therapies hold great promise for the treatment of rare genetic diseases through their ability to deliver genetic cargo to a variety of tissues. However, their applicability has been hindered by liver toxicity in several otherwise-successful gene therapy trials. One of these, the ASPIRO trial for X-linked myotubular myopathy (XLMTM), was placed on clinical hold after four patient deaths due to liver failure. This led to the discovery that cholestatic liver disease is a clinically significant feature of XLMTM, which has been further demonstrated in both zebrafish and mouse models of the disease. This pre-existing liver phenotype is further exacerbated by AAV viral treatment. Therefore, it is important that gene therapies for XLMTM can be delivered in a way that protects the liver from further injury. With this goal in mind, we performed a high-throughput drug screen in hepatocyte spheroids to find small-molecules that would block AAV8 entry. Our screen yielded 13 hits, one of which was Osimertinib, a previously approved EGFR inhibitor. After further validation and testing of small-molecule drugs, monoclonal antibodies, PROTACs, and siRNAs against EGFR, we found that approaches that target the intracellular domain of EGFR are sufficient to reduce GFP fluorescence but do not reduce viral entry as measured by ddPCR. On the other hand, extracellular targeting or siRNA knockdown effectively reduce both viral entry and transgene expression. We are currently testing our lead candidate, a GalNAc-conjugated siRNA against EGFR, in a mouse model of XLMTM to assess whether viral entry is reduced and whether signs of liver toxicity following AAV8 injection are ameliorated. This therapeutic strategy holds promise because we can specifically de-target the liver while boosting expression to desired tissues, which may even allow for dosing at an overall lower viral titer. Additionally, compared to approaches where the viral capsid is altered, this strategy is compatible with existing AAV-based therapies.

888 Interrogating the Dependency of AAV Capsids on AAVR for Retinal Transduction following Intravitreal or Subretinal Injection

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While there have been successes using adeno-associated virus (AAV)-based gene therapies to treat human disease, certain indications remain unaddressed, in part because the target tissue is relatively difficult to transduce. We continue to learn more about the underlying mechanisms of transduction by various AAV capsids and, in so doing, expand the AAV vector toolkit to overcome these hurdles. Recently a factor involved in transduction, AAV Receptor (AAVR), was discovered. The purpose of our study was to determine the role of AAVR in retinal transduction by different AAV capsids following either intravitreal (IVtI) or subretinal (SRI) injection in mice. Vectors containing CBA-GFP, were injected in littermate $AAVR^{+/-}$ and $AAVR^{-/-}$ mice at either high (5e9vg/eye) or low (5e8vg/eye) dose and transduction was evaluated at 3 and 6 weeks post-injection (p.i.) via fluorescent fundoscopy. For IVtI, retinal transduction by AAV2, AAV6, and AAV9 was completely dependent on the presence of AAVR. Retinal transduction by SRI AAV2 required AAVR, whereas AAV6, AAV8, and AAV9 demonstrated partial dependency. In contrast, AAV5 exhibited robust retinal transduction following SRI that was independent of AAVR at the high dose and partial dependency at the low dose, indicating this dependency can be overcome at high

doses. The observation that AAV8 and AAV5 both transduced photoreceptors and retinal pigmented epithelium (RPE) in an AAVR-independent manner following SRI contrasts the observation that these capsids rely on AAVR to transduce various tissues following systemic injection. To further explore this discrepancy, $AAVR^{+/-}$ and $AAVR^{-/-}$ mice were intraperitoneally injected with AAV5, AAV8, or AAV9 vectors (1e11 to 2e11 vg) containing smCBA-luciferase to determine if this observation was unique to the retina. At both 3 and 7 weeks p.i., fluorescent imaging revealed that AAV8 and AAV9 were partially dependent on AAVR for transduction of systemically targeted tissues. AAV5 remained completely independent, a result that contrasts previous in vitro research. We also investigated the relationship with known AAV capsid cellular receptors and AAVR dependency. AAV6ΔHS and AAV2ΔHS capsid mutants devoid of heparin sulfate proteoglycan (HSPG) binding sites, [AAV6 (K531E) and AAV2 (R487G, R585S, R588T), respectively] were subretinally injected in $AAVR^{+/-}$ and $AAVR^{-/-}$ mice. AAV6ΔHS (4e9vg) displayed reduced transduction in $AAVR^{-/-}$ mice compared to $AAVR^{+/-}$ mice, similar to parental capsid, AAV6. Interestingly, SRI of AAV2ΔHS (5e9vg) displayed strong transduction in $AAVR^{-/-}$ mice, in stark contrast to its parental capsid, AAV2, indicating a potential co-dependency of HSPG and AAVR for transduction by AAV2. Future retinal gene therapies will need to consider how to leverage the requirement for AAVR depending on the route of administration.

889 Non-Invasive Monitoring of rAAV-Mediated Gene Expression Using Chemical Exchange Saturation Transfer (CEST) MRI

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Introduction: Recombinant adeno-associated virus (rAAV)-mediated gene therapy is a promising strategy for treating genetic disorders. To enable detection of rAAV transgene expression in vivo, we developed a superCESTide reporter gene, engineered to generate contrast in chemical exchange saturation transfer (CEST) MRI. This novel approach enables non-invasive, real-time, in vivo monitoring of gene expression using MRI. The objective of our study was to determine the efficacy of rAAV-mediated delivery of the superCESTide for CEST MRI-based imaging of transgene expression in the liver.

Methods: rAAV vectors were engineered to express either superCESTide or tdTomato genes, both under the enhanced chicken beta-actin (CBA) promoter and pseudotyped with an AAV8 capsid (Figure 1a). Five groups of C57Bl/6 mice were studied across two experimental rounds. In Round 1, two groups received either 8×10^{12} gc/kg of tdTomato (Group 1 Td, n=5) or a combination of superCESTide + tdTomato (Group 1 SCT, n=5). In Round 2, two additional groups were injected with a higher dose (1.4×10^{13} gc/kg) of tdTomato (Group 2 Td, n=4) or superCESTide+tdTomato (Group 2, SCT, n=4), with an untreated control group (n=5). In vivo CEST MRI experiments were conducted using a Bruker 11.7 T scanner with steady-state saturation via a Gaussian pulse (saturation power, 2.4 μT; pulse length, 100 ms) and interleaved FLASH readouts with radial sampling. Transgene expression was verified using fluorescence imaging with IVIS Spectrum CT. Gene expression levels were quantified through reverse transcriptase-polymerase chain reaction (RT-PCR). CEST contrast was assessed using magnetization transfer ratio asymmetry (MTR_{asym}) analysis, with regions of interest (ROIs) drawn over the liver to evaluate superCESTide expression.

Results: Fluorescence imaging confirmed hepatic expression in rAAV-injected mice (Figure 1b). CEST MRI scans showed significantly higher contrast in the superCESTide groups compared to controls (Figures 1c-h). Mean MTR_{asym} values were significantly different across groups (One-Way ANOVA, $P = 0.0054$). Upon pairwise comparison between all groups using unpaired parametric t-tests with Welch's correction (Figure 1i), the MTR_{asym} for the mice injected with higher dosage of superCESTide+ tdTomato, had significantly higher values. RT-PCR was performed to determine superCESTide transgene expression levels relative to actin (Figure 2a, difference between Group 2 SCT to other groups, *, $P<0.05$), tdTomato expression level relative to actin and groups with higher dosage of both transgenes (Figure 2b, no statistical difference). Also, the superCESTide gene expression levels were significantly higher in the liver compared to the kidneys, showing organ specific targeting (Figure 2c). Linear

regression analysis between MTR_{asym} values and log_{10} values of superCESTide expression revealed a strong linear relationship ($R^2 = 0.6$), with non-zero slope ($m=0.8P < 0.0001$) (Figure 2d).

Conclusion: This study demonstrates a clear correlation between CEST MRI contrast and superCESTide expression, supporting its potential as an MRI reporter gene for non-invasive gene therapy monitoring. The findings highlight the feasibility of real-time, in vivo tracking of rAAV-mediated transgene expression, advancing the field of molecular imaging in gene therapy.

Acknowledgments
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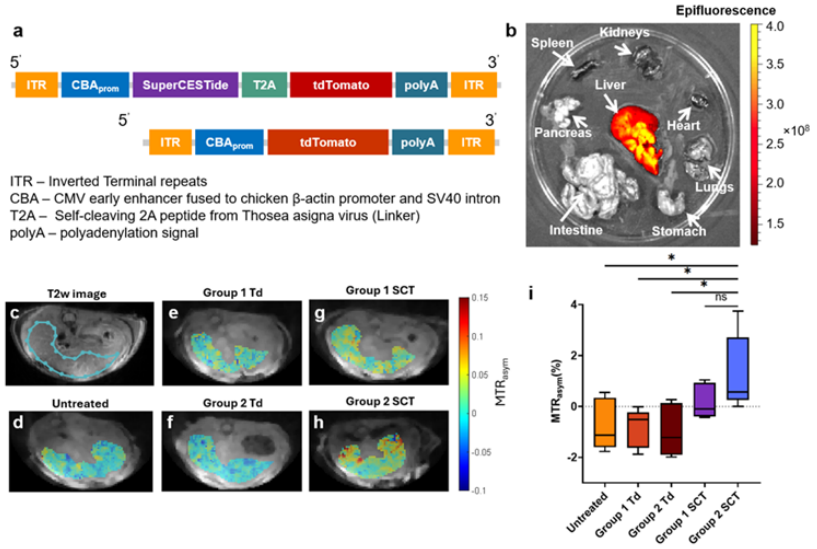

Figure 1. rAAV-mediated transgene expression and CEST MRI contrast in the liver.
(a) Schematic of rAAV vectors encoding either the SuperCESTide reporter gene or tdTomato control under the enhanced CBA promoter, packaged in AAV8 capsids. (b) Fluorescence imaging confirm hepatic expression in rAAV-injected mice. (c) T2-weighted image defining the region of interest (ROI) for CEST MRI analysis. (d-h) CEST MRI MTRasym maps overlaid on respective CEST M0 images at week 5 post-injection for (d) untreated (UT) mice, (e-f) tdTomato-injected mice (Group 1 Td, Group 2 Td), and (g-h) superCESTide + tdTomato-injected mice (Group 1 SCT, Group 2 SCT); ; SuperCESTide expression levels in Untreated, Group 1 Td, Group 2 Td, Group 1 SCT and Group 2 SCT mice are 0.75, 0.05,0.16, 1.67 and 133.43 respectively in figures c-h, (i) Distribution of MTR_{asym} along the five study groups. Statistical analysis using an unpaired parametric t-test with Welch's correction between control and SuperCESTide groups, (*, $P < 0.05$)

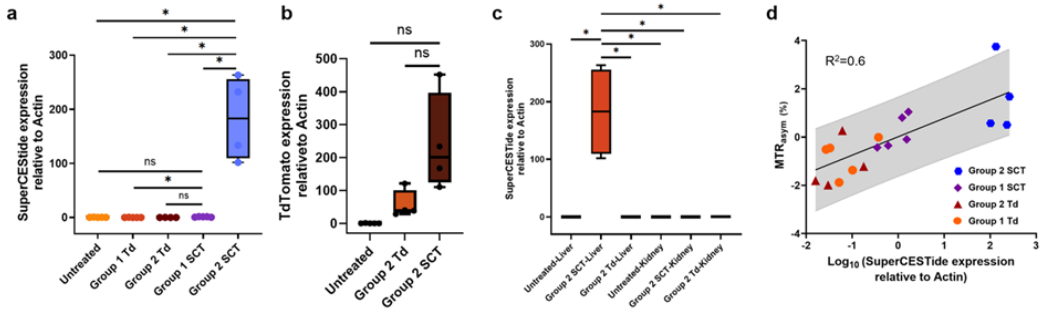

Figure 2. RT-PCR analysis of SuperCESTide and tdTomato transgene expression and correlation with CEST MRI contrast.
(a) Relative SuperCESTide expression normalized to Actin across all groups, showing significantly higher expression in the high-dose SuperCESTide group compared to others (*P < 0.05). (b) Relative tdTomato expression normalized to Actin, with no significant differences observed between groups. (c) SuperCESTide expression levels in liver and kidney for Untreated, Group 2 Td and Group 2 SCT mice. (d) Linear regression analysis between MTR_{asym} values and the $\log_{10}$ of SuperCESTide expression showing strong correlation ($\mathbf{R^2} = 0.6$, slope = 0.8, $\mathbf{P} < 0.0001$)

890 Engineering a gene dosage compensation circuit through competitive dimerization for gene therapy

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Synthetic gene circuits offer considerable potential for engineering human cells to perform complex functions for innovative therapeutic applications. However, current approaches are limited by the lack of versatile, scalable and modular molecular components compatible with the limited packaging capacity of viral vectors used in gene therapy, such as AAV. We overcame these barriers by developing a novel class of Modular Artificial Dimerizing transcription factors (MADTFs) whose activity is dynamically regulated through interaction with competitive inhibitory proteins (ΔTFs). These DTFs competitively heterodimerize with MAD-TFs preventing their homo-dimerization and thus their function. This dual strategy enables dynamic and reversible regulation of gene expression, establishing a novel technological platform to build synthetic circuits and program cell behaviour. The compact and modular design of MADTFs and ΔTFs not only facilitates their integration into a variety of biotechnological applications but also enhances their potential for clinical translation.

We first implemented two different biomolecular circuits for doxycycline-inducible gene expression using the MADTF/DTF technology and then tested them by transient transfection in HEK293T cells. These circuits rely on the Coherent Inhibitory Loop (CIL) topology, which can be used to achieve high-performance inducible gene expression in mammalian cells while reducing "leaky" basal gene expression. Thanks to the combination of low leakiness and high maximal expression, the CIL circuit confers a 200-fold higher increase in gene expression, in response to doxycycline administration, while the state-of-the-art TRE3G tet-on system's performance is one order of magnitude lower.

After having demonstrated the capabilities of the technology, as a proof of concept for potential applications to gene therapy, we are now building a gene dosage compensation circuit (GDC), i.e. a synthetic circuit capable to regulate the expression of a gene within a cell in a specified range independently of plasmid copy number or virus uptake. The GDC is able to mitigate the overxpression-related toxicity risks associated with gene replacement therapy approaches for some conditions such as RETT syndrome and Friedrich's Ataxia. In our implementation, MADTF drives expression of the gene of interest (in our case a GFP fused to a transcriptional activator), which in turns control the expression of the ΔTF. The construct fits within the AAV packaging capacity allowing the whole circuit to be delivered in a single AAV vector. The balance between MADTF and ΔTF ensures that the expression of the

gene of interest is maintained within a tight expression window independently of the plasmid copy number. Our work aims to establish a foundational technology for precision-engineered synthetic circuits enabling transformative advances in both biotechnology and medicine.

891 Small Molecule Inhibition of SUMOylation Increases Expression from AAV Vectors Both During and Long After Initial Transduction in Mice

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Recombinant adeno-associated virus-based vectors (rAAV) are in clinical development for human gene therapy, but a common limitation is a low level of transgene expression after vector delivery. Multiple mechanisms have been proposed that silence expression of unintegrated DNA, such as AAV episomes. This makes it challenging to maintain therapeutic levels of gene expression, particularly because readministration with AAV is not feasible due to immune responses. One potential silencing mechanism is the conjugation of the small ubiquitin-like modifier (SUMO) to proteins. Previous studies have suggested that SUMOylation may influence both AAV capsid function and host cellular antiviral pathways. We thus set out to identify clinically relevant modulators of SUMOylation useful in improving AAV transduction. TAK-981 is a small molecule inhibitor of the SUMO-activating enzyme (SAE) complex in clinical development. We thus tested TAK-981 for potentially boosting expression from AAV vectors. To test this in vitro, HeLa or IMR90 cells were treated with TAK-981 prior to transduction with AAV-eGFP and expression was quantified with microscopy and flow cytometry. Boosting of GFP expression was observed, with a 14-fold (HeLa) and 26-fold (IMR90) increase at the highest optimal dose. Vector DNA copy number was not significantly altered, and the ratio of mRNA copies to vector template was increased. To evaluate the effect of TAK-981 in vivo, BALB/cJ or Hemophilia A/CD4KO mice were treated with TAK-981 or vehicle control. TAK-981 was administered during or long after AAV delivery of vectors expressing luciferase (AAV-luc) or human factor VIII (AAV-hFVIII). Expression was quantified by bioluminescence (luciferase) or hFVIII antigen ELISA. DNA vector copies and transgene mRNA from mouse livers were measured by qPCR and RT-qPCR. In BALB/cJ mice treated with TAK-981 at time of AAV-luc delivery, luciferase expression increased 200-fold over controls at 3 days post-AAV administration and returned to control levels by day 7. When BALB/cJ mice were delivered AAV-luc with no drug and treated with TAK-981 a month after transduction, luciferase expression increased 100-fold over controls for 2-3 days, decreasing thereafter to control levels. A similar increase was observed with repeated dosing of TAK-981 at later times. Mice treated with drug during AAV delivery were later exposed to TAK-981 or vehicle at 1.5 months post transduction. All mice receiving TAK-981 had a 150-fold boost in luciferase expression, regardless of initial treatment. In hemophilia A/CD4KO mice transduced with AAV-hFVIII in the presence of TAK-981, treatment boosted hFVIII expression 4-fold and increased hFVIII RNA copies per DNA template. Integration site distributions were quantified using ligation-mediated PCR and the AAVengeR pipeline. Analysis of human cell lines and mouse livers did not show significant changes in integration frequency or distribution, or vector DNA rearrangements at integration junctions. In summary, SUMOylation inhibition with TAK-981 can boost expression when given during or long after AAV transduction. Repeated dosing with TAK-981 results in enhanced transgene expression at each administration. We postulate that TAK-981 modulates AAV transcription, rather than vector genome synthesis or integration. As TAK-981 is undergoing clinical testing in human patients, this may represent a strategy for boosting AAV transgene expression to improve human gene therapy outcomes.

892 An Enhancer-based AAV Toolbox for Precise Neuronal Targeting

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Recombinant AAV (rAAV) can drive long-term gene expression in vivo and has become a popular gene delivery vector for research and gene therapy. However, to better leverage rAAV as a research and therapeutic tool, the

expression of transgene needs to be regulated in a cell-type specific or context dependent manner. To address this, significant efforts have been made to modify the AAV capsid. However, the extent of specificity capsid choice can offer is relatively limited. Enhancers are non-coding elements that can regulate gene expression and control the activity or spatial-temporal pattern of gene expression. In recent years, we and others have identified a number of enhancers that, when incorporated into rAAV vectors, can restrict the transgene expression to particular neuronal populations. Yet, viral tools to access and manipulate fine neuronal subtypes are still limited. Here, we developed a highly efficient computational pipeline to identify cell-type specific enhancers from single-cell ATAC-seq and RNA-seq data. We incorporated these regulatory elements into rAAV vectors and built a set of viral tools that are highly specific for distinct neuronal populations. Importantly, we showed that the route and age of injection can have significant impacts on the performance of these enhancer-based rAAV vectors. These enhancers, when used in the context of different effectors, can target (fluorescent proteins), observe activity (GCaMP) and manipulate (opto- or chemo-genetics) specific neuronal subtypes. We also validated our enhancer-AAV tools across species. Thus, we provide the field with a powerful set of tools to study neural circuits and functions and to develop precise and targeted therapy.

893 Novel Rationally Designed Promoters Surpass CAG in Mouse and Human Models

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Introduction: Promoters are essential for gene expression and can be engineered to develop optimized gene therapies. Ideal promoters are short in length and confer tissue specificity as well as potent transgene expression. Approximately 50% of gene therapy clinical trials rely on the CAG or CMV promoters. However, high doses of gene therapies have recently led to serious adverse events in patients. Thus, there is a need to increase gene therapy vector potency to improve the safety profile of gene therapies. Accordingly, we aimed to engineer novel CAG-based constitutive promoters which are both shorter than the original sequence and lead to stronger transcriptional activity. CMV was excluded because it reportedly inactivates *in vivo.* These optimizations would allow for a lower effective dose which could potentially reduce immune responses after treatment and lower the cost of goods for gene therapy applications.

Methods: The CAG promoter is a hybrid promoter, 1.8 kb in length, consisting of the CMV immediate early enhancer, the chicken β-actin promoter, and a hybrid rabbit β-globin intron. We rationally designed a series of 82 new CAG promoter variants by systematically introducing modifications to each of the promoter elements and tested them in human and mouse *in vitro* and *in vivo* models. In our library of engineered CAG promoter variants, 51 are smaller than the original CAG, of which 22 are fewer than 1000 base pairs in length.

Results: In HEK293T cells, 67 CAG promoter variants are stronger than the original CAG sequence with the strongest promoter exhibiting 13-fold improvement in potency. In both primary human hepatocytes and primary human myotubes, four CAG variants drove 15-fold higher transgene expression than the original while being ~40% shorter in length. These four variants and an additional strong CAG variant were packaged into AAV9 and administered either systemically or directly into the gastrocnemius muscle of C57BL/6 mice. After systemic administration, one variant in particular, C178, was found to be stronger than the original CAG in the muscle, liver, heart, and brain while being 700 base pairs shorter in length. In the mouse muscle, four of these variants led to 0.5 to 2.5-fold stronger transgene expression three months after direct injection. In contrast, muscle-specific promoters that are used in the clinic such as Ck8e are significantly weaker than the original CAG promoter when assessed using the same bioimaging paradigm. Expression will continue to be evaluated to determine if these potent promoters have durable activity *in vivo*.

Conclusions: Here, we have shown several rationally designed CAG-based constitutive promoters which are both shorter than the original CAG sequence and yield higher transcriptional activity in human and mouse *in vitro* and *in vivo* models. These promoters show potential for the development of future gene therapies. In particular, these smaller, more potent CAG variants could be advantageous for the design of gene therapies for the muscle and central nervous system where transgenes are typically large, and packaging constraints are of major concern.

894 KT-Splice: Regulation of AAV Transgene Expression by Manipulation of Alternative Splicing

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In the development of gene therapies using Adeno-associated viral (AAV) vectors, engineered capsids and tissue-specific promoters allow drug developers to restrict vector expression to specific tissue types. While these advancements in vector design offer some control over where the therapy is expressed, they lack the ability to regulate the level or the timing of therapeutic transgene expression. Further modifications to AAV vectors to allow for sequence-specific regulation of transgene expression would improve safety and efficacy while expanding potential indication options. To address this need, we have developed KT-Splice, which is a system to regulate transgene expression through control of alternative splicing. This system uses delivery of a small molecule to induce a targeted change in RNA splicing thereby altering expression of an AAV transgene in a sequence-specific manner.

We first conducted an RNA-sequencing based small molecule screen to identify candidate small molecules that demonstrated splice-switching of RNA in a sequence-dependent context. We then generated the KT-Splice system by cloning the splice-switches (i.e. sequences that demonstrated alternative splicing upon drug treatment) into a Green Fluorescent Protein (GFP) reporter construct and assessed induction of GFP in vitro after small molecule treatment. Following further optimization of the splice-switch sequence, we examined the activity of KT-Splice in vivo.

The KT-Splice switch was packaged into AAV9 and systemically injected into mice at 2 vector doses, 1e11 or 1e12 vector genomes per animal, and 4 weeks post-injection the small molecule was introduced by oral gavage at two concentrations, 10 or 20 milligrams per kilogram. We collected liver and hearts from these animals and tested for levels of vector DNA and alternative slicing of the switch sequences in these tissue types in animals treated with drug versus vehicle. We observed a significant increase in RNA splicing of the optimized KT-Splice vector in mice only after small molecule delivery in both vector doses. These data demonstrate that KT-Splice is potentially a transformative system to control the timing, location, and expression level of an AAV transgene and provides a mechanism for the next generation of AAV gene therapies to treat more common diseases.

895 Ultra-Compact Synthetic Transcription Factor Platform for Small-Molecule Regulated Control of AAV Transgene Expression

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Gene therapies rely on ubiquitous or tissue-specific promoters to drive constitutive transgene expression, but these approaches lack the flexibility needed for precise therapeutic control. In many applications, the ability to fine-tune transgene expression in response to patient-specific needs is crucial for optimizing efficacy. Dynamic control over gene expression allows for dose adjustment and temporal regulation of therapeutic activity, reducing the risk of under- or over-treatment. Inducible gene expression systems offer this level of precision, yet the packaging constraints of widely used AAV vectors limit the implementation of existing systems. Developing compact, small-molecule-inducible systems would address these challenges and enhance the versatility of gene therapies across a range of clinical applications.

In this study, we describe the development of new ultra-compact, potent, and tunable, small molecule-induced gene regulation systems optimized for use in AAV vectors. We pioneered the adaptation of minimal zinc finger transcription factors based on previous technology[1] to make them controllable by FDA-approved drugs[2], which we termed minimal inducible synthetic transcription factors (mini-synTFs). We demonstrated the ability to employ

multiple small-molecule controllable modalities to conditionally regulate transgene expression. These demonstrations include initial prototyping via transient transfection of plasmids to explore design space, followed by validation of promising designs via transduction of neuronal cells with a single AAV vector encoding both the transgene and regulatory components. This approach identified promising small-molecule controllable systems that enable dynamic and regulatable AAV-mediated gene expression. Our toolkit offers valuable solutions for researchers and drug developers seeking precise control over transgene expression in gene therapy applications.

References
1. Donahue, P. S. *et al.* The COMET toolkit for composing customizable genetic programs in mammalian cells. *Nature communications* **11**, 779 (2020).
2. Li, H.-S. *et al.* Multidimensional control of therapeutic human cell function with synthetic gene circuits. *Science* **378**, 1227-1234 (2022).

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896 Controlling the Expression of Dose-Sensitive AAV Transgenes with Chemically Induced Proximity

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AAV-based gene therapy is a growing field with recent approvals for diseases ranging from hemophilia B to aromatic L-amino acid decarboxylase (AADC) deficiency. However, the majority of AAV vectors employed in clinical trials use constitutive vectors with expression profiles that cannot be altered after vector administration. The lack of post administration control of the transgene limits the applicability of the AAV delivery in cases where clinical success requires precise protein expression within a narrow therapeutic window. In previous work, we have demonstrated that the expression profiles of AAV vectors can be controlled by the co-localization of endogenous transcriptional machinery and AAV genomes through chemically induced proximity (CIP). Specifically, by embedding a small, humanized synthetic DNA binding protein (DBP) and cognate binding array in the AAV transgene cassette, we show in human cells that the epigenetic and transcriptional activator protein BRD4 can be recruited to the AAV genome through the use of a heterobifunctional small molecule with affinity for both BRD4 and the DBP. Here, we extend this work by screening seven different organizations of the CIP AAV dose control platform, and identify a new lead vector with a balanced profile of dynamic range and total gene expression. For the first time, we show that the CIP approach to controlling AAV expression after transduction can be applied to biologically impactful transgene cargo in the context of VEGF blocking therapy. The VEGF decoy receptor is an efficient inhibitor of VEGF signaling and subsequent angiogenesis that has been explored for applications including diseases of the eye such as wet age-related macular degeneration and cancers such as metastatic colorectal carcinoma. However, toxicity associated with VEGF decoy receptor therapy has contributed to the slow adoption of this therapeutic strategy to a gene delivery platform such as AAV. In this work we apply the lead CIP AAV control vector to the delivery of the VEGF decoy receptor, and show dose-dependent activation of the transgene payload in colorectal cancer cells and primary human endothelial (HUVEC) cells concentrations. To measure the impact of the CIP-controlled VEGF decoy receptor on angiogenesis, we performed a tube formation assay with primary HUVEC cells. We show that CIP activation of AAV-mediated delivery of the VEGF decoy receptor significantly reduced HUVEC tube network formation compared to AAV treatment alone. These studies provide evidence that chemical recruitment of endogenous upregulating machinery to AAV genomes can be used to titrate the expression of therapeutically relevant transgenes in human cells. Future preclinical *in vivo* studies will determine the extendibility of this platform to the development of a new class of dynamically-dosable AAV vectors.

897 Differential Usage of Transcription Factor Binding Sites to Boost Synthetic Promoter Activity

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Transcription factor binding sites (TFBS) play a crucial role in regulating gene expression and can either enhance or repress expression depending on their function and context. Here, we designed a library of short synthetic promoters to evaluate the effect of differential TFBS usage in N2A, mouse neuroblastoma, and C2C12, mouse myoblasts, cell lines.

A massively parallel reporter assay was created to assess the transcriptional strength of 244,000 synthetic promoter sequences. These 182 bp sequences include a variable regulatory upstream region and one of six core promoters with known tissue specificity. Two ubiquitous, JeT and AdML, two muscle-specific, MCK and DES, and two liver-specific promoters, mTTR and AAT, were chosen to assess the impact of alternative TFBS usage on promoter specificity. Regulatory regions were generated to evaluate (i) the effect of each TFBS in isolation, and (ii) the combinatorial effect of selected and tissue-enriched TFBS. Promoter activity in each cell line was calculated as a normalized expression score, referred to as SoR, using Illumina-based sequencing data. Subsequently, the TFBS fold change, or Boost SoR, over the activity of the parent core promoter without any regulatory region was calculated.

Using Boost SoR, we categorized all TFBS as activators (Boost SoR $\geq$ 0.1, n=85,711), repressors (Boost SoR $\leq$ -0.1, n=53,146), or as having minimal impact on promoter performance (Boost SoR between -0.1 and 0.1, n=13,814). The top five activating TFBS tested with muscle-specific MCK achieved a four-fold or greater increase in expression compared to MCK alone, with the top boosting TFBS exhibiting a six-fold Boost SoR. The five most repressive TFBS tested with MCK reduced transcription by over five-fold. For JeT, the five strongest activating TFBS increased expression by three-fold or more, while the five most repressive TFBS decreased expression by four-fold or more. In total, 21.6% of all TFBS combined with JeT were characterized as activators with the strongest activators boosting expression by more than four-fold. Except for DES, all core promoters exhibited the highest boost in expression through homotypic, or repetition of the same TFBS, designs rather than through combinatorial approaches. Amongst the two hundred strongest activating homotypic TFBS designs for each of the core promoters, only six were consistently observed across promoters, emphasizing their high degree of selectivity. To further evaluate TFBS-promoter selectivity, we correlated the Boost SoR of TFBS across core promoters. On average, TFBS tested with JeT and MCK exhibited a lower correlation with other core promoters (R^2_{JeT} = 0.14, R^2_{MCK}=0.23), suggesting a higher TFBS selectivity of those promoters. Conversely, the constitutive promoter, AdML, showed a significantly higher correlation with the liver promoter, mTTR, and MCK, a muscle promoter (R^2_{mTTR} = 0.29, p_{mTTR}=1.4E-81; R^2_{MCK} = 0.28 p_{MCK}= 9.6E-77).

We successfully characterized TFBS that positively and negatively boost the expression of core promoters. By quantifying the boosting effects of differential TFBS, we demonstrated the ability to enhance the performance of the widely used and optimized JeT promoter. Furthermore, we identified TFBS uniquely associated with each promoter and sequences that should be excluded from active regulatory element design. This analysis represents a key asset when designing stronger synthetic promoters for gene therapy.

898 Characterization of AAV ITR-proximal regions (IPRs) from different AAV serotypes reveals tissue-specific enhancer and promoter activities

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While AAV-based gene therapy has made significant strides through capsid engineering and regulatory cassette optimization, the functional contributions of native AAV genetic elements have received relatively little attention. Previous studies have shown that inverted terminal repeats (ITRs) possess weak promoter-like activity, while the ITR-proximal region (IPR) at the 3' end of the wildtype AAV2 genome exhibits tissue-dependent enhancer-like function. These findings suggest that native AAV elements can be harnessed to improve gene therapy vectors. Building upon our unpublished work, which demonstrated that IPRs from AAV8 and AAVrh.39 can enhance transcription *in vitro* and *in vivo*, we expanded our investigation to systematically evaluate the enhancer and promoter activities of IPRs from multiple AAV serotypes (AAV1, AAV2, AAV3, AAV3b, AAV4, AAV5, and AAV6) in human hepatocytes and HEK293 cells.

We cloned IPR sequences (IPR1, IPR2, IPR3, IPR3b, IPR4, IPR5, and IPR6) into three distinct configurations: 1) at the 5' end, upstream of the human phosphoglycerate kinase (*hPGK*) promoter; 2) at the 3' end, downstream of the polyA signal in *hPGK*-driven luciferase expression cassettes; and 3) at the 5' end, in a promoter-less luciferase expression cassette. These designs allowed us to systematically evaluate the enhancer and promoter activities of the IPRs in regulating transgene expression. Our initial transfection studies in HEK293 cells revealed distinct enhancer activities for specific IPR elements. When positioned at the 5' end of the *hPGK* promoter, IPR1, IPR2, IPR3, IPR3b, IPR4, and IPR5 enhanced transgene expression by approximately two- to four-fold relative to controls. Similarly, placement of IPR1, IPR2, IPR3, IPR3b, IPR4, and IPR6 at the 3' end of the polyA signal yielded comparable increases in expression. However, none of the IPRs exhibited significant promoter activity in HEK293 cells. In human hepatocytes, all IPRs except IPR5 functioned as enhancers at both the 5' end of the *hPGK* promoter and the 3' end of the polyA signal, increasing expression at levels similar to those observed in HEK293 cells. Notably, IPR1 demonstrated dual functionality in hepatocytes, acting as an enhancer and exhibiting promoter-like activity when placed upstream of a promoter-less luciferase cassette. Expression levels conferred by IPR1 was comparable to those achieved by the *hPGK* promoter. Our current findings are consistent with previous observations by Ian E. Alexander's group, which focused on IPR2. These results underscore the tissue-dependent regulatory potential of IPRs, particularly their ability to fine-tune transgene expression in hepatocytes. While further validation is ongoing, these *in vitro* findings provide a robust framework for further translational gene therapy studies. Based on their consistent enhancer activity and hepatocyte-specific promoter capability, IPR1, IPR3b, and IPR4 have been prioritized as lead candidates for *in vivo* validation, which is currently in progress to assess their capacity in optimizing gene expression.

In conclusion, our work reveals the untapped potential of native IPRs to precisely modulate transgene expression in a tissue-specific manner. These findings underscore the value of mining native AAV biology, a strategy that complements capsid engineering and promoter optimization, to develop next-generation gene therapy vectors with tailored efficacy. [@]Corresponding authors, *equal contributing authors

899 A Platform for Next-Generation Synthetic Promoter Design and Engineering

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The promoter is a critical component of any gene therapy payload, determining cell and tissue specificity, transgene expression levels, and stability. Its selection directly impacts safety and efficacy. Synthetic promoters enable precise control to maximize therapeutic benefits, minimize off-target effects, can reduce dosing, and repress transgene expression during manufacturing.

We have created a proprietary computational platform that enables rapid generation of highly tissue-targeted synthetic promoters with controllable activity. Our platform quantitatively surveys the different transcriptional landscapes of all human cell types to mine subsets of transcriptionally active building-block DNA sequences that are assembled *in silico* into novel, patentable, synthetic promoters with desirable functionality. Our synthetic promoters are designed to be specific to a given cellular/disease context, possessing functional characteristics that overcome the inherent limitations of natural and viral promoters.

To demonstrate its function and versatility, we applied our platform to engineer synthetic promoters for immunotherapeutic payload expression in natural killer (NK) cells exhibit a broad range of activity (between 5 to 100% of hCMV-IE), in B-lymphoma and liver cell lines (Ramos and HepG2, respectively). Liver-targeted synthetic promoters exhibit a >10-fold activity range from ~17% to ~200% of hCMV-IE in HepG2 cells and up to 27-fold that of liver-specific promoter currently used in a commercial gene therapeutic. Negligible activity in differentiated muscle C2C12 cells was observed and promoter activity in HepG2 cells correlates well with promoter activity in cultured primary hepatocytes ($r2 = 0.97$, $p<0.0001$). In contrast, skeletal muscle targeted synthetic promoters exhibit high activity in C2C12 cells (up to 93% of hCMV-IE) with minimal activity in HepG2 cells (3.4% of hCMV-IE).

Importantly, these novel synthetic promoters were designed for high transcriptional efficiency (small DNA footprint to minimise utilization of the limited AAV payload) and minimal interference with the host-cell transcriptional landscape.

In summary, synthetic promoters rationally engineered *de novo* for disease-specific targeted expression offer significant advantages over the limited repertoire of natural (endogenous/viral) promoters with poorly-defined activity and specificity. As a key component of the therapy, the use of safe and efficacious synthetic promoters should be a primary consideration for vector design.

900 A novel AAV2 variant designed for targeting of retinal tissue via intravitreal injection

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Purpose: Eyes possess unique autoimmune privilege properties that make them favorable targets for gene therapy. However, current gene therapy methods utilizing adeno-associated virus (AAV) vectors face challenges. For example, AAV vectors demonstrate limited transduction efficacy in retinal tissue and RPE-choroid when administered via intravitreal injection. The purpose of the current study is to develop a AAV variant with high transduction efficiency to retina tissue.

Methods: Guided by rAAV viral structural biology principles and receptor binding, a 7-peptide sequence was "rational design" and incorporated into variable region VIII of AAV capsid to augment its binding affinity for cellular receptors. AAV variants were screened and AAV2.M1 was found to have high transduction efficiency and selected to do further assessment.

Results: An EGFP report gene was packaged into wild-type AAV2, AAV2.M1 and a positive control AAV variant. Compared with wild-type AAV2 and the positive control AAV variant, the M1 variant exhibited comparable packaging efficiency but demonstrated a marked increase in transduction efficiency to ARPE19 cells. Following intravitreal injection at a dose of 2e9 vg/eye, the AAV2.M1 variant outperformed both wild-type AAV2 and the positive control AAV variant in mice, achieving higher transduction levels in RPE and photoreceptor cells. In rhesus monkeys, the transgene expression was found 1915.64 ng/g in the animals' retinal tissue in AAV2.M1 variant group after intravitreal injection, significantly higher than the positive control group which is 11.8 ng/g.

Conclusion: The AAV2.M1 variant represents a promising vector candidate in ophthalmic gene therapy. Its potential to advance the gene therapy industry is substantial.

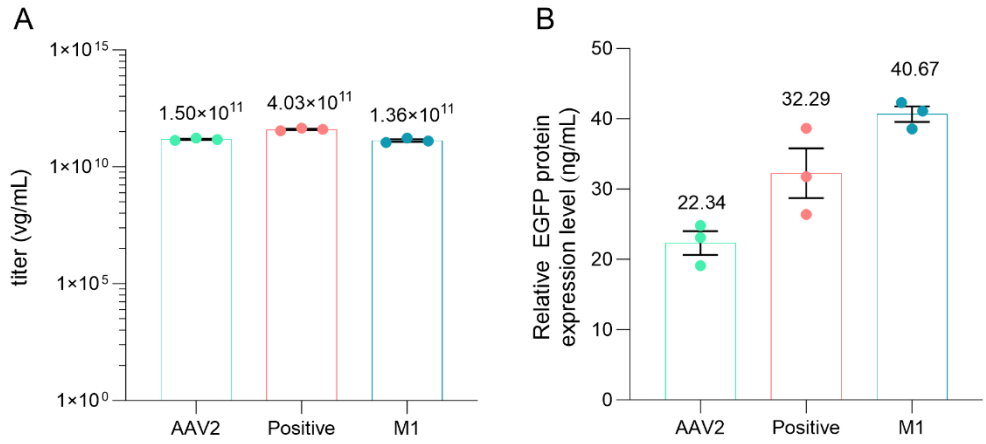

Fig.1 A, packaging efficiency of three AAV vectors; **B**, EGFP reporter gene was packaged into three AAV vectos and ARPE19 cells were transduced with these vectors the EGFP protein expression was detected by ELISA.

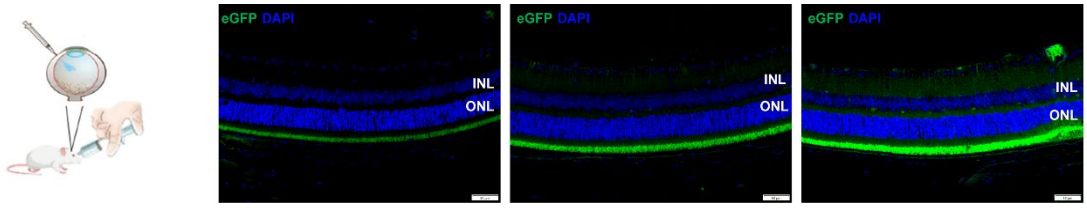

Fig.2 Intravitreal injection of different AAV variants vectors include AAV2-EGFP, Positive control, and AAV2.M1-EGFP were performed in mice eyes at a dose of 2.5E9 vg/eye. Mice were sacrificed at day 14 post-injection, retinal cross-sections and immunofluorescence (EGFP, green) analyses were conducted. (Scale bars: 50 μm; INL: inner nuclear layer, ONL: outer nuclear layer)

901 Identification of a novel AAV capsid variant with enhanced transduction efficiency in mice

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Purpose: The transduction efficiency of AAV vectors to retinal tissues has not yet met the expectations of researchers when administrated via intravitreal injection. The purpose of this study is to identify AAV variants with high transduction efficiency to the retina tissue. **Methods:** A capsid library was constructed using a rational design approach. This library underwent three rigorous rounds of screening in cellular, murine, and primate models, to identify highly enriched candidate rAAV capsids (Fig.1). **Results:** the AAV variant M4 exhibited the highest enrichment score during the screening process and was subsequently confirmed to have a higher transduction efficiency than positive benchmark variant in ARPE19 cells (Fig 2A). A transgene of sCD59/EGFP were packaged into this novel AAV variant to generate the AAV2.M4-sCD59 AAV2.M4-EGFP vectors. In a head-to-head study the sCD59 expression level in AAV2.M4-sCD59 group was 2.45 times higher than wild-type AAV2-sCD59 vector group when transduced these vectors into ARPE19 cells (Fig.2B). Following intravitreal injection into C57BL/6 male mice of these two vectors, the transgene expression was found 2.46 times higher than that of AAV2-sCD59 in animals' retinal tissues (Fig.2C). Immunofluorescence staining revealed significantly higher EGFP fluorescence intensity in the AAV.2M4-EGFP group compared to the AAV2 group (Fig.2D). **Conclusion:** Our newly isolated

AAV2.M4 variant has high transduction efficiency to the retinal tissues in mice, making it a promising vector candidate for advancing rAAV-based gene therapy in clinical applications.

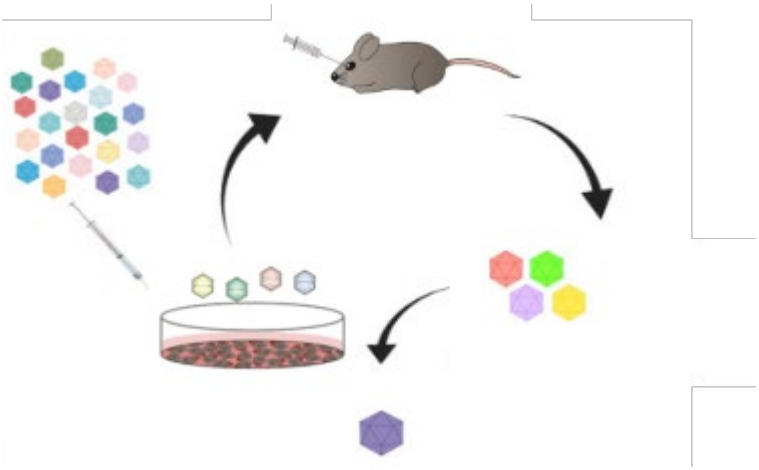

Fig.1 Schematic flowchart for the screening of rAAV variants

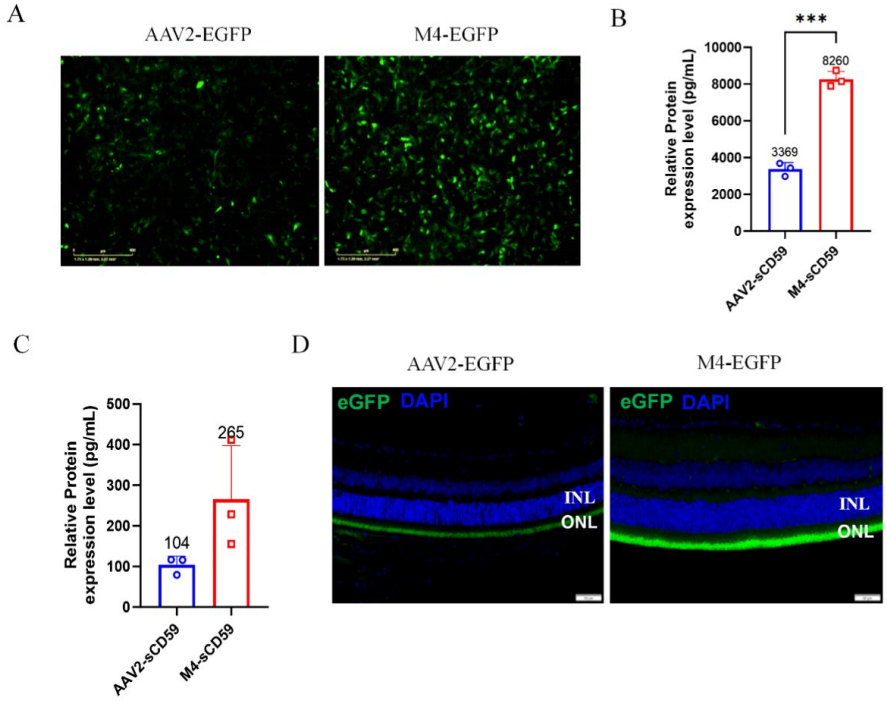

Fig.2 A. The AAV2-EGFP and AAV2.M4-EGFP vectors were transduced into ARPE19 cells, and the fluorescence images were taken 48 hrs post transduction. **B.** The cells were harvested and the EGFP protein level was measured in cell lysis. **C** AAV2-sCD59 and AAV2.M4-sCD59 vectors were administrated into mice eye via intravitreal injection. Mice retinal tissues were collected at day-13, the relative sCD59 expression level was measured by ELISA. **D** Cross-section and immunofluorescence (EGFP, Green) analyses of mice retina treated with IVT injection of AAV2-EGFP(left) and M4-EGFP(right). (Scale bar: 100μm, GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer)

902 A Novel AAV Variant for Efficient RPE Transduction via Intravitreal Injection

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Purpose: Effectively delivering of therapeutic drugs to target cells, particularly RPE and photoreceptor cells, poses a significant challenge in AAV-mediated gene therapy for ophthalmic diseases. Intravitreal (IVT) injections offer a simpler method to directly deliver the therapeutic drug into retina in an outpatient clinic setting. However, IVT injections necessitate a high transduction efficiency of the AAV vector to effectively target the key cells. The aberrant activation of complement cascade is believed to play a key role in the development of Dry age-related macular degeneration (AMD). CD59 is a complement regulator which is capable of protecting RPE cells from complement-induced cell destruction. The purpose of this study is to investigate the transduction efficiency of our novel AAV variant when administered via IVT injection.

Method: A novel AAV2 variant was created by capsid engineering technology. And a soluble CD59 (sCD59) was packaged into this capsid to generate a recombinant AAV-sCD59 vector. The parental AAV2 wild-type serotype was used as control. The transduction efficiency was evaluated in nonhuman primates (Rhesus Monkey). Basically, intravitreal injection was performed at a dose of 1E11 vg/eye in both the experimental group (n=4) and control group (n=2) respectively. At 14- and 28-days post-injection, retinal tissues were collected, the vector genome copies and the transgene expression level were measured by qPCR and ELISA respectively.

Result: On the 28th day post-injection, the concentration of sCD59 protein in animals' aqueous humor tissues, retina and vitreous humor were significantly higher than in the wild-type AAV2 group. Besides, our novel AAV variant showed 11 times higher than that of the wild-type AAV2 capsid in terms of vector genome copies. (Fig. 1)

Conclusion: We report a novel AAV2 variant with high transduction efficiency following IVT injection in a Rhesus Monkey model. The evaluation of the therapeutic effect of the AAV-sCD59 in the treatment of dry-AMD is ongoing.

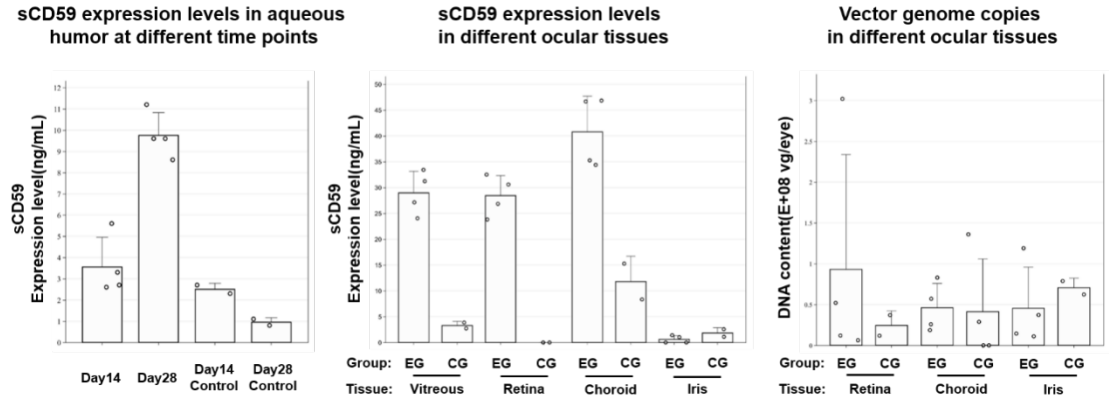

Figure 1. The expression levels of sCD59 protein at various time points (left) and in diverse ocular tissues (middle), as well as the vector genome copies in the aqueous humor (right) of monkey eyes, were detected by ELISA and qPCR following transduction. EG stands for the Experimental Group, and CG stands for the Control Group (wild-type AAV2).

903 Engineered AAV capsids for transduction of the retina by intravitreal injection

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Recombinant adeno-associated viruses (rAAV) are a promising gene therapy candidate, in particular for treating Inherited retinal diseases (IRDs). While subretinal delivery of curative transgenes via AAV vectors is well established, exemplified by the market approval for Luxturna, it remains a complex procedure performed by highly trained specialists. To address broader needs, many IRDs will require delivery of the AAV capsid via the simpler and more accessible route of intravitreal (IVT) injection. This is particularly relevant for those IRDs that compromise the structure of the retina, resulting in a pathology that renders it highly sensitive to complications following subretinal injection. AAV capsids available to date display limited transduction of the retina following IVT administration leaving patient needs unmet and increasing the demand for novel AAV capsids. To fill this gap, we performed RNA-driven biopanning of AAV peptide libraries following intravitreal injection in mice. We deployed this approach performing three rounds of selection, isolating a variety of lead capsid candidates that confer strong retinal tropism. Several of these candidates, delivered intravitreally, substantially outperform AAV2.7m8, a leading candidate for retinal gene therapy, achieving up to 10-fold increased retinal transduction. Furthermore, our candidate capsids show distinct transduction profiles towards different layers of the retina. A head-to-head comparison of our lead candidate FSp0026 with AAV2.7m8 revealed a 7-fold greater transduction of the inner nuclear layer at an equivalent viral dose also outperforming AAV2.NN and AAV2.SS. Beyond this, FSp0026 displayed high production fitness, yielding titers about 40-fold higher than AAV2.7m8, demonstrating the translational potential of our lead candidate candidates isolated via our RNA-driven biopanning pipeline.

Our pipeline yielded additional candidates outperforming AAV2 and AAV2.7m8, following intravitreal injection at a low titer (1E+08 vg/eye) quantified via immunohistochemistry (IHC) and automated image quantification. Moreover, one capsid was able to efficiently transduce anterior eye structures including stroma cells and keratocytes. To the best of our current knowledge, this would be the first AAV capsid that could be leveraged to address corneal diseases or open the opportunity for keratocytes to serve as a cellular factory for the production of therapeutic proteins.

These experiments confirmed that lead candidates outperform the literature references in both the outer and inner nuclear layer and retinal ganglion cells, showcasing our ability to derive novel AAV-capsids from our pipeline in an in vivo context. Following this selection we further expanded our biopanning pipeline to human retinal organoids, demonstrating its capacity to rapidly evolve novel AAV capsids in clinically relevant model systems.

904 Systematic Evaluation of AAVs in Two Non-human Primate Models Following IVT Injection Identifies AAV(Eye) as a Promising Candidate for Further Capsid Engineering

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Gene therapy holds great promise for the treatment of eye diseases. Adeno-associated virus (AAV) serotypes, such as AAV2, AAV5, and AAV8, are widely used for retinal gene delivery. AAV2 is predominantly utilized for intravitreal injection (IVT), while AAV5 and AAV8 are often used for subretinal injection (SRI). Due to its minimally invasive nature and the ability to be performed in an outpatient setting, IVT represents a safe and promising approach for ocular gene therapy. IVT delivers AAV vectors into the vitreous space, primarily targeting the ganglion cell layer (GCL) and other inner retinal cells. However, the inner limiting membrane (ILM) poses a significant barrier, restricting the penetration of natural AAV serotypes into deeper retinal layers beyond the GCL.

To overcome these limitations, engineered AAV2 variants such as AAV2-YFs, AAV2-7M8, and AAV-4D-R100 have been developed through directed evolution, enabling improved delivery to the outer retina. However, significant challenges remain when translating these advancements to large-animal retinas, which differ substantially from rodent retinas. Large-animal retinas, such as those in dogs and non-human primates (NHPs), feature specialized high-acuity regions (e.g., the area centralis in dogs and the fovea in primates), a thicker vitreous, and a more robust ILM. Consequently, AAV2 may not be the optimal serotype for retina-targeting capsid engineering in NHPs using the IVT route.

To identify potential AAV capsids for further engineering, we systematically evaluated commonly used AAV serotypes (AAV2, AAV5, AAV6, AAV8, AAV9, and AAVrh.74) and a series of AAV2 variants (AAV2-Y444F-Y500F, AAV2-Y444F-A493V-Y500F, AAV2-GL, AAV2-NN, and AAV2-7M8) in the whole retina of Rhesus Macaque using barcode technology. The results showed that among the commonly used serotypes, AAV2 exhibited the highest DNA levels in the retina, followed by AAV5, AAV9, AAV8, and AAV6. AAV2-YFs slightly increased DNA levels. AAV2 variants selected from large-animal models (AAV2-7M8) demonstrated a ~10-fold increase in DNA levels compared with AAV2. However, AAV2 variants evolved using mouse models (AAV2-NN and AAV2-GL) showed either comparable or reduced DNA levels.

At the RNA level, AAV6 exhibited an ~8-fold increase in expression compared to AAV2. Notably, AAV2-7M8 demonstrated a several-hundred-fold increase in RNA expression relative to AAV2. Moreover, we identified a novel AAV capsid, designated AAV(Eye), which achieved a 3.4-fold increase in DNA levels and a 47-fold increase in RNA expression compared to AAV2. Additionally, AAV(Eye) demonstrated greater specificity in the retina compared to AAV2-7M8. Importantly, AAV(Eye) also exhibited a ~12-fold increase in RNA expression in Cynomolgus Monkeys compared to AAV2. Using single-cell molecular sequencing, we aim to identify the specific target cells for this capsid.

In summary, AAV(Eye) demonstrates strong potential as a template for future AAV capsid engineering efforts, providing an efficient platform for targeted retinal gene delivery especially in NHP models.

905 Development of a Novel Antibody Targeting Engineered AAV2 Capsids for Gene Therapy Applications

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Introduction: Wild-type AAV2 is commonly used in gene therapy but has limited tropism to deeper retinal regions, which hinders its effectiveness in treating retinal diseases. To address this, we engineered a novel capsid, AAV2.N54, that exhibits enhanced specificity and efficiency in targeting these regions. The ability to accurately detect engineered capsids is crucial for evaluating their therapeutic potential and ensuring safety in clinical applications. Here, we present an antibody that specifically recognizes AAV2.N54, enabling precise monitoring of its distribution following intravitreal administration.

Methods: To engineer the novel AAV2.N54 capsid, we employed directed evolution techniques, optimizing its tropism for deeper retinal regions. An antibody specific to AAV2.N54 was generated using hybridoma technology, enabling the isolation of high-affinity clones. Characterization of the antibody was conducted through ELISA and Western blot assays to confirm its specificity and binding affinity to AAV2.N54, demonstrating no cross-reactivity with wild-type AAV2 or any other AAV serotypes. Statistical analyses were performed to validate the significance of the observed differences in antibody binding and tropism across different capsids.

Results: Results indicate that AAV2.N54 demonstrates improved tropism compared to wild-type AAV2 and the engineered capsid AAV2.7m8 across all four species studied. This is evidenced by efficient delivery to the macular retina and reduced targeting of the ciliary body. AAV2.N54 has been successfully utilized in toxicology studies and clinical trials, with comprehensive analysis conducted to determine the identity and empty-to-full ratio of the capsid. Developing a specific antibody for the AAV2.N54 capsid is crucial; it exhibits high specificity with no cross-

reactivity with wild-type AAV2 or any other AAV serotypes (1, 2, 3, 5, 6, 7, 8, 9, and 10). This has been validated through indirect and sandwich ELISA assays. Additionally, we possess an antibody that recognizes wild-type AAV2, creating an optimal combination for quantifying AAV2.N54 while ensuring accurate measurement in various applications. The antibody's specificity and sensitivity position it as a valuable tool for monitoring AAV2.N54 in both research and therapeutic contexts.

Conclusions: In conclusion, the engineered AAV2.N54 capsid represents a significant advancement in ocular gene therapy, offering enhanced tropism for deeper retinal regions and improved therapeutic potential. The development of a specific antibody that accurately detects AAV2.N54, without cross-reactivity to wild-type AAV2 or other serotypes, facilitates precise monitoring of this novel capsid's distribution and efficacy in clinical applications. Together with the wild-type AAV2 antibody, these tools enhance our ability to quantify and assess the safety and effectiveness of AAV2.N54 in therapeutic settings, paving the way for future advancements in retinal disease treatment.

906 Directed Evolution of an Intravitreally Delivered Random Mutagenesis Library of the AAV2 VP1u Region Generates Novel Capsids with Enhanced Retinal Transduction by Increasing Cellular Trafficking and Transcription Efficacy

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Adeno-associated virus (AAV) vectors are the most utilized vehicle for gene therapy. Thus, there is a growing demand for capsids with specific tropism profiles, high transduction efficacies, and enhanced safety. To meet these demands, the field continues to discover novel capsids by engineering the viral protein sequence of AAVs, specifically the variable regions within the VP3 region. Our previous study has shown that AAV2.MC1, an AAV2 capsid variant with naturally mutated residues in the VP1 unique (VP1u) region, significantly increases retinal cell transduction in murine and canine retinas following intravitreal (IVT) injections. The VP1u region, located at the N-terminus of the VP1 protein, is a poorly characterized sequence involved in AAV cellular trafficking and transgene transcription. The sparse knowledge of VP1u constrains efforts to rationally modify the region, highlighting the need for further investigation.

To explore the role of VP1u in enhancing vector transduction and uncover its underlying mechanism, we constructed a VP1u-specific mutagenesis library by introducing random mutations into the region via error-prone PCR, yielding 4.93×10^5 unique VP1u variants. The resulting plasmid library was packaged into the first AAV library, which was intravitreally injected into mouse eyes. Four weeks post-injection, retinal tissue transcripts of enriched VP1u variants were extracted, converted to cDNA, repackaged into a second AAV library, and subjected to the IVT injection. Following another four weeks, transcripts from enriched VP1u variants in the retina were converted to cDNA and analyzed by NGS to estimate variant diversity and enrichment. NGS analysis identified six VP1u variants with better enrichment scores than wild-type AAV2 VP1u. These variants were then individually evaluated in mouse retinas via IVT injection. The top-performing capsid (VP1u1) showed a ~6-fold higher transduction than the parental AAV2 capsid and ~ 3-fold higher transduction than the well-established retinotropic capsid, AAV2.7m8. VP1u1 also showed a ~2-fold higher increase in photoreceptor transduction compared to AAV2.7m8. A second variant, VP1u2, showed ~4-fold and ~2-fold higher transduction than AAV2 and AAV2.7m8, respectively. Interestingly, VP1u1 has a single amino acid mutation in a poorly described N-terminal region of AAV2 VP1u with an unknown function. To uncover this region's function, we tested its impact on transcription by luciferase reporter assays. Overexpressing the VP1u region of AAV2 increased reporter expression from the hPGK promoter in different cell lines, while VP1u1 and VP1u2 further increased reporter expression by 2- to 2.5-fold compared to AAV2. We are currently validating these findings with ChIP assays.

This study demonstrates that VP1u is a promising target for AAV capsid engineering, offering significant potential for improving vector performance. Moreover, our results provide further insights into the previously uncharacterized domains of the AAV capsid, expanding opportunities for innovative applications in gene therapy.

907 Breaking Through the ILM: An Evolved AAV Variant for Efficient Photoreceptor Targeting

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Photoreceptors are a compelling target for gene therapy due to their essential role in vision and their involvement in retinal degenerative diseases, such as retinitis pigmentosa, Stargardt disease, and Leber's congenital amaurosis. Effective gene delivery to photoreceptors typically relies on subretinal injection, an invasive procedure that involves penetrating the retinal layers and detaching the neural retina from the retinal pigment epithelium. This approach carries significant risks, including collateral damage, and often fails to achieve widespread photoreceptor transduction. In contrast, intravitreal injection offers a less invasive alternative with the potential for broader retinal coverage. However, efficient gene delivery to photoreceptors via this route remains challenging due to physical barriers such as the inner limiting membrane (ILM), which restricts viral vector penetration. To overcome this challenge, we employed directed evolution to engineer a novel adeno-associated viral (AAV) vector with enhanced tropism for murine photoreceptors via intravitreal injection. Through high-throughput mutagenesis and Darwinian selection, we identified key amino acid residues that improve photoreceptor infection efficiency across retinal layers. Our results demonstrate that the newly developed AAV variant can bypass the ILM and achieve robust photoreceptor transduction via intravitreal administration, offering a promising strategy for non-invasive retinal gene delivery. In this presentation, we will highlight the enhanced transduction specificity of our evolved AAV vector compared to its wild-type parent vector and multiple widely used variants. Furthermore, we will discuss its therapeutic potential in animal models. These findings provide valuable insights into the structure-function relationship of AAV capsid proteins and highlight the potential of engineered AAV vectors for gene therapy applications targeting retinal degenerative diseases.

908 Tailoring AAV Vectors for Gene Therapy of Inner Ear Disorders by Directed Evolution

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There is a pressing need to advance treatments for hearing loss (HL) and deafness, which is a currently incurable disease affecting 20 % of the global population. The treatments most commonly used for HL include hearing aids and cochlear implants. However, they require some functionality remaining in the inner ear of the patient and, furthermore, no hearing restoration is achieved with these treatments. On the other hand, success has been seen in the first clinical trial using Adeno-associated virus (AAV) vectors to treat HL, where the therapeutic transgene, *OTOF*, is being delivered to the inner ear of DFNB9 patients.

Thus far, AAV variants being tested in the inner ear today originate from libraries screened in other target tissues (e.g. CNS, eye), have been *in silico* generated (e.g. Anc80L65), or use the wildtype capsid. In contrast, we are aiming to optimize the AAV vector system for inner ear directed gene therapy by focussing on the inner ear as target structure. Therefore, we generated AAV peptide display libraries based on the AAV1, AAV2 and AAV6 capsid backbones. All libraries presented random unique 7-mer peptide inserts at variable region VIII of the capsid protein

with diversities (number of different capsid variants) ranging from 80,000-622,000 (maximum likelihood estimate, MLE). We conducted high-throughput *in vivo* selection screens in the inner ear of adult mice (n = 4, per library), testing alternative administration routes that demand overcoming robust biological barriers, in order to reduce the invasiveness of the eventual treatment.

Distinct variants were found to be accumulated to up to 5% for AAV2-based variants and up to 2.5% for AAV1-derived capsids after two rounds of *in vivo* selection. A total of 20 top candidates from the AAV1 and AAV2 libraries were produced as vectors packaging the dTomato transgene. Individual administration into the adult mouse ear showed the variants outperformed the parental serotypes and showed distinct expression patterns in the cochlea. Three promising variants had the ability to transduce outer hair cells, a challenging cell type to infect, and many also targeted inner hair cells. Almost half of the variants also strongly transduced all layers of the stria vascularis – a viable target tissue for the treatment of age-related HL – and 4 variants were highly specific for spiral ganglion neurones.

Top AAV candidates have also been confirmed to reach the cells in 10-11 week old pig cochleae (n = 6) using the less invasive administration route, with three clear top performers - Var1, Var3 and Var4. Furthermore, cDNA analysis of the cochleae showed clear and strong expression from Var4 after only 7 days post-administration. Var4 is now being investigated to carry a therapeutic transgene as it works cross-species and in mice has been shown to infect OHCs alongside IHCs and SGNs and shows a mild but widespread transduction of the SV

909 A High throughput, high-efficient AAV evolution system for tissue targeting AAV screening

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Introduction: Numerous AAV capsid optimization approaches have been developed to improve tissue-specific tropism and expression efficacy, including direct evolution and rational design. These methods suffer from low screening efficiency due to low library abundance or poor underlying science. Only very few valuable capsids specific for muscle or CNS were obtained from these engineering systems. To overcome these issues, we developed an innovative evolution system for tissue specific AAV screening. Human genome contains all the information of the ligands to receptors on cell membrane. We hypothesize that human genome displaying AAV can gain the cell entry function of these ligands. Through appropriate screening systems, any tissue specific AAV capsid can be attained from this library.

Methods: Random peptides from human genome were inserted between amino acids 588 and 589 in AAV9 library capsid. The library was enriched in vitro and in vivo (mice and NHP) for several rounds. Capsid variants enriched relative to the virus library at the DNA and RNA level in various tissues were identified by target-NGS for each round. New motifs from the enriched sequences will be identified by a score-based approach. Leading candidates will be further optimized by combining motif-based machine learning with additional rational design to enhance the targeting properties and transduction efficiencies. Individual capsids with 3 unique barcodes will be further confirmed in NHP.

Results/Outlook: Approximately 8 x 10^{10} unique nucleotide sequences library was packaged into rAAVs. After several rounds of enrichment in iPSC-derived myotubes and muscles of animals (mice or NHP). Multiple new motifs have been enriched with RGD listed as one of TOP 10 motifs. Multiple top new motif-containing sequences exhibited 2-3 times higher specificity to muscles than RGD-containing sequences in mice. Motif-based machine learning was utilized to optimize tissue specificity of the sequences from in vitro and in vivo experiments. Leading candidate sequences specific to muscles are under investigation in DMD mice and the results will be available in Mar 2025. In summary, this innovative Human Originated Peptide Evolution (HOPE) AAV platform enables the findings of new motifs specific to different tissues (CNS, kidney, lung, et al), which can extremely expand the scope of AAV gene therapy in future.

910 Nanobody Incorporation Confers Precise Infectivity to Adeno Associated Virus

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Adeno-associated virus (AAV) is a non-pathogenic virus that is widely used in both research and the clinic for delivery of genetic payloads to cells of interest. Several AAV serotypes are in use because of their slightly varying tissue tropism. Recombinant AAV (rAAV) variants such as AAV-DJ and AAV-PHP.eb have been engineered to expand on AAV's natural tropism. This tropism can be attributed to interactions of the virus with proteoglycans and polysaccharides found on most cell surfaces. Mutating the rAAV capsids at residues that bind to these common polysaccharides results in the virus losing most of its infectivity and by extension its natural tropism. rAAV is amenable to domain and peptide insertions for retargeting to target cells while maintaining capsid integrity and infectivity. Previous work in our lab has revealed positions in the AAV-DJ capsid that tolerate incorporation of various domains. This has been used in combination with antibodies or nanobodies to re-engineer viral tropism. Incorporation of a nanobody targeting the Fibroblast activating protein (FAP) receptor showed a boost in infection for target cells. In this work, we report the incorporation of a nanobody targeting a metabotropic glutamate receptor (mGluR5), a receptor of clinical significance in neuroscience research. First, we show that the nanobody can be incorporated in either the VP1 or VP2 protein of the AAV-DJ capsid without impinging on capsid integrity and packaging efficiency. Mutating the Heparan binding domain (HBD) in the capsid results in a loss in infectivity, seen in both immortalized cell lines and primary hippocampal mouse neurons. However, virus capsids that incorporated the nanobody against the HBD background can rescue infection. In addition, these engineered variants show a preference to infect neurons over glial cells in primary culture. Next, we replicated incorporation of the nanobody in AAV-PHP.eb and show that nanobody mediated infectivity boost can be extended to other AAV serotypes. In conclusion, we show that nanobody incorporation is tolerated in multiple AAV serotypes and confers specificity to virus infection, re-directing AAV tropism and allowing AAV to be used as a precise delivery vehicle to target neural circuits.

911 AAV capsid engineering for improved AAVR binding to enhance transduction efficiency

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Immunogenicity and the consequent toxic side effects present major obstacles to broader AAV usage, and this can become especially problematic with the highest doses used in the clinic (E13-E14 vg/kg). The engineering of adeno-associated virus (AAV) capsids for enhanced transduction would be beneficial for reducing vector dose. AAV transduction includes two key mechanistic events: i) Capsids binding to cell surface receptors, followed by internalization, ii) intracellular trafficking of AAVs including steps such as endosomal escape. The universal AAV receptor, AAVR, was identified as a key receptor for AAV infection, playing an important role in intracellular AAV trafficking. In this study, we generated AAV9 virus-like particles (VLPs) and performed saturation mutagenesis at the receptor-binding interface. We then screened the generated variants bearing single substitutions as well as combinations, via surface plasmon resonance (SPR). Ten single or combinational AAV9 variants with improved AAVR affinity, ranging from ~2-114 fold, were characterized and structures of virus like particles containing these substitutions, in complex with a truncated AAVR, were determined by CryoEM.

We then packaged these variants with a GFP reporter payload to study their transduction efficiency *in vitro*. We observed up to ~15-fold improvement in transduction efficiency (%GFP x Mean fluorescent intensity), compared to cells transduced with WT AAV9. Notably, among the top three AAV9 variants (ER, QK and ER/QK), the degree of improvement in transduction efficiency was correlated with the degree of increased AAVR binding affinity. However, there is not a strong overall correlation between binding affinities of the variants, with transduction efficiency. Particularly the variant AN/ER/QK with the highest AAVR affinity (~114 fold) only showed a low level of improvement in transduction efficiency. This could be due to the existence of an optimal affinity window.

To further investigate the mechanism of action of the top three AAV9 variants (ER, QK and ER/QK), we studied the intracellular trafficking of the virus particles by immunofluorescent imaging of assembled capsids. We observed a trend of increased signal of assembled capsid staining in cells transduced by these three variants at 6hr post transduction, compared to the cells transduced with WT AAV9. The variant AN/ER/QK with the highest affinity showed a similar level of capsid staining as the top three variants with more puncta appearance, some of which co-stained with phagolysosome-like structures in the cytoplasm, which could potentially lead to the degradation of virus particles.

Overall, this study provided insight into the interaction of AAV capsid variants with AAVR and their impact on transduction efficiency. We have demonstrated that engineering the viral capsid to modulate its binding affinity to AAVR can be advantageous. This approach may also be compatible with and complementary to orthogonal capsid engineering efforts focusing on enhanced tropism, and can be used in a broad range of gene therapy applications.

912 Conjugation of Peptide Ligands to AAV Towards Pancreatic Cell AAV Gene Delivery

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Adeno-associated virus (AAV) is a small DNA virus that has been closely studied for its potential for human gene therapy. Multiple serotypes of AAV and recombinant AAV possess differential transduction efficiency and specificity towards different tissues; however, no cell type-specific AAV vectors have been created to date. In order to understand and improve cell/tissue transduction efficiency and specificity, our lab continues to explore unnatural amino acid (UAA) substitution on the AAV capsid that allows for site-specific modification via click-chemistry to conjugate aptamers or a small molecule ligand to the AAV (Puzzo et al., 2023). In this study, we seek to demonstrate an expansion of these capabilities to other cell types, particularly pancreatic cells, by conjugation of peptide ligands that bind to surface receptors known to be expressed on those cells. Specifically, we selected the ligands that bind to GLP1R (glucagon-like peptide 1 receptor, for targeting pancreatic endocrine cells) or SCTR (secretin receptor, for targeting pancreatic exocrine cells). After optimization using the technologies previously developed in our lab (Puzzo et al, 2023), the peptides were successfully conjugated to the UAA-substituted AAV-DJR/A-A587. The secretin peptide-conjugated AAVs (SCT-AAV) demonstrated a several-fold increase in transduction efficiency towards cell lines exogenously expressing SCTR when compared to unconjugated AAVs or when compared to transducing control cell lines not expressing SCTR. We are currently establishing whether the improvement in transduction efficiency was due to an increase in viral entry into the SCTR-expressing cells. In contrast, in transducing GLP1R-expressing cells, the GLP1 peptide-conjugated AAVs demonstrated a decrease in transduction efficiency as compared to unconjugated AAV. This reduction in transduction efficiency was also seen in our control cells (non-GLP1R expressing cells). The cause for this reduction is unclear. Future steps include interrogating how the AAV-ligand-receptor interaction improves transduction, understanding why GLP1 peptide-conjugated AAVs have decreased transduction efficiency overall and if steps can be taken to reverse this, as well as further validating this 1) *in vitro* in cell lines that endogenously express the surface receptors of interest and eventually 2) *in vivo* to evaluate whether this improved transduction efficiency is translatable in tissue/organs and whether this improved efficiency is specific to cells expressing the corresponding receptors.

913 An Approach to Facilitate Multiple Peptide Insertions at Different Locations on the Surface of AAV Viral Particle

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Adeno-associated virus (AAV) has been successfully used for *in vivo* delivery of gene therapy drugs to treat multiple clinical indications. However, this gene delivery vehicle still faces some limitations and challenges, including low tissue specificity or insufficient transduction in certain target tissues, and/or tissue toxicity due to high accumulation in non-target organs.

One common strategy to overcome these limitations is to insert a peptide into the common VP3 region of AAV capsid proteins to retarget the virus to a specific cell or tissue type. Not all the surface sites of AAV particles are suitable for surface display of foreign peptides. Site I-587, I-588, and I-453 of AAV2 (or corresponding locations in other AAV serotypes) are the most frequently used positions for inserting foreign peptides. These sites can be used either individually or as a combination of two sites. The simultaneous use of three or more sites for inserting foreign peptides in VP proteins is more difficult due to steric hindrance and have not been explored thoroughly.

We sought to utilize multiple affinity tags, including the 8 aa Strep-tag II, the 10 aa c-Myc tag, and the 9 aa HA tag, to explore this possibility. The tags were first individually inserted to 130 surface sites of AAV9 VP3 proteins and every corresponding mutant AAV was generated in 293 HEK cells and purified. The yield of the mutant AAVs was first quantified by qPCR to determine whether the insertion of the foreign peptides interfered with viral production.

The mutant AAVs were further tested in pulldown assays and biolayer interferometry (BLI) assays to determine whether the tags were well presented at the surface of viral particles and retained their original binding properties. In pulldown assays, Strep-tag II, c-Myc tag, and HA tag-containing viral particles were bound to BeaverBeads Magrose Strep-Tactin, Pierce anti-c-Myc magnetic beads, and HA-beads, and were eluted with D-Desthiobiotin, c-Myc peptide, and HA tag elution peptide, respectively. In BLI assays, each tag's binding kinetics to its corresponding antibody was analyzed on the GatorPrime biolayer interferometry system, a label-free analysis instrument based on the biolayer interferometry technology, using anti-c-Myc, anti-HA, and anti-Strep-tag II antibodies.

Through these experiments, we discovered many sites suitable for peptide insertion to the AAV9 VP3 region. Among them, multiple sites could be used together to facilitate simultaneous insertion of two different peptides with a very mild impact on viral production and little interference on peptide presentation. The simultaneous insertion of three tags proved to be difficult, with most of the site combinations leading to either a substantial loss of viral production or a disruption of the inserted tag's antibody-binding property. However, we found that two of the site combinations could be used for the simultaneous insertion of three peptides with a mild loss of viral production and a good presentation of all three inserted peptides at the surface of the viral particles.

Foreign peptides have been successfully introduced into AAV to ablate the binding of a natural receptor, to mediate transduction through a novel AAV receptor, to facilitate downstream purification, to escape pre-existing neutralizing antibody, or to modulate immune response. The above findings could expand our abilities to improve current AAV vectors for a more desirable clinical outcome.

914 Assembly-based RNA Packaging Using Adeno-associated Virus Capsids

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Adeno-associated virus (AAV) is a powerful vector for efficient tissue-specific and bodywide gene delivery. While persistent AAV-mediated gene expression is ideal for many gene therapy applications, this is not desirable for applications which require transient expression. One salient example is CRISPR editing. Long-term expression of the bacterial-derived editing enzyme (such as Cas9) increases the risk of untoward genome modifications and cellular immune responses. Encapsidation of an mRNA into AAV capsids would allow robust delivery and transient expression. Efficient RNA-protein conjugation can be achieved using an RNA aptamer and the corresponding aptamer-binding protein (ABP). We hypothesized that an ABP engineered in the inner surface of the AAV capsids would allow recruitment of an aptamer-containing RNA into AAV capsids during assembly. To test this hypothesis, we inserted N22, a 22 residue ABP to positions 192 and 210 in VP2 of AAVMyo. Structural modeling suggests that these two positions are oriented toward the interior in the assembled capsids. AAVMyo was selected for its superior muscle targeting. Dot blot and western blot suggest that inserting N22 into VP2 did not interfere with AAV assembly and packaging. To evaluate RNA packaging, we extracted RNA from purified AAV particles and quantified the spliced mRNA by TaqMan PCR with or without reverse transcription, in the presence and absence of DNase and/or RNase. No RNA was detected when no plasmid or a conventional ITR-containing plasmid was used

in packaging. Interestingly, we detected a low amount of RNA packaging with a plasmid that did not carry BoxB, the N22-matching aptamer. Similarly, a low amount of RNA was detected using unmodified AAVMyo capsids. Collectively, these results suggest that AAV capsids are permissive to RNA packaging. Strikingly, when the VP2-N22 modified AAVMyo and the BoxB-containing plasmids were used together, we achieved 60 to 90-fold enhancement in RNA encapsidation. In summary, our results suggest that AAV capsids can be adapted to carry RNA. Further development of this technology may broaden AAV gene therapy applications. (This work was supported by the NIH grants AI177600 and NS131416, as well as the University of Missouri Research Excellence Program)

915 Engineering AAV2 Capsids Using a Novel Synthetic Biology Approach to Enhance Genome Packaging Efficiency and Reduce Empty Capsid Ratios

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Recombinant adeno-associated virus (rAAV) has emerged as a leading vector for gene therapy, due to its safety and efficacy. However, current rAAV manufacturing systems are burdened by the excessive production of empty capsids- viral particles lacking the therapeutic gene of interest. These empty capsids are considered as product-related impurities that reduce the infectivity of full particles and may even trigger undesired immune responses. Thus, reducing the empty capsid ratio in rAAV preparations is crucial to enhance the efficiency, safety, and affordability of AAV-based gene therapies. Various techniques, such as analytical ultracentrifugation (AUC) and anion-exchange (AEX) chromatography, are available to quantify and separate empty capsids, however, these methods are labour-intensive, time-consuming, and costly.

Here, we introduced a novel synthetic biology-based approach to enhance AAV2 genome packaging efficiency, thereby increasing the full-to-empty capsid ratio. Using a rational engineering strategy, plasmids encoding novel fusions of native AAV2 proteins were incorporated into standard transfection procedures. Transfections were performed in suspension HEK293F cells at a 1 mL scale in 6-well plates via transient transfection. Viral genome and capsid titers were quantified using crude lysates by quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The percentage of full capsids was inferred using the qPCR-to-ELISA ratio. Among the 16 novel plasmids tested, 10 exhibited a higher full capsid ratio than WT AAV2, with 7 showing statistically significant improvements, and the highest ratio observed was 19.8%, compared to 7.1% for WT AAV2. Four of the novel capsids, showing the highest full capsid ratios, were scaled up to 50 mL cultures in shake flasks, and crude lysate data from qPCR and ELISA confirmed consistent improvements in full capsid ratios similar to the small-scale experiments. The purification of vectors was then performed using Dynabeads CaptureSelect AAVX magnetic beads, and the full/empty capsid ratio of purified vectors was determined via negative-staining transmission electron microscopy (TEM). TEM analysis, conducted by counting over 500 capsids per vector, revealed that the best-performing fusion capsid brought about a 28.9% full capsid ratio, a 1.9-fold increase compared to WT (15.1%). Despite these promising results, all fusion capsids displayed a significant reduction in transducing unit titers (TU/mL), with reductions ranging from approximately 2-fold to 17-fold compared to WT AAV2, as determined by transduction of HEK293T cells followed by the flow cytometry. Ongoing studies are focused on addressing this reduction by further modifying the variants to rescue their infectious abilities. Overall, our findings showed promise to advance AAV bioprocessing, offering a fresh perspective on developing of more efficient AAV production systems.

916 Advancing AAV Capsid Engineering for Targeted Gene Therapy Using PackGene's π-Icosa Platform

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Adeno-associated virus (AAV) is a widely used gene delivery vector, but its clinical utility is constrained by limitations in tissue specificity, immune evasion, and transduction efficiency. To address these challenges, we developed the π-Icosa Capsid Engineering System, a platform combining rational design, directed evolution, and AI-driven prediction models to engineer AAV capsids with enhanced targeting, manufacturability, and reduced off-target effects. The platform integrates an AI-based targeting prediction model, built on a transformer architecture and pre-trained using protein language models, which achieved an AUC of 0.83 on directed evolution datasets for accurate tissue-specific tropism prediction.

Building on these advancements, the π-Icosa platform employs a proprietary algorithm to design and screen diverse capsid libraries, followed by iterative in vivo testing to identify optimized variants. This approach has yielded novel AAV capsids with improved tissue tropism. Muscle-targeting variants PG016, PG017, and PG018 demonstrated superior transduction compared to AAV9 and MyoAAV 4A. CNS-targeting variant PG008 in mice, along with additional ongoing library screening, has resulted in many candidates that achieved 10^3-10^6-fold higher reporter gene expression in NHPs. Retinal-targeting variants PG021 and PG022 efficiently transduced retinal ganglion cells (RGCs), Müller glial cells, and inner nuclear layer (INL) cells. For ex vivo T-cell therapy, variants PG009, PG010, PG012, and PG013 exhibited 10x higher infectivity in primary human T cells compared to wildtype AAV6.

Furthermore, to complement the engineering of these advanced AAV capsids, a manufacturability prediction model was developed to generate AAV mutants with high packaging efficiency (up to 97%), 2-3 times higher yields than wildtype AAV, and enhanced sequence diversity, enabling the discovery of novel capsids with improved properties. These results demonstrate the potential of integrating AI-driven approaches with rational design and directed evolution to overcome key gene therapy challenges. The π-Icosa platform enables the development of highly specific, efficient, and manufacturable AAV variants, advancing the field toward safer and more effective therapeutic applications.

917 Generative Transformer Models for Designing Diverse and Viable AAV Capsid Sequences

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Designing novel adeno-associated virus (AAV) vectors is necessary for addressing the limitations of AAV gene therapies, but most AAV capsid sequence variants fail to assemble, creating a major roadblock. High-throughput sequencing of mutational capsid libraries has enabled a data-driven assessment of the fitness landscape of AAV capsids, and some studies have combined machine learning models with ad-hoc algorithms to design novel and viable capsid sequences. Recently, transformer models have revolutionized the field of deep-learning and led to the creation of large language models, such as generative pre-trained transformers (GPTs) from OpenAI, and protein language models (PLMs), such as ESM2 from Meta which excels in a range of protein structure prediction tasks. However, the potential of GPTs and PLMs in designing novel AAV capsids remains largely unexplored. In this study, we adapted ESM2 to predict assembly of AAV2 capsid sequence variants and trained a GPT model to generate diverse and viable capsid sequences.

Methods: We used the AAV2 capsid library data (N=296,970 sequences consisting of 52% viable capsids) from Bryant et al. (*Nature Biotechnology*, 2021) and targeted mutations within amino acid positions 561–588 of the VP1 protein. The data was evenly split into training and validation sets to develop a classifier model, where the ESM2 model was trained on the training set and evaluated on the validation set. We then trained a new generative model, based on OpenAI's GPT-2 architecture, to generate viable or non-viable capsid sequences with user-specified edit distances from the wild-type AAV2 sequence, guided by specific input prompts. Finally, the ESM2 classifier was used to assess the viability of the GPT-2-generated capsid sequences.

Results: On the validation dataset, our ESM2 classifier showed a high accuracy of 96%, with 96.7% precision and 95.4% recall. We prompted the trained GPT-2 model to generate 100k viable and 100k non-viable capsid sequences with edit distances between 2 and 29. After removing any overlaps with the initial capsid library, we kept 74.8k and 53.5k, putatively viable and non-viable novel capsid sequence candidates, respectively. The putative viable and non-

viable sequences had a mean edit distance of 10.2 (SD=4.4) and 15 (SD=6.6) from the wild-type AAV2 sequence, respectively. The ESM2 classifier predicted 99% of the putative viable capsids to be viable and predicted 82% of the putative non-viable capsids to be non-viable.

Conclusions: Our evaluation shows that the ESM2 classifier can accurately assess a capsid sequence for assembly viability, making it suitable for evaluating our generative model. Our GPT-2 model can generate novel capsid sequences that are diverse from the wild-type AAV2 sequence and have a high likelihood of assembly. This approach differs from the previous studies that have used a classifier model to screen billions of randomly mutated capsid sequences and iteratively mutate promising candidates, which makes it difficult to design capsids with multiple desired traits. In contrast, our generative model can directly generate capsid sequences with multiple desired properties, such as viability and edit distance. This highlights the potential of generative transformer models in designing capsids that can simultaneously optimize for other AAV traits such as lower immunogenicity or increased liver transduction, thus accelerating the development of novel gene therapies.

918 Artificial Intelligence Enables Rapid and Efficient Identification of Adeno-Associated Vectors with Improved Transduction of Target Tissues

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Introduction: Clinical success has been achieved with several AAV-based therapies; however, the inconvenient truth remains that the current generation of capsids are unsuitable for many target tissues. As a result, there remains a significant unmet burden of disease, meaning gene therapies are either not possible or require large doses – placing patients at risk of serious adverse events (such as immune reactions), and increasing manufacturing costs dramatically. More efficacious vectors are urgently required for tissues that are otherwise ideal targets for gene therapy, including but not limited to the brain, kidney and lungs.

Methods: Sendatu Therapeutics leverages a unique combination of specialised know-how and patent-pending technological approaches to develop novel delivery mechanisms for safe and effective targeted genetic therapies. Synergistically, our artificial intelligence engine accelerates the search for the 'needle in the haystack' by designing optimal peptide libraries not only for the intended cell/tissue target, but also for the desired functional characteristic(s), such as transduction efficiency, titre, immunological properties. This reduces the in vivo experimentation required to find suitable candidates, reducing R&D costs, all while producing AAV capsids with improved chance of clinical success.

Results: To demonstrate the power of our platform, we generated a 10-mer peptide insertion AAV library and performed capsid selection in HuH-7 cells, generating a combined score that uses packaging, cell entry and transgene expression data. Our trained ML models subsequently scored 120 million possible peptides and identified the 1,000 top-performing peptides. We then vectorised the top N=10 ML-designed capsids and N=10 top AAV variants from the physical, lab-based selection. These vectors were used to package a GFP cassette and transduce HuH-7 cells; their performance was monitored by fluorescence activated cell sorting (FACS). All ten ML-designed variants performed better than the original vector (with a median fold-change of 1.8), and all were also better than the median performance of the physical selection group. We additionally tested a 7-mer peptide insertion library in mice, to select for capsids that target the kidney while simultaneously de-targeting from the liver. After training on packaging, cell entry and transgene expression datasets for kidney and liver, our ML models scored 120 million peptides for kidney transduction and liver detargeting. Following synthesis of the predicted top 1000 peptides, several candidate capsids were identified, with kidney expression up to 200-fold higher than liver expression as measured by next-generation sequencing.

Conclusion: Sendatu Therapeutics' functional transduction platform, allowing for parental capsid modification and generation of capsid packaging, entry (DNA) and expression (RNA) datasets in a cell-and tissue-specific manner, combined with artificial intelligence models, has enabled the prediction and validation of capsids that have preferentially demonstrated desired characteristics including enhanced transduction and targeting specific tissues.

919 From Data to Delivery: AI-Driven Design of Optimized AAV Vectors for Gene Therapy

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Traditional directed evolution with randomized AAV libraries has been a cornerstone of AAV engineering. However, this approach is constrained by the limited diversity of AAV libraries, restricting the exploration of the vast sequence space of capsid, which consists of approximately 735 amino acids. The disparity between the theoretical diversity of capsid and the practical library size (~10^8 variants) presents significant challenges in developing optimized AAV vectors for specific applications. Recent advancements in capsid engineering using artificial intelligence (AI) have shown promise in addressing these limitations by learning and predicting capsid properties, enabling more efficient and targeted capsid design. In this study, we developed deep learning-based AI models to predict capsid properties. These models were employed as cross-validation tools to enhance prediction accuracy and evaluate potential capsid variants *in silico*, significantly narrowing down candidates for *in vitro* and *in vivo* validation. This streamlined approach enhanced the efficiency of variant selection for functional testing. A key focus of this work was analyzing the gradients of feature maps within the trained models to identify key amino acids contributing to specific capsid properties. This analysis provided insights into how individual amino acids influence AAV functionality, guiding rational capsid design. Additionally, we optimized data preprocessing to uncover meaningful patterns, thereby improving model performance. By leveraging AI to explore previously uncharted sequence space, we successfully designed an optimized AAV vector with enhanced cell tropism. This AI-driven vector significantly increased transduction efficiency across several animal models compared to its wild-type parent and several widely used engineered vectors. Our findings highlight the potential of AI as a powerful tool for advancing capsid engineering, overcoming the limitations of traditional engineering approaches, and accelerating the development of next-generation gene therapy vectors. In this presentation, we will introduce and discuss our AI-driven approach and the successful outcomes achieved, providing insights into the future of AI-powered AAV engineering.

920 Machine Learning-Driven AAV Capsid Engineering for Enhanced Tissue Targeting and Optimized Production Efficiency

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Adeno-associated viruses (AAVs) are widely used as gene therapy vectors due to their high safety profile and long-term gene expression. However, optimizing AAV serotypes for efficient blood-brain barrier (BBB) penetration, muscle targeting, and other tissue-specific delivery remains a significant challenge. In this study, we employ convolutional neural networks (CNNs) and other machine learning (ML) models to design and predict AAV capsid variants with enhanced tissue tropism and high production efficiency. By training ML models on extensive datasets of AAV sequences and experimentally validated functional capsids, we generate novel serotypes that demonstrate superior targeting efficiency to specific organs while maintaining high viability and full capsid ratio.

Furthermore, our approach optimized to minimize cross-packaging events, ensuring precise vector identity and reducing unwanted recombination. This strategy enhances AAV vector homogeneity and production consistency, critical factors in clinical applications. By integrating ML-driven sequence design with experimental validation, we establish a scalable pipeline for developing next-generation AAV vectors optimized for therapeutic applications.

Our findings provide a robust framework for engineering AAV capsids with tailored tropism, improved manufacturability, and enhanced safety for gene therapy.

921 Investigating the Effect of Age on AAV2 and AAV8 Delivery and Transgene Expression via Subretinal Injection in Mice

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Retinal disorders, such as inherited retinal diseases (IRDs) and age-related macular degeneration (AMD), affect patients of all ages. However, therapeutic options remain limited and thus far are only able to slow disease progression. Gene therapy offers a promising approach and has previously shown great potential to treat retinal disorders, as evidenced by the first FDA-approved ophthalmic gene therapy (Luxturna). Despite the wide range of age in patient demographics, the impact of age on the delivery, expression, and tolerability of adeno-associated virus (AAV)-mediated therapies remains poorly understood. Notably, while younger patients, especially those diagnosed with IRDs, are thought to benefit most from early intervention, clinical practices often prioritize adult patients due to safety and ethical concerns. This leaves pediatric, as well as geriatric, populations underrepresented in gene therapy trials, creating critical gaps in our understanding of how age-specific factors affect the effectiveness of retinal gene therapy.

In this study, we investigate the effect of age on the delivery and safety of two AAV vector serotypes (AAV2, AAV8) when considered independently from the efficacy of specific therapeutic genes that employ the use of these vectors. AAV2 was selected for its previous clinical precedence and established tropism for retinal cells along with AAV8, which has been reported to have greater transduction efficiency, especially in the photoreceptors. We have administered AAV2-CAG-EGFP and AAV8-CAG-EGFP (1 uL; $1x10^{12}$ vg/mL) bilaterally via subretinal injection in C57BL/6J mice at different ages. In Phase I, we investigated AAV2 and AAV8 delivery in adult (6-10 weeks) and aged (≥78 weeks) mice. We evaluated vector delivery and spatial expression of the vector's transgene using fundus autofluorescence imaging and immunohistochemistry. Additionally, tolerability was assessed through ophthalmoscopic examination, optical coherence tomography, and electroretinography to monitor functional retinal outcomes. Phase II will extend this work to young mice at postnatal day 21, a developmental stage comparable to human infants at approximately 2-3 years. At this stage, the mouse retina is sufficiently mature while still reflecting the unique challenges of treating young patients with developing immune and retinal systems. We will evaluate whether the immature retinal environment affects vector tolerability or transgene expression. By systematically analyzing how age affects AAV vector tolerability and transgene expression, this study provides insights for the development of age-specific gene therapies. Results from this research will inform the design of safer and more effective therapies, particularly for pediatric and geriatric populations. Furthermore, the findings will serve as a preliminary step toward translational studies in larger animal models (e.g., minipig, non-human primate). Ultimately, this work aims to help optimize the delivery of gene therapies for diverse patients, from early-onset IRDs to age-related retinal degeneration.

922 A new dosing regimen to treat Bardet-Biedl Syndrome 1 (*BBS1)* retinal degeneration with AXV-101 (AAV9-BBS1) improves histological and functional photoreceptor survival in *Bbs1* M390R mice. Finding the right balance between bleb number, dose per bleb and total dose per eye.

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BBS1 patients become blind before they reach adulthood. We have previously demonstrated that AXV-101, an AAV9 vector expressing a human codon-optimised copy of BBS1 (hCOBBS1) was able to rescue retinal degeneration in the *Bbs1* M390R mouse model when deliver subretinally. To improve histological and functional rescue, we devised a new strategy by increasing the numbers of blebs, reducing the dose per bleb while increasing the total viral particles per eye. Retinae from mutant Bbs1 M390R mice were unilaterally dosed subretinally with AXV-101 at day 7 to 9 (P7-9) with two blebs, one superior and inferior regions of the retina, leaving the untreated contraleral eye as control. Three cohorts with three different dosed were injected; 7.5x109, 4x109 and 1.3x109 viral genomes per bleb (vg/bleb), for a total of 1.5x1010, µ and 2.6x109 total genomes per eye (vg/eye). Animals were followed for 6 months, monitoring the structure of the retina with Optical coherence tomography (OCT) and function by Electroretinogram (ERG) and hCOBBS1 transgene expression 6 months after dosing. We tried 3 doses (low-2.6x109 vg/retina) medium 8x1010 vg/retina) and high (1.5x1010 vg/retina), in two blebs of 1ml per retina in treated and M390R animals. At 2 months, low (27.20µm) and medium (25.34µm) M390R treated eyes display thicker ONL thickness compared to buffer treated M390R eyes (21.16µm) with high dose (37.83 µm, $p<0.0001$) showing a significant rescue in ONL thickness (Figure 1). At 4 months M390R treated eyes display a trend in dose response in rescue of mean ONL thickness with low, medium and high dose at 24.43µm ($p=0.0062$), 26.81µm ($p<0.0001$) and 37.55µm ($p<0.0001$), respectively, all showing significant rescue compared to buffer treated M390R eyes (13.38µm). At 6 months post-treatment the treated M390R eyes maintain the increased ONL thickness compared to buffer treated M390R eyes (low $p<0.0001$, medium $p=0.0849$, high $p<0.0001$) (Figure 1). In the WT buffer treated and in M390R treated eyes (in all doses) there is slight decline in ONL thickness with time, but this is not significant while in M390R buffer treated and untreated contralateral eyes show a significant decline in ONL thickness over 6 months. The functional rescue of the M390R animals is even better than the histological rescue of the ONL, showing a clear dose dependent response. The cone responses with of photopic b-wave at maximum stimulus showed significant differences at 4 month after treatment for low, medium and high doses was at 40%, 39%, 83% of WT buffer treated eyes and by 6 months post-treatment these were at 24%, 34% and 65%, respectively. For scotopic b-wave at 6 months we saw a 30%, 40% and 68% of buffer treated WT function compared with the 18% of the M390R buffer treated eyes (Figure 2). To assess level of hCOBBS1 transgene expression in the retinal tissue eyes were collected at 6 months post-treatment and were used for RT-qPCR of hCOBBS1. This analysis showed expression of hCOBBS1 with medium (11564-fold) and high (13522-fold) doses compared to housekeeping genes. These results demonstrate that an increased bleb strategy, with reduced vital titre per bleb but increase total titre per eye is more effective. That the histological rescue and halting of the photoreceptor death can be observed with lower doses than the functional rescue. That AXV-101 can sustain a high level of functional rescue over time and it is a prime candidate for first-in-human clinical trials.

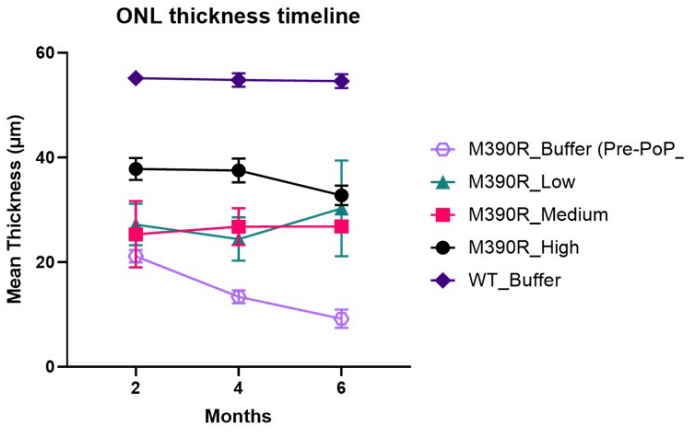

Figure 1. The ONL thickness is sustain over time. Change in mean ONL thickness ±SEM for M390R low, medium and high dosed and WT buffer and M390R buffer treated eyes from 2 months to 6 months post-injection. Data were analysed by 2-way ANOVA with post hoc Šídák's multiple comparisons.

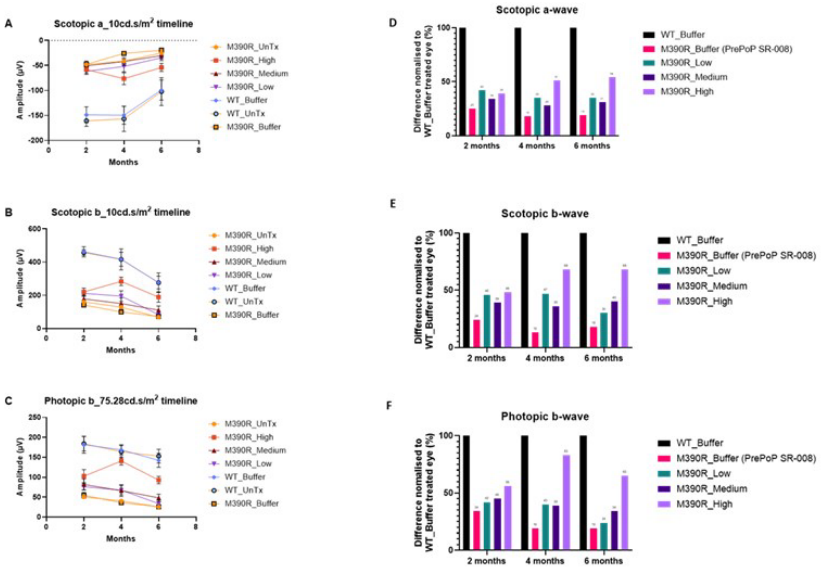

Figure 2. Temporal changes in retinal function measured by ERG at maximum stimulus (A-C) Changes in (A) Scotopic a, (B) Scotopic b, and (C) Photopic b-wave response (mean amplitude ±SEM) at maximum stimulus over 6 months period for untreated contralateral (UnTx), M390R low, medium and high treated and WT buffer treated eyes as well as M390R buffer treated eyes. (D-F) Percentage response of M390R treated eyes for (D) Scotopic a, (E) Scotopic b, and (F) Photopic b-wave at maximum stimulus. Stimulus response is normalised to WT buffer treated eyes at respective timepoints.

923 INS1203: RNA-End Joining Technology Enables Full-length ABCA4 Expression In Mouse and NHP Retinal Photoreceptors

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Stargardt Disease is the most prevalent form of inherited childhood blindness, affecting about 1:10,000 individuals in the United States. Stargardt Disease is caused by mutations in the ABCA4 gene encoding a large transmembrane protein that clears retinoids from photoreceptors. Loss of ABCA4 leads to accumulation of toxic byproducts that cause degeneration of the photoreceptors and apposing retinal pigment epithelium. Despite a well characterized disease mechanism, treatment for Stargardt patients is restricted to symptom management, as there is currently no approved cure or method for stalling progressive vision loss. A major challenge in gene therapy has been the ~5-kilobase cargo size limitation of AAV which restricts the ability for efficient and safe delivery of large genes like ABCA4 (6.8kb coding sequence).

The RNA-end joining (REJ) technology described here aims to address the size limitation related to AAV-based gene therapy by delivering a gene of interest as fragments in two (or more) individual AAVs and subsequently reconstituting the full-length coding sequence on the mRNA level in the target cell. In REJ, each half-coding sequence is expressed from a separate AAV vector, refered to as REJ-donor (RD) and REJ-acceptor (RA). The RD and RA transcripts are equipped with short RNA elements that mediate strong non-covalent binding of the two and through recruitment of the cellular splicing machinery efficient joining of the two RNA molecules in trans. REJ is therefore uniquely positioned to potentially address a challenge of conventional gene therapy approaches, and in particular advance the use of AAV strategies in large gene related ocular diseases such as Stargardt disease.

With INS1203, we have developed and characterized a therapeutic REJ-AAV8-ABCA4 gene replacement vector formulation that expresses Abca4 protein from two AAV8 vectors in a 1:1 ratio. Each construct drives expression of the N- or C-terminal half of the protein by the human GRK1 promoter. The two fragments join, and elements designed to inhibit fragment expression function to block translation of truncated protein from either single AAV.

Following subretinal injection in the Stargardt Disease mouse model of INS1203, we have shown that Abca4 protein expression is restored to physiological levels, specifically expressed in photoreceptors, and predominantly localizes to the photoreceptor outer segment, showing the intended biodistribution and cellular localization. Here we report functional studies in the mouse model demonstrating dose-dependent reduction of the disease biomarker A2E in the eye. Additionally, in the non-human primate (NHP) eye, INS1203 was generally well-tolerated when delivered via subretinal injection. Using a junction spanning RNA-probe, the INS1203 derived full-length abca4 transcript is found to be abundant and widespread in the NHP retina. These results demonstrate that REJ can be used to deliver large genes to the mouse and primate retina. Taken together, these results support further study of INS1203 as potential ABCA4 gene replacement therapy for Stargardt Disease.

924 Evaluation of MERTK gene therapy in RCS rats following a single bilateral subretinal injection.

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Retinitis pigmentosa (RP), a group of inherited retinal dystrophies affecting 1 in 3000 – 7000 people, is characterized by disruption of rod photoreceptors, leading to rod-cone degeneration. Mutations in the proto-oncogene tyrosine-protein kinase MER (*MERTK*) gene cause ~3% of RP cases. Loss of *MERTK* function leads to disruption of phagocytosis by the RPE, followed by rod cell death. OPGx-*MERTK* (AAV2-VMD2-h*MERTK*) is a gene augmentation approach developed to help patients impacted by *MERTK*-related RP. In this study, we evaluated the efficacy of OPGx-MERTK in Royal College of Surgeons (RCS) rats which exhibit a retinal pigment epithelium (RPE) phagocytosis defect due to a natural mutation in *mertk*, following a single bilateral subretinal injection.

At P14, male and female animals (n=10-12 per group) received a single subretinal injection of vehicle (group 1) or 5E8 (low dose, group 2) or 1E10 (high does, group 3) vg/eye OPGx-MERTK. Study parameters included clinical observations, body weights, ophthalmic examinations (slit lamp microscopy and indirect ophthalmoscopy), optical coherence tomography (OCT), fundus imaging, full field electroretinography (ffERG), and ocular histopathology and immunohistochemistry. Slides from the eye were stained with hematoxylin and eosin (H&E) for histopathology, and duplicate slides were labeled for immunohistochemistry (IHC) for rhodopsin, R/G opsin, and MERTK

There were no test article-related effects on clinical observations or body weights. Ophthalmic exam observations on 1 day post injection (dpi) were associated with the dosing procedure, and included bleb formation, vitreous and sub-bleb hemorrhage, which resolved at 21 dpi. OCT and fundus evaluation showed retinal degeneration of the photoreceptor layer on day 21, historically observed in RCS rats. On day 21, 13/14 eyes, 9/9 eyes and 2/10 from group 1, 2 and 3, respectively showed photoreceptor debris. On Day 42, similar findings of retinal degeneration were noted in Group 1, while in Groups 2 and 3, 8/14 and 1/10 eyes showed photoreceptor debris, respectively. Group 3 animals had detectable outer nuclear layer (ONL) on days 21 and 42, while Group 1 ONL was reduced on day 21 and generally not quantifiable by Day 42, and Group 2 ONL was reduced on day 21 and detectable only in a few eyes on day 42. Measurement of outer nuclear layer thickness exhibited significant preservation of ONL at 42 dpi (Vehicle: 1.870±3.60; Low dose: 3.60±4.51; and High dose: 21.28±12.94 µm) in the high dose cohort ($p<0.0001$, n=10). ffERG measurements showed a significant improvement in scotopic-a (at 39.8 cd.s m^{-2}: Control-18.76±13.79 µV, High dose-53.32±27.93 µV, $p<0.05$, n=10) and scotopic-b (at 39.8 cd.s m^{-2}: Control-44.08±14.90 µV, High dose-170.57±86.38 µV, $p<0.0001$, n=12) amplitudes.

In conclusion, subretinal administration of 0, 5E8, or 1E10 vg/eye OPGx-MERTK on P14 in the RCS rat model of retinal degeneration was well tolerated. Model induction in this pilot study was as expected, with retinal degeneration, photoreceptor debris, thinning of the ONL, and flat ERG waveforms observed in the vehicle group on Days 21 (P35) and 42 (P56). These data demonstrate that in the RCS rat model of retinal degeneration, 1E10 vg/eye OPGx-MERTK effectively preserved the photoreceptors and retinal function.

925 Inhibition of Laser Induced Choroidal Neovascularization (LCNV) by Blocking Complement Activation Cascade Using AAV Vectors Carrying Complement Inhibition proteins

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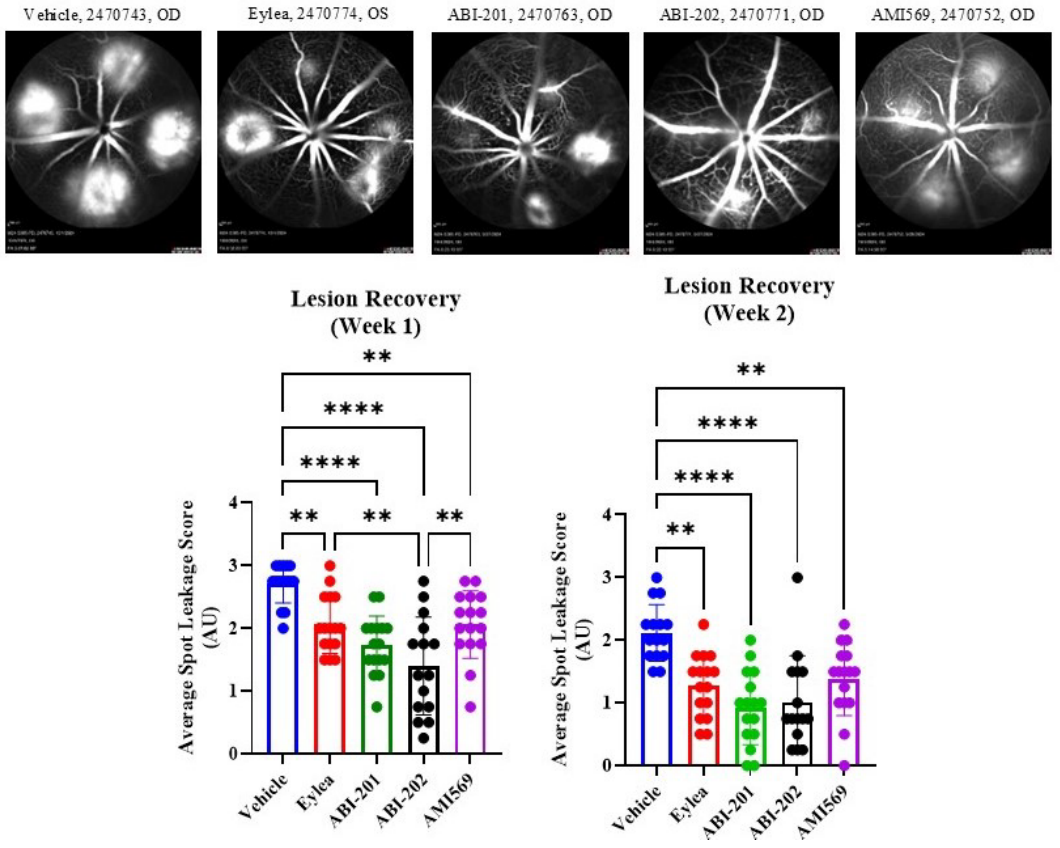
Introduction: This study aimed to determine whether a mouse laser-induced choroidal neovascularization (LCNV) model, treated with constructs carrying the complement protein sCD59 and a complement 3 inhibition (C3i) fusion protein, could elicit non-VEGF-dependent anti-angiogenesis and anti-inflammatory responses. The study also evaluated the localized therapeutic effects of these constructs compared to VEGF inhibition using Eylea as a control.

Methods: C57BL/6J mice received bilateral intravitreal (IVT) injections of either Vehicle, Eylea (40 µg/mL), ABI-201 (sCD59+C3i fusion protein), ABI-202 (sCD59+C3i fusion protein), or AMI569 (C3i+sCD59) at a dose of 1.0E+09 vg/eye. Laser injury was induced in the retinal tissue 28 days after dosing to model choroidal neovascularization (CNV). Lesion recovery was assessed at 7 and 14 days post-injury using fundus angiography and by measuring average spot leakage scores. In vivo protein expression levels in the whole eye, serum, and other organs were quantified using custom in-house ELISA assays to evaluate the localized or systemic distribution of the therapeutic constructs.

Results: Mice treated with the vehicle exhibited the largest lesion sizes and highest intensity of injury, confirming the model's sensitivity. Among the treatments, ABI-202 (sCD59+C3i fusion protein) demonstrated the greatest reduction in lesion size and leakage intensity, likely due to its dual mechanism of complement inhibition and the protective effects of the fusion protein. ABI-201 (sCD59+C3i fusion protein) was the next most effective, showing enhanced lesion healing through a combination of complement inhibition and protective properties of the fusion protein. AMI569 (C3i+sCD59) provided notable reductions in lesion intensity by complement inhibition alone, comparable to Eylea, which served as the VEGF antagonist control. The ranking of treatments based on efficacy revealed: ABI-202 — The most effective, combining complement inhibition with additional protective effects from the C3i fusion protein. ABI-201 — Provided robust healing, coupling complement inhibition with further benefits of the C3i fusion protein. AMI569 — Delivered solid outcomes by targeting complement activation exclusively. At 14 days post-injury, lesion intensity and size reductions remained consistent with trends observed at the 7-day time

point. The highest expression of gene products was observed in ocular tissues, confirming the localized activity of the constructs. Serum and other systemic tissues showed minimal or undetectable protein levels, suggesting a targeted and localized therapeutic effect.

Conclusion: This study demonstrates that complement inhibitors (C3i, sCD59, and their combinations with a C3i fusion protein) are comparable to anti-VEGF treatments like Eylea in reducing laser-induced retinal injury in a mouse LCNV model. These findings highlight the therapeutic potential of complement inhibitors to address inflammation, oxidative stress, and angiogenesis in retinal diseases such as wet AMD. By targeting complement activation, these therapies offer a broader mechanism of action beyond VEGF inhibition, providing new possibilities for standalone or combination therapies. This is especially relevant for patients who do not respond to anti-VEGF treatments. Furthermore, the results validate the LCNV model as an effective tool for studying complement-driven ocular diseases and exploring novel, targeted therapies for preserving vision.



926 Development of Gene Therapy Vectors to Deliver *MYO7A*, *PCDH15*, *CDH23*, and *USH2A*

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Usher syndrome is a genetic disorder that affects both vision and hearing. While at least nine genes are linked to Usher syndrome, mutations in four genes—*USH2A*, *MYO7A*, *PCDH15*, and *CDH23*—account for over 90% of cases in the United States. Adeno-associated virus (AAV) is a safe and efficient gene delivery vehicle and is currently the most broadly used viral vector in retinal gene therapies. However, the aforementioned Usher syndrome

genes are all larger than the AAV vector's packaging capacity, making conventional single AAV-based gene therapies unsuitable for the vast majority of Usher syndrome patients.

We have developed a strategy for delivering large genes using up to four AAV vectors, combined with a CRE-lox-mediated unidirectional DNA recombination system to reconstitute full-length genes. We applied this strategy to the four most commonly mutated Usher syndrome genes and created gene therapy vectors for the treatment of retinal degeneration. For *MYO7A* and *PCDH15*, dual AAV vector systems were designed to deliver the full-length genes, while a tripartite system was developed for *CDH23*. For *USH2A*, which has a coding sequence spanning 15,606 bp, we devised three sets of triple AAV vectors and one set of quadruple AAV vectors to deliver "shortened" *USH2A* (*sUSH2A*) genes with different deletions. *In vitro* studies in 293T cells confirmed that all these AAV vectors successfully produced the intended full-length proteins. *In vivo* studies in mouse retinas are currently underway. These gene therapy vectors provide a foundation for advancing to larger animal models to evaluate their therapeutic efficacies in preclinical studies and future clinical trials.

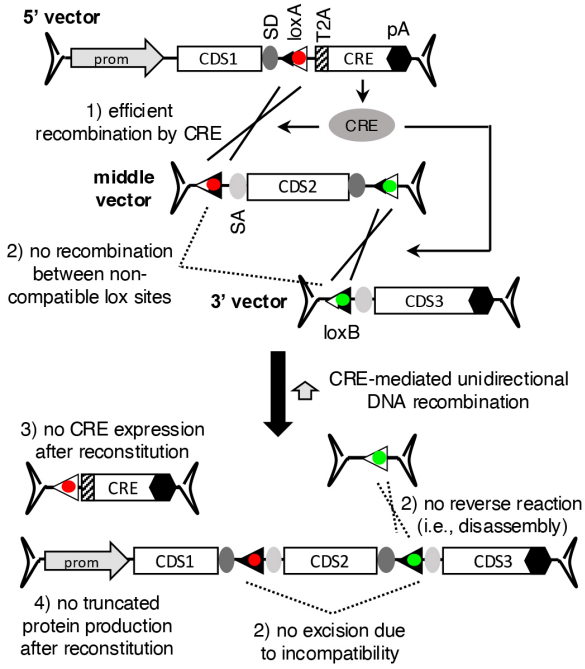

Figure 1. Delivery and reconstitution of large therapeutic genes using multiple AAV vectors and CRE-lox-mediated unidirectional DNA recombination. Advantages of this approach are indicated (1-4).

927 Insulin-Assisted AAV Gene Therapy for Inherited Retinal Diseases

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Purpose: Adeno-associated viruses (AAVs) are proven efficient and relatively safe for intraocular delivery with subretinal injections. However, this method is not without therapeutic limitation in that only the retinal area injected has access to AAV and complications arise like detachment of retina, traumatic macular holes, subretinal neovascularization, and late-appearing retinal atrophy in treated patients with voretigene neparvovec-rzyl (Luxturna). Intravitreal injection attempts have largely failed to transduce the retina efficiently and carry concern for inflammatory response with residual virus left in the eye. To treat the retina more completely, without the physical

trauma of subretinal injection and late atrophy, we propose a novel approach that delivers AAV to the pre-retinal surface after removal of the vitreous, accelerates it into the retina with adjuvant insulin coadministration, and then removes the vector 30 minutes later to avoid residual AAV in the eye. With this approach, we hypothesize that insulin-assisted AAV pre-retinal delivery can transduce outer retina photoreceptors. To model parts of this approach pre-clinically, we have conducted in vivo murine intravitreal injections, ex vivo murine retinal explant cultures, and in vitro cell culture that demonstrates an AAV transduction efficiency dose response with increasing doses of insulin.

Methods: Mouse eyes were treated with an intravitreal injection of 7.2x1010 viral genomes/eye of AAV9.CB.GFP in 2 microliters with insulin doses of 10, 20, and 30ng and balanced salt solution as control in contralateral eye for 4 weeks. Ophthalmic exams including in vivo fundus imaging, optical coherence tomography (OCT) and electroretinography (ERG) were performed to assess retinal structural integrity and function, respectively. Retina morphology, GFP transduction and expression were evaluated using immunohistochemistry and GFP-ELISA. To test AAV transduction on the order of minutes, both murine retinal explants and retinal pigment epithelium (RPE) cell lines were treated with AAV9.CB.GFP and the same doses of insulin as above[TM1] for only 30 minutes before rinsing. We evaluated GFP transduction in explants and RPE cultures on the 3rd, 5th and 7th day.

Results: Our results demonstrate a dose response of AAV transduction to increasing concentrations of insulin. GFP expression was observed robustly throughout both inner and outer retinal cells after intravitreal injection, including in the photoreceptors, with increasing transduction as more insulin is added. Retinal integrity and function were retained on OCT and ERG, respectively. In retinal explants and primary RPE cell cultures, only 30 minutes of AAV plus insulin exposure resulted in similar dose response curve.

Conclusions: We developed a novel insulin-assisted AAV gene therapy approach that significantly increases transduction efficiency to the order of minutes and effectively treats the outer retina. The short 30 minutes of residence time on the pre-retinal surface will facilitate successful transduction and then AAV removal in larger pre-clinical models during typical retina surgery time constraints. Our preliminary data from pigs also demonstrated robust transduction in outer retina. This gene agnostic approach may well be applied to any AAV therapy or conceivably non-viral therapy, allowing it to be utilized for genes larger than AAV carrying capacities and future gene editing approaches.

Figure 1

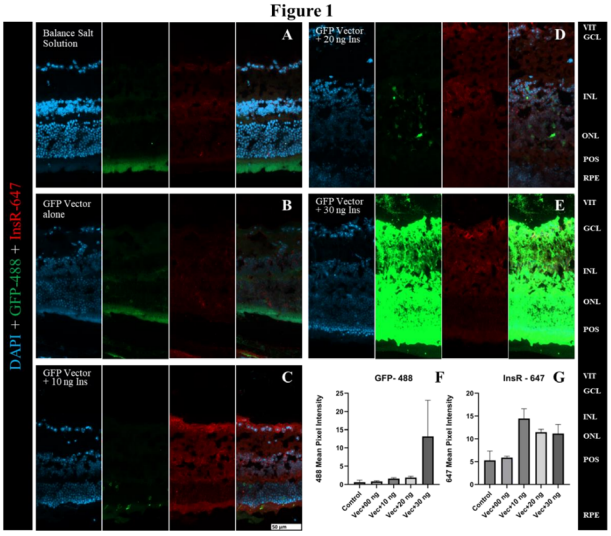

Figure 1. Retinal sagittal sections with dose response curve to insulin. Representative confocal images of eyes injected intravitreally with salt-balance, a control with auto fluorescent RPE **(A)**, AAV9.CB.GFP only **(B)**, AAV9.CB + 10ng insulin **(C)**, AAV9.CB + 20ng insulin **(D)**, and AAV9.CB + 30ng insulin **(E)** labeled with anti-GFP (green) and anti-insulin receptor (red) antibodies. Nuclei counterstained with DAPI (blue). **(F)** quantifies GFP expression by mean pixel intensity using 488 channel in region of interest. **(G)** quantifies InsR expression by mean pixel intensity using 647 channel in region of interest. Representative individual channels are shown along with a merged image for each experimental dose. Scale bar = 50 µm. AAV, adeno-associated virus; GFP, green fluorescent protein; InsR, insulin receptor; VIT, vitreous; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; POS, photoreceptor outer segment; RPE, retinal pigmented epithelium.

928 Inhibition of IGF1R with KRIYA-586: A Novel AAV Gene Therapy Expressing an Anti-IGF1R Antibody Demonstrates Comparable Pharmacodynamic Activity to Teprotumumab

Rachel Eclov[1], Ruth Castellanos[2], Angela Ambakhutwala[2], Cigdem Yasar[3], Chris Lassiter[1], William Chappell[4], Lauren Parker[1], Benjamin Johnson[5], Tanika Williamson[6], Chaitali Basole[1], Jiewu Liu[2], Pilar Prentiss[1], Jessica Raubenolt[1], Ashlyn Bassiri[7], Andrew Murphy[8], Andrea Kossler[9], Michele Stone[10]
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Teprotumumab is a human IgG1 monoclonal antibody that inhibits the insulin growth factor 1 receptor (IGF1R) and is approved for treatment of acute and chronic Thyroid Eye Disease (TED). Increased expression and activation of IGF1R on orbital fibroblast contributes to the pathophysiology of TED and leads to fat accumulation and muscle expansion behind the eye, driving the physical phenotypes of the disease. Inhibition of IGF1R in TED reduces orbital inflammation and proptosis. Adverse reactions have been reported with the current intravenous teprotumumab treatment regimen, including hearing impairment, hyperglycemia, and muscle spasms. To address these limitations, we have developed KRIYA-586, an investigational adeno-associated virus (AAV) gene therapy engineered to express an anti-IGF1R similar to teprotumumab. AAV produced anti-IGF1R reduces IGF1R signaling and expression in cancer cell lines and TED patient derived fibroblasts, and KRIYA-586 intratumoral treatment reduces IGF1R in a tumor xenograft model similar to recombinant teprotumumab.

Excreted anti-IGF1R from immortalized human embryonic kidney (HEK293T) cells transduced with KRIYA-586 was evaluated using either supernatant or enriched protein isolated by Protein A Chromatography (verified for heavy and light chain molecular weight (immunoblot) and total protein quantified (Gyros and Electrochemiluminescence method)). The KRIYA-586 produced protein was evaluated for amount bound to IGF1R (Insulin Signaling MSD ELISA), reduction in total IGF1R (flow cytometry and immunocytochemistry) and IGF1R phosphorylation signal in fibrosarcoma (HT1080) cells and/or TED fibroblasts. Fibroblasts were isolated from adipose decompression samples that would have otherwise been discarded from consenting TED patients. To evaluate KRIYA-586 in vivo pharmacodynamic activity, xenografted nude mice bearing IGF1R+ human colon carcinoma (Colo 205) tumors were given a single intratumoral injection of KRIYA-586 (7.75E11 vg/tumor) or weekly intraperitoneal injection of 6 mg/kg recombinant teprotumumab. At Days 7, 14 and 21 (N = 3 per day) and Day 31 (N = 8) post treatment, animals were sacrificed to assess tumors (size, IGF1R protein, anti-IGF1R protein, vector genomes) and serum (anti-IGF1R protein).

KRIYA-586 produced anti-IGF1R had similar detection of human IgG1 heavy and light chains ratios compared to recombinant teprotumumab. A comparable quantity of vectorized anti-IGF1R and recombinant teprotumumab bound to IGF1R with similar inhibitory activity in HT1080 and TED fibroblast cells. In the xenograft model, anti-IGF1R was detected in serum within 14 days of KRIYA-586 administration and a significant and similar decrease of IGF1R levels in tumors between KRIYA-586 or weekly recombinant teprotumumab were observed.

These data demonstrate that KRIYA-586 generates functional anti-IGF1R with pharmacodynamic activity in vitro, ex vivo and in vivo, equivalent to exogenously administrated teprotumumab. This supports further evaluation of the potential translation of KRIYA-586 gene therapy to impact the course of TED.

929 Periorbital Delivery of AAV Therapeutics for the Treatment of Orbital Diseases

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Orbital diseases, including thyroid eye disease, orbital cellulitis, idiopathic orbital inflammatory syndrome and dacryoadenitis affect tissues surrounding the eye, which are not typically targeted by intraocular or AAV gene therapies. Some of the tissues involved in these diseases are small and not feasible for intra-tissue injections, such as extraocular muscles. To address this issue, we explored periorbital AAV delivery via retrobulbar injection in Göttingen minipigs as a novel route targeting local delivery of AAV to orbital tissues. We selected Göttingen minipigs due to their anatomical similarities to humans.

Minipigs negative for AAV neutralizing antibodies were administered AAV8.mCherry or AAV9.mCherry via retrobulbar injection into the muscle cone at a dose of 1E12 vg/eye in 250uL, or AAV9.mCherry (3.6E11 vg/eye in 12 uL) via intramuscular (IM) injection to the lateral rectus extraocular muscle. Three weeks post-administration, orbital and systemic tissues were examined for vector genomes (qPCR), transgene expression (RT-qPCR), and mCherry protein via immunohistochemistry (IHC). Retrobulbar administration of either AAV8 or AAV9, yielded robust mCherry staining in >75% of extraocular muscles and 51-75% of adipocytes with limited spread to the optic nerve or peripheral tissues. Vector genome analysis shows that animals treated with retrobulbar AAV8 and AAV9 had high vector genome in the extraocular muscles and adipose tissues (1E5-2E6 vg/µg DNA), both superior to IM AAV9.mCherry at 3E4 vg/µg DNA.

Retrobulbar AAV delivery achieved superior transduction of orbital tissues compared to intramuscular injection in large animal models, with minimal systemic distribution. This targeted approach represents a promising novel therapeutic strategy for treating orbital diseases and establishes proof-of-concept for clinical translation.

930 Intracochlear Administration Methods Across Species

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[1]CILcare, Cambridge, MA, [2]Cilcare Inc., Andover, MA, [3]CILcare, Montpellier, Hérault, France, [4]CILcare, Lexington, MA
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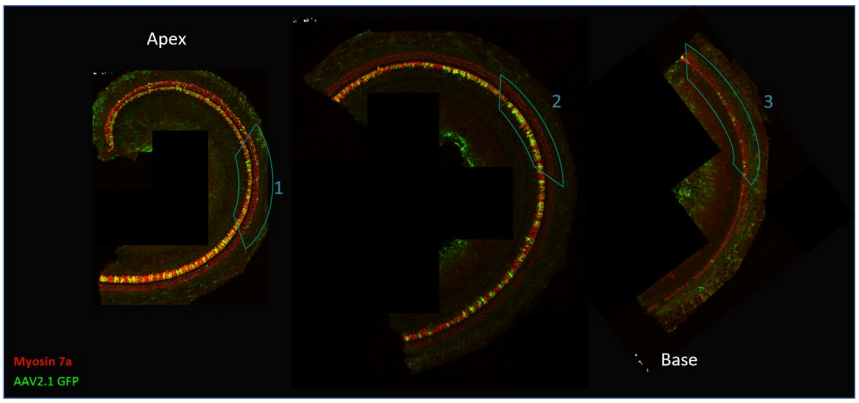
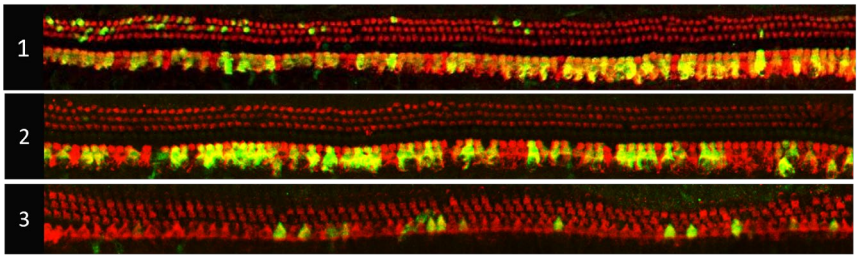
The delivery of gene or cell therapy to the inner ear represents a challenge due to the blood-labyrinth barrier, necessitating local delivery approaches, especially for expensive therapy with limited therapeutic range or susceptibility to systemic side effects. This is particularly relevant for viral gene transfer and cell-based therapies. Adeno-associated virus (AAV) vectors emerge as a prominent choice for gene therapy due to infection efficiency, low toxicity, sustained gene expression, and cost-effectiveness.

This study aimed to compare different intracochlear (IC) injection techniques in mice and guinea pigs. Mice were administered via posterior semicircular canal (PSCC) and round window (RW) injections, while guinea pigs received cochleostomy infusion, RW injection (RWI) or deposit (RWD) due to anatomical differences. Auditory function effects of IC administration were evaluated in both species via auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) measurements.

Following AAV delivery, GFP expression from AAV-mediated cell transfection enabled the identification of transfected cochlear regions. Both PSCC and RW injections successfully targeted hair cells, albeit with variations in transfection patterns and intensity depending on the injection method and AAV subtypes. Finally, impacts on auditory function varied among injection methods, with PSCC and IC routes showing less effect on DPOAE amplitudes and ABR thresholds compared to the RW route.

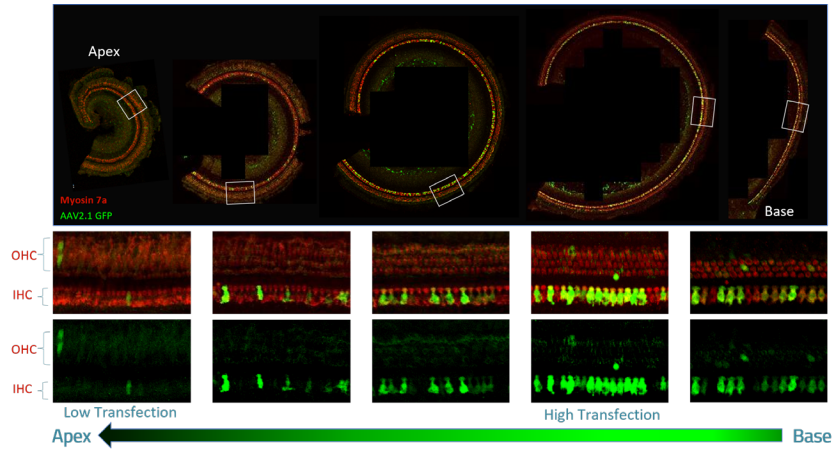
In conclusion, our study highlights the importance of refined intracochlear delivery method in animals accordingly to the delivered product and targeted inner ear disorders. While all methods effectively reached cochlear hair cells, variations in transfection patterns or in product concentration at targeted cells, and effects on auditory function highlight the need for careful consideration of injection strategies. It is worth mentioning that the posterior semicircular canal (PSCC) and cochleostomy routes, though effective in animals, lack translational relevance to humans. Moving forward, further research into optimizing delivery methods will be essential for advancing therapeutic interventions in auditory disorders.





Injection parameters

- *Nanoliter pump and glass capillary*
- *Adenovirus : AAV2.1 at 9x10[12] vg/ml*
- *Volume: 1 µL*
- *Rate : 200 nL/min*
- *Duration injection ~5 min*



Injection parameters

- *Adenovirus : AAV2.1*
- *Concentration : 9x10[12] vg/ml*
- *Description : Serotype 1 with the eGFP gene*
- *Volume injected : 5 µL*
- *Rate : 0.5 µL/min*
- *Duration injection : 10 min*

931 AAV mediated gene delivery to the aged and young mice lung

Lu Song[1], Agnieszka Anya , Wendorff[2], Alexander Li[2], Varahram Shahryari[2], Aaron Stebbins[2], John Patino[2], Andy Chang[2], Astrid Gillich[2], James Lee[2], Calvin Jan[2], Putianqi Wang[1]
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Adeno-associated virus (AAV) mediated gene delivery provides a more rapid method for in vivo validation of aging-related target genes, compared to the development of transgenic animal models. Aging is the primary risk factor for many chronic lung diseases, including idiopathic pulmonary fibrosis, chronic obstructive pulmonary

disease and acute respiratory distress syndrome. Many studies have investigated gene delivery to the lungs of young mice; however, the physiological decline of tissues in aged animals presents a major challenge for gene delivery. Here, we compared two AAV serotypes, AAV6.2FF and AAV9, transduced in young and aged mice. AAV6.2FF shows high transduction efficiency in the epithelial cells of both young and aged mouse lungs. AAV9 is more biased towards infecting fibroblast cells in the lungs of young and aged mice. Comparing AAV-mediated gene transduction in young and aged animals will facilitate understanding of AAV gene delivery in the aged pulmonary system, and define research tools to understand aging-related lung diseases.

932 A Head-to-Head Comparison of AAV9 Biodistribution in Mice: Routes of Administration and Age Dependence

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**Corresponding Author*

Adeno-associated virus serotype 9 (AAV9) has broad systemic delivery, the ability to cross the blood brain barrier, and a low toxicity profile, making it widely used as a gene delivery vector. Despite the extensive use of AAV9 in translational research, variability in vector constructs, injection volumes, treatment ages, and routes of administration make the existing body of work regarding AAV9 biodistribution fractured and often studies are difficult to directly compare. We designed the following study to provide a head-to-head comparison of biodistribution of AAV9 based on the route of administration in mice, with internally controlled groups treated at various developmental timepoints. Due to the emergence of AAV9 as the dominant AAV serotype used to target the central nervous system, we concentrated our analysis on central nervous system tissue (cerebrum, cerebellum, lumbar spinal cord, and cervical spinal cord) with a secondary focus on key peripheral organs (liver, heart, and sciatic nerve). We evaluate transgene expression of a self-complementary AAV9 vector expressing GFP driven by a strong ubiquitous promoter (CBh) via four different routes of administration at four different ages. Bilateral intracerebroventricular, intravenous, lumbar intrathecal, and lumbar intrathecal intracerebroventricular combination injections were performed in C57BL/6J mice at postnatal day 1, 5, 10, and 28. Mice were treated with a total dose of either $2.5x10^{11}$ or $5.0x10^{11}$ viral genomes, which allowed for direct comparison across treatment groups. All tissue was collected at 8 weeks of age to allow for peak gene expression and brain maturation to at least adolescent levels before immunohistochemistry and quantitative polymerase chain reaction analysis. As targeted therapies continue to improve through engineered viral vector optimizations, it will be important to establish standardized benchmarks to gold standards like AAV9, for accurate comparison.

933 *Pre-Clinical Safety and Efficacy of AAV9 gene therapy for Mucolipidosis type IV (ML4)*

Tyler Mola[1], Olivia Morales[2], Simon Wentworth[3], William Callahan[2], McKenna Watson[4], Ananya Uppalapati[2], Ana Rita Batista[2], Miguel Sena-Esteves[2]
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Mucolipidosis type IV (ML4) is an autosomal recessive lysosomal storage disease caused by mutations in the *MCOLN1* gene, encoding for the transient receptor potential channel, mucolipin 1 (TRP-ML1). Evidence has shown that TRP-ML1 acts as a non-selective cation channel whose channel activity is pH- and Ca^{2+}- dependent and that it regulates lipid trafficking by regulating late endosomal/lysosomal fission and fusion events. However, the exact function of TRP-ML1 and how the mutated versions interfere with lysosomal function is still unknown. It has been shown that AAV-mediated CNS gene transfer of human *MCOLN1* results in rescue of motor deficits and delayed time to paralysis with correction of brain pathology in ML4 mice (*Mcoln1*$^{-/-}$). We are conducting a blinded long-term dose escalation efficacy study in *Mcoln1*$^{-/-}$ mice treated neonatally (P0-P1) by bilateral intracerebral ventricular (ICV) injection of 1E10, 3E10 or 1E11 vg of scAAV9-JeT-h*MCOLN1* vector or PBS. Behavioral performance is assessed at 2 and 6 months of age in the Open Field and rotarod tests to assess efficacy and durability of effect. Interim data analysis of survival (**Fig. 1**) showed that all animals have perished in group B with a median survival of

200 days. The identity of group B was unblinded prior to conclusion of the study and corresponds to mice treated with PBS. For the other groups there appears to be a dose effect with two groups (A and C) with several animals that have reached the humane endpoint (median survival in group C is 251 days), and one group (Group D) where all mice remain alive at 292 days of age. Performance in the rotarod test was comparable across groups at 2 months of age, but it declined significantly in PBS-treated animals by 6 months of age. Performance was significantly better in AAV-treated mice compared to PBS-treated mice at 6-months of age and remained unchanged between 2 and 6 months of age for groups A and D. Importantly there have been no adverse events in age-matched carrier and wild type littermates that also receive AAV vector at birth. Molecular and histological analyses of tissues are ongoing.

Conclusions: Our survival and behavioral data show that neonatal ICV treatment with scAAV9-JeT- h*MCOLN1 vector* has a substantial therapeutic effect, rescuing motor deficits and increasing survival of ML4 mice. We are currently analyzing tissues for MCOLN1 expression and impact of treatment on storage material and myelination, which is defective in ML4 mice. We are also developing a *MCOLN1* knockout HEK293T cell line to assess vector potency across AAV vector batches. The interim results show a durable benefit of neonatal treatment with scAAV9-JeT-MCOLN1 vector and support transition of this AAV gene therapy to a phase I/II clinical trial in ML4 patients.

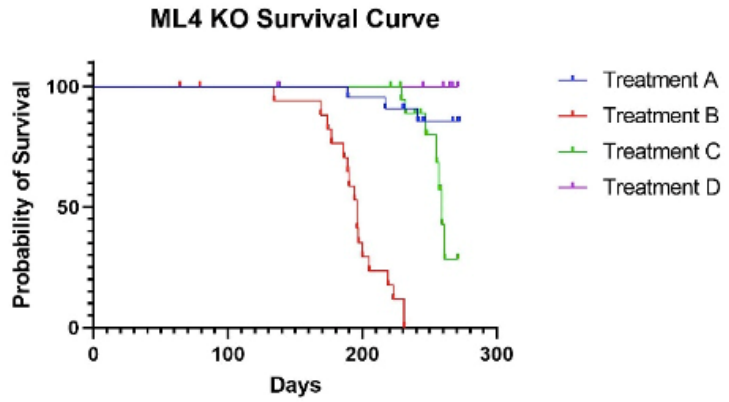

Figure 1. Survival of ML4 mice treated neonatally by ICV injection of AAV9-hMCOLN1 vector or PBS (Group B).

934 *Neonatal Intravenous Delivery of AAV-F in a Gaucher disease mouse model*

Ozge Cetin[1], Anna Keegan[2], Ellie Chilcott[3], Juan Antinao Díaz[4], Amy Geard[5], Giulia Massaro, Ahad Rahim, Simon Waddington, Rajvinder Karda*
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While AAV9 has demonstrated clinical success in treating diseases such as Spinal Muscular Atrophy (SMA), there is some limitations. One major challenge is the requirement for high concentrations of AAV9 to achieve therapeutic efficacy, which can lead to off-target effects and dose-limiting toxicity. To address these challenges, the AAV-F capsid has emerged as a promising candidate for gene therapy studies, due to its widespread central nervous system (CNS) targeting capabilities and greater transduction efficiency.

A comparative biodistribution profiles of AAV-F and AAV9 was conducted using a CAG promoter driving the green fluorescent protein (GFP) reporter gene. After administering titre-matched AAV-F and AAV9-CAG-eGFP ($1.9x10^{10}$ vg/pup) via single intravenous neonatal delivery, GFP analysis at six weeks post-injection revealed that AAV-F exhibited significantly greater neuronal and astrocytic transduction targeting throughout the brain compared

to titre-matched AAV9. Additionally, AAV-F demonstrated higher transduction levels in visceral organs including heart and liver, compared to titre-matched AAV9.

The therapeutic efficacy of AAV-F was evaluated using a knock-out (KO) mouse model of Gaucher disease (GD), a severe neurometabolic disorder caused by mutations in the *GBA1* gene. Which manifests with severe neurodegeneration and visceral pathology. The most acute neuronopathic form (nGD), has no effective treatment options and leads to death in early development. The GD KO mice display neurological and visceral pathology, leading to premature death between postnatal days P12-P14.

Newborn GD KO mice were administered with titre-matched AAV-F and AAV9-CAG-*hGBA1* ($6x10^{10}$ vg/mouse), as well as a higher dose of AAV9-CAG-*hGBA1* ($6x10^{11}$ vg/mouse) via a single intravenous injection. The therapeutic outcomes were evaluated based on survival, body weight, enzyme activity, and behavioural assessments. Both AAV-F and AAV9 treatments resulted in a significant extension of lifespan, compared to PBS KO control group. Rotarod behavioural assessment, resulted in comparable results between AAV-F LD (low dose), AAV9 HD (high dose), and WT control group. AI behavioural assessment using DeepLabCut showed similar results. Analysis of GCase enzyme activity in the brain revealed that AAVF-LD resulted in comparable GCase enzyme levels to AAV9-HD.

Our proof-of-concept study showed that a single intravenous administration of AAV-F-CAG-*hGBA1* to a GD KO mouse model demonstrated significant improvement in survival, weight and GCase enzyme activity. Thus, providing early evidence of the use of AAV-F as a potential treatment for nGD at a lower vector copy number compared to AAV9.

935 AAV-Mediated Beta-Cell-Specific Smad2 Silencing: Bridging Transgenic Mouse Evidence to Gene Therapy for Hyperglycemia

Madison Thomas[1], Ashley Diehl[2], Mohammad Tariq[1], Lydia Liszewski[1], Ting Zhang[3], Vinitha Dhamotharan[1], Krishna Prasadan[1], George Gittes[4]
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Introduction:
Hyperglycemia resulting from beta-cell dysfunction is a hallmark of type 2 diabetes mellitus (T2DM), a leading global health challenge. Chronic high-fat diet (HFD) exposure exacerbates beta-cell exhaustion, leading to dedifferentiation, senescence, and apoptosis. Smad2, a key mediator of TGF-β signaling, negatively regulates beta-cell proliferation while promoting beta-cell dysfunction under hyperglycemic stress. Studies using transgenic mice with congenital beta-cell-specific Smad2 knockout have demonstrated improved beta-cell function and protection against diet-induced hyperglycemia. Furthermore, conditional deletion of Smad2 in adult beta cells showed similar protection against and reversal of diet-induced hyperglycemia. This study aims to translate these findings by employing adeno-associated virus (AAV)-mediated Smad2 silencing as a potential gene therapy for hyperglycemia.

Methods:
Beta-cell-specific Smad2 knockout (Smad2-fx/fx; InsCreERT) mice were fed an HFD (60 kcal/kg) from 6 weeks of age and treated with tamoxifen or vehicle at 18 weeks. Glucose homeostasis was assessed using glucose tolerance tests (GTT), insulin tolerance tests (ITT), glucose-stimulated insulin secretion (GSIS), and HbA1c measurements. Islets were harvested for RNA sequencing and functional assays. Based on these results, to create a therapeutic system applicable to humans with T2DM, we created an rAAV vector expressing Smad2-targeted short hairpin RNA (shRNA) controlled by the rat insulin promoter (RIP) for beta-cell-specific Smad2 gene silencing. The vector included EGFP for tracking. In vitro validation was performed using MIN6 beta cells, and in vivo delivery was achieved via pancreatic ductal infusion in high-fat-fed CD1 mice. Smad2 expression, infection efficiency, and functional outcomes were evaluated.

Results:
Transgenic Smad2 knockout mice demonstrated significant improvements in glucose homeostasis, as evidenced by

enhanced GTT, ITT, GSIS, and reduced HbA1c compared to controls. RNA sequencing of isolated islets revealed upregulation of beta-cell maturity markers and downregulation of ER stress and mitochondrial dysfunction pathways.
In vitro validation of rAAV-mediated Smad2 silencing in MIN6 cells resulted in a 65% reduction in Smad2 mRNA. In vivo, CD1 mice treated with the Smad2-targeted rAAV showed a 75% reduction in Smad2 expression, with viral infection primarily localized to islet peripheries. Ongoing studies aim to evaluate physiological outcomes and optimize infection efficiency.

Conclusion:
AAV-mediated Smad2 silencing effectively mirrors the benefits observed in transgenic models, including enhanced beta-cell function and improved glucose regulation in high-fat-fed hyperglycemic mice. These findings support the potential of AAV-based Smad2 silencing as a therapeutic strategy for hyperglycemia. Future work will focus on optimizing viral delivery and assessing long-term physiological impacts.

936 Functional Elevation of Computationally Designed IL-10 in IL-10 KO mice

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Increasing evidence suggests that IL-10 has therapeutic effect for the treatment of inflammatory diseases. However, it is challenging to use IL-10 as a therapeutic drug since it also has pro-inflammatory functions. To reduce the proinflammation effects of IL10, we have designed 3 IL-10 mutants (A, B and C) using recently developed structure-based computational-design technology. We first tested the function of these mutants in human PBMCs. Western blot analysis showed that intracellular pSTAT3 (mediator of IL-10 signaling) levels in IL-10 mutant treated PBMCs were lower than that in IL-10W treated cells. To quantitatively evaluate the functions of the IL-10 mutants, we have generated 3 cell lines (THP-luc, U937-luc and RAW-luc), in which the reporter gene (luciferase) are responsive to IL-10 signaling pathway through pSTAT3. Using these cell lines, we showed that our IL-10 mutants are functional, yet have lower activity than IL-10W. We next generated recombinant adeno-associated virus (rAAV8) vectors expressing wild type or mutant IL-10 molecules (W, A, B, and C). We tested our vectors in IL-10 knockout (IL-10 KO) mice, which are on Balb/c background (BALB/cAnNTac-Il10em7Tac) and spontaneously develop inflammatory bowel disease (IBD). We IP injected cohorts of IL-10 KO mice with rAAV8 vectors (10^{11}vg/mouse) expressing W, A, B, C, or GFP, or PBS (as model control) at 8 weeks of age. Results from this study showed that our vectors mediated high and sustained levels of transgene expression detected at 2 and 4 weeks after the vector injection. The body weight gains in A, B, and C groups were significantly higher than in GFP (the vector control) group. Importantly, the accumulative disease activity index (DAI) scores in W, A, B and C groups were significantly lower than GFP group and were similar to that in Balb/c mice (normal control group). In addition, the colon injury in W, A, B, C group were significantly lower than that in GFP and PBS group. In summary, our IL-10 mutants have similar protective effect to IL-10W in IL-10KO mouse model. These results indicate that gene therapy of IL-10 mutants may be safer and more effective than IL-10W for the treatment of IBD.

937 InstaGTx Amplifies AAV Tropism and Is Safe in Non-human Primates

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Increasing targeting and thereby decreasing off-targeting of systemically-administered AAV remains a challenge for hard-to-transduce sites such as skeletal or cardiac muscle. Typically, this challenge is addressed on an organ-by-organ basis, with engineered capsids meant to be delivered to a single target organ most effectively. Direct evolution or other methods are often employed for this purpose; however, these methods are expensive and time-consuming, and must be replicated for each organ of interest. Here, we present a tool that enhances AAV targeting, agnostic to AAV serotype. We have engineered a cargoless, polymeric nanoparticle enhancer, InstaGTx, that bolsters different AAV serotypes' tropism to their target organ when administered prior to systemic AAV injection. We have

previously demonstrated InstaGTx's efficacy for heart targeting of AAV1 and AAVrh74.[1] Although we have already shown efficacy of InstaGTx, its safety has not yet been demonstrated in non-human primates (NHPs), a necessary step towards clinical translation. Here, we show that InstaGTx exhibits a favorable safety profile in African Green monkeys (Figure 1a). In this non-terminal study, subjects between 5 and 8 years old who were prescreened for anti-AAV neutralizing antibodies had blood drawn after a baseline saline injection. They were injected with InstaGTx 28 days later and their blood was collected and processed for serum 1 hour and 1 week post injection. Serum was analyzed with a clinical chemistry panel for liver enzymes and assays for inflammatory cytokines,chemokines, and complement factors. The results show that InstaGTx injection does not increase liver enzymes or inflammatory cytokines and chemokines, nor does it activate complement C3a and C5a. We have also validated an in vitro-in vivo screening assay, in which NHP serum is added to human iPSC-derived cardiomyocytes simultaneously with AAV1-Luc. The addition of InstaGTx-injected NHP serum increased transduction relative to saline-injection serum control (Figure 1b), suggesting a serum-derived factor as a driving force of increased in vivo transduction. Excitingly, this assay could allow future high-throughput screening of AAV serotypes for tissue-targeting enhancement, by testing InstaGTx-injected NHP serum with serotypes of interest in in vitro transfections. Further, screening nonviral gene delivery vehicles (polymeric or lipid nanoparticles, etc) with this assay will determine whether InstaGTx can bolster uptake of these different modalities as well. Our data from an in vivo-in vitro NHP study suggests that InstaGTx technology may be translatable for human gene therapy. By improving the targeting of existing AAV serotypes and other therapeutic delivery vehicles, the effective administered dose of treatment will be reduced. This dose reduction increases safety and decreases the cost of goods per dose of AAV (or other delivery vehicle) and decreases the cost of manufacturing. InstaGTx technology holds the potential to expand treatable indications for AAV gene therapies—and potentially other delivery modalities—while streamlining candidate screening, improving safety, and accelerating the timeline to IND submission.

1. Switala, L. et al. Engineered nanoparticles promote cardiac tropism of AAV vectors. J. Nanobiotechnology 22, 223 (2024).

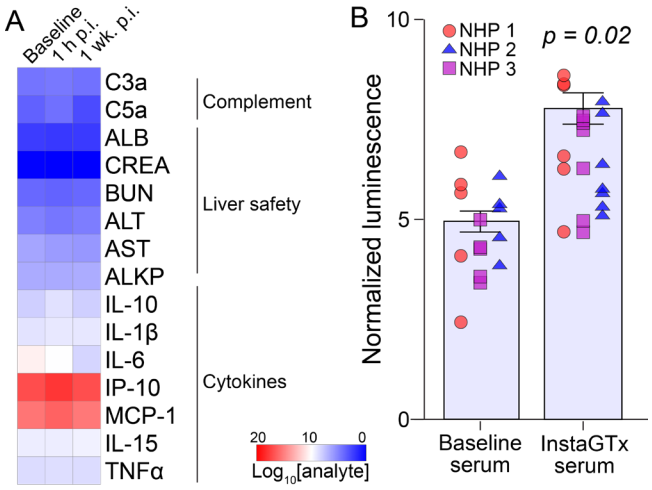

Figure 1. A) Heat map depicting immune and liver markers at baseline and post injection (p.i.) of InstaGTx in 3 NHPs. **B**) AAV1-Luc transduction in human iPSC-derived cardiomyocytes in the presence of NHP serum.

938 Broad Biodistribution of AAV5 and AAV9 Following ICV Administration Compared to ICM in Non-Human Primates.

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Due to the lack of suitable AAV capsids for intravenous delivery, gene therapies for CNS disorders typically rely on local administration routes. Intraparenchymal injections are ideal for targeting specific brain areas, while cerebrospinal fluid (CSF) injections are preferred for diseases affecting multiple, distant brain regions. Delivering AAV to the CSF can be achieved by administration to the lateral ventricles (i.e. intracerebroventricular, ICV) or to the cisterna magna (ICM). The relative benefits of each route in achieving optimal brain biodistribution remains unclear. This study aims to compare the biodistribution of two AAV serotypes, AAV5 and AAV9, following ICV or ICM delivery in non-human primates (NHP) with unilateral intra-thalamic dosing as a control. A tool cargo expressing a secreted protein under the control of a CAG promoter was used to compare the effect of route of administration and serotype on expression of vector DNA and mRNA across brain regions. Additionally, CSF neurofilament and glial fibrillary associated protein levels were measured in NHPs as markers of neuronal damage and gliosis. Overall, the results showed a broad expression of vector DNA and mRNA following ICV administration for both serotype and localized to spinal cord regions following ICM administration. Furthermore, AAV5 administration led to higher vector DNA expression than AAV9, independent of the route, but resulted in similar mRNA expression levels, suggesting potential difference in cellular tropism. Overall, these findings support the preference for ICV administration when targeting higher brain regions, and ICM for lower brain regions and spinal cord.

939 Biodistribution in ocular tissues after suprachoroidal injection of KH658 in cynomolgus monkeys

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Purpose: The suprachoroidal injection is a recent breakthrough in the retinal gene-delivery landscape and may provide a unique opportunity to perform less invasive surgeries. KH658 is an anti-VEGF gene therapy product that employs a novel AAV capsid variant (AAVv128), delivered by suprachoroidal injection for the treatment of neovascular age-related macular degeneration (nAMD). The purpose of the present study was to assess the ocular tissue distribution following a single suprachoroidal injection of KH658 in cynomolgus monkeys.

Methods: Cynomolgus monkeys were sacrificed 13 weeks following a single suprachoroidal injection (100µl/eye) of 1E12vg/eye KH658, and ocular tissues were collected for subsequent analysis. The quantification of transgene and transgene product was performed by qPCR and ELISA, respectively.

Results: The transgene was present at a concentration of 1.81E7 vg/g in the retina, 1.17E10 vg/g in the choroid, and 2.18E10 vg/g in the sclera. The transgene product was found to be 8,700 ng/g in the retina, 7,680 ng/g in the choroid, and 162 ng/g in the sclera. The transgene and transgene product were below the limit of quantification in the other ocular tissues, including the cornea, lens, conjunctiva, vitreous body and optic nerve.

Conclusions: The transgene and transgene product of KH658 were predominantly distributed within the retina, choroid, and sclera, which is consistent with the characteristics of suprachoroidal injection. The high concentration of anti-VEGF transgene products within the retina suggests that suprachoroidal administration of KH658 may represent a treatment option for nAMD.

940 Exploring the Transduction Efficiency of Suprachoroidal Injection of AAV in the Treatment of Neovascular Age-related Macular Degeneration through A Twice-laser Modeling

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AAV-antiVEGF is a replication-deficient recombinant AAV variant, which is contained a gene that encodes for a VEGFR-Fc fusion protein intended to treatment for neovascular age-related macular degeneration (nAMD) via suprachoroidal space (SCS) injection. SCS injection has lower invasiveness and fewer complications than subretinal injection (SR) and intravitreal injection (IVT). It also has less impact on ocular structure and vision. However, physiological barriers may limit AAV drug diffusion. nAMD is caused by choroidal neovascularization (CNV) that damages the blood - retinal barrier (BRB). Therefore, in the pathological state of nAMD, the retinal permeability may be increased, which is conducive to the transduction of AAV. To explore whether suprachoroidal injection can increase the transduction efficiency of AAV in the pathological state, we have explored a design of a twice - laser modeling. Two lesions were laser induced 14 days prior to the administration of AAV-antiVEGF in rhesus monkey, in order to induce CNV to mimic the compromised barriers of eyes under the pathological state of nAMD.

At Day 0, laser photocoagulation in the peri-macular region of the retina to induce CNV (1st modeling) to mimic the compromised barriers of eyes under the pathological state of nAMD. Single suprachoroidal space injection of AAV-antiVEGF was performed 14 day (Day 14) after the first modeling (1st modeling), with doses of 3E11 vg/eye and 1E12 vg/eye. At Day 42, 28 days after injection, the laser induced CNV will be performed again (2nd modeling). At day 56 and day 70, the percentage of grade-4 lesions, the fluorescein leakage (FL) areas and the retina thickness will be determined to evaluate the efficacy. Compared with the historical data of single - modeling, the results showed that AAV-antiVEGF can effectively inhibit the formation and development of Grade 4 lesions, reduce the area of the fluorescein leakage (FL) region and the thickness of the retina.

The results of this study suggest that in the pathological state of nAMD, suprachoroidal injection of AAV can transduce the retina more efficiently, thereby achieving a therapeutic effect.

941 Biodistribution and Expression of Ixoberogene Soroparvovec (Ixo-vec) in Non-Human Primates: A Comprehensive Study on Intraocular and Systemic Distribution

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Ixo-vec is an investigational intravitreal gene therapy designed to express aflibercept, a VEGF inhibitor, as a treatment for neovascular age-related macular degeneration (nAMD). Clinical studies, Phase 1 OPTIC (NCT03748784) and Phase 2 LUNA (NCT05536973), demonstrated that a single intravitreal administration eliminated or significantly reduced the need for supplemental anti-VEGF injections in patients who required frequent IVT injections prior to study initiation. LUNA data has confirmed the potential best-in-class profile of Ixo-vec at the 6E10 vg/eye dose, demonstrating robust and sustained aflibercept levels, industry leading annualized reduction in anti-VEGF injection rates, BCVA preservation, CST reduction and a greater than 90% patient preference for Ixo-vec compared to their prior standard of care.

Non-human primate studies reported here evaluated the tissue biodistribution and expression of Ixo-vec using doses of 3E10 vg/eye and 1E11 vg/eye which are the primate doses equivalent to the human doses used in Phase 2 LUNA (6E10 and 2E11 vg/eye). Results showed that aflibercept mRNA was limited to intraocular tissues and was below the limit of detection in all other tissues at both doses, suggesting no systemic exposure to the ectopically expressed aflibercept protein. Aflibercept protein levels were 4- to 7-fold higher in the retina than in the aqueous humor and uvea, suggesting that the retina, where VEGF causes neoangiogenesis, is the tissue location for the highest levels of aflibercept protein production.

Vector genome DNA was shown to be localized to intraocular tissues, including the retina, choroid, anterior chamber tissues, and parts of the visual system containing retinal ganglion cell axons (optic nerve, optic chiasm, optic path, and LGN). Systemic tissue levels were significantly lower, with the highest levels detected in lymphatic

tissues. At the lower dose (3E10 vg/eye), systemic tissue levels were below the limit of quantification while at the higher dose (1E11 vg/eye) only very low levels of vector genome DNA were detected in liver and lung tissues.

An additional study tested whether there is a transfer of Ixo-vec to fellow eyes. Animals were dosed in one eye with supra-therapeutic doses of 2E12, 6E12, and 2E13 vg/eye.. The study found no evidence of vector genome transfer between eyes. This was supported by the absence of aflibercept or anti-capsid antibodies in the un-dosed fellow eyes. These data also support clinical trials to assess dosing the second eye of patients with bilaterial nAMD.

In summary, these data continue to demonstrate the safety profile of Ixo-vec at the 6E10 human equivalent dose, which has been selected for evaluation in registrational studies, as transduction occurred only in ocular tissues with no systemic expression detected. Additionally, these data confirm the potential to safely dose the second eye for patients with bilateral nAMD.

942 Preclinical Safety Evaluation of AAV5-hRORA (OCU410-ST) a Gene Therapy Candidate for Stargardt's disease and other *ABCA4* related retinopathies

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Background: Mutations of the ATP binding Cassette 4 (*ABCA4)* gene lead to its functional deficiency, resulting in defective processing and transportation of all-trans retinaldehyde from the photoreceptors to retinal pigment epithelium (RPE) cells. This deposition and subsequent accumulation of oxidized products triggers complement activation and leads to retinal degeneration, ultimately resulting in the development of disease phenotypes such as Stargardt disease 1 (STGD1), Cone Rod Dystrophy 3 (CRD), and Retinitis pigmentosa 19 (RP19). Our prior studies revealed that Retinoic acid-related orphan receptor α (RORA) regulates many of the pathways impacted in STGD, including cellular response, metabolism, and complement activation. OCU410ST (AAV5-*hRORA*) is a modifier gene therapy platform that is being developed to treat individuals with Stargardt disease and other *ABCA4* related retinopathies.

Methodology: A total of 12 cynomolgus monkeys were treated with a single dose of either formulation buffer as vehicle control or with OCU410ST at doses of 8.61×10^{10}, 1.75×10^{11} or 3.50×10^{11} Vg/eye, respectively via subretinal injection. Parameters evaluated in this study included clinical observations, body weight, food consumption, clinical pathology (hematology, coagulation, and clinical chemistry), immunogenicity (AAV5 binding antibody, RORA protein binding antibody, antigen-specific T-cell immunoreactivity, and cytokines), tissue biodistribution of vector DNA, target gene mRNA and protein expression, virus shedding. In addition, detailed ophthalmic examinations, including general ocular examination, electroretinography (ERG), intraocular pressure (IOP), optical coherence tomography (OCT), fundus fluorescein angiography (FFA), and fundus photography (FP) were performed throughout the study.

Results: The *hRORA* transgene was mainly distributed in ocular tissues. In tissues positive for OCU410ST, the *hRORA* mRNA expression was at highest levels in the Iris-ciliary complex, retina, and retinal pigment epithelium (RPE)-Choroid Complex. The hRORA specific DNA was detected in tears in a dose-dependent manner up to one-week post-dosing. In toxicological assessment, OCU410ST appeared to be well tolerated at all dose levels. There were no unscheduled deaths or notable changes in body weight, food consumption, hematology, coagulation, or clinical chemistry for the duration of the study. No related systemic toxicity and changes in IOP were observed across different dose groups. Pigment deposition, local depigmentation, local fluorescence leakage, and multifocal pigmentation with blocked fluorescence were observed in all groups, including control, and were related to the subretinal injection procedure. No ADA response was observed against the hRORA protein. A dose dependent response was observed against the AAV5 capsid. However, no systemic clinical signs due to an increase in anti-AAV5 antibodies were observed. There was no immunogenic response detected in the PBMCs against hRORA or AAV5 capsid.

Conclusions: Our results demonstrate that OCU410-ST is safe and tolerable at tested doses. Based on these results, 3.50×10^{11} Vg/eye was identified as the maximum tolerable dose for further planned clinical studies.

943 Bilateral sequential injection of KH631 in rhesus monkeys showed good safety profile

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Purpose: In the real world, neovascular age-related macular degeneration (nAMD) can occur in either one eye or both eyes, with unilateral onset being relatively more common. As time progresses, the other eye also has a risk of developing the disease. Therefore, it is necessary to investigate the safety of repeated drug administration in the contralateral eye.

Method: KH631 Ophthalmic Injection is a replication-deficient recombinant AAV variant, which is contained a gene that encodes for a VEGFR-Fc fusion protein intended to treatment for nAMD via subretinal injection. In our study, on the first day, KH631 was injected into the rhesus monkey right eyes with dose of 2.5E11 vg/eye, 1E12 vg/eye and 5E12 vg/eye. After a 13-week interval, the left eyes were injected again with same dose. The safety and tolerance of the eye and the whole system after sequential re-administration were examined for 52 weeks.

Results: Throughout the 52-week study period, the results demonstrated that under the conditions of this experiment, there were no statistically significant differences ($P>0.05$) in immune-related indicators such as immunoglobulins (IgM, IgG), complements (C3, C4), T cell subsets (CD45+CD3+, D45+CD3+CD4+, CD45+CD3+CD8+), and circulating immune complexes (CIC) when compared with the control group. No obvious abnormalities were found in cytokines such as IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, and IL-10. There were no test article-related abnormal changes were observed in the general condition, body weight, food intake, body temperature, electrocardiogram and blood pressure, neurological function and behavior, hematology, blood biochemistry, routine urine test, bone marrow smear, organ weight/coefficient, and histopathological examination of organ tissues of each system except the eyeball in monkeys of each dose group.

Conclusions: The research results indicate that after repeated administration to the contralateral eye, the systemic safety is good and no new adverse reactions emerge compared with the first administration. This provides strong support for repeating the administration to patients in the real world.

944 KH631 consistently expressed in rhesus monkey eyes over 152 weeks

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Purpose: KH631 is a clinical-stage anti-VEGF gene therapy product designed to treat neovascular age-related macular degeneration (nAMD). In previous studies, KH631 demonstrated favourable efficacy and safety in pharmacodynamic, pharmacokinetic and toxicological studies in non-human primates (NHPs). The purpose of the present study was to investigate the long-term expression stability of KH631, continuous transgene product assays were conducted in rhesus monkeys.

Methods: Rhesus monkeys 1M002, 1F002 and 3F001 received bilateral administration of KH631 at a dosage of 3E10, 1E11 and 5E11vg/eye, respectively. The transgene product of KH631 in the aqueous humour was collected and detected at 53, 104 and 152 weeks. The aqueous humour, with a volume of between 50 and 100µl per eye, was collected from the eye using a 0.3 mL insulin syringe with a 27G needle. The transgene product was detected by a validated ELISA method.

Results: The transgene product of KH631 at 53, 104 and 152 weeks for 1M002 (3E10 vg/eye) was 19.4, 32.2 and 17.7 ng/mL in OS and 22.6, 27.6 and 17.2 ng/mL in OD. For 1F002 (1E11 vg/eye), the concentrations were 53.0, 43.9, and 31.3 ng/mL in OS and 36.1, 45.5, and 24.9 ng/mL in OD. For 3F001 (5E11 vg/eye), the concentrations were 96.3, 119.5 and 62.5 ng/mL in OS and 126.0, 84.9 and 91.8 ng/mL in OD. Longer expression of KH631 is currently under investigation.

Conclusions: The results demonstrated that KH631 was consistently expressed in rhesus monkey eyes for 152 weeks at all three doses, suggesting that a single subretinal injection of KH631 has the potential to offer a one-time treatment for nAMD patients.

945 Evaluating the impacts of pre-existing NAb and ADA in rhesus monkeys on the efficacy of subretinal injection of KH631

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Purpose: Currently, AAV represents the most common vector for the delivery of *in vivo* gene therapy. However, the presence of pre-existing anti-AAV neutralising antibodies (NAb) may impede the ability of AAV to transduce target tissues, potentially leading to unfavorable results. Furthermore, the formation of anti-drug antibodies (ADA) will result in the removal of the therapeutic proteins expressed by AAV, which will consequently impact the final therapeutic outcome. The purpose of the present study was to evaluate the impact of pre-existing NAb and ADA on the therapeutic efficacy of subretinal injection of KH631, a clinical-stage anti-VEGF AAV gene therapy product.

Methods: On day 1, a dosage of between 3E9 and 5E11 vg/eye of KH631 was administered subretinally to rhesus monkeys (26 eyes in total). Serum were collected on days 0 and 27 and analysed for the presence of anti-AAV NAb and ADA. On day 28, a laser photocoagulation procedure was conducted to create a model of laser-induced choroidal neovascularisation (CNV). Subsequently, on day 42 and 56, the percentage of grade-4 lesions was then determined by means of fluorescein fundus angiography (FFA).

Results: On day 0, three rhesus monkeys were identified as exhibiting pre-existing anti-AAV NAbs. On day 27, five rhesus monkeys were found to be ADA positive, one day prior to the laser photocoagulation procedure. Nevertheless, 100% suppression of the CNV grade-4 lesion was observed in these rhesus monkeys on day 42 and 56.

Conclusions: As with other gene therapy products administered via subretinal injection, there was no negative effect of pre-existing anti-AAV antibodies on the therapeutic efficacy of KH631. Moreover, the presence of ADA did not impede the efficacy of KH631 as observed in the monkey CNV model.

946 Prevalence of Neutralizing Antibodies Against Adeno-Associated Virus (AAV) 8 and 9 in a Specific Pathogen-Free (SPF) Colony of Cynomolgus Macaques

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Systemic administration of recombinant adeno-associated virus (AAV) vectors is the preferred delivery route to achieve broad vector distribution and therapeutic transgene expression in preclinical and clinical settings. However, pre-existing humoral immunity against wild type AAV negatively affects transduction by systemically administered recombinant AAV vectors, severely impacting treatment efficacy and durability. It is well established that there is a strong negative correlation between the levels of pre-existing anti-AAV neutralizing antibodies (NAb) and AAV

gene therapy efficacy. Screening of NAb titers in preclinical animal models prior to vector dosing is therefore paramount to ensure seronegativity and enable a maximal response to the administered therapy. We screened 132 cynomolgus macaques (*Macaca fascicularis*) from the Bioculture Group®'s 100% specific pathogen-free (SPF) colony in Mauritius to determine pre-existing NAb titers against AAV serotypes 8 and 9. Anti-AAV8 and AAV9 NAb responses were analyzed in the sera using a cell-based assay, with serum dilutions ranging from 1:5 to 1:20, according to standard operating procedures. Our results demonstrate that 45% and 56% of the SPF cynomolgus macaques presented with a titer of <1:5 for both AAV8 and AAV9, respectively, and were seronegative. Forty percent of the animals presented with a low seropositivity titer (1:5 - 1:10) against AAV8, and 11% against AAV9. Only 14% of the animals evaluated presented with a seropositivity rate of ≥1:20 against AAV8, and 33% against AAV9. In conclusion, about 50% of the cynomolgus macaques screened were suitable for preclinical studies involving systemically administered AAV8 and AAV9 serotypes.

947 Integrating Raman Spectroscopy and Genome-Scale Modeling to Enhance AAV Upstream Manufacturing

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Viralgen is a Contract Development and Manufacturing Organization (CDMO) focused on the production of adeno-associated viruses (AAVs). We utilize an innovative and standardized manufacturing platform based on Pro10™ cells, which allows the production of diverse AAVs and the capability to scale up to 2000L bioreactors. This platform is structured into two main phases: upstream (USP) and downstream (DSP). Within the final stage of USP, conducted in a bioreactor, two critical processes take place: cell expansion, which ensures a sufficient cell population, and subsequent virus production, initiated by the transfection of the expanded cells.

In both final stages of USP (cell expansion and viral production), cells require precursors (such as amino acids) and energy (ATPs), generated as outputs from metabolic pathways after metabolizing nutrients from the culture medium. Therefore, the cell's performance in generating biomass and subsequently AAVs should correlate with the levels of precursors and energy produced as a result of cellular metabolism.

Although the manufacturing process is based on a standardized platform, the output exhibits variability associated with several factors, including the product itself (which may vary according to client specifications), measurement inaccuracies, and the degrees of freedom of the Critical Process Parameters (CPPs) within pre-established ranges. Among these CPPs are Viable Cell Density (VCD) and metabolites, which are measured off-line every 24 hours. The consumption of metabolites could serve as a basis for estimating the generation of necessary precursors and energy, correlating them to understand the activities related to cell expansion and AAV production.

However, two main barriers exist to achieve this correlation:

1. A mathematical model is required to simulate cellular metabolism and estimate precursor and ATP levels based on consumption ratios. Such models will not only estimate cell productivity but also help identify active subsystems and metabolic bottlenecks, potentially guiding feeding strategies to alleviate these constraints or inefficiencies.

2. The off-line measurements taken every 24 hours lack the necessary frequency to accurately calibrate and simulate a metabolic model.

To address these barriers, Viralgen has implemented Raman spectroscopy in 3L development bioreactors, generating a chemometric model that estimates the concentration of five essential metabolites (glucose, lactate, glutamine, glutamate, and ammonium) every 30 minutes from the acquired spectra. Additionally, a preliminary version of a

genome-scale metabolic model (GEM) has been developed and simulated using consumption ratios quantified by Raman spectroscopy.

Currently, efforts are focused on calibrating the metabolic model to reduce prediction errors. This involves calibrating the model with flow data from four of the five essential metabolites measured by Raman (leaving lactate free) and with online VCD measurements captured by an ABER capacitance probe. The lactate estimation is compared against actual measurements to assess predictive capabilities.

At present, the prediction error for lactate is approximately 40%, indicating excessive degrees of freedom in the model. Future steps will include incorporating transcriptomics to calibrate reactions governed by genomic regulation and continuing to refine the model with new experimental flow data measured by Raman in upcoming experiments.

948 Improving VP1 Ratios Impact on CQAs in rh10 AAV Manufactured Through Sf9 Platform.

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Adeno-associated virus (AAV) capsids can support successful gene therapies, but the impact of VP ratios on product CQAs is still poorly understood. We optimized our Sf9-baculovirus manufacturing process for 3 AAV programs and performed analytical characterization. Vector genome titers were obtained by ddPCR, while capsid purity and VP ratios were analyzed by CE-SDS. Moreover, gene of interest (GOI) potency was evaluated at the mRNA or protein level, depending on the program. Our data demonstrates a linear correlation between VP1 content and GOI potency at a protein level from rh10 AAVs produced by a baculovirus process. The increase in VP1 ratios resulted in higher genome titer and potency expression. Comparability experiments suggest that, after manufacturing process optimization, Sf9 AAVs and HEK293T AAVs are not statistically different in terms of genome titer and potency. The optimization of Lexeo's Sf9-baculovirus process demonstrates that knowledge of the relationship between VP ratios impact on CQAs, improving VP1 amount in the capsids, capsid purity and potency. Moreover, the current baculovirus manufacturing platform at Lexeo yields AAVs that display similar purity and potency profiles as the widely available HEK-based platform. In addition, the ease of production level scaling and the favorable cost-of-goods underscores the many advantages baculovirus platform can offer to address the unmet needs for many rare disease indications.

949 msDNATM: A Superior DNA Vector For Improved rAAV Manufacturing

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Background: Recombinant adeno-associated viruses (rAAV) are essential and important tools for the treatment of genetic disease. However, the manufacturing of rAAV vectors remains challenging, particularly due to the inclusion of unwanted sequences in the packaging process. These contaminants—such as plasmid backbone or RepCap sequences—result in suboptimal potency, increased immune responses, and difficulties in purification. To address these challenges, we propose the use of a next-generation miniDNA vector, ministring DNA (msDNA™), as a superior alternative to conventional plasmid DNA (pDNA). msDNA™ is a linear, covalently closed, double-stranded DNA vector that is devoid of bacterial sequences, such as antibiotic resistance genes and origins of replication, thereby reducing the risk of erroneous packaging.

Methods: rAAV vectors were produced using a triple transfection approach with either isogenic msDNA™ or plasmid-based vectors for rAAV2 and rAAV9 serotypes. Transfections were performed in HEK293 cells, followed by crude collection at day 2, benzonase treatment of cell lysates, and purification by affinity capture and ion exchange chromatography. Viral genome titers (qPCR), capsid protein expression (ELISA), and in vitro potency (GFP expression via flow cytometry) were assessed. A Design of Experiment (DOE) approach was used to optimize transfection parameters, including DNA mass, reagent type, reagent ratio, and vector molar ratios. Crude and purified rAAVs were analyzed using qPCR and next-generation sequencing (NGS) to detect bacterial sequence mispackaging. Purified vectors were evaluated for in vivo bioactivity in mice via RT-PCR and western blotting.

Results: Optimization revealed that msDNA™ required unique transfection conditions for maximum yield. Using JetOptimus as the transfection reagent led to a 5-fold increase in crude viral genome titer for msDNA™-derived rAAV and a 2-fold increase for pDNA-derived vectors compared to Fectovir-AAV. Adjusting the molar ratio of msDNA-RepCap relative to cis and helper vectors further enhanced crude viral genome titers by 2X, an effect not observed with pDNA. In vitro potency of crude msDNA™-derived vectors was comparable to, or better than, crude pDNA-derived vectors, even in cases where viral genome titers were lower. Alignment of Illumina NGS reads to pDNA backbone collected from of purified rAAV2 vector genomes, demonstrated a dramatic reduction (10^4-fold) in bacterial sequence mispackaging for msDNA™-derived rAAVs compared to pDNA-derived vectors. However, qPCR of crude rAAV vectors revealed residual mispackaging of RepCap sequences in both msDNA™ and pDNA-derived vectors. In vivo studies confirmed equivalent or moderately higher potency of msDNA™-derived rAAVs in liver, central nervous system.

Conclusion: msDNA™ represents a significant advancement in rAAV manufacturing, offering a bacterial-sequence-free DNA vector platform that enhances vector purity and potency. By reducing mispackaging of bacterial sequences, msDNA™ improves the quality of rAAV products, reducing the risk of immune activation and enhancing therapeutic potential. Further optimization, such as increasing RepCap size or modifying the p5 promoter, may further minimize RepCap mispackaging and improve final product purity.

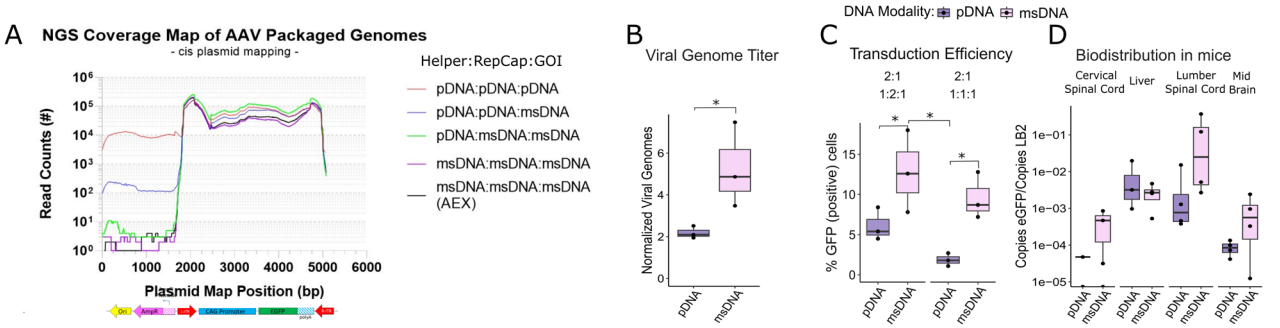

Figure 1: msDNA™-Derived rAAV Vectors Exhibit Negligible Backbone Sequence Packaging and Comparable or Enhanced Bioactivity (A) Illumina read counts aligned to the Cis- Gene of interest plasmid reveal a negligible signal for backbone sequences when msDNA™ vectors progressively replace traditional plasmid (pDNA) vectors in triple transfection. (B) Relative viral genome titer in crude preps of optimized conditions was higher for msDNA-derived rAAV2 vectors. (C) in vitro potency was greater in msDNA™-derived rAAV2 preps. (D) Relative mRNA copy number in liver and CNS tissues of mice treated with msDNA™- or pDNA-derived rAAV2 via intrathecal (IT) injection shows comparable copy numbers in the liver and moderately higher levels in CNS tissue with msDNA-derived AAV2 and AAV9 vectors.

950 Plasmid Engineering and Process Development to Improve Self-Complementary AAV Productivity

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Self-complementary AAV (scAAV) vectors were developed by mutating one of the ITRs to form double-stranded genome. Unlike traditional single-stranded AAVs (ssAAV), scAAVs can enhance gene expression efficiency by

bypassing the need for second-strand synthesis. Therefore, it has been widely used in clinical and commercial stages. In this study, we first presented design improvements in pHelper and pRepCap plasmids that can increase scAAV titers. We also presented a case study for a client-specific Gene of Interest (GOIs), where we apply our Lonza plasmid system. We further improved the scAAV productivity more than 7 fold at the upstream production stage by significantly decreasing transgene plasmid. In summary, we utilized both plasmid engineering and process development strategy to improve self-complementary AAV productivity with clinically relevant GOI.

951 Investigation of the Variations in Viral Protein 2 Stoichiometric Ratio and Deamidation of Viral Protein 1 and Their Impacts on Transduction Efficiency of Adeno-Associated Virus 8 Vectors

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Corresponding Author

In the upstream process of adeno-associated virus (AAV) vector manufacturing, HEK293 cells, HEK293 cell-derived cells, such as adherent HEK293T cells or suspension HEK293 cells, are employed in the production system via the transient transfection of plasmids. To mitigate the potential risk, HEK293 cells without the T-antigen protein and/or T antigen-encoding nucleotide sequence are preferred for the AAV vector production. However, limited information is available on the variation of quality attributes (QAs), during manufacturing using the HEK293 suspension cell line system, which is suitable for small- to large-scale production using bioreactors.

Although several QAs of AAV vectors that were potential CQAs have been reported, information is limited on the variations in QAs of the AAV vector product under similar production and purification procedures and the corresponding impacts on the transduction efficiency of the produced AAV vector. To better understand these relationships, we manufactured 10 AAV vector products using the HEK293 suspension cell line system under the conditions, with the only differences being the culture volume and container type, and examined 14 QAs, including particle properties, particle size distribution, viral protein properties, impurities, and transduction efficiency.

Obvious variations across products were observed in several QA parameters. We investigated the relationship between QAs with significant variation and the transduction efficiency using the following correlation analysis. To extract the QA parameters that can be used for the correlation analysis, one-way analysis of variance (ANOVA) was used to evaluate significant differences in the mean values of 18 QA parameters obtained from 14 QAs across the 10 products. For the 14 QA parameters that showed a significant difference in mean values, the correlation matrix was used to investigate whether there was a correlation between QA parameters. Correlation analysis of the QAs revealed the attributes that could affect the transduction efficiency. Notably, the VP2 stoichiometric ratio was positively correlated with the transduction efficiency. The deamidation ratios of N57 and N94 in VP1 had a negative tendency to the transduction efficiency. Consistently, the accelerated stability testing showed a negative correlation of the time-dependent increase in the deamidation ratios of the two asparagine residues (N57 and N94) with the transduction efficiency. Our study clearly demonstrated that the VP2 stoichiometric ratio and deamidation ratios of N57 and N94 could be pCQAs of AAV vector products in terms of gene transduction.

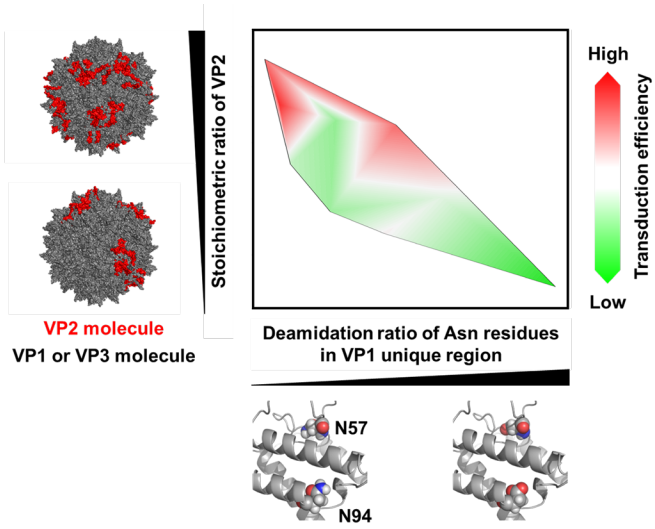

Figure. Schematic representation of the relationship between the stoichiometric ratio of VP2, the deamidation ratio of Asn residues in VP1, and the transduction efficiency.

952 High Productivity HEK293 AAV Production Platform Enabled by Novel Transfection Reagents and Proprietary Plasmid Expression Systems

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Adeno-associated virus (AAV) continues to be a central modality in vehicles for gene therapy, having a known safety profile, variation in tissue tropism and optionality to dose by local or systemic injections depending on serotype. While many gene therapies have proven to be safe and effective, the primary bottleneck to move the industry to the next phase of maturity is high manufacturing costs. Tenaya conducted studies intended to increase productivity, while preserving safety, purity, and potency of AAV gene therapy drug product. Tenaya HEK293 producer Cell lines utilized in these studies demonstrated comparable productivity to a benchmark commercial cell line and development work with transfection and expression systems combined has notably improved titer up to 6x as well as upstream % full capsid up to 3x controls. These improvements to AAV productivity and quality were achieved through studies testing transfection protocols and reagents, including in-house developed novel transfection complexes, as well as propriety transient expression systems, including triple, dual, and single plasmid formats.

Tenaya demonstrated improvement in productivity with small scale production models which report comparable purity, *in vitro* and *in vivo* protein expression. By using high productivity HEK293 producer cell lines, scaling productivity to stirred tank reactors and *in vivo* data reporting comparable protein expression, Tenaya demonstrated its HEK293 manufacturing process has the potential to support the development of cost-effective AAV gene therapies.

953 A Scalable, DoE-based Approach to Improving Plasmid Ratios for AAV Transfection

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Adeno-associated viruses (AAVs) are a leading modality for gene therapy owing to their safety and broad tropism. As the use of these applicates increase, optimal process development is critical to improve production titers and critical quality attributes. Here, we demonstrate the use of a design of experiment (DoE) approach to investigate the effect of plasmid DNA ratio on capsid and genomic titer using the AAV8 and AAV9 serotypes using an Ambr15 system. For both serotypes, jmp software was used to design a mixture-design experiment to investigate the plasmid ratio used in a triple transfection for AAV production on lysate capsid and genome titer. The results were used to generate a serotype-specific model that uses plasmid ratio to predict titer production. The plasmid ratio that optimizes production was then tested at the bench-scale using the Ambr250 and Mobius 3 L bioreactors.

The DoE analysis led to the generation of predictive models maximizing the desired outcome of increased titer production. A statistical analysis of the actual and predicted log capsid titer returned root mean square error (RMSE) values of 0.2188 and 0.2388 with P values < 0.0001 for AAV8 and AVV9 respectively. Similarly, the genomic titer RSME values for AAV8 and AAV9 with P<0.0001 were 0.4618 and 0.505 respectively. For AAV8, the model-generated pDNA ratio predicts a production of 3.74E+12 capsids/mL and 1.18E+12 genome copies/mL (GC/mL). Similarly, for AAV9, the generated ratio predicts production of 9.30E+11 capsids/mL. and 5.27E+11 GC/mL. When applied to bench scale processes, the plasmid ratios were scalable. Average AAV8 production in the Ambr250 system was 3.74E+12 capsids/mL and 1.18E+12 GC/mL and average production in the 3 L Mobius was 4.35E+12 capsids/mL and 1.38E+12 GC/mL. For AAV9, average production in the Ambr250 was 9.30E+11 capsids/mL and 5.27E+11 GC/mL and average production at the 3 L scale was 1.637E+12 capsids/mL and 5.58E+11 GC/mL.

The DoE approach utilized here resulted in optimal transfection pDNA ratios that maximized AAV8 and AAV9 capsid and genome titer production. The generated models were able to predict the viral production at two different scale bench scale bioreactor systems. Therefore, the systematic use of DoE modeling to assess the pDNA mixture ratio in microbioreactors was able to maximize titer production at larger scale.

954 High-Yield, Pan-Serotype Plasmid System for Manufacturing Adeno-Associated Virus Gene Therapies: Cost and Efficiency Benefits for R&D and Commercial Processes

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A major challenge in development of adeno-associated virus (AAV)-based gene therapies is the low manufacturing yield and poor packaging efficiency of AAV capsids. While the enhanced tropism of novel capsids has reduced the total required dose, capsid modifications can have negative effects on manufacturability. Therefore, we design and screen for high yielding capsids during the capsid discovery phase. Even after discovering novel capsids with good manufacturability, a high-yield, capsid-agnostic manufacturing method could drastically decrease the cost and difficulty of screening and production of these capsids and unlock low-yielding capsids, such as AAV2, as viable delivery vehicles.

Affinia therapeutics has developed a plasmid system which led to an increased yield during initial screens for all tested wild-type and novel capsids. Directed optimizations can increase vector genome yield more than 10-fold relative to traditional plasmid systems. Packaging efficiency increases of 4-fold are commonly achieved in poorly packaging serotypes. This system has shown consistent effectiveness across all tested wildtype and novel capsids, all evaluated commercially available helper plasmids and all tested transfection reagents.

These manufacturability enhancements decrease total upstream batch size requirements and increase capacity on the downstream unit operations by minimizing the presence of empty capsids which consume purification yield to remove. In 2024, Affinia Therapeutics implemented this system, achieving transformative results in both capsid discovery and drug development manufacturing. In R&D, the Affinia Therapeutics Vector Core average clonal production titer increased 740% in 2024 when compared to 2023 clonal productions, decreasing average batch size

and increasing production throughput, and thereby significantly reducing cost of goods per unit. For pipeline products with more development focus, even higher yields were achieved; an intensified production process for one of Affinia's lead novel capsid has achieved over 5e15 vg/L at bioreactor harvest which should lead to lower cost of goods manufactured for clinical products.

In summary, this innovative plasmid system addresses critical manufacturing challenges, enabling more efficient production of AAV gene therapies and unlocking the potential of diverse capsid platforms for development, clinical, and commercial use.

955 Overcoming Challenges in AAV Plasmid Preparation: Advanced Solutions for Superior ITR Integrity and High-Yield Production

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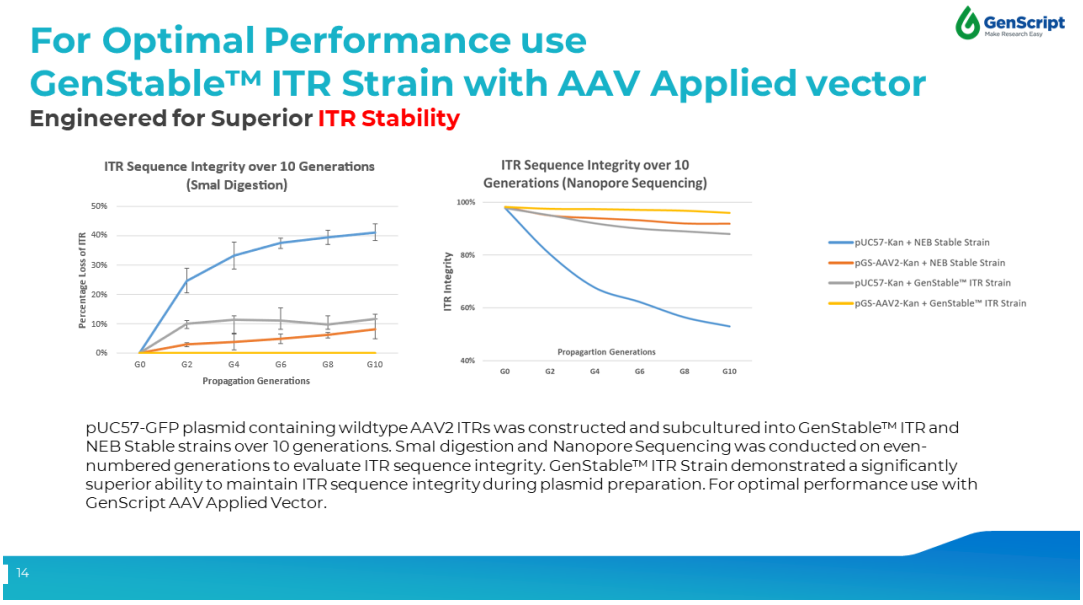
The field of gene and cell therapy, particularly adeno-associated virus (AAV)-based therapies, is revolutionizing the treatment of genetic disorders, cancers, and rare conditions. AAV vectors are highly valued for their ability to deliver genetic material with high specificity and low immunogenicity, making them a cornerstone in therapeutic applications. However, the preparation of high-quality plasmids for AAV production presents unique challenges, particularly in maintaining the integrity of AAV inverted terminal repeat (ITR) sequences due to its repetitive and high GC-content nature.

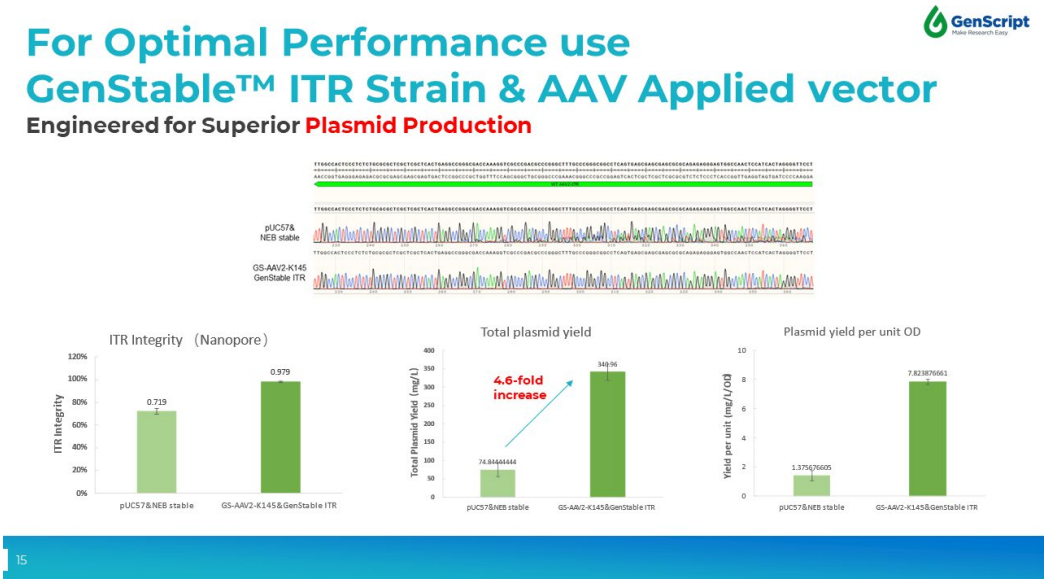
ITRs are essential components of AAV plasmids, playing a critical role in viral genome replication, packaging, and integration. Regulatory agencies such as the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) require verification of intact ITR sequences for clinical applications, as mutations or truncations can compromise vector performance and therapeutic efficacy.

To overcome these challenges, GenScript has developed a comprehensive suite of plasmid solutions specifically designed for AAV plasmid propagation. Our proprietary GenStable™ ITR strain and AAV applied vectors with built-in ITR sequences are engineered to preserve ITR integrity, significantly enhancing sequence stability and plasmid yield. Together, these solutions achieve an impressive >95% success rate in generating ITR intact clones through 10 generations, representing a 100% improvement over conventional tools, while boosting plasmid yields by more than fourfold.

In addition, GenScript offers advanced quality control technologies to ensure precise ITR sequence analysis, addressing the limitations of traditional methods such as standard Sanger sequencing and enzyme digestion, which often fail to accurately evaluate ITR integrity. Our proprietary ITR Sanger sequencing technology provides full reads of the ITR regions with clean and reliable peaks, ensuring comprehensive verification of sequence integrity. Complementing this, ITR Nanopore sequencing delivers detailed insights into sequence purity and population heterogeneity, offering a deeper understanding of plasmid quality.

By integrating cutting-edge plasmid solutions with sequencing technologies, GenScript enables researchers to overcome challenges in AAV plasmid preparation. Our solutions streamline workflows, reduce costs, and facilitate the production of high-quality AAV vectors, accelerating the development of safe and effective gene therapies.





956 Development and Optimization of a Transient Two-Plasmid AAV Manufacturing Platform

Anna Prendeville[1], Omar El-Ghouch[2], Kate Dray[3], Jeffrey McMahan[4], Will Johnson[5], Liliana Wroblewska[1], Ta-Chun Hang[1]
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INTRODUCTION: Adeno-associated viral (AAV) vectors are amongst the most widely used viral manufacturing production systems for gene therapies. The current gold standard for transient viral vector manufacturing utilizes a 3-plasmid system. However, this manufacturing process approach has significant limitations, including suboptimal genomic titer and low packaging efficiency. To address these limitations, we combined an optimized refactored 2-plasmid transient process with a proprietary, monoclonal HEK293 cell line possessing properties of minimal aggregation and nutrient consumption. Our 2-plasmid system enables us to conduct design space characterization of

input factors (i.e., plasmid ratio studies) with greater experimental reproducibility compared with the standard 3-plasmid system.

METHODS: The refactored 2-plasmid system consists of the following: the first plasmid contains Rep, Cap, and the gene of interest (GOI). The second plasmid contains the Helper genes along with a genetic booster to improve productivity. We applied a combination of machine learning (ML) and upstream process development approaches to identify optimal transfection and process conditions for 5 different AAV serotypes (AAV2, AAV5, AAV6, AAV8, AAV9) utilizing GFP as the GOI. Bayesian Optimization (BO), Design of Experiments (DOE), and subject matter expert (SME)-driven approaches were assessed in parallel to iteratively evaluate regions of the design space over five experiments (Figure 1, Figure 2A). Experiments were conducted at different scales (e.g., shake flasks, ambr15, and ambr250) to evaluate the impact of transfection conditions (e.g., plasmid ratios, transfection reagent, total DNA) and process conditions (e.g., agitation rates, dissolved oxygen, inoculation densities) on titer (vg/mL) and full capsids (%). After each experimental iteration (Figure 2A), Bayesian Optimization was trained on available data and mapped to a multi-objective acquisition function balancing design space exploration and exploitation.

CONCLUSION: Using our integrated computational and experimental methodology, we identified key levers within the upstream process development area that maximizes key manufacturing metrics of genomic titer (1e+12 vg/mL) and percent full capsid (35%) (Figure 2B). These results demonstrate the utility of BO for PD optimization. Furthermore, we estimate that we were able to reduce experiments by 50% and accelerate timelines to achieve a target spec. We believe our approach can be applied to optimize AAV manufacturing processes and be a tool for SMEs to plan and integrate experimental results. Future work includes using the BO framework to continue optimizing the AAV Edge system performance across an expanded set of GOIs.

Figure 1. Workflow diagram of how BO, DOE, and SME work together.

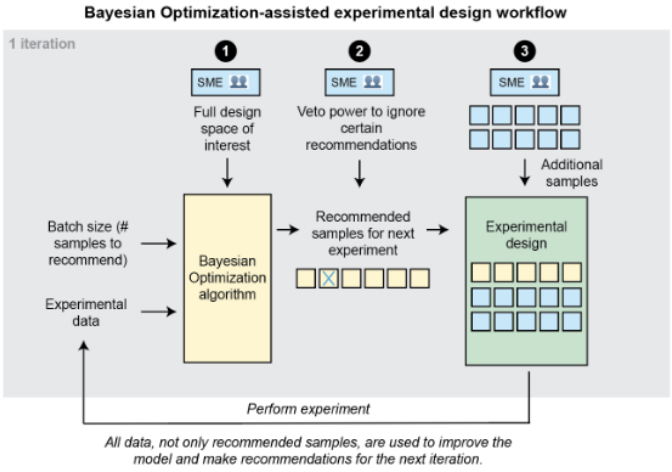
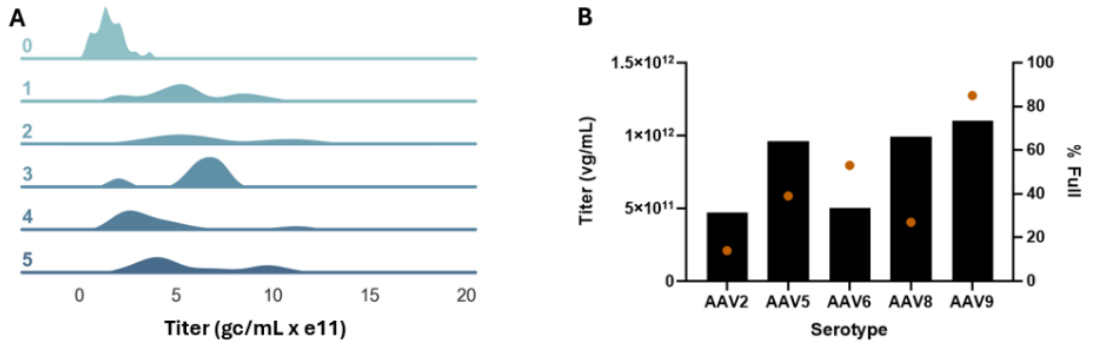


Figure 2. Performance across iterative study designs. Pareto front as a ridge plot visualization of genomic titer demonstrated progressive improvement in identification of design space parameters using BO, DOE, and SME (A). Titer and %full were achieved for all serotypes (B).



957 Streamlining Recombinant AAV Production: A Single Plasmid Approach for Higher Yields and Reduced Costs

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High manufacturing costs for complex biologics, driven by low product yields, excessive impurities, and scaling issues, currently hinder our ability to meet market demands. To address these challenges, significant resources are being invested into innovative strategies aimed at maximizing yields per cell and enhancing the production of genome-containing capsids. One critical process in manufacturing recombinant adeno-associated virus (rAAV) involves a transfection step that often requires three or four plasmids to be manufactured independently. All these plasmids must enter not only the cell cytoplasm but also the nucleus to enable successful production. Typically, rAAV is produced through a triple transient transfection method, which necessitates the purification of the three plasmids, optimization of their ratios, and complexation of the total plasmid DNA with a transfection reagent. In this context, we demonstrate that combining the adeno helper genes, the AAV replication (rep) and capsid (cap) genes, along with a gene of interest into a single plasmid offers a practical alternative to the conventional triple transfection process and presents a highly advantageous solution to produce rAAV. Around 1E6 cells/mL of HEK293SF-3F6 suspension cells cultured in serum-free conditions, in 20 mL working volume in shake flasks were transfected with various concentrations of plasmid DNA. Triple transient transfection was performed using pAdDeltaF6 helper plasmid, pRepCap6, and pAAV-CAG-GFP (Addgene) at equal molar plasmid ratios. PEI-25K (Polysciences) was used as the transfection reagent at a ratio of 2:1 PEI: DNA. Productions with the conventional triple transfection and the new single transfection methods were assessed at 48 and 70 hours post-transfection (hpt). Clarified crude lysate was used to determine viral genome content (VG/mL) by droplet digital PCR. The functional viral particle quantification (TU/mL) was determined using a cell-based assay where GFP-positive cells are assessed via flow cytometry and the transducing units are calculated. Production of rAAV was carried out using the single plasmid at various DNA concentrations and compared to triple transient transfection. Plasmid DNA concentrations of 1 µg/mL, 0.75 µg/mL, and 0.5 µg/mL were tested with the single plasmid and the control triple transient transfection was performed using 1 µg/mL. A higher viral genome titer was obtained using the single plasmid compared to the control, as well as a higher cell viability at the time of harvest. The single transfection condition using 0.75 µg of plasmid DNA/mL demonstrated an increase of 2 times in viral genome titer compared to triple transfection, for production harvested at 48 hpt and 5 times when harvested at 70 hpt. Also, the production of functional rAAV particles was 2.3 times higher (48 hpt) and 4.6 times higher (70 hpt) using 0.75 µg/mL of the single plasmid than the control triple transfection. We demonstrated significant increases in the viral genome titer of AAV6-GFP and in the functional titer when produced using the single plasmid method. Additionally, we identified a 25% to 50% reduction in the total plasmid DNA required, which also led to a corresponding 25% to 50% decrease in the amount of

transfection reagent needed. Furthermore, since the single plasmid method requires only one purification step, it substantially lowers the cost of transfection, making it more efficient for the successful production of rAAV.

958 Assessment of Novel RepCap Vectors for Improved AAV Titers along with Superior Product Characteristics

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Introduction: Recombinant adeno-associated viral vectors (rAAV) are an essential tool in AAV-mediated gene therapy. A continuous challenge in this field is to produce a high-titer, high-potency vector to achieve a better therapeutic effect. Some key aspects of vector production include improved plasmid design, which involves optimizing and configuring AAV genes, promoters, and regulatory elements. The recently discovered membrane-associated accessory protein (MAAP), encoded by an alternative open reading frame within the AAV capsid VP1 protein, has been shown to influence AAV packaging and production. MAAP features an N-terminal basic and hydrophobic region, a central threonine/serine (T/S)-rich domain, and a C-terminal hydrophobic and basic region. This study evaluates over 15 novel RepCap plasmids with single or combinatorial mutations in MAAP within the VP1 ORF protein, termed as RepCapExcel vectors. This evaluation was carried out across four AAV serotypes: AAV5, AAV6, AAV8, and AAVrh10. The goal of this study was to evaluate the impact of the Catalent RepCapExcel vectors on AAV productivity and characteristics.

Methods: The RepCapExcel plasmids were assessed using triple transfection in Catalent's baseline UpTempo^{SM} process in shake-flasks using the Catalent clonal HEK293 suspension cell line, along with Catalent's® Off-the-shelf (OTS) helper plasmid and a model gene-of-interest plasmid (pGalacorin™). AAV particles produced with these RepCapExcel plasmids, were compared to those generated with Catalent OTS RepCap plasmids. Viral genome titer and particle titers were quantified by droplet digital PCR and particle ELISA. A single step affinity purification was completed prior to evaluation of AAV product characteristics.

Results: Several of the novel RepCapExcels demonstrated a 3- to 7-fold increase in AAV vector genome and particle titers across multiple serotypes. Importantly, the several-fold increase in titers was achieved without increasing residual hcDNA (host cell DNA) contaminant or compromising the full capsid percentage. The purity of the viral capsid proteins (VP1, VP2, and VP3) showed consistent ratios (VP1:VP2: VP3) between AAV particles produced with the RepCapExcel plasmids, when compared with the control OTS RepCap plasmids as determined by CE-SDS (Capillary Electrophoresis-SDS). To further ensure product identity, capsid proteins and their post-translational modifications (PTMs) were characterized via intact mass LC-MS (Liquid Chromatography-Mass Spectrometry) and peptide mapping LC-MS analysis respectively. Intact mass analysis confirmed alignment between the observed molecular weights and theoretical predictions for all serotypes, with no significant differences between AAV particles produced with the novel RepCapExcels and OTS control RepCap plasmids. AAV PTMs, including acetylation, deamidation, oxidation, and phosphorylation, were consistent with those obtained using the Catalent OTS RepCap plasmids.

Conclusions: Preliminary findings suggest that novel RepCapExcel vectors are promising candidates for achieving high AAV titers along with superior quality attributes during AAV production for gene therapy applications.

959 One Stone, Two Birds: Engineering Rep-Cap Plasmids to Enhance AAV Manufacturability and Potency

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Recombinant adeno-associated virus (rAAV) vectors are pivotal in gene therapy due to their proven safety and efficacy for therapeutic gene delivery. However, low production yields and vector heterogeneity remain significant barriers to scalable, high-quality manufacturing. In this study, we introduce a novel strategy to address these challenges through the engineering of Rep-Cap plasmids, a critical component of the triple-plasmid transfection system.

Using rational design and synthetic biology approaches, we optimized Rep-Cap plasmid sequences by incorporating regulatory elements for synchronized gene expression, fine-tuning the VP1/VP2/VP3 protein ratio, optimizing codon usage, and eliminating unstable or recombinogenic regions. Engineered plasmid variants were assessed in proprietary and commercial suspension HEK293 cell platforms across multiple AAV serotypes, including AAV2, AAV6, and AAV8.

Our optimized Rep-Cap plasmids yielded substantial improvements in AAV production and quality. Specifically, vector yields increased by 2- to 10-fold, exceeding 2E15 VG/L, while the ratio of full-to-empty capsids improved by 50%, indicating enhanced vector integrity. Transcriptomic analyses revealed balanced and sustained expression of Rep and Cap proteins, reduced cellular stress, and improved packaging efficiency. Comprehensive characterization, including TEM, AUC, CE, and NGS assays, confirmed the quality of the AAV vectors produced using these plasmids.

Beyond improving production, the engineered Rep-Cap plasmids demonstrated scalability across serotypes in suspension-based systems and retained robust functional performance. In vitro and in vivo functional assays validated the enhanced bioactivity and safety of the resulting vectors, highlighting the translational potential of this approach.

Our findings underscore the dual benefits of engineering Rep-Cap plasmids: enhancing AAV manufacturability and potency. This strategy offers a scalable, versatile solution to current manufacturing challenges, meeting the growing demand for high-quality AAV vectors in clinical and commercial applications.

960 Novel RepCap Vectors with a Mutated Membrane-Associated Accessory Protein for Recombinant AAV Titer Improvement

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Introduction: Recombinant adeno-associated viral (rAAV) vectors have emerged as one of the powerful tools in the field of human gene therapy based on their excellent safety profile and ability to achieve long-term efficacy. The single-stranded AAV genome contains genes such as Rep (Replication), Cap (Capsid), and AAP (Assembly), flanked by inverted terminal repeats (ITRs) required for transgene packaging. The Cap gene encodes for the viral capsid proteins (VP1, VP2 and VP3) actively involved in cell binding and internalization. A 119 amino acid viral protein named membrane-associated accessory protein (MAAP) was discovered in 2019 as the expression product of an alternative open reading frame that utilizes an abnormal start codon with Cytosine instead of Adenine in CTG (Cytosine, Thymine Guanine). MAAP functions as an egress factor limiting AAV production through competitive exclusion. In addition, MAAP facilitates AAV replication and has a role in AAV infection. MAAPs were found in all currently reported natural serotypes. The structure of MAAP is highly conserved across different serotypes. This study aimed to investigate the role of the conserved protein MAAP in AAV production and assess the impact of targeted mutations to the protein on recombinant AAV titers.

Methods: AAV vector production involves transient co-transfection of human embryonic kidney (HEK) 293 cells with three plasmids: pHelper, pGOI, and pRepCap. The quality and titer of AAV product can be correlated with improved vector design. To improve AAV titer, we developed novel RepCap plasmids with MAAP substitution mutations termed as RepCapExcels. Changes included 15 unique mutations in serotype specific regions as well as conserved areas across serotypes. Specific changes included stop and start codon substitutions, amino acid

substitutions, and combinations thereof. The MAAP mutated RepCapExcel vectors were evaluated for the production of several AAV serotypes, including AAV5, AAV6, AAV8, and AAVrh10, and it was ensured that, while MAAP was mutated, the VP1 protein amino acids remained unchanged.

Results and Conclusions: Compared to the standard pRepCap with WT-MAAP (wildtype MAAP), most of the mutants tested contributed to an improved titer without significant increase in residual packaged hcDNA (host cell DNA) in all tested serotypes. Particularly, we identified an amino acid mutation variant RepCapExcel 5 and a substitution variant RepCapExcel 2 that played a critical role in MAAP function in all the serotypes tested, with up to 3-7-fold increase in rAAV production. Variant RepCapExcel 5 exhibited the highest vector genome titer increase (for example 7-fold increase for AAV5) as well as the highest AAV particle titer increase, compared to other tested mutants in various serotypes tested. These findings demonstrate a critical role of these RapCapExcel vectors in MAAP functionality and highlight potential for optimizing AAV production.

961 Enhancing AAV Production Efficiency through Plasmid Modification and Dual-Plasmid Systems

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Adeno-associated virus (AAV) vectors are a cornerstone of gene therapy, offering a safe and effective means to deliver therapeutic genes. However, high production costs and lengthy timelines hinder their widespread application. To address these challenges, PackGene has developed an optimized AAV production platform that significantly improves yield, purity, and cost-efficiency. Key innovations include the engineering of a high-efficiency PCS3.0 suspension cell line, the design of a novel high-yield rep-cap (RC) plasmid, and the development of a dual-plasmid production system.

The novel RC plasmid, incorporating a proprietary non-coding regulatory element, increased AAV yield by 3-8 times across multiple serotypes in both adherent and suspension cultures, while reducing plasmid DNA encapsulation to 0.1-0.2%. Additionally, PackGene's dual-plasmid system, which integrates RC and helper functions into a single plasmid (TP011), demonstrated superior AAV production efficiency compared to traditional triple-plasmid methods. The TP011 system achieved over 3x higher AAV9 titers and reduced residual host cell DNA (HCD) by more than 3x, while maintaining comparable plasmid DNA impurity levels.

Further optimization of transfection reagents and process parameters within PackGene's ℝ?-Alpha 293 High-yield AAV Production Platform ensured consistent and scalable production. Case studies confirmed the dual-plasmid system's ability to produce high-quality AAVs with low empty capsid ratios, making it a cost-effective solution for IND to BLA and commercial manufacturing.

These advancements collectively reduce production timelines and costs, paving the way for broader accessibility to AAV-based gene therapies. PackGene's platform represents a significant leap forward in AAV production technology, enabling the efficient and scalable manufacturing of gene therapies for treating genetic disorders.

962 rAAV Manufacturing Solutions: Strategic Designs of Engineered rAAV Two Plasmid Systems for Cost Effective Scaling and Product Safety

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Recombinant adeno-associated virus (rAAV) is becoming an increasingly common delivery system for a variety of gene therapy applications. Advances in rAAV engineering have led to the development of novel AAV capsids with significantly improved target tissue tropism, higher transduction efficiency, and lower immune toxicity, enabling safer, more effective gene therapies. More than ever, these newly engineered AAV capsids are being investigated as

vehicles to treat a variety of genetic diseases for which there are currently limited or no treatment options available. As the demand for treatments grows, innovative manufacturing solutions are essential to meet scaling needs, boost production, and reduce costs while maintaining efficacy and safety for patients. Currently, transient transfection of mammalian cells with three plasmids containing rAAV components is the most widely used method for manufacturing rAAV vectors. While effective, the three plasmid system has a high cost of raw materials, is complex to optimize, and may introduce variability between runs, making it less ideal for scalable manufacturing.

We initially developed a proof-of-concept two plasmid rAAV transfection system, which displayed improved productivity and packaging efficiency compared to our three plasmid system. Although the system met evaluation criteria, key AAV components appeared in a wild-type-like configuration (ITR-GOI-ITR-Rep-Cap), thereby increasing the risk of producing replication-competent AAV. To mitigate this risk, we designed, constructed, and evaluated various plasmid configurations that separate these components. Additionally, we isolated specific adenovirus helper open reading frames (ORFs) to reduce plasmid DNA size and enhance transfection efficiency. Leveraging an internally developed, high throughput functional screening method with 24-deep well suspension culture transfection, we evaluated these numerous sequence modifications and plasmid configurations to identify the most effective combinations. We simultaneously evaluated our two plasmid system using a diverse range of transgenes to gain further insights into its characteristics and functionality. Preliminary results from these studies demonstrate that the newly configured two plasmid system sustains productivity while minimizing risks of replication competent rAAV. We aim to take our two plasmid system to the next stage of development, which includes upstream optimization and scaling for production in stir-tank bioreactors using our suspension platform.

963 An Engineering Biology Design Approach to Improving Genome Upload into AAV Capsids

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Introduction: AAV capsids are understood to be filled with single-stranded DNA genome payloads via a series of molecular encounters between viral proteins within the host cells. The Rep52 and Rep40 proteins encounter and bind ATP molecules. ATP-bound Rep proteins then encounter and bind payloads. The Rep-ATP-DNA complex must then diffuse and collide with an empty capsid. Once everything is correctly positioned, the Rep proteins hydrolyse ATP molecules to power the mechanical process of threading the DNA into the capsid interior. This intricate dance of molecules finding each other through diffusion and maintaining specific binding interactions ensures that the viral genome is properly packaged. While the cellular environment helps bring these components together in a confined space, and specific binding interfaces ensure correct interactions, the process still fundamentally relies on molecules randomly encountering each other through diffusion until they find their correct partners. We sought to test the hypothesis that engineering the capsid Viral Protein 2 (VP2) with the ability to bind payload directly, without the need for diffusion/collision with or among Rep proteins, could increase the level of capsid filling with payload at the time of viral harvest.

Method: We co-transfected standard AAV production plasmids with a selection of novel plasmids encoding the central repeating domain (CRD) of the Transcription Activator-Like Effector (TALE) at the VP2 N-terminus with flexible linkers of varying lengths in a set of VP2-TALE fusion plasmids. Different numbers of TALE CRD target sequences were also inserted into various positions within the self-complementary payloads to facilitate binding to the TALE CRD at the VP2 N terminus and the payload during the rAAV2 assembly and packaging process. The rAAV2 variants were produced using combinations of VP2 fusion variant and payload variant constructs, supplemented with either wild-type Rep-Cap plasmids or VP2-silenced Rep-Cap plasmids. The variants were evaluated for viral genome titres (qPCR), capsid titres (ELISA), infectious titres (Flow cytometry), and full:empty capsid ratios inferred therefrom. Selected variants were characterised by TEM imaging.

Results and conclusions: We first demonstrated that the presence of TALE targets inserted at three different positions within the payload genome was compatible with infectious viral particle production, with no notable

reduction in titres. We used a novel, 4-plasmid transfection procedure consisting of standard Helper and Rep-Cap plasmids, a novel payload plasmid with three TALE target sequence inserts, and a CMV-driven fusion plasmid, encoding VP2-TALECRD. This novel method resulted in an 18% filling, compared to 5% filling with conventional AAV production, as determined by ELISA and qPCR. Viral particles from this novel method have comparable *in vitro* infectious titres and morphology comparable to particles produced using a conventional method. These observations are consistent with our hypothesis that the presence of VP2-TALECRD fusions within the viral particles are increasing the frequency of genome-capsid collision events per unit time. Future work, outside the scope of this current study, will directly characterise viral particles to confirm VP2-TALE fusion incorporation and CRD:DNA binding in this context.

964 Assessing the Impact of AAV Genome Size on Vector Productivity and Quality: A Comprehensive Analysis using Automated Small Scale AAV Production

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Recombinant Adeno-associated virus (AAV) has emerged as a leading vector in gene therapy, with several approved therapies and numerous clinical trials underway. Enhancing AAV productivity and product quality are critical for gene therapy advancements, as systemic treatments often require high doses (up to 1E14 vector genomes per kg of patient body weight), and the presence of empty capsids poses manufacturing, regulatory, and clinical challenges.

AAV is a small, non-enveloped, single-stranded DNA virus with a packaging capacity of approximately 4.7 kb. The impact of genome size on the critical quality attributes of recombinant AAV vectors remains unclear, with studies showing conflicting results. Some research suggests that extending the transgene length close to the natural virion packaging capacity improves quality attributes, while other studies indicate reduced productivity and capsid packaging efficiency with full-capacity vectors compared to smaller genomes. As the demand for AAV therapies grows, a deeper understanding of how cargo size affects the production and quality of recombinant AAV vectors is essential.

To address this, we automated AAV production using a liquid handler at a 96-well plate scale, facilitating consistent, high-throughput AAV production. We generated a panel of recombinant AAVs with various genes of interest (GOIs) for five different AAV serotypes, with cargo sizes ranging from 3 to 4.8 kb. Additionally, for one construct, we investigated the inclusion of a stuffer sequence to increase the cargo size to the wild-type virion capacity. These small-scale lysates were purified via automated bead-based purification and analyzed for viral genomes per mL, infectivity, percent full capsids, residual host cell DNA, and residual plasmid DNA.

This study illustrates the impact of genome size on AAV productivity and quality and highlights the potential of incorporating automation into the AAV production workflow. Automation enhances experimental design by improving throughput and reducing variability, enabling efficient data generation for screening at sample volumes sufficient for relevant analytical analyses.

965 Removal of Additional P5 and VA RNA II Elements from AAVone Enhances AAV Productivity, Increases Full Particle Ratio, and Reduces Impurities

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Adeno-associated viruses (AAV) are widely used as gene delivery vectors in clinical applications, with several AAV-based treatments already approved. However, high production costs and impurity-related challenges limit

broader use. Our single-plasmid AAV production system, AAVone, streamlines vector generation by integrating adenoviral helper genes (E2A, E4orf6, and VA RNA), AAV helper genes (Rep and Cap), and the AAV vector genome into a compact 13-kb plasmid, pAAVone. Compared to the traditional triple-plasmid system, AAVone achieves 2–4 times higher yields while reducing plasmid-related impurities. The original pAAVone plasmid (pAAVone1.0) features the following structure: F1 ori-KanR-Ori-(L)ITR-GOI-(R)ITR-VARNA-P5-Cap-Rep-P5-E4orf6-E2A, with an additional P5 promoter located between VA RNA II and Cap PolyA. We found that removing the additional P5 and VA RNA II elements in pAAVone1.0 to generate pAAVone1.1 significantly increased AAV2 productivity by 36–50% in HEK 293T and HEK 293 cells. Additionally, this modification improved the AAV2 full particle ratio (increasing from 28.7% to 34.4%), as determined by mass photometry analysis. Most importantly, pAAVone1.1 significantly reduced VA RNA-related impurities located outside R-ITR compared to pAAVone. Further evaluation of the effects of pAAVone1.1 on AAV productivity, full particle ratios, and impurities with AAV6, AAV8, and AAV9 is ongoing. Taken together, optimizing the AAVone design has the potential to further enhance AAV productivity, improve the full/empty capsid ratio, and reduce plasmid-related impurities. These advancements significantly improve overall vector quality, reinforcing AAVone1.1 as a promising platform for large-scale, GMP-compliant AAV production.

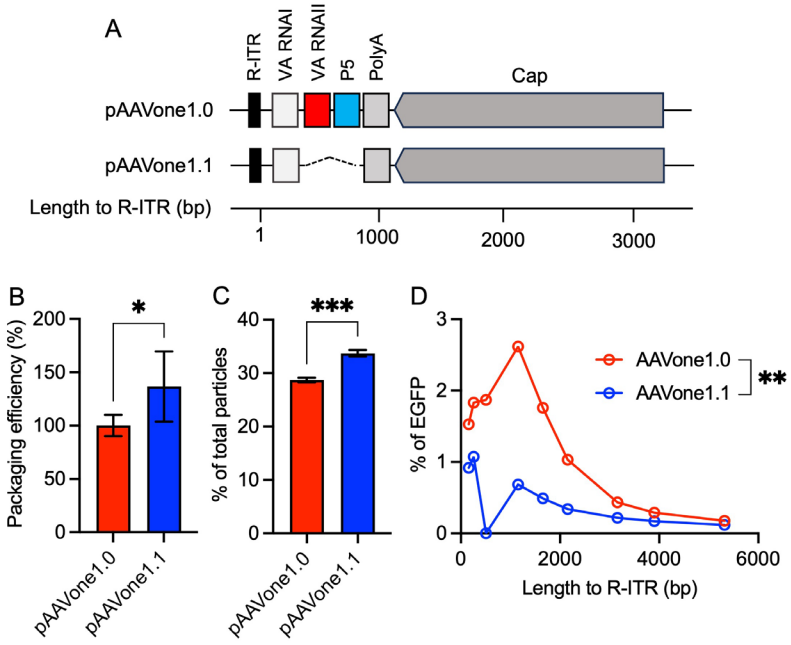

Fig. 1: Removal of the additional P5 and VA RNA II elements from pAAVone1.0 to generate pAAVone1.1 (A) improves AAV packaging efficiency (B), increases the full/empty capsid ratio (C), and reduces VA RNA-related impurities (D).

966 Application of DoE method to improve the rAAV Packaging Titer of Large GOIs

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Recombinant adeno-associated virus (rAAV) has been widely used as an effective vector in gene therapy. However, Due to the limited packaging capacity of rAAV (4.5 kb excluding ITRs), the viral titer drops significantly when the gene of interest (GOI) exceeds this size. Increasing the packaging titer of large GOIs remains a critical challenge in the field.

To improve rAAV packaging titer, we employed a systematic approach using design of experiment (DoE) method to optimize transfection conditions for a therapeutic cytosine base editor (CBE101) with 4.8 kb long. Previously, we conducted single factor experiments to screen suitable cell lines, total mass/proportion, and transfection reagent concentrations. Under optimal conditions, the packaging titer reached to 1E+11 vg/mL. FectoVIR®-AAV, a novel transfection reagent from Polyplus, has been reported to increase transfection efficiency of small GOI AAV vectors. Here, the DoE method was applied to investigate the optimal transfection conditions for CBE101. This DoE study contained two factors and their interaction effects: (1) DNA amount (micrograms per 10^6 cells) ranging from 0.2 to 0.6 µg; and (2) DNA-to-FectoVIR®-AAV ratio (µg:µL) ranging from 0.5 to 1.5. Using response surface methodology, a total of 12 experiments were conducted, including positive controls. The results indicated a significant positive correlation between rAAV titer and DNA amount ($P = 0.031$). Additionally, there was a significant quadratic relationship between the titer and the DNA-to-FectoVIR®-AAV ratio ($P = 0.023$). The highest titer was achieved at a 1:1 ratio, with deviations leading to lower titers. Model predictions suggest a 40% increase in maximum titer compared to single factor optimization, reaching 1.4E+11 vg/mL. Robust testing confirmed these results within the 95% confidence interval, demonstrating a 30%-50% improvement in titer. These findings indicate that the use of FectoVIR®-AAV and DoE method can effectively improve transfection efficiency and rAAV packaging titer for large GOIs, providing valuable insights for future research in this area.

967 Small Scale AAV Bioreactor Optimization Demonstrates Iterative Titer Gains of rAAVrh74 Serotype EPI-321, a CRISPR-mediated Epigenetic Therapy

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Although recombinant AAV is a viable option for delivering gene therapies with a high degree of tropism, high cost of goods remains a significant obstacle in the field. At Epicripsr Biotechnologies, we have developed EPI-321, an epigenetic therapy to treat Facioscapulohumeral muscular dystrophy (FSHD), the most common adult muscular dystrophy caused by the misexpression of DUX4 gene in skeletal muscle. It works by encoding an ssDNA sequence of a small transcriptional repressor tethered to a miniaturized Cas molecule and a unique sgRNA sequence within an rAAVrh74 capsid. Our technology can target the hypomethylated D4Z4 locus of DUX4 gene, thereby inhibiting its ability to activate downstream signaling cascades which cause rapid cell death and subsequent muscular wasting. The discovery and engineering of such a medicine is challenging; generating patient-ready rAAV poses further obstacles, including, but not limited to, process flexibility, productivity, and scalability. rAAV production can be triggered through the introduction of essential AAV genes and the gene therapy cassette of interest through a triple transfection system in mammalian HEK293 cells, the most widely executed method today largely due to its process flexibility. However, scalability remains a pain point as HEK293 culture requires thousands of liters to manufacture, thus confounding matters of rAAV titer productivity and reproducibility. To address these reservations, small scale bioreactor production of rAAV offers insight into scaling. Furthermore, increasing productivity from conception is a boon since it tends to drop at each stage of scaling. One way to enhance productivity in the triple transfection system is by selecting the transfection reagent that stably delivers plasmids into host cells; often, commercial versions of a cationic polymer known as polyethylenimine (PEI) are used. For each reagent screened, parameter optimizations including, but not limited to, PEI volume, DNA level (ug/mL), plasmid ratios, PEI to DNA ratios, and transfection complex formation times, are often required. Here, we first screened Reagents A-D at a smaller 125 mL shake-flask scale to identify the best-performing system (Note: Reagent B is our control transfection method). Then, we executed DoE using our top performing Reagent A to evaluate the effects of adjusting the parameters previously described. Furthermore, we evaluated the impact of the addition of a small molecule Enhancer X with both Reagents A and B and after observing comparable results, decided to move forward with Enhancer X alongside Reagent A only. Following our shake flask studies, we scaled the use of Reagents A and B to 2 L and 5 L bioreactors. Next, we screened for the optimal AAV harvest timepoint for Reagent A and assessed that 3-days post transfection with Reagent A works best. With all iterations of testing considered, we conclude that Reagent A mediated transfection with addition of Enhancer X increases titer (vg/mL) by nearly 1 order of magnitude compared to using Reagent B alone. These gains in productivity can lead to significant cost savings when scaled up appropriately. Our current framework provides us with a process to efficiently generate EPI-321 but also leaves room for further growth until full tech transfer to large scale manufacturing is achieved.

968 Scalable Production of an AAV Manufacturing Platform: Transitioning from Adherent to Suspension-Based Triple Transfection

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Adeno-associated virus (AAV) gene therapy holds immense potential for addressing genetic causes of frontotemporal dementia (FTD). Clinical supply of Passage Bio's PBFT02, an AAV1-based gene therapy candidate for treating FTD-GRN and FTD-C9orf72, has been produced to date using a triple transfection adherent process in HEK293 cells. To improve the scalability and efficiency of PBFT02 manufacturing, a high-productivity, suspension-based process has been developed.

This presentation describes the output of development activities that enabled the adaptation of HEK293 cells to a suspension process under serum and animal component-free conditions, and focuses on the optimization of key process parameters to maximize yield; these parameters include cell density at transfection, DNA ratios, transfection reagents, and harvest time.

The suspension process conducted at a 200-liter scale demonstrated a significant increase in vector yield per cell (vg/cell) and more than a 20-fold increase in volumetric productivity (vg/L) relative to the adherent process. Furthermore, key quality attributes were observed to be comparable or improved.

The successful demonstration of this suspension based triple transfection process is a significant milestone in the development of PBFT02, as it enables a commercially viable process that is both robust and efficient. Additionally, this high productivity and adaptable suspension process represents a significant step towards the establishment of a scalable platform for the manufacturing of future AAV products.

969 Capillary Isoelectric Focusing (cIEF) Platform for Characterization of Charge Variants of Adeno-Associated Virus (AAV) Capsids and Impact on Their Transduction Efficiency

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Background: Adeno-associated virus (AAV) vectors comprised of a capsid protein encapsulating a deoxyribonucleic acid (DNA) transgene have been used in the gene therapy field in an effort to treat a range of genetic diseases. Improved methods in the field of gene therapy should be used to deepen understanding of AAVs, particularly regarding charge heterogeneity of capsid species to assess stability from a chemistry, manufacturing, and controls (CMC) perspective. CMC is a crucial aspect of drug development to maintain quality and consistency during manufacturing. Objective: In this study, a versatile approach was used to investigate the charge heterogeneity of a variety of AAV capsid serotypes. Capillary isoelectric focusing (cIEF) coupled with native fluorescence imaging detection were used and underwent exhaustive validation. Methods: Seven different AAV serotypes (AAV1, AAV3, AAV4, AAV5, AAV6, AAV8, and AAV9) from multiple manufacturing platforms were analyzed and demonstrated its platform nature. Vectors contained either enhanced green fluorescent protein (EGFP) or luciferase gene of interest (GOI). The distinctive profiles generated for each AAV serotype served as valuable indicators for both identity confirmation and stability assessment. Results: It was shown that thermal (4 °C, 25 °C, 45 °C, and 100 °C) and oxidative (hydrogen peroxide treatment) stress conditions played a role in increasing acidic charged variants over time, affecting the charge heterogeneity of the tested AAVs. High thermal stress conditions resulted in consistently increasing acidic variants for all AAV serotypes, but total acidic species was serotype specific. Increase in acidic variants for each serotype was statistically significant ($p<0.0001$). Peptide mapping LC-MS was used to qualitatively assess the common post translational modifications (PTMs) following thermal and oxidative stress conditions, which include deamidation, oxidation, phosphorylation, and acetylation. Oxidation was the primary PTM used to quantitatively assess acidic charged variants and effects on in vitro potency using an AAV9 vector. Diminished protein expression levels at high thermal and oxidative stress were statistically significant ($p<0.0001$). Conclusions: The cIEF method developed successfully analyzed a variety of AAV serotypes. Increasing trends of

acidic variants and PTMs (such as oxidation) were associated with a reduced level of in vitro potency. The AAV serotypes showed comparable trends to each other relating to how acidic charge variant totals changed with increased thermal stress at various timepoints. In quantitative evaluations using AAV9, oxidation resulted in an increase of overall acidic variants and both stress conditions demonstrated a correlative trend of decreasing in vitro protein expression levels that were statistically significant ($p<0.0001$). These findings suggest that ensuring AAV manufacturing processes do not inadvertently increase acidic variants during the later stages of purification may be an important factor in generating a more potent AAV final product.

970 AAV9 Process Optimization and Robustness Evaluation in Cloud-native 250mL Bioreactors

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Gene therapy has significantly advanced the treatment of rare diseases previously deemed incurable. Among the various delivery methods for gene therapy, adeno-associated virus (AAV) vectors are the most utilized due to their safety and efficacy.
While significant strides have been made in the large-scale production of AAV vectors to improve yields and reduce costs, there remain opportunities for further improvement as the demand for AAV therapies increases.

During process development, it is crucial to gain a comprehensive understanding of the process design space. This can be effectively achieved through Design of Experiments (DoE) studies, which systematically investigate the interactions between various process parameters and their impact on desired outcomes. For AAV systems, two key goals are to maximize viral titers and to increase the proportion of fully packaged capsids.

We sought to explore the impact of process parameters on production of AAV9-GFP in a HEK293 suspension-based product system, with the goal of optimizing for viral titers and full capsids. To do this, we leveraged the Gibco™ AAV-MAX Helper-Free AAV Production System and Culture Biosciences' cloud-native proprietary bioreactors. In our initial DoE, we adjusted dissolved oxygen (DO), pH, agitation, and viable cell density (VCD) at the time of transfection while keeping other process parameters constant. Genome titers and percent full analysis of AAV9-GFP particles showed no negative impact on titer across all parameter ranges tested, demonstrating the robustness of the production system. However, statistical analysis suggested that increasing pH and VCD at time of transfection positively influenced AAV9-GFP titers. Consequently, we evaluated pH effects on viral titers and found that higher pH at time of transfection resulted in higher viral genome titer up until around a pH of 7.3. However, we also observed that percent full levels decreased at these higher pH levels, indicating a tradeoff between optimizing production parameters for titer and percent full levels. By employing Design of Experiments (DoEs) methodologies, manufacturers can systematically explore the effects of these critical parameters, leading to higher yields, improved product quality, and more consistent outcomes. This guidance serves as a valuable example for AAV manufacturers aiming to enhance their production strategies and achieve significant improvements in their overall process performance through data-driven optimization.

971 Leveraging High-Throughput Virology and Design of Experiment Expertise for Rapid Transfection Reagent Optimization for AAV/LV Production

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Background: The Design of Experiments (DoE) methodology is a powerful tool for linearizing testing parameters, significantly enhancing the efficiency of complex workflows. Traditional trial-and-error methods can be time-

consuming and costly, but DoE enables researchers to test multiple variables concurrently, accelerating the experimental process. In our comprehensive study, we used DoE methodology in our high throughput virology experiments to optimize transfection processes. Our objective was to identify and analyze key factors significantly affecting the production of recombinant adeno-associated virus (rAAV/AAV) and lentivirus (LV).

Method and Result: Adhering to DoE methodologies, the key design space was confirmed for the three key drivers of viral production - cell concentration, total DNA, and transfection reagent ratio. This allowed the generated model to be leveraged to specify key factor levels for optimal functional viral production using the cationic polymer polyethyleneimine (PEI). These optimal factor levels were then assessed at a larger scale to validate the optimal functional AAV/LV production. Initially, we adopted a standard set of parameters, widely referenced in existing literature, to establish a baseline for our experiments. This approach enabled us to achieve the anticipated AAV/LV production levels. However, our optimization process revealed that by reducing the amount of DNA while increasing the cell concentration, we obtained yields of functional AAV/LV that were either similar to or exceeded those produced under the standard conditions.

Conclusion: This result challenges long-held assumptions about the amounts of DNA necessary for successful transfection. This finding is especially significant given that DNA is one of the most expensive raw materials in cell and gene therapy. Our DoE studies clearly show that through careful optimization, ideal conditions can be identified that lower DNA consumption and maintain or improve virus production performance. Consequently, these advancements contribute to enhancing the overall efficiency and cost-effectiveness of processes within the field of gene therapy, making treatments more accessible and economically viable to potentially millions of patients.

972 One Size Does NOT Fit All: Impact of AAV Serotype and GOI on Choosing the Optimal Transfection Reagent

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Adeno-associated virus (AAV) has emerged as a leading platform for gene delivery in the treatment of a variety of human diseases. Transient transfection of suspension 293 cells is the most common method for large scale AAV production. High titer manufacture of AAV is more complicated and unpredictable compared to the delivery of a conventional reporter plasmid DNA construct such as green fluorescent protein (GFP). This is due to the complex regulation of the expressed viral genes, the intricate coordination of AAV capsid protein trafficking, assembly and genome loading, and the variety of therapeutic molecules that are required to treat genetic diseases. Often, GFP is used as a representative gene of interest (GOI) during the manufacture of recombinant AAV. This is due to its ease of detection; the efficiency of transfection can be monitored during AAV production representing one of the plasmids that has been transfected. We have observed that when generating an AAV carrying a GFP genetic payload, high GFP expression was necessary but not sufficient for high titer AAV production.

To investigate the role of the GOI in AAV production, we synthesized a therapeutic GOI encoding retinal pigment epithelium-specific 65 kDa protein (RPE65) and tested it with different capsid serotypes. The transfection reagent is a critical raw material in recombinant AAV production and can have profound effects on the overall titers obtained. When comparing GOIs, such as GFP and RPE65, we found that different transfection reagents can result in > 2-fold different relative AAV production efficiencies using the same experimental parameters. This underscores the need to test different transfection formulations for therapeutic GOIs when maximizing the AAV production conditions. Additional optimization was performed to reduce the amount of plasmid DNA, resulting in equal or higher titers when utilizing 50-75% the amount of input plasmid DNA. Proof-of-principle experiments were done at the 2L scale to demonstrate scalability. Altogether, we demonstrate an enhanced experimental workflow that offers an option to increase AAV titers and reduce pDNA input to generate a lower cost-per-dose AAV therapeutic.

973 Quantifying DNA Point Mutations in Commercial AAV products

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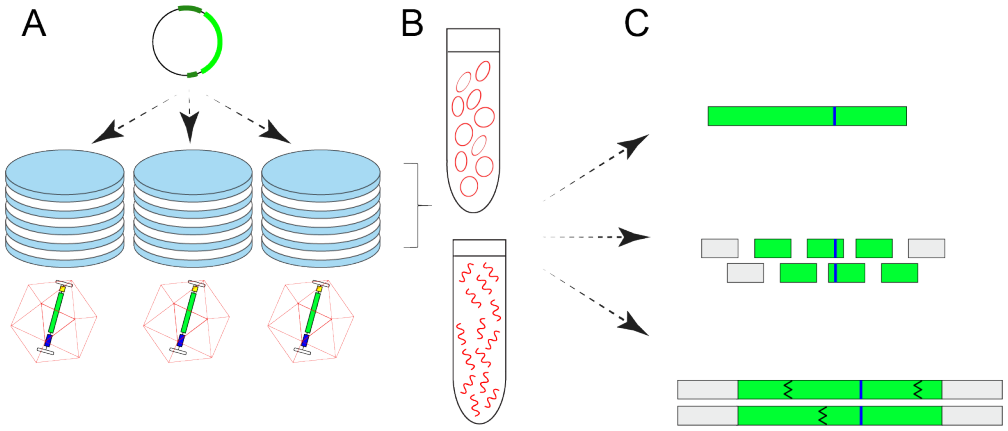
Background: Manufacturing adeno-associated virus (AAV) products may result in a sub-population of capsids with genomes that contain non-target DNA derived from producer cell and/or plasmid DNA, have major genomic rearrangements (e.g., truncations/chimeras), or which harbor single nucleotide point mutations. Although single-point mutations are the most difficult to detect, they can change a codon or binding site, which may reduce transgene expression, resulting in a sub-optimal product. Currently, there is no standardized protocol that identifies point mutations in AAV vector DNA with high accuracy and confidence.

Methods: To compare the accuracy and potential utility of three sequencing methods for quantifying point mutations in AAV products, we sequenced the green fluorescent protein (GFP) transgene from two commercial AAV9 reporter vectors and their respective producer plasmids using single template amplification followed by Sanger sequencing (STA-Sanger), a lower-throughput STA-Sanger method (commonly used in HIV research to study individual viral genomes), as well as two next generation sequencing (NGS) platforms (Illumina and Oxford Nanopore). We purchased three lots of AAV products produced from the same plasmid stock from two different suppliers (Figure 1A), extracted DNA from the AAV virions (Figure 1B), and sequenced the producer plasmid and vector DNA using STA-Sanger and NGS (Figure 1C). After strict filtering, the per-base mutation rates for the GFP gene were calculated for each sample/platform. For the NGS data, the majority variant frequency (MVF) was calculated for each position in the GFP gene.

Results: Significantly more STA-Sanger vector GFP sequences (10.2%) contained ³1 point mutation compared to plasmid GFP sequences (1.3%; $p<0.001$). The average overall vector per-base mutation rate was similar for both STA-Sanger (0.018%) and Illumina (0.013%) GFP sequences, although much higher in Nanopore (2.5%). In addition, the vector per-base mutation rate was higher than the plasmid per-base mutation rate for all platforms. For both NGS platforms, the MVF was strongly correlated among vector lots for Nanopore ($R^2=0.88$) and Illumina ($R^2=0.47$) across GFP positions; although no correlation was found for the same vector lot, or plasmid, between platforms ($R^2<0.05$).

Conclusions: The vector point mutation rate was higher than expected, requiring additional studies to determine the underlying mechanisms. The consistency of the per-base mutation rate between two markedly different sequencing approaches (Illumina and Sanger) was encouraging, as was the strong correlation of positional frequency of variants among different vector lots for each NGS platform. On the other hand, the lack of MVF correlation between the two NGS platforms and the much higher per-base mutation rate for Nanopore, highlight the limitations of NGS applied to AAVs. As the AAV industry is pivoting to genomic sequencing to ensure they manufacture genetically homogeneous and efficacious products at scale, our results demonstrate that systematic studies are needed to validate sequencing approaches before they can be reliably incorporated into cGMP.



974 Leveraging Short- and Long-Read NGS Technologies In-House for Optimal Quality Control in Manufacturing of Gene and Cell Therapies

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Biopharmaceutical companies are increasingly adopting next-generation sequencing (NGS)-based assays in the cell and gene (CGT) therapy manufacturing process to assess Critical Quality Attributes (CQAs), such as the integrity and safety of the product. Regulatory agencies, such as the FDA or EMA, can request thorough information from biopharmaceutical companies about the product CQAs during the IND application.

Besides the broad adaptation of short-read technology, long-read sequencing has further advanced the field of NGS by enabling the sequencing of long DNA fragments. Long-read NGS-based assays can be used for CGTs to, for example, detect fusion or truncation events. This ensures product safety and efficacy as well as fully sequenced plasmids and vectors to verify the orientation of gene inserts so that correct and error-free adeno-associated viruses (AAVs) are packaged into capsids.

Here we report on how to automate, validate in-house, and optimally use both short- and long-read sequencing in manufacturing QC assays of CGTs. We show how both techniques complement each other to achieve optimal outcomes. We demonstrate how to simplify and streamline all NGS workflows in-house in a GMP environment, ensuring reliable results, faster turnaround times, and improved ROI. This contributes to safer, more efficient, and more ethical biotherapeutic development.

975 Mapping of common vector genome breakpoints and non-specific DNA fragments in single-stranded and self-complementary AAV vectors by nanopore sequencing

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Recombinant adeno-associated viral (rAAV) vectors have become a cornerstone of human gene therapy. A commonly encountered challenge in rAAV preparations is the high degree of vector heterogeneity, not just driven by empty, partial and full particles, but also often characterized by unwanted byproducts such as host genome contaminants and truncated vector genomes. This heterogeneity reduces therapeutic potency and necessitates higher vector doses, which can lead to dose-dependent toxicity and increase the risk of adverse effects. Application of nanopore sequencing to directly interrogate vector genome content and obtain full-length inverted terminal repeat (ITR)-to-ITR resolution of encapsidated genomes has been reported and adapted by several groups. In particular, identification of truncation hotspots in vector genomes similar to SMRT sequencing has been demonstrated. Further bioinformatic pipelines for processing long-read data from AAV directed evolution experiments have also been developed.

Here, we build on these efforts using the Oxford Nanopore Technologies (ONT) MinION platform to enhance the resolution of vector genome profiling. Custom code is implemented to investigate common vector genome breakpoints with the goal of mapping specific nucleotide sequences that are prone to generating truncated vector genomes. We also pair ONT sequencing with Canu sequencing alignment to generate consensus maps de novo. Using an ONT workflow - “What’s in My Pot” (WIMP), we evaluate non-specific packaging of undesirable host DNA content with a particular focus on Adenoviral helper gene and bacterial plasmid fragments within AAV vectors. Together, this workflow introduces rapid and low-cost assessment of AAV vector genomes that can be readily adapted for research validation across GT pipelines and potentially help avoid common pitfalls in AAV vector genome design.

976 Enabling Innovation Through Transition to the FUEL™ Platform: Regulatory Feedback on the Comparability of Ad Helper Plasmid pEMBR™ 1.2 vs. pEMBR™ 2.0

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Forge's FUEL™ platform provides an accelerated foundation for adeno-associated virus (AAV) development and includes innovative technologies to deliver faster, potentially safer, and more efficient AAV manufacturing. As part of the FUEL™ platform, Forge developed the pEMBR™ 2.0 adenovirus (Ad) helper plasmid. pEMBR™ 2.0 is one of the smallest Ad helpers commercially available and has demonstrated an increase in manufacturing efficiency as well as an improved safety profile by reducing the adenoviral components that are unnecessary for AAV production (Adsero et al., 2024). Before the release of pEMBR 2.0™, products manufactured using the Forge platform utilized the predecessor, pEMBR™ 1.2 helper plasmid. For historical clients wanting to adopt the pEMBR™ 2.0 plasmid, Forge developed a risk assessment and comparability strategy to support the change in Ad Helper plasmid and gained regulatory feedback on the approach. Leveraging a Type D meeting with US FDA, Forge submitted a meeting package that presented: 1) proposed AAV critical quality attributes (CQAs), 2) a risk assessment on the impact of the plasmid change to each of the CQAs, and 3) a comparability study outline that evaluates each of the AAV CQAs with a ranking of moderate to high risk of impact from the plasmid change. Importantly, the comparability study plan leverages a surrogate vector (AAV9-GFP) manufactured in Forge's platform process for broad applicability across client programs. This presentation will discuss the details of the submission content as well as FDA feedback received on the approach. Overall, the approach was well-accepted by FDA. Key guidance from the agency included a request for the development of an AAV9-GFP potency assay (abstract submitted to ASGCT by Forge) to support client comparability packages, as well as improvements to the methodology for the selection of the study's sample size and acceptance criteria. FDA accepted the platform study design and the potential to support the change in multiple products. As a result, the innovative FUEL™ Platform has been de-risked and provided a path to be incorporated into existing and historical client manufacturing programs, enabling more efficient manufacturing. Forge is uniquely positioned to support clients through regulatory interactions to gain feedback on new innovative technologies before they reach the production records.

977 Optimizing AAV Production: Accelerating Discovery of Small Molecule Enhancers using High Throughput Virology and Design of Experiments

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Adeno-associated virus (AAV) has become essential in the advancement of cell and gene therapies due to its ability to deliver genetic material safely and efficiently to target cells. However, the production of AAV for gene therapy applications is a complex process where final yields and product quality are often a manufacturing bottleneck. Process optimization is traditionally a time-consuming process, yet necessary to reach robust yields and product quality, and ultimately, commercial viability.

With process optimization playing a large factor in therapeutic success, methods of maximizing yields and quality on expedited timelines are of critical importance. Virica Biotech has developed a panel of proprietary small molecules called Viral Sensitizers (VSE™) which enhance the production yields of viruses by transiently dampening cellular antiviral defenses. These defenses are triggered upon the introduction of foreign particles and nucleic acid into a cell, consequently limiting viral transfection, assembly and replication. We have combined our yield-enhancing VSE technology with our expertise in Design of Experiments (DoE) and high-throughput virology capabilities to achieve rapid process optimization and scale-up validation for increased AAV production.

Our high-throughput, 96-well viral production platform leverages liquid-handling automation and luciferase-based transduction quantification methods to rapidly produce and quantify functional AAV2 yields for process optimization and VSE screening. Coupling this high-throughput approach with DoE statistical modelling methodologies allowed for rapid AAV yield enhancement screening of up to 360 unique conditions. These conditions comprised six novel VSEs at various concentrations in the presence or absence of other transfection kit-associated additives, to understand compound synergy and antagonism while identifying novel small molecule enhancers. The top VSE molecules/combinations from small-scale screening were identified in a single DoE

iteration and then refined in additional iterations to yield up to 3-fold improvement in functional AVV2 titer. This formulation was then successfully scaled up directly to Culture Biosciences 250mL cloud-connected bioreactor, where a consistent enhancement in functional AAV2 titer was achieved.

By combining our proprietary small molecule enhancing technology with high throughput production screening methods and DoE statistical expertise, we identified a robust AAV-enhancing VSE formulation and demonstrated performance at a 250 mL scale. Our novel approach to rapid production optimization has the potential to substantially reduce both timelines and costs for AAV production optimization, addressing a fundamental bottleneck for the cell and gene therapy industry as demands continue to rise.

978 Optimizing AAV Production: Advances in Transfection Agents, Additives, and Clonal Cell Line Development

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AGC Biologics is a leading global Contract Development and Manufacturing Organization (CDMO), specializing in the development and production of therapeutic proteins, plasmid DNA, viral vectors, and genetically engineered cells derived from both mammalian and microbial systems. Our expertise on viral vectors originally focused on LVVs and RVVs, has expanded to the production of Adeno-Associated Virus (AAV) vectors via transient transfection in HEK293 cells, utilizing both adherent and suspension cultures for applications in ex vivo and in vivo gene therapies.
AGC Biologics has developed the BravoAAV™ platform, a scalable and adaptable system capable of supporting multiple AAV serotypes. To keep pace with market demands for higher productivity, enhanced purity, and compliance with increasingly stringent regulatory standards, the development of this platform is undergoing continuous optimization. In this study, we present the evaluation of novel transfection agents, specific additives, and optimized packaging plasmid designs, each contributing to improved vector yields. In addition, the continuous optimization of key process parameters—including the amount of DNA used in transfection, cell seeding density, and plasmid ratio—has proven to be a successful strategy for further enhancing overall productivity.
Furthermore, regulatory expectations are progressively shifting toward the use of clonal cell lines for vector production, ensuring greater batch-to-batch consistency and product uniformity. In response to this need, AGC Biologics has developed a proprietary suspension-adapted clonal HEK293 cell line, specifically selected to enhance AAV production. This clonal line has been established in GMP and successfully compared not only to its bulk parental population but also to other cell lines used in AAV manufacturing.
Some of the above mentioned process improvements have been tested at 2L and 50L scales, yielding successful results and demonstrating their readiness for scaling up to higher production volumes (200L-1000L).
This work highlights the advancements we made in AAV production through strategic process optimization and the development of robust cell lines, paving the way for more efficient, reproducible, and scalable manufacturing solutions that AGC can offer for gene therapy applications.

979 Increasing rAAV productivity through selection of a high producing HEK293 transient cell line and addition of an enhancer

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Adeno-associated virus (AAV) has emerged as a very popular viral vector for gene therapy due to its low pathogenicity, ability to transduce many cell types, and long-term gene expression. The increase in demand for these vectors to meet dose requirements for clinical trials has created manufacturing challenges. To increase productivity and to reduce cost of production, the industry has shifted to use of suspension based HEK293 cells and 3-plasmid transient production in large scale bioreactors, plasmid engineering, and the use of feeds and enhancers. Here, we

highlight our screening and selection of a high producing HEK293 transient cell line and the use of an enhancer to boost rAAV titers for platform process development.

980 Cell Cycle Modulation as a Strategy to Enhance AAV Production Across Serotypes

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Adeno-associated virus (AAV) vector production remains a key bottleneck in the development of gene therapies due to low titers. Here, we examined the relationship between cell cycle status and AAV productivity in suspension HEK293 cells. Further, we identified cell cycle–modulating strategies that substantially enhance vector output. By applying a targeted additive at key points in the production process, we increased AAV titers and specific productivity across multiple serotypes. These findings highlight a biomanufacturing strategy to enhance AAV vector production, providing a scalable and cost-effective solution for both clinical and commercial manufacturing processes.

981 Small samples, big insights: accelerating AAV stability studies with high-quality, low-volume analytics

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Adeno-associated virus (AAV) products are at the forefront of gene therapy innovation, yet their inherent complexity poses significant challenges for characterization and regulatory compliance. In response, we have developed an extensive portfolio of in-house analytical capabilities tailored to the needs of gene therapy product developers.

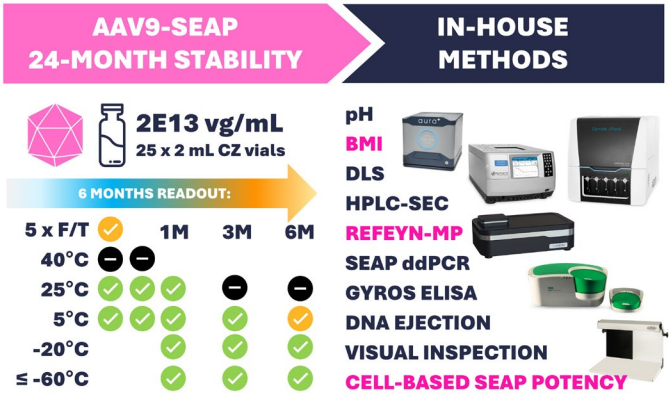
To showcase these capabilities, we conducted a stability study that encompasses a comprehensive set of methods and quality parameters. By employing low-volume techniques such as backgrounded membrane imaging (BMI) and mass photometry, we minimized sample requirements for traditionally resource-intensive assays (e.g., subvisible particles, full-empty characterization). This allowed detailed analysis at earlier time points and extended the study duration to 24 months, using only 25 vials and less than 30 mL of an AAV9-SEAP drug product at 2E13 vg/mL stored in industry-standard 2 mL CZ® vials with a fill volume of 1.1 mL.

At the 6-month interim read out, the study showed that storage at −20°C sufficiently maintains the stability of AAV and that storage and ≤ -60°C is not required. Dynamic light scattering (DLS) results indicated low particle dispersity across all temperature levels, even after six months at room temperature and five freeze-thaw cycles. Visual inspection also demonstrated absence of visible particulates. However, BMI, a high-throughput, low-volume method requiring only 150 µL per measurement, revealed subvisible particle formation particularly after one and five freeze-thaw cycles down to ≤ −60°C. Interestingly, no significant increase in subvisible particles was detected after a single freeze-thaw cycle at −20°C. This finding was confirmed by HPLC-SEC, which detected smaller aggregates, and by increased SYBR-GOLD fluorescence, indicating ejected DNA, after one or repeated freeze-thaw cycles at ≤ −60°C, but not after storage at -20°C.

Despite these observations, no reductions in capsid and genome titers were detected after one or multiple freeze-thaw cycles. Furthermore, our in-house cell-based SEAP reporter gene assay demonstrated that vector efficacy remained unaffected by repeated freeze-thaw stress. Loss of functional transduction during liquid storage was only observed after exposure to stress temperature or after prolonged storage (40°C; 3 months at 25°C, 6 months at 5°C).

Our in-depth analytical expertise enables faster development timelines, a better understanding of the product and

greater confidence in meeting regulatory requirements. We provide AAV developers with the insights and data they need to successfully advance their AAV therapies. Innovative approaches and the provision of cutting-edge analytics ensure that we remain at the forefront of the gene therapy landscape.



982 Characterization of Plant-made AAVs and a Side-by-side Comparison with HEK-derived AAV

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Introduction: Gene therapies hold immense promise for treating genetic disorders, however, their widespread adoption is hindered by major manufacturing challenges, high costs, complex production processes, and scalability limitations. Current manufacturing platforms, primarily based on mammalian and insect cell systems, are expensive, slow, and often difficult to scale, contributing to therapy costs that exceed millions of dollars per patient, making these life-saving treatments inaccessible to many. Given the urgency of delivering these therapies efficiently, there is a pressing need for a new, scalable, cost-effective, and reliable manufacturing platform. We have developed an innovative plant-based alternative that reduces costs, shortens production timelines, and ensures consistent vector quality that is crucial for the future of gene therapy. A new platform must integrate seamlessly with existing purification methods while offering flexibility for different vector types and therapeutic applications. By overcoming current limitations, such a system could make gene therapies more accessible, accelerating their potential to transform global healthcare.

Methods: We produced AAV2 and AAV9-CMV-GFP in Nicotiana benthamiana by infiltrating leaves with Agrobacterium carrying the necessary plasmids. After five days, leaves were harvested, homogenized, and purified using tangential flow filtration, affinity capture, and iodixanol gradient purification mirroring HEK293-based methods. Both plant- and HEK-derived AAVs were characterized via qPCR, ELISA, TEM, SDS-PAGE, sterility, endotoxin, and mycoplasma testing. Infectivity was assessed in vitro, ex vivo, and in vivo, and post-translational modifications were analyzed via mass spectrometry.

Results: Agrobacterium carrying recombinant plasmids successfully infiltrated N. benthamiana leaf cells, enabling AAV vector assembly in plants yielding more than 1e14 viral genomes per kilogram of biomass. Using standard AAV purification methods, we obtained comparable recovery rates to mammalian cell-derived AAVs. Quality assessments confirmed ~90% full particles, endotoxin levels well below FDA guidelines, and absence of bacterial, fungal, or mycoplasma contamination similar to HEK293T-derived AAVs. Both Plant and HEK-derived AAVs exhibited proper VP1, VP2, and VP3 ratios on SDS-PAGE, with ~90% full capsids confirmed by TEM and ELISA. We observed better transgene expression and reduced neutralizing antibody profile from plant-produced AAV in Non-human Primates compared to HEK-derived AAVs, despite identical transduction rates. Finally, a thorough post-translational modification analysis showed a very similar profile besides some differences in phosphorylation.

Conclusion: We successfully demonstrated transient AAV production in plants, reducing costs by over tenfold and significantly shortening scale-up time. Since each plant serves as an independent, reproducible production unit, scalability is highly reliable. Additionally, the plant-based platform integrates with existing purification workflows used in AAV production, making it easily adaptable. A head-to-head comparison of the plant produced AAVs with those from the HEK origin showed similar analytical profile, and better activity in the Non-Human Primates, making the plant platform more interesting and worth exploring. We strongly believe this innovation addresses key challenges in the CGT field, offering a cost-effective and scalable solution.

983 Increasing the Susceptibility of Cell Lines to a Broad Range of Adeno-Associated Viral Vectors to Enable in Vitro Potency Assays

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Adeno-associated virus (AAV)-based vectors are promising tools for gene therapy, but robust cell-based assays are needed to quantify their potency in transgene expression for clinical development. However, many clinically relevant AAV serotypes exhibit poor in vitro transduction and often contain cell type-specific promoters that remain inactive in commonly used cell lines. To address these limitations, we employed a multi-faceted approach to enhance *in vitro* AAV transduction by (1) overexpressing the AAV receptor (AAVR/KIAA0319L), (2) preventing transcriptional silencing by the HUSH complex, and (3) using CRISPR activation to drive transgene expression. For the latter, we designed guide RNAs targeting the conserved AAV2 inverted terminal repeat sequence present in most AAV transfer vectors. This strategy led to the development of engineered cell lines with significantly increased transduction efficiency across a wide range of AAV serotypes, including those with cell type-specific promoters. These optimized cell lines enable the robust determination of AAV functional (infectious) titers, and facilitate standardized potency assays across diverse AAV preparations. The enhanced susceptibility of these engineered cell lines to AAV transduction could provide a valuable platform for standardization of *in vitro* quantitative assays in AAV-based gene therapy development.

984 Development of High-performing Stable Producer Cell Lines for Therapeutic AAV Vector Manufacturing

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Attributed to their safety profiles and transduction efficiencies, recombinant Adeno-associated virus (AAV) vectors are widely used for treatment of human genetic diseases. However, the high cost and low productivity in AAV vector manufacturing process limit the clinical and commercial development of these life-changing therapies for more patients. Recently, we reported the development of high-performing, HEK293 cell based, helper virus-free AAV stable producer cell lines (PCL) that significantly increased the AAV productivity (>1E12 vg/mL at harvest) compared to transient transfection based process. In this new case study, we describe the generation of high-titer stable PCL for AAV vectors using a clinically relevant transgene and an engineered capsid provided by our partner. The beta test results confirmed the robust AAV PCL development process from stable pools to selected single cell clones, which produced significantly higher titers than the transient transfection process. Therefore, our Xcite® AAV stable PCL represents a next-generation technology to enable large-scale AAV manufacturing at markedly reduced cost and complexity.

985 Generation and Characterization of a Novel HEK293 Suspension Cell Line for rAAV Production.

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Advanced therapeutic medicinal products (ATMP) have led to an increased focus on biologics produced in mammalian cells. The HEK293 cell line is currently used for the manufacturing of several therapeutic proteins and viral vectors. Several HEK293 derivatives are commercially available for R&D activities and for the GMP-manufacturing of biologics. However, we still have limited knowledge on the genetic composition of these cells and on how to improve their properties.

In this study, we generated a novel HEK293 cell line specifically selected for the efficient transfection of plasmids and for rAAV production. Single-cell clones derived from a polyclonal parental HEK293 cell line were selected based on their high transfection efficiency, growth profile in suspension culture, capacity for efficient single-cell sorting and growth in selected culture media, and ultimately for improved rAAV production efficiency following triple-plasmid transfection. Several clones were characterized both genomically and functionally. A single clone displaying a homogeneous and stable genomic structure was selected and its performance was compared to that of currently available HEK293 cell lines used in pre-clinical and clinical studies. The selected HEK293 clone was fully characterized at the genomic level using PacBio's High-Fidelity long-read sequencing and Bionano's optical genome maping, and at the transcriptomic level using high-throughput Illumina's paired-end short-read sequencing. This analysis revealed fewer genomic rearrangements in the selected clone compared to the parental heterogeneous HEK293 cell line. In addition, the selected clone was genetically stable over 55 generations, making it an ideal choice for long-term and bioreactor cell culture applications.

The novel HEK293 clone produced similar or higher rAAV titers as compared to either the parental polyclonal cell line or currently available leading HEK293 cell lines. This effect was demonstrated using various transgenes or culture volumes, and it was consistently observed over 55 generations of culturing. In addition, the new clonal cell line produced 2-fold higher ratios of DNA-containing vs empty capsids when compared to the materials produced by a leading HEK293 cell line, as well as comparable or higher degree of AAV genome integrity as assessed by high-throughput sequencing.

In conclusion, we have generated and characterized a novel cell line specifically optimized for the production of high quality rAAV gene therapy candidate vectors.

986 Optimization of rAAV Production Using an In-House Suspension HEK293 Cell Line

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Recombinant adeno-associated virus (rAAV) vectors have emerged as a leading platform for gene therapy, necessitating the development of efficient and scalable production methods. One routinely used manufacturing approach for these vectors involves the transient transfection of plasmid DNA into mammalian cell lines such as HEK293. Many HEK293 cell lines are available commercially and have been used to produce rAAV with varying levels of success. These commercial HEK293 cell lines offer advantages such as their adaptability to suspension culture and their availability at GMP-grade, but they are often burdened with challenges such as low productivity, inconsistent quality, and high cost. To address these limitations, we have developed a manufacturing approach using a novel, in-house suspension HEK293 cell line optimized for rAAV production.

Developing this process first involved comprehensive screening of serum-free, chemically defined media formulations to support robust cell growth and high rAAV titers. In addition, transfection conditions were also optimized, including the evaluation of novel transfection reagents and enhancers. To mitigate cell clumping, various anti-clumping strategies and reagents were tested, resulting in a solution that reduced cell aggregation by greater than 75%. Lastly, we were able to increase both vector yield and quality through targeted plasmid engineering. We have also demonstrated the scalability of this cell line and process and have established scaled-up production methods in single-use bioreactors at both the 2L and 10L scales through targeted optimization of key parameters

such as pH, dissolved oxygen, and agitation rate. Through this work, our proprietary HEK293 cell line and optimized process meets or exceeds the bar set by commercially available cell line options, achieving rAAV titers of 1.5E15 genome copies per liter in crude lysate across multiple serotypes. In addition, vector quality was improved, with a proportion of full capsids in crudeupstream lysate greater than 40% in some serotypes. This optimized platform offers a robust, scalable solution for high yield and high quality rAAV production, allowing for reduced costs compared to commercially available HEK293 cell lines and improving the manufacturability of the rAAVs needed as a critical vector for gene therapy.

987 Recombinant Adeno-Associated Virus (rAAV) Production in Spodoptera Frugiperda (Sf9) Cells: Viral Cathepsin Mediated Capsid Cleavage and Mitigation Strategies

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Currently, the two most common methods of recombinant adeno-associated virus (rAAV) production are plasmid transfection of human embryonic kidney 293 (HEK293 cells) or infection of Spodoptera frugiperda (Sf9) insect cells with recombinant baculovirus (rBV). Our group focuses on rAAV production in Sf9 cells, and we have used this method to produce naturally occurring and novel rAAV capsids. While many capsids are produced without issue in the Sf9 system (such as AAV2, AAV9, and STAC-BBB), some AAV capsids are prone to additional viral protein (VP) banding observed by SDS-PAGE or Western blotting (such as AAV8 and some novel capsids). Peptide sequencing confirmed that the additional bands are cleavage fragments from the VPs. Viral cathepsin (VCATH*)* is a known cathepsin L-like proteinase expressed by baculovirus as a late viral gene. Galibert et al. demonstrated that some naturally occurring capsids are sensitive to VCATH-mediated VP cleavage during manufacturing in the Sf9 system, and such degradation can be prevented by eliminating or inhibiting VCATH (PMID: 30440025). We tested the addition of a known VCATH proteinase inhibitor (E64) and glycosylation inhibitor (tunicamycin) in Sf9 AAV productions with two affected capsids (capsid A and B).

For capsid A and B, we performed multiple small-scale Sf9 productions with 50µM of E64 (n=7 or 4 respectively) or 10µg/mL of tunicamycin (n=2 or 3 respectively) vs. standard productions (n=10 or 7 respectively). We analyzed the AAV for VP cleavage products, yield, VP ratio, and transgene potency (as an orthogonal measure of capsid integrity). Our results showed that tunicamycin did not eliminate the cleavage products and instead caused additional aberrant capsid protein staining by SDS-PAGE for both capsids. E64 addition successfully reduced or eliminated the VP cleavage products. However, E64 addition negatively impacts AAV yield for capsid A, while capsid B slightly improved (58% and 1.08% of the average absolute yield). Adding E64 improved the VP1 content for both capsids and more closely aligned the VP ratio to the ideal 1:1:10 (1:5:14 to 1:2:11 for capsid A and 1:5:34 to 1:2:14 for capsid B). Finally, we assayed the activity of a zinc finger repressor transgene from productions with E64 or tunicamycin vs. control productions. Adding either inhibitor improved the transgene expression and target repression for both capsids, but most notably for capsid A. For capsid A, average transgene expression improved 4x with tunicamycin vs. 13x with E64, corresponding to 300x vs. 459x repression of the native gene target, respectively. For capsid B, average transgene expression improved 1x with tunicamycin vs. 2x with E64, corresponding to 6x vs. 2x repression. There is no consensus recognition sequence for VCATH, so capsid cleavage cannot be easily predicted before manufacturing or preemptively prevented by capsid engineering. However, our results demonstrate that E64 successfully inhibits VCATH-mediated cleavage of VP proteins with largely beneficial effects on AAV production when VCATH is present in the rBV backbone. Additionally, this work supports the adoption of VCATH null rBV to produce rAAV.

988 Engineering a stable cell line for AAV production through synthetic biology

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Adeno-Associated virus (AAV) vectors have emerged as highly promising tools for in-vivo gene therapy in clinical applications. However, the large-scale industrial bioproduction of AAV faces challenges in terms of efficiency and scalability, primarily due to its reliance on transient transfection of HEK293 cell lines. Efforts to develop more scalable systems, such as AAV producer cell lines endowed with inducible viral gene expression system to initiate viral vector production "on demand" have been hindered by the toxicity associated with the leaky expression of viral genes. We previously designed the CASwitch, a novel inducible gene expression system with minimal leakiness. It combines the CRISPR-Cas endoribonuclease CasRx with the state-of-the-art Tet-On3G inducible gene system and can act as a switch for gene expression in response to doxycycline administration [https://doi.org/10.1038/s41467-024-47592-y]. Specifically, in the absence of doxycycline, the CasRx cleaves a short sequence (direct repeat - DR) placed in the 3'UTR of the target gene's mRNA thus leading to the loss of its polyA tail and subsequent degradation, thus eliminating leaky gene expression. In the presence of doxycycline, the tet-transcativator fully expresses the target gene while repressing the CasRx, thus enabling the target gene to be maximally expressed.

Here, we developed a stable AAV producer HEK293T cell line by using the CASwitch to regulate the inducible expression of Helper genes (E2A, E4, and VA RNAI) derived from Human Adenovirus 5 (HAdV-5). To this end, we first engineered a minimal Helper gene cassette with a doxycycline inducible promoter driving a minimal Helper gene cassette (pTRE3G-E2A[DBP]-IRES-E4[Orf6]). We then integrated this cassette, together with the VA RNAI and the CASwitch components in the genome of HEK203T cells by PiggyBack and Sleeping Beauty transposases to obtain an AAV producer cell line. Cell viability and growth analyses indicated a higher metabolic burden when the integrated cells were continuously induced with doxycycline compared to both uninduced cells, and to wild type cells grown in doxycyline, suggesting that constant expression of the helper genes for prolonged periods causes a decrease of cell viability.

We then proved the inducer cell line can efficiently be used to produce AAV vectors on demand. To this end, we first expanded cells in a cell stack with medium without doxycycline. Once the desired confluence was reached, only the Transgene and Packaging plasmids were transfected, as opposed to the traditional triple transfection method. We achieved a production efficiency of 80% compared to the triple transfection in the presence of doxycycline.

We demonstrated that integrating the CASwitch control network into a stable HEK293T cell line for AAV production significantly enhances scalability and production efficiency by minimizing the reliance on transient transfection. This stable system offers a more efficient and consistent approach for AAV vectors suitable for clinical applications.

989 Transient vs stable cell lines for AAV manufacturing: a cost modeling approach

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To address the growing demand for industrial-scale adeno-associated vector (AAV) production, we recently launched an innovative solution for stable, helper virus-free AAV production. The ELEVECTA™ producer cell line incorporates all the necessary components for AAV production stably integrated into its genome, enabling high-titer AAV production upon induction with an inducing agent.

Stable cell lines like the ELEVECTA producer cell line hold the potential to reduce the manufacturing costs of AAV-based viral vector therapies. Cost modeling can be a valuable tool in understanding how their use would impact manufacturing costs and, consequently, the cost per dose. Using the BioSolve software from Biopharm Services Limited, we established a cost model for AAV manufacturing with the current ELEVECTA producer cell line.

In this study, we modeled two different processes: a transient process based on our in-house, best-performing data corresponding to average industry standards and a process using the ELEVECTA producer cell line based on the

average process performance achieved thus far. These two processes were compared in terms of manufacturing costs for two different yearly throughputs of viral genomes, based on modeled diseases, including a sensitivity analysis on plasmid DNA cost.

ELEVECTA is a trademark of Global Life Sciences Solutions USA LLC or an affiliate doing business as Cytiva. BioSolve Process is a trademark of Biopharm Services Limited.

990 Generation of a Universal AAV Packaging Cell Line for use in Commercial Gene Therapy Manufacturing

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The urgency to generate large quantities of high-quality viral vectors while simultaneously reducing costs is critical to support both rare disease indications and broader therapeutic applications. The SKPT-US suite of cell lines includes the validation of a cGMP-ready, clonally derived, universal viral production cell line, SKPT-HEK293-4G9, for plasmid-based transient viral vector production, supporting programs from research to commercial scale. Using a common parental cell, the serum-free, suspension-adapted SKPT-HEK293 cell line, the SKPT-HEK293 AAV packaging cell line has been developed with stable integration of Rep, Capsid, and Ad Helper elements under the control of a doxycycline-inducible promoter system. Transfection with a gene-of-interest (GOI) cis-plasmid enables single-step AAV production upon induction, eliminating the need for inefficient multi-plasmid-based transient transfection. This strategy reduces process complexity, production costs, and batch variability. Here, we present the workflow for generating stable pools and, subsequently, single-cell clones using the Cytena F.Sight platform for cell printing. Our results demonstrate stable expression and comparable production efficiency to transient triple transfection assays. Our off-the-shelf stable AAV capsid product offerings, combined with our robust manufacturing, quality, and regulatory expertise, provide an attractive solution for large-scale commercial manufacturing.

991 Enhanced AAV Vectors for Targeted and Efficient Gene Expression in Brain Microglia

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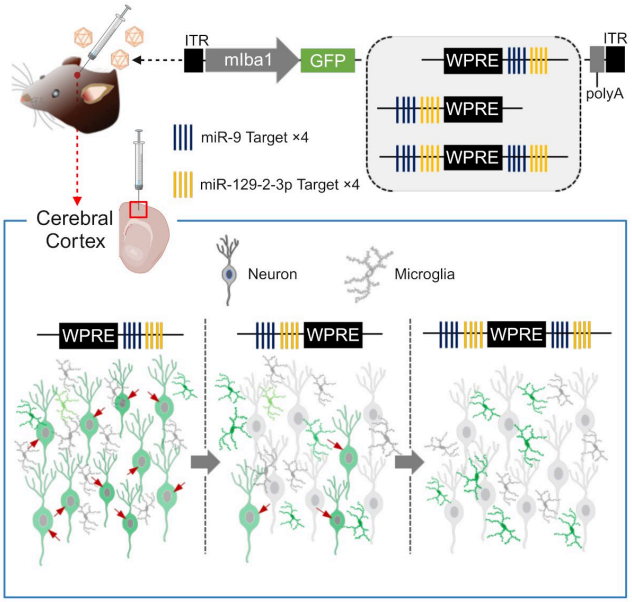
Microglia are immune cells of the central nervous system that originate from yolk sac progenitors during embryonic development. They interact with synapses and axons, monitoring and regulating neuronal function. Upon pathological conditions, microglia become activated, migrating to lesion sites, phagocytosing damaged cells and amyloid-beta (Aβ), and releasing soluble factors. Microglia play a crucial role in the onset and progression of central nervous system disorders such as Alzheimer's disease and multiple sclerosis, making them a promising therapeutic target. However, since microglia function as immune cells that eliminate invading viruses in the brain, efficient gene delivery via viral vectors remains highly challenging.

We previously reported an AAV vector capable of microglia-specific gene expression by combining the microglia-specific mIba1 promoter with microRNA target (miR.T) sequences. While the mIba1 promoter selectively functions in microglia, it also exhibits activity in neurons. To overcome this limitation, we incorporated complementary sequences for miR-9 and miR-129-3p—microRNAs expressed in neurons and astrocytes but absent in microglia—into the vector. In neurons, transcribed mRNA containing complementary sequences binds to endogenous microRNAs and undergoes degradation. In contrast, in microglia, where miR-9 and miR-129-3p are absent, the mRNA remains intact, enabling gene expression. When injected into the brain parenchyma, this AAV vector successfully achieved microglia-specific gene expression in the cerebellum and striatum. However, in the cerebral

cortex, while microglia-specific expression was maintained for up to one week post-injection, neurons exhibited substantial transgene expression by the third week.

In this study, we further optimized microglial targeting by repositioning the miR.T sequences from the 3′ to the 5′ side of WPRE, significantly improving specificity and efficiency in the cortex. Additional enhancement was achieved by placing miR.T sequences on both sides of WPRE, resulting in 90% specificity and 60% efficiency of GFP expression three weeks post-injection. This optimized vector enabled GCaMP expression in cortical microglia, allowing real-time visualization of calcium dynamics and microglial process movement. Furthermore, intravenous administration of an AAV-9P31 capsid mutant led to widespread transgene expression in microglia throughout the brain, although off-target expression in vascular endothelial cells was observed. Our findings demonstrate that this AAV system ensures long-term, specific, and efficient gene expression in cortical microglia, offering new insights into their pathophysiological roles.



992 Identification of common AAV surface residuals responsible for AAV pre-existing antibody recognition

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AAV has been widely regarded as one of the most promising vectors for in vivo gene therapy. However, 30%–60% of individuals have pre-existing anti-AAV antibodies, which could compromise the therapeutic effect or exclude some patients from AAV treatment. How to solve the pre-existing antibody issues remains one of the biggest challenges for AAV gene therapy.

In our efforts to systemically map all the AAV9 surface functional units, we utilized insertional mutagenesis strategy to disrupt these units and purified the corresponding mutant AAVs. We then injected these mutant AAVs into non-human primates to monitor any functional loss. We also tested these mutant AAVs for their reaction with pre-existing AAV antibodies in a panel of human serums from 48 different individuals. Three key sites on AAV surface showed unique features from these two assays. The corresponding AAV mutants were able to evade the recognition by this panel of AAV pre-existing antibodies. Next, we performed a functional test, in which these selected mutant AAVs and the wild-type AAV9 control virus were incubated with serially diluted human serum, followed by

infection of HEK293 cells. All these mutants, but not wild-type AAV9, were able to infect 293 cells at higher concentration of serums. As a comparison, without human serum incubation, all mutants and wild-type AAV9 could infect 293 cells. To further prove these sites are universal among different AAV serotypes, we mutated similar sites on AAV6. These AAV6 mutants also escaped recognition by this panel of pre-existing antibodies. Similarly, AAV2 variants containing these mutation sites displayed resistance to pre-existing antibodies too.

To further validate these findings and to apply these findings to more AAV variants, we inserted different length of tags from 2 amino acids to 12 amino acids at these sites. All the corresponding mutants display similar resistance to pre-existing antibody binding.

Furthermore, knowing the complex nature of the pre-existing antibody, we tested these mutants in different serums from different backgrounds. In both Asian groups and North American groups, these mutants displayed similar resistance to pre-existing antibody recognition.

These findings will be helpful for understanding the mystery of pre-existing antibody generation, and possibly benefit the patients with a high titer of pre-existing antibody who currently can't receive AAV gene therapy.

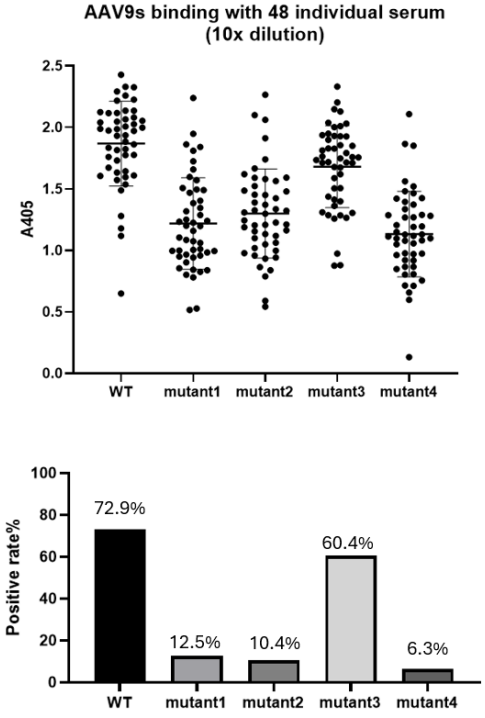

Fig 1 Binding assay of AAV9 and its mutants with individual serum containing pre-existing antibody. AAV9 with mutation in different positions (Mutant 1, mutant 2 and mutant 4) but not AAV9 wild type and other irrelevant mutant (AAV9 and mutant3 can escaping recognition of about 80% pre-existing antibody from 48 individual serums. Interestingly no mutants, can escaping all the pre-existing antibody binding

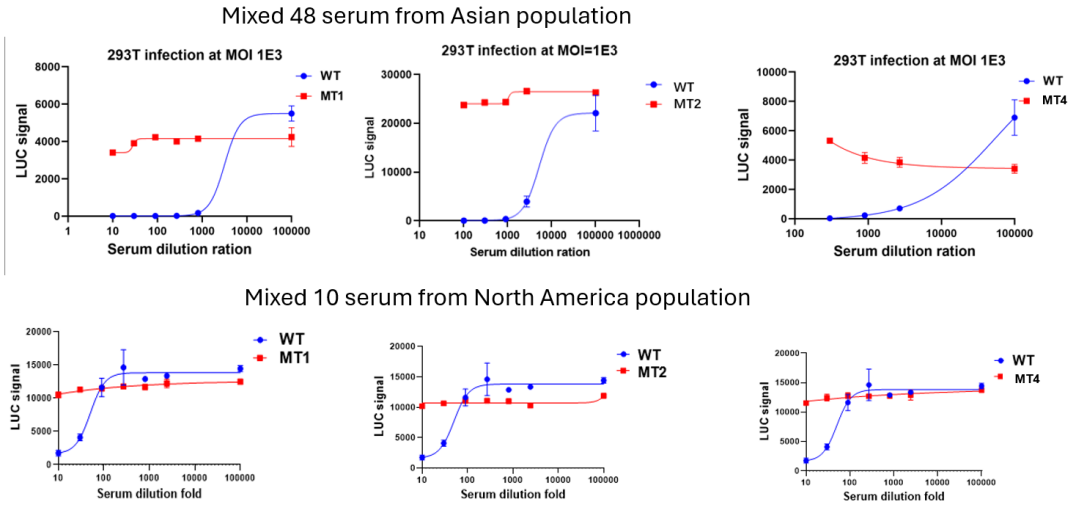

Fig 2 Function test: AAV infect 293 cells with incubation of serial diluted mixed human serums from different populations. AAV9 and three mutants were incubated with serial diluted mixed serums from 48 individuals from Asia and 10 individuals from North America, containing pre-existing AAV antibody. WT: wild type AAV9, MT1, MT2, MT4, three AAV mutants containing disrupted AAV pre-existing antibody recognition sites.

993 Evaluation of Multiple Immunoassay Formats for the Detection of AAV Capsid Specific Antibodies

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Background: AAV capsid-specific antibodies may impact clinical efficacy and safety of gene therapies and thus need to be monitored in clinical trials. Monitoring includes both pre-existing antibodies that arose from previous exposure to natural AAVs in the environment and post-treatment immune responses following AAV gene therapy administration.

Methods: We compared several different clinical assay formats to detect capsid antibodies against a proprietary AAV capsid, including an electro-chemiluminescent (ECL) sandwich assay, a colorimetric bridging ELISA, and ECL bridging formats with either sequential or simultaneous addition of capture and detection reagents. Assays were characterized for sensitivity, precision, and other performance parameters, allowing for a comprehensive comparison.

Results: The sandwich ECL assay was the least sensitive of all evaluable methods, as judged by the limits of detection (LOD) for the surrogate positive control (SPC) antibodies across assay formats. Limited sensitivity was largely attributable to increased background signals in negative human serum, likely due to binding of anti-human kappa/lambda detection antibodies to non-specifically captured total human IgG. In sharp contrast, bridging ECL and ELISA methods had much lower background signals, resulting in the ability to detect positive control anti-AAV antibody concentrations below 100 ng/mL, the recommended minimal sensitivity limit per health agency guidance. Furthermore, bridging formats were more advantageous from a practical point of view, since this format does not rely on species-specific antibody detection, allowing for use of a single detector with both clinical samples and non-human positive-controls to monitor performance of the assay over time. Intra- and inter-assay precision of all assay formats was largely comparable.

Conclusion. Bridging immunoassays utilizing either electro-chemiluminescent or colorimetric technology can allow for highly sensitive detection of AAV-specific antibodies, while sandwich immunoassays may be more restricted in achieving sensitivity of sub-100 ng/mL. The ultimate utility of each assay format to detect pre-existing AAV antibodies that may be relevant for gene therapy eligibility needs to be established in clinical trials.

994 Structural Characterization of Antibody Interactions from Infants with AAV2-associated Hepatitis to Guide Rational Capsid Redesign

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Pre-existing neutralizing antibodies (NAbs) against the capsids of adeno-associated virus (AAV) vectors pose a significant challenge for AAV-mediated gene therapy, resulting in patients being excluded from receiving these therapeutics. These antibodies originate from prior exposure to naturally circulating AAVs. Recently, a global outbreak of acute pediatric hepatitis concomitant with an AAV2 infection has been reported. For this study, infants who presented with an AAV2-associated hepatitis at the Children's Hospital at Westmead, Australia showed high anti-AAV2 IgG endpoint titers. Subsequently, monoclonal antibodies (mAbs) were reconstituted from these patients enabling the characterization of the natural antibody response towards the AAV capsids.

Reported here, are six human derived anti-AAV2 mAbs, that have been confirmed by dot blots to recognize the AAV2 capsid. Additionally, cross-reactivity studies showed that three of the mAbs recognized AAV3 and one recognized several AAV serotypes. For further insights, the binding sites of the fragment antigen binding (Fab) regions of the anti-AAV2 mAbs were determined to the AAV2 capsid using cryo-electron microscopy. To date, two AAV2-Fab complex structures have been reconstructed from 83,248 and 262,343 particles to ~2.2 Å resolution and showed different binding modes. While one of the antibodies bound to the capsid two-fold depression, the other bound at the side of the 3-fold protrusion and 2/5-fold wall towards the 5-fold canyon. Due to its binding position, the 2-fold binding antibody does not conform to the icosahedral symmetry of the capsid and required localized reconstruction to resolve the specific interactions to the capsid. The contact information of these Fabs is being used to guide site-directed mutagenesis efforts to develop a novel AAV2 capsid capable of escaping these pre-existing Nabs, potentially expanding the patient pool eligible to receive AAV2 biologics such as Upstaza®. Furthermore, regions occluded by the Fab provide insights into the mechanism by which these NAbs inhibit transduction.

995 Redesigning Immunogenic AAV Epitopes to Prevent Cell Surface Display by Human Leucocyte Antigens

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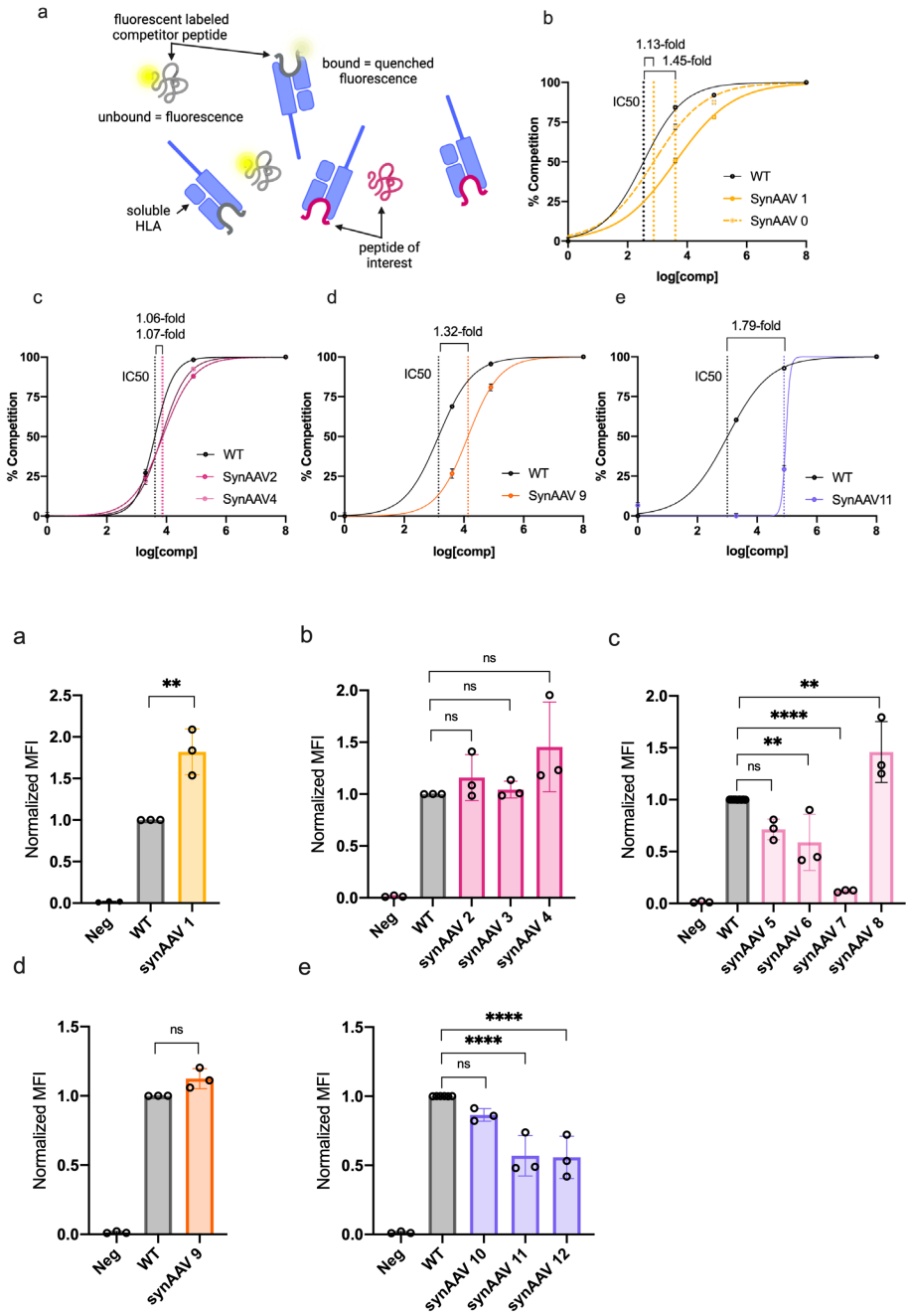
AAV's have proven to be feasible and effective gene delivery vehicles, as evidenced by their approval for treating various diseases, such as Leber congenital amaurosis (retinal dystrophy), spinal muscular atrophy, and Hemophilia B. Nevertheless, the field faces a significant challenge in addressing capsid immunogenicity, given that approximately 90% of the adult population has prior exposure to wild-type AAVs. This prior exposure leads to cytotoxic T-lymphocyte (CTL) destruction of transduced cells by memory CTLs, as seen in several clinical trials. To tackle this issue, we aimed to engineer the AAV capsid to evade recognition by memory CTLs by introducing point mutations to decrease binding affinity and presentation of immunogenic epitopes on MHC I. Leveraging previously published mutagenesis data and rational protein design techniques, we identified and tested potential mutations within the AAV capsid for decreased HLA presentation of immunogenic epitopes. Mutant AAV vectors were synthesized in HEK293T cells and tested for capsid assembly and transduction efficiency. Furthermore, mutations at MHC I anchor residues within each peptide were tested for change in molecular binding affinity and CTL recognition using human PBMC samples through ELISpot assays. Mutations exhibiting a similar transduction

efficiency to wild-type AAV and decreased MHC I binding affinity were identified for several immunogenic epitopes. These findings demonstrate a proof of concept for addressing CTL responses towards AAVs in the context of gene therapy.



996 The Seropositivity Dilemma for AAV Gene Therapy

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Anti-capsid antibodies inhibit transduction and effectiveness of AAV gene therapy, creating a major hurdle in the transformative potential of these therapies, as antibody-positive individuals are often excluded from AAV GT programs. Parameters that may impact transduction include antibody titer, serotype, dose, route of administration, and percentage of full capsids. Each sponsor must create an assay, define the specifications, and test against the individual product for efficacy. In addition, the anti-capsid antibody assays are generally not publicly available for people with these conditions, creating challenges for families to understand and compare between treatment options. Altogether, this has resulted in a real "seropositivity dilemma".

To explore the impact of seropositivity further, we characterized the total binding antibody titers in individuals with Ornithine Transcarbamylase Deficiency (OTC), Glycogen Storage Disease type Ia (GSDIa) and Wilson Disease (WD), as these are the populations that are under investigation for DTX301, DTX401 and UX701 AAV gene therapies, respectively. We also performed an AAV gene therapy study in cynomolgus macaques that were all neutralizing antibody negative, but some animals were total antibody positive.

Six of 31 (19.4%) evaluable individuals with GSDIa had detectable anti-AAV8 titers, with levels ranging from 1:47 to 1:6275. Eight of 17 (47.1%) evaluable individuals with WD had detectable anti-AAV9 titers, with levels ranging from 1:36 to 1:4854. The one individual with OTC did not have detectable anti-AAV8 titers.

A cynomolgus macaque intravenous AAV gene therapy study was conducted to determine the impact of dosing total antibody (TAb)-positive (+) or TAb-negative animals at two doses (1e13 Vector Genome (VG)/kg and 7e13 VG/kg). Animals were pre-screened and neutralizing antibody (NAb)-negative prior to study entry. In the 1e13 VG/kg group, four animals were TAb seronegative, and two had titers of 1:200. In the 7e13 VG/kg group, three animals were TAb seronegative, and three had titers of 1:100, 1:1600, and 1:12,800. Following AAV GT administration, no clinical observations were noted, and there was no apparent change in platelets eight days after dosing. Whole blood vector genome levels were lower in the TAb+ group compared with the TAb-negative group at the 1e13 VG/kg dose; there was no difference in the 7e13 VG/kg dose TAb groups. All animals had detectable VG DNA and mRNA in the liver.

We demonstrated that individuals with GSDIa and WD may have a 3-log range of pre-existing anti-AAV titers. In cynomolgus macaques, we demonstrated that AAV GT can deliver VG to the liver of TAb+ animals. Additional work to assess the safety of dosing TAb+ humans with AAV GT is needed. These data support further investigation into more frequent AAV TAb testing and will inform discussions about the possibility of dosing low TAb+ individuals in future AAV GT trials.

1000 tRNA at the Frontier: Developing Medicines to Treat Stop Codon Disease

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Alltrna is developing a class of engineered transfer RNA (tRNA) based drugs that read through nonsense mutations, also known as premature termination codons (PTCs), and re-introduce the wildtype (WT) amino acid into the growing protein chain during translation. PTCs within an mRNA prevent synthesis of the full-length WT protein often resulting in severe disease due to complete loss, diminished or altered protein function. Nonsense mutations are the underlying cause of approximately 10% of all genetic diagnoses in humans. The fact that a given nonsense mutation can be found in multiple disease-causing genes opens the possibility of developing a drug to target PTCs across multiple indications. In recent years, tRNAs engineered to read through PTCs and restore continued synthesis of the polypeptide chain have gained attention as an emerging novel therapeutic approach to potentially unify the treatment of thousands of rare and ultrarare PTC diseases, also known as Stop Codon Disease, including liver diseases with high unmet needs.

To develop tRNA therapeutics, Alltrna has built a unique platform based on (i) high-throughput screening of engineered tRNA oligonucleotides combining optimized sequence and chemical modification patterns and (ii) rational and machine learning-guided design principles. In parallel, we have developed clinically relevant disease

mouse models to assess tRNA performance and delivery to the relevant target tissue and characterize pharmacokinetic and pharmacodynamic relationships that can support preliminary human dose predictions.

Preclinical data demonstrated productive restoration of full-length functional protein in vitro and in vivo, confirming preclinical proof of concept. Preclinical data studying Alltrna's drug candidate AP003, a chemically modified, engineered tRNA oligonucleotide (tRNA-3) in a lipid nanoparticle (LNP), showed that a single dose of AP003 tRNA leads to robust *in vivo* restoration of protein production to clinically meaningful levels in two transgenic Stop Codon Disease mouse models, methylmalonic acidemia (MMA) and phenylketonuria (PKU).

In the MMA mouse model, a single dose of the tRNA-3 in a LNP delivery system restored in vivo protein levels of methylmalonyl-CoA mutase (MMUT) over 25% of wild type levels at Day 4, well above the 1-2% protein rescue considered to be clinically meaningful in MMA by key opinion leaders.

In the PKU mouse model, a single dose of AP003 demonstrated phenylalanine hydroxylase (PAH) restoration to 7% of wild type levels within 72 hours, exceeding the clinically relevant 3% protein rescue target defined by key opinion leaders. Further, this PAH protein restoration resulted in a meaningful 76% reduction of phenylalanine (Phe) levels from baseline, as PAH is the enzyme responsible for breaking down Phe. When PAH is mutated or deficient, Phe accumulates to toxic levels, causing the hallmark elevated Phe levels observed in PKU.

Additionally, tRNA levels and dose response data were collected through several preclinical studies that together form the basis of translational PKPD models that are used for preliminary human dose predictions in support of clinical trial design.

Enabled by the development of a tRNA platform, Alltrna's preclinical data with AP003 show the potential of tRNA therapeutics as a novel drug modality for the treatment of Stop Codon Disease patients across multiple rare diseases in liver

1001 AAV-mediated Gene Knock-down and Replacement Therapy for PKU was Effective with High Efficacy and Low Dosage

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Background: Phenylketonuria (PKU) is an autosomal recessive disorder of amino acid metabolism caused by mutations in the phenylalanine hydroxylase (PAH) gene, causing impaired phenylalanine (Phe) metabolism. The standard care for PKU requires life-long strict dietary restrictions, whose compliance is poor. Attempts to improve the life-quality of PKU patients have met with limited successes. Restoring PAH in 10 to 20% hepatocytes in mice is sufficient to normalize plasma Phe concentration, but clinical trials with gene therapies have not been very successful. Because PAH functions as a tetramer and most of the PAH mutations in PKU patient are missense mutations instead of null mutations, we reasoned that we should reduce endogenous mutant PAH at the same time when we introduce exogenous wild type (WT) PAH for gene therapy. **Results**: We utilized two PKU mouse models, the PAH-KO (PAH null) and ENU2 (PAH F263S mutant) models. When we introduced PAH-expression only vector to these mouse models, the magnitude of Phe decreases in PAH-KO mice was larger than that in ENU2 mice. Instead, when we introduced a vector to simultaneously express WT PAH and silence mutant PAH, the magnitude of Phe decreasing in ENU2 was slightly larger than that in PAH-KO mice. These results support a novel approach for PKU treatment. After rounds of vector optimization, we obtained GS1168 as our candidate. GS1168 uses a single AAV to simultaneously expresses a therapeutic RNAi-resistant PAH protein and a miRNA to knocks down the endogenous mutant gene. In the ENU2 male mouse models, we achieved the therapeutic effects (Phe<360uM) at a dosage as low as 3E11 vg/kg. We also observed a dose and sex dependent effect after a single injection of GS168. Toxicological studies showed GS1168 was safe and well-tolerated in both mice and non-human primates (NHP). **Conclusions**: By reducing the mutant PAH at the same time of introducing a functional PAH, GS1168 provides an effective AAV-based gene therapy for PKU. Our innovative strategy sets GS1168 apart from existing gene therapies, offering new hope to improve the quality of life of PKU patients.

1002 LY-M003, An AAV Gene Therapy Product with Copper Responsive Engineering of ATP7B Expression (CREATE) for Wilson Disease

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Wilson disease (WD) is a rare autosomal recessive metabolic disorder primarily caused by mutations in the human *ATP7B* gene, located on chromosome 13 (13q14.3). This genetic aberration results in diminished ATP7B activity, leading to the progressive accumulation of copper in vital organs, including the liver, brain, and kidney. The consequential organ damage underscores the severity of WD, manifests as multi-organ involvement and a deteriorating clinical presentation. The fundamental approach to addressing Wilson's disease involves restoring ATP7B protein function. LY-M003, designed to hepatic-specifically produce a modified ATP7B^{LY} protein in a copper-dependent manner, emerges as an investigational adeno-associated virus (AAV)-based gene therapy for Wilson's disease.

A novel modified ATP7B^{LY} protein was optimized through exhaustive screening from single MBD to maximum four MBD combinations with nucleotide sequence optimization, and validated to remain copper-dependent intracellular translocation and exhibit comparable functionality to the full-length wild-type ATP7B. LY-M003 emerges as an investigational AAV8-based gene therapy intended for Wilson Disease, employing Copper Responsive Engineering of ATP7B Expression (CREATE) scheme to regulate the expression of the ATP7B^{LY} in a copper dependent manner. *In vitro* study demonstrated that LY-M003 could controllably express ATP7B^{LY}, offering a finely tuned response to cellular copper levels, and displayed high level of copper exportation capacity. LY-M003 was further found to efficiently protect hepatocytes from copper mediated toxicity and restore ceruloplasmin synthetical pathway, indicating a successful reversal of abnormal copper metabolism.

Preclinical efficacy studies were conducted in *Atp7b*$^{-/-}$ mice and *Atp7b*$^{M1/M1}$ mice models, both of which were confirmed detrimental copper accumulation in liver, abnormally high urinary copper excretion, and deficient serum ceruloplasmin activity. Comparative analyses demonstrated that LY-M003 with CREATE scheme exhibited more potent therapeutic effect than the corresponding control with regular hepatic-specific promoter in male *Atp7b*$^{-/-}$ mice. In *Atp7b*$^{M1/M1}$ mice, treatment with LY-M003 brought hepatic-specific ATP7B^{LY} expression and restoration of fecal copper excretion pathway. Pharmaceutical study of LY-M003 dose-dependently resulted in decrease of hepatic copper level, reduction of 24-hour urinary copper level and normalization of serum ceruloplasmin activity following a 13-week therapeutic regimen. Moreover, LY-M003 could stably expression ATP7B^{LY} and exhibit efficacy in *Atp7b*$^{M1/M1}$ mice model up to 8 months. Toxicology studies of LY-M003 were conducted in both SD rats and Cynomolgus macaques with dose-ranging investigation, the results of which demonstrated high safety and tolerability of LY-M003. Significantly, copper metabolism in healthy animals remained unchanged after LY-M003 administration.

The comprehensive preclinical data strongly support the safety and long-term therapeutic efficacy of LY-M003, warranting the further study of human clinical trials. An Investigator Initiated Trial (IIT) to evaluate the safety and efficacy of a single intravenous infusion of LY-M003 in patients with WD is ongoing.

1003 Mitochondria Targeted rAAV Gene Therapy for Cytochrome C Oxidate Deficiency

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Cytochrome c oxidase 20 (COX20) deficiency is a rare autosomal recessive disorder caused by mutations in the *COX20* gene. The COX20 protein functions as a chaperone, stabilizing the COX2 subunit on the inner mitochondrial membrane, which is critical for assembling mitochondrial complex IV and maintaining respiratory

chain function. To date, over 30 cases have been reported, with patients experiencing symptoms including sensory neuropathy, early onset ataxia, dysarthria, hypotonia, dystonia, and ophthalmoplegia. Despite the growing recognition of this disorder, its underlying pathomechanism remains poorly understood. Additionally, the absence of Cox20 animal models that replicate human disease phenotypes has impeded the establishment of an effective therapeutic intervention. In this study, we developed a recombinant adeno-associated virus (rAAV)-based gene therapy for COX20 deficiency and assessed its efficacy in a newly engineered COX20-deficient mouse model.

Our first-generation rAAV9-hCOX20 vectors utilized the ubiquitously active chicken beta-actin (CB) promoter and expressed one of the two isoforms of the human *COX20* gene (201 and 203). hCox20 expressed from rAAV9.CB-hCOX20 localized to mitochondria and rescued the mitochondrial defects in COX20 deficient HEK293T cells, as demonstrated by Western blot and Seahorse Mito stress tests, suggesting its proper functionality. In a proof-of-concept study, rAAV9.CB-hCOX20 vectors were administered intravenously to neonatal wild-type mice. Mitochondrial localization of the hCOX20 protein in the brain, liver, and heart was confirmed by immunofluorescence analysis. Safety evaluations including complete blood count (CBC), basic metabolic panel (BMP) blood tests, and histological analysis conducted six months post-treatment revealed no pathological findings. rAAV9.CB-hCOX20 treated wild-type mice exhibited a reduction in body weight compared to untreated mice, possibly due to increased complex IV activity and energy consumption associated with hCox20 overexpression driven by the CB promoter. To avoid the risk of unintended side effects resulting from excessive protein expression, we developed second-generation rAAV9-hCOX20 vectors using endogenous COX20 regulatory elements derived from human COX20 promoters, which are currently being evaluated for their expression and toxicity profiles.

Given the embryonic lethality caused by complete COX20 loss, we generated a conditional COX20 knockout mouse model. Induction of ubiquitous *COX20* knockout resulted in rapid body weight loss starting around day 40 post-tamoxifen injection, followed by phenotypes including abnormal hair appearance, kyphosis, and cataract, ultimately leading to death between 80 to 100 days post-induction. To evaluate therapeutic efficacy, both first- and second-generation rAAV9-hCOX20 vectors were delivered at different stages of disease progression, and their ability to prevent or reverse disease phenotypes is currently being studied.

In summary, our ongoing study presents a potential and safe rAAV-based gene therapy approach for COX20 deficiency and suggests that improved specificity with an endogenous promoter might be advantageous. Additionally, this work may contribute to uncovering the mechanisms underlying other mitochondrial-related disorders.

1004 Correction of a Recurrent Pathogenic Variant in Methylmalonic Acidemia Using Adenine Base Editing

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Methylmalonic acidemia (MMA) is a recessive genetic disease caused by variants in *MMUT* (mitochondrial enzyme methylmalonyl–CoA mutase) or by defects in transport or metabolism of *MMUT* cofactor (5' deoxyadenosylcobalamin), including the *MMAB* gene. MMA is diagnosed by routine newborn screening, and the first metabolic decompensation event typically occurs within the first few days of life, when toxic compounds accumulate in the blood. MMA can result in serious complications, such as an enlarged liver, kidney failure, vision problems, and intellectual and motor disability. The current standard of care is liver transplantation, yet patients typically need to reach at least one year of age to undergo the procedure, and elevated toxic metabolites often remain even after transplantation. Thus, there is a pressing need for improved therapeutic strategies to treat patients with MMA. Previous studies suggest that there is a survival advantage of cells with corrected alleles in the livers of patients with MMA, and that correction in the liver may provide enough functional enzyme to slow or halt disease progression. Here, we show corrective editing of a recurrent pathogenic variant (*MMAB* R186W) using adenine base editing in cell lines containing the pathogenic variant.

First, we developed a cell line using the human hepatoma HuH-7 cell line as a background and containing four pathogenic variants for MMA, all of which are amenable to adenine base editing. These variants were inserted into the HuH-7 cells via lentiviral vector transduction. We tested a variety of adenine base editors (ABEs) in combination with different guide RNAs (gRNAs) and selected the best performing pairs. For one of the recurrent variants, *MMAB* R186W, we achieved substantial corrective editing (≈30%) with minimal bystander editing using standard plasmid transfection. mRNA transfection using the best performing ABE/gRNA pair yielded robust editing at the desired locus (≈76%). The other three variants displayed less corrective editing and more bystander editing upon initial screening, so we prioritized *MMAB* R186W to pursue a therapeutic ABE strategy. In parallel, we have been developing a cell line that incorporates this pathogenic variant into the endogenous *MMAB* locus via prime editing, with a plan to validate our best performing ABE/gRNA pair. To assess off-target editing at other locations in the genome with the ABE/gRNA pair, we used ONE-seq to generate a list of candidate genomic sites and are in the process of verifying/ruling out the list of nominated sites in hepatocytes.

Once the ABE/gRNA pair has been validated *in vitro* using cell lines, we will dose a humanized murine model containing the human pathogenic variant with the ABE/gRNA pair encapsulated in lipid nanoparticles (LNPs). We plan to measure levels of on-target, bystander, and off-target editing in the mouse model via next generation sequencing (NGS), assess for improved survival, and measure biochemical markers of MMA such as serum levels of methylmalonic acid and propionylcarnitine.

While *in vivo* gene editing may provide an elegant solution for the correction of many pathogenic variants associated with metabolic and other genetic diseases, there are currently no approved *in vivo* gene editing therapies in the clinic. If results from our murine studies and off-target analyses are favorable, this study will provide a new approach to treating a rare genetic metabolic disease.

1005 AAV9 -Mediated Gene Therapy of MMAB Deficiency: Preclinical Efficacy in Mouse models of Cobalamin B Type Methylmalonic Acidemia

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Introduction: Cobalamin B-type (*cblB*) methylmalonic acidemia (MMA) is a rare and severe autosomal recessive inborn error caused by pathogenic variants in the MMAB gene, which encodes the enzyme ATP:cob(I)alamin adenosyltransferase (ATR). ATR catalyzes the synthesis of 5'- deoxyadenosylcobalamin (AdoCbl), the essential cofactor for methylmalonyl-CoA mutase (MMUT). MMAB deficiency can be clinically severe, characterized by lethality and severe biochemical perturbations, with massively elevated methylmalonic acid levels in the blood. Despite medical and dietary management, some patients require elective liver or liver-kidney transplantation. There are currently no established therapies for MMAB deficiency, which is an indication for the Platform Vector Gene Therapy (PaVe-GT) Program.

Methods: We studied a cohort of 17 patients with MMAB deficiency identified through the natural history study of MMA (NCT00078078). Two severe mutations, the orthologue of a recurrent missense mutation in patients who received LT/LKT (p.Arg186Trp), and a deletion of exons 3-7 to mimic null phenotype, were engineered into the *Mmab* locus. Additionally, a germline rescue transgene was created to restore MMAB expression in the skeletal muscle under the control of the murine muscle creatine kinase (MCK) promoter, knocked into the ROSA26 locus ($Tg^{MCK\text{-}Mmab}$), generating a hypomorphic model ($Mmab^{D3\text{-}7/D3\text{-}7}$; $Tg^{MCK\text{-}Mmab}$). AAV vectors expressing *MMAB* under the EF1L or EF1s promoters in either single-strand (ss) or self-complementary (sc) genome configurations (NCATS-BL2680), respectively, were tested for in vivo efficacy.

Results: $MMAB^{R180W/R180W}$ mice exhibited growth retardation, lethality around weaning, and massively elevated plasma methylmalonic acid (pMMA) levels (1777 +/- 693 uM, 1399 x WT), resembling patients with severe forms of *cblB* MMA. $Mmab^{D3\text{-}7/D3\text{-}7}$;$Tg^{MCK\text{-}Mmab}$ mice survived with near-normal growth but exhibited significant MMAemia (496+/- 91 uM, 203x Het). Neonatal or adolescent treatment of $MMAB^{R180W/R180W}$ mice with AAV

vectors (systemically delivered at a dose of 1E11 GC/pup on DOL1 or 1E13 GC/kg on DOL 21) resulted in complete rescued from lethality (Figure 1), normalized body weight, and reduced pMMA levels.

Young adult Mmab$^{D3\text{-}7/D3\text{-}7}$;Tg$^{MCK\text{-}Mmab}$ mice treated with either vector at a dose of 1E13 GC/kg showed substantial reductions in pMMA levels (27 +/- 6.7 uM for ss, 18 +/- 4.9 for sc at 6 months post treatment) and restoration of ^{13}C propionate oxidation to wild type levels. In contrast, treatment with 1E12 GC/kg revealed different outcomes: sc-treated mice showed similar reductions in pMMA (33 +/- 7.5 uM) as those treated with 1E13 GC/kg ss vectors, while ss treatment at 1E12 GC/kg had a minimal effect (330 +/- 112.5 uM) (Figure 2). Older mice treated with 1E12 GC/kg sc vectors demonstrated marked improvement in hepatic pathology.

Conclusions: We have developed a series of clinically relevant MMAB animal models and demonstrated that AAV9-mediated gene therapy can rescue survival, reduce of pMMA levels, and improve pathological outcomes. Notably, sc AAV vectors provided superior correction compared to ss vectors at low doses. These models provide a platform for neonatal and adult proof-of-concept therapeutic studies and hold promise for advancing AAV gene therapeutics for MMAB deficiency, a severe form of MMA.

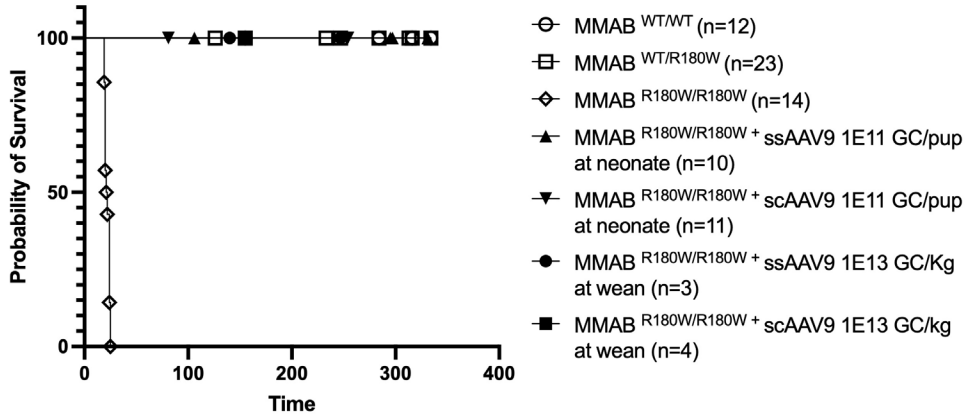

Figure 1

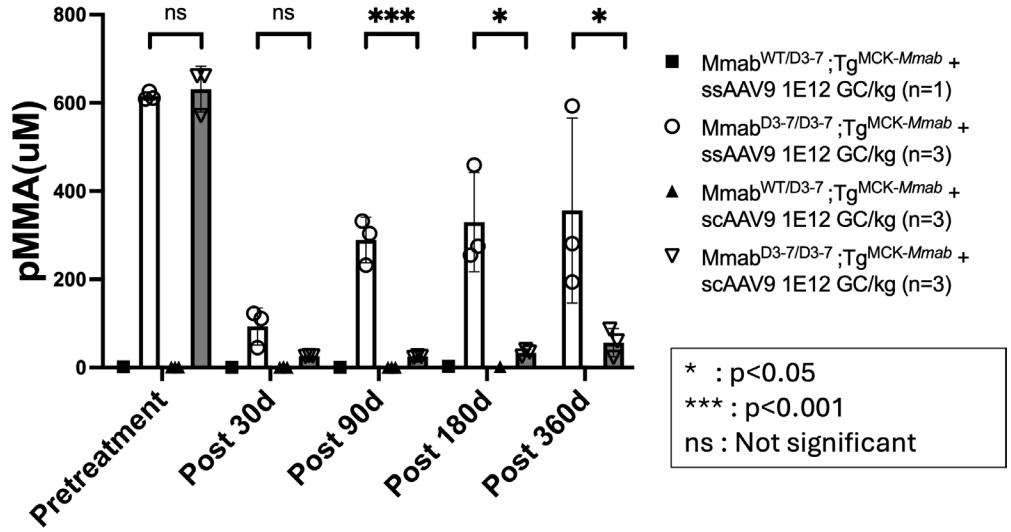

Figure 2

1006 The Mouse Peroxisome Research Resource for Testing Gene and Cell-based Therapies

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Peroxisomes are metabolic membrane-bound organelles that play essential roles in the development and physiological functions of all mammalian organ systems. This is exemplified by Zellweger spectrum disorder (ZSD), a group of rare multisystemic disorders caused by inherited defects in peroxisome assembly, structure, and replication. ZSD and X-linked adrenoleukodystrophy, the most common inherited peroxisomal disorder, can be detected by newborn screening prior to irreversible organ damage in many patients. This provides therapeutic windows for targeted gene and cell-based therapies to address unmet needs of patients. Nevertheless, the limited number of well-annotated publicly available mouse models and their often-suboptimal genetic backgrounds has slowed the pace of translational peroxisome-related research. Here, we discuss recent progress made by the NIH-funded Mouse Peroxisome Research Resource (MPRR), a central resource for mouse models for basic and translational research relevant to peroxisome biology and disorders caused by peroxisome dysfunction. This community-driven effort leverages expert knowledge of mouse model development, gene editing, and peroxisome biology to accelerate the generation, distribution, and proper use of high-impact mouse models for peroxisome research. All deposited mouse models are on standardized genetic backgrounds and publicly available as well-annotated resources with minimal required legal restrictions. The models produced or in production include strains expressing reporter proteins to visualize peroxisomes in mouse tissues or immunopurify peroxisomes from cell extracts. Furthermore, a host of conditional knockout alleles and single copy CAG-lox-stop-lox (LSL) regulated human cDNA transgenes for the tissue-specific elimination or tissue-specific over-expression of specific peroxisomal genes, respectively, have been generated. Mice with humanized copies of whole genomic regions encompassing peroxisome genes in safe harbor sites are also under development. The MPRR captures baseline data such as measurement of peroxisomal metabolite levels, including those of very long-chain and branched-chain fatty acids in addition to lifespan determinations of these new models. Overall, the MPRR has substantially increased the number of available mouse models for peroxisome research and accelerated preclinical testing of rationally designed therapeutic interventions for disorders caused by peroxisome dysfunction.

1007 Systemic lentiviral and AAV gene therapies correct several metabolic imbalances in the brain of the maple syrup urine disease mouse model

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Maple syrup urine disease (MSUD) is an inborn error of metabolism that is caused by mutations in any of the constituents of the branched chain ketoacid dehydrogenase complex, thereby impairing the metabolism of branched chain amino acids (BCAAs). It is associated with severe neurologic sequalae, including intellectual disabilities, seizures and ataxia. We have investigated the efficacy of VSVg-pseudotyped lentiviral (LV) and AAV9 vectors in the intermediate MSUD mouse model, which is a knock-out (KO) for the endogenous *Dbt* gene. Both vectors encode a codon-optimised human DBT transgene under the control of a synthetic liver-specific promoter, and were administered intravenously to pups at P1-P2 at doses of 1.4E11 TU/kg (n=9) and 1.0E14 vg/kg (n=9), respectively. The mice were sustained on diets with normal protein content.

Both therapies significantly ameliorated the general phenotype. Untreated and AAV-treated KOs had median survivals at P20 and P73 respectively, whereas seven of the LV-treated KOs survived beyond the study's P120 endpoint. LV-treated animals at P120 demonstrated complete normalisation of speed (15.2±1.2 vs 15.0±0.6 cm/s;

p>0.9999), hind limb swing speed (52.0±1.9 vs 53.6±1.2 mm/s; p=0.9712), stride length (5.0±*0.2* vs 5.5±0.2 cm; p=0.1839) and regularity index (89.3%±0.7% vs 90.0%±1.6%; p>0.9999) compared to wildtypes (WTs), respectively. LV lowered plasma leucine, isoleucine and valine levels in KOs by 54% (p<0.0001), 35% (p=0.0074) and 52% (p<0.0001), and AAV by 37% (p=0.0012), 44% (p=0.0006) and 36% (p=0.0003), respectively. In adult subjects that survived past the study's mid-timepoint of P60, LV and AAV were found to have mean hepatic vector copy numbers of around 0.6±0.1 and 9±2 vector copies per cell, which mediated expression at around 26%±4% and 8±3% of WT *DBT* mRNA levels, respectively.

Metabolomic brain analysis revealed that these therapies corrected many of the MSUD-associated bioenergetic imbalances. Although most glycolytic intermediates remained stable in untreated KOs, levels of around 35% of 17 TCA cycle-related metabolites including pyruvate were significantly altered in P15-P20 subjects. While only one of 66 fatty acids (1.5%) was significantly affected at that timepoint, around 33% of 42 fatty acyl carnitine and CoA ester, 41% of 46 sphingolipid and 14% of 236 phospholipid metabolite reads exhibited significant differences from age-matched WTs. LV- and AAV- treated KOs in contrast exhibited significant differences from adult WTs in around 0% and 12% of TCA cycle, 7% and 12% of fatty acyl carnitine and CoA ester, 6.5% and 2% of sphingolipid, and 7% and 10% of phospholipid metabolite reads, respectively. These results suggest that systemic gene therapies may exert their efficacy via mechanisms beyond correction of plasma BCAA levels.

1008 AAV-Mediated Restoration of Hepatic *CYP27A1* Expression in Chenodeoxycholic Acid-Pretreated Mice with Cerebrotendinous Xanthomatosis

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Cerebrotendinous xanthomatosis (CTX) is an autosomal recessive disease resulting from mutations in the *CYP27A1* gene, which encodes the mitochondrial enzyme sterol 27-hydroxylase. This enzyme plays a critical role in the conversion of cholesterol into bile acids. Loss-of-function mutations in *CYP27A1* disrupt bile acid synthesis, leading to the accumulation of toxic intermediates such as cholestanol and 7α-hydroxy-4-cholesten-3-one (7αC4) in blood, eyes, tendons and brain. These deposits result in clinical manifestations such as infantile-onset diarrhea, juvenile cataracts, adolescent-onset xanthomas, and adult-onset neurological impairments like ataxia, dementia, and peripheral neuropathy. The standard treatment for CTX is oral administration of chenodeoxycholic acid (CDCA), which works by downregulating hepatic *CYP7A1* enzyme and the synthesis of bile acids. It effectively reduces toxic metabolite levels and alleviates symptoms but may not fully prevent neurodegeneration, highlighting an unmet medical need for improved therapeutic strategies. In this sense, liver-directed gene therapy becomes a valuable treatment alternative to improve patient outcomes. We first developed an adeno-associated virus (AAV) vector serotype 8, VTX-806(8), with high potency and low immunogenicity carrying a codon-optimized CpG-depleted *CYP27A1* sequence under the control of a liver-specific promoter. Treatment of CTX mice with 5E12 VG/kg of VTX-806(8) achieved a full correction of disease biomarkers, including circulating 7αC4 levels, expression of hepatic bile acid synthesis-related genes and hepatomegaly, as well as the levels of 7αC4 in the brain, tendons and liver. We then determined that the therapeutic dose of chenodeoxycholic acid (CDCA) able to reduce hepatomegaly, expression levels of *Cyp3a11* in the liver and 7αC4 levels in blood in CTX mice, without any associated toxicity, was 0.25% admixed in the diet (0.375-0.75 µg/g/day). After diagnosis is confirmed, CTX patients are immediately placed under treatment with the standard of care CDCA. Therefore, to mimic the conditions in which a CTX patient under CDCA could potentially be treated with VTX-806(8), an efficacy study of VTX-806(8) in mice pre-treated

with CDCA was performed and the interaction and comparison of these two therapeutic approaches were assessed. Four-week-old CTX mice were fed with 0.25% CDCA admixed in the diet for 4 weeks and then received a single intravenous administration of VTX-806(8) at 5E12 VG/kg. Four weeks later, CDCA was removed in a group of mice and maintained in another group. Healthy animals and untreated CTX animals, as well as groups treated with each of the therapies alone were included as controls. The levels of serum disease biomarkers were followed over time in all the groups of mice to assess and compare the efficacy of the different therapeutic regimens. Moreover, hepatomegaly, expression of *Cyp7a1* and *Cyp3a11* and the levels of 7αC4, cholestanol and 27-hydroxycholesterol in the brain were quantified after sacrificed at 16 weeks of age. In conclusion, our study determined the therapeutic dose of CDCA for CTX mice to be 0.25% admixed in the diet and evaluated the efficacy of VTX-806(8) in combination with CDCA treatment. This comprehensive approach allowed for a thorough comparison of different therapeutic regimens, providing valuable insights into the interaction of CDCA and gene therapy treatments, mimicking potential clinical scenarios for CTX patients.

1009 Improvement of Progressive Familial Intrahepatic Cholestasis Type 2 Via AAV-Mediated Hepatic BSEP Expression in Mice

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Progressive familial intrahepatic cholestasis type 2 (PFIC2) is an uncommon genetic condition inherited in an autosomal recessive manner, resulting from alterations in the *ABCB11* gene. This gene encodes the hepatic bile salt export pump (BSEP), which transports bile salts (BS) from hepatocytes to the bile canalicular lumen. BSEP deficiency or dysfunction leads to impaired BS excretion and their buildup in the liver, causing severe hepatic damage. PFIC2 patients typically exhibit symptoms in early childhood, including cholestasis, growth retardation, jaundice, hepatomegaly, and pruritus. Many develop early-onset fibrosis and end-stage hepatic disease, with an elevated risk of developing hepatocellular carcinoma. Treatment options include symptom management, surgical bile diversion, or liver transplantation, which is currently the only curative option. We demonstrated the safety and therapeutic benefits of adeno associated virus (AAV)-mediated liver directed gene therapy for the treatment of PFIC2 in an *Abcb11*$^{-/-}$ mouse model of PFIC2. After developing VTX-802(8), an AAV8 vector containing a codon-optimized human *ABCB11* sequence controlled by a liver-specific promoter, a dose range finding study of the therapeutic vector was performed. Five-week-old female PFIC2 mice received a single intravenous injection of VTX-802(8) at three different doses and the therapeutic efficacy was monitored up to five months of age. Treated mice showed a dose-dependent improvement in serum transaminase levels maintained over time. These mice also exhibited partial but significant correction of hepatomegaly, and increased BS transport from the liver to bile and small intestine at 15 weeks post-injection, indicating partial restoration of normal bile secretion. These findings suggest that VTX-802(8) may have therapeutic potential for PFIC2 patients. Current treatment with IBAT inhibitors limit treatment to certain patient profile and specific mutations, therefore combination with gene therapy could improve treatment efficacy to expand population. Developing a long-lasting cure for PFIC2 by restoring long-term hepatic BSEP expression and physiological bile secretion would provide a transformative option for these pediatric patients with significant unmet medical needs.

1010 New murine models of propionic acidemia caused by PCCB deficiency allow for the testing of genomic therapies in both severe and mild disease phenotypes

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Propionic acidemia (PA) is rare autosomal recessive metabolic disease caused by defects in the mitochondrial localized enzyme propionyl-CoA carboxylase (PCC). This enzyme is composed of six α- and six β-subunits with causative variants occurring at equal frequencies in either the nuclear encoded *PCCA* or *PCCB* genes. PA patients can suffer from stunted growth, potentially lethal metabolic crises, cardiomyopathy, and chronic kidney disease. The poor prognosis for PA patients has led to the exploration of gene therapy as a new treatment option. To explore the etiology and to test new therapies for PA, we have developed and characterized 5 new murine models of PCCB deficiency. Gene editing of the 7th and 14th exon of the murine *Pccb* gene was used to create loss of function alleles: an 8 bp deletion in the 7th exon, and a 4 bp deletion in the 14th exon. Both alleles result in a frameshift and premature stop codon in the *Pccb* gene and no detectable PCCB protein. $Pccb^{E7del/E7del}$ and $Pccb^{E14del/E14del}$ homozygotes manifest an immediate neonatal lethal phenotype accompanied by elevations of disease related metabolites, 2-methycitrate (2-MC) and 3-hydroxypropionate (3-OHP). Because of the severe lethality displayed by the homozygous *Pccb* frameshift stop mutations, we designed a germline transgene to express the murine *Pccb* cDNA under the control of a muscle specific promoter ($Tg^{Mck\text{-}Pccb}$) and bred to rescue the $Pccb^{E14del/E14del}$ mice. The resultant $Pccb^{E14del/E14del}$;$Tg^{Mck\text{-}Pccb}$ animals are rescued from neonatal lethality but perish by 2 months of age. These mice express low levels of PCCB only in the skeletal and cardiac muscle, and have milder biochemical perturbations of 2-MC and 3-OHP in comparison to the $Pccb^{E14del/E14del}$ mice but are severely growth retarded, very much like patients with PA. To generate a murine model with a late onset form of PA, we used gene editing to insert the c.C689T (p.P230L) mutation into the murine *Pccb* gene. PA patients who are homozygous for the orthologous missense mutation in the human *PCCB* c.C683T (p.P228L) are mildly affected, but symptomatic when stressed. Subsequently, $Pccb^{P230L/P230L}$ homozygous were bred to $Pccb^{WT/E7del}$ mice to create compound heterozygous $Pccb^{P230L/E7del}$ mice. Both $Pccb^{P230L/P230L}$ and $Pccb^{P230L/E7del}$ mice express a dysfunctional PCCB protein, display attenuated metabolic changes and less impaired growth in comparison to $Pccb^{E14del/E14del}$;$Tg^{Mck\text{-}Pccb}$ mice. However, a lethal metabolic crisis can be induced when the $Pccb^{P230L/P230L}$ or $Pccb^{P230L/E7del}$ mice ingest an increased protein diet. To demonstrate the utility of these models to test new therapies, $Pccb^{E14del/E14del}$ were treated with AAV-PCCB at a dose of 1e11 vg/pup at birth, which resulted in a significant increase in survival in comparison to untreated mutant controls ($p<0.01$). Similarly, when $Pccb^{E14del/E14del}$;$Tg^{Mck\text{-}Pccb}$ mice were treated at weaning with a dose of 1e14 vg/kg of AAV-PCCB , there was a significant increase in survival and growth in comparison to untreated controls. These new murine models replicate the clinical and biochemical spectrum of PA, and can be used to test the effects of new genomic and small molecule therapies targeting both mild and severe forms PA caused by PCCB deficiency.

1011 AAV capsid prioritization in normal and steatotic human livers maintained by machine perfusion

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Adeno-associated virus (AAV) vectors have revitalized gene therapy. In the liver, AAV vectors proved to be effective and safe for hepatocyte-targeted gene therapy of hemophilia A and B, with vectors produced with the AAV5 capsid being the first to receive FDA approval. However, whether the reach of AAV gene therapy can be extended to other liver diseases is uncertain because most require a higher fraction of hepatocytes to be transduced than hemophilias, which cannot be safely achieved by vector dose escalation. Using clinical trial data to predict the hepatocyte transduction efficiency of AAV capsids in the human liver is challenging because of variability in vector dose and design, and lack of liver biopsies. Similarly, even advanced preclinical models, such as mice repopulated

with human hepatocytes or non-human primates, produce contradictory results. Moreover, it is unknown whether AAV capsids target functionally distinct hepatocyte zones or other cell types in the human liver, which has implications for efficacy and safety of liver gene therapy. It is also unknown how the tropism of AAV capsids is impacted by hepatic steatosis, which affects more than a third of the global population and is a common comorbidity in gene therapy patients. To address these uncertainties, we established side-by-side comparison of AAV vectors produced with different capsids in normal and steatotic human livers maintained under physiological conditions by normothermic machine perfusion. We analyzed transduction of hepatocytes and other liver cell types at the DNA, RNA and protein level, including by single-cell RNA sequencing. Focusing on the capsids most commonly used in clinical trials, we found that AAV-LK03 transduces hepatocytes much more efficiently than AAV5, AAV8 and AAV6; AAV-LK03 is also better than AAV-NP59, which is more efficient at transducing human hepatocytes engrafted in immune-deficient mice. In normal human liver, AAV-LK03, and to a lesser extent AAV8, preferentially transduces periportal hepatocytes, a tropism lost in steatotic liver. Conversely, AAV5 preferentially transduces pericentral hepatocytes only in steatotic human liver. While AAV-LK03 is specific for hepatocytes, AAV5 and AAV8 also efficiently transduce liver sinusoidal endothelial cells. In addition, we discovered that the AAV capsid influences vector episome formation, which determines the durability of gene therapy, with AAV5 delaying concatemerization. We also found that steatosis impairs vector episome formation. Our findings inform capsid choice in clinical trials of hepatocyte-targeted AAV gene therapy, enabling advanced strategies that consider disease-specific zonation and steatosis. Moreover, our findings establish an opportunity for physiological gene therapy of hemophilia A targeting liver sinusoidal endothelial cells. Our approach facilitates the development of AAV capsids that transduce hepatocytes or other therapeutically relevant cell types in the human liver with maximum efficiency and specificity.

1012 Impact of liver fibrosis on AAV-mediated gene transfer to hepatocytes

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Liver fibrosis, characterized by the accumulation of scar tissue in response to various insults, poses a significant challenge for liver-directed gene therapy. Patients with liver fibrosis are currently excluded from clinical trials and fibrotic liver diseases are hindered to enter the clinical pipeline because intact liver architecture is postulated to be required for safe and efficient gene transfer to hepatocytes by adeno-associated viral vectors (AAV), the platform of choice for vivo gene therapy.

In this study, we investigated the biodistribution and hepatocyte transduction of AAV8-based vectors in the context of liver fibrosis. Studies in three mouse models, with induced or genetic liver fibrosis and different fibrotic patterns, revealed that AAV8 vectors transduce fibrotic livers with reduced efficiency. This reduction primarily arises from diminished vector uptake by the liver rather than vector genome loss due to hepatocyte proliferation. Additionally, liver fibrosis led to changes in blood vector clearance and biodistribution in extra-hepatic organs, with a model-specific impact on increased viral particle uptake by spleen, lung, and kidney. At the cell-type level, fibrosis resulted in decreased vector genome content in hepatocytes and increased content in non-parenchymal liver cells, as well as in splenic macrophages and dendritic cells. Finally, we demonstrated that the impact of liver fibrosis on AAV-

mediated transduction is capsid-dependent, with the AAV-KP1 variant maintaining efficient transduction in fibrotic livers comparable to non-fibrotic ones.

Overall, this work emphasizes the need for careful evaluation of AAV administration in patients with liver fibrosis and underscores the importance of more extensive safety assessments, while highlighting the potential of AAV as a platform to broaden gene therapy applications to include fibrotic diseases.

1013 Determination of the Minimum Effective Dose of AAV9-LSP-hPYGL in a Glycogen Storage Disease Type VI Mouse Model

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Glycogen Storage Disease Type VI (GSD VI), also known as Hers disease, is a rare inherited metabolic disorder caused by a deficiency or dysfunction of hepatic glycogen phosphorylase (PYGL), a key enzyme in glycogen breakdown in the liver. PYGL is activated via phosphorylation by phosphorylase kinase, converting the inactive phosphorylase b into the active phosphorylase a, which catalyzes glycogen degradation to glucose 1-phosphate (G1P). PYGL deficiency results in impaired glycogenolysis and hepatic glycogen accumulation, leading to hepatomegaly, ketotic hypoglycemia, growth retardation, elevated hepatic transaminases, hypertriglyceridemia, and hypercholesterolemia. While GSD VI is often considered a mild disorder, severe cases may progress to liver cirrhosis. Current treatment is limited to dietary management, including uncooked cornstarch and high-protein diets. The recently characterized Pygl knockout (KO) mouse model recapitulates key symptoms and pathophysiological features of the disease. Over time, hepatic glycogen accumulation in these mice leads to liver damage, inflammation, elevated hepatic transaminases, and collagen deposition, increasing the risk of liver fibrosis. In this study, we aimed to determine the minimum effective dose of an adeno-associated virus (AAV) vector carrying the human PYGL gene (hPYGL) under the control of a liver-specific promoter (LSP) and packaged into an AAV9 capsid (AAV9-LSP-hPYGL) in Pygl KO mice. Both male and female Pygl KO mice were administered AAV9-LSP-hPYGL intravenously at three doses (1E+12, 3.16E+12, and 1E+13 vg/kg), with outcomes assessed after two weeks. Results revealed a dose-dependent response with sex-specific differences. At 1E+12 vg/kg, male mice showed minor improvements, while female mice showed no noticeable effects. At 3.16E+12 vg/kg, disease phenotypes improved in males, although the effects on females were minimal. In contrast, at 1E+13 vg/kg, both male and female mice exhibited significant improvements across various disease indicators. These improvements included decreased liver size, normalized expression of PYGL, increased enzyme activity, reduced hepatic glycogen accumulation, and normalized serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Overall, our findings suggest that the minimum effective dose of AAV9-LSP-hPYGL that produces positive effects in both male and female Pygl KO mice is 1E+13 vg/kg. This study provides critical insights for optimizing AAV-based gene therapy strategies for patients with GSD VI.

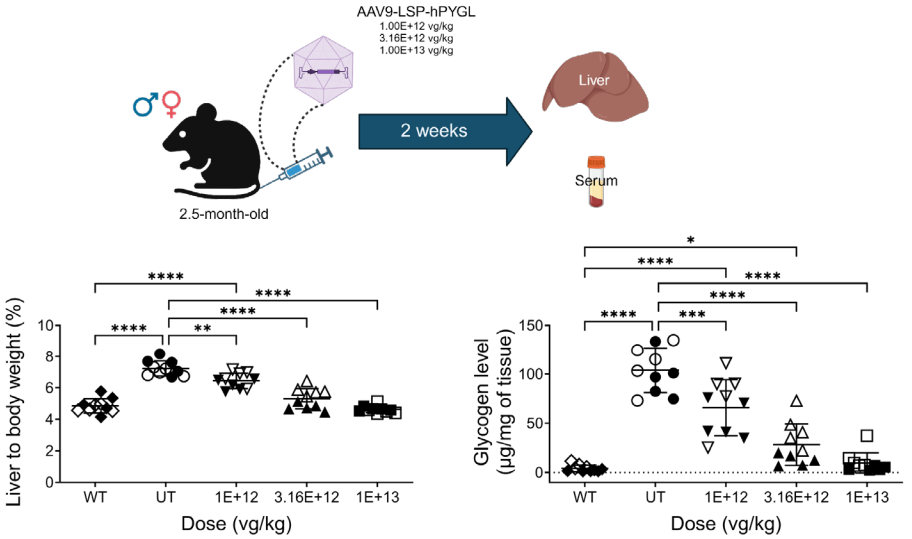

Figure Legend

2.5-month-old male (n=5, closed symbols) and female (n=5, open symbols) Pygl KO mice were intravenously administered AAV9-LSP-hPYGL via tail vein injection at three doses (1E+12, 3.6E+12, and 1E+13 vg/kg). After two weeks, liver and serum samples were collected to evaluate disease phenotypes. Age- and sex-matched untreated Pygl KO (UT) and wild-type (WT) mice served as controls. Liver size and hepatic glycogen levels decreased in a dose-dependent manner, with observed sex-specific differences.

1014 *Ass1* Targeted Gene Insertion in Newborn Mice Provides Effective, Long Term Disease Correction in a Lethal Mouse Model of Citrullinemia Type I

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Citrullinemia Type I (CTLN1) is a life-threatening urea cycle disorder impacting approximately 1 in every 57,000 live births. It is a monogenic condition arising from deficiency of argininosuccinate synthetase (*ASS1*). Left untreated, CTLN1 can lead to life threatening ammonia elevation and metabolic decompensation. Today CTLN1 is included in the newborn screening panels of all 50 states in the US, and several additional countries worldwide. These screening programs have helped support rapid diagnosis of CTLN1, enabling prompt initiation of dietary protein restriction and nitrogen scavenger therapy. However, lifelong dietary protein restriction and frequent administration of nitrogen scavenger medication pose significant adherence challenges, leaving patients at risk for periodic bouts of hyperammonemic crises and metabolic decompensation. These events lead to progressive accumulation of irreversible neurological, behavioral, and cognitive sequelae. A gene therapy approach that can be administered early in life to provide durable protection is critical to prevent the irreversible neurological manifestations associated with CTLN1.

To address known durability challenges of AAV episome-based liver gene therapies in pediatric settings, we have developed an alternative, CRISPR-based platform for hepatic gene replacement that can be administered early in life, even as the liver is undergoing rapid growth. We aimed to apply this technology to develop a durable and effective liver-directed, native locus gene insertion treatment for CTLN1 that can be administered during childhood. Our approach transiently delivers Cas9 mRNA and a guide RNA via a lipid nanoparticle, which is co-administered with an AAV that contains a promoterless human ASS1 partial cDNA. Together these components mediate targeted insertion of the ASS1 partial cDNA into the native Ass1 gene locus to restore expression of wild type ASS1 protein. Gene insertion treatment of *Ass1*^{fold} citrullinemic mice, dosed either as juveniles (P28) or as newborns (P7) resulted

in rapid, durable, and complete normalization of blood ammonia levels throughout a 20-week study period. Blood citrulline was reduced to levels seen in CTLN1 patients that have received a human liver transplant. Histological and molecular analyses of the liver revealed robust targeting and correction of the *Ass1* gene. In contrast, episomal AAV delivery of *ASS1* in newborn *Ass1*^{fold} pups failed to durably reduce blood ammonia levels. Importantly, *Ass1* native locus gene insertion led to preferential expression of ASS1 protein in peri-portal hepatocytes with high urea cycle activity, reflecting the natural *Ass1* zonation pattern of expression. Collectively, these results demonstrate that *ASS1*gene insertion may provide an effective and durable treatment that can be administered early in life to promptly halt the progression of irreversible damage caused by high ammonia levels in CTLN1.

1015 Long-term Efficacy of VTX-806(8) for the Treatment of Cerebrotendinous Xanthomatosis: Sustained Correction of Metabolic Abnormalities in Mice

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Cerebrotendinous xanthomatosis (CTX) is a rare recessively inherited metabolic disorder caused by mutations in the *CYP27A1* gene, which encodes the sterol 27-hydroxylase enzyme. This deficiency leads to impaired bile acid synthesis that results in a dramatic decrease in 27-hydroxycholesterol and to the accumulation of toxic metabolites like 7 alpha-hydroxy-4-cholesten-3-one (7αC4) and cholestanol in blood, eyes, tendons and brain resulting in a diverse array of clinical manifestations. CTX is characterized by the presence of xanthomas in multiple tissues, particularly in the brain and tendons and the early symptoms include chronic diarrhea, bilateral juvenile cataracts, and progressive neurological dysfunction. Chenodeoxycholic acid (CDCA) is the standard treatment for CTX and has shown efficacy in halting disease progression but its effectiveness in improving or stabilizing neurological symptoms varies between patients, with some individuals still experiencing neurodegeneration despite CDCA treatment. This highlights the potential need for alternative approaches such as gene therapy. We have previously shown that adeno-associated virus (AAV)-mediated gene supplementation of *CYP27A1* in the liver corrects disease biomarkers in a mouse model of CTX. VTX-806(8) is an AAV8 vector carrying an optimized expression cassette including a liver-specific promoter and a codon-optimized *CYP27A1* sequence depleted of CpG motifs to avoid the potential activation of an innate immune response. VTX-806(8) demonstrated the ability to correct hepatomegaly, normalize the expression of *Cyp7a1* and *Cyp3a11* genes and the levels of 7αC4 in blood, brain, liver and tendons of CTX mice one month after the administration of the vector. In order to evaluate the capacity of VTX-806(8) to maintain a sustained therapeutic effect, CTX male and female adult mice were treated with a single intravenous administration of the vector at the therapeutic dose of 5E12 VG/kg and the levels of serum disease biomarkers were followed over a period of 9 months, showing at 4 months a sustained normalization of 7αC4 levels. Mice were sacrificed 9 months after treatment and hepatic transduction, transgene expression, hepatomegaly, expression of hepatic genes and the levels of 27-hydroxycholesterol and toxic metabolites in tissues were evaluated.

To summarize, this study demonstrates the long-term efficacy of VTX-806(8) in a mouse model of CTX. A single administration of VTX-806(8) at 5E12 VG/kg resulted in sustained correction of serum and tissue biomarkers, reduced hepatomegaly, and restored proper expression of hepatic bile acid synthesis-related genes. These findings suggest that VTX-806(8) holds promise as a potential long-term therapeutic option for CTX, addressing the underlying genetic defect and correcting associated metabolic abnormalities.

1016 Exploring Cell-Free DNA Signatures as Biomarkers for Disease Progression in Metachromatic Leukodystrophy

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Metachromatic leukodystrophy (MLD) is a rare, inherited lysosomal storage disorder characterized by progressive demyelination and neurodegeneration. Hematopoietic stem cells gene therapy (GT) has shown remarkable success in early-onset pre-and early symptomatic patients. Unfortunately, nowadays it is difficult to predict disease progression in presymptomatic MLD patients because the clinical course cannot be followed by validated biochemical assays, challenging the clinical decision-making for treatment. Therefore, there is a strong need for the identification of novel, reliable and non-invasive biomarkers for the diagnosis and assessment of MLD disease severity.

Here we explore the role of cell-free DNA (cfDNA) as biomarker for MLD. We recently showed that cfDNA levels were at a pathological level in MLD patients compared to healthy donors (HDs) before GT ($p=0.01$, 5-fold), and these values decreased progressively to normal levels after GT, only in those patients with a positive therapeutic outcome[1]. Elevated cfDNA levels have been described in disease conditions associated with inflammation and increase in cell death rate. Therefore, the high cfDNA concentration observed in these patients may reflect the number of dying cells due to the toxic accumulation of lysosomal compounds at the different stages of disease progression. Identifying the specific cells contributing to cfDNA production could provide deeper insights into the biological mechanisms underlying tissue degeneration during disease progression. Methylation-based approaches have been utilized to determine the cellular origin of cfDNA, as cell-specific epigenetic signatures are preserved within cfDNA fragments. To determine the cellular origins of cfDNA, we applied enzymatic approaches to profile CpG methylation, followed by high-throughput sequencing. For tissue deconvolution, we refined an existing non-negative least squares model to enhance performance on low-coverage sequencing data based on cell type-specific methylation atlas. Studies performed on cfDNA samples collected from HDs demonstrate that we can reproduce published data showing that cfDNA mainly originates from leukocytes (80%)- particularly granulocytes, monocytes/macrophages, and megakaryocytes. To demonstrate the accuracy and specificity of our approach, we analyze cfDNA profiles from patients with hematological malignancies, revealing a clear shift in cellular contributions compared to HDs. Indeed, patients with T-cell leukemia or B-cell tumors showed a marked increase in fragments derived from T (50% T-frag vs. 2% in HD) and B-cell (30% B-frag. vs. 0.5% in HDs), respectively. Our preliminary analysis of 4 untreated MLD patients showed a significantly different pattern compared to HDs, with neuronal and oligodendrocyte derived-fragments present at higher relative ($p=0.03$, 4-fold) and absolute levels ($p=0.01$, 25-fold), reflecting damage in these cell types associated to disease pathology. Further studies in larger cohorts of pre- and symptomatic patients associated with different disease variants and monitored overtime will be required to validate these findings and establish cfDNA as a predictive biomarker in MLD. If validated, this approach may provide a non-invasive tool that can be used to monitor disease progression, guide clinical intervention to improve patient care.

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1017 Development of a Durable Gene Therapy for Targeting CNS and Visceral Pathologies in Acid Sphingomyelinase Deficiency

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Acid Sphingomyelinase Deficiency (ASMD) is a lysosomal storage disorder caused by mutations in the sphingomyelin phosphodiesterase (*SMPD1*) gene. The resulting deficiency of ASM activity leads to an accumulation of sphingomyelin in both visceral and central nervous system (CNS) tissues. ASMD patients exhibit visceral manifestations in the liver, spleen, lung, and heart, as well as severe, progressive neurodegeneration. The current enzyme replacement therapy (ERT), Olipudase alfa, is limited to treating non-CNS pathologies due to its inability to cross the blood-brain barrier (BBB). We have developed a novel BBB-crossing antibody-targeted therapeutic by fusing the ASM enzyme with a single chain variable fragment (scFv) that binds to transferrin receptor 1 (TfR1). Hepatocyte-specific gene insertion of this anti-TfR1scFv fusion results in the expression and secretion of the fusion enzyme into the bloodstream, facilitating transcytosis and uptake into both visceral and CNS tissues. Our results demonstrate that liver insertion of anti-TfR1:ASM significantly reduces sphingomyelin accumulation in the brains of ASMD mice, an effect not observed with untargeted ASM. Additionally, anti-TfR1:ASM gene therapy reduces sphingomyelin accumulation in the heart, spleen, and lungs. Anti-TfR:ASM expression also results in robust improvements in behavioral performance as measured by rotarod and balance beam, in addition to preventing cerebellar neurodegeneration. This innovative gene therapy approach not only reduces the need for frequent ERT infusions required by the current standard-of-care but also effectively targets and mitigates CNS deficits in ASMD, making it a potential therapeutic to address unmet need in neurovisceral ASMD.

1018 Engineering T Lymphocytes for Enzyme Replacement Therapy

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Background: Enzymopathies are metabolic disorders caused by enzyme deficiencies or dysfunctions, leading to toxic substrate accumulation and metabolic imbalances. Among these, lysosomal storage diseases (LSDs) result from defective lysosomal enzymes, causing progressive, multi-system pathology. Mucopolysaccharidosis type I (MPS I) is a severe LSD caused by a deficiency in alpha-L-iduronidase (IDUA), an enzyme essential for degrading glycosaminoglycans (GAGs). Without functional IDUA, GAG accumulation leads to widespread tissue damage, including skeletal abnormalities, organ dysfunction, neurological decline and early mortality. Current treatments, including hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT), provide limited efficacy, particularly for neurological symptoms, due to poor blood-brain barrier (BBB) penetration. T cells are a promising platform for enzyme delivery due to their longevity, high protein secretion, and ability to migrate into tissues, including across the BBB. These properties make them ideal for sustained in vivo enzyme replacement, enabling targeted IDUA delivery to affected organs.

Objective: To develop a novel cell-based therapy using autologous, engineered T cells for sustained and systemic IDUA delivery, addressing the limitations of current therapies and improving outcomes for MPS I patients.

Methods: As a proof-of-concept, human T cells were engineered to express IDUA using homology-directed repair with AAV-mediated delivery for targeted knock-in at the AAVS1 safe harbor locus and successfully evaluated in an MPS I NSG mouse model. To improve clinical scalability, we recently developed a non-viral, transposon-based engineering approach for IDUA expression. Preclinical studies utilizing MPS I T cells assessed transgene integration, enzyme secretion, cell viability, and cross-correction of IDUA-deficient cells in vivo/in vitro.

Results: In a pilot study, engineered human T cells expressing IDUA were evaluated in an MPS I NSG mouse model. A single dose led to sustained IDUA activity above heterozygous levels, significantly reducing GAG accumulation and improving MPS I-associated pathology. By 10 weeks post-treatment, peripheral GAG levels matched heterozygous controls, and histological analysis showed improved liver and brain morphology, supporting the therapeutic potential of T cell-based enzyme delivery. Building on these findings, we developed a non-viral

transposon system to generate IDUA-expressing T cells using patient-derived MPS I T cells, achieving >95% engineering efficiency with an average of four transposon copies per cell. These cells exhibited a >300-fold increase in IDUA secretion compared to healthy, non-MPS I T cells. Engineered cells remained viable and expanded 30-fold over seven days ex vivo. In vitro studies confirmed that secreted IDUA effectively cross-corrected IDUA-deficient cells, further supporting the feasibility of T cell-based enzyme delivery for addressing manifestations of MPS I.

Conclusion: These findings highlight the potential of engineered T cells as a transformative, durable, and effective therapy for MPS I. By providing sustained enzyme replacement, this platform could significantly improve patient outcomes. Future studies will focus on evaluating long-term persistence, immune response, and BBB penetration in additional MPS I models, further advancing this approach toward clinical application.

1019 Penetrating the blood-brain barrier: Utilization of the PS Gene-editing System to encode a novel fusion β-galactosidase for the treatment of GM1 gangliosidosis

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GM1 gangliosidosis is a lysosomal disorder caused by β-galactosidase (β-gal) deficiency, leading to the accumulation of GM1 and GA1 gangliosides, particularly in the central nervous system (CNS). Currently, there are no effective therapies available due to the inability of therapeutic enzymes to cross the blood-brain barrier (BBB). The PS Gene-editing (PSG) system has previously demonstrated efficacy in producing sustained therapeutic levels of enzyme that prevent the onset of cognitive decline in a murine model of GM1 gangliosidosis. However, there is still room for improvement in CNS uptake of the enzyme. This study explores the use of a novel β-gal-ApoE fusion enzyme to enhance BBB penetration and improve CNS pathology in the murine model of GM1 gangliosidosis.

In this study, the PSG system was employed to integrate the β-gal-ApoE fusion cDNA into intron 1 of the albumin gene of hepatocytes via two adeno-associated viruses (AAVs), administered intravenously into β-gal$^{-/-}$ mice at P0-P2. Over the course of the study, plasma was collected to measure and monitor β-gal enzyme activity. At six months of age, motor and cognitive function were assessed using the Barnes maze, balance beam, accelerating rotarod, and pole test. At 10-12 months of age, tissue β-gal enzyme activity was measured, and ganglioside levels were quantified in the hippocampus, cerebellum, and cerebral cortex. Plasma β-gal enzyme activity was sustained for up to 7 months post-dosing, averaging 1284- and 692-fold higher than heterozygous levels in female and male PSG-β-gal-ApoE-treated β-gal$^{-/-}$ mice over the 7 months. This significant elevation of plasma enzyme activity resulted in significant increase in β-gal enzyme activity in the liver, heart, spleen, kidney, muscle, bone marrow, spinal cord and brain of β-gal$^{-/-}$ mice who received treatment. Importantly, this elevation of brain β-gal enzyme activity was 12-fold higher than previous experiments using the non-fusion enzyme. Normalization of GM1 and GA1 gangliosides in the brain was observed. In behavior testing, mice that received PSG-β-gal-ApoE maintained normal cognitive and motor function.

In conclusion, the use of the PSG system encoding the novel β-gal-ApoE results in permanent, high levels of circulating β-gal enzyme, which is effectively taken up throughout the body. Additionally, these results demonstrate that the β-gal-ApoE fusion enzyme effectively crosses the BBB, restoring enzymatic function in the CNS and reducing the pathogenic gangliosides, resulting in improved neurological outcomes. These findings further demonstrate the power of the PSG system as a platform technology for treating not only lysosomal disorders, but any disorder that could benefit from circulating proteins.

1020 Biological Pacemaker Activity Induced by AAV-TBX18

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Background: Bradycardia is a clinical disorder characterized by an abnormally slow heart rate and a common cause of fatigue and fainting. Patients with this condition often need an electronic pacemaker. Although it is the current standard of care, these devices can have complications. A biological pacemaker has been created by adenovirus expressing the transcription factor T-box 18 (TBX18) as an alternative to electronic pacemakers. However, adenoviruses are immunogenic, limiting the duration of expression.

Objective: We investigated whether expressing TBX18 in the less inflammatory adeno-associated virus (AAV-TBX18) represents a viable alternative to induce somatic reprogramming and sustained pacemaker activity.

Methods: AAV9 containing GFP or TBX18 was directly injected into the myocardium of eight-week-old male Sprague Dawley rats (n=70) at low ($1x10^9$ GC), mid ($1x10^{10}$ GC) and high ($1x10^{11}$ GC) doses. Transient AV block was induced by tail vein injection of adenosine. A permanent complete AV block was induced by ablation of the his bundle. Hearts were harvested for optical mapping, mRNA analysis and immunofluorescence. Yorkshire-SPF farm pigs were subjected to a surgery to induce AV block by ablation of the his bundle, and a backup electronic pacemaker was implanted (VVI: 50 beats/min). Viral vectors were injected into the ventricular septum using a minimally invasive percutaneous approach.

Results: Six weeks after injection, all doses of AAV9-TBX18 had increased heart rate compared to AAV9-GFP when we induced AV block using adenosine. AAV9-GFP and -TBX18 mid dose injected rats underwent surgery to induce complete AV block. TBX18 group presented superior heart rate over the 24 hours monitored. 6-lead EKG analysis of the QRS axis in the frontal plane revealed that the ventricular ectopic beats from the AAV9-TBX18-injected group originated from the injection site. Activation maps further confirmed that the injection site originated those beats in TBX18-injected rats only. Successful reprogramming of adult ventricular myocytes into SAN-like cells was confirmed by transcriptional profiles, resembling the native SAN. A nonlinear analysis of the heart rate variability reavealed a reduction in the sympathetic overstimulation in the AAV9-TBX18-injected rats. In a clinically relevant porcine model of AV block, the TBX18 group presented a superior heart rate, and reduced dependency on the backup pacemaker. AAV-TBX18 injection also induced transcriptomic changes consistent with cellular reprogramming in the pigs' heart.

Conclusion: Collectively, these data indicate that the AAV-based somatic reprogramming induced by TBX18 leads to sustained biological pacemaker activity in different AV block models. Long-term studies are needed to characterize the long-term effects of sustained TBX18 expression.

1021 Gene Therapy Targeting the Hippo Pathway for In Vivo Cardiac Regeneration in Ischemic Heart Failure

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This preclinical study evaluates a first-in-class gene therapy designed to stimulate cardiomyocyte cell division and coordinate cardiac regeneration to treat ischemic heart failure with reduced ejection fraction (HFrEF). Current treatments, including pharmacotherapy and mechanical interventions, fail to reverse ischemia-induced myocardial damage. Consequently, there is an urgent unmet need for novel therapies that promote cardiac regeneration involving cardiomyocyte proliferation, vascular recruitment, and a reduction in fibrosis.

We developed YAP101, a non-replicating recombinant adeno-associated virus serotype 9 (AAV9) vector encoding a cardiac-specific troponin T promoter to drive the expression of three short hairpin RNAs (shRNAs) that suppress Salvador-1 (*SAV1*), a core Hippo pathway component. By reducing *SAV1* expression, YAP101 increases nuclear localization of YAP, activating multiple endogenous cardiomyocyte-directed repair pathways that promote cardiac regeneration.

To assess efficacy, safety, and biodistribution, we conducted preclinical studies in mouse and pig models using a clinical-grade YAP101 formulation. We evaluated efficacy in a pig model of myocardial infarction (MI)-induced heart failure using a catheter-based delivery system to administer 15 transendocardial injections of YAP101 directly into the myocardium surrounding an infarct. We also conducted pharmacology and, biodistribution, and toxicology studies over 3 and 6 months in healthy pigs, 3 months in MI pigs, and 3 months in healthy mice to gather essential preclinical safety data.

Results demonstrated robust pharmacological activity across species. Mechanistic and efficacy studies revealed that YAP101 effectively suppressed Hippo pathway activity, leading to functional cardiac improvement. In MI pig models, YAP101 administration enhanced ejection fraction, reduced infarct fibrotic scar size, stimulated cardiomyocyte cell division, and activated revascularization of the myocardium. Notably, YAP101 did not induce cardiac hypertrophy or arrhythmias at any tested clinically relevant doses.

Pharmacokinetic and biodistribution studies demonstrated sustained cardiac expression of YAP101. Biodistribution studies in mice and pigs confirmed that YAP101 localizes to the heart, and we detected transgene DNA in the myocardium up to six months post-administration. We also detected transgene RNA localized to the heart, which demonstrates the specificity of the cardiac-specific troponin T promoter. Importantly, YAP101 did not induce adverse immune responses beyond transient antibody formation, which did not impact efficacy. Toxicology studies across species confirmed that YAP101 is well tolerated, and we observed no organ toxicity or long-term adverse effects.

These findings support advancing YAP101 into clinical development as a first-in-class gene therapy targeting the Hippo pathway for cardiac regeneration. By coordinating in vivo cardiomyocyte cell division, vascular recruitment, and fibrosis reduction, YAP101 activates cardiac regeneration, leading to improved cardiac function and offering a potential breakthrough treatment for ischemic heart failure.

1022 TN-501 Gene Editing Therapy for PLN-R14del-Associated Cardiomyopathy

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PLN-R14del-associated cardiomyopathy, caused by a pathogenic mutation in the *Phospholamban (PLN)* gene, disrupts calcium handling in cardiomyocytes, leading to life-threatening arrhythmias, progressive heart failure, and high mortality rates. Current treatment options, limited to standard heart failure and arrhythmia management, fail to address the underlying genetic defect. TN-501 is an in vivo gene editing therapy designed to specifically inactivate the mutant *PLN-R14del* allele, eliminating the "poison peptide" effects of the mutant protein while preserving wild-type allele function.

TN-501 employs an adeno-associated virus 9 (AAV9) vector to deliver a cardiac-specific Cas9-sgRNA system. This vector ensures precise editing and transient Cas9 expression through tissue-specific promoters, self-inactivation elements, and liver-detargeting mechanisms. Preclinical studies in patient induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) and murine models demonstrate that TN-501 effectively prevents mutant PLN protein aggregation, reduces cardiac fibrosis, improves cardiac function, and significantly enhances survival. These findings highlight its potential to prevent sudden cardiac death, halt heart failure progression, and reduce the need for advanced interventions such as heart transplantation.

With its targeted approach, TN-501 represents a significant advancement in the treatment of PLN-R14del-associated cardiomyopathy. By addressing the genetic root cause of the disease, this therapy offers a promising, durable, one-

time intervention for a condition with high unmet medical need. TN-501 is currently in preclinical development, with further studies planned to refine its safety and efficacy and inform future clinical trials.

1023 AAV-Based Gene Therapy Targeting p53 Signaling as a Therapeutic Strategy for Arrhythmogenic Right Ventricular Cardiomyopathy Type 5

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Introduction: Arrhythmogenic right ventricular cardiomyopathy type 5 (ARVC5) is the most aggressive form of ARVC, caused by a fully penetrant missense mutation (p.S358L) in TMEM43. The disease is characterized by progressive dilation of the cardiac chambers and fibro-fatty replacement of the myocardium, leading to heart failure and sudden cardiac death. The function of TMEM43 and its role in ARVC5 pathogenesis remain largely unknown, hindering the development of effective therapeutic options.

Methods: We performed single-nucleus RNA sequencing (snRNA-seq) on cardiac tissue from TMEM43-S358L transgenic mice (TMEM43mut mice) at three different disease stages (early, mild and severe disease) to investigate the molecular drivers of ARVC5 progression. Based on these findings, we developed an adeno-associated virus (AAV)-based gene therapy to selectively deliver GSE56 (AAV-GSE56), a dominant-negative p53 mutant, to cardiomyocytes. Eight-week-old TMEM43mut mice were treated systemically via femoral vein with either AAV-GSE56 or AAV-Luciferase (control). Cardiac function was assessed through electrocardiography (ECG) and echocardiography until 16 weeks of age.

Results: snRNA-seq analysis revealed a marked depletion of cardiomyocytes and an expansion of fibroblasts and inflammatory myeloid cells in TMEM43mut mice. A subset of mutant cardiomyocytes displayed upregulation of genes linked to the DNA damage response (DDR) and p53 signaling activation in 5-week-old mouse hearts, preceding overt functional impairment. Western blot analysis confirmed these findings, showing increased levels of γH2AX, ATM/ATR, and phosphorylated p53 in TMEM43mut hearts. Therapeutic inhibition of p53 signaling via AAV-GSE56 significantly improved cardiac function compared to AAV-Luciferase controls, as evidenced by increased left ventricular ejection fraction, reduced left ventricular volume, and enhanced right ventricular performance. Additionally, treated mice exhibited shortened QRS duration and increased QRS amplitude, indicative of improved electrical conduction.

Conclusion: Our findings establish DDR activation and p53 signaling as key contributors to ARVC5 pathogenesis. AAV-mediated inhibition of p53 signaling in cardiomyocytes effectively slows disease progression, preserving cardiac structure and function. These results provide novel mechanistic insights into ARVC5 and highlight p53 inhibition as a promising gene therapy strategy for this highly lethal disease.

1024 A Novel Investigational AAV Gene Therapy for Treatment of BAG3 Dilated Cardiomyopathy

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Gene therapy with adeno-associated virus (AAV) vectors is emerging as a promising therapeutic platform for the treatment of cardiovascular disease. Unfortunately, effective gene delivery to the heart and safety concerns have been major limitations to clinical translation. Achieving therapeutically relevant levels of cardiomyocyte transduction with intravenous-delivered AAV vectors at doses that are well tolerated has been the field's most pressing challenge. We have identified novel AAV capsids by a machine learning-guided rational design approach that demonstrates significantly improved cardiac transduction at lower doses compared to wild-type AAV9. Dilated

cardiomyopathy (DCM) is defined as dilation and systolic impairment of the left ventricle and is the most common cause of heart failure (HF) in the young and most frequent indication for heart transplantation. More than 50% of patients with DCM have a genetic predisposition. B-cell lymphoma 2 (Bcl-2) associated anthanogene-3 (BAG3), a cochaperone that interacts with members of the heat shock protein (HSP) family, has been identified as a DCM-causing gene. BAG3-DCM represents a significant unmet medical need in patients with rapidly progressive cardiac dysfunction, for whom no treatments targeting the disease mechanism exist. We are developing AFTX-201, an AAV-mediated gene replacement therapy to treat BAG3-associated DCM using a novel cardiotropic capsid with an improved safety profile. Here we report therapeutic benefit in a genetically relevant mouse model and achieve safe, therapeutic levels of BAG3 in nonhuman primates at low doses.

To evaluate therapeutic efficacy in a disease-relevant context, we employed a conditional *BAG3* KO mouse with cardiac-specific haploinsufficiency (*BAG3* cKO$^{+/-}$). Cardiac-specific heterozygous (+/-) deletion in BAG3 provides a model that mirrors the biology (reduced BAG3 protein in heart) and phenotype (decreased ejection fraction and a dilated heart) seen in the presentation and natural history of BAG3 Dilated Cardiomyopathy (DCM) patients. Mice received a single IV injection of AAV containing full length human BAG3, similar to the proposed first-in-human Ph1/2 trial, under the control of a cardiac promoter at a timepoint in which mice exhibit significant cardiac dysfunction as measured by echocardiography. We demonstrate that *BAG3* cKO$^{+/-}$ mice show significant improvement in cardiac function following systemic administration of our vector with no safety findings. A dose range finding study performed in NHPs demonstrated levels of BAG3 protein comparable to efficacious *BAG3* cKO$^{+/-}$ mouse protein measurements in the heart at very low dose levels of AFTX-201. Histological analysis showed no evidence of test article-related changes in any tissue analyzed. Immunohistochemistry confirmed BAG3 expression in >70% of the NHP cardiomyocytes and expression throughout the heart with little to no expression in other tissues.

In summary, we propose AFTX-201 as an investigational gene therapy to safely achieve broad, therapeutic levels of BAG3 in the heart, while effectively detargeting other tissues. The proposed First-in-Human Phase 1/2 trial will be a multicenter, open-label, dose-escalation study with an expansion cohort. This trial aims to evaluate the safety, tolerability, and pharmacodynamics of AFTX-201, administered as a single intravenous dose to adult patients with symptomatic BAG3 mutation-associated dilated cardiomyopathy (DCM).

1025 Gene Replacement Therapy for Barth Syndrome Using a Novel Cardiotropic Capsid

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Barth syndrome is a pediatric onset, X-linked mitochondrial cardiomyopathy caused by loss of function of tafazzin (TAZ), a protein highly expressed in cardiac and skeletal muscles. TAZ is critical to maintaining the mitochondrial inner membrane structure, and thus cellular energy levels, through its role in cardiolipin metabolism. Heterozygous females are generally asymptomatic, but affected boys develop cardiomyopathy during infancy, a mild proximal myopathy, and neutropenia. Many patients do not respond to conventional heart failure treatments and 12% require heart transplantation at a median age of 1.7 years. The severity and rate of deterioration means that Barth syndrome represents a severe unmet medical need that may be suitable for AAV-mediated gene replacement therapy. By varying the position of an RGD motif within the 7 amino-acid peptide capsid insert, our group recently developed novel AAV variants that exhibit a spectrum of cardiotropic and myotropic properties in mice and NHPs, thereby providing new translational opportunities for targeting either heart or heart and skeletal muscle. Our lead cardiotropic capsid for Barth syndrome demonstrates a 30-fold increase in cardiac transduction (mRNA levels) compared to a clade F benchmark in nonhuman primates, with >50% of cardiomyocytes positive for TAZ transcript by *in situ* hybridization 90 days after intravenous (IV) dosing at 2E13 GC/kg. Upon assessing the efficacy of the candidate in a TAZ knock-down mouse model, IV vector administration at 1E13 GC/kg led to improvements in heart-to-body-weight ratio, echocardiography-based measures of cardiac function, and disease-specific biomarkers, namely GDF-15 in serum (a mitochondrial stress biomarker) and monolysocardiolipin-to-cardiolipin ratio in cardiac

tissue. We evaluated the pharmacology and safety of our lead candidate in a good-laboratory-practice compliant toxicology study in cynomolgus macaques to test three dose levels (2E12, 6E12, 2E13 GC/kg; 2 animals per group) at Day 90 or 180 timepoints. Robust cardiac transduction was observed at 6E12 and 2E13 GC/kg, and no dose-limiting toxicity was detected. These data suggest our candidate exhibits a promising risk-benefit profile that warrants further clinical development for the treatment of Barth syndrome.

1026 Human iPSC-Derived Cardiomyocyte Disease Modeling of Friedreich's Ataxia and Duchenne Muscular Dystrophy for Therapeutic Assessment

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The reprogramming of patient-derived fibroblasts into human induced pluripotent stem cells (hiPSCs) and their subsequent differentiation into cardiomyocytes (hiPSC-CMs) has enabled the development of physiologically relevant in vitro models for human cardiac diseases. Here, we present data from models of Friedreich's ataxia (FRDA) and Duchenne muscular dystrophy (DMD), demonstrating a drug-screening platform with closer fidelity to human pathophysiology. These models recapitulate key cellular disease phenotypes, offering advantages over murine and other animal models.

Control and disease-specific (FRDA and DMD) hiPSC-CMs were differentiated using controlled stirred-tank bioreactors, facilitating large-scale production under tightly regulated conditions (e.g., pH and gas exchange). This approach minimized batch-to-batch variation and yielded consistently pure hiPSC-CMs in sufficient quantities for high-throughput drug screening.

FRDA is characterized by reduced frataxin levels, while DMD results from the absence of dystrophin. To assess these deficiencies, we quantified frataxin and dystrophin levels using ELISA and immunocytochemistry (ICC), respectively. As expected, FRDA patient-derived hiPSC-CMs exhibited significantly lower frataxin expression compared to controls, whereas DMD hiPSC-CMs showed an absence of endogenous dystrophin.

A key pathological feature of FRDA is elevated reactive oxygen species (ROS) levels. Using flow cytometry with a CellROX probe, we detected a significant increase in ROS levels in FRDA hiPSC-CMs relative to controls. Additionally, both FRDA and DMD hiPSC-CMs exhibited aberrant calcium handling. Calcium transients recorded via the FDSS/μCell kinetic reader (Hamamatsu) revealed prolonged calcium transient duration and decay in FRDA hiPSC-CMs. In DMD hiPSC-CMs, impedance-based contractility measurements and calcium transient recordings showed significantly reduced calcium transient amplitude and duration compared to controls.

To mitigate the functional deficits observed in FRDA and DMD hiPSC-CMs, cells were transduced with adeno-associated virus (AAV) vectors encoding Frataxin or Dystrophin. ELISA and ICC confirmed successful overexpression of frataxin and dystrophin, respectively. Functionally, FRDA hiPSC-CMs exhibited a significant reduction in calcium transient duration and ROS levels following frataxin overexpression, while DMD hiPSC-CMs showed significant recovery of calcium transient amplitude upon dystrophin restoration.

In conclusion, patient-derived hiPSCs differentiated into cardiomyocytes provide a robust platform for in vitro modeling FRDA and DMD. By leveraging controlled stirred-tank bioreactors, we achieved high-yield, high-purity hiPSC-CM production suitable for large-scale screening of new therapeutics. Our findings confirmed expected molecular deficiencies in FRDA and DMD models and highlighted disease-specific phenotypic abnormalities. Importantly, we demonstrated the potential of this platform for therapeutic assessment by successfully rescuing key disease phenotypes through AAV-mediated gene delivery of Frataxin or Dystrophin.

1027 A gene regulatory element modulates myosin expression and alters cellular stress response

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A hallmark of heart disease is gene dysregulation. Reactivation of fetal programs has compensatory effects during heart failure, depending on the type and stage of the underlying cardiomyopathy. We identified and characterized a potentially therapeutically relevant gene regulatory element essential for regulating cardiac fetal myosin, *MYH6*, a gene silenced during stress and heart failure. We identified the myosin enhancer through ATAC sequencing and RNA sequencing. The intergenic enhancer increases in accessibility and transcription with increased *MYH6* expression. CRISPR-mediated enhancer activation increases *MYH6* expression (238%, P_{adj} <0.0001) and repression reduces expression (49% of control, P_{adj} =0.0003). The enhancer interacts with the *MYH6* promoter based on human cardiac Hi-C data. Repression or activation of the enhancer invitro can reproduce the various chromatin conformations in the human heart. Activation of the enhancer attenuates the functional declines and alterations in iPSC-derived cardiomyocytes following stress generated by the hypertrophy-inducing and disease-related peptide endothelin-1. Endothelin-1 treatment reduced *MYH6* expression to 30% of control, and enhancer activation significantly increased expression to 59% of control under stress (P_{adj} =0.0003). Increased *MYH6* expression led to higher expression of other factors essential for cardiac function under basal and stressed conditions: NPPA (P_{adj} <0.0001), NPPB (P_{adj} =0.007), TNNT2 (P_{adj} =0.0001), and ATP2A2 (SERCA2A, P_{adj} <0.0001). Stress reduced the expression of function factor ATP2A2 to 71% of the control (Padj =0.0006), while stressed enhancer-activated cells maintained higher expression than the unstressed control (119%, P_{adj} <0.0001). The enhancer's function and pleiotropic effects are conserved in the mouse's endogenous context, tested in the mouse cardiomyocyte cell line HL-1. The alteration in gene expression resulted in functional differences in the iPSC-derived cardiomyocytes and reduced functional alterations in polyploidization and calcium dynamics induced by endothelin-1. Enhancer activation reduced stress-induced polyploidization (P_{adj} =0.0026) and reduced the resulting stress-related cellular hypertrophy. Stressing control iPSC-derived cardiomyocytes significantly decreased calcium amplitude (P_{adj} =0.0057), necessary for generating contractile force, while enhancer-activated cells saw no such decline with stress (P_{adj} =0.96). Finally, enhancer activation prevented stress-induced declines in sarcomere protein density (P_{adj} = 0.019*)*. Using chromatin conformation assays combined with epigenome editing, activating this key enhancer reduces stress-driven alterations in chromatin looping (Padj = 0.023) and maintains higher expression of *MYH6* (P_{adj} =0.0003). These results identify regulatory mechanisms of cardiac gene expression programs that modulate the cellular stress response, providing insights for developing future therapeutics to maintain or improve cardiac function during failure.

1028 Developing *In Vivo* Prime Editing as a Potential Treatment Option for Heart Disease

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Heart disease is the leading cause of death in the world, and the unmet need for curative therapies that target the underlying causes of heart disease is high. Heart disease comes in many forms, affects individuals at many ages, and can be a result of many factors, including cardiomyocyte malfunction caused by genetic mutations. Gene editing technologies have the potential to correct the underlying genetic causes of cardiac conditions, and prime editing (PE) approaches are particularly attractive for its versatility and precision. However, the large size of standard PE machinery exceeds the packaging capacity of a single AAV, posing challenges to cardiac delivery.

To enable *in vivo* cardiomyocyte PE, we employed our proprietary, small, strong, cardiomyocyte-specific promoters, optimized dual-AAV cassette arrangement, and novel cardiomyocyte-targeting AAV capsids. When tested in mice targeting a reference genome locus, *Dnmt1*, our prototype corrected 22% of all genome copies in the heart at a high dose, providing proof of concept for dual AAV vector-based PE in the heart. The percentage correction of cardiomyocyte genome copies is likely higher than the measured value, as our PE machinery expresses specifically in cardiomyocytes, but genome copies from all cell types were collected and contributed to the denominator. Further optimization of the PE machinery resulted in enhanced *in vivo* editing efficiency, potentially benefiting hard-to-edit loci within the genome and/or allowing clinically meaningful editing at a lower dose. To demonstrate the therapeutic potential of *in vivo* PE, we developed PE cassettes targeting a mouse *Rbm20* region corresponding to the mutation hotspot identified in human *RBM20* dilated cardiomyopathy (DCM) patients and dosed the heterozygous *Rbm20* R636Q (corresponding to human *RBM20* R634Q mutation) DCM mouse model. Our *Rbm20* PE treatment drove *in vivo* correction of the mutated *Rbm20* allele, correction of the resulting transcripts, and successful improvement of cardiac function. The results of our *in vivo* studies suggest that our PE strategy may be a potential treatment option for heart disease caused by genetic mutations. We plan to focus future efforts on improving PE efficiency and tailoring our PE strategy for specific human genetic mutations known to cause heart disease. (WL and LR contributed equally to this work.)

1029 Comparison of New and Existing Promoters for Cardiac Gene Expression and Specificity

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Gene delivery has offered new treatment options for cardiovascular diseases. When using DNA carrying vectors such as AAV for gene delivery, an appropriate choice of promoter is an important aspect in designing gene therapy vectors, as this determines both expression level and off-target expression.

To compare and improve promoters used in cardiac gene therapy, we have tested benchmark promoters. This included several promoters used in cardiac gene therapy trials such as the muscle promoter SPC5-12 and the cardiac-specific promoter TNNT2, as well as novel promoter iterations and promoter-enhancer combinations designed based on sequence and epigenetic state analysis (cell type-specific accessibility, level of H3K27ac). We used neonatal-rat ventricular myocytes to compare cardiomyocyte gene expression, while using C2C12 mouse myoblasts, HEPG2 human hepatocytes and HEK293T producer cells for off-target expression quantification. Cells were transfected with plasmids containing the nano-luciferase reporter driven by the selected promoter-enhancer sequences. The read-out was performed using a nano-luciferase assay. In neonatal rat ventricular myocytes, CMV, SPC5-12 and derivatives/combinations thereof were 5 fold more active compared to TNNT2. SPC5-12 also showed high activity in C2C12 and HEK293T cells compared to TNNT2, while maintaining low activity in HEPG2, indicating partial specificity. Using deletion assays based on chromatin state, we identified a 200bp shorter version of TNNT2 that retained cardiac activity as well as specificity, being inactive in C2C12, HEPG2 and HEK293T. Additionally, we identified a 294bp promoter-enhancer combination with significantly higher cardiac and HEK293T activity when compared to TNNT2, while showing similar activity in C2C12 and HEPG2 cells.

In conclusion, we have identified several promoter candidates which provide useful expression profiles for specific cardiac gene therapy applications where vector length is highly limiting or where transgene expression in producer cells is unwanted, for example, due to transgene toxicity.

1030 Gene Expression Alterations in HIV-Associated Chronic Obstructive Pulmonary Disease: Insights into Molecular Pathways and Inflammation

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Background: Chronic obstructive pulmonary disease (COPD) poses a significant health challenge for individuals living with HIV, with the prevalence of COPD among this population emerging as a serious concern. HIV-infected individuals exhibit a higher susceptibility to COPD, potentially due to immunological and genetic factors. This study aims to conduct preliminary screenings of genes that may contribute to the pathogenesis of HIV-associated COPD, seeking to identify molecular pathways that could inform future therapeutic interventions.

Methods: We conducted experiments *in vitro* and *in vivo* using primary human bronchial epithelial (NHBE) cells re-differentiated at an air-liquid interface (ALI) and tissue samples from HIV-infected human lungs and SP-C Tat Transgenic mice model, respectively. NHBE ALI cultures were grown on a trans-well infected with the R5-tropic viral strain (HIV BaL) at a concentration equivalent to 5 ng/mL of p24. In addition, NHBE ALI cultures were also treated with HIV-Tat. Total RNA was extracted, and the expression of POFUT1, ZNF451, and RUNX1 were assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR), and Western blotting was employed to quantify protein levels. Additionally, various pro-inflammatory cytokines were measured using an enzyme-linked immunosorbent assay (ELISA).

Results: The expression levels of POFUT1, ZNF451and RUNX1 were significantly reduced in HIV-infected lung tissue and SP-C Tat mice lung tissue samples, indicating these genes' involvement in recurrent lung inflammation. The mRNA expression levels of Pofut1, ZNF451and RUNX1 were significantly reduced in HIV-infected lung tissue and SP-C Tat mice lung tissue samples, indicating these genes' involvement in recurrent lung inflammation. Similar downregulation of mRNA levels is also observed in HIV-infected NHBE cells, and HIV-Tat treated NHBE cells. In addition, Pofut1 expression is markedly reduced in smoker and human COPD lung tissue samples.

Conclusion: Downregulation of POFUT1 destabilizes the Notch signaling that affects lung development and maintenance and can lead to developmental defects and various lung pathologies. In addition, altered expression of the ZNF451 and RUNX1 genes can significantly impair lung function and exacerbate pulmonary inflammation. Reduced expression of ZNF451 leads to increased fibroblast activation and fibrosis, while RUNX1 dysfunction can disrupt immune responses and promote chronic inflammation, resulting in severe respiratory complications.

1031 Single-Cell RNA Sequencing Reveals Lung T and B Cell Dysregulation in SP-C Tat Transgenic Mice Models and Cigarette Smoke Exposure

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Background: Chronic obstructive pulmonary disease (COPD) is marked by chronic lung inflammation and immune dysregulation, with T and B cells playing central roles in shaping the immune microenvironment. T cells persist in COPD lungs, exhibiting cytotoxicity and promoting inflammation, while B cells produce autoantibodies that contribute to lung injury through immune complex formation and complement activation. The interplay between HIV Tat protein and cigarette smoke (CS) exposure may exacerbate immune dysregulation, leading to accelerated lung damage. This study aims to explore gene expression changes in T and B cells in the lungs of COPD mouse models using single-cell RNA sequencing (scRNA-seq).

Methods: Four experimental groups of mice were analyzed: control mice, SPC-TAT mice, wild-type (WT) mice exposed to cigarette smoke, and SPC-TAT mice exposed to cigarette smoke. Single-cell suspensions were prepared from mouse lungs using the gentleMACS™ Octo Dissociator with Heaters and the Miltenyi Biotec mouse lung dissociation kit. 10,000 cells per lung sample were processed for library preparation using the Chromium platform

(10X Genomics). Libraries were sequenced on a NovaSeq 6000 system (Illumina) at a mean sequencing depth of 30,000 reads per cell. Differential gene expression analysis was performed to identify dysregulated genes in T and B cell populations across experimental groups.

Results: scRNA-seq analysis revealed significant dysregulation of genes in both T and B cell populations. T cells in SPC-TAT mice showed increased expression of genes associated with cytotoxicity and inflammatory responses. SPC-TAT mice exposed to cigarette smoke exhibited an exacerbated dysregulation of both CD4+ and CD8+ T cell-related genes compared to control mice. Similarly, B cells demonstrated enhanced gene expression in maturation, activation, and autoantibody production. Notable changes were observed in mediators linked to immune complex formation and complement activation in COPD lungs. These findings were most pronounced in the SPC-TAT with cigarette smoke group, indicating a synergistic effect of HIV Tat protein and cigarette smoke in driving immune dysregulation.

Conclusions: This study highlights the molecular mechanisms by which HIV Tat protein and cigarette smoke contribute to immune dysregulation in COPD. Dysregulated gene expression in T and B cell populations provides insights into their roles in chronic lung inflammation and injury, with implications for therapeutic targeting in COPD patients with HIV. Future research will focus on validating these findings and identifying specific pathways that mediate the combined effects of Tat and smoke exposure.

1032 CRISPR-Cas9-Mediated *ATXN3* Gene Inactivation as a Promising Gene Therapy for Machado-Joseph Disease

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Machado-Joseph disease (MJD) is an autosomal dominantly-inherited neurodegenerative disorder, caused by an over-repetition of the polyglutamine-codifying region in the ataxin-3 (*ATXN3*) gene. The resulting mutant ATXN3 protein is prone to aggregation, leading to cellular dysfunction and death. Currently, no treatment able to reverse or stall MJD progression has been developed. Gene suppression strategies have shown promise in preclinical models. However, these strategies do not directly target the underlying genetic cause of the disease, resulting in incomplete and/or transient therapeutic effects. CRISPR-Cas9 has emerged as a powerful tool to permanently inactivate disease-associated genes, offering the potential for the development of definitive treatments for a wide range of diseases.

In order to prevent the downstream toxic pathways, we developed a CRISPR-Cas9-based approach to efficiently inactivate the human *ATXN3* gene. A panel of single guide RNA sequences targeting an early exon of the human *ATXN3* gene was designed. Functional characterization was performed in a human cell line and the top-ranked sequence was selected for further *in vivo* experiments.

Recombinant adeno-associated viruses (rAAV1/2) encoding CRISPR-Cas9 sequences were delivered into the striatum of a lentiviral-based mouse model of MJD by intracranial injection. This resulted in a marked reduction of aggregates and the preservation of neuronal function. Further validation in a transgenic mouse model of MJD, through the cerebellar administration of rAAVs encoding the CRISPR-Cas9 system, demonstrated a 75% reduction in the number of cells with intranuclear ATXN3 accumulation in deep cerebellar nuclei. However, this was insufficient to rescue motor deficits of treated animals in the open-field test. To achieve a broader brain transduction and early intervention, we administered CRISPR-Cas9-encoding rAAVs into the cerebrospinal fluid of neonatal transgenic mice. This approach successfully preserved locomotor function, in contrast to untreated mice, which showed a deterioration of their motor performance over time.

Our findings demonstrate the efficacy of CRISPR-Cas9-mediated *ATXN3* inactivation in two MJD mouse models, supporting its potential as a novel therapeutic strategy for this incurable disorder.

1033 CRISPR-hfCas12Max-Mediated DNA Editing Restores Motor Function in Spinocerebellar Ataxia Type 3

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Background: Spinocerebellar ataxia type 3 (SCA3), a rare autosomal dominant neurological disorder that significantly impairs motor functions and quality of life and typically manifesting symptoms between 30 and 40 years of age. The progressive nature of the disease imposes substantial psychological and economic burdens on patients and their families. Increasingly, early disease onset is being observed, driven by genetic factors. SCA3 is caused by a CAG repeat expansion of the poly Q tract in exon 10 of the *ATXN3* gene, producing toxic ataxin-3 protein that aggregates and triggers neuronal death in the cerebellum, brainstem, spinal cord, and other neural tissues. Despite advances in understanding molecular pathogenesis, effective therapeutic options remain unavailable, with current treatments limited to clinical symptom management. Therefore, there is an urgent need to develop disease-modifying therapies for SCA3.

Methods: Here, we developed a potential therapeutic strategy using a high-fidelity Cas12Max (hfCas12Max) system to target exon 10 of the *ATXN3* gene using an engineered CRISPR-Cas12i variant that we show has increased activity and specificity. A panel of guide RNAs (gRNA) targeting *ATXN3* exon 10 was screened in HEK293T cells for editing efficiency and specificity. The potential off-target sites were nominated using Cas-OFFinder and assayed in treated HEK293T cells. The optimal *ATXN3*-targeting gRNAs (g*ATXN3*) were packaged with hfCas12Max into a single AAV vector (AAVhfCas12Max-g*ATXN3*). Neonatal humanized SCA3 (84Q) transgenic mice received intracisterna magna (ICM) injection of AAVhfCas12Max-g*ATXN3* to evaluate editing efficacy in the forebrain (FB), cerebellum (CB), and brain stem (BS). The motor function in these mice was evaluated monthly in the rotarod test after ICM injection.

Results: Eighteen gRNAs targeting human *ATXN3* were designed and cloned into the hfCas12Max plasmid and transfected into HEK293T cells. *ATXN3* gRNA4 exhibited the highest editing efficiency (41.12±2.54%) in HEK293T cells. No detectable off-target editing was detected when sequencing DNA from the treated cells at the 23 nominated sites, highlighting the specificity of *ATXN3* gRNA4. In SCA3 (84Q) transgenic mice, the AAVhfCas12Max-g*ATXN3* achieved robust editing efficiencies of 74.17±3.49%, 48.33±15.17%, and 40.20±12.37% in FB, CB, and BS, respectively, at 4 weeks post ICM injection (**Fig. 1b**). Correspondingly, the mutant human Ataxin-3 protein levels were reduced to 57.5±1.1%, 62.0±15%, and 51.2±10.4% in the FB, CB, and BS, respectively, compared to PBS-treated controls (**Fig. 1c** and **1d**). Importantly, rotarod performance, a measure of motor coordination, showed significant improvement in 3-month-old and 4-month-old AAVhfCas12Max-g*ATXN3* treated SCA3 (84Q) transgenic mice compared to PBS-treated controls (**Fig. 1e**).

Conclusions: AAV-mediated delivery of hfCas12Max-g*ATXN3* demonstrates high on-target editing efficiency and specificity, effectively reducing toxic ataxin-3 protein levels and improving motor function in a preclinical model of SCA3. Our findings position hfCas12Max-based gene editing as a promising therapeutic approach for SCA3 and warrant further investigation.

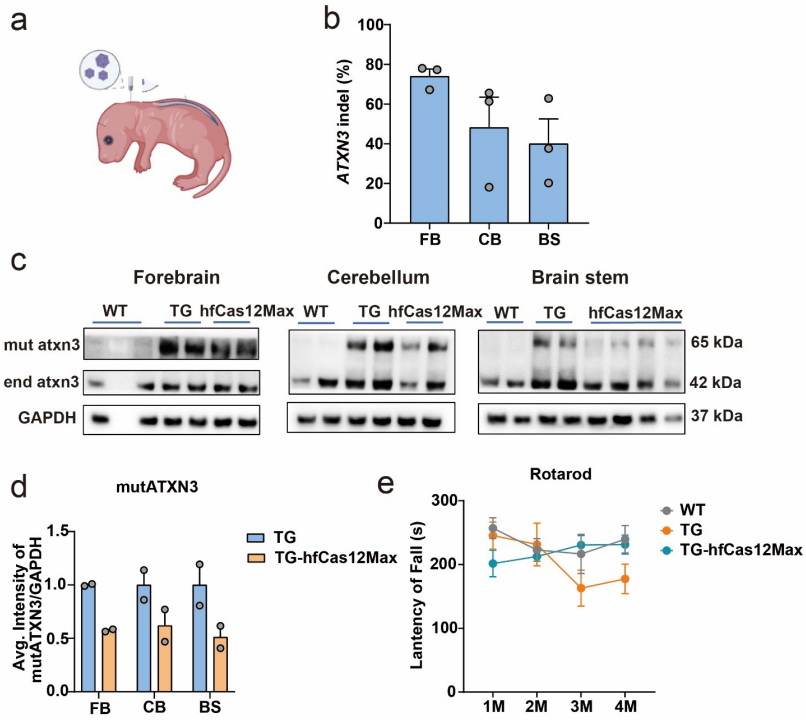

Figure 1 The edit efficiency of *ATXN3* and the motor function of the SCA3 (84Q) mice after AAV dosing.

1034 The Use of AAV2.retro to Deliver Transgenes to Both Upper and Lower Motor Neurons in the Rat for the Treatment of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is fatal disorder affecting both upper and lower motor neurons. The onset of this disease is generally between 40 and 60 years of age, and median survival from diagnosis is approximately three years. The pathophysiology of the disease is poorly understood, but it likely includes mitochondrial defects, inflammation, changes in pro- and anti-apoptotic signals, and defects in axonal transport. Experimental therapies, including gene therapy, have been evaluated for this disease. Most treatments either support motor neuron health and function or target one of the dozens of genes associated with this disease for degradation. However, efficiently treating both upper and lower motor neurons in these patients remains a challenge.

We have begun to investigate the use of an adeno-associated viral vector, AAV2.retro. This adeno-associated viral capsid was previously identified by Tervo *et al*. using an *in vivo* evolution strategy, selecting for the ability to achieve retrograde transduction in GABAertic and glutamatergic neurons when injected into the central nervous system. Since upper motor neurons are glutamatergic, injections of AAV2.retro vectors in the spinal cord should transduce both the lower motor neurons at the site of injection and the upper motor neurons projecting to that level. In healthy adult male rats, injection of an AAV2.retro vector expressing GFP into the lumbar spinal cord provided robust transduction at the site of injection. Transduced cells were also found in the cervical spinal cord. The location of these neurons suggested that they are propriospinal neurons. These neurons are involved in coordinating forelimb and hindlimb movement and project axons long distances up and down the spinal cord. In addition, GFP-positive axons were observed in the dorsal columns, particularly the gracile fasciculus, and in the pyramidal tracts, consistent with retrograde transport from the lumbar spinal cord. In the brain, substantial transduction was observed in the red nucleus and primary motor cortex, as well as in pontine nuclei.

Both ALS patients and animal models of this disease exhibit axonal transport defects that could negatively affect therapeutics based on the AAV2.retro capsid. We are currently assessing the potency of AAV2.retro vectors in the $SOD1^{G93A}$ rat model of ALS, both before and after disease onset. Spinal cord transduction in animals treated presymptomatically yielded a distribution similar to that of wild type animals. Quantitation of transduction in the brain and spinal cord for both wild type and SOD1 animals is currently ongoing. The current results suggest that AAV2.retro is a promising vector for ALS therapeutics.

1035 Exploring the Role of Inverted Terminal Repeats in AAV Vector Performance for CNS Gene Therapy

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Diseases of the central nervous system (CNS) are difficult to treat due to the blood-brain barrier preventing many therapeutic drugs from crossing. Gene therapy using adeno-associated viruses (AAVs) offers a promising solution with the advancement of new capsids including AAV-DJ, which has been demonstrated to cross the blood-brain barrier and achieve targeted and effective expression in CNS tissues. AAVs are well suited for CNS treatments because they can achieve long-term gene expression with minimal immune response, and are ideal for conditions such as Alzheimer's, Huntington's, and Parkinson's diseases. The AAV genome consists of a transgene construct flanked by 145bp inverted terminal repeat (ITR) sequence. Previous studies have demonstrated that inverted terminal repeats (ITRs) are essential for AAV packaging; however, they are prone to mutations and deletions during bacterial propagation, leading to inconsistent experimental results. While advances in capsid engineering have led to enhanced ability for AAVs to cross the blood-brain barrier, the instability of the ITRs remains a major concern.

The impact of five common ITR mutations, which exhibited varying degrees of integrity and insert length, were evaluated. HEK293T cells were co-transfected with the transfer, Rep/Cap (serotype AAV-DJ), and helper plasmids for viral packaging. AAV-ITR Sanger sequencing verified the ITR mutations in the transfer plasmid sequence in comparison to the wild-type. Following iodixanol purification, viral titers were assessed using quantitative PCR (qPCR), and transduction efficiency was evaluated through fluorescence microscopy of a GFP marker. To further investigate the effects of these mutations, each mutation was repaired to the wild-type sequence, and the titer and fluorescence were re-evaluated. To assess brain-wide transgene expression and the impact of ITR mutations, AAVs targeting the CNS tissues were performed and evaluated using a triple-labeling immunofluorescence technique with DAPI, NeuN, and GFP to analyze AAV-mediated gene expression in the mouse brain.

The results show recombinant AAV genomes with partial or significant deletion of the 5’-ITR had reduced titers compared to wild-type; even a single base pair deletion resulted in decreased titer. This drop in viral titer was more pronounced in constructs with significant ITR deletions and larger transgene cassette cargo. A marked decrease was also noted in GFP fluorescence, which correlated to the severity of the ITR deletion and transgene size. AAV mutant constructs retained the capability to cross the blood-brain barrier, with wild-type vectors achieving the highest efficiency.

In conclusion, this study demonstrates the critical role of the 5’-ITR in the efficacy of recombinant AAV vectors. Even minor alterations, such as single base pair deletions, can significantly affect viral titers and transduction efficiency. These findings provide valuable insights into the design of AAV vectors for gene therapy applications, emphasizing the need to carefully consider ITR integrity at all stages of design and development, and transgene size to maximize therapeutic potential.

1036 Novel Noninvasive Gene Therapy for Parkinson's Disease Using Viral Encoded Single-Chain Antibody Treatment

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Parkinson's disease is primarily characterized by the degeneration of dopaminergic neurons from the substantia nigra of the brain. Intracellular inclusions composed mainly of phosphorylated alpha-synuclein (aSyn) are present in affected neurons and play a key role in driving cellular dysfunction and neurodegeneration. Evidence in various animal and cell models suggest that pathological aSyn can be transmitted from cell-to-cell in a prion-like manner. Blocking either the aggregation of pathological proteins or their transmission across the brain could represent effective therapeutic strategies. We generated small antibodies in the form of secreted single-chain variable fragments (scFv) that bind to aSyn. We validated the specificity of our antibodies with dot blots and ELISA and identified one scFv specific for phosphorylated aSyn, and one recognizing both phosphorylated and non-phosphorylated aSyn. We then encoded the single-chain antibodies in an AAVCap-B10 viral vector, a serotype that can cross the blood-brain barrier when injected intravenously (IV) in mice. After IV injection, we verified the expression of the antibodies in the brain with immunohistochemistry for the Myc tag expressed by the antibody fragments. To model Parkinson's disease and the spreading of aSyn pathology, we injected human pre-formed fibrils (PFF) of aSyn into the dorsal striatum of transgenic mice overexpressing human mutated aSyn (M83 mouse line). Seven days later, we administered an AAV encoding the aSyn scFvs or a control scFv. Control groups were also injected with PBS instead of PFF. We closely monitored the motor impairments arising after the injections with a clinical scoring system. Results show a strong protective function of the scFvs against motor impairments. Moreover, aSyn brain pathology was significantly reduced in mice who received the AAV-scFvs against aSyn compared to the mice who receive the control scFv. We also show that our secreted scFvs can penetrate brain cells and possibly act on intracellular aSyn. Finally, we did not observe signs of immune reaction and inflammation following the administration of our AAV-scFvs in mice. In conclusion, we developed scFvs targeting pathological aSyn and proved that, when administered as a gene therapy through a viral vector, they can modulate aSyn pathology in a mouse model of PD. Our future steps toward clinical development include the humanization of the antibodies, and the development of a brain penetrating AAV vector for efficient biodistribution in the human brain.

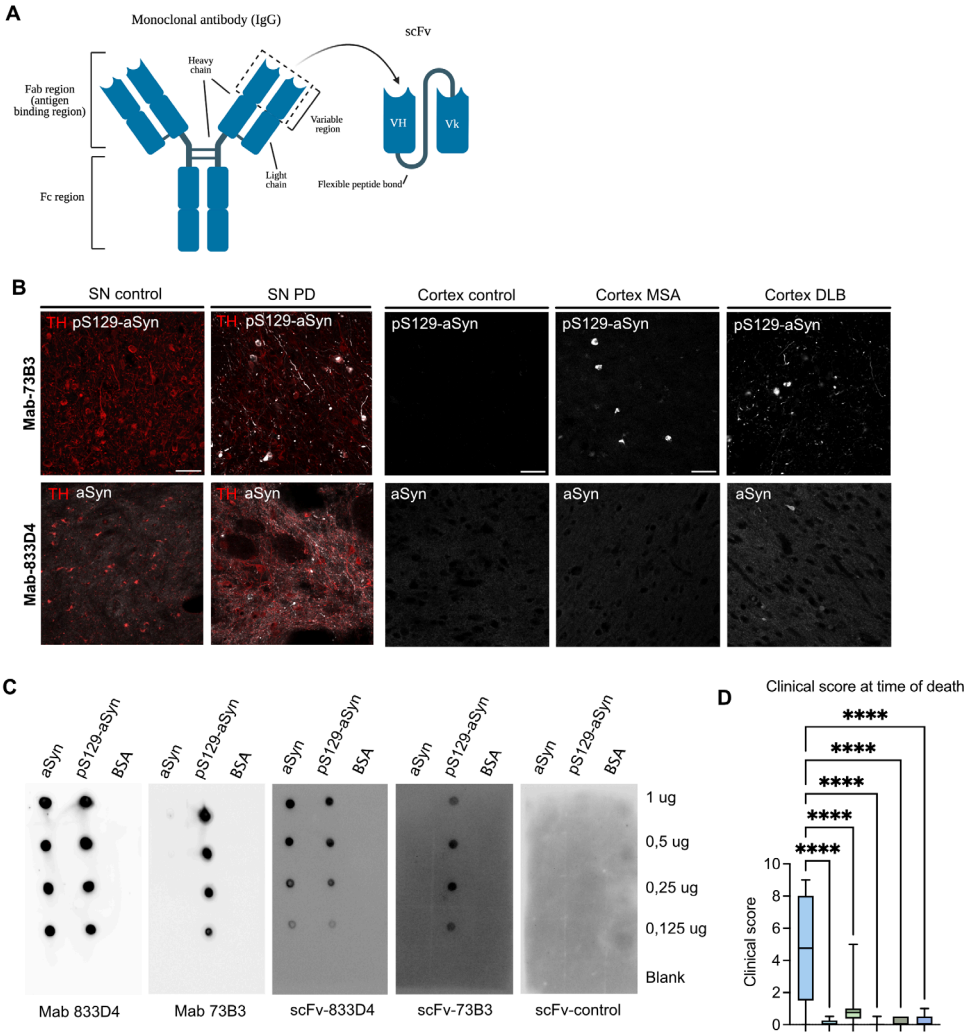

Figure 1: Single-chain variable fragments (scFvs) against alpha-synuclein were generated from murine monoclonal antibodies and show neuroprotective potential in a PD mouse model. **A)** Schematic representation of an IgG and its scFvs derivative containing the variable regions. **B)** Confocal images showing the binding of IgG 73B3 and 833D4 to pathological aSyn aggregates in PD, MSA and DLB human brain sections, in comparison to control brains. **C)** Dot blot performed on aSyn WT or pS129-aSyn monomers showing the preservation of the binding specificity of scFv 833D4 and 73B3. **D)** Clinical score at the time of sacrifice in mice that received aSyn preformed fibrils 12 weeks prior, and were treated with anti-aSyn scFvs compared to a control scFv.

1037 RSwitch Enabled Gene Therapy to Fine Tune Frataxin Expression for the Treatment of Friedrich's Ataxia

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Improvements in engineered AAV capsids are enabling the development of next-generation therapeutics for unmet medical needs. However, there remains a pressing need for transgene control features to improve the safety and tractability of gene therapy. Amongst advancements in transgene engineering, exogenous tunability of transgene expression has long been a sought-after technology. While methods exist, they typically rely on engineered transcription factors and repurposed pharmacology, not specific to the switching system. Rgenta has developed a

platform for imparting gene therapy vectors with regulation by orally bioavailable small molecules. These DNA-encoded elements, called RSwitches, can be selectively modulated by synthetic small molecules, called RDrugs, in a dose-dependent manner. Tailored to interact with each RSwitch, the RDrugs direct specific pre-mRNA splicing events needed for therapeutic payload production. Our collection of synthetic small molecule RDrugs is built to meet the needs of each indication with a range of pharmacokinetic properties. We have generated a variety of RSwitches and demonstrated their ability to functionally regulate transgene expression in vitro and in vivo. Gene therapy for Friedrich's Ataxia (FA) is a prime use case for exogenous regulation of transgenes. FA is an autosomal recessive disorder where expansion of GAA repeats in intron 1 of the Frataxin gene (FXN) result in loss of expression. Frataxin loss inhibits iron-sulfur (Fe-S) cluster biogenesis, leading to mitochondrial dysfunction and cell death. FA patients experience debilitating symptoms brought on by neurodegeneration and cardiomyopathy. While constitutive replacement of FXN by AAV vectors has shown promise in animal models, multiple studies have demonstrated the cardiotoxic effects of Frataxin overexpression. We, therefore, hypothesized that AAV-RSwitch control of an FXN transgene could facilitate safe and efficacious levels of gene rescue while maximizing tissue transduction coverage in the heart. Given the need for titratable Frataxin expression, we have optimized RSwitch DNA elements fused to human FXN cDNAs. This process has been aided by massively parallel sequence library screening and deep learning-driven design. Successive rounds of in vitro engineering yielded a system that displays a highly dynamic Frataxin expression, dose-responsive to a brain-penetrant RDrug molecule (Cp7). Complementation of Frataxin loss to WT levels occurs at single-digit nanomolar levels of Cp7 in cultured cells. When part of an AAV vector, the RSwitch performs similarly, providing functional Frataxin protein to transduced cells in response to Cp7. Dose-range finding studies in MCK Cre cardiac models of FA demonstrate the relationship between AAV-RSwitch-FXN vector dose, Cp7 daily oral dose, and steady-state levels of Frataxin in mouse tissues. We are now pursuing an efficacy study in MCK Cre mice to demonstrate how sustained RDrug Cp7 dosing after systemic AAV administration: 1) maintains non-toxic levels of exogenous Frataxin expressed in the mouse heart and 2) rescues the morbidity that the mice develop at ~10 weeks of age. Overall, we foresee RSwitch, with the highly drug-like RDrug molecules, will improve the safety and clinical success of gene replacement therapies for diseases such as FA. The technology could create therapeutic opportunities for a variety of gene therapy applications needing temporal control of transgene expression levels.

1038 Muscle-Mediated Klotho Secretion Outperforms Spinal Overexpression in Mitigating ALS Progression

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive degeneration of motoneurons (MNs), leading to muscle weakness, paralysis, and ultimately respiratory failure. In the $SOD1^{G93A}$ mouse model, we targeted the secreted form of α-Klotho (sKL) to skeletal muscles or the spinal cord at an early disease stage. Skeletal muscle overexpression was achieved using systemic MyoAAV delivery and the desmin promoter, while spinal cord targeting used intrathecal AAV9 delivery with the CMV promoter.

Muscle-targeted sKL therapy significantly improved motor function, delayed disease onset, and preserved muscle mass. Treated mice showed enhanced neuromuscular function, with increased compound muscle action potential (CMAP) amplitudes, larger hindlimb motor units, and preserved motor endplate innervation. Connectivity between corticospinal and spinal MNs was improved, as evidenced by preserved motor-evoked potentials (MEPs), and lifespan was extended. In contrast, spinal cord overexpression of sKL produced milder benefits, primarily in

females, where CMAPs, MEPs, and muscle mass were partially preserved. Functional improvements were observed in both sexes, but other parameters showed limited benefit in males.

To uncover the mechanisms of action in our muscle-targeted strategy, we employed RNA sequencing and proteomics on the lumbar spinal cord and gastrocnemius muscles. In muscles, sKL therapy restored pathways associated with muscle contraction and development while reducing fibrosis and atrophy. In the spinal cord, sKL enhanced synaptic activity, reduced neuroinflammation, alleviated mitochondrial dysfunction, and modulated RNA processing, proteostasis, and apoptosis, offering key insights into its protective effects.

Our results demonstrate that muscle-driven sKL delivery is more effective than spinal cord overexpression in preserving neuromuscular function and delaying ALS progression, supporting the dying-back hypothesis in this model. These findings highlight sKL's therapeutic potential and uncover mechanisms relevant to ALS and related disorders.

1039 Supraphysiological expression of SMN1 protein leads to compromised heart function improvement and cardiac thrombosis in mice

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G.Gao, J.Xie. are co-corresponding authors.

Spinal muscular atrophy (SMA) is an autosomal recessive disease, which compromises the α-motor neurons located in the spinal cord, progressively leading to symmetrical muscle atrophy and weakness. SMA affects approximately 1 in 11,000 newborns and remains the leading genetic cause of infant deaths. Onasemnogene abeparvovec (Zolgensma®), a self-complementary adeno-associated virus serotype 9 (scAAV9) vector that expresses the native *SMN1* transgene is an intravenously administered gene therapy. The heart and liver had also been found to be the main target organs of Zolgensma® toxicity in pre-clinical studies. Except the liver damage, signs of cardiac damage also emerged among the most frequently adverse events reported, such as the increase in troponin I.

We previously developed a 2nd-generation SMA gene therapy expressing a codon-optimized *hSMN1* transgene driven by a promoter derived from the native *hSMN1* gene. This vector restored SMN expressions close to physiological levels in the central nervous system and other major tissues of a severe SMA mouse model. Here, we investigated the 2nd-generation vector and a benchmark vector with a design identical to Zolgensma on the heart toxicity in SMA and FVB mice.

We assessed the heart function of SMA mice received these two vectors at the dose of 3.3xE14 vg/kg at P1 by facial vein by echocardiography on day 30. Compared to healthy carriers, the benchmark vector group showed compromised ejection fraction, fractional shortening, and stroke volume, whereas mice that received the 2nd-generation vector did not show any heart function abnormalities. Premature ventricular contractions were observed in the benchmark vector-treated animals but were not observed in the 2nd-generation vector group. This data demonstrated that the benchmark vector was unable to rescue heart function in SMA mice. In contrast, the 2nd-generation vector-treated mice normalized heart function to the level comparable to healthy carriers. Notably, we detected more SMN expressions in the heart from benchmark vector-treated mice than in the 2nd-generation vector treatment on day 30. At day 90, the SMN expression in the benchmark group is ~5-fold higher than the healthy carriers, but the 2nd-generation vector treatment showed indistinguishable SMN expression to the healthy carriers in the heart. By ICV administration, the benchmark vector significantly extended the survival of SMA mice but was still less effective than the 2nd-generation vector. In the seven surviving animals (total of 16) from the benchmark group, we observed cardiac thrombosis in 3 of them but not in all eight surviving mice (total of 12) from the 2nd-generation vector group. To further illustrate the need to maintain physiological expression, we replaced the coding sequence of SMN1 with the codon-optimized sequence in the benchmark vector genome to maximize SMN1 expression. In the ICV-treated SMA and wildtype mice, the codon-optimized vector group developed left atrial

thrombosis more rapidly at a higher frequency than the benchmark group. By facial vein at P1, strikingly, all the FVB mice manifested atrial thrombosis and died in two weeks after receiving the codon-optimized vector at 1.0E+14 vg/kg, also increased with SMN protein in left atrium. These data suggest that supraphysiological SMN expressions in the heart are detrimental to cardiac function and safety.

1040 Preclinical Efficacy and Potency Assay Development of An Optimized AAV-hUPF1 Gene Therapy for Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD)

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Amyotrophic lateral sclerosis (ALS) is a devastating progressive neurodegenerative disease that damages motor neurons in the brain and spinal cord. While the pathogenesis of ALS can result from either sporadic or familial inherited mutations, the underlying pathology in over 95% of cases is associated with dysregulation of TAR DNA-binding protein 43 (TDP-43). Here, we present the optimization and preclinical efficacy of AAV-hUPF1, a central regulator in nonsense-mediated RNA decay (NMD), in multiple ALS models. Our data and the mechanism of Up-frameshift 1 (hUPF1) action suggest that the efficacy of this therapy would be agnostic to patients' genetic background.

To improve AAV packaging and thus convey manufacturing benefits, we reduced the size of the original hUPF1 construct by 1.5kb while improving therapeutic efficacy in both in vitro and in vivo models. Using an efficient capsid for CNS delivery and a highly potent expression cassette, our therapeutic strategy is to minimize AAV dosage while maintaining a high level of expression and subsequent efficacy. The optimized AAV-hUPF1 effectively mitigated toxicity in TDP-43 and C9orf72 iNeuron models and restored the normal function of neurons derived from C9orf72 patient cell lines at a lower multiplicity of infection (MOI) than used for the original. Mechanistically, UPF1 target engagement was confirmed in transduced C9orf72 patient-derived neurons by the downregulation of known NMD targets. In a Fused in Sarcoma (FUS) mouse model of ALS, optimized AAV-hUPF1 significantly improved motor neuron survival to levels comparable to wild-type controls at approximately 36% transduction.

To further develop the AAV-hUPF1 program, we piloted different TDP-43 induced toxicity models as in vitro potency assays for manufacturing release. To induce cell death, TDP-43 was overexpressed in primary mouse neurons using AAV9 transduction, and in human SH-SY5Y cells using the doxycycline-inducible TET-ON system. While toxicity was reliably and robustly induced in the primary neuronal model, UPF1 co-expression did not result in a measurable global effect and thus did not meet the requirements for our assay development. In contrast, repeated experimentation in the stably transduced and differentiated SH-SY5Y cells showed that TET- inducible overexpression of TDP-43 caused significant toxicity in only about 50% of the cases, demonstrating that the TET-inducible system is too variable to be implemented as a reliable potency assay. After exploring gross neuronal toxicity as a strategy to establish a validated potency assay, we now aim to explore other approaches that are based on the proposed underlying mechanism of action for UPF1, including RNA homeostasis. Developing a robust potency assay is a major component in the manufacturing of clinical therapeutics and our goal towards treating ALS patients safely and effectively with AAV-hUPF1.

Collectively, the data from in vitro and in vivo studies using FUS, TDP-43, and C9orf72 models demonstrate that hUPF1 holds significant potential for treating the majority of patients with ALS and other degenerative diseases caused by TDP43 pathology such as Frontotemporal Dementia, as we continue the development of this program.

1041 AAV-mediated delivery of an intrabody targeting TDP-43 for Amyotrophic Lateral Sclerosis therapy

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by progressive motor neuron degeneration, leading to muscle atrophy, and ultimately death. The vast majority of ALS cases involves the pathological mislocalization and aggregation of TAR DNA binding protein 43 (TDP-43), a key player in RNA metabolism. Loss of motor neuron nuclear TDP-43 disrupts its role splicing regulation, further disrupting the neuronal transcriptome, all contributing to ALS pathology. Therefore, targeting TDP-43 pathophysiology presents a promising avenue for therapeutic intervention. Here, we present an AAV vector encoding a novel intrabody designed to selectively target and neutralize TDP-43 aggregates while preserving TDP-43 nuclear function. We demonstrated the therapeutic efficacy of the vectorized intrabody in iPSC-derived ALS models, by showing target engagement with a reduction in TDP-43 aggregates, correction of loss-of-function of TDP-43 and ALS transcriptome, and restoring motor neuron survival. In addition, we showed that the therapeutic intrabody has preferential binding to cytoplasmatic TDP-43 in disease-relevant specimens. To enable widespread motor neuron targeting, we developed an AAV-mediated delivery ensuring efficient transduction in central nervous system regions implicated in ALS pathology. Collectively, our findings suggest that AAV-mediated intrabody delivery targeting TDP-43 is a viable therapeutic strategy for ALS and paves the way for clinical development in ALS and other TDP-43-related disorders.

1042 Preclinical Evaluation of LY-N001 in an innovative GBA1-Associated Parkinson's Disease Model

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Parkinson's disease (PD), a globally prevalent neurodegenerative disease characterized by striatal dopamine (DA) deficiency and intracellular inclusions containing aggregated α-synuclein, leading to motor and cognitive impairments. Increasing evidence indicates that loss of function mutations in GCase are a significant risk factor for Parkinson's disease. GBA1 related PD (GBA1-PD) typically presents at an earlier age than idiopathic PD, with clinical symptoms progressing more rapidly, including motor dysfunction and cognitive decline. This highlights the urgent need for therapeutic interventions aimed at restoring GCase levels in the brain to slow or even halt the progression of GBA1-PD. Numerous transgenic GBA1 related mouse models have been employed to study GBA1-PD, however, these models have notable limitations in replicating human clinical symptoms. Most models exhibited GCase deficiency, substrate accumulation, some with slightly a-synuclein deposition, while absence of neurodegeneration and behavior abnormalities.

Acknowledging the complexity and heterogeneous characteristics of PD, we developed an innovative compound rat model that combines both GBA1 genetic deficiency and a low-dose 6-hydroxydopamine (6-OHDA)-induced chemical lesion. This model exhibited diminished GCase activity in the striatum and substantia nigra, along with a more pronounced degree of degeneration of tyrosine hydroxylase (TH)-positive neurons compared to the 6-OHDA model in wild type (WT) rats, closely mirroring the progression and severity of GBA1-PD. LY-N001, an innovative adeno-associated virus (AAV9)-based gene therapy, was designed to constitutively express a codon-optimized GBA1 gene, aiming to rectify the intrinsic genetic defect causing lysosomal dysfunction and potentially altering the disease's trajectory. Administration of LY-N001 in 6-OHDA *GBA1*$^{M1/M1}$ rat model resulted in widespread distribution of GCase enzyme activity throughout cerebral regions, including cortex, striatum, hippocampus and

substantia nigra. Immunohistochemistry results further affirmed the distribution of GCase in CNS. Notably, LY-N001 effectively mitigated the degeneration of tyrosine hydroxylase (TH)-positive neurons in the striatum and substantia nigra, accompanied by functional and behavioral improvement. Additionally, an 8-week Non-GLP toxicology study in Cynomolgus monkeys was conducted via intra cisterna magna (ICM) injection and preliminary results indicated the dose-dependent GCase biodistribution in CNS and endorsed LY-N001's safety profile, exhibiting exceptional tolerability and absence of toxicity. In summary, the extensive preclinical data in both rodent model and NHPs demonstrate the therapeutic potential of LY-N001 for GBA-PD, potentially ushering in a new era in the treatment of this complicated neurodegenerative disease.

1043 Characterisation of a dnajc6^{R857G} Knock-in Mouse Model of PARK19 Towards Gene Therapy Development

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Parkinson's disease (PD) is the second most common neurodegenerative disorder with up to 10% of PD with genetic aetiology, including PARK19/ *DNAJC6.* The DNAJC6 gene encodes auxilin, a neuronal cochaperone required for uncoating clathrin-coated vesicles during clathrin-mediated endocytosis (CME). It is crucial for synaptic vesicle recycling and neurotransmission, and bi-allelic loss of function mutations in the *gene* result in juvenile Parkinsonism or early onset PD. We previously reported a paediatric cohort of individuals with *DNAJC6* related juvenile parkinsonism with rapidly progressive parkinsonism, and broader neurological symptoms including cognitive decline and epilepsy. There is a major clinical need to develop novel therapies for this disorder, as no disease-modifying treatments exist. Our collaborators recently reported generation of *DNAJC6* patient derived induced pluripotent stem cell (hiPSC)-derived midbrain dopaminergic (mDA) neuronal model and demonstrated proof of principle of *in vitro* lentiviral gene therapy for this disorder (Abela et al. Brain 2024).

To progress gene therapy approach for this disease we have characterised the dnajc6^{R857G} knock-in mouse model, performing a comprehensive behavioural battery, including open field locomotor activity, vertical pole descent, rotarod, and neuroscore assessments. At six months of age, these mice displayed significant motor coordination deficits, impaired balance, and seizures recapitulating human disease phenotype. Additionally, brain immunohistochemistry showed dopaminergic neuronal degeneration in the *substantia nigra*. These findings pave the way for *in vivo* preclinical studies aimed at developing novel genetic therapeutic strategies to address synaptic dysfunction in juvenile and early-onset PD.

1044 Targeted Delivery to Putamen via MRI-Guided Surgery: Nonhuman Primate versus Göttingen minipig®

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Introduction: Recent advances in clinical approaches to delivering targeted gene or cell therapies to the brain using MRI-guidance have necessitated in vivo safety studies applying the similar procedures used in the clinical studies. While the standard preclinical animal model, to date, has been the nonhuman primate (NHP), the Göttingen minipig® has emerged as an important human-relevant preclinical model for brain and central nervous system (CNS) targeted gene or cell therapies. The minipig is a more human-relevant model than rodents while balancing the monetary cost and debate sometimes associated with using NHPs. The NHP and minipig brain are similar to the human brain developmentally, anatomically, and neurochemically and represent valuable models for therapies intended to target the CNS. Neurodegeneration in the putamen as indicated in Parkinson's and Huntington's disease requires precise treatment for gene replacement or disease reversibility. By targeting current surgical approaches to disease-specific parenchymal structures, one can minimize broader distribution and focus the therapy to the intended area.

Methods: In this work, we used magnetic resonance imaging (MRI)-guided Clearpoint catheter-based infusion to directly target parenchymal structures, including the putamen, in NHPs and Göttingen minipigs®. A 1.5T MRI scanner in conjunction with ClearPoint® Navigation software was used to scan the brain and plan the bilateral trajectories to the putamen. Clearpoint® catheter based infusion of 2% Gadoteridol and MRI scanning during infusion confirmed the correct trajectory and dosing of the targeted structure. After surgery, animals were taken to necropsy where 4 mm coronal sections of the brain were examined for correct target and dose placement.

Conclusions: The surgical procedures were well tolerated with no neurological or clinical impairments during the surgical procedures. Together, these data offer a path forward when testing the delivery of cells or cell specific gene therapies directly to the putamen and can provide a clinically relevant method for targeted brain delivery with accuracy in NHPs and Göttingen minipig®.

1045 Next-Generation AAV-Based Gene Therapy for Spinal Muscular Atrophy: Safety and Efficacy of EXG001-307 in Clinical Trials

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***Introduction*:** Gene therapy has transformed the treatment landscape for spinal muscular atrophy (SMA). Onasemnogene abeparvovec (Zolgensma), approved by the FDA in 2019, offers significant clinical benefits but is associated with off-target toxicities, including liver failure, as highlighted in its black box warning. This underscores the need for next-generation therapies with improved tissue-specific transgene expression and reduced toxicity.

***Methods*:** We developed EXG001-307, an AAV-based gene therapy engineered to enhance neuronal transgene expression. A Phase 1/2 open-label, multicenter, single-arm, dose-escalation clinical trial was initiated to evaluate its safety and efficacy in Type 1 SMA patients.

***Results*:** Thirteen patients were enrolled across two dose cohorts (3 patients in Low-dose Cohort [1.1×10^{14} vg/kg] and 10 patients in High-dose Cohort [1.5×10^{14} vg/kg]. Clinical efficacy was compelling, with all patients demonstrating rapid and sustained improvements in motor function: (1) CHOP-INTEND scores exceeded 40 in 100% of patients in the High-dose cohort within 5 months post-dosage; (2) 44% of patients in the High-dose cohort with over two months of follow-up were able to sit independently, achieving the primary endpoint; (3) one patient also demonstrated the ability to stand with assistance at 16.6 months of age. Notably, only one patient in the High-dose cohort has reached 18 months of age so far, and we anticipate additional developmental milestones as more patients reach this age. Two patients in High-dose Cohort experienced thrombotic microangiopathy (TMA), which resolved following treatment with *eculizumab* (anti-C5 antibody). Notably, both patients presented with concurrent infections within three days of AAV administration, involving either encapsulated bacteria or coronavirus. These infections have been recognized as potential triggers for TMA, suggesting a plausible association between the timing of the infections and the onset of TMA in the context of AAV administration. Most of other treatment-related adverse events (AEs) were mild, transient, and resolved without intervention.

***Conclusions*:** EXG001-307 demonstrated remarkable efficacy, enabling Type 1 SMA patients to achieve critical motor milestones, such as sitting and standing, which are unattainable in the natural history of the disease. This novel therapy offers a promising, targeted approach to SMA treatment, advancing the standard of care for patients with this devastating condition.

1046 Nonclinical Evaluation of AAV Gene Therapy EXG001-307 for Spinal Muscular Atrophy

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***Introduction*:** Spinal muscular atrophy (SMA) is a rare genetic disorder and the leading inherited cause of mortality in children under two years old. While the current gene therapy has demonstrated significant benefits, safety concerns related to off-target effects highlight the need for advanced, tissue-specific therapies. EXG001-307 is being developed to address these challenges by providing enhanced precision, efficacy, and safety.

Methods: EXG001-307, an AAV-based gene therapy, was administered directly into the cerebrospinal fluid (CSF) in preclinical animals. Efficacy and dose range-finding studies in SMA mice (SMNΔ7) were conducted to establish therapeutic potential. A pilot safety study was performed in wild-type rats, followed by a 6-month GLP-compliant toxicology study in nonhuman primates (cynomolgus macaques) to evaluate safety, toxicokinetics, biodistribution, shedding, and immunogenicity.

***Results*:** Preclinical studies demonstrated exceptional efficacy and a favorable safety profile for EXG001-307. SMA mice treated with the highest dose (2×10^{11} vg/animal) exhibited a median survival of 373 days, with a maximum survival of 501 days, compared to a maximum survival of 17 days in vehicle-treated controls. Biodistribution studies revealed robust AAV genome presence and transgene expression in the central nervous system (CNS) while minimizing expression in the liver and heart, resulting in an improved therapeutic profile. GLP toxicology studies in cynomolgus macaques confirmed safety and tolerability at all tested doses (1×10^{13} vg and 3×10^{13} vg/animal).

***Conclusion*:** These findings strongly support the clinical development of EXG001-307 for intrathecal administration in SMA patients. This approach has the potential to treat all SMA subtypes across various ages while addressing critical limitations of existing therapies. Regulatory communication with the FDA has been initiated, and IND submission is targeted for June 2025.

1047 Development of Tissue Engineered Tregs for the Treatment of Ischemic Stroke and Neurodegenerative Diseases

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Stroke represents a significant global health burden, ranking a leading cause of disability and death with a staggering prevalence of nearly a million individuals affected annually in the United States alone. Current reperfusion therapies used for stroke treatment, provide life-saving treatment but fail to address neuro-repair, therefore offering little promise for reducing the burden of long-term disability often associated with stroke patients. Regulatory T cells (Tregs) play a central role in establishing and maintaining immune tolerance, with more recent discoveries suggesting a subset of these cells, dubbed “Tissue Tregs”, are additionally involved in orchestrating tissue homeostasis, repair and regeneration. Tissue Tregs, enriched in tissues following injury but rare in circulation, have been shown to play a critical role for controlling neuroinflammation and promoting neurogenesis following injury. We report the generation of engineered Tissue Tregs (Tissue EngTregs) using a gene editing approach from bulk CD4+ T cells resulting in the stable expression of FOXP3, a chemically inducible signaling complex providing tunable IL-2 signaling in the edited cells, and an alarmin responsive transgene imparting tissue Treg-like phenotype.

The resulting engineered cells displayed a robust Treg phenotype as measured by the expression of key Treg-associated proteins including FoxP3, CD25, CD39, HLA-DR, TNFRII, and increased suppressive ability relative to control mock-engineered cells. Tissue EngTregs also upregulated key chemokine receptors required for homing and anchoring to inflamed tissue (including CCR4, CCR5, and CCR8). When exposed to an acute inflammatory cytokine milieu, tissue EngTregs facilitated sequestration of inflammatory cytokines (e.g. TNFα, IL-2, IL-4, IL-33) in vitro. Finally, Tissue EngTregs can uniquely express neuro-repair associated growth factors such as Osteopontin, an important biomarker of stroke known to improve BBB function and promote microglia mediated white matter repair in models of ischemic stroke. To validate the off-the-shelf efficacy of this approach, we used surrogate allogeneic polyclonal murine Engineered Tregs (mEngTreg) in the transient middle cerebral artery occlusion (tMCAO) mouse model of ischemic stroke. Briefly, mEngTregs were generated by Cas-9 mediated editing of MND in the Foxp3 locus of C57BL/6 (autologous) or CB6F1 (allogeneic) CD4 splenocytes and administered IV to tMCAO mice. tMCAO mice treated with autologous or allogeneic mEngTregs but not disease controls, showed enhanced motor skills and sensory function as assessed by multiple readouts including accelerated rotarod, foot fault and adhesive removal tests. Impressively, allogeneic Tissue mEngTreg treated mice showed improvements in key learning and memory readouts at four to five weeks after administration of allogeneic cells and injury onset as assessed by activity in the Morris Water Maze and passive avoidance tests. Finally, Tissue mEngTregs showed injury specific engraftment as they were enriched only in the injured segment of the brain but not in the contralateral hemisphere or other uninjured tissue. Altogether, the ability of mEngTregs to ameliorate disease as measured by key translatable biomarkers supports the potential for developing a potent off-the-shelf allogeneic engineered Treg cellular therapy for ischemic stroke with potential efficacy in additional neuroinflammatory and neurodegenerative indications.

1048 Development of deficits in attention and cognitive functions in Spinal Muscular Atrophy mouse model with non-physiological restoration of SMN1 transgene expression

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Spinal muscular atrophy (SMA) is a genetic neuromuscular disorder characterized by the degeneration of alpha-motor neurons, primarily caused by the loss of functional survival motor neuron gene 1 (SMN1) and retention of SMN2. While the FDA-approved gene therapy using adeno-associated virus vector (AAV) has marked a significant advancement in SMA treatment, concerns persist regarding potential toxicities and phenotype alternations with long-term benefits still to be seen. This study investigates the therapeutic potentials of neuromuscular, cognitive, and mental functions of a novel AAV vector (cceAAV) in a humanized SMA mouse model. We tested various doses and vector designs for profiles on hSMN1 expression, survival, and body weight, as well as behavioral assessments for motor function, coordination, locomotor activity, gait patterns, and cognitive and attention-/anxiety-like behaviors. A dose-dependent increase of therapeutic benefits was observed, reaching the highest at 1x1011 vg/g for cceAAV9 with a lifespan of 2.5-fold of untreated SMA, a level comparable to parallel controls treated with conventional self-complimentary vector (scAAV9) at A the same dose. Interestingly, when separating treated SMA mice into short ($/_S$, lifespan<6 weeks) and long-survivor groups ($/_L$), all AAV-treated SMA groups, except for SMA SC$/_S$, exhibited normalized motor functions like heterozygous littermates (Δ7Het) at Day 14 of age in behavioral tests for surface righting reflex, negative geotaxis test and hind-limb posture. However, only the SMA CCE$/_L$ group showed horizontal activities comparable to normal controls on Day 16 of age in the open-field test. Such normalized locomotor activities were also observed in the SMA CCE$/_L$ group but not in SMA SC$/_L$ mice at 2 months, with significantly fewer rearing activities. Markedly, all SMA mice treated with either vector displayed a complete loss of marble burying reflex at 4 weeks of age when normal or significantly higher horizontal mobilities were detected compared to Het controls. Such abnormal behaviors indicated the possible involvement of attention and anxiety deficits in AAV-treated SMA. Moreover, unlike normal controls exhibiting habituation to the environment with a 50% reduction of horizontal activities in the 3rd trial during the repeated open-field tests, the AAV9-treated SMA mice failed to habituate with only a 7% reduction, suggesting cognitive abnormalities in treated mice. Notably, such abnormalities in non-motor functions were not detected in age-matched normal littermates injected with the same dose of AAV vectors. Investigations are underway to determine potential disfunctions/dysregulations in the cerebrum of AAV-treated SMA mice. The data not only presents defects of neuropsychiatric and cognitive

functions in outlived SMA mice after AAV gene therapy but also reveals novel neurological phenotypes with undefined pathogenesis that might uniquely affect the outcomes and quality of life for treated SMA patients.

1049 Chemogenetic silencing of hyperexcitable neurons demonstrates sustained pain relief in a preclinical model of lumbosacral radiculopathy

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Lumbosacral Radiculopathy (LSR) is a chronic pain condition that affects 3-5% of the US population and results from compression or irritation of lumbar nerve roots leading to neuropathic back pain along with sensory and motor dysfunction. Treatments, including anti-inflammatory drugs, physical therapy, and epidural steroid injections typically fail to provide pain relief, leading to invasive surgical intervention or nerve ablation as final options which often yield variable and transient results.

A novel therapeutic approach to address the unmet medical need in LSR and potentially other chronic pain indications is through the application of chemogenetics. Using AAV, a chimeric receptor/ion channel can be introduced into target sensory neurons designed to alter membrane potential and reduce overactive firing associated with pain following treatment with a high affinity receptor ligand. The efficacy of AAV carrying a modified nicotinic α7 receptor coupled to a glycine receptor (AAV.m-α7-GlyR) was evaluated in a rat peripheral neuropathic pain model of LSR. AAV.m-α7-GlyR is designed to drive expression of a glycine channel that is activated by the FDA approved, high affinity, nicotinic agonist drug varenicline (VT). AAV.m-α7-GlyR + VT is expected to decrease lumbar nerve root hyperexcitability following focal administration to dorsal root ganglia (DRGs) representing a potential new and durable treatment option for chronic pain.

The preclinical efficacy of AAV.m-α7-GlyR + VT was evaluated in the chronic constriction injury (CCI) rat model of neuropathic pain. Nerve ligation surgeries resulted in mechanical allodynia as measured by a reduction in Paw Withdrawal Threshold (PWT). Three to four weeks prior to ligation surgery, AAV.m-α7-GlyR was administered using a transforaminal epidural (TFE) administration at doses ranging from 1E9 to 1E10 vg/animal. Efficacy was measured using von Frey filament assays to determine PWT between 2 and 8 weeks after model induction. Animals were divided into three treatment groups: AAV + VT (active gene therapy); AAV without VT (inactive gene therapy); and vehicle + VT (control). PWT was measured at baseline and 1 to 24 hours post VT treatment (0.05 to 0.1 mg/kg, PO).

Animals treated with AAV.m-α7-GlyR + VT demonstrated significant improvement in mechanical hypersensitivity. PWT at baseline were between 2 and 4 g, which increased to 6 to 10 g between 1 to 6 hours post-VT ($P < 0.01$); PWT returned to near baseline levels within 24 hours. This efficacious effect of AAV.m-α7-GlyR + VT was sustained throughout the study duration. Neither AAV.m-α7-GlyR alone nor VT alone demonstrated significant changes in PWT. There were no surgery or treatment related changes in PWT measured on the contralateral side. Analysis of DRGs showed dose-dependent vector DNA and transgene mRNA expression, with the highest expression observed in targeted DRG neurons that correlated with efficacy. These results support further development of AAV.m-α7-GlyR + VT as a novel therapeutic approach for LSR and other chronic pain conditions.

1050 AAV9-h*PLA2G6* Gene Therapy Improves Survival in a Preclinical Mouse Model of Infantile Neuroaxonal Dystrophy

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Infantile neuroaxonal dystrophy (INAD) is a rare neurodegenerative disorder affecting approximately 5,000 children globally. Pathology arises from autosomal recessive loss-of-function variants in the *PLA2G6* gene, leading to a rapid loss of vision, motor, and cognitive function. The disease is severe, with children not surviving beyond ten years of age. Currently, no approved treatment exists, highlighting the urgent need for effective therapies. As such, the primary goal of this work is to develop a gene therapy intervention for INAD using adeno-associated virus serotype 9 (AAV9) mediated delivery into cerebrospinal fluid. Our preclinical studies use the B6.C3-Pla2g6^{m1J}/CxRwb mouse model (mutant *Pla2g6* mouse) of INAD, which exhibits a severe loss-of-function allele of *Pla2g6*. Like INAD patients, mutant mice develop phenotypes that progressively worsen including tremor, body weight loss, unstable gait, loss of righting ability, histopathological findings in the central nervous system, and most notably, a significantly shortened lifespan. We compared neonatal intracerebroventricular delivery of an AAV9 vector expressing the full-length human isoform of *PLA2G6* driven by either the ubiquitously expressed EF1α- or the neuronal-specific SYN1 promoter. mRNA expression levels were measured by qRT-PCR in both spinal cord and brain of mice receiving either vector. In the brain, homozygous *Pla2g6* mutant mice had approximately 2% *Pla2g6* mRNA compared to wild-type controls. Human *PLA2G6* expression ranged from 4 - 38 fold higher than endogenous wild-type mouse expression, whereas spinal cord expression ranged from 0.07 - 1.24 fold of wild-type expression. There was a strong sex-dependent difference in median survival between male (17 weeks) and female mice (28 weeks) in the vehicle-treated homozygous mice cohorts ($p < 0.01$). AAV9-EF1α-hPLA2G6 treatment extended median survival to 23 weeks for homozygous males and 30 weeks for homozygous females ($p > 0.05$ in males and $p < 0.05$ compared to vehicle-treated homozygotes). However, expression from the SYN1 promoter further extended survival to a median of 25 weeks in male mice and 35 weeks in female mice (both $p < 0.05$ compared to EF1α promoter). Nevertheless, SYN1-mediated expression of membrane-bound PLA2G6 was not sufficient to completely restore survival or motor defects in homozygous *Pla2g6* mice. We conclude that the central nervous system delivery of AAV9 vectors expressing the membrane-bound isoform of PLA2G6, from either a neuron-specific or a ubiquitous promoter, provides a credible candidate for preclinical development and have commenced dose dependency and toxicology studies towards this goal.

1051 Human patient-specific FOXG1 syndrome mouse model reveals roles of protein homeostasis and neuroinflammation in neurodevelopmental disorder

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Neurodevelopmental disorders (NDDs) are characterized by disrupted brain development, leading to cognitive, behavioral, and neurological impairments. FOXG1 syndrome (FS), caused by heterozygous mutations in the *FOXG1* gene, exemplifies a severe monogenic NDD. To investigate FS pathogenesis, we generated a patient-specific W300X mouse model carrying a truncation mutation. Our findings reveal that the truncated FOXG1 protein is more stable and almost exclusively nuclear-localized relative to the full-length protein, suggesting potential underlying mechanisms for its pathogenic effects. W300X-heterozygous (W300X-Het) mice exhibited profound dentate gyrus (DG) abnormalities, including disrupted neurogenesis, impaired granule cell migration, and altered dendritic morphology. Transcriptomic analyses identified widespread dysregulation in synaptic and protein homeostasis pathways, highlighting impaired ribosomal biogenesis, translational machinery, and proteostasis. The disruption of the FOXG1-MYCN pathway, critical for robust protein synthesis required in neural stem cell (NSC) division, synaptogenesis, and synaptic plasticity, emerged as a key mechanism underlying these defects. Furthermore, microglial activation and inflammation were markedly increased in the DG, contributing to a pro-inflammatory environment that exacerbates neurogenic and structural deficits. Mirroring hippocampal dysfunction in FS patients,

W300X-Het mice exhibited significant spatial learning and memory impairments. This study highlights disrupted protein homeostasis and neuroinflammation as central mediators of FS pathogenesis, providing a framework for developing therapeutic strategies targeting these pathways.

1052 Amsterdam Oncology and Neuroscience Research: ADORE is breaking barriers in therapy and diagnostics research in the Netherlands

Mark Mizee, Amsterdam UMC, Amsterdam, Noord-Holland, Netherlands

ADORE is a revolutionary initiative embedded in the Netherlands' largest academic hospital, Amsterdam UMC. Our unique approach has a clear mission: by fostering collaboration between oncology and neurology researchers, we aim to accelerate progress in the fight against cancer and neurological diseases. This requires us to think and act beyond conventional boundaries. Our leading clinicians, cancer researchers and neuroscientists, both from the Netherlands and abroad, collaborate in this research center to unravel the most pressing questions in cancer and neurological diseases. The goal is to enable earlier diagnoses, develop better and more effective personalized treatments, and reduce the risk of cancer and neurological diseases.

To enable our more than 2000 scientists to reach this goal, ADORE utilizes both public and private funding and has realized a state-of-the-art research center in Amsterdam, housing shared labs, infrastructure, and workspaces. Combining existing and new infrastructure and established expertise, 7 core focus areas are currently in development, with a focus on next generation modalities like cell and gene therapy:

Bioanking
Proteomics and Genomics
Total Body PET-CT
Clinical Trial Unit
Immuno- and cellular therapy
AI and Biocomputation
Target and Drug discovery

Within these core focus areas, an ever-evolving group of investigators will determine allocation of project and infrastructure funds, initiate interdisciplinary research initiatives, and utilize their network for public-private partnerships. Besides enabling our researchers to develop novel therapies and diagnostics, a dedicated team of business developers is actively pursuing worldwide research collaborations with biotech and pharma. Our aim is to drive breakthroughs needed to improve patient care, both now and in the future, at an accelerated pace.

1053 Characterization of a SOD1 Rat Model of ALS using Behavior, Electrophysiology, and Biomarkers: An Ideal Model to Test Novel Gene and Cell Therapies

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Amyotrophic lateral sclerosis (ALS) is a fatal neurological disorder characterized by progressive motor neuron degeneration resulting in muscle weakness, paralysis, and eventually death. Superoxide dismutase 1 (SOD1) gene mutations are associated with familial and sporadic forms of ALS. In collaboration with Taconic Biosciences, PsychoGenics characterized a SOD1 G93A overexpressing (NTac:SD-Tg(SOD1G93A)L26H) transgenic rat model of ALS. A total of 30 wildtype (WT) and 30 transgenic SOD1 mixed-sex rats (15 per sex in each group) were obtained at 12 weeks of age, and survival was measured up to 38 weeks of age. Body weight (BW) was measured weekly, and phenotypical characterization began at 14 weeks of age. Locomotor activity was assessed by open field,

grip strength, rotarod, and gait was assessed using PsychoGenic's automated platform, NeuroCube®. Neuromuscular responses were assessed by EMG. Additional biomarker analysis was performed at necropsy.

SOD1 rats exhibited a progressive decrease in BW and hindlimb grip strength starting at 20-22 weeks of age in both males and females, whereas decreased locomotor and rearing activities occurred at 29 weeks of age, only in males. Deficits in rotarod performance were also seen in the SOD1 rats starting at 22 weeks of age, clearly manifesting by 25 weeks. In general, behavioral deficits were more robust in male SOD1 rats compared to female rats, and female rats survived longer than male rats. NeuroCube® analysis showed a clear age-dependent discrimination of gait features between WT and SOD1 rats. Some of the gait changes seen in SOD1 rats were step length, stance and stride duration, and average speed. Assessment of compound muscle action potential (CMAP) found that starting at 24 weeks of age, onset latency, which corresponds to the time from initiation of nerve stimulus to response, was increased in SOD1 rats. Peak amplitude and neuromuscular conduction velocity were reduced in SOD1 rats compared to WT rats starting at 24 weeks of age.

Analysis of cerebrospinal fluid (CSF) and plasma showed an increase of Neurofilament light (NFL) and the inflammatory marker IL-6, in the plasma and CSF of SOD1 rats. Immunohistochemistry analysis showed significant astrocyte (GFAP+)- and microgliosis (Iba1+) accompanying pFTAA positive SOD1 aggregate load in the spinal cord and brain stem. Additional histological marker analysis of brain and spinal cord are ongoing (ChAT, SOD1, NeuN markers). Together, these results suggest that the SOD1G93A transgenic rat model can be used for screening novel therapeutics, such as gene and cell therapies, for ALS treatment.

1054 Morc2a Variants Cause Hydroxyl Radical-Mediated Neuropathy and Are Rescued by Restoring GHKL ATPase

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**Corresponding Author*

Mutations in the Microrchidia CW-type zinc finger 2 (MORC2) GHKL ATPase module cause a various of neuropathies, however, the aetiology and therapeutic strategy are not understood. Previously, we reported that the Morc2a p.S87L mouse exhibited neuropathy through DNA damage accumulation. In this study, we investigated the pathological pathway using Morc2a p.S87L mouse embryonic fibroblasts and human fibroblasts in MORC2 p.R252W. We revealed that Morc2a p.S87L causes a protein synthesis defect, resulting in the loss of function of Morc2a and high hydroxyl radical levels. We considered the Morc2a GHKL ATPase domain as a therapeutic target because it complements hydroxyl radical level. We used the adeno-associated virus (AAV)-PHP.eB, to express Morc2a or Morc2a GHKL ATPase domain protein *in vivo*. Notably, AAV gene therapy ameliorated neuropathy in Morc2a p.S87L mouse. Loss of function in Morc2a p.S87L were also noted in human MORC2 p.S87L or p.R252W variants, indicating the correlation between mouse and human pathogenesis. In summary, this study demonstrates that the Morc2a p.S87L variant causes hydroxyl radical-mediated neuropathy, which can be rescued through AAV-based gene therapy.

1055 A humanized *Atl1* mouse model for Spastic Paraplegia 3A exhibits accelerated clinical phenotypes in a *Reep1* KO background.

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Introduction: Hereditary spastic paraplegias (HSPs) encompass a diverse group of rare diseases hallmarked by a progressive spasticity and weakness in the lower limbs. These disorders can arise from genetic mutations in a variety of different genes and cellular pathways. Mutations in the gene Atlastin-1 (Atl1) gives rise to spastic paraplegia 3A (SPG3A), an autosomal dominantly inherited disease accounting for about 10% of hereditary spastic paraplegia cases. *Atl1* functions in the fusion of smooth endoplasmic reticulum (ER) tubules at three-way junctions, which plays a vital role in cellular trafficking and communication. Mutations in *Atl1* fail to properly distribute the tubular ER, which severely impacts larger cell types. In the case of SPG3A, the long corticospinal axons of the central nervous system are unable to operate effectively due to a disrupted ER network failing to support the full length of the axons.

Methods: Mouse models for human diseases have played a pivotal role in the development of new treatments. A major hurdle for developing new mouse models is phenocopying the human disease in a nonhuman organism. In the case of *Atl1*, mutant mice develop a progressive loss of hindlimb function, albeit at a much slower rate compared to the developmental timeline of the disease in humans. One way to overcome delayed onsets in mouse models is to compound the defective pathway with a sensitizing genetic background or mutation. Zhu, *et al* demonstrated this approach by crossing a mouse pathogenic *Atl1* mutation into a *Reep1* KO background, resulting in paraplegia onset occurring within a few weeks compared to several months in the single mutant. Reep1 binds Atl1 and also functions in shaping the tubular ER network, and its dysfunction similarly produces a human HSP, SPG31. By generating a mouse model that contains the human exon harboring the offending SPG3A mutation, insights gained from mouse studies are much more translatable to the clinic. Here, we employ a humanization strategy to introduce the human genetic sequence, along with a severe pathogenic p.M408T mutation, into a mouse model. We then use the mutational load of *Reep1* KO to accelerate the disease onset. Using the human mutation to establish and then correct the disease state offers fewer regulatory hurdles when moving treatments into the clinic.

Results: Here, we demonstrate that a humanized *Atl1* mouse model harboring the p.M408T patient mutation produces the SPG3A phenotypes previously seen in the mouse *Atl1* mutant in a *Reep1 KO* background (B6.Cg-Reep1$^{Gt(OST398247)Tigm}$; Atl1^{em4Lutzy}, referred to as Reep1 KO; Atl1 hM408T). Significant decline in several phenotypes, including progressive loss of rotarod performance, hindlimb grip strength, and limb clasping, recapitulate the disease presentation in humans. Characterization of this mouse has also revealed an additional clinical phenotype, in the form of prominently reduced compound muscle action potentials (CMAPs).

Conclusions: Taken together, these readouts validate the Reep1 KO; Atl1 hM408T model as a functional platform for SPG3A study and therapeutic testing.

Acknowledgements
Kids with SPG3A, Carter Foundation for Neurologic Disease

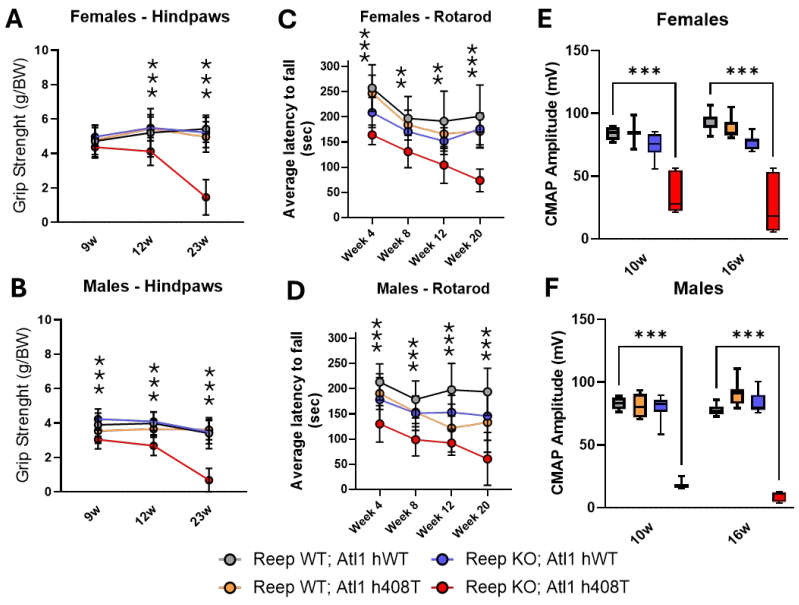

Figure 1: Key Phenotypes in a humanized Alt1 M408T mouse model

*(**A,B**) Hindlimb grip strength assessment in female and male mice. Animals were assessed across 3 trials each at 9w, 12w and 23w of age. n = 5-10 for all groups for grip strength (**C,D**) Rotarod latency to fall for female and male mice. Mice were longitudinally assessed at 4w, 8w, 12w, and 20w of age for their ability to remain on the rotating rod. n ≥ 5-10 for all groups for rotarod. Data represents average of 3 trials with 1m rests between runs. **(E,F)** Longitudinal CMAP signal in control and Reep1 $^{-/-}$/ Atl1 $^{hM408T/hM408T}$. Mice were anesthetized and electrodes placed on either side of the sciatic nerve on the proximal thigh. Stimulus intensity was increased until CMAP was maximized). Data presented as peak-to-trough amplitude average. n ≥ 6 for all groups. Data presented as mean ± SD. Asterisks indicate P≤0.05(*), 0.01(**), 0.001(***), two-tailed unpaired t-tests with Welch correction.*

1056 A PURA Knock-out mouse model for PURA syndrome exhibits fatal seizures

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Introduction: The Pur-alpha protein (Purα) is a DNA-RNA-binding protein involved in various cellular processes such as DNA replication and the regulation and control of transcription and translation. This 322 aa protein is highly conserved from bacteria to humans. *PURA* is ubiquitously expressed throughout the body, with highest found in the prefrontal cortex, and immune system. *PURA* plays a crucial role for brain development, supporting growth and division of neurons. A microdeletion in *Pura* leads to PURA syndrome. This rare disease is caused by *de novo* mutations leading to a wide range of symptoms, including but not limited to neonatal hypotonia, developmental delay, and seizures. The mechanism is believed to be haploinsufficiency with no direct treatment available. Physical, occupational speech and language therapies are currently the only options. To date, few mouse studies have investigated PURA deficiency. Here, we describe a new model for PURA that produces phenotypes making it ideal for therapeutic investigations.

Methods: Mouse models for human diseases have played a pivotal role in the development of new treatments. A major hurdle for developing new models is phenocopying human disease into a nonhuman organism. We generated a new mouse knockout model to study PURA syndrome using CRISPR/Cas9. RNA expression was utilized to establish the mechanistic underpinnings of any physiological or behavioral phenotypes. To interrogate the mouse model, we established phenotyping cohorts of heterozygous (Het) and homozygous (Hom) animals. Body weight and survival were monitored weekly. Additionally, home cage monitoring was utilized to record mouse activity in cages that tracked the animals 24/7 to capture seizure events.

Results: In this study, we identified several patient-relevant phenotypes in our PURA mouse model. After confirming Het animals had reduced mRNA expression in whole brain tissue, animals were assessed for body

weight, survival. We observed that Hom mice were leaner at birth compared to Het and wild-type counterparts. Hom mice experienced early attrition and died prematurely by 20 day of age. Het mice also exhibited a reduction in survival, with an increased death rate between 8 and 13 weeks of age. By 13 weeks, 90% of mice had died. To better understand the nature of this early attrition, Het and wildtype littermates were housed in camera-equipped DIVA cages, allowing for 24/7 recording of the animals. We found that all Het animals that succumbed to early death experienced a massive, terminal seizure event.

Conclusion: These results indicate that the PURA mouse model exhibits phenotypes consistent with human disease. Our initial findings show that PURA KO mice exhibit reduced PURA mRNA expression compared to controls. Additionally, we see that the terminal event for the mice demonstrates a massive seizure event, indicating abnormal neuronal activity in this model and resulting in shortened lifespan. It is worth noting, previous Het PURA models did not exhibit this attrition, nor reduced survival, making this new model an attractive platform for therapeutic interventions. Future investigations using VEEG/EMG to establish neuronal activity are currently underway. This platform will provide support for the scientific community in further understanding the role of PURA and exploring potential therapeutic strategies such as gene therapies.

1057 Gene Therapy Development for DDX3X Syndrome

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Background: DDX3X syndrome is a neurodevelopmental disorder first described in 2015 and today is thought to be one of the leading known genetic causes of intellectual disability in females. The syndrome is caused by mutations in the Dead-Box Helicase 3 X-Linked gene (*DDX3X*), which encodes an abundant and ubiquitously expressed RNA helicase. Despite the clinical burden of DDX3X syndrome and the known genetic cause, there is no effective treatment for these patients. We have previously shown that intracerebroventricular administration of an AAV9-mediated gene therapy delivering a functional human optimized *DDX3X* transgene in mice at postnatal day 1 is safe but results in minimal efficacy, warranting further studies to understand if the loss of DDX3X during embryonic development results in permanently altered postnatal behavior.

Methods: To determine if gene reinstatement after birth can ameliorate postnatal phenotypes, we generated a Ddx3x reinstatement mouse using the flip excision (FLEx) genetic technology. With this model reactivation of *Ddx3x* under endogenous conditions occurs after two steps: 1. Cre mediated inversion of a stop cassette by homologous recombination to turn *Ddx3x* off after conception, and 2. tamoxifen inducible FLPO excision of the stop cassette postnatally at a user defined timepoint to turn *Ddx3x* back on. The stop cassette used in this mouse models heterozygous *DDX3X* variants resulting in haploinsufficiency, which accounts for at least half of the patient population who have known loss-of-function mutations. *Ddx3x* was reinstated at postnatal day (P) 1 via a subcutaneous injection or P21 via an intraperitoneal injection of tamoxifen to assess if there is a time window necessary for treatment. A battery of behavioral tests at 3 months of age assessing motor function, anxiety, and other disease relevant behaviors was conducted.

Results and Conclusions: The mice treated with tamoxifen had varying degrees of *Ddx3x* reinstatement dependent on tissue type as measured by digital PCR. *Ddx3x* in the liver, ear, and brain showed about 90%, 60%, and 35% reinstatement (respectively), 4 months after a P1 injection of tamoxifen. Four months after a P21 injection of tamoxifen, *Ddx3x* in the liver, ear, and brain showed about 90%, 60%, and 15% reinstatement, respectively. At both reinstatement timepoints phenotypes were not rescued or ameliorated. These phenotypes included anxiety as measured by both duration in the center and frequency to enter the center of an open field, repetitive behavior as measured by number of marbles buried during a marble burying paradigm, and weight. Tamoxifen related toxicity was observed in relation to motor readouts, making it difficult to understand the effect of gene reinstatement on motor coordination and function. While it is possible that higher reinstatement in the brain could ameliorate phenotypes, the data suggests that mutations in *Ddx3x* cause phenotypes that will be difficult to treat postnatally with current vector technology such as AAV9. A caveat to these experiments is the relatively low degree of brain-

specific reinstatement that was achieved, and it remains possible that a higher proportion of rescued cells could alter the course of the disease.

1058 AAV Gene Therapy for a Newly Identified Inherited Disorder of Copper Transport

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CTR1 (*SLC31A1*) is a high-affinity importer of the trace metal copper that is required for proper infant brain development and that enhances the metastatic potential of certain copper-dependent cancers. Our report of twin males homozygous for a novel *SLC31A1* missense variant, R95H, provided the first detailed description of human CTR1 disease (Batzios et al., 2022). These patients manifested a distinctive clinical phenotype: profound infantile-onset neurodegeneration characterized by seizures, developmental delays, hypotonia, and brain atrophy. To futher characterize this disorder, we are conducting detailed clinical, biochemical and molecular studies, and experimental therapeutics in a CRISPR/Cas9-generated *SLC31A1*^{R95H} mouse model, as well as in cerebral organoids derived from iPSCs generated from cultured fibroblasts of affected individuals. Initial data reveal that R95H homozygous affected mice have shortened lifespans, drastically reduced brain copper levels, impaired growth, and neurological abnormalities, including seizures, features that recapitulate findings in affected human subjects. We are assessing the clinical and biochemical impacts of systemic AAV9-*SLC31A1*(dose ranging from 2.6 x 10^{12} vg/kg to 2.6 x 10^{13}vg/kg). In addition, we are performing transcriptomic and proteomic profiling of cerebral organoids derived from patient fibroblasts harboring CTR1^{R95H} or other variants (CTR1^{L79P}, CTR1^{Q125K}) from other recently identified subjects. These studies include CRISPR-corrected isogenic controls as well as complete CTR1 knockout brain organoids. The evaluation of the impact of AAV6-*SLC31A1* gene transfer on cerebral organoid phenotypes is also in progress, since the AAV6 serotype has high tropism for cerebral organoids. Considering the absence of CTR1 deficiency models, our combined studies provide valuable insight concerning 1) brain-associated functions of CTR1, 2) disease mechanisms related to CTR1 deficiency, and 3) proof of principle for AAV-mediated gene therapy in affected patients.

1059 Dissecting Molecular Mechanisms Underlying Blood-Brain Barrier Deficits in Tuberous Sclerosis Complex

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Tuberous sclerosis complex (TSC) is a multisystem rare genetic disorder. It is caused by mutations in either *TSC1* or *TSC2*, encoding for Tuberin and Hamartin, respectively. Tuberin and Hamartin interact to form a complex that negatively regulates cell growth by inhibiting signal transduction mediated by the mammalian target of rapamycin (mTOR). TSC patients not only develop benign growths in multiple organs such as skin, lungs, and brain, but also show a high incidence of refractory epilepsy, which adversely impairs cognitive abilities. Current treatment options include the use of mTOR inhibitors, although they have multiple limitations such as suppressing the immune system and show variable efficacies in patients. Perivascular inflammation and dysfunction of the blood-brain barrier (BBB) have been reported in TSC patients. Specifically, several studies have reported focal perivascular leakage of serum proteins in cortical tubers, which elicit neuronal hyperexcitability and may trigger the onset of seizures. Brain pericytes play an important role in the establishment and maintenance of the BBB. They control key physiological functions such as regulating BBB permeability and relaying inflammatory responses. In addition, pericyte dysfunction has been implicated in several neurological disorders including epilepsy, although their role in TSC pathogenesis has been understudied.

To investigate BBB dysfunction in TSC, we adopted a method to differentiate pericytes from patient-specific human induced pluripotent stem cell (iPSC) lines and have characterized their function in an in vitro BBB model. We have also generated *TSC2* KO pericytes and show they exhibit an increased propensity towards a calcified phenotype,

suggesting hyperactive mTOR signaling may contribute to tuber calcification in vivo. Moreover, we generated isogenic iPSC cell lines from the TSC patients with CRISPR-Cas9 techniques, thereby providing rigorous controls for our studies. In summary, this study will broaden our understanding of molecular mechanisms underlying BBB deficits in TSC and will reveal novel mechanistic insights into the role of pericyte-mediated calcification during disease pathogenesis, thus providing new strategies for therapeutic development for TSC.

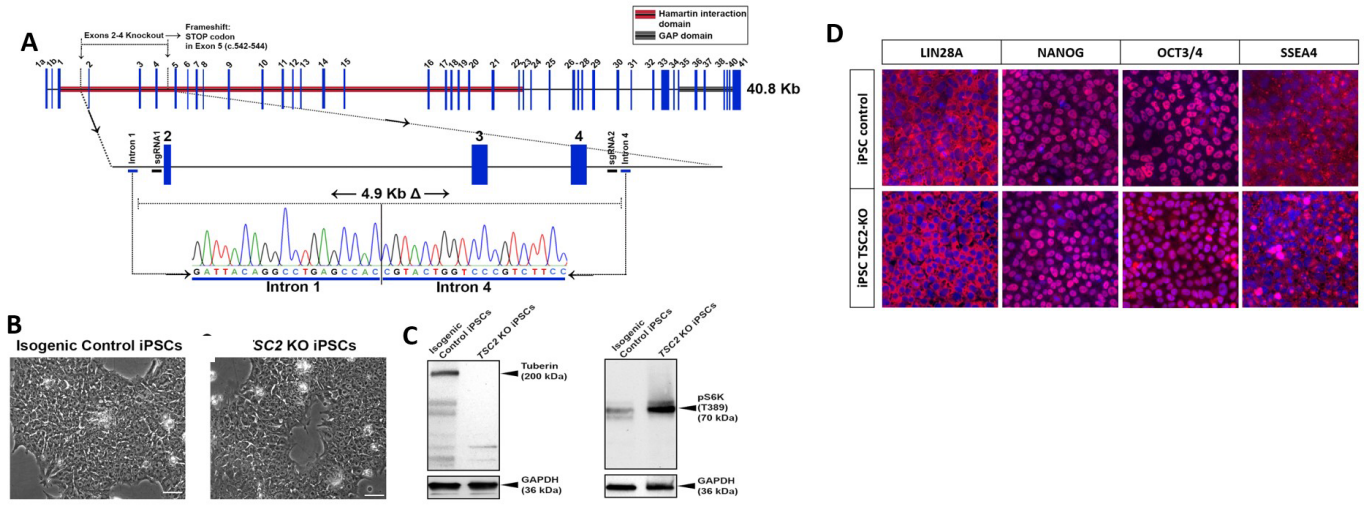

Figure 1| *TSC2* CRISPR Cas9 KO strategy and characterization of iPSCs. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from patient and control blood samples and underwent Sendai virus transduction with Yamanaka factors (OCT3/4, SOX2, KLF4, c-MYC) to produce induced-pluripotent stem cells (iPSCs). **(A)** *TSC2* genome locus, CRISPR Cas9 knock-out strategy and Sanger's sequencing results showing the 4.9kb deletion. **(B)** Phase contrast image of control and *TSC2* KO hiPSCs. **(C)** Western blot analysis showing TSC2 (Tuberin) and pS6 kinase expression in control and *TSC2* KO iPSCs. **(D)** Immunofluorescence staining of pluripotency markers in control and *TSC2* KO iPSCs showing LIN28a, NANOG, OCT3/4, and SSEA4.

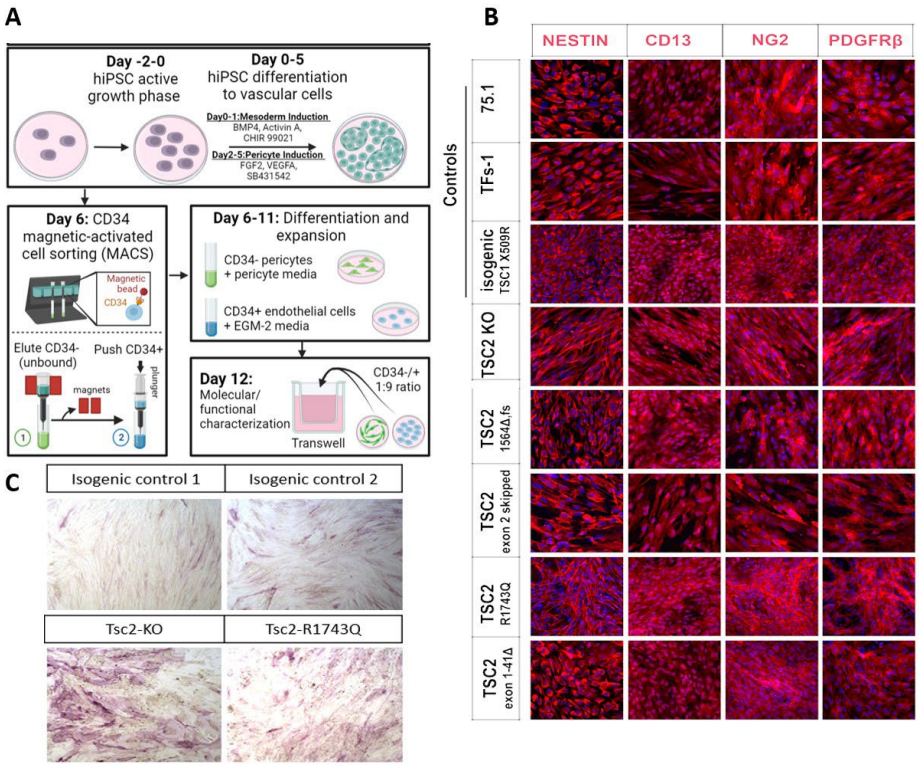

Figure 2 | Strategy to differentiate iPSCs into pericytes and endothelial cells and subsequent molecular characterization for pericytes. **(A)** Schematic showing the protocol to differentiate iPSCs into pericytes and endothelial cells. **(B)** Immunofluorescence staining of pericyte specific markers (NESTIN, CD13, NG2, PDGFRβ) in control and *TSC2* KO and TSC2 patient-derived iPSC lines. **(C)**Alkaline phosphatase staining of control and TSC1/2 deficient pericytes after a two-week culture in calcification induction media.

1060 Redefining TSC2 Gene Therapy: Development of a Novel Split Intein Payload and a Translational Potency Assay

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Introduction: Tuberous sclerosis complex (TSC) is a haploinsufficiency disorder affecting approximately 42,000 individuals in the United States, with ~70% of cases caused by *TSC2* mutations. These mutations lead to hyperactive mTOR signaling, contributing to a range of disease manifestations, including epilepsy and neurodevelopmental symptoms. TSC2 mutations are highly heterogeneous, making a single gene-editing approach impractical; instead, gene supplementation is the most viable therapeutic strategy. Importantly, overexpression of *TSC2* has not been associated with toxicity, meaning that restoration of *TSC2* protein levels does not carry the risk of a "goldilocks effect" requiring strict dose control. However, gene therapy for *TSC2* presents two major challenges: (1) its coding sequence (5.4 kb) exceeds AAV packaging limits, and (2) its delivery requires widespread distribution across the brain, as no single brain region or cell type is uniquely responsible for seizure generation. While minigene-based approaches have been explored to overcome the size limitation, they have failed to restore mTOR regulation in preclinical studies. To evaluate therapeutic potential preclinically, we developed a cell-based potency assay to assess *TSC2* payload function in vitro. This assay revealed that a previously published *TSC2* minigene was unable to restore mTOR regulation. Notably, this same minigene failed to demonstrate a therapeutic effect in an established *in vivo* disease model, underscoring the need for alternative strategies. In parallel studies with *TSC1*, payloads that restored function in the potency assay also demonstrated therapeutic effects in vivo, reinforcing the potential translational utility of this approach. Here, we present a novel split intein-based *TSC2* payload that enables full-length protein reconstitution, alongside a cell-based potency assay and a clinically relevant delivery strategy using an intravenously administered, BBB-penetrant capsid.

Methods: A phospho/total p70S6K-based potency assay was developed to assess therapeutic potential and guide payload optimization. Five split intein *TSC2* payloads were designed to splice within key domains, preserving critical folding and functional regions. *In vitro* studies were conducted in *TSC2* knockout (KO) HEK293 cells via co-transfection and viral co-transduction to assess protein expression and mTOR regulation. Comparative studies included 21 novel minigene constructs, as well as one previously published minigene.

Results: Despite efficient protein expression in *TSC2* KO cells, none of the 21 novel minigenes nor the previously published minigene restored mTOR regulation. Likewise, the previously published minigene failed to demonstrate a therapeutic effect in an established animal model. The split-intein payloads tested demonstrated expression and function in vitro, paving the way for future in vivo studies.

Conclusion: We report the successful development of a functional *TSC2* payload using split inteins, supported by a cell-based potency assay with clear preclinical and regulatory applications. The potential predictive value of this potency assay has been reinforced by successful in vivo studies in *TSC1*, supporting its utility for gene therapy development. These findings establish a viable path forward for *TSC2* gene therapy, with next steps including in vivo validation.

1061 Design of Synthetic Promoters for AAV-Driven Treatment of Limb-Girdle Muscle Dystrophy Type R1

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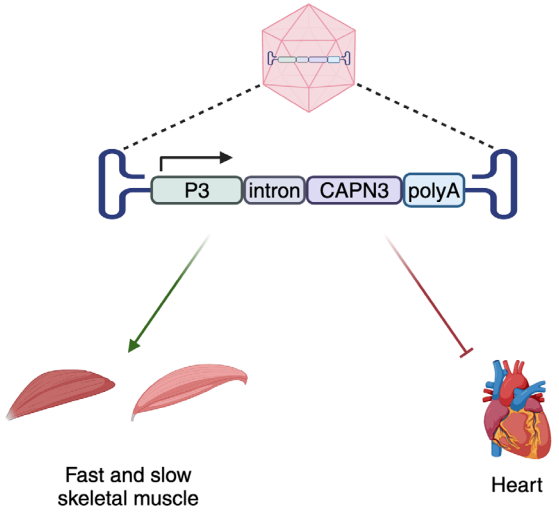
Limb-girdle muscle dystrophy type R1 (LGMDR1) is an autosomal recessive, progressive muscle-wasting condition caused by mutations in the *CAPN3* gene. *CAPN3* encodes for Capn3, a muscle-specific member of the calpain family of calcium-dependent cysteine proteases. Patients with LGMDR1 typically begin to exhibit clinical symptoms in their second decade of life. The muscle wasting is progressive and often leads to wheelchair dependence within 10 to 20 years after diagnosis. Since LGMDR1 is a loss-of-function disease, AAV-delivery of a healthy *CAPN3* gene should represent a promising therapeutic strategy for affected individuals. However, due to the absence of Capn3 expression in cardiac tissue, it was necessary to develop an artificial promoter that avoids expressing Capn3 in the heart while promoting high expression in both fast and slow skeletal muscle tissue. Currently, no existing promoters meet this specific expression pattern. To address this challenge, synthetic promoters were designed using various approaches, including machine learning, which was trained on known skeletal muscle enhancers. We packaged each engineered promoter that drives the mouse Capn3 gene into the myotropic vector AAVMyo and then assessed Capn3 expression across a wide range of muscle types as well as in the heart using capillary western analysis. Any promoters that resulted in Capn3 expression in the heart were excluded from further studies. This process identified promoter #3 (P3), which showed the strongest expression of Capn3 across both fast and slow muscle types, with no detectable expression in the heart. At the highest in vivo dose of 2e14 vg/kg, P3 achieved over 100% restoration of Capn3 across all tested muscle tissues, including the soleus. Additionally, Capn3 was not detected in either the heart or liver at this highest dose, and no signs of toxicity were observed in muscle and heart tissues expressing this elevated level of Capn3. A preliminary dose-response assessment using dosages of 1e13 vg/kg, 5e13 vg/kg, and 2e14 vg/kg indicated that even at the lowest dose of 1e13 vg/kg, at least 75% restoration of Capn3 occurred across various muscle types. Future studies will focus on establishing the relationship between Capn3 levels and the functional restoration of the LGMDR1 phenotype.



1062 Novel Muscle-Targeted Gene Therapy Vectors with Increased Potency based on Combinatorial Assembly of Evolutionary Conserved Human Transcriptional C*is*-Regulatory Elements (CRE) Identified by Comprehensive Transcriptome-Wide Data-Mining

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In vivo gene therapy based on adeno-associated viral vectors (AAV) is a promising modality to treat hereditary muscle disorders by directly tackling the root cause of the disease. Nevertheless, current AAV vectors are characterized by a relatively narrow therapeutic window and often result in low expression, toxicity and even mortality in clinical trials, especially with higher vector doses. To overcome these limitations of muscle-directed AAV gene therapy, we aimed to maximize transcription of the therapeutic genes in the afflicted target tissues. To achieve this, we have validated novel combinations of transcriptional cis-regulatory elements (CREs) that serve as "molecular turbo-chargers" to specifically boost expression of the therapeutic genes in the diseased target cells including skeletal muscle, heart and diaphragm. These CREs were identified by transcriptome-wide data-mining of genes that are highly expressed in human muscle, heart and/or diaphragm, providing a selected short-list of evolutionary conserved transcriptional elements. The potency and specificity of these CREs was validated based on in vivo AAV9 gene delivery in various murine disease models of severe genetic muscle disorders, including Duchenne muscular dystrophy (DMD), limb girdle muscular dystrophy (LGMD) and Pompe disease. Notably, incorporation of these CREs led to a significant increase in transcription of luciferase gene expression when combined with desmin and Spc5-12 promoters and outperforming potent muscle-targeted promoters such as MHCK7. These CREs also boosted expression of therapeutically relevant genes including micro-dystrophin or myostatin in the case of DMD, beta-sarcoglycan for LGMD or a-glucosidase (GAA) for Pompe. The extent of the increased transcription varied depending on the gene and/or target tissues attaining a significant 10- to 100-fold increase in transcriptional activity. The increased transcription correlated with a concomitant increase in protein expression levels of the respective therapeutic genes. The increased expression of the therapeutic protein was consistent with the normalization of nuclear localisation. Most importantly, systemic administration of the CRE-containing AAV9 vectors expressing the respective therapeutic genes resulted in sustained phenotypic correction in the DMD, LGMD or Pompe mouse models. In particular, Pompe mice treated with the CRE-containing vectors exhibited a normalisation of glycogen accumulation in skeletal muscle, diaphragm and heart resulting in complete functional correction of skeletal muscle, diaphragm and cardiac (S-T interval) function using comprehensive functional assays (i.e. hanging assay, grip tests, diaphragm force assay, electrocardiogram). In contrast, control vectors without CRE only yielded partial phenotypic correction, consistent with the lower GAA expression levels. Finally, the elevation of gene expression by these CREs was not confined to these murine models but was also confirmed in transfected human primary cardiomyocytes. Taken together, these preclinical results underscore the potential broad therapeutic implications of using CRE-containing vectors for the treatment of severe hereditary muscle disorders by improving the therapeutic window and overcome some of the manufacturing constraints.

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1063 RNA-guided Transcriptional Upregulation of Neuromuscular Disease Modifiers Using CRISPR-DREAM

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Duchenne Muscular Dystrophy (DMD) is an X-linked neuromuscular genetic disease caused by mutations in the gene that codes for dystrophin which results in no expression or the expression of nonfunctional protein. This disease affects 1 in 5,000 male births and results in muscle degradation leading to weakness, cardiac and respiratory complications, loss of ambulation, and premature death with a median life expectancy of 28 years of age (1). The CRISPR-dCas9 recruited enhanced activation model (CRISPR-DREAM) can potently increase transcription of

target genes. CRISPR-DREAM is a CRISPR-dCas9 system that makes use of human derived transcriptional activation domain plasmids (TADs) which may elicit lesser immune responses in humans than those derived from viruses (2). To demonstrate broad utility in skeletal muscle, we examined the following target genes. *UTRN* is a paralog of dystrophin and has been shown to alleviate the impact of dystrophin protein deficiency, and increased *UTRN* RNA expression causes increased protein production to alleviate the effects of dysfunctional dystrophin protein (3). IL-10 is of interest due to its inhibition of immune response, thus working to reduce inflammation when upregulated (4). Klotho is of interest due to its role in metabolism and aging (5). This study aims to determine optimal use of CRISPR-DREAM in skeletal muscle tissue for guide sequences of the gene targets to treat DMD in mouse skeletal muscle myoblast cells (C2C12). Target sequences in the mouse genes Utrophin (*Utrn)*, *IL-10*, and *Klotho* have been transformed into CRISPR-DREAM plasmids for transcriptional regulation. Multiple guide sequences were designed for each genetic target and transformed into CRISPR-DREAM plasmids for genetic regulation. Protospacer sequences in the respective DREAM plasmids were confirmed through sanger sequencing with whole plasmid nanopore sequencing also confirming the identity of all cloned plasmids. The C2C12 cells were then transfected with lipofectamine to deliver the therapy. Afterwards, RNA transcriptional efficacy was observed through quantitative real-time polymerase chain reaction (qRT-PCR) of the media of the treated cells, showing increased *UTRN* expression for both TADs with higher performance from guide sequences upstream of the gene's promoter region rather than downstream showing potential as a tool in DMD applications. Due to successful epigenetic regulation of the C2C12 cell line using CRISPR DREAM, a constitutively expressed CRISPR-DREAM C2C12 model cell line is being developed through lentiviral transduction to allow for rapid testing of several new targets. Through this design, optimal combinations of genetic targets for holistic gene therapy approaches to treating DMD will be tested in conjunction with each other to test for the most efficient combination.

References
Padmaswari et al. *Biophysics Reviews, 2023*
Mahata et al. *Nature Methods*, 2023
Wu et al. *Fundamental Research*, 2022
Saraiva et al. *The Journal of Experimental Medicine*, 2020
Castner et al. *Nature Aging*, 2023

1064 RNA Polymerase II Transcription of RNAi Sequences for Myotonic Dystrophy Gene Therapy

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INTRODUCTION: We are developing a gene therapy approach for the dominant genetic disease myotonic dystrophy (DM), a rare genetic disease affecting ~1 in 9000 adults. Individuals with DM experience progressive muscle weakness and myotonia (delays in muscle relaxation) and other multisystem effects such as sleep disturbances, executive function deficits, gastrointestinal discomfort, and heart conduction failure that can lead to sudden death. DM type I is caused by a CTG repeat expansion, with larger generational expansions (anticipation) that lead to accumulation of the repeat-expanded myotonic dystrophy protein kinase gene (*DMPK*) mRNA in the nuclei of cells and subsequent mis-regulation of splicing of hundreds of different mRNAs.

BACKGROUND: Previous proof of principal studies have demonstrated the potential for systemic AAV6-RNAi for reduction of the *DMPK* expanded repeat mRNA. We have pioneered delivery of the key splicing factor MBNL1 to mice for additional phenotypic correction, while preventing damaging over expression in heart. We have shown 2.5-fold upregulation in the quadriceps muscle of the HSALR mouse model of DM1.

CURRENT WORK: Our current focus is development of RNA polymerase II expression of DMPK RNAi sequences and we are currently testing *β*-actin promoter driven RNAi hairpin expression in the HSALR mouse model. Intramuscular injection of modified cassettes was performed and comparison with RNA polymerase III U6 expression of RNAi will be presented. Our goal is to combine RNA polymerase II limited expression of RNAi with supplemental MBNL1 protein as a dual therapeutic approach for DM1.

1065 Developing Suppressor-tRNAs to Treat Duchenne Muscular Dystrophy

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Nonsense mutations are point mutations that convert a sense codon into a premature termination codon (PTC) in the mRNA. One promising strategy to address these mutations is the use of suppressor transfer RNAs (sup-tRNAs) that can induce PTC readthrough during translation, thereby restoring full-length protein production. This approach has long been considered a viable gene therapy for approximately 11% of human pathogenic mutations caused by nonsense mutations. Recently, we reported the *in vivo* safety and therapeutic efficacy of AAV-NoSTOP, an adeno-associated virus (AAV)-delivered sup-tRNA targeting the TAG nonsense mutation, in the Hurler syndrome's mouse model (Wang J. et al. *Nature* 2022). Building on this work, the current study aims to expand the AAV-NoSTOP toolbox to target the TAA nonsense mutation, the most prevalent type of nonsense mutation in patients living with Duchenne muscular dystrophy (DMD) (Torella A. et al. *PLOS One* 2020). We hypothesized that AAV-NoSTOP could be an effective treatment for nonsense-mediated DMD because 10-15% of *DMD* mutations are nonsense in nature, and this strategy circumvents the challenge of delivering the full-length *DMD* cDNA, which exceeds the packaging capacity of AAV. The mdx mouse model, carrying a TAA nonsense mutation in the *Dmd* gene resulting from a glutamine codon (Gln)-to-TAA change, provides an ideal system for testing dystrophin restoration through AAV-NoSTOP, as this mutation represents a significant proportion of nonsense mutations in DMD patients. (Torella A. et al. *PLOS One* 2020). Therefore, we focused on developing UAA-sup-tRNA^{Gln}. Using a dual reporter plasmid with a Gaussia luciferase (*Gluc*) gene and a mutant *EGFP* gene disrupted by the TAA premature stop codon, we were able to assess the UAA-sup-tRNA's ability to readthrough the PTC by measuring the reporter protein levels. In HEK293 cells, we found that 11 out of 18 UAA-sup-tRNA^{Gln} designs achieved ≥30% readthrough. To assess whether the top UAA-sup-tRNA^{Gln} candidates could induce UAA readthrough *in vivo*, we co-delivered the reporter and sup-tRNA plasmids into wild-type mice via hydrodynamic injection. Interestingly, we observed a significant discrepancy between the readthrough activity in HEK293 cells and the mouse liver, suggesting that readthrough capacity in HEK293 cells is not a reliable predictor of sup-tRNA functionality *in vivo*. This may be due to the altered tRNA and aminoacyl-tRNA synthetase regulation in cancerous cell lines like HEK293, which may create a cellular environment more permissive to sup-tRNA biogenesis and function. Importantly, we identified one UAA-sup-tRNA^{Gln} candidate that exhibited ~9% UAA readthrough *in vivo*. We are now proceeding with the generation of AAV.NoSTOP reagents and will test their ability to restore dystrophin expression in *mdx* mice. (WF and MX: co-first authors)

1066 Long-term Follow-up and Evaluation of Multi-Exon Skipping by CRISPR/Cas9-based Genome Editing in a Canine Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe and progressive genetic disorder caused by mutations in the DMD gene, leading to the absence of dystrophin protein. Genome editing has emerged as a promising therapeutic approach for DMD, with ongoing validation in animal models. However, long-term studies assessing genome editing in large animal models of DMD are still lacking. In this study, we performed CRISPR/SaCas9-mediated multi-exon skipping genome editing in beagle-based Canine X-linked Muscular Dystrophy (CXMD). This model carries a point mutation at the splice acceptor site of exon 7, located in a hotspot N-terminal region of the DMD gene. We followed up for one year to evaluate the efficacy and safety of the treatment.

We developed an all-in-one recombinant adeno-associated virus 9 vector-based CRISPR/SaCas9 system, which included a spc 5.12 muscle-specific promoter and dual guide RNAs designed to excise exons 6-8 of the DMD gene. After validating the vector in immortalized DMD canine muscle cells, we intravenously administered the vector (2.0×10^{14} GC/kg) to 4-week-old DMD dogs (n=3). Age-matched DMD dogs (n=3) received saline as a control group.

Molecular analysis conducted one year post-treatment showed sustained exon skipping and dystrophin expression in various skeletal muscles and the heart. In the treated skeletal muscle tissues, in-frame exon skipping ranged from 1-22%, as detected by RT-PCR, with varying levels between muscle regions or individuals. Western blot analysis revealed 1-6% restoration of dystrophin protein of healthy levels. In the heart, in-frame exon skipping ranged from 5-33%, and dystrophin expression was restored by 2-17%. No significant changes in dystrophin expression were observed in temporalis muscle biopsy samples taken at 8 weeks and one year post-treatment. Histopathological analysis showed improved conditions in the treated skeletal muscles, including larger fiber size, fewer centrally nucleated fibers, and reduced fibrosis compared to untreated controls. Functional assessments at 1, 2 and 4 months post-treatment, including the 15-meter running test and standing test, showed promising trends in motor function. At one year, the treated group demonstrated improved isometric dorsiflexion torque, an indicator of hind limb muscle strength. Mild elevations in liver enzymes were observed in the treated group, but no other adverse events were noted. Over the one-year follow-up, there were no significant changes in serum creatine kinase levels between the treated and untreated groups.

These results demonstrate that our CRISPR/Cas9-based multi-exon skipping strategy induces long-term dystrophin expression in skeletal muscle and the heart, leading to improvements in muscle function. Our findings provide valuable insights for the future clinical application of gene editing therapies for DMD.

1067 Advancing the Novel CasX-Editor for Precise Genome Editing to Address Muscular Dystrophies

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Duchenne Muscular Dystrophy (DMD) is a severe, progressive neuromuscular disease caused by loss-of-function mutations in the DMD gene, leading to life-threatening respiratory and cardiac failure. Recent advances in AAV-mediated gene therapy, including the FDA-approved micro-dystrophin, offer promise for alleviating disease severity. However, challenges remain, as the micro-dystrophin encodes for a truncated, partially functional form of dystrophin that may also elicit immune responses, diminishing long-term efficacy. This underscores the urgent need for genome-editing strategies to directly address DMD mutations. Most notably, over 70% of DMD mutations are amenable to exon skipping or reframing to restore the expression of a nearly functional protein product, and advances in these approaches could provide mechanisms towards more effective and safe genetic solutions.

This study presents novel genome-editing strategies using a highly engineered CRISPR-CasX paired with a guide RNA molecule called the X-Editor (XE) to target mutations in DMD. We demonstrate that XE has a unique deletion pattern allowing for highly effective exon skipping by perturbing key splicing elements to produce a functional, near-full-length, truncated dystrophin protein. We first demonstrate highly efficient targeting of the 5' intron-exon boundary of DMD exon 53. In myoblasts derived from patients with a deletion of exon 52, we achieved ~90% editing efficiency, resulting in near-complete exon 53 skipping and robust dystrophin restoration in myotubes. Next, we set out to identify additional mechanisms of exon skipping other than canonical 5' exon boundary targeting. We predicted enhancer splicing elements in exon 53 in an engineered DMD exon 53 reporter line to demonstrate that XE-based genetic modification at these sites can induce exon skipping at levels comparable to or higher than those achieved via the canonical splice acceptor targeting strategy. Building on these in vitro findings, we evaluated AAV-mediated delivery of XE into muscle tissue in vivo. XE's compact size enabled efficient packaging into a single all-in-one AAV vector with different muscle-specific regulatory elements. Systemic delivery of these AAVs encoding the XE transgene into wild-type mice resulted in saturated editing in both cardiac and skeletal muscle at

exceptionally low viral genome levels (1-2 copies per diploid cell). To our knowledge, this is the first demonstration of saturated editing in muscle tissue at very low AAV doses.

Our findings establish XE as a powerful and versatile genome-editing technology for neuromuscular diseases like DMD, with the ability to target various splicing elements to enable therapeutic levels of exon skipping or reframing. By enabling precise and efficient AAV-mediated genome-editing in vivo, XE addresses critical challenges in neuromuscular gene therapy, offering a promising path toward scalable, durable treatments that could redefine the standard of care for DMD and other inherited muscle disorders.

1068 Development of Self-Delivering Gene Editors for Enhanced Non-Viral Delivery in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe genetic disorder characterized by progressive muscle degeneration, leading to loss of mobility and premature death due to cardiac and respiratory failure. Despite advances in gene therapy and exon-skipping treatments, there is still no curative therapy. AAV-based gene therapy has shown potential in delivering microdystrophin, but challenges remain regarding long-term efficacy, immune responses, and restricted access due to pre-existing immunity. Non-viral gene delivery offers a promising alternative but currently lacks the efficiency needed for effective skeletal muscle targeting.

This study presents a novel approach to overcoming these challenges by developing a "self-delivering" gene-editing platform. We designed a CRISPR-Cas9 delivery system modified with cell-penetrating peptides (CPPs) using cysteine-disulfide chemistry, improving tissue-specific delivery and enhancing cellular uptake while preserving Cas9's gene-editing functionality. Solvent-accessible surface area (SASA) analysis identified key residues (V743, S204, R1078, and L551) as ideal modification sites for conjugation with CPPs *via* reversible thiol-maleimide chemistry. Protein purification results using Ni-Sepharose affinity chromatography show that the Cys modified Cas9 proteins were successfully expressed and purified. Western blot analysis confirmed the successful modification of Cas9, with the mutants displaying a band at 160 kDa. In vitro DNA cleavage assays demonstrated that the cysteine-modified Cas9 variants retained full gene-editing functionality, comparable to commercial Cas9, with efficient DNA cleavage activity.

This strategy provides an efficient, non-immunogenic alternative to viral-based delivery systems, ensuring that Cas9 retains its activity post-delivery, making it suitable for gene therapy applications in muscle tissues. This platform holds potential not only for DMD but also for a wide range of genetic diseases requiring precise gene-editing tools. Future work will focus on preparing the Cas9 library with CPPs and testing them on transwell and microphysiological systems (MPS). The developed MPS will have broader applications in studying the biology of biomacromolecular drug delivery through the vascular endothelium. This study aims to establish a versatile toolbox of self-delivering gene editors optimized for muscle tissue delivery, addressing key barriers in non-viral gene therapy and paving the way for restoring muscle function in DMD and other genetic muscle disorders.

1069 MM201-Mediated Gene Activation Therapy Enhances Utrophin Expression and Improves Muscle Function in Duchenne Muscular Dystrophy

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Background: Duchenne Muscular Dystrophy (DMD) is a severe neuromuscular disorder primarily affecting young males, that leads to progressive muscle degeneration, loss of ambulation, and premature mortality. Given the devastating impact of DMD on individuals and their families, there is an urgent need for effective therapeutic strategies. Utrophin is a protein that shares structural and functional similarities with dystrophin, partially compensating for dystrophin loss. Increasing utrophin expression levels may improve muscle function, enhance quality of life, and potentially alter the disease's progression. We investigated a novel therapeutic approach using MM201-miniVPR, a Cas protein-mediated gene activation tool delivered via a single AAV vector to upregulate utrophin expression. This approach is mutation-independent, potentially benefiting a wide range of DMD patients.

Methods: Two DMD mouse models were generated: one with exon50 deletion ($DMD^{\Delta E50E51,hE50KI}$), and the other with nonsense mutation in exon23 ($DMD^{\Delta E23,hE23KI}$) (Fig. 1A). Muscle pathology in the tibialis anterior (TA), diaphragm (DI), and heart was assessed as dystrophin expression using immunofluorescence and H&E staining (Fig. 1B and C). MM201-miniVPR-mediated utrophin activation was evaluated in human HEK293T cells, mouse N2a cells, and porcine PK15 cells. Based on these in vitro results, AAV vectors encoding MM201-miniVPR were injected intramuscularly into two DMD mouse models. Utrophin expression and therapeutic outcomes were analyzed 7 weeks post-injection.

Results: Both DMD mouse models exhibited a complete loss of dystrophin expression compared to wild-type mice (Fig. 1B). Hematoxylin-eosin (H&E) staining revealed disorganized muscle fibers and severe fibrosis in the muscle tissues (Fig. 1C), displaying phenotypes closely resembling those of human patients. In vitro studies demonstrated MM201-miniVPR increased utrophin expression ~10-fold in N2a cells, ~6-fold in HEK293T cells, and ~4-fold in PK15 cells (Fig. 1D). Intramuscular administration of MM201-miniVPR-containing AAV vectors significantly increased utrophin expression in TA muscle, with over 4-fold and 6-fold increases in the $DMD^{\Delta E50E51,hE50KI}$ and $DMD^{\Delta E23,hE23KI}$ mouse models, respectively (Fig. 1E–G). Studies with a newly developed DMD pig model are ongoing to validate the effectiveness of MM201-miniVPR-mediated utrophin upregulation in a larger organism.

Conclusions: MM201-miniVPR-mediated utrophin activation offers a novel therapeutic strategy for DMD. By leveraging a mutation-independent mechanism, MM201-miniVPR could address the needs of a broad patient population. This study highlights MM201-miniVPR as a promising intervention to improve muscle function and mitigate the progression of DMD.

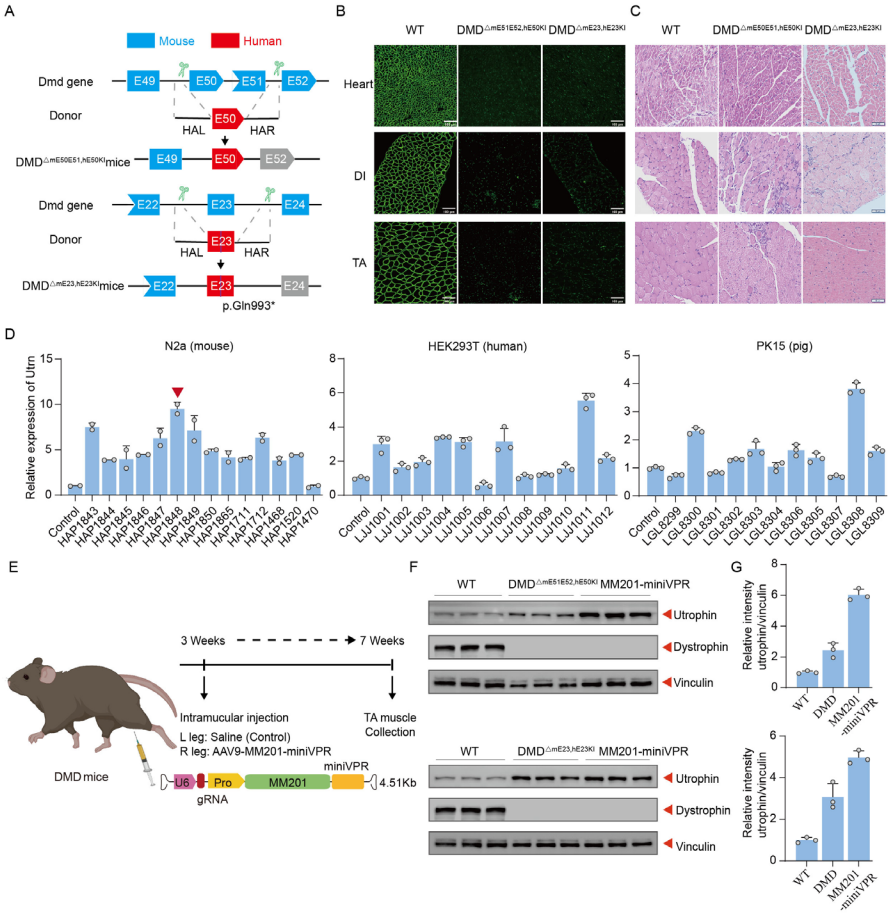

Figure 1. MM201-miniVPR-Mediated Utrophin Upregulation in Duchenne Muscular Dystrophy
A. Strategy for generating two DMD mouse models. The shape and color of the exon boxes indicate the reading frame. **B.** Dystrophin immunohistochemistry results from the indicated muscles of wild-type (WT) and DMD$^{\Delta mE50E51,hE50KI}$ mice. **C.** Hematoxylin-eosin (H&E) staining of the tibialis anterior (TA), diaphragm (DI), and heart muscle from WT and DMD$^{\Delta mE50E51,hE50KI}$ mice. **D.** sgRNA screening of MM201-miniVPR-mediated utrophin expression in N2a, HEK293T, and PK15 cells. **E.** Overview of the in vivo intramuscular injection of AAV9-MM201-miniVPR into the tibialis anterior muscle of the right leg in 3-week-old DMD mice, with the left leg receiving a saline injection as a control. Black arrows indicate the time points for tissue collection post-injection. **F.** Western blot analysis of utrophin, dystrophin, and vinculin expression in TA muscles, conducted 4 weeks after injection with MM201-miniVPR or saline. **G.** Quantification of utrophin expression from Western blots, normalized to vinculin expression. Each dot represents an individual biological replicate. Data are presented as mean ± s.e.m.

1070 Design and Evaluation of FKRP Dimer Expression for Restoration of Functional Matriglycan in a LGMD 2I/R9 Mouse Model

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Defects in the glycosylation of the membrane protein α-Dystroglycan result in numerous muscular dystrophies termed dystroglycanopathies. Of these, Limb Girdle Muscular Dystrophy 2I/R9 results from mutation-bearing Fukutin-related protein (FKRP) having little or no functional capacity as ribitol-phosphate (RboP) transferase to elongate the core M3 glycan (matriglycan) which is required to bind laminin and other proteins, securing the muscle membrane and extracellular matrix. The reduced or absent matriglycan expression results in destabilized cardiac and skeletal muscle with fiber damage followed by degeneration, inflammatory response, and regeneration. Decreased regenerative capacity coupled with ongoing inflammatory response results in fibrosis and intramuscular fat infiltration which reduce muscles' function. A number of AAV-mediated hFKRP transgene replacement constructs have been developed and demonstrated in LGMD mouse models to be effective at restoring matriglycan expression to levels comparable to that of unaffected wild-type mice, but at high AAV doses elevating risks of severe to lethal side effects. Recently, Kuwabara et al characterized the crystal structures of FKRP in monomeric, and dimeric form, with the FKRP dimer being most efficient in enzymatic activity.

We have designed an AAV9 vector encoding an FKRP dimer to replicate the naturally occurring dimer conformation. Systemic administration at $1x10^{13}$ VG/Kg of this FKRP dimer expression vector resulted in dramatic levels of matriglycan restoration detectable in skeletal, cardiac, and diaphragm muscles after four weeks and amelioration of the early stages of disease progression, as compared to the untreated LGMD 2I/R9 animals. Remarkably, the effect of this dimer-FKRP expression does result in an increased efficiency (~1.5 x) compared to monomer-FKRP at the same dose, as observed through immunofluorescence and western blot. This observed improvement in the functional matriglycan expression is also correlated with improved muscle histology including reductions in centrally nucleated fibers as well as monocyte infiltration and improvement in fiber size distributions. This novel approach to FKRP gene replacement has the potential to restore greater levels of matriglycan expression at lower doses of AAV thereby reducing dose related toxicity.

1071 In vivo overexpression of muscleblind-like 1 leads to cardiac toxicity in mice

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Myotonic Dystrophy Type 1 (DM1) is the most common adult-onset muscular dystrophy, caused by an autosomal dominant CTG repeat expansion in the 3' noncoding region of the *DMPK* (DM1 protein kinase) gene. This expansion leads to the formation of secondary RNA structures that sequester splicing regulators, such as the muscleblind-like protein family (MBNL), resulting in widespread splicing defects. DM1 is a multisystemic disorder primarily affecting skeletal muscle, with hallmark symptoms including myotonia and muscle weakness.

Preclinical studies in mice have shown that MBNL1 overexpression is either partially effective (Yadava et al) or insufficient (Kanadia et al) to reverse the phenotypes caused by CUG RNA toxicity. To evaluate gene therapy potential, we tested an AAV-based MBNL1 approach in vitro and in vivo, utilizing a muscle-specific promoter and a surrogate muscle tropic MYO-AAV capsid (Tabebordbar et al). In vitro, MBNL1 expression in myocytes from DM1 patients successfully restored splicing defects in patient derived myotubes. However systemic administration of MYO-AAV-MBNL1 to C57BL/6 mice caused significant toxicity, including weight loss and euthanasia by day 17 post injection. Postmortem heart analysis revealed necrosis, inflammation, and cell infiltration, indicating toxicity from MBNL1 overexpression. While partial restoration of splicing defects was observed in vitro, the in vivo results demonstrated unbalanced splicing likely due to supra-physiological expression levels.

These findings suggest that achieving therapeutic benefits of MBNL-1 based gene therapy will require precise regulation of gene expression levels to avoid toxicity and ensure safety, highlighting the need for controlled approaches in future studies.

1072 Editing of Satellite Cells and Postmitotic Muscle for Continuous Correction of Dystrophin Expression in Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is a fatal X-linked muscle wasting disease caused by mutations in the *dystrophin* gene, characterized by progressive muscle weakness, loss of ambulation, cardiorespiratory insufficiency, and premature death. Several DMD gene therapy clinical trials deliver functional miniaturized dystrophin using systemic adeno-associated virus (AAV) delivery. However, the lifelong workloads of skeletal muscles may cause therapeutic efficacy to decrease over time due to muscle remodeling in response to growth, maintenance, exercise, or trauma. An alternative strategy is to use genome editing to correct DMD-causing mutations in both postmitotic muscle and satellite cells (SCs), restoring long-term capacity of muscle to endogenously express functional

dystrophin. Our approach leverages base editors delivered by myotropic AAV vectors. So far, we have screened five myotropic AAV capsid variants to identify the most potent capsids for achieving optimal muscle transduction and editing of both muscle fiber nuclei and stem cells. To achieve specific editing in SCs we have designed miniaturized AAV-size-compatible gene regulatory cassettes (RCs) comprised of highly conserved regulatory regions from the human *PAX7* and *MYOD1* genes. These SC-RCs exhibit a range of activities in primary SC-derived myoblasts *in vitro* and in FACS-isolated SCs from wild-type and dystrophic mice. *In vivo*, prime SC-RC candidates display multifarious myogenic transcriptional patterns, ranging from preferential activity in SCs to more widespread activity in both SCs, myoblasts and postmitotic striated muscle. Encouragingly, our synergistic dystrophin gene editing approach is showing great promise, resulting in restoration of near normal dystrophin expression levels (>90%) in certain muscle groups, and with SC-RC-mediated editing boosting dystrophin gene correction in SCs over 100-fold compared to postmitotic muscle-specific editing alone using our CK8e RC.

Importantly, we believe achieving efficient SC gene correction to be crucial for addressing current DMD gene therapy longevity concerns, and that effective SC-editing methods could prove highly beneficial for the treatment of many muscle wasting disorders.

1073 Novel Circular RNA Scaffolds Enable Efficient Circularization and Expression of Full-Length Dystrophin as an Advanced Therapeutic Approach for Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is a severe, progressive genetic disorder caused by mutations in the DMD gene, resulting in the absence of functional dystrophin protein and premature death. Due to the large size of the dystrophin coding region (11 kilobase, 11kb), current therapies can only partially restore the dystrophin protein function.

Circular RNA (circRNA), known for its high intracellular stability and low immunogenicity, is emerging as a promising novel modality for protein replacement therapy. However, conventional scaffolds for RNA circularization are optimized for sequences up to 3 kb and are unsuitable for longer RNAs which limit the therapeutic applications of circRNA. We developed a novel scaffold specifically designed for the circularization of long RNA fragments. This advanced scaffold successfully circularized full-length DMD (circDMD) and enhanced the circularization efficiency of various lengths of circDMD (6.6, 10 and 11.7 kb). Transfection of full-length circDMD into cells resulted in robust expression of functional full-length dystrophin protein. The circDMD payloads were encapsulated in lipid nanoparticles (LNPs) to efficiently deliver and express the full-length dystrophin protein in the muscles of DMD *Del52/mdx* mice.

This breakthrough paves the way for more effective and comprehensive therapeutic strategies for DMD, offering a significant advancement over current treatments by enabling the expression of full-length dystrophin protein.

1074 AAV-Delivered U7snRNA Restores Full-Length Dystrophin in Patient Cell Lines with *DMD* Intronic Pseudoexon Mutations

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X-linked Duchenne muscular dystrophy (DMD) is caused by a null allele of the *DMD* gene, resulting in absent or altered dystrophin expression. Deep intronic mutations within the *DMD* locus represent an under recognized class

of pathogenic variants, accounting for 1-7% of all DMD patients. These are typically point mutations that create splice acceptor or donor sites, resulting in the inclusion of an intronic fragment as a pseudoexon within the *DMD* mRNA. Because such patients possess all of the canonical *DMD* coding exons, efficient exclusion of the pseudoexon from the mature mRNA restores a full-length transcript. To induce such skipping in patient-derived cells, we employed a modified U7 small nuclear ribonucleotide (U7snRNA) that targets either a splice acceptor (SA), splice donor (SD), or exon splice enhancer (ESE) sequence of the frame-shifting pseudoexons. Human MyoD-transformed cells with the pathogenic variants c.2292+1024 G>T (intron 18), c.2949+909 C>T (intron 22), c.5448+67 A>G (intron 38), or c.9163+2510 G>A (intron 61) were treated with increasing doses of AAV1 vectors containing a U7snRNA antisense sequence targeting the respective pseudoexon. Treatment resulted in dose-dependent pseudoexon skipping. In cells carrying the c.2292+1024 G>T variant, skipping efficiency reached 93%, resulting in 34% of wild-type dystrophin protein levels. Our preliminary data confirm that the pseudoexon class of *DMD* mutations is amenable to U7snRNA treatment and studies in additional cell lines are underway. This approach addresses an orphan class of *DMD* mutations, and demonstrates the potential of bespoke gene therapies to restore full-length dystrophin in individual patients.

1075 Optimized triple-vector gene therapy system enhances full-length dystrophin restoration in DMD mice

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Gene therapy for Duchenne muscular dystrophy (DMD) has traditionally relied on adeno-associated virus (AAV) to deliver micro-dystrophin (μDys). However, μDys lacks critical functional domains of full-length (FL) dystrophin, providing only partial protection for striated muscles. To overcome this limitation, we recently developed a triple-vector system capable of delivering FL-dystrophin to skeletal and cardiac muscles, achieving efficient protein assembly and functional restoration. Building on this foundation, we optimized the system to further enhance FL-dystrophin expression and improve therapeutic outcomes. FL-dystrophin was divided into three fragments linked by two orthogonal pairs of split inteins and subjected to a series of rational engineering to improve the expression and assembly. Western Blot analyses showed that these modifications resulted in the shift of an optimal ratio of the three fragments from 4:2:1 to a more desirable 1:1:2, as well as a 3- to 5-fold increase in FL-dystrophin expression levels *in vitro*. The optimized transgenes were packaged into a myotropic AAV (MyoAAV4A) and delivered to male *mdx*^{4cv} mice. The optimized system improved gastrocnemius muscle force and wire hanging time and further reduced serum creatine kinase (CK) levels, reflecting enhanced muscle integrity. These results demonstrate that the optimized triple-vector system significantly enhances FL-dystrophin expression and functional outcomes in DMD mice. This mutation-independent approach represents a promising direction for further clinical development.

1076 Administration of AAV9-Midlength-Dystrophin Gene Therapy Demonstrates Functional Efficacy in a Mouse Model of DMD

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An AAV9-midlength-dystrophin construct in development creates a 248 kilodalton (kDA) dystrophin for the treatment of DMD versus the more common ~140 kDA micro-dystrophin by utilizing RNA end-joining (REJ) technology to piece together two RNA fragments within a cell. This work opens the possibility of developing potential improved dystrophin constructs that may have therapeutic benefit in muscles affected by the dystrophic pathology experienced in patients with DMD.

When dosed systemically at postnatal day (p)1, *mdx* mice treated with AAV9-midlength-dystrophin demonstrated significant dose-response improvement in hindlimb strength when assessed at ~p115 as well as a later time point of

~p275, relative to *mdx* (-) controls. Using the Aurora Scientific Whole Animal System for Mice, physiology measurements were performed to assess contractile torque of the plantar flexion muscles, which includes the gastrocnemius muscle. Peak plantar flexion twitch torque and peak tetanic torque measurements were both improved by ~100%, relative to *mdx* controls following administration of the higher dose in the study. This magnitude of improvement was observed at both ~p115 and ~p275 timepoints, indicative of durable biological activity and functional benefit following early administration of midlength-dystrophin. Significant impairment of muscle strength in *mdx* (-) control mice was evident, relative to WT mice, as early as ~p28 and declined over time. When AAV9-midlength-dystrophin was administered to *mdx* mice via the cerebrospinal fluid at ~p28, muscle strength was maintained when tested at ~p115 showing improvement of over 90% in both peak plantar flexion twitch torque and peak tetanic torque measurements, relative to *mdx* (-) controls.

In accordance with functional improvements, successful RNA end-joining within skeletal and cardiac muscle tissue from *mdx* mice dosed with AAV9-midlength-dystrophin was confirmed using ddPCR to detect joined RNA and by immunostaining to demonstrate robust expression of midlength-dystrophin protein. Ongoing studies are designed to test the optimal timing of administration, route of administration and length of dystrophin construct.

1077 Evaluate DWORF Gene Therapy in the Diaphragm of Dystrophin-deficient mdx Mice

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Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy and is caused by an absence of the protein dystrophin in muscle. Without dystrophin, patients experience severe muscle weakness and degeneration. One cause for rapid muscle degeneration is high calcium concentrations within the cytosol of DMD muscle fibers that activate cellular proteases and phospholipases. Dwarf open reading frame (DWORF) is a positive regulator of the sarco-endoplasmic reticulum calcium ATPase (SERCA), a pump responsible for moving cytosolic calcium into the sarcoplasmic reticulum (SR) of muscle cells. SERCA function is reduced in DMD. Increasing SERCA activity by DWORF is one avenue for clearing aberrant cytosolic calcium in DMD. We recently demonstrated that adeno-associated virus (AAV)-mediated DWORF overexpression significantly attenuated dilated cardiomyopathy in aged mdx mice, a symptomatic DMD mouse model. Diaphragm is the most severely affected muscle in mdx mice. Here we examined whether AAV DWORF therapy can attenuate dystrophic pathology and force loss in the mdx diaphragm. We delivered 6 x 10^{12} vg/mouse of the AAV9.DWORF vector to 6-week-old male mdx mice via the tail vein. We examined DWORF expression, diaphragm histology (fibrotic area, myofiber size, and % of centrally nucleated myofibers), and diaphragm function (twitch force, tetanic force and resistance of eccentric contraction-induced muscle force loss) when mice reached 6 months of age. Surprisingly, we did not detect significant improvements in the parameters we analyzed. Future studies are needed to understand the discrepancy in the therapeutic outcomes between the diaphragm and the heart.

1078 Characterization of New Dystrophic Mouse Models of Duchenne Muscular Dystrophy as a Tool for Therapeutic Development

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Objective: Duchenne muscular dystrophy (DMD) is a severe, X-linked muscle wasting disorder affecting newborn males. DMD is characterized by progressive muscle degeneration caused by mutations in the dystrophin gene. Gene

therapies targeting exon skipping have shown promise, but developing personalized treatments for unique mutations has been hindered by the lack of specific mouse models. This study aimed to comprehensively characterize multiple DMD mouse models to elucidate the disease's underlying mechanisms, paving the way for tailored gene therapies.

Methods: We examined three dystrophic mouse models in comparison with wild-type C57Bl/6 mice. The models consisted of the previously characterized Dup2 mouse and two novel models: Del18-41, featuring a large exon 18-41 deletion, and Dup2Del18-41, combining both mutations. Our analysis consisted of survival studies, electrophysiological assessments, including measurements of absolute and specific forces, eccentric contraction fatigue in the tibialis anterior muscle, and morphological and histopathological examinations of muscles and organs collected after euthanasia. This comprehensive approach enabled a thorough evaluation of the dystrophic mouse models.

Results: Our study revealed significant reductions in longevity among all dystrophic mouse models compared to C57Bl/6 mice. *In situ* physiology assessments demonstrated substantial declines in muscle function, including in specific force and force retention during eccentric contractions (ECC). These declines were statistically significant across various time points (3-12 months) and consistent among all three dystrophic models. In addition, muscle histopathology showed progressive dystrophy, characterized by increased central nucleated fibers. Ongoing studies include behavioral testing and additional histopathological analyses to further elucidate the disease phenotype.

Conclusion: Our study provides preliminary characterization of two novel dystrophic mouse models (Del18-41 and Dup2Del18-41), revealing significantly reduced lifespans and impaired muscle function in all DMD mice. Electrophysiological analysis of the tibialis anterior muscle showed decreased resistance and endurance in response to tetanic and eccentric contractions, correlating with negligible or low dystrophin levels. These findings set the stage for future behavioral studies to further elucidate the disease phenotype in these models.

1079 AAV-expressed Microdystrophin Containing Extended C-terminus Improves Muscle Function and Protects Against Injury in a Mouse Model of Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is a fatal, X-linked disease resulting from mutations in the *DMD* gene, leading to progressive loss of skeletal and cardiac muscles. Muscle weakness in DMD initially affects proximal muscles, presenting as difficulties in walking and running. As the disease progresses, patients lose the ability to walk and eventually face respiratory or cardiac failure. In developing dystrophin replacement or restorative gene therapies, preserving muscle function that resists injury in early stages is essential to slowing the degenerative progression of the disease. Specifically, gene replacement therapy via viral vectors has demonstrated restoration of muscle strength in rodent models using shortened “microdystrophins” (µDys), which are accommodated by the 4.7kb packaging limit of AAV vectors. Yet debate exists regarding the selection of which dystrophin domains to include that optimize muscle function improvement. Inclusion of an extended portion of the C-terminus (CT) of dystrophin in the µDys has been shown to improve resistance to force loss from repeated eccentric injury compared to µDys lacking the CT domain.

To further characterize the CT domain’s role in improving µDys efficacy via rescuing muscle function, we tested two AAV vectors expressing µDys with and without the extended CT domain (µDysCT194 and µDysCT48 respectively). Vectors were administered i.v. via tail vein at 5 weeks of age in male *mdx* mice, which lack dystrophin. At 6- and 12- weeks post-dosing, mice expressing µDysCT194 showed significantly improved forelimb grip strength, exhaustion treadmill performance, and distance traveled in the open field compared to mice expressing µDysCT48.

To determine whether these effects were mediated through direct protection of myofibers expressing µDysCT194, we used Evans blue dye to visualize damaged muscle following an *in vivo* plantar flexion eccentric injury assay. We

found that µDysCT194-treated mice showed significantly less force loss from repeated eccentric injuries compared to the µDysCT48 group, concomitant with significantly fewer number of Evans Blue positive (i.e. damaged) myofibers in the µDysCT194 animals. To address natural motions experienced by muscles, we developed the downhill treadmill running assay, a physiologically relevant eccentric injury model based on movement that is discouraged for DMD patients. This assay produced a severe phenotype in mdx mice, indicating its utility for preclinical studies of µDys gene therapies, opening an important future direction of REGENXBIO's research.

In summary, across numerous behavioral and physiological assays, we observe that animals treated with the extended CT domain of dystrophin (µDysCT194) demonstrate superiority in motor function, muscle force, and resilience to eccentric injury compared to animals treated with µDys lacking the extended CT domain. These findings suggest a critical function of the dystrophin CT domain in improving the therapeutic benefit of µDys gene therapies.

1080 CSF Delivery of INS1201 AAV9-Micro-Dystrophin Demonstrates Long-Term Durability of Efficacy in a Mouse Model of DMD

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INS1201 is an AAV9-based micro-dystrophin gene therapy construct being evaluated as a potential treatment for Duchenne muscular dystrophy (DMD). This vector expresses a truncated form of dystrophin, which has demonstrated therapeutic efficacy in the *mdx* mouse model of DMD and is delivered via a single intrathecal (IT) injection. INS1201 was administered to *mdx* mice via the cerebrospinal fluid (CSF) at postnatal day (p) 28 with subsequent evaluation of its effects on functional efficacy, histopathology, vector genome biodistribution, and micro-dystrophin expression at approximately one-year post-administration.

Following intracerebroventricular (ICV) administration of INS1201 in *mdx* mice at p28, significant long-term functional improvements were observed in hindlimb muscle strength. Using the Aurora Scientific Whole Animal System for Mice, physiology measurements were performed to assess contractile torque of the plantar flexion muscles, which includes the gastrocnemius muscle. At approximately 400 days of age, INS1201-treated mice exhibited substantial enhancements in peak plantar flexion twitch torque, with improvements of 87%, 67%, and 104% at doses of 4.0E+11, 8.0E+11, and 1.6E+12 vector genomes (vg), respectively, compared to *mdx* (-) controls. Furthermore, when assessed at stimulation frequencies of 80-160 Hz, INS1201-treated mice showed notable improvements in plantar flexion peak torque across these doses relative to controls.

Histopathological analysis of gastrocnemius muscle at p400 revealed significant reductions in dystrophic pathology, including improved structural organization, fewer inflammatory cells, and reduced fibrosis in INS1201-treated mice. These findings were consistent across all doses evaluated, with the highest dose demonstrating the most pronounced effects. Importantly, micro-dystrophin expression was sustained in the gastrocnemius muscle of treated mice at this late time point at all doses, confirmed by immunostaining and Western blotting. Quantitative Jess Western analysis revealed a significant increase in micro-dystrophin expression at the 1.6E+12 vg dose relative to controls. Vector genome analysis using ddPCR showed a positive correlation between vector genome presence and micro-dystrophin expression. Sustained micro-dystrophin expression was also observed in cardiac muscle tissue at p400, as confirmed by immunostaining and Western blotting, with a significant dose-dependent increase in protein expression at all doses evaluated. Biodistribution analysis of INS1201 vector genomes in the heart correlated with protein expression levels, with mean values of 0.758, 1.509, and 3.225 vg/diploid genome at the respective doses.

In conclusion, these results demonstrate that CSF delivery of INS1201 in *mdx* mice leads to long-term (at least one year) durability of micro-dystrophin expression and vector genome biodistribution in both skeletal and cardiac muscle, along with sustained functional benefits. These findings support ongoing studies to evaluate the potential of INS1201 as a therapeutic option for DMD.

1081 Comprehensive analysis of longitudinal SV95C measurements, an e-digital mobility assessment in a real-life Duchenne population in the GNT-014-MDYF natural history study

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Duchenne muscular dystrophy (DMD) is characterized by a wide range of clinical manifestations, and its progression can vary greatly. Hospital-based, physiotherapist-assisted functional assessments, including the North Star Ambulatory Assessment (NSAA), the Six Minute Walk Test (6MWT), the 10 Meter Walk Test (10MWT), and the Time to Raise from Floor (RFF), are widely utilized for monitoring disease progression in both clinical and research settings. Stride Velocity at the 95th Centile (SV95C), captured via a wearable device, provides a continuous measure of daily mobility in real-life environments. This method reduces dependence on patient cooperation (e.g., young patients) and minimizes inter-evaluator variability. Recently, the European Medicines Agency (EMA) has qualified SV95C as a primary endpoint for clinical trials in DMD. The current analysis aims to evaluate the validity and establish normative data for SV95C within the context of a natural history (NH) study.

Seventy-seven boys with DMD, aged 5-9 years, receiving steroids and achieving a NSAA score of ≥18, were enrolled in the prospective NH study GNT-014-MDYF. Both SV95C and other clinical functional outcomes (e.g., NSAA, RFF, 10MWT) were measured biannually. Data were described by age subgroups (5-8 years and >8 years). The accuracy of SV95C was evaluated and compared to both age-matched healthy controls (n=66) and DMD populations (n=107) from the EMA SV95C Qualification Dossier. Reliability was assessed using the intra-class correlation coefficient (ICC), with SV95C data separated into two half-recording periods. Spearman's correlation was employed to assess the consistency between different functional outcomes.

From baseline to 2 years, SV95C demonstrated high reliability, with ICC ranging from 0.96 to 0.98. The mean SV95C values in DMD patients were distinctly separated from those of healthy controls and were consistent with the data reported in the EMA SV95C Qualification Dossier. At the 1-year follow-up, the mean changes in SV95C were -0.097 m/s and -0.162 m/s for the 5-8 years and >8 years age groups in GNT-014-MDYF, respectively, compared to -0.263 m/s and -0.226 m/s for the corresponding age groups in the DMD population from the EMA Dossier.

While the overall trend in SV95C change was consistent, the changes observed in GNT-014-MDYF were numerically lower, which may be attributed to differences in baseline characteristics. Strong correlations were observed between SV95C and other clinical outcomes, with correlation coefficients of 0.70, 0.56, -0.73, and 0.83 for NSAA, 6MWT, RFF, and 10MWT, respectively. Notably, these correlations appeared to improve over time. The minimal clinically important difference (MCID) for NSAA, defined as a 3-point change, anchored the SV95C change in this natural history population to -0.140 m/s, with values of -0.125 m/s and -0.221 m/s for the 5-8 years and >8 years age groups, respectively.

This analysis demonstrated the validity of SV95C in a NH study with standardized follow-up, confirming its consistency with previously published data. It further suggested that SV95C is a sensitive measure for detecting clinical changes and showed strong correlations with other clinical assessments, supporting its potential application in the clinical development of innovative treatments. The predictive value of SV95C is currently being explored, and additional analyses will be conducted to better understand its MCID.

1082 Patient-Specific 3D Myofiber Cultures as Model for Therapeutic Development

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Understanding the functional impact of patient-specific disease-related mutations in muscle often requires functional assessments, particularly regarding contractility. However, culture of patient myoblasts and differentiation into myotubes is typically performed in two-dimensional (2D) monolayer cultures that preclude contractility assessments. In vivo studies are limited as most private mutations are not found in animal models, and the cost of repeatedly creating patient-specific models is prohibitive. As an alternative, 3D cultures offer the possibility to better represent myofiber organization in muscle and to model diseases in vitro. 3D cultures include cell-cell and cell-extracellular interactions which may be important in the formation of myofibers. In addition, 3D cultures methods that allow assessment of contractility provide the opportunity to measure the responses of pathological skeletal muscle to different therapies.
We have used the Mantarray platform (CuriBio) to generate preliminary studies of patient-derived cells from our large Wellstone P50-funded Cell Line bank, which includes 37 primary myoblast lines and 149 transdifferentiatable FibroMyoD (FM) lines. Mantarry integrates 3D myofiber cultures with a magnetic sensing system to measure contractile forces in response to spontaneous contraction or electrical stimulation as well as small chemical treatments. Specifically, 3D myofiber cultures of iPSC-derived myoblasts, primary myoblasts and MyoD induced FM lines under tension to generate aligned myofibers. By comparing their morphology, gene expression, and contraction to those of wild type myofibers, we are able to assess the pathological and functional characteristics associated with a range of mutations responsible for Duchenne and Becker muscular dystrophy, facioscapulohumeral muscular dystrophy, and congenital myopathies. Our preliminary data suggest that 3D myofiber culture is a promising model for both characterizing functional impact of mutations and assessing treatment effects with AAV vectors.

1083 Targeting of ribitol metabolic pathway using AAV to enhance glycosylation and therapeutic efficacy of metabolites in the FKRP-P448L mutant mouse.

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The glycosylation of alpha-dystroglycan is crucial in binding extracellular matrix proteins to provide structure and stability to the muscle membrane. There are currently 18 known genes involved in the glycosylation of alpha-dystroglycan and mutations in any of these can result in the loss or reduction of glycosylation causing a subset of muscular dystrophy known as dystroglycanopathies. Mutations in Fukutin-related protein (FKRP) are the primary cause of limb-girdle muscular dystrophy 2I/R9. FKRP functions as a ribitol-5-phosphate transferase working in tandem with fukutin to add ribitol-5-phosphate to the core M3 glycan chain, a necessary step for completing an elongated glycan chain of repeating units of glucuronic acid and xylose referred to as matriglycan.

The full biosynthetic pathway of ribitol-5-phosphate is unknown but advancements have been made to identify several different proteins involved. FGGY has been shown to convert ribitol to ribitol-5-phosphate but other reductases have also been identified to have this activity. The aldo-keto reductases AKR1A1 and AKR1B1 have also been shown to be involved in the conversion of ribose to ribitol and/or ribose-5-phosphate to ribitol-5-phosphate with varying degrees of efficiency. Our lab has shown that administration of ribitol to the FKRP-P448L mutant mouse increases levels of ribitol-5-phosphate and subsequently ameliorates the disease phenotype. Similarly, it has been shown that administration of ribose can have beneficial effects to FKRP-related muscular dystrophies.

However, the recovery of glycosylation is incomplete, and attempts are being made to improve the effectiveness of metabolite administration. Here we use AAV9 delivery of 1E13vg/kg and 5E13vg/kg of FGGY, AKR1A1 and AKR1B1, with and without exogenous metabolite administration, to examine their potential of improving alpha-dystroglycan glycosylation and metabolite effectiveness. Analysis of heart, TA and diaphragm tissues 1 month post administration shows increased levels of alpha-dystroglycan glycosylation in the FKRP-P448L mutant mouse. By targeting the biosynthetic pathway of ribitol-5-phosphate the effectiveness of ribitol treatment can potentially be improved for treating FKRP-related muscular dystrophies.

1084 Development of dosing protocol to reduce the required dose of rAAV using adult stem cells

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Background: AAV vectors are safe and allow long-term expression of therapeutic genes. rAAV-based gene therapy is being tested for genetic disorders such as SMA, XLMTM, and Duchenne muscular dystrophy (DMD). In clinical trials, the typical intravenous rAAV dose is $3x10^{13}$~$1x10^{14}$ vg/kg body weight.
DMD is a congenital disease in which skeletal and cardiac muscles are gradually lost due to mutations in the dystrophin gene. We previously reported a therapeutic effect of bone marrow-derived MSC (BM-MSC) pretreatment followed by a low-dose ($2x10^{12}$ vg/kg BW) rAAV-μDys administration in DMD dogs ($CXMD_J$), and a clinical trial is planned to use this administration method. In this presentation, we investigated the safety of AAV-μDys in combination with adult mesenchymal stromal cells (MSCs), which are used for cell therapy in children with DMD, using non-human primates.
Methods: Normal cynomolgus monkeys were intravenously injected with MSCs and rAAV8-LacZ ($2x10^{12}$ vg/kg BW). Seven days after injection, MSCs were administered systemically again. Eight days after the first injection, the same monkeys were injected intramuscularly with rAAV8-LacZ. Blood samples were collected over time for biochemical analysis and neutralizing antibody titers against rAAV8. Muscle biopsies were performed at 8, 16, and 24 weeks after rAAV injection.
Results: When rAAV was administered to non-human primates according to the above protocol, no weight loss was observed, and no blood biochemical abnormalities were detected with AAV vector and/or MSCs administration.
Conclusion: These results indicate that the combination of MSCs and rAAV is safe in nonhuman primates. This dosing protocol appears to be a practical approach for analyzing transgene expression and function *in vivo*. These findings support the future potential of rAAV-mediated protein replacement strategies in the treatment of DMD and various genetic disorders.

1085 Base Editing Screen For Endogenous Activation of Zeta Globin Expression As A Treatment For Alpha Thalassemia Major

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Alpha Thalassemia is an inherited blood disorder caused by mutations or deletions in the alpha-globin locus. The most severe form, Alpha Thalassemia Major (ATM) occurs when all four alpha globin genes (HBA1 and HBA2) are absent. In some cases, deletion of HBA1 and HBA2 results in upregulation of the nearby alpha like zeta globin gene (HBZ), which may compensate for the loss of HBA and rescue the lethal phenotype. In normal erythropoiesis, transcriptional control of HBZ, HBA1, and HBA2 is mediated by common enhancer elements. The embryonic HBZ globin is expressed almost exclusively in primitive erythroid cells and silenced in definitive erythroid cells. Known regulators of HBZ expression include leukemia/lymphoma-related factor (LRF) and B-cell lymphoma/leukemia 11A

(BCL11A), which coordinate HBZ repression via binding in enhancer and promotor regions of the HBZ gene. To uncover activator elements that can be leveraged to induce HBZ expression in definitive erythroid cells, we designed a sgRNA library that encompassed the entire alpha globin locus. The library included sgRNAs targeting the alpha globin locus (HBZ, HBA2 and HBA1; ~7500), positive control sgRNAs targeting the LRF (ZBTB7A) and BCL11A gene (100), and non-targeting sgRNAs controls (500). We constitutively expressed an adenine base editor (ABE 8.20) and a single sgRNA in HUDEP-2 cells, an erythroid test cell line and isolated high expressing HBZ cells. sgRNA enrichment was quantified and top individual sgRNA targets were validated in HUDEP-2, primary CD34+ human cord blood and adult hematopoietic stem cells. Positive control sgRNAs targeting the LRF and BCL11A gene induced HBZ expression. sgRNAs targeting HBA2 and HBA1 gene induced HBZ expression, suggesting reciprocal control between these genes. Overall, this approach identified numerous sgRNAs with varying degrees of HBZ induction in normal models of erythropoiesis. Future studies test the functional significance of endogenous HBZ induction on the accumulation of toxic beta-globin dimers observed in ATM cell models.

1086 Identification of Genetic Variants Associated with T-Cell Lymphoma: A Case-Control SNP Analysis

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Introduction: T-cell lymphoma (TCL) is a rare and heterogeneous group of malignancies characterized by poor prognosis and limited therapeutic options. While the role of genetic predisposition in TCL remains poorly understood, recent advances in genomic studies suggest that specific single nucleotide polymorphisms (SNPs) may contribute to disease susceptibility. This study aims to identify SNPs associated with TCL by comparing genetic variants between patients diagnosed with TCL and healthy controls.

Methods: This retrospective case-control study was conducted at a single institution, analyzing genetic data from 31 TCL patients and 301 healthy controls. A targeted SNP analysis was performed to assess differences in allele and genotype distributions. Pearson's Chi-square and likelihood ratio tests were utilized to determine statistical significance. The SNPs included in the analysis were selected based on prior associations with immune regulation, inflammation, and tumorigenesis.

Results: A total of 10 SNPs demonstrated statistically significant differences between TCL patients and controls. Among these, rs308395 (FGF2, $p<0.001$) and rs3025039 (VEGFA, $p<0.001$) exhibited the most significant associations, suggesting their potential role in TCL susceptibility through pathways related to angiogenesis and tumor progression. Additionally, rs2228001 (XPC, $p=0.028$), a variant linked to DNA damage repair, and rs2167270 (LEP, $p=0.029$), associated with immune regulation and metabolism, showed significant differences between cases and controls. Variants in key inflammatory and immune-related genes were also identified, including rs1800890 (IL10, $p=0.050$), rs1800629 (TNF, $p=0.029$), and rs1790192 (CXCR5, $p=0.041$), all of which have been implicated in cytokine regulation and immune surveillance. Moreover, rs17757541 (BCL2, $p=0.034$), a critical regulator of apoptosis, and rs1800683 (LTA, $p=0.140$), involved in lymphotoxin signaling, showed trends toward significance, warranting further investigation into their roles in TCL pathogenesis.

Conclusions: Certain SNPs exhibit varying frequencies between TCL patients and controls. These findings highlight potential genetic markers for TCL risk, which could inform future gene-targeted therapies and precision oncology approaches.

1087 Gene Editing Primary T Cells to Enable CRISPR-On-Demand Therapy in an "Every Patient Brings Their Own Mutation" Setting: the Use Case Scenario of Hemophagocytic Lymphohistiocytosis

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The toolbox of CRISPR gene editing enables targeted repair of disease-causing mutations, but particular genetic disorders where every patient has a private mutation pose a profound challenge for nonclinical development. We are developing a platformized gene editing approach to treat hemophagocytic lymphohistiocytosis (HLH) - a rare and life-threatening immunological disorder marked by persistent hyperinflammation stemming from consequences of defective cytotoxic function in immune cells, particularly T and NK cells, with inability to terminate the immune response. Ca 40% of newly diagnosed patients fail to survive under standard care, with only a few months available to develop an on-demand therapy for each patient's unique mutations. In the case of fHLH caused by mutations in the PRF1 gene, most patients are compound-heterozygous for loss-of-function mutations of different severity, and de novo mutations are initially classified variants of uncertain significance (VUS). Before therapeutic editing can occur, a viable platform requires the ability to rapidly determine mutation pathogenicity and which allele(s) to target for repair.

Here we describe such an approach: we used gene editing to introduce PRF1 variants of uncertain significance (VUS) found in a patient with HLH at UCSF [c.112G>A (p.Val38Met, VUS1) and c.133G>A (p.Gly45Arg, VUS2)]. To assess the functional consequence of these VUSs and enable VUS derisking in < 1 week, VUS1 and VUS2 were individually introduced into the endogenous PRF1 locus using CRISPR targeted HDR integration of a partial cDNA bearing one or the other VUS translationally linked to a reporter gene that allows rapid isolation of mutation-bearing cells. Functional assays demonstrated significant reductions in perforin expression (60% for VUS1 and 90% for VUS2) and corresponding defects in CTL cytotoxic function. This demonstrated clear pathogenicity of these variants and sets a methodology for studying other PRF1 VUSs. Given the exceptionally narrow window of time available for clinically impactful mutation repair, we developed a gene-editing approach to correct PRF1 mutations that relies on the same gene editor - nuclease Cas9 (rather than mutation-bespoke isoforms of other editors - such as base or prime - each of which would need additional derisking pre-dosing). We combine Cas9 RNPs with ssODN HDR templates to attain locally efficient mutation repair in PRF1 in primary T cells at numerous different gRNA targets; a non-exhaustive tiling approach across the PRF1 locus identified multiple optimal gRNAs to attain therapeutically-relevant repair within a given window.

Taken together our work establishes, for the first time, an end-to-end platform of rapid derisking a VUS to nominate it for clinical repair and then repairing it using a set of genome editing tools that are clinic-grade and where the transition from mutation 1 to mutation 2 would not require changing the protein subunit of the genome editor, potentially accelerating the path of such a CRISPR-on-demand approach through regulatory review.

1088 A base editing platform optimised for efficient editing retains stemness and differentiation potential of hematopoietic stem cells

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Hematopoietic stem and progenitor cells (HSPCs) are a foundational cell type for the development of engineered therapies. Since engineered HSPCs are meant to persist throughout a patient's lifetime and are sensitive to DNA damage, it is important to utilize a technology which minimizes genotoxicity and safeguards engineered cell

functionality. Nuclease-induced double strand breaks (DSBs) trigger a DNA damage response which may lead to chromosomal aberrations, decreased viability and cell function, and ultimately reduce the efficacy and safety of engineered therapies. Base editors are not reliant on DSBs and represent an effective mitigation strategy but must be optimised for efficient delivery into HSPCs. Our proprietary Pin-point^{TM} platform is a flexible modular base editor typically comprising a DNA binding Cas, a DNA modifying deaminase, and a sequence-targeting guide RNA which assembles at the target locus, and demonstrates an advanced safety profile compared to nuclease-based technologies. This makes it well suited to the development of cell and gene therapies in sensitive cell types such as HSPCs. In this study we used a Pin-point base editing system composed of Rat APOBEC1 mRNA, SpCas9 nickase mRNA and a synthetic aptamer-containing gRNA to edit CD34+ HSPCs with 80% efficiency. Flow cytometry of edited cells revealed an unaltered frequency of hematopoietic stem cells (HSCs) compared to the control and high levels of gene knock out in both the bulk and HSC population which was retained over 5 weeks. Moreover, primitive hematopoietic cells edited with the Pin-point platform retain their stemness capacity as validated in a Long-Term Culture-Initiating Cell (LTC-IC) assay. Additionally, no significant DNA damage response was detected, further supporting the gentler activity on cells. Next, we validated the technology on two separate loci known to reactivate γ-globin expression and achieved high levels of base editing that corresponded with an increase in γ-globin mRNA and protein expression as a relevant therapeutic outcome. The ability to base edit HSPCs efficiently and safely, while maintaining their stemness and differentiation potential, demonstrates the strength of the Pin-point platform as a tool for the generation of advanced cell therapies using sensitive multipotent cell types.

The Pin-point™ base editing platform technology is available for clinical or diagnostic study and commercialization under a commercial license from Revvity.

1089 A new model using humanized mice to explore the fate of HIV DNA in secondary lymphoid and hematopoietic organs during the early phase of acute infection

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The HIV-1 replication cycle has been extensively studied through biochemical analysis, yielding valuable insights into the replication cycle. However, unveiling the potential of viral reservoirs for elimination necessitates more efficient techniques to identify these cells *in vivo*. Traditional methods restrict the assessment of new therapeutic strategies for the HIV cure.

Recent advances in imaging technologies have provided new tools for studying the HIV-1 life cycle and reservoir formation. We have developed a combination of imaging microscopy with fluorescently labeled viral genomes that enable the investigation of HIV-1 biology at the single-cell level. Here, we present a unique approach by fluorescence microscopy-based method, using a humanized (HIS) mouse model sensitive to HIV-1 to live-track the final products of the reverse transcription. Our technology employed a bacterial-derived fluorescence technique (OR-GFP) to tag the replication-competent virus. The genetically modified mice expressing the OR-GFP protein in CD4+ T-cells can reveal, by confocal or in live, individual double-stranded full-length HIV DNA forming bright nuclear puncta in the nucleus. Combined with the RNA-FISH, this method distinguished between non-transcribing and actively transcribing viral DNA from lymph nodes, spleen, and bone marrow extracted from infected mice. Our results were consistent with the notion that viral reservoirs are established during the early stages of viral infection. Of paramount significance, our innovative *in vivo* system not only provides a sensitive and fresh approach for live tracking the full-length viral DNA genome in infected cells without relying on cell culture but also holds the potential for mapping the trajectories of infected cells throughout various phases of HIV infection. This aspect of our research is promising, as it opens up new possibilities for understanding the dynamics of HIV infection and developing new treatments targeting early reservoir cells and potential latent viral forms.

1090 Induction of Immune Tolerance in a Food Allergy Model Utilizing Platelet-Targeted Therapy

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Background: Food allergy is a significant public health concern driven by a failure in oral immune tolerance, with no definitive cure. Our previous studies demonstrated that platelet-specific gene therapy induces antigen-specific IgG-mediated immune tolerance in hemophilia models. Additionally, we found that desialylated platelets suppress immune responses. This study aims to develop a novel strategy to induce immune tolerance to allergens and mitigate allergic reactions. *Methods*: Using ovalbumin (OVA) as a model allergen, we investigated two mouse models: the OVA-specific OT-II model (98% OVA-specific CD4 T cells) and IL4ra^{F709} mutant mice (prone to allergic responses). For preventive therapy, we transduced hematopoietic stem cells (HSCs) with a 2bOVA lentivirus, followed by transplantation. Platelet-OVA expression was confirmed via ELISA. Mice were sensitized weekly with OVA (10 mg/kg) and staphylococcal enterotoxin B (SEB, 0.4 mg/kg) for eight weeks before a high-dose OVA challenge (6 g/kg). For treatment studies, OVA-containing platelets from 2bOVA transgenic mice were transfused into pre-sensitized mice before rechallenge. Anaphylaxis was assessed via body temperature changes, and immune tolerance was evaluated using tissue analysis. *Results*: Platelet-OVA expression levels were 11.49 ± 7.49 ng/10^8 platelets in 2bOVA-transduced OT-II recipients and 6.82 ± 6.89 ng/10^8 platelets in IL4ra^{F709} recipients. Following OVA challenge, body temperature dropped significantly in control mice (1.89 ± 1.29ºC in OT-II; 2.05 ± 0.59ºC in IL4ra^{F709}) but remained stable in 2bOVA-transduced animals. Anti-OVA IgE titers increased in controls but remained unchanged in the 2bOVA group. Flow cytometry revealed significantly lower Th2 cell percentages in 2bOVA mice (0.87 ± 0.60% OT-II; 0.40 ± 0.33% IL4ra^{F709}) compared to controls (3.18 ± 2.11% OT-II; 2.89 ± 1.39% IL4ra^{F709}), while Treg and Tr1 cells were markedly increased. Importantly, mast cell protease-1 levels were significantly reduced in 2bOVA-transduced recipients compared to control (2bGFP or untransduced) groups under the same immune challenge protocol. Notably, transfusion of desialylated 2bOVA platelets effectively suppressed anaphylaxis via IgE regulation. *Conclusion:* Our findings demonstrate that platelet-targeted gene therapy induces immune tolerance by modulating the Th2/IgE pathway and preventing anaphylaxis. Furthermore, transfusion of allergen-expressing platelets mitigates anaphylactic responses, with suppression of mast cell activation, highlighting a novel therapeutic approach for food allergy treatment.

1091 An iPSC-derived 3D liver model for safety assessment of AAV-based gene therapies

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Adeno-associated viral (AAV) vectors have evolved as one of the key delivery methods in the growing field of gene therapy. However, systemic administration of AAVs can lead to accumulation not only in the desired target tissue, but also in other organs such as the liver. Due to its particular anatomy, the liver is prone to take up AAVs and it has been observed in several clinical studies that AAVs may lead to severe liver toxicity and even fatalities. This raises significant concerns for AAV programs as the mechanism of AAV-driven liver toxicity is not understood and there are currently no good biomarkers to predict who is at risk. Furthermore, conventional preclinical in vitro liver models that do not encompass the complexity and individual variability in humans have failed to recapitulate AAV-mediated liver toxicity. To tackle this issue, the aim of this study was to develop a patient-specific, iPSC-derived, multi-cell type liver-immune organoid model that is able to predict liver cells transduction and AAV-mediated liver toxicity. We made use of peripheral blood mononuclear cells (PBMC)-derived induced pluripotent stem cells (iPSC) that were differentiated into hepatocytes, stellate cells, endothelial cells and monocytes - the four main cell types of the liver. We co-cultured these cells as organoids and characterized the model by immunohistochemistry and qRT-PCR for liver-specific markers. We then exposed the organoids to GFP-labeled wild type AAVs and could show that they can be transduced by different AAV serotypes. Our model is able to recapitulate the transduction of the

different cell types by various rAAVs. However, AAV-mediated hepatotoxicity could not be observed following AAV transduction in this configuration. We therefore established a co-culture of the organoids with PBMCs from the same donor to reflect the immune component. Using such a multicellular, immunocompetent liver organoid model we sought to perform T Cell priming experiments in combination with multi-omics assessment of the liver-immune system. While still in an exploratory stage, the results from this innovative approach are encouraging and provide deeper insights into the mechanisms and individual patient-factors that contribute to rAAV transduction and AAV-driven liver toxicity, eventually leading to the development of more effective and safer gene therapies.

1092 Generation of Functional Liver Organoids Using CD34+ Cord Blood-Derived GMP Friendly iPSCs

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Background & Aim: Liver transplantation is the standard treatment for patients suffering from acute and chronic liver disease caused by congenital diseases, infectious agents, and environmental factors. Recent progress in liver organoid technology has opened a new opportunity for organ transplantation and cell therapy to treat liver diseases. However, current methods relying on stochastic self-organizing potential produce mixed cell populations of differentiated cell types, raising concerns about ectopic tissue formation. Induced pluripotent stem cells (iPSCs), which can differentiate into three-dimensional (3D) liver organoids, present a promising alternative. Moreover, the application of homozygous human leukocyte antigen (HLA) donor-derived iPSCs minimizes the risk of immune reaction post-transplantation. This study aims to develop reproducible GMP-compliant liver organoids from HLA-homo iPSCs and assess their potential for clinical cell therapy applications.

Methods: Human HLA-homo GMP iPSCs were used to generate gut endoderm cells (GECs) via a reproducible, scalable protocol. GECs were further differentiated into two-dimensional (2D) hepatocytes and 3D liver organoids using stepwise differentiation media. The generated cells were analyzed for hepatic functionality, including gene expression of alpha-1 antitrypsin (AAT), transthyretin (TTR), albumin (ALB), and secretion of human albumin. Functional comparisons were performed between 2D hepatocytes and 3D liver organoids to evaluate their maturation and suitability for clinical applications.

Results: The differentiation protocol successfully produced 2D hepatocytes and 3D liver organoids from HLA-homo GMP iPSCs. Hepatic genes, including AAT, TTR, and ALB, are highly induced in generated liver organoids. Human albumin reached as high as 3 µg/ml/24hr per million cells in 3D liver organoids, which is 10-fold higher than induced 2D monolayer hepatocytes.

Conclusion: This study demonstrates that liver organoids derived from HLA-homo GMP iPSCs offer scalable, reproducible, and functionally enhanced alternatives for clinical cell therapies targeting end-stage liver disease. These findings highlight the feasibility of using GMP-compliant iPSCs to strengthen organogenesis and advance next-generation liver regenerative medicine.

1093 An Optimized Protocol to Transduce Photoreceptors in Human Retinal Organoids Using AAV

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In vitro models, such as retinal organoids, are increasingly used to evaluate novel gene therapy strategies. While we have demonstrated the feasibility of infecting retinal organoids with AAV vectors, outcomes vary significantly depending on factors such as organoid age and infection medium, both of which influence infection efficiency and

organoid development. Therefore, optimizing these parameters is crucial to achieving high expression levels without compromising organoid integrity.

Using RT-ddPCR quantification and RNA sequencing, we monitored gene expression and organoid development post-infection. The infection process was notably affected by the presence of serum in the medium, which competes with AAV receptors. Additionally, organoid age played a key role: infecting immature organoids risked impairing post-infection development, whereas mature organoids with segment structures exhibited lower infection efficiency due to the presence of a mechanical barrier.

Our results underscore the importance of considering seemingly minor factors, such as age and infection medium, to ensure successful AAV transduction in retinal organoids.

1094 Advanced Base and Prime Editing Strategies to Correct Common ALS-causing *SOD1* Mutations

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Objective: Develop innovative base and prime editing therapies to achieve precise somatic gene correction for common missense mutations in *SOD1*.

Background: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with no effective treatment, leading to death within five years of diagnosis. Approximately 10% of ALS cases are familial, with mutations in *SOD1* being the second most common genetic cause. These mutations drive toxic gain-of-function properties in SOD1 protein, accelerating motor neuron degeneration. Our goal is to develop cutting-edge base and prime editing approaches to correct prevalent *SOD1* mutations. We will test and optimize next-generation domain-inlaid base editor strategies with enhanced efficiency, compact size to facilitate AAV delivery, and minimized bystander editing. We will also optimize different prime editor systems with optimal editing efficiencies and low off-target editing. Additionally, we will optimize adeno-associated virus (AAV)-mediated delivery of these gene editing tools for *in vivo* application.

Design/Methods: We generated *SOD1* mutant HEK293T cells via prime editing and will systematically screen base and prime editor strategies in these mutation-carrying cells. Optimized editors will then be evaluated in patient-derived fibroblasts and *in vivo* mouse models. The therapeutic impact of gene correction will be assessed by analyzing molecular, cellular, and motor phenotypes linked to both gain- and loss-of-function consequences of *SOD1* mutations.

Results: Preliminary data show 30% correction of the A5V mutation in patient fibroblasts and A5V-HEK293T cells using unoptimized base editing, with minimal bystander editing. Additionally, prime editing without nicking guides achieved 15% correction of the A5V mutation in patient fibroblasts.

Conclusions: We propose that AAV-mediated somatic gene correction using base or prime editing to target *SOD1* mutations (A5V and G94A) will mitigate toxic gain-of-function pathology, restore wild-type SOD1 protein levels, and rescue motor deficits. This strategy offers a promising therapeutic avenue for SOD1-ALS.

1095 Restoration of MeCP2 Function by converting the R270X Nonsense STOP Mutation to Tryptophan Through A-to-I RNA Editing

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Rett syndrome (RTT) is a severe neurodevelopmental disorder primarily caused by single-nucleotide variants in the *MECP2* gene, with approximately 70% involving C-to-T mutations. Among the most severe forms are nonsense mutations, where a CGA codon is mutated to TGA, creating a premature stop codon (e.g., R168X, R255X, and R270X). This truncates the MeCP2 protein, leading to severe RTT phenotypes due to the loss of MECP2 function. To address this, we plan to utilize REPAIR, a programmable RNA editing tool that fuses the ADAR2 deaminase domain with a catalytically inactive Cas13b domain to achieve adenosine-to-inosine RNA editing with high specificity. Our goal is to convert the UGA stop codon into UGG using REPAIR, effectively substituting the stop codon with tryptophan. We hypothesize that this approach could restore MeCP2 function and alleviate the RTT phenotype associated with MECP2 nonsense mutations.

To evaluate the potential of this approach, we used R270X as a test case and generated R270W knock-in mice, where the R270 residue was replaced with tryptophan. This allowed us to assess whether the tryptophan substitution affects MeCP2 function. Behavioral analysis revealed that MeCP2 R270W mice exhibit no significant differences compared to their wild-type littermates. At the cellular level, a pathological hallmark of MeCP2 loss is the disruption of its interaction with alpha-thalassemia/mental retardation X-linked (ATRX) at heterochromatic foci. Immunohistochemistry analysis demonstrated that MeCP2 R270W retains normal interactions with ATRX. Together, these findings suggest that tryptophan substitution at R270 maintains MeCP2 function. To further develop a therapeutic approach for in vivo STOP-to-Trp conversion using the Cas13-based REPAIR system, we developed an in vitro reporter system and identified the most optimal guide RNA for subsequent in vivo testing. Altogether, these findings indicate that STOP-to-Trp conversion through RNA editing holds significant potential as a viable therapeutic strategy for MeCP2-R270X, offering a reversible, precise, and potentially safer alternative to DNA editing.

1096 Development of a Humanized Mouse Model for PACS1 Syndrome and Evaluation of Base Editing as a Therapeutic Approach

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PACS1 Syndrome is a rare neurodevelopmental disorder caused by a de novo mutation (c.607C>T) in the *PACS1* gene, resulting in a gain-of-function arginine to tryptophan substitution. PACS1 is involved in trans-Golgi membrane trafficking, but the pathogenic mechanism remains unclear, and there is currently no cure for the disorder.

We developed a humanized mouse model with the R201W amino acid substitution to study PACS1 Syndrome and investigate base editing as a potential therapy. Due to embryonic lethality in constitutive R201W knock-in mice, we generated a conditional knock-in model carrying a humanized exon 4 with the R201W substitution and an upstream lox-STOP-lox cassette. We used a Nes-Cre driver to express the mutation specifically in neurons. Phenotyping assays revealed hyperactivity, anxiety-like behavior, and increased seizure susceptibility in Nes-cre;$Pacs1^{PACS1(R201W)}$ mice. Female mice were more prone to seizures, and both sexes showed reduced survival starting at 18 weeks, but no changes in learning and memory were observed.

We tested seven base editing strategies *in vitro* using $Pacs1^{PACS1(R201W)}$ mouse embryonic stem cells (mESCs), including both correction using A-base editors (ABE) and gene disruption via stop codon conversion using C-base editors (CBE). We identified a lead CBE design that resulted in robust and specific stop codon installation in our in vitro assay. To support proof-of-concept in vivo experiments, we developed split-intein dual AAV vectors. We achieved up to 80% base editing in transfected ES cells. We have produced AAV particles and are testing editing efficiency and therapeutic efficacy in postnatal $Pacs1^{PACS1(R201W)}$ pups via intracerebroventricular injection. In summary, our model highlights behavioral phenotypes and a promising base editing strategy for potential therapeutic intervention for PACS1 syndrome.

1097 Base Editing Strategies for Restoring Functional Collagen VII Expression in Recessive Dystrophic Epidermolysis Bullosa

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a devastating genetic disorder marked by extreme skin and mucosal fragility, resulting in chronic blistering, slow-healing wounds, and premature mortality. This condition is caused by mutations in the *COL7A1* gene, which encodes collagen VII (C7), a critical structural protein for dermal-epidermal cohesion. In Brazil, exon 54 mutations, particularly c.5047C>T, are predominant and result in reduced or absent C7 expression.

Our study focuses on developing a gene-editing strategies to restore functional C7 expression in cells derived from individuals with RDEB harboring exon 54 mutations. Two complementary approaches are being investigated: (i) exon 54 skipping to bypass the pathogenic mutation and (ii) precise correction of the c.5047C>T mutation. Both strategies leverage advanced adenine base editors (ABEs), which offer enhanced specificity and safety compared to conventional CRISPR/Cas9 systems.

Preliminary experiments using the BJ-hTERT cell line harboring the c.5047C>T mutation demonstrated successful optimization of the gene-editing protocol. Nucleofection of ABE8e-mRNA and guide RNAs (gRNAs) yielded high transfection efficiency while maintaining cell viability. Targeted editing at the c.5047 locus achieved A-to-G conversion efficiencies of 80–90% with gRNA-04 and gRNA-05. Additionally, exon skipping was induced with approximately 80% efficiency using gRNA-02. In these approaches all bystander mutations are silent and the induced indel levels were quite low.

The optimized gene-editing protocols are being currently tested in primary fibroblast from patient's skin biopsies and the edited cells will be used to generate three-dimensional skin equivalents comprising dermal and epidermal layers. These bioengineered skin fragments, expressing functional C7, will serve as prototypes for autologous skin grafts aimed at accelerating wound healing in RDEB patients and improving their quality of life. By the conclusion of this project, we aim to deliver a validated, safe, and efficient protocol for restoring C7 expression in fibroblasts and keratinocytes, laying the groundwork for future clinical applications in gene therapy for RDEB.

1098 Prime Edited T-regulatory Cells for Immune Therapy

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T-regulatory cells (Treg), CD4+25hi127neg T-cells, are immune suppressive and as such useful as cellular therapy to control adverse immune responses including graft-versus-host disease (GVHD). Since Tregs do not produce interleukin-2 (IL-2), systemic infusion of recombinant IL-2 (rIL-2) is required to maintain cell viability and function. However, exogenous IL-2 can promiscuously activate and expand T-cells resulting in toxicity. To minimize rIL-2 side-effects, previous work generated an orthogonal IL-2 system and showed that a two amino acid conversion (H134D Y135F) in the interleukin-2 receptor beta (IL-2Rβ) gene enabled selective responses to an orthogonal IL-2 ligand (orthoIL-2)[1].

Initial studies employed lentiviral ortho-IL-2Rβ delivery[2], and here we pursued a DNA prime editing (PE) strategy. PE uses a programmable nickase fused to a reverse transcriptase and a user-specified sequence encoded by an engineered prime editor guide RNA (epegRNA)[3] to install all base substitutions, insertions, deletions, and

combinations thereof. PE operates without inducing significant double-stranded DNA breaks (DSB) thereby minimizing the cellular/genotoxicity associated with gene editing nucleases and lentiviral vectors[4,5].

We designed an epegRNA to mediate the PE conversion of H134D Y135F at the endogenous IL-2Rβ locus (Fig. 1A). Using FACS-purified peripheral blood Tregs (n=2 donors) we electroporated a synthetic epegRNA, complementary-strand nicking guide RNA, PE6c[6] mRNA, and mRNA of a dominant negative version of the cellular mismatch repair protein MLH1[7]. Following gene transfer, Tregs were cultured in orthoIL2 (STK009; labeled "PE S100", 100 nM) or native IL-2 (Proleukin, 300 IU/ml; labeled "PE P300"). Genomic analysis showed H134D Y135F prime edit installation, demonstrated by Sanger (Fig. 1B) and Illumina sequencing with a low frequency of insertions and deletions (indels) (Fig. 1C), consistent with the high allelic purity expected of a PE strategy.

PE Tregs maintained their characteristic FOXP3^{+} CD127neg phenotype (Fig. 1D) and suppressed, to a similar degree, the proliferation of activated T-cells in an *in vitro* co-culture assay (Fig. 1E) in the presence of PE orthoIL2 (PE S100) or native IL-2 (Proleukin (PE P300)).

Sub-lethally irradiated, immune-deficient NSG-SGM3 mice (n=5) were given HLA-A2^{+} PE Treg alone or with STK009 (20 µg/dose, 3 doses/week) along with HLA-A2^{-} peripheral blood mononuclear cells (PBMC) (Fig. 2A). We observed superior in vivo PE Treg *in vivo* Treg expansion in the presence vs absence of STK009 orthoIL2 (Fig. 2B; p=0.004). Preliminary studies showed improved GVHD prevention, evidenced by less pronounced weight loss (Fig. 2C), and improved survival in PE Treg STK009-treated mice over the PBMC treated or unedited Treg only control mice (Fig. 2D; p=0.021).

PE mediated the conversion of the endogenous IL-2Rβ locus to an orthogonal version with minimized formation of DSBs and indels than are generated using nuclease-based strategies. PE Tregs expanded in vitro and in vivo with STK009 orthoIL2 ligand injection. PE Treg also retained the ability to respond to Proleukin, consistent with intact IL2Rb signaling and PE Treg capacity to respond to activated donor T-cell derived IL-2.

In conclusion, our data shows that PE is a promising approach for Treg engineering that should facilitate immune therapy without employing lentiviral vectors or generating DSBs.

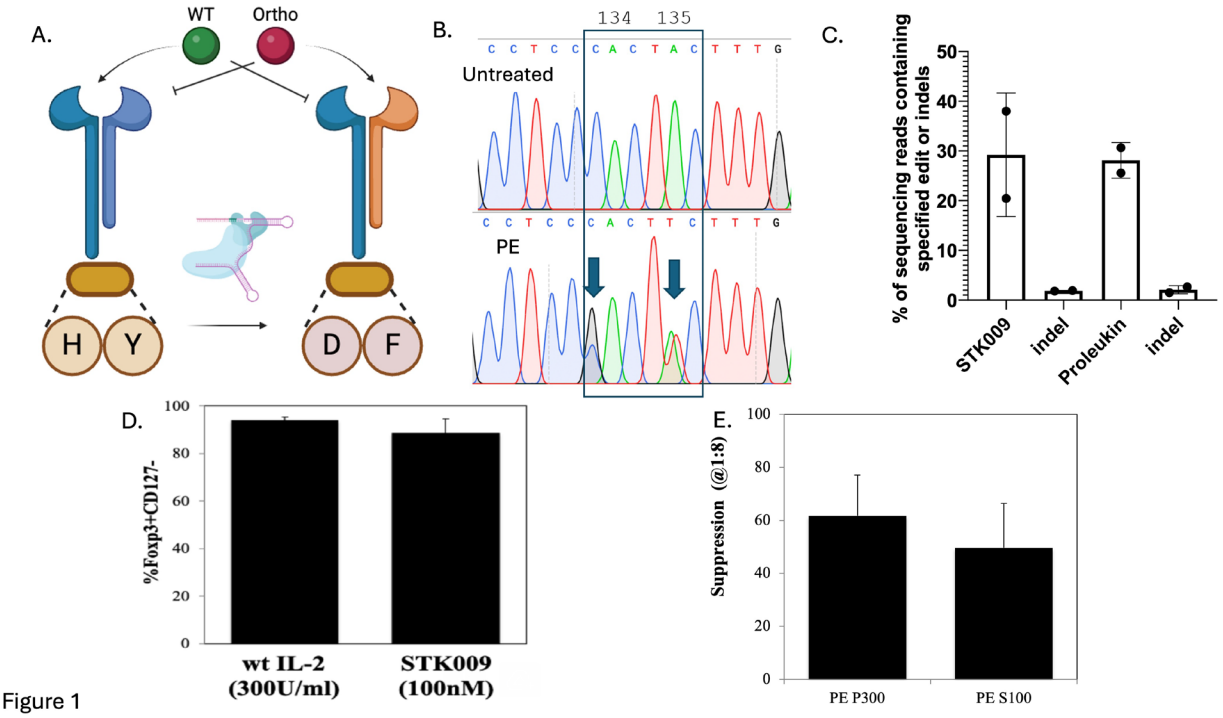

Figure 1

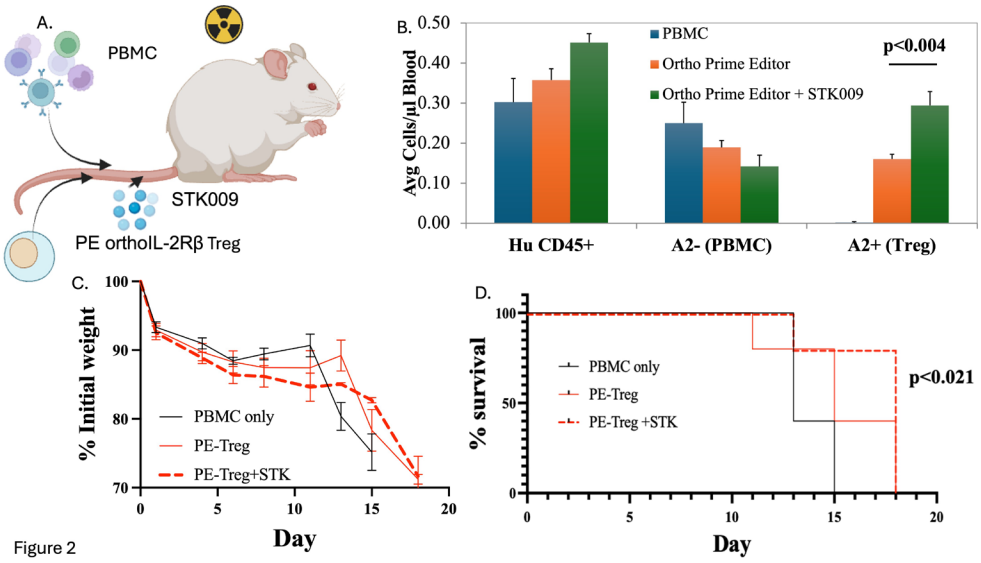

Figure 2

1099 Paired Prime Editors to excise GAA repeats in the Friedreich's Ataxia gene

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Background: Friedreich's ataxia (FRDA), a rare autosomal recessive neurodegenerative disorder, belongs to the group of genetic disorders known as Repeat Expansion Diseases (REDs), in which repeats of three nucleotides beyond a critical threshold lead to developmental, neurological, or neuromuscular disorders. In the case of FRDA, the repeat unit is the triplet GAA•TTC, located in the first intron of the frataxin (FXN) gene. The GAA repeats can form an R-loop, which potentially affects histone modification and leads to a reduction in the transcription of FXN. FXN protein plays a pivotal role in maintaining mitochondrial function, facilitating the conversion of iron into heme and iron-sulfur clusters. Disruption of this process results in oxidative stress, thus contributing to damage in the nervous system and muscle weakness with scoliosis. Patients with severe FRDA typically do not live past the age of 40 due to cardiomyopathy or arrhythmia. The prevailing therapeutic approach aims to increase FXN protein levels, but it does not address the underlying causes of FRDA. Thus, the optimal therapeutic solution to treat FRDA would be to correct the genetic cause at its root by removing the triplet repeat. Prime editing (PE), a Cas9 nickase paired with a polymerase and a guide RNA, could install any substitution, small insertion or deletion without inducing double-stranded breaks (DSBs). Previous data from our lab show that Prime Editors-Cas9-based deletion and repair (PEDAR) could produce precise and efficient 10kb deletion. Here, we utilized paired prime editors to precisely contract GAA repeats in cells.

Methods and Results: We designed two engineered pegRNAs (epegRNAs) that target distinct regions flanking the GAA repeats. The reverse transcription template (RTT) sequence with AttB facilitates a 1.2 kb deletion and precise integration of the AttB sequence in HEK293T cells. Editing rate measured by ddPCR is around 27%. We also showed different lengths of RTT, 6xGAA repeats or 6xGAA repeats flanking with 20nt 3' end hangover, can efficiently contract GAA repeats. Illumina sequencing (NGS) showed perfect editing can achieve around 92.79% and the indel is 7.21%. We also electroplated GM03816 FXN patient-derived fibroblasts with two synthetic pegRNAs (38bp AttB as RTT) and PE nickase mRNA. Our paired PE system for 2.2kb GAA repeats contraction has around 12.85% editing efficiency in patient fibroblasts and the perfect editing rate is around 91.41%.

Conclusions: These results demonstrate paired PE system can contract 1.2kb and 2.2kb GAA repeats both in HEK293T cells and patient-derived fibroblasts with highly precise editing at FXN locus. This work provides a foundation to explore PE-mediated deletion as a strategy of FRDA treatment.

1100 A compact RT editor built from the metagenome

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To grow the list of treatable genetic diseases beyond the liver, it is essential to develop compact genome editors deliverable by size-constrained approaches like adeno-associated viruses (AAVs). AAVs allow for efficient targeting of many tissues, including heart, muscle and brain, but impose a strict size limit that precludes delivery of many highly active, large Cas enzymes. The size limitation is particularly limiting for the delivery of reverse transcriptase (RT) genome editors, which show exceptional therapeutic promise for introducing highly specific genomic edits without creating double stranded breaks, as the addition of the RT enzyme consumes valuable real estate in the AAV vector.

To build an RT editor capable of fitting in a single AAV vector, we searched through our metagenomic sequence database to identify deep branching examples of nucleases containing both RuvC and HNH domains (RHNs) and a diverse set of RTs. Through biochemical and *in vitro* screening, we found RHNs <800aa that were active in human cells, and RTs as small as 300 aa supporting high levels of RT editing. After multiple rounds of mutagenesis of each enzyme, we combined the most active candidate enzymes into new RT editors and observed significant precise editing in both mouse and human primary cells following AAV delivery.

Our small RT editor enables a path toward unlocking potential new treatments for genetic diseases in tissue types beyond the liver without the need for manufacturing or delivering multiple viral vectors.

1101 Cas13-Mediated RNA Base Editing Using mxABE Restores Heart Function in a Humanized Hypertrophic Cardiomyopathy Model

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Background: Hypertrophic Cardiomyopathy (HCM) is the most common inherited heart disease, affecting 0.2% to 0.5% of the population (15-20 million people worldwide). About half of the cases are linked to gene mutations, with 25-40% caused by mutations in the *MYH7* gene (5-8 million people), including the c.1208G>A (p.R403Q) variant. These mutations disrupt normal heart function and energy consumption, increasing the risk of arrhythmias, heart failure, and premature death. Current treatments, such as Aficamten and Mavacamten, do not address the underlying genetic cause and have significant side effects. Base editing techniques can correct disease-causing mutations in vivo and in vitro, potentially rescuing disease phenotypes. RNA base editing offers a safer, reversible alternative to correct disease-causing mutations without altering the DNA. This study investigates the potential of a mini CRISPR-Cas13X-mediated adenosine base editor (mxABE) to treat HCM by correcting the MYH7 mutation in a preclinical humanized mouse model.

Methods: To create a genetically humanized HCM mouse model carrying the human *MYH7* c.1208G>A (p.R403Q) pathogenic mutation, we replaced part of the mouse *Myh6* exon 13 sequence with the corresponding sequence from human *MYH7* exon 13, which contains the c.1208G>A (p.R403Q) missense variant (Figure 1A). In the in vitro studies, gRNAs targeting the mutant allele were screened in HEK293T cells co-expressing the *MYH7* mutation, identifying g8 as the most efficient gRNA. An all-in-one adeno-associated virus (AAV) vector encoding mxABE

base editor and g8 was injected into 2-week-old *Myh6*$^{hR403Q/+}$ mice. Heart function and histology were analyzed 6 months post-treatment.

Results: The homozygous *Myh6*$^{hR403Q/hR403Q}$ mice exhibited high mortality within two weeks of birth (Figure 1B). The heterozygous *Myh6*$^{hR403Q/+}$ mice developed severe ventricular hypertrophy and fibrosis by 6 and 9 months of age, closely mimicking human HCM (Figure 1C). We co-transfected the *MYH7* gene carrying the mutant allele and the candidate gRNAs in HEK293T cells. In the in vitro studies, g8 gRNA achieved an average RNA editing efficiency of 63.69% for the c.1208G>A mutation (Figure 1D). We injected into 2-week-old *Myh6*$^{hR403Q/+}$ mice with either saline or mxABE and then monitored their development for over 6 months (Figure 1E). The results demonstrated that mxABE achieved high A-to-I editing rate in cardiac tissues (Figure 1F). Furthermore, AAV-mxABE-treated mice exhibited significantly improved cardiac function, including restored ejection fraction, fractional shortening, and reduced ventricular wall thickness, effectively reversing disease progression (Figure 1G).

Conclusions: This study demonstrates the successful application of mxABE, a CRISPR-Cas13-based RNA base editor being developed for HCM treatment by correcting the *MYH7* mutation. The AAV-mxABE therapy effectively prevented heart remodeling and restored heart function in a humanized *Myh6*$^{hR403Q/+}$ mouse model, highlighting its potential as a safe and targeted therapeutic strategy for hereditary cardiomyopathies.

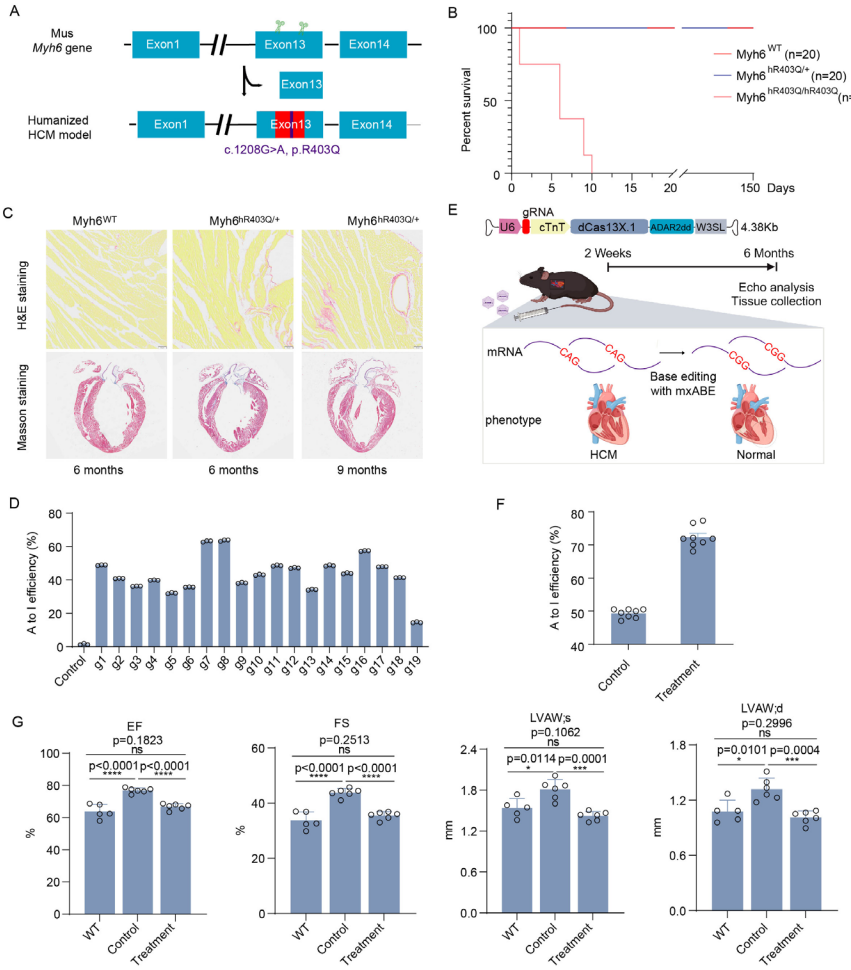

Figure 1. Intravenous delivery of the mxABE system for treating humanized HCM mice
A. Schematic of humanized HCM mouse model. **B.** Kaplan-Meier curve for WT (n = 13), Myh$^{6h403/+}$ mice (n = 13), Myh6$^{hR403Q/hR403Q}$ mice (n = 8). **C.** Representative Masson staining and hematoxylin and eosin (H&E) staining of heart sections of the humanized mouse model for the WT (left) and heterozygous (right) genotypes at 6 and 9 months of age. **D.** Deep sequencing of the RNA transcribed from the reporter vector. **E.** Schematic of the HCM therapeutic vectors and RNA-base editor strategy. **F.** EdiR analysis of the sanger sequencing results in the Myh6$^{hR403Q/+}$ mice injected with AAV-mxABE and the corresponding uninjected (n=8). **G.** Echocardiographic measurements for ejection fraction (EF), fractional shortening (FS), left ventricular end-systolic anterior wall thickness (LVAW;s) and left ventricular end-diastolic anterior wall thickness(LVAW;d) (n=6). Dots and bars represent biological replicates and are mean ± SEM. Different asterisks represent statistical significance (P< 0.05) using unpaired two-tailed Student's test (* P< 0.05, ** P< 0.01, *** P< 0.001, **** P< 0.0001) .

1102 A prime editing approach to rapidly model rare neurological disease variants in wild type animals

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Genetic diseases have traditionally been difficult to treat owing largely to heterogeneous genetic underpinnings. These disorders are a collection of common symptoms with highly varied etiology, a phenomenon well represented by genetic epilepsies. More than 30% of epilepsies are caused by genetic perturbations in one or more of 977 distinct genes, any one of which may harbor up to hundreds of unique pathogenic loci. Standard preclinical animal modeling methods are not well suited to handle this level of diversity due to the cost and time required to produce a single strain. With the goal of expediting timelines for rare disease variant modeling, we have developed a toolset and pipeline for precise in vivo genome manipulations using prime editing. We begin with a webtool, PegAssist.app, that expedites the design of pegRNAs with cloning compatibility for a publicly available plasmid set. To confirm in vivo prime editing functionality in the target cells and provide a visual assessment of editing efficiency, we designed a deliverable reporter for prime editing. This reporter makes use of an externally expressed, editing-dependent fluorophore, providing compatibility with various downstream assays for cell/tissue function or assessment of editing outcomes. We then use this validated editing approach to introduce rare epilepsy variants in wild type mice. We show precise integration of the target variants via Next-Generation Sequencing and functionally confirm gene activity. The low error rate makes this approach amenable to direct use in vivo, eliminating lengthy and costly screening steps. Most strikingly, a subset of mice with these introduced variants displays frequent spontaneous seizures, a result that is upheld across multiple genetic targets. Utilizing this feature, we demonstrate a proof-of-concept experiment for drug screening in a personalized mouse model of genetic epilepsy. The results of this work demonstrate an alternative modeling strategy for genetic diseases that could serve as patient surrogates for n-of-one trials of candidate drugs. Demonstrable efficacy could justify off-label use for approved therapeutics and greatly benefit an underserved patient population.

1103 Base Editing Premature Stop Codons Within the *ATXN2* Repeat Reduces Pathogenic Polyglutamine Proteins.

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Spinocerebellar Ataxia Type 2 (SCA2) has an incidence of 1 in every 50,000 individuals, and causes ataxia, dementia, and premature death. SCA2 is caused by greater than 33 CAG trinucleotide repeats in exon 1 of the *ATXN2* gene (*mATXN2*). Previous work from our lab has shown that targeting *mATXN2* with a nuclease via AAV delivery can result in genomic integration of AAV DNA following repair of the induced double stranded break. To advance therapies that avoid genomic integration events, cytosine base editing using a Cas9 nickase and cytosine deaminase to alter CAGs into premature termination codons (PTCs) via C:T conversion was tested. Unlike Cas9 nuclease editing, base editing modifies DNA without a double stranded break, thereby reducing insertions, deletions, translocations, and integrations. Here, we exploited the ability of cytosine base editors to make C: T substitutions within a broad window of nucleotides, increasing the likelihood for multiple PTCs. Screening base editors in human cells identified guides that inserted PTCs at multiple sites along the repeat at greater than 50% efficiency at any one repeat, and up to 6 repeats consecutive repeats. In a model N2A reporter line containing m*ATXN2* exon 1 at various repeat lengths the C:T conversion reduced target protein levels by 35% and 17% for 58 and 114 CAG repeats, respectively. For in vivo work, base editor mRNA and guides packaged in lipid nanoparticles (LNPs) resulted in similarly edited consecutive repeats. Ongoing work is focused on testing of LNP and AAV delivery modalities in two distinct SCA2 mouse models for impact on disease phenotypes.

1104 Regulation of the *ABCD2* uORFs as a Therapeutic Approach to Adrenoleukodystrophy

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Adrenoleukodystrophy (ALD) is an X-linked disorder with a wide phenotypic spectrum, ranging from adrenal insufficiency to a highly fatal childhood or adult presentation of rapid demyelination – known as cerebral ALD (cALD). The disease is caused by mutations in the *ABCD1* gene encoding the peroxisome ALD associated transporter. As a result, the peroxisome is less able to transport and oxidize very long chain fatty acids (VLCFAs), which accumulate in tissue. To date, there are no cures for ALD. Treatment options, including hematopoietic stem cell transplantation, are limited by early detection and associated morbidities. Upstream Open Reading Frames (uORFs) have been identified as gene suppressors in various loci, preventing the translation of their associated downstream ORFs. The *ABCD2* gene and protein is homologous to the *ABCD1* gene and has been shown in prior studies to offer some rescue of function when expressed in mutated Abcd1 cells and mice. Therefore, in this study, we have designed and optimized adenine base editors of *ABCD2* uORFs to disrupt their recognition by the translation machinery, thus allowing the translation of the *ABCD2* region to compensate for loss of *ABCD1*. This novel therapeutic strategy has the potential to be applied to any of the thousands of *ABCD1*-associated pathogenic variants. The efficiency of different base editing packages was tested by Next Generation Sequencing 72 hours after electroporation. *In vitro* analysis and validation of gene edited human ALD derived fibroblasts was conducted to determine expression of *ABCD2* via Western blot, qPCR, and immunohistochemistry, as well as accumulation of VLCFA (C26:0 lysophosphatidylcholine) measured by liquid chromatography-tandem mass spectrometry – the gold standard ALD diagnostic test. Reductions in VLCFA accumulation, as well as increases in *ABCD2* expression have been observed. Additional guides will be designed to target the homologous region in mouse, and tested in N2a cells prior to AAV9 packaging and delivery to the *Abcd1* knock-out mouse model of ALD. The mouse ALD model exhibits a robust behavioural and biochemical phenotype, and will offer a platform to determine the viability of *Abcd2* uORF modulation as a treatment to ALD. Further studies are required to determine the potential of this therapy to mitigate demyelination and slow the progression of cALD. In absence of a reliable cALD mouse model, patient derived iPSCs and organoids may offer translational relevance.

1105 Design and Assembly of Modular Pin-point™ Base Editors for Precise Correction of Pathogenic SNVs

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Single nucleotide variants (SNVs) account for the majority of pathogenic mutations associated with monogenic diseases. These single-base changes can disrupt protein function, alter gene regulation, or interfere with splicing, leading to severe and often life-threatening conditions. The ability to correct such mutations at the DNA level has long been a goal of precision genome engineering. However, due to the diversity of rare disease causing SNVs, this remains an ongoing challenge. Targeted genome editing technologies, such as CRISPR-Cas9 nucleases, enable the programmable editing of diverse DNA sequences required to tackle this challenge but rely on double-strand breaks (DSBs) and homology-directed repair (HDR) which can be error-prone, and lead to genomic instability.

Base editors have emerged as next-generation genome editing tools that precisely and efficiently correct SNVs in post-mitotic cells with undetectable levels of DSBs and without requiring donor DNA templates. However, a major challenge faced by base editors is their potential to introduce unintended 'bystander' edits adjacent to target DNA bases. As a solution to this challenge we have developed the Pin-point™ base editing platform, which enables the modular assembly of DNA binding Cas and DNA modifying deaminase components via an aptamer encoded in the sequence-targeting guide RNA (gRNA). This modularity allows the flexible design and assembly of diverse base editor configurations with defined base conversion activities, protospacer-adjacent motif (PAM) specificities, and

target sequence editing preferences. These characteristics make the Pin-point platform a powerful tool for assembling bespoke base editors tailored to correct SNVs with high efficiency and specificity.

Here we present our pipeline for the design and development of bespoke modular Pin-point base editors with defined editing activities across therapeutic DNA target sequence space. We have established an arrayed screening platform and optimised gRNA libraries to characterise the editing activities of diverse combinations of Cas and deaminase modules at scale. Knowledge obtained from arrayed screens is used to guide the design of massively parallel screens to empirically determine the efficiency and specificity of favourable base editor assemblies across pooled libraries covering all targetable pathogenic SNVs, with promising candidates validated and further optimised in cellular models harbouring SNV target sequences. In addition to generating an inventory of validated base editor assemblies with strong potential for further preclinical development the knowledge obtained from our screening activities is synthesised in predictive models of base editor activity. These predictive capabilities enable both the identification of additional candidate targets not empirically analysed through screening and inform the strategy of future screening campaigns with novel modular base editor assemblies.

Disclaimer: The Pin-point base editing platform technology is available for clinical or diagnostic study and commercialization under a commercial license from Revvity

1106 Optimized RESTORE+ oligonucleotides for an efficacious and safe RNA base editing treatment for Alpha-1 antitrypsin deficiency

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Endogenous ADAR-mediated RNA editing is a transformative technology enabling precise A-to-I editing of mRNA. Unlike DNA editing, RNA editing is transient, reversible, and redosable, ensuring safety and flexibility. Using the RESTORE+ platform, we developed fully chemically modified oligonucleotides to optimally recruit endogenous ADAR and enhance RNA editing therapeutics through improved potency and delivery.

Our optimized GalNAc delivery system, combined with advanced chemical modifications, significantly enhances RNA editing efficiency and durability while minimizing potential safety risks. Specifically, we designed oligonucleotides targeting the SERPINA1 mRNA to treat Alpha-1 Antitrypsin Deficiency (AATD). By catalyzing precise A-to-I conversion, the platform corrects the PiZZ mutation (E342K), a G-to-A substitution responsible for misfolding and aggregation of the Z-AAT protein, leading to liver and lung pathology in AATD. In primary mouse hepatocytes, we achieved >90% editing efficiency in vitro, and >40% editing in vivo in the NSG-PiZ mouse model 7 days post-dose. This restored wild-type (M-AAT) and total AAT levels above therapeutically meaningful thresholds (>14 μM and >17 μM, respectively). Long-term studies demonstrated sustained M-AAT levels (>14 μM) with multiple doses over 8 weeks, alongside improvements in liver health and pathology.

Pharmacokinetics (PK) and safety profiling in non-human primates (NHPs) showed 3-4x extended oligonucleotide half-life compared to mice, with no observable toxicity. This PK enhancement aligns with the durability and potency improvements observed in siRNA therapeutics across species. We have established a robust and competitive disease modifying RNA-based therapeutic for AATD with the RESTORE+ platform enabling rapid development of RNA editing therapies for other diseases with high unmet needs.

1107 Correction of Multiple Dominant-Negative Mutations in Collagen VI-Related Muscular Dystrophy via ADAR-Mediated RNA Editing

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Introduction: Collagen VI-related muscular dystrophies (COL6-RD) are a group of muscle disorders characterized by progressive muscle weakness, distal joint laxity, and proximal joint contracture. COL6-RD is caused by mutations in any of the three genes encoding collagen VI, with inheritance patterns either dominant or recessive, depending on the mutation and its location. The most common mutations are missense variants that result in the substitution of glycine within the conserved Gly-x-y repeat sequences of the triple helical (TH) domain. These mutations impair collagen VI assembly by incorporating aberrant chains into the secreted tetramer. Allele-specific gene silencing has emerged as a potential therapeutic approach for COL6-RD, with early-stage developments focusing on antisense oligonucleotides (ASOs) and small interfering RNA (siRNA) technologies. In this context, we present an alternative therapeutic strategy for COL6-RD involving ADAR-mediated RNA editing. ADAR is a site-specific RNA editing enzyme that converts adenosine (A) to inosine (I) in RNA transcripts. Since I is recognized by the translation machinery as guanine (G), A-to-I editing of G-to-A transition mutations effectively corrects the mutation at the transcript level. Unlike ASO and siRNA approaches, which are limited to specific mutations, ADAR editing offers the potential to target multiple adjacent mutations using a single guide RNA. This is particularly advantageous in COL6-RD, where dominant-negative mutations are clustered in the N-terminal end of the TH domain. We hypothesize that a single guide RNA could be designed to target several nearby mutations, thereby expanding the applicability of ADAR-based therapies to a larger cohort of patients.

Methods: Two fibroblast lines derived from patients with COL6-RD harboring commonly observed missense mutations in the TH domain, c.850G>A (p.G284A) in exon 9 and c.868G>A (p.G290A) in exon 10, were utilized in this study. Our previous work using circularized guide RNAs achieved approximately 20% editing efficiency at the c.850G>A mutation site; however, no significant editing was observed at the c.868G>A site. In this study, we introduced multiple mismatches and structural loops, along with different configurations of ADAR-recruiting domains, into the guide RNAs to enhance editing efficiency. Candidate guide RNA sequences were cloned and delivered into fibroblasts using an AAV-based system. RNA editing efficiency was assessed using RNA-seq by comparing read counts of wild-type and mutant transcripts.

Results: Several guide RNAs capable of inducing A-to-I editing at both mutation sites were identified. Both the guide RNA sequence and secondary structure affect recruitment of ADAR and editing efficiency. The most effective guide RNA achieved an editing efficiency of 35% at the c.850G>A site and approximately 20% at the c.868G>A site. The introduction of a mismatch 35 bp upstream of the target site and the inclusion of ADAR-recruiting domains were found to be the key to editing efficiency.

Conclusions: We demonstrate feasibility of using a single guide-RNA to target A-to-I editing of multiple adjacent mutations in COL6A1. Our next experiments will next determine editing efficiency of our most effective guide RNAs on other nearby mutations with goal to identify a single guide RNA to treat most of these mutations. These experiments support development of ADAR editing as a therapy for COL6-RD.

1108 MM201-miniVPR: A Novel Single AAV-Delivered Multiplexed Gene Activation System for Therapeutic and Research Applications

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Background: CRISPR-based activators (CRISPRa) have expanded the potential of CRISPR technology by enabling precise control over gene regulation. These systems use catalytically inactive Cas proteins fused to transcriptional activators, such as VPR, miniVPR, SunTag, or SAM, to upregulate target genes. In many therapeutic and research contexts, such as somatic cell reprogramming into iPSCs or regenerative therapies, multiplexed gene activation targeting multiple promoter sites, such as *Oct4, Sox2, Klf4, and c-Myc*, is essential. However, the large size of traditional Cas proteins (e.g., Cas9 and Cas12a) and their associated guide RNA scaffolds limits packaging into a single adeno-associated virus (AAV) vector. Cas12f, though smaller, lacks efficient self-processing of sgRNAs, further complicating multiplexed activation. Here, we develop MM201-miniVPR, a novel compact CRISPRa tool capable of multiplexed gene activation using a single AAV delivery system.

Methods: We used a computational pipeline to analyze bacterial genomes and identify novel, compact Cas proteins (<630 aa), leading to the discovery of wtMM201, a protein with self-processing crRNA capabilities. Using a dual-fluorescence reporter system, we optimized the activation efficacy of wtMM201 through mutagenesis and combinatorial modifications of its key domains, generating MM201-miniVPR. We evaluate MM201-miniVPR's single and multiplex gene activation efficiency against dhyperCas12a-miniVPR, dCasMINI-miniVPR, and wtMM201-miniVPR at 28 endogenous sites in HEK293T cells. Additionally, we compared MM201-miniVPR with dhyperCas12a-miniVPR for multiplexed activation using pooled or crRNA arrays.

Results: We identified and engineered wtMM201, a previously undocumented compact Cas protein with binding activity and self-processing crRNA abilities in mammalian cells. Through multiple rounds of protein engineering, we generated MM201-miniVPR with higher efficiency of single and multiplexed gene activation based on the dual-fluorescence reporter genes and endogenous genes in HEK293T cells (Fig. 1b and c). MM201-miniVPR exhibited significantly improved gene activation efficiency (Fig. 1d) compared to dCasMINI-miniVPR and wtMM201-miniVPR. It achieved comparable activation efficiency to dhyperCas12a-miniVPR at endogenous sites with 5'-NTTN PAMs (Fig. 1d). Additionally, MM201-miniVPR enabled efficient simultaneous activation of three genes using a multiplex crRNA array, comparable to the activity of dhyperCas12a-miniVPR (Fig. 1e). Importantly, MM201-miniVPR's compact size allows packaging into a single AAV vector with up to 10 crRNAs, addressing a major limitation of existing CRISPRa tools (Fig. 1a).

Conclusions: MM201-miniVPR is a powerful and versatile CRISPRa platform capable of robust single and multiplexed gene activation. Its compact size, high efficiency, and compatibility with a single AAV delivery make it a promising tool for basic research and therapeutic applications.

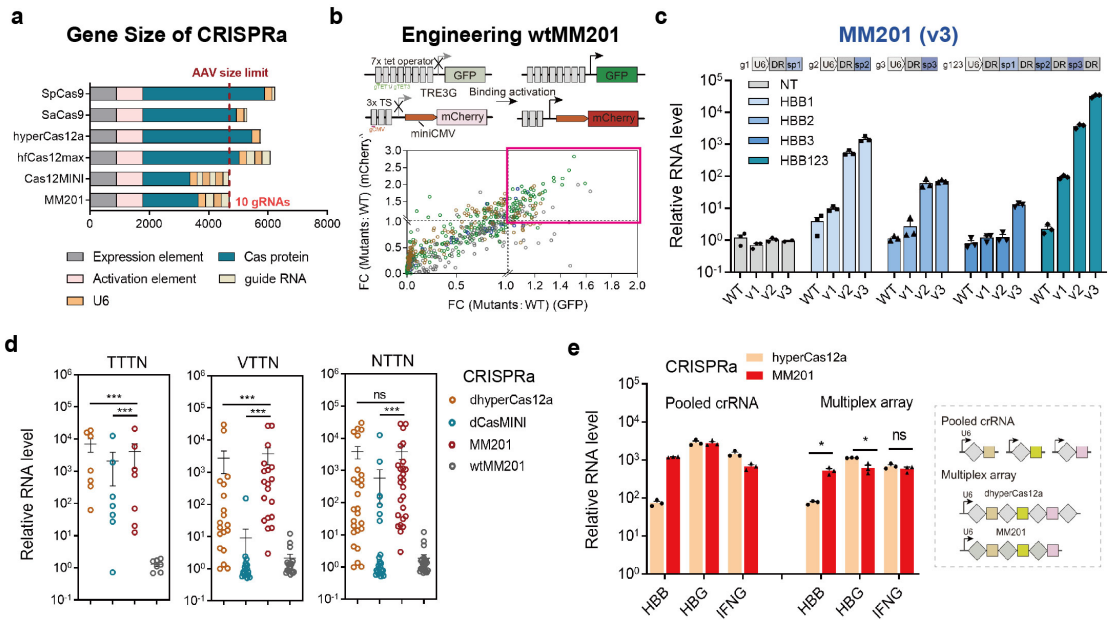

Figure 1. Engineered MM201-miniVPR mediated efficient multiplexed gene activation. a, Comparison of the gene sizes of CRISPRa derived from MM201 and other Cas effectors from the Cas9 or Cas12 family. **b,** Iterative protein engineering to generate efficient variants for reporter gene activation from wtMM201. **c,** Increased activity of single and multiplexed endogenous gene activation mediated by MM201-miniVPR. **d,** Comparison of dhyperCas12a-, dCasMINI-, wtMM201- and MM201-miniVPR for the activation of 28 endogenous genes in HEK293T cells. Each dot represents the average activation activity at each endogenous target site from three independent biological replicates. **e,** dhyper-Cas12a-miniVPR and MM201-miniVPR for multiplexed activation of endogenous genes in HEK293T cells. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. NS, no significant. n=3.

1109 A Miniature CRISPR Editor for Epigenetic Rescue in Human Models of ZC4H2-associated rare disorders

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Females undergo random X chromosome inactivation to regulate gene dosage. In X-linked intellectual disability, this process creates a mosaic pattern in affected tissues, such as the brain, where cells express either the wild-type or mutant allele. ZC4H2 Associated Rare Disorders (ZARD) is an ultra-rare X-linked intellectual disability caused by a spectrum of inherited and *de novo* mutations in the *ZC4H2* gene. This gene encodes a zinc finger protein that is highly expressed in the brain during early neurodevelopment, yet its direct role in regulating neurodevelopment remains unclear. Current understanding of ZARD pathophysiology is predominantly based on non-human models, with limited insights from human models. Identifying the molecular and cellular mechanisms underlying ZARD is essential for elucidating its neuropathology and potential therapeutic interventions. In our study, we have established female patient iPSC-derived neural stem cells to explore the cellular and molecular perturbations underlying ZARD pathology and to assess the feasibility of reactivating wildtype *ZC4H2* using epigenetic tools. To determine whether ZARD NSCs are primarily self-renewing or differentiating into neurons, we performed RNA sequencing and immunocytochemistry (ICC). RNA sequencing data from ZARD neural stem cells revealed >2000 differentially expressed genes. Notably, Gene Ontology enrichment analysis identified significant enrichment of pathways related to neuron differentiation, neuron generation, and stem cell differentiation, suggesting a pro-neural fate at the transcriptomic level. Protein-level analysis showed elevated expression of both immature and mature neuronal markers, including TUJ1, PAX6, and MAP2, in ZARD NSCs compared to controls. Orthogonal shRNA-mediated knockdown of *ZC4H2* in a wildtype NSC line recapitulated the significant increase in MAP2 mRNA expression ($p<0.05$), reinforcing the critical role of *ZC4H2* in maintaining the balance between NSC self-renewal and differentiation. Conversely, pathways regulating BMP-signaling, cellular responses to BMP signaling, and SMAD

protein phosphorylation were downregulated, providing a potential molecular mechanism underlying ZARD pathogenesis. We then sought to investigate a newly engineered miniature CRISPR-Cas system to demonstrate the feasibility of reactivating *ZC4H2* in this clinically relevant cell type. Here we demonstrate that single guide RNA (sgRNA) paired with dCasMINI-VPR transfections of NSC successfully reactivated using two independent sgRNAs ($p<0.05$). Multiplexing sgRNAs did not result in increased mRNA levels. In conclusion, our study reveals that ZARD NSCs are proliferating faster and maturing more rapidly than sex-matched control cell lines, likely driven by aberrant BMP-signaling. We also demonstrate the potential of smaller Cas systems for reactivating X-linked genes, expanding therapeutic options.

1110 Compact DNA Demethylase-Activator combination Modulators for CRISPR-Mediated Epigenetic Gene Activation

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Epigenetic silencing of gene expression is a well-characterized mechanism that has been successfully harnessed for therapeutic applications, particularly in CRISPR-based approaches. In contrast, the development of epigenetic tools for robust and sustained gene activation has lagged, representing a significant barrier to advancing transformative therapies for human disease. While DNA demethylation, mediated by enzymes such as TET family members, has shown promise in de-repressing silenced genes, two major challenges remain: (1) the large size of current DNA demethylation editors, which limits their delivery via adeno-associated viruses (AAVs), and (2) the variability in gene activation across distinct chromatin states and transcriptional contexts. To address these limitations, we developed a new class of compact, AAV-compatible DNA demethylating enzymes, termed "miniTETs," which are 33% smaller than conventional catalytic domains. These miniTETs can be co-packaged with guide RNAs and tissue-specific promoters into a single AAV vector, facilitating efficient in vivo delivery. We demonstrate that dCas9-miniTET variants are capable of stable reactivation of silenced loci in vitro, with sustained target gene expression observed for up to 100 days in HEK293T cells following transient transfection. Additionally, we engineered an ultra-compact dCas variant, dCasONYX (<500 amino acids), and showed that a fusion to miniTET domains is highly active in human cells, and capable of epigenetically reactivating a silenced locus. Remarkably, dCas-miniTET fusions with transcriptional activators yielded synergistic reactivation of a silenced synthetic reporter gene, with stable expression maintained for 30 days post-transfection. These findings provide a foundation for the next generation of CRISPR-based tools for epigenetic gene activation, tailored for therapeutic applications. Our work establishes a robust platform for precise, durable, and tissue-specific control of gene expression in vivo.

1111 CRISPR Epigenetic Reprogramming of Neuroprotective Factors as Therapy for Alzheimer's Disease

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Background. Alzheimer's disease (AD) is a neurodegenerative condition imposing a significant burden on healthcare systems globally. Despite extensive efforts, it remains incurable, with only moderately effective treatments available for some patients. Gene therapy, particularly using CRISPR-Cas, holds promise as an advanced alternative. The ability to reprogram cell phenotypes by regulating gene expression via epigenetic reprogramming using the CRISPR-dCas9 variant has widened our options for complex disease therapies, like AD. It's known that exercising modulates genes encoding for neuroprotective factors that can induce neurogenesis, reduce neuropathology, and lead to fewer cognitive deficits in AD. We hypothesized that using CRISPR-dCas9 epigenetic reprogramming to upregulate exercise-responsive (*FNDC5, BDNF*) and other neuroprotective genes (*NGF, GDNF, KL, GRN, NTF3,* and *NTF4)* could mimic the beneficial effects of exercise in the brain, potentially slowing AD progression. As CRISPR delivery methods advance, there's optimism that this strategy could not only delay the onset of AD and its progression but also reduce cognitive decline in patients.

Methods. We used a variety of CRISPR-dCas9 activation domains known to recruit epigenetic effector proteins or directly modifying CpG methylation and histone residues. We evaluated four to six single-guide RNAs (sgRNAs) designed around 200 bases upstream of the top neuroprotective genes' transcription start sites. The cells used were iPS-derived neural progenitor cells (NPCs). sgRNAs and mRNAs were transfected with cationic lipids. The readouts were mRNA expression measured by TaqMan RT-qPCR and ELISA for the secreted proteins. The specificity of the leading sgRNAs was assessed by mRNA sequencing. We also tested different effector domains known to upregulate gene expression in different configurations (SunTag or fusioned to dCas9) either at N- or C-terminus of dCas9.

Results. For the tested sgRNAs, we obtained 3× to 487×-fold increase in mRNA expression of the target genes compared to controls (Fig. 2AB). The leading sgRNAs for the upregulated neuroprotective factors were assessed for specificity using mRNA sequencing. The results showed specific upregulation of *FNDC5* using the dCas9-VPR system for at least one of the leading sgRNAs (Fig. 2C). ELISA from the supernatants showed parallel upregulation for most of the neuroprotective factors tested (Fig. 2D). The gene and protein expression were upregulated for at least seven days for some of the targets (Fig. 2D).

Conclusions. In summary, the sgRNAs located upstream of the transcription start sites showed upregulation of the target transcripts and protein levels for most of the neuroprotective factors chosen. The leading sgRNAs showed good specificity, and different effector domains and their configurations lead to different upregulation of the neuroprotective factors. Next experiments will evaluate if the upregulation of these factors can avoid or dampen neuronal cytotoxicity in vitro. In the future, in vivo experiments on Alzheimer's disease mouse models will measure the effect of this approach on disease progression, memory, and neuropathology.

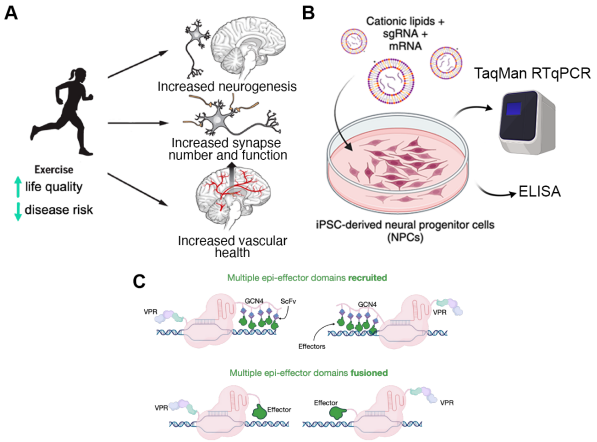

Figure 1 – Rationale for choosing exercise-responsive neurotrophins (A), delivery strategy and study model (B), and strategy to screen different effector domains to upregulate target genes (C).

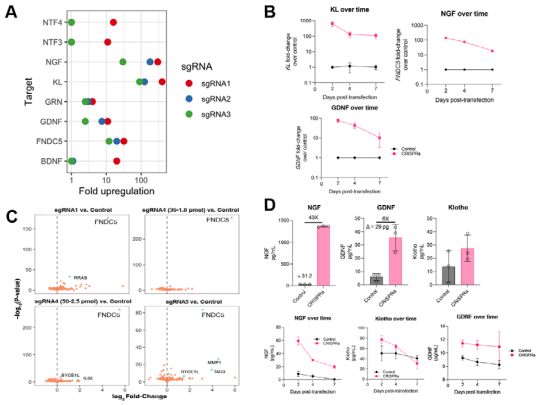

Figure 2 – Upregulation of gene expression of different neuroprotective factors (A). Longitudinal assessment of gene expression for selected neuroprotective factors (B). Volcano plots showing the differentially expressed genes from RNA-sequencing after activation with CRISPR-dCas9-VPR plus FNDC5's leading sgRNAs (C). ELISA protein quantification of neuroprotective factors from neural progenitor cells' supernatants after transfection with CRISPR-dCas9-VPR and lead sgRNAs.

1112 Epigenetic CRISPR Editors Encoded as mRNA for Engineering Primary Human T Cells

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Autologous T cell therapies for cancer, including CAR-T and TILs, have transformed cancer care by engineering the endogenous immune system. However, these therapies are limited by the heterogenous subpopulations of the cell product, with high prevalence of an exhaustion phenotype which limits efficacy and durability. We have developed CRISPR screening platforms to interrogate perturbations in a high-throughput manner. Previous screens using stable lentiviral expression of CRISPR activators and repressors have focused on identifying genes which modulate IL7R expression in primary human T cells, as IL7R is an important marker of a more stem-cell like memory phenotype. Here, we designed CRISPR epigenetic editors as messenger RNA (mRNA) and delivered the mRNA to healthy T cells to screen for transcription factors which can improve T cell phenotype after transient perturbation. mRNA-based delivery may offer a better safety profile for clinical translation due to the lack of genomic integration and transient expression of epigenome editing machinery. We validated our mRNA constructs against IL7R and saw a decrease in expression for 7 days using ZIM3-KRAB-dCas9 (CRISPR inhibition) and an increase in expression for 3 days using 2xVP64-dCas9 (CRISPR activation). We performed screens using gRNAs against all human transcription factors and delivered either CRISPRi or CRISPRa as mRNA, and sorted cells based on IL7R expression at both 2 and 10 days post-delivery. Sequencing of cells which had low or high IL7R expression revealed many significant transcription factor hits on day 2, but this effect was lost by day 10. Future work will investigate using CRISPR constructs which aim to cause a permanent epigenetic change using targeted DNA methylation or demethylation. These constructs, delivered as mRNA, may be more robust for generating stable, durable changes to cell phenotype without genomic integration or continuous transgene expression – and improve the phenotype of cells used in adoptive T cell therapies.

1113 Programmable Epigenome Editing by Transient Delivery of CRISPR Epigenome Editor Ribonucleoproteins

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Programmable epigenome editors offer a powerful approach to precisely modulate gene expression by altering the local chromatin environment at target loci without introducing DNA breaks. These tools hold great promise for advancing both fundamental research and therapeutic applications. However, their large size poses a challenge for delivery, limiting their broad use in gene and cell therapy applications. To address this limitation, we developed Robust ENveloped Delivery of Epigenome-editor Ribonucleoproteins (RENDER), a platform for delivering CRISPR-based epigenetic repressors (CRISPRi, DNMT3A-3L-dCas9, CRISPRoff) and activators (TET1-dCas9) as ribonucleoprotein complexes into human cells.

Through rational engineering, RENDER achieves durable epigenetic silencing of endogenous genes in a wide range of human cell types. Importantly, our results show that RENDER enables target gene silencing in primary human T cells with significantly lower cytotoxicity compared to conventional mRNA electroporation methods, highlighting its suitability for therapeutic applications. Furthermore, we demonstrate the therapeutic potential of RENDER by reducing the level of neurodegeneration-associated V337M-mutated Tau protein in human stem cell-derived neurons. Our findings establish RENDER as a transformative platform for precise and robust modulation of gene expression without altering DNA sequences. By overcoming key delivery challenges, RENDER broadens the accessibility of CRISPR-based epigenome editing technologies, paving the way for their use in fundamental research, immunotherapy, and the development of novel gene and cell therapies.

1114 Programmable RNA Editing Enables Precise Protein Modulation in the Central Nervous System

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Therapeutic RNA editing enables programmable, genomically-scarless genetic manipulation to treat complex human disease. To realize the full therapeutic potential, precise nucleotide-level manipulation must be combined with targeted delivery to reach organs beyond the liver. ShapeTX's RNAfix® platform uses generative machine learning to design efficient and precise gRNAs that recruit Endogenous Adenosine Deaminase Acting on RNA (ADAR) to virtually any target sequence, resulting in functional A-to-G base substitutions. RNAfix gRNAs are delivered via AAV for sustained, high-level expression that maximizes the durability and therapeutic impact of a single administration and harnesses the tissue targeting potential of AAV. ADAR is highly active in the central nervous system (CNS) and thus, can be harnessed to treat a wide range of neurological diseases due to the diversity of ways that editing transcripts can modulate protein expression and function. Here we present preclinical data highlighting the potential of RNAfix to both restore protein function and reduce pathogenic proteins implicated in neurological diseases. We also demonstrate robust RNA editing activity in the CNS of both mice and non-human primates (NHPs).

Rett syndrome is a devastating neurodevelopmental disease caused by loss-of-function mutations of the X-linked *MECP2* gene, with over 30 percent of causal mutations being nonsense mutations that generate premature termination codons (PTCs). In heterozygous female Rett patients, ADAR-mediated editing could restore functional MECP2 protein by converting the PTC into a tryptophan and allowing full-length protein translation. Using RNAfix gRNAs, we demonstrate mutant allele-specific editing of *MECP2* in human Rett patient iPSC derived neurons of up to 43% and associated full-length MECP2 protein restoration in 38% of cells. MECP2 gRNAs delivered via a single systemic injection of AAV achieve broad distribution throughout the mouse brain (average of 66% gRNA-positive cells) and mutant allele-specific editing of 52-69%, restoring full-length protein in an average of 39% of cells.

Beyond genetic diseases, RNA editing can regulate protein levels in patients with more diverse and complex etiology by targeting transcripts encoding pathogenic proteins. For example, we applied RNAfix to knockdown two genes implicated in serious neurodegenerative diseases. By designing gRNAs targeting a canonical translation

initiation site (**A**UG>**G**UG) or a splicing signal, we found that target editing led to dose-dependent protein knockdown by both mechanisms. We achieved >50% editing/splicing and >70% protein knockdown in iPSC-derived glutamatergic neurons and >30% editing/splicing and >50% protein knockdown in the mouse brain following a single systemic AAV dose.

Delivery is critical to maximize translatability from rodent to NHP and ultimately, to human patients. We previously demonstrated high efficiency editing in the NHP brain using direct intraparenchymal delivery of gRNAs packaged in AAV9. Building on this data, we developed novel brain-penetrating capsids and achieved up to 88% RNA editing in focal brain regions following systemic delivery. Collectively, the data presented here demonstrate the broad applicability and enormous therapeutic potential of the RNAfix platform for CNS diseases.

1115 Direct Reprogramming of Primary Human Astrocytes to Functional Neurons with CRISPR-Based Epigenome Editing

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Neurodegenerative disorders and brain injuries impact millions and are steadily increasing in prevalence. Thus, there is a dire need for regenerative therapies able to restore the neuronal loss resulting from these conditions. Direct reprogramming of endogenous astrocytes into neurons has emerged as a promising approach for replenishing these lost neurons. However, current approaches are limited in efficacy and efficiency, preventing clinical utility. Here, we establish a CRISPR activation (CRISPRa)-based epigenome engineering strategy to reprogram primary human astrocytes to neurons and conduct high-throughput CRISPRa screens of all human genes encoding transcription factors (TFs) to identify novel and efficient astrocyte-to-neuron reprogramming factors.

To establish a CRISPRa-based epigenome editing system for astrocyte-to-neuron reprogramming, we first activated known neurogenic TFs ASCL1, NeuroG2, and NeuroD1 in primary human astrocytes. These perturbations resulted in widespread transcriptome remodeling and outperformed cDNA overexpression of the same factors in a direct comparison, leading to higher expression of downstream neuron marker genes. However, reprogramming efficiency (percent of cells converted) remained limited. Therefore, we next devised a strategy for pooled CRISPRa screening in primary cells without requiring cell-line engineering and utilized it to functionally interrogate the reprogramming capability of all TF genes in the human genome in astrocytes from multiple donors. In total, we screened 1,627 TF genes and identified >60 novel hit TFs, with top hits performing vastly more efficiently than previously established reprogramming factors (58% vs. 5%). To map the fidelity and determine the subtype of induced neurons resulting from activation of each TF, we next performed a single cell RNA-sequencing CRISPR (perturb-seq) screen and mapped the resulting transcriptomes to multiple atlases of the human neurodevelopment. This screen revealed that single novel factors reprogram primary human astrocytes into multiple neuronal subtypes with distinct cell type-specific gene signatures. We functionally characterized the electrophysiology of the resulting neurons using multi-electrode-arrays and found that they display subtype-specific firing patterns. Further, we investigated the robustness of hit TFs, demonstrating that they successfully reprogram astrocytes to neurons via orthogonal systems, including CRISPRa and ORF overexpression, and across multiple contexts, including both human and mouse primary astrocytes as well as directed differentiation of iPSCs to neuron. Finally, we conducted combinatorial CRISPRa screens to identify co-factors that cooperate to enhance reprogramming efficiency and neuronal subtype-specificity. ATAC- and RNA-sequencing of induced neurons generated by identified TF combinations elucidated modes of neurogenic TF interaction and resulted in chromatin remodeling and increased expression of key synaptic genes. In sum, here we demonstrate a modular platform for primary cell reprogramming screens and apply it to discovery of factors capable of reprogramming primary astrocytes to neurons, widely expanding the list of potential targets for neuroregenerative therapies.

1116 In silico prediction of domain insertion sites for the engineering of inducible CRISPR gene editors

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Domain insertion engineering is a powerful approach to juxtapose otherwise separate biological functions and is frequently used to engineer switchable proteins including inducible CRISPR effectors. For instance, insertion of a photoreceptor or ligand-binding domain (LBD) into a CRISPR-Cas nuclease can yield gene editors whose activity is regulated by light or small molecules. In these cases, a trigger-mediated conformational change of the receptor domain is transmitted to the acceptor protein, thereby activating or deactivating it. However, identifying suitable allosteric sites for domain insertion typically requires extensive screening and optimization, explaining the small number of potently switchable Cas variants. We present ProDomino, a novel machine learning pipeline to rationalize domain recombination and showcase its application to the efficient engineering of potent inducible CRISPR genome editors and modulators.

Our approach builds on a novel curated protein dataset based on naturally occurring intradomain insertion events fetched from public protein databases, exceeding the size and complexity of existing experimentally derived datasets by orders of magnitude. Using this diverse dataset, we leveraged ESM-2-derived protein sequence representations and a novel masking strategy to train ProDomino (protein domain insertion optimizer), a powerful model for accurately predicting domain insertion sites in proteins (Fig. 1a-b). We extensively validated ProDomino *in silico* and *in vitro* as showcased by a comparison with experimental insertion tolerance data for the protein AraC (Fig. 1c). As expected, the model correctly distinguished insertion-permissive from non-permissive sites as indicated by an AUROC of 0.84. To test whether ProDomino would facilitate low-N engineering of switchable genome editors, we predicted the domain insertion tolerance for all amino acid positions of the widely employed *S. pyogenes* Cas9 and *M. bovoculi* Cas12a orthologs (Fig. 2a, c). Next, we inserted the blue light-responsive *As*LOV2 photoreceptor domain at four high-ranking positions of a dCas9-VPR transactivator fusion and tested the resulting hybrids in a luciferase CRISPR activation assay in HEK293T cells (Fig. 2b). Consistent with our predictions, all four dCas9-LOV2 hybrids were still active in the dark and strongly induced luciferase expression. Excitingly, three of the four variants were highly photosensitive, resulting in strongly decreased luciferase activity when incubated under blue light (Fig. 4c).

Finally, we sought to apply ProDomino to engineer Cas12a variant responsive to a clinically relevant drug via insertion of a glucocorticoid receptor 2 (GR2) LBD. We introduced the receptor at two promising ProDomino-inferred insertion sites, K487 and N1153, followed by experimental validation of the resulting protein hybrids by targeting several endogenous loci in HEK293T. Remarkably, we observed potent, cortisol-induced gene editing for both hybrid proteins in the presence of the ligand, while editing efficiencies in the absence of cortisol were close to the detection limit (Fig. 2d). Our Cas12a receptor hybrids represent the first single-chain switches of a Cas12a ortholog and their robust response to cortisol was achieved without any further optimization. Taken together, we believe that ProDomino will enable scientists to more effectively engineer allosteric proteins including novel on-demand inducible CRISPR genome editors with tailored function

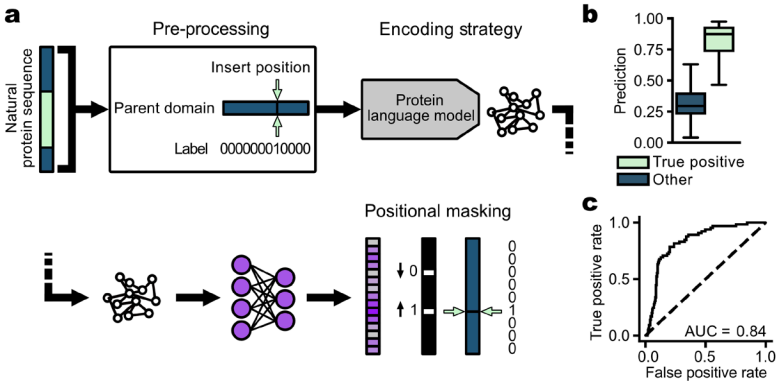

Figure 1: A machine learning model to infer domain insertion sites in proteins. **a**, Schematic of the machine learning pipeline for protein insertion site prediction. **b**, Boxplots showing prediction scores for true insertion site (green) and negative labels (no insertion present in natural proteins; blue) on a test set. **c**, Receiver operating characteristic of the insertion site prediction for AraC for which experimentally validated ground truth data was available.

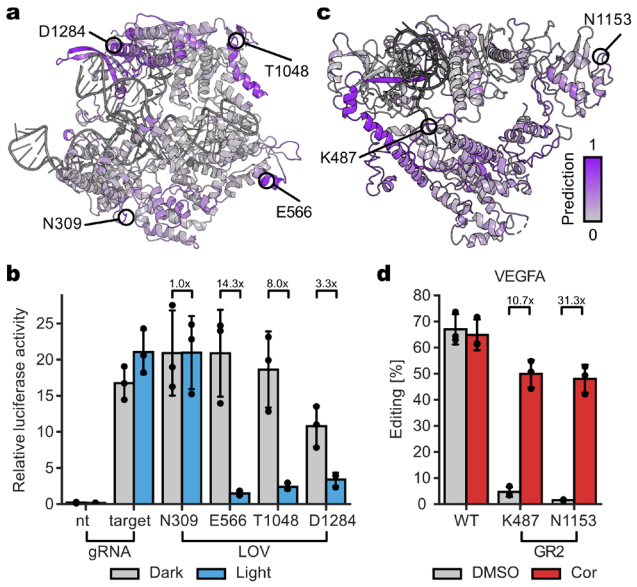

Figure 2: ProDomino confidently predicts potent opto- and chemogenetic Cas9 and Cas12a variants. **a**, **c**, The structures of Cas9 (**a**) or Cas12a (**c**) are color-coded based on the insertion scores predicted by ProDomino. PDB IDs: 4UN3, 6IV6. **b**, HEK293T cells were transfected with vectors encoding (i) the indicated Cas9/VPR-LOV hybrid variant (or Cas9/VPR as control), (ii) a TetO-targeting sgRNA together and a *Renilla* luciferase and (iii) a firefly luciferase preceded by multiple TetO repeats. Samples were incubated under blue light or in the dark for 48 h, followed by luciferase assay. **d**, HEK293T cells were transfected with vectors encoding (i) the indicated Cas12a-GR2 hybrid variant (or wild-type Cas12a as control), and (ii) a gRNA targeting the endogenous VEGFA locus. Samples were treated with cortisol or DMSO as indicated. 72 h post-transfection, InDel frequencies were assessed by NGS. **b**, **d**, Bars indicate means, error bars the standard deviation, and black dots individual data points from n=3 independent experiments. Cor, cortisol.

1117 Phenotypic Correction of Ornithine Transcarbamylase Deficiency by Knock-in Genome Editing with Single Adeno-associated Virus Vector in Mice

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Background: Ornithine transcarbamylase (OTC) deficiency is the X-linked hereditary urea cycle disorder, often presenting with severe neonatal phenotypes and hyperammonemia. Genome editing to insert the target gene offers potential long-term solutions. However, the size limitation of adeno-associated virus (AAV) vectors makes it challenging to simultaneously package both a donor sequence and a genome-editing tool. To address these limitations, we previously developed an engineered AsCas12f (enAsCas12f), enabling all necessary knock-in components to fit into a single vector (Cell 2023; 186: 4920-4935).

Aim: To develop a gene knock-in genome editing strategy using a single AAV vector for OTC deficiency.

Methods: We constructed an AAV8 vector harboring enAsCas12f, a guide RNA, its target sequence, and a segment of the murine albumin (Alb) intron conjugated with a 2A-fused cDNA. The vector was administrated into mice. Genome editing efficiency was assessed by immunohistochemistry or next-generation sequencing. Phenotypic correction was evaluated in male $Otc^{spf\text{-}ash}$ (low expression) mice on a high-protein diet, and in heterozygous female OTC-knockout mice generated by genome editing. Body weight and blood ammonia levels were monitored following vector injection.

Results: Compared with homology-directed repair (HDR) targeting Alb intron 14, non-homologous end joining (NHEJ)-mediated knock-in targeting Alb intron 13 significantly enhanced the knock-in efficiency in mice treated with a single AAV vector harboring enAsCas12f and the donor sequence (HDR: 2.4 ± 0.4% in neonates, <1% in adult mice; NHEJ: 5.8 ± 0.6% in neonates, 29.6 ± 3.4% in adult mice). Administration of a single AAV vector harboring enAsCas12f and OTC cDNA in adult $Otc^{spf\text{-}ash}$ mice significantly prevented body weight loss and hyperammonemia induced by a high-protein diet. Enzymatic activity, mRNA expression, and protein expression of OTC in liver tissue increased in a dose-dependent manner. Female heterozygous OTC-knockout mice showed growth retardation and mild hyperammonemia even on a normal diet. *In vivo* genome editing using a single AAV vector harboring enAsCas12f and OTC cDNA improved body weight and reduced blood ammonia levels.

Conclusion: A single AAV vector harboring enAsCas12f with the donor sequence successfully inserted the target sequence in liver cells *in vivo* and restored the phenotype of OTC deficiency in mice.

1118 A novel and empowered TARGETED Gene Addition Approach at a Relevant Microglia Locus for the Treatment of X-linked Adrenoleukodystrophy – Mechanistic Insights

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Adrenoleukodystrophy (X-ALD, OMIM: 300100) is an X-linked genetic disorder caused by mutations in the ABCD1 gene, which disrupt peroxisomal beta-oxidation and result in the accumulation of saturated very long-chain fatty acids (VLCFAs) in body tissues. This pathological accumulation primarily affects the Central Nervous System (CNS). The pediatric form, Childhood Cerebral ALD, remains a major cause of severe neuropathology and early mortality during infancy. While allogeneic Hematopoietic Stem and Progenitor Cell (HSPC) transplantation and lentiviral gene therapy (GT) are valuable therapeutic options for childhood cerebral X-ALD, their effectiveness is constrained by the slow engraftment of transplanted cells in the CNS, which is outpaced by the rapid progression of neurodegeneration. Moreover, side effects are associated to the use of either a specific lentiviral design in gene therapy, with recent FDA reports identifying cases of hematological malignancies likely associated with clonal expansion events following vector integration, or of an allogeneic stem cell source, i.e. graft-versus-host disease. We have identified CX3CR1, a chemokine receptor expressed on microglia that binds fractalkine (CX3CL1), as a key

target for enhancing the ability of hematopoietic stem and progenitor cells (HSPCs) to generate microglia-like cells (MLCs) following transplantation. CX3CR1 plays a critical role in regulating microglial recruitment to sites of neuroinflammation and microglial ontogeny. Our studies demonstrated that transplantation of CX3CR1-haploinsufficient HSPCs leads to faster and more robust engraftment, improved generation of MLCs, and more efficient acquisition of a mature microglia-like phenotype compared to wild-type cells. Building on these findings, we developed a CRISPR/Cas9 gene editing strategy to precisely insert the human ABCD1 cDNA at the CX3CR1 locus. This approach simultaneously creates haploinsufficiency at the CX3CR1 locus and drives therapeutic cassette expression under its control. By leveraging this strategy, we aim to achieve accelerated microglia replacement by gene-corrected cells while ensuring microglia-specific, regulated expression of the therapeutic gene. Additionally, this method mitigates safety concerns associated with the use of integrating vectors. We developed a promoterless splice-trapping cassette encoding hABCD1 cDNA, targeting the CX3CR1 intron to achieve highly efficient targeted insertion and regulated transgene expression in microglial cell lines and human HSPCs. Our results demonstrated successful transgene integration in up to 60% of targeted alleles in human HSPCs. Furthermore, we observed robust, CX3CR1-promoter-regulated hABCD1 expression in HSPC progeny cells upon myeloid differentiation, alongside the establishment of CX3CR1 haploinsufficiency, which retained a functional copy of the gene. We are currently assessing the efficacy of CX3CR1-edited human HSPCs in relevant immunodeficient models to generate proof-of-concept data for correcting disease-associated biochemical abnormalities, specifically using the NSG-SGM3 ABCD1-KO (Δ897) disease model, as well as testing protocols compatible with large scale manipulation. Moreover, to investigate the hyper-functional phenotype of the HSPCs edited at the CX3CR1 locus, we are generating CX3CR1 mutant iPSC lines (WT, haploinsufficient, KO). As single-cell RNA sequencing (scRNA-seq) data from our lab suggest migratory pathways as key drivers of this

1119 Optimizing gene editing for erythroid-specific therapeutic protein expression using HSPCs

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We previously described an *ex vivo* editing approach to achieve efficient gene targeting in human hematopoietic stem/progenitor cells (HSPCs) and robust expression of clinically relevant proteins in the erythroid lineage. Using CRISPR-Cas9, we integrated various transgenes under the transcriptional control of the endogenous α-globin promoter, recapitulating its high erythroid-specific expression. Erythroblasts derived from targeted HSPCs secreted therapeutic proteins that retained enzymatic activity and cross-corrected patient cells.

To validate the erythroid-specific expression platform in vivo, we obtained a humanized mouse model, where the mouse α-globin locus was replaced with the human α-globin locus (~120 kb), and we optimised editing of murine HSPCs. First, we confirmed that the sequence of the human HBA locus was correct, using nanopore DNA sequencing, and functional, confirming human alpha globin expression by HPLC analysis. We then nucleofected murine HSPCs with CRISPR-Cas9 and human gRNA (RNP) and confirmed the efficient generation of indels at the expected target site.

To perform DNA-targeted integration, we combined RNP nucleofection with the transduction of an AAV6 coding for a promoterless ΔNGFR reporter gene, expressed by the endogenous HBA promoter, and a PGK.GFP cassette flanked by homology arms to the genomic locus. Combination of RNP and AAV6 resulted in good DNA integration and transgene expression, but it was associated with some cellular toxicity. Therefore, we first compared different RNP nucleofection protocols in terms of indel efficiency and cellular toxicity. Then, we tested the addition of a caspase inhibitor, Z-VAD, and observed a 1.5-fold increase in targeted integration. We are now exploring Cas9/gRNA delivery using different lipid nanoparticles (LNPs).

Concerning donor DNA delivery, we screened a library of AAVs with or without polyvinyl alcohol (PVA) as enhancer of transduction, including AAV6 mutants previously shown to enhance transduction of human HSPCs, and

identified one mutant that outperformed natural AAV6 of about 3- fold. To improve DNA integration and reduce toxicity, we are currently comparing AAV with novel integration-deficient lentiviral vectors (IDLVs) and naked DNA delivery methods. In addition, we demonstrated that chemical modulation of cellular DNA repair resulted in a 1.5-fold improvement in DNA integration.

Finally, we aim to perform targeted integration of a therapeutic gene in HSPCs and transplant edited cells into a $Lal^{-/-}$ mouse model that recapitulates the severe lipid accumulation seen in lysosomal acid lipase (LAL) deficiency. This will allow us to evaluate the therapeutic benefit of erythroid-derived LAL transgene expression. Currently, we are evaluating different conditioning regimens, including busulfan and irradiation, to improve the engraftment of edited HSPCs in recipient mice. This analysis will include evaluating mouse survival, body weight, and normalization of lipid accumulation in liver and spleen.

Overall, we have optimised DNA targeted integration in murine HSPCs in terms of efficacy and toxicity of CRISPR/Cas9 editing and DNA template delivery to be able to perform the in vivo experiment required to move our universal HSC-based gene therapy platform to the clinics, thus paving the way for in vivo testing of other HSC based treatments.

1120 In vivo therapeutic gene knock-in for hemophilia A and B using LNP and AAV

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Hemophilia treatment has advanced through therapeutic gene expression and rebalancing therapies, yet challenges such as immune responses, temporary efficacy, and inadequate control of acute bleeding remain. To address these limitations, we developed a combined strategy of rebalancing and therapeutic gene knock-in (KI) using lipid nanoparticles (LNPs) and adeno-associated viruses (AAVs) for hemophilia A and B. Targeting the anticoagulation pathway at the SerpinC1 locus, we achieved sustained downregulation of antithrombin (AT) and integration of human Factor 9 (hF9) and Factor 8 (hF8) coding sequences. In hemophilia B models, LNP-CRISPR and AAV-donor achieved 20% indel efficiency in SerpinC1, reducing AT levels by 67%, with hF9 KI restoring coagulation activity and producing hFIX at therapeutic levels for up to 63 weeks. Similarly, in hemophilia A models, low-dose AAV and 244-cis LNPs enabled safe and effective hF8 KI, improving coagulation and survival for 40 weeks without adverse effects. The proliferation of genetically edited hepatocytes ensured sustained therapeutic benefits, even following partial hepatectomy. These results highlight a promising, safe, and long-term genome editing approach for treating hemophilia and protein-deficient genetic disorders.

1121 Autologous MPO Knock-out HematopoieticStem and Progenitor Cells for Pulmonary Arterial Hypertension

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Pulmonary Arterial Hypertension (PAH) is a progressive condition for which there is no cure. Even with substantial pharmacologic advances in the modern treatment era, existing treatments provide only symptomatic relief and survival remains unacceptably poor. Patients who fail to respond to medications usually have the worst prognosis and patients with persistently elevated pulmonary pressure and right heart failure usually die within 5 years. PAH may be a severe complication of Systemic Sclerosis (SSc) affecting approximately 8-12% of patients. SSc affects 13.5–44.3 per 100,000 individuals and is complex and diverse, with limited treatment options. SSc-associated PAH is the second largest group of patients with PAH. Once diagnosed, PAH becomes the leading cause of mortality among this patient population. Although the exact cause of PAH is not known, there is extensive evidence for a pathogenic role for neutrophil myeloperoxidase (MPO) in PAH disease. PAH patients with higher levels of MPO have a poorer outcome. Studies in murine models of PAH demonstrate significant disease amelioration by

elimination of neutrophil MPO by hematopoietic stem and progenitor cells (HSPC) transplantation with HSPC lacking *MPO* from germline *MPO* gene knock-out.

Based on this evidence, we developed a gene therapy approach to PAH in which HSPC undergo gene editing using CRISPR/Cas9 to achieve bi-allelic disruption of the *MPO* gene followed by auto-transplantation. Autologous transplantation has been shown to limit SSc progression by interfering with inflammation. Transplantation of autologous HSPC in which the *MPO* gene is disrupted is expected to further prevent progression of PAH, due to the essential role of neutrophil MPO activity in disease development. We identified a sgRNA that leads to high frequency disruption of *MPO* in human CD34+ HSPC, leading to efficient elimination of MPO protein in the resultant neutrophils. That sgRNA was assessed for genome-wide off-target and showed no detectable off-target sites, validating it as a lead candidate for safe gene editing. In an *in vitro* neutrophil differentiation model, neutrophils produced from *MPO* KO CD34+ HSPC exhibited reduced neutrophil extracellular trap formation (NETosis) and absence of MPO protein. Outside of the MPO-related function, the differentiated neutrophils retained normal functions such as phagocytosis and degranulation. A clinically-compatible HSPC gene editing protocol was optimized and showed consistent ≥80% MPO allelic disruption. The edited cells retained hematopoietic potential and showed no skewing in the generated lineages in both *in vitro* colony-forming assay and *in vivo* humanized mouse model. We developed a surrogate gene editing protocol for murine cells and optimized electroporation to achieve high allelic disruption of the murine *Mpo* gene in lineage-negative murine HSPC. In a murine model, transplant of these cells showed reduction of MPO plasma level over the control group. An *Mpo* KO rat line was generated to confirm the results in a second *in vivo* model. Both models are currently being tested for the effect of MPO reduction on SSc-PAH.

The limited therapeutic options and severity of PAH, and specifically SSc-PAH compels developing new approaches. Based on clinical evidence implicating MPO in the pathogenesis of PAH, the gene therapy protocol developed shows promising results. This approach would help to understand the development of PAH but also bring a new therapeutic option.

1122 Highly Efficient Gene-editing with SaCas9 and AsCas12a in Patient Hematopoietic Stem and Progenitor Cells for Treating Sickle Cell Disease

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Introduction: Sickle cell disease (SCD) is caused by a single nucleotide mutation (A>T) in β-globin gene (*HBB*). CRISPR/Cas9 mediated gene-editing has emerged as a promising approach to cure SCD by either correcting the sickle mutation or reactivating fetal hemoglobin (HbF) in hematopoietic stem and progenitor cells (HSPCs). Most advances in gene-editing therapies including the FDA-approved Casgevy have utilized *S. pyogenes* Cas9 (SpCas9). However, for *in vivo* gene editing therapy of SCD, it is important to evaluate smaller Cas orthologs, such as *S. aureus* Cas9 (SaCas9) and *Acidaminococcus sp*. Cas12a (AsCas12a). We investigated the editing efficiency of SaCas9 and AsCas12a in targeting three critical loci for SCD therapy, *HBB*, the *HBG* promoter, and the *BCL11A* erythroid enhancer, and compared their performance to SpCas9.

Methods: HSPCs derived from peripheral blood of five SCD donors were gene-edited. For correcting the sickle mutation, SpCas9 or SaCas9 ribonucleoprotein (RNP) complexes with single guide RNA (sgRNA) and single-stranded oligodeoxynucleotide (ssODN) donor templates were delivered via electroporation. To reactivate HbF, RNPs of SpCas9 and AsCas12a were used to disrupt the *HBG* promoter, while RNPs of all three orthologs were used to target the *BCL11A* erythroid enhancer. Editing efficiency was measured using next-generation sequencing (NGS) and edited HSPCs were differentiated into erythroid cells over 14 days. The percentage of F-cells (cells expressing HbF) was analyzed by intracellular staining and flow cytometry, while HbS, HbA, and HbF levels were quantified by high-performance liquid chromatography (HPLC). Unintended editing outcomes, including off-target editing, large deletion, and chromosomal rearrangements, were evaluated using GUIDE-seq, ddPCR, LongAmp-seq, and CAST-seq, respectively.

Results: We found that SaCas9 showed a higher rate of sickle mutation correction (53%) than SpCas9 (49%), resulting in increased adult hemoglobin (HbA) levels (81% vs. 75%) and fewer chromosomal rearrangements (5 vs. 11 sites detected by CAST-seq). At the *HBG* promoter loci, AsCas12a outperformed SpCas9 in cutting efficiency (37% vs. 34% at *HBG1* and 60% vs. 44% at *HBG2*), and generated more F-cells (80% vs. 72%) and higher HbF levels (69% vs. 59%) than SpCas9. However, it induced a higher frequency of 4.9 kb intergenic deletions (54% vs. 25%) measured by ddPCR. Neither SpCas9 nor AsCas12a showed detectable chromosomal rearrangements by CAST-seq. At the *BCL11A* erythroid enhancer loci, SaCas9 had the highest editing rate (92%), followed by AsCas12a (84%) and SpCas9 (79%). F-cell percentages were similar across the orthologs (51% for SpCas9, 50% for SaCas9, and 53% for AsCas12a), with HbF levels being comparable (47% for SpCas9, 44% for SaCas9, and 50% for AsCas12a). SaCas9 showed no rearrangements by CAST-seq, whereas SpCas9 and AsCas12a showed rearrangements at three and five sites, respectively.

Discussions: This study demonstrates that SaCas9 and AsCas12a offer high efficiency and reduced off-target effects compared to SpCas9 when editing *HBB*, *HBG*, and *BCL11A* erythroid enhancer for treating SCD, and their smaller size provides a distinct advantage for *in vivo* delivery using adeno-associated virus (AAV) or lipid nanoparticle (LNP) vectors. These findings highlight the therapeutic potential of SaCas9 and AsCas12a as viable alternatives to SpCas9 for the development of efficient and safe gene-editing therapies for SCD.

1123 *In Vivo* CRISPR Editing of Genetic Regulatory Regions Results in Functional Upregulation of Target Protein and Meaningful Reduction of Disease-Associated Biomarker in Mice

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CRISPR-Cas is a transformative gene editing technology with the potential to enable durable genetic medicines for diseases with severe unmet needs. CRISPR-Cas-based gene editing can be used to knock down expression of disease-causing proteins. However, knockdown can also be achieved with established modalities, such as RNA interference. Editas' differentiated strategy aims to edit regulatory regions of genes to induce functional upregulation of disease-mitigating target proteins. Our strategy for this specific target is genetically validated in humans by a naturally occurring variant of the gene that results in upregulation of the target protein expression and reduction of a disease-causing pathway biomarker.

Here, we demonstrate proof of concept for upregulation of a liver target using *in vivo* CRISPR editing with both AsCas12a and SpCas9 nucleases. *In vitro* guide RNA (gRNA) screens were conducted in primary human hepatocytes to delineate critical regulatory regions and demonstrated up to 9-fold upregulation of target protein expression. Proof of upregulation *in vivo* was achieved by lipid nanoparticle (LNP) delivery of RNA-encoded gene editing cargo in mice, which resulted in up to 3.5-fold target protein upregulation in the liver and clinically relevant reduction (up to 56%) of a disease-associated biomarker.

Our data provide proof of concept for *in vivo* gene editing–based upregulation therapeutic strategies resulting in meaningful reduction of a disease biomarker. These results support advancing this approach to non-human primates for further validation.

1124 Targeting MSH3 for Triplet Repeat Diseases Treatment using CasPlus

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Huntington's disease (HD) and myotonic dystrophy type 1 (DM1) are progressive disorders caused by DNA triplet repeat expansions in the *HTT* and *DMPK* genes, respectively. Human genome-wide association studies have identified MSH3, a DNA mismatch repair gene, as a modifier of disease onset/severity in both HD and DM1. Moreover, studies in preclinical disease models have established that MSH3 is a driver of somatic triplet repeat expansion, which in turn is increasingly regarded as underlying disease pathology. Therefore, modulation of MSH3 function has been drawing attention as an attractive therapeutic strategy for treatment of multiple neurodegenerative diseases, and indeed several approaches are currently under evaluation. These include gene therapy approaches, although a key bottleneck remains the ability to deliver such therapeutic agents to the CNS.

At Script Biosciences, we are developing a one-and-done therapeutic agent that combines the CNS tropism of our proprietary lipid nanoparticle delivery platform with our unique CasPlus gene-editing technology to knock out MSH3 in the brain. Herein, we show that CasPlus enables high knockout efficiency of MSH3 (70 - 90%) in HEK293 cells, and unlike Cas9-based approaches, CasPlus displayed virtually no on-target deletions flanking the editing site. We also show that CasPlus knockout of MSH3 in HEK293 results in robust attenuation of MSH3 protein levels as judged by immunoblot assay (Fig. 1A), in-house developed Meso Scale Diagnostics (MSD) assay (Fig. 1B), and by immunofluorescence microscopy (Fig. 1C). Likewise, we have also been able to achieve ~70% knockout of MSH3 in mouse NIH3T3 cells.

In order to deliver MSH3-targeting CasPlus components to the brain, we have used Script's proprietary CNS-specific lipid nanoparticle (LNP) platform that has been validated for delivery of reporter mRNAs and CRISPR components directly to the mammalian CNS, with minimal accumulation in the liver, spleen or other peripheral organs. With these CNS-tropic LNPs, we have succeeded delivering Cas-Plus targeting hMSH3 to HEK293 cells, achieving >60% MSH3 knockout at the genomic level, paving the way for validation of LNP-mediated delivery of MSH3-targeted CasPlus in HD and DM1 pre-clinical mouse models. Script's innovative Cas-Plus platform and CNS-specific LNP technology offer a promising strategy to address unmet medical needs in triplet repeat diseases like HD and DM1 that could complement therapeutic currently developed by other biopharmaceutical companies.

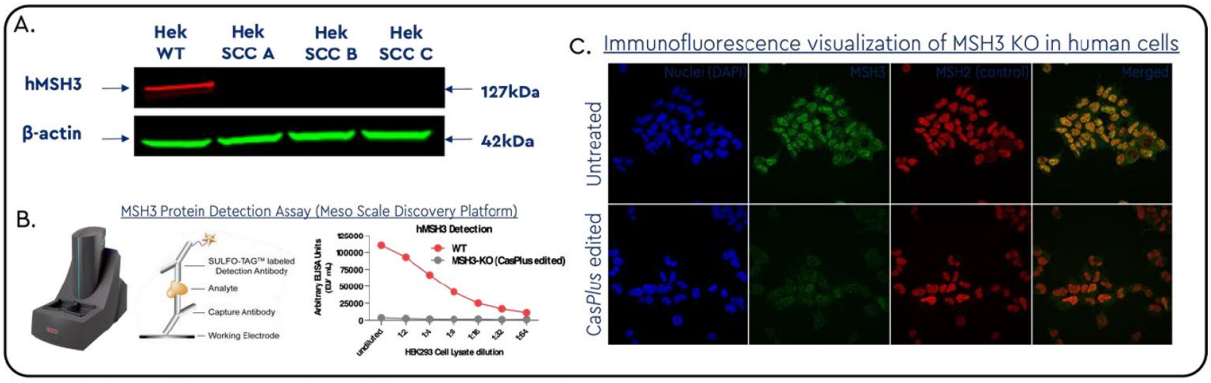

Figure 1 : Assay development for hMSH3 protein expression assessment

1125 Cas12a CRISPR Hybrid RNA-DNA (chRDNA)-Mediated *In Vivo* Genome-Editing Technology Elicits Functional Hepatic Gene Disruption with Long-term Durability

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Familial hypercholesterolemia (FH) is an autosomal dominant inherited disease characterized by high circulating low-density lipoprotein (LDL) cholesterol. Affected patients quickly develop atherosclerosis, leading to peripheral and coronary artery disease often followed by heart attack, stroke, or other cardiovascular complications. Transthyretin amyloidosis (ATTR) is a progressive life-threatening disease characterized by the accumulation of misfolded transthyretin (TTR) protein in nerves and heart tissue leading to neurodegeneration and cardiomyopathy. While significant progress has been made to demonstrate the utility of Class 2 Type II Cas9 for *in vivo* genetic intervention, the *in vivo* potential of Class 2 Type V Cas12a has not fully been realized. In these proof-of-concept studies, three clinically validated gene targets for FH and ATTR, proprotein convertase subtilisin/kexin type 9 (*Pcsk9*), angiopoietin-like 3 (*Angptl3*), and transthyretin (*Ttr*), were targeted using Cas12a chRDNA genome-editing technology. It has been previously shown that CRISPR hybrid RNA-DNA guides (chRDNAs), designed with a combination of RNA and DNA nucleotides, can be leveraged with either Cas9 or Cas12a to enhance nuclease specificity and significantly reduce off-target genome editing. Upon identification of high-activity gene targeting Cas12a chRDNA guides, optimization of the Cas12a mRNA sequence, and refinement of our lipid nanoparticle (LNP) formulation, we sought to demonstrate functional gene disruptions in wild-type Balb/c mice. A single intravenous dose of Cas12a mRNA and chRDNA guides optimized to target *Pcsk9* resulted in nearly 60% hepatic editing, followed by a 50% reduction in circulating PCSK9 protein and a 30% reduction of plasma cholesterol in wild-type mice. Similarly, targeting *Angptl3*, we demonstrated >70% hepatic editing efficiency followed by a >70% reduction of plasma ANGPTL3 protein, accompanied by a 30% reduction in plasma cholesterol. Surprisingly, dual knockout of *Pcsk9* and *Angptl3* did not further reduce circulating LDL cholesterol compared to single gene disruptions. Lastly, we observed 70% gene disruption targeting *Ttr* in liver with a single administration, leading to a 98% reduction in circulating TTR protein levels. Notably, gene editing of *Ttr* demonstrated long-term durability, with effects persisting over one year following single low-dose administration. With all three targets, we did not detect significant liver toxicity or chronic cytokine release. Moreover, analysis of *in silico* predicted off-target sites by nextgeneration sequencing (NGS) did not identify off-target genome editing. In addition, no significant peripheral organ on-target genome editing was detected following tissue isolation and NGS analysis in mice. Early preclinical rat tolerability studies suggest that the chRDNA guides may offer improved tolerability compared to all-RNA guide designs, supporting their potential for clinical use. Furthermore, we observed highly efficient editing across multiple clinically relevant targets, highlighting the broad therapeutic potential of this platform. Our studies demonstrate that LNP delivery of Class 2 Type V Cas12a mRNA in conjunction with an optimized chRDNA guide is a powerful tool for efficient and functional genome editing in hepatocytes.

1126 Generation of a *de novo* intronic junction in the *DMD* gene through CRISPR/Cas genome editing as a potential therapy for a high proportion of Duchenne muscular dystrophy patients.

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Background: Duchenne Muscular Dystrophy (DMD) is caused by mutations across the *DMD* gene. The subsequent absence of dystrophin protein compromises muscle stability and contractility and gives rise to progressive muscle wasting. Different gene therapies are being investigated, such as AAV micro-dystrophin delivery, premature termination codon read-through, exon-skipping and utrophin upregulation. Nevertheless, these therapies require repeated administration, could carry an adverse immunological risk and some are restricted by mutation specificity. Such problems may be circumvented with genome editing.

Aims: We aim to create a *de novo* junction between introns 18 and 55 (deleting exons 19-55), using CRISPR/Cas, to express an internally truncated functional dystrophin from the endogenous *DMD* locus. The gRNAs are designed to target introns and produce a ~800 kbp deletion. It is estimated that this strategy would eliminate ~81% of total DMD mutations.

Methods/Materials: To produce the deletion of exons 19-55, gRNAs for *Staphylococcus aureus* (*Sa*)Cas9 were designed and screened individually *in-vitro* for cleavage efficacy using TIDE analysis. The optimal gRNAs for each intronic site in murine *Dmd* were multiplexed into an *Sa*Cas9 construct. Top multiplexed candidate gRNAs were

tested in Neuro2A and C2C12 cells to confirm edited deletion by PCR and Sanger Sequencing. Gene editing constructs were packaged into AAV9 vectors, locally injected into 2-months old *mdx* mice and functional efficacy assessed by muscle electrophysiology. As a positive control, a cDNA construct expressing D19-55 dystrophin was generated and tested in *mdx* mice for protein expression.

Results: The positive control plasmid significantly increased dystrophin positive fibres *in vivo* by plasmid intramuscular injection, indicating that D19-55 dystrophin is expressable and has the potential for beneficial effects when expressed in sufficient levels. Deletion of exons 19-55 was confirmed at DNA level *in vitro*. Multiplex construct and co-delivery of gRNAs packaged into AAV9 were assessed *in vivo* but no beneficial effects on muscle physiology was seen likely as a result of inability to detect a deletion in treated muscle samples using the techniques employed.

Conclusion: This study shows the development of a universal genome editing strategy from theory to *in-vivo* testing, exploring limitations of achieving a large deletion *in-vivo* and highlighting potential functionality of a new truncated dystrophin.

1127 Genome Editing with Fast Degrading Cas9 Restores Dystrophin Expression in mdx Mice

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Adeno-associated virus (AAV)-mediated CRISPR-Cas9 gene editing holds great promise in treating Duchenne Muscular Dystrophy (DMD). This technology can be adapted to precisely edit the DMD gene and restore dystrophin expression. However, previous studies have revealed that Cas9-specific immune responses can eliminate edited fibers, impeding successful treatment. Thus, there is an urgent need to address Cas9-specific immune responses for AAV CRISPR therapy. One such approach is through employing immune-evasive variants of Cas9. Fast degrading Cas9 (fdCas9) is a variant of SpCas9 that contains a pentapeptide motif that directs its degradation towards the lysosomal pathway. This motif, otherwise known as a KFERQ-like motif, is recognized by the HSC70 chaperone and trafficked to lysosome associated membrane protein 2A (LAMP2A) for chaperone-mediated autophagy (CMA). Importantly, proteins degraded under the CMA pathway produce peptide fragments that are loaded onto MHC class II complexes instead of MHC class I. By avoiding MHC-I, fdCas9 can remain less detectable by cytotoxic CD8+ T cells, which are largely responsible for the Cas9-specific immune response in AAV CRISPR therapy. Here, we assess the application of fdCas9 and its potential for DMD gene therapy by evaluating its characteristics *in vitro* and *in vivo*. First, we transfected HEK293 cells with plasmid constructs encoding for CK8.fdCas9 or CK8.SpCas9. RNA and protein extracts were harvested at 24h, 48h, and 72h. We detected ample RNA transcripts for both constructs, but low to no protein expression for fdCas9 compared to SpCas9. For the *in vivo* murine study, we utilized gRNAs that target exon 23 of the mdx mouse model as reported by Long et al. (Science 351:400-403, 2016). We packaged the mdx gRNA vector, CK8.fdCas9 vector, and CK8.SpCas9 vector in AAVMYO. As a pilot study, we co-injected 1×10^{11} vg/muscle/vector of the mdx gRNA vector with CK8.fdCas9 vector into the left and right tibialis anterior (TA) of mdx mice. Immunostaining revealed robust dystrophin restoration in injected tissues. Contrary to *in vitro* data, we also detected high fdCas9 protein expression in muscle injected with CK8.fdCas9 vector. We hypothesized that the amount of fdCas9 delivered may be too high in expression for basal CMA activity to efficiently degrade. Subsequently, we tested 4 different doses of CK8.fdCas9 vector: 1×10^{10}, 1×10^{9}, 1×10^{8}, and 1×10^{7} vg/muscle. We detected dystrophin restoration at 1×10^{10} and 1×10^{9} vg/muscle doses, but not at the lower doses. Futheremore, we detected some fdCas9 protein expression at the 1×10^{10} dose, but no fdCas9 protein expression at the 1×10^{9} dose. For a direct side-by-side comparison, we co-injected 1×10^{11} vg/muscle of the mdx gRNA vector with 5×10^{9} vg/muscle of CK8.fdCas9 vector or CK8.SpCas9 vector into the left and right TA of mdx mice. We are currently processing and evaluating the tissues collected from the study. We will report our findings at the annual conference (Supported by University of Missouri Life Science Fellowship and NIH grants NS131416 and AI177600).

1128 Sequencing analysis of *in vivo* editing of the DMD gene using homology-independent targeted integration

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Background: We developed a gene editing system using homology-independent targeted integration (HITI) to correct N-terminal mutations causing Duchenne muscular dystrophy. The system is based on insertion into the native intron 19 of a new promoter and superexon composed of the coding sequence of exons 1-19, effectively bypassing transcription from the 5' end of the gene. The system is delivered using a pair of adeno-associated virus (AAV) vectors. Cas9 is expressed from the first vector, then cleaves the genomic DNA and the second vector, which contains the donor construct. The donor construct is integrated into the genome through non-homologous end joining.

Methods: The editing system was evaluated *in vivo* in a mouse model of exon 2 duplication. Molecular analyses were performed in the heart, skeletal muscles, and liver at 1 month and 3 months post-treatment. Editing outcomes were evaluated by ddPCR and by targeted long-read sequencing. This was accomplished using a custom panel of oligonucleotides to capture integrated and episomal AAV vectors, followed by Nanopore sequencing.

Results: The highest therapeutic editing efficiency was seen in the heart, reaching 3%. In skeletal muscles, efficiency was lower and further declined between 1 and 3 months post-treatment. Breakpoints between the donor vector and genomic DNA at the target site primarily coincided with the expected cleavage sites on the vector, but integration occurred in either the forward or reverse orientation. Integration of Cas9 vectors was also observed, though at a lower frequency. Episomal concatenation occurred both between identical (Cas9-Cas9 or donor-donor) and different (Cas9-donor) vectors. Partial or complete loss of the inverted terminal repeats were common in both integrated and episomal vectors.

Conclusions: Therapeutic editing can be achieved using HITI with AAV-based delivery, but its reliance on non-homologous end joining can lead to unintended editing outcomes, including reverse integration and integration of the Cas9 vector.

1129 ARCUS-Mediated Gene Editing Excision of Exons 45-55 of the Human Dystrophin Gene using PBGENE-DMD Leads to Functional Dystrophin Protein and Durable Restoration of Skeletal Muscle-Function In Vivo for the Treatment of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a genetic disorder associated with mutations in the dystrophin gene that prevent expression of functional dystrophin protein in skeletal, smooth, and cardiac muscle tissues. Dystrophin protein is essential for stabilizing muscle cells by connecting cytoskeletal actin to the cell membrane-bound dystrophin-associated protein complex. The absence of functional dystrophin protein leads to inflammation and fibrosis, resulting in progressive loss of muscle function, and eventual death in the second or third decade of life due to worsening cardiomyopathy and respiratory insufficiency. DMD occurs in 1 in 3,500-5,000 male births, and current treatments, such as gene therapies which express a synthetic, miniature dystrophin protein missing many important dystrophin protein domains, have shown limited improvement to disease prognosis and have not met primary clinical endpoints. Further, all gene therapies have potentially limited durability, particularly in pediatric patients due to cell turnover, immunological responses, and silencing over time. By contrast, the permanence of a gene editing approach could allow for lifelong benefits of muscle retention and function. We have previously described an approach to restore native dystrophin function by utilizing two ARCUS nucleases, delivered using a single AAV, to excise exons 45-55 of the dystrophin gene, resulting in a natural form of the dystrophin protein

(del45-55 dystrophin) that is functionally competent and found in a subset of Becker Muscular Dystrophy patients who are often asymptomatic. Up to 60% of DMD patients exhibit pathogenic mutations in the 45-55 exon region, potentially allowing for broad applicability of this therapeutic approach. Here, we demonstrate functional improvement using PBGENE-DMD, clinical candidate ARCUS nucleases packaged together in a single AAV, in a humanized DMD mouse model. Following AAV delivery, we observed dystrophin protein in target tissue types such as heart and skeletal muscle. Importantly, in skeletal muscle the ARCUS-edited dystrophin transcript was observed in $PAX7^+$ cells, a marker for muscle satellite stem cells, supporting the potential for long-term durability. The maximum force output (MFO), a key functional readout, was significantly improved in vivo 3 months after ARCUS treatment. Additionally, PBGENE-DMD-treated DMD mice displayed significant resistance to eccentric injury, reaching 66% improvement compared to untreated DMD mice. Over the course of a full 6-month time interval, the disease progression of untreated DMD mice caused the MFO to drop from 72% to 58% of levels observed in healthy mice. In contrast, PBGENE-DMD-treated DMD mice sustained functional improvement over the 6 months, maintaining 88-93% of the MFO observed in healthy mice, demonstrating strong durability of the ARCUS gene editing approach. Moreover, between 3 and 6 months, we observed similar improvements in MFO between ARCUS-treated DMD mice and healthy mice, in stark contrast to continued disease progression in untreated DMD mice. This in vivo durability and efficacy study further supports the therapeutic potential of PBGENE-DMD for the treatment of DMD and ongoing development toward clinical trials.

1130 Targeted Integration of Large DNA Cassettes in the *B2M* locus of induced Pluripotent Stem Cells

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Targeted integration of large DNA fragments using CRISPR/Cas9 and other nucleases remains challenging in induced pluripotent stem cells (iPSCs). Serine site-specific recombinases like Bxb1 can integrate large DNA fragments in a specific locus when short sequences (*attB* and *attP* sites) are present in the genome and in the donor. This reaction is irreversible and unidirectional, making this ideal for stably integrating expression cassettes into the genome. Several safe harbor loci have been used to integrate and express transgenes in iPSCs, including *AAVS1* and *CLYBL*. However, many targeted constructs have shown heterogenous expression and promoter silencing when iPSCs are differentiated in specific cell lineages. The *B2M* gene is expressed in most cell types (including iPSCs) as it encodes the β-2 microglobulin protein, which is a component of MHC class I molecules. Moreover, B2M knockout doesn't impact cell fitness or pluripotency, and it has been widely used to generate hypoimmune iPSC for off-the-shelf cell therapy.

Here, we hypothesize that inserting a Bxb1 landing pad in the *B2M* locus in iPSCs will result in stable transgene expression in different lineages upon site-specific recombination. This approach will allow the targeted integration of large DNA payloads and the rapid generation of monoallelic or biallelic transgenic lines, without the introduction of DNA double-stranded breaks.

We first tested and validated our approach in immortalized cell lines (HEK293 and K562). We used prime editing to introduce a 38 bp *attB* sequence downstream of the *B2M* promoter, and then used Bxb1 recombinase to integrate the cassette. We designed a reporter plasmid containing the *attP* site followed by a promoterless GFP and a constitutively expressed puromycin resistance-TagBFP reporter. This donor allowed us to easily recognize on-target vs. off-target integration, as GFP is expressed only upon integration at the *B2M* locus.

After transfection of prime editing components, Bxb1, and DNA donor followed by puromycin selection we were able to detect co-expression of GFP and TagBFP in HEK293 and K562 by flow cytometry. We confirmed on-target integration in GFP expressing clones by PCR amplification of the 5' and 3' ends of the cassette. Importantly, we detected *attL* and *attR* sites flanking the integration by Sanger sequencing, indicating successful site-specific recombination.

Following the functional validation of our approach, we used the same prime editor components to create iPSC lines with monoallelic and biallelic *attB* insertion at the *B2M* locus. We confirmed the presence of *attB* sites in individual

iPSC clones and verified genome integrity and pluripotency. We then targeted our GFP-TagBFP reporter to the *B2M* locus using Bxb1. After puromycin selection and clone isolation, we verified on-target recombination with functional assays and DNA analysis. We are currently performing tri-lineage differentiations with a targeted iPSC clone to monitor GFP/TagBFP expression from the *B2M* locus in neuronal, pancreatic, and blood progenitors.

In summary, we have generated iPSC lines with monoallelic and bi-allelic *attB* sites in the *B2M* locus using prime editing. We successfully integrated a large DNA cassette (5.6 kb) in the *attB* landing pad using Bxb1 and confirmed functional expression of the cassette. Future plans include the targeted integration of other relevant DNA donors, including a Dox-inducible *NGN2* construct to generate iPSC-derived neurons.

1131 Enhanced human NK cell function by CLICK, an efficient non-viral CAR integration on *CISH* locus with a mini-circular single stranded DNA

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Natural killer (NK) cells are promising for adoptive cell therapy due to their potent innate cytotoxic abilities. Early clinical trials have shown their safety and efficacy in treating cancer and autoimmune diseases. Enhancing NK cells with chimeric antigen receptors (CARs) improves their target specificity, positioning them as viable off-the-shelf allogeneic cell therapy products. However, engineering NK cells, whether through viral or non-viral methods, poses significant challenges. Here, we present a novel non-viral approach for CAR NK cell engineering using a mini-circular single-stranded DNA-based target genome editing system. This system led to the development of our CAR-NK engineering platform, named CLICK (*CISH* Locus Integrated CAR Killer), which efficiently integrates CD19CAR sequences into a metabolic checkpoint *CISH* locus. NK cells derived from peripheral blood and engineered with CLICK exhibited high and stable CAR expression throughout NK cell expansion, with specific cellular enrichment at the CISH locus. CLICK-engineered CAR NK cells showed potent cytotoxicity compared to *CISH*knockout NK cells and demonstrated strong anti-leukemic activity *in vitro* and *in vivo* using a xenograft leukemia mouse model. Importantly, CLICK engineered CAR-NK cells exhibited extended lifespans and reduced exhaustion *in vivo*, maintaining durable anti-tumor functions. Our findings indicate that the CLICK NK cell engineering platform is a highly efficient, safe, flexible, and cost-effective method for non-viral engineering of functional CAR NK cells, making it a compelling approach for scalable allogeneic immune cell therapy against cancer and autoimmune diseases.

1132 Developing a Gene Insertion Strategy for Homozygous Familial Hypercholesterolemia

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Homozygous Familial Hypercholesterolemia (HoFH) is a severe genetic disorder characterized by excessively high levels of low-density lipoprotein (LDL) cholesterol and premature atherosclerotic vascular disease. HoFH is caused by loss-of-function mutations in the LDL receptor (*LDLR*), a critical gene that removes excess cholesterol from circulation by the liver. A recent attempt at human gene therapy for HoFH using an Adeno-Associated Virus (AAV) was unsuccessful, likely due to immune responses to the vector or transgene silencing. The goal of this project is to develop a gene insertion strategy for *LDLR* in the liver in which correctly edited hepatocytes can be selectively expanded overtime through transient inhibition of an essential gene. To accomplish this, we generated AAV8 vectors to express *Ldlr* driven by the hybrid-liver promoter (HLP) to test the optimal configuration for integration into the genome. Specifically, we tested AAV8 co-expressing tdTomato and LDLR in *Ldlr* knockout mice to determine whether LDLR would tolerate a P2A skipping peptide on its N- or C-terminus. We injected four groups of

mice (n=3) with one of four AAV8 vectors: HLP-tdTomato-2A-*Ldlr*, HLP-*Ldlr*-2A-tdTomato, HLP-tdTomato, or HLP-*Ldlr*, and collected plasma prior to initial injection (t=0) and once weekly for four weeks. We measured cholesterol levels in plasma and analyzed protein expression in the liver by western blotting. We found that LDLR protein expression is independent of P2A position, however LDLR with P2A peptide on the N-terminus failed to lower plasma cholesterol. Based on these studies we conclude that LDLR best tolerates a P2A skipping peptide at its C-terminus, as this vector configuration lowered plasma cholesterol on par with overexpression of the native LDLR protein. This data will be used to further develop a permanent cure for HoFH through targeted integration of the *LDLR* gene into the genome and selective expansion of *LDLR*-corrected cells.

1133 Precise and Efficient Immune Cell Engineering via Pin-pointTM Base Editing Platform: Insights from TotalSeq Single-Cell Multiomic Analysis

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Many gene editing platforms, such as CRISPR-Cas9, rely on nuclease activity to introduce a targeted protein knockout through the introduction of DNA double-strand breaks (DSBs). While this revolutionary platform has been used in a wide array of cell and gene therapy applications and at varying stages of pre-clinical research up through recent FDA approvals, the technology does not come without risks. DSBs introduced by nucleases can lead to unwanted genomic alterations and cytotoxicity. These effects are magnified when more complex genomic perturbations, such as editing at multiple genomic sites, are introduced. Here, we demonstrate efficient multiplex gene editing using the Pin-point base editing platform as a safe and suitable technology for the creation of CAR-T cell therapies. The Pin-point platform can efficiently knockout four or more protein targets simultaneously with limited impact on cell viability in T cells and can also be utilized for a number of iPSC and HSPC therapeutic engineering applications. Through single-cell multiomic approaches, we can characterize the effect of editing with the Pin-point platform at the RNA and protein level. We see that the platform can efficiently edit multiple subpopulations of cells, for example, CD4, CD8, and dual CD4/CD8 positive T-cells, with high on-target efficacy and with no detectable off-target phenotypic impacts identified via interrogation of the whole transcriptome and a highly-multiplexed protein panel. From discovery to pre-clinical development, the Pin-point and TotalSeq platforms are well suited for engineering and characterizing next generation cell and gene therapies.

1134 Signature of base editor RNA edits in the transcriptome and reduction of RNA editing

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As base editor therapeutics progress through the clinic, better metrics are needed to understand the guide-independent mutational burden they place on the transcriptome. CRISPR-derived base editors are used to alter specific bases in the target DNA sequence in order to introduce a stop codon, alter expression of a gene through splice site disruption, or repair a disease-causing mutation. However, the deaminases used for A-base editors are widely evolved from proteins that recognize and deaminate tRNAs. Activity at the DNA level is a feature that must be engineered into these proteins. As deaminases increase their ability to recognize DNA, it is thought their activity on RNA should decrease inversely. Quantification of RNA deamination by evolved deaminases remains difficult due to high rates of naturally occurring, endogenous RNA editing. Here we present a method that can separate the signature of RNA edits made by base editors from endogenous RNA editing. Using mammalian cell systems including primary cells, we demonstrate that RNA editing from base editors is dose-dependent and transient. Further, we show that targeted engineering of the deaminase enables a significant reduction in RNA editing. We believe this approach enables the design and engineering of improved base editor systems, which in turn supports

the advancement of improved genomic medicines with higher on-target efficacy while minimizing off-target RNA editing.

1135 Improving Precision and Specificity of CRISPR Gene Editing with RGEN-seq and RGEN-mod

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CRISPR nuclease-based genome editing (GE), along with zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), has emerged as a powerful molecular tool across various biological disciplines, including gene therapy. However, off-target effects pose a significant challenge, highlighting the need for sensitive genome-wide detection methods to establish reliable gene therapy platforms. Since its inception, considerable efforts have been made to enhance the specificity of CRISPR nuclease GE and minimize off-target activity to ensure its safety for therapeutic applications. While no single method currently fully eliminates off-target effects, ongoing advancements continue to improve precision.

Here, we present RGEN-seq [1], a sensitive, amplification-free next-generation sequencing method designed for unbiased detection of double-stranded breaks induced by RNA-guided CRISPR endonucleases and other nucleases. Originally developed on the Illumina platform, this amplification-free digital method is now compatible with all major sequencing platforms, including Illumina, DNBSEQ, Nanopore, and PacBio, enhancing its versatility and applicability across various laboratory settings. RGEN-seq optimizes workflows by eliminating genomic coverage bias and gaps typically associated with PCR and enrichment procedures, providing a robust foundation for correlating DNA cleavage scores with the likelihood of off-target mutations in cells. We also introduce the RGEN-seq-based RGEN-mod method, a novel approach that combinatorially functionalizes guide RNA libraries to select CRISPR-sgRNA complexes with minimal off-target activity. RGEN-mod utilizes phosphoramidite chemistry to incorporate succinimido moieties during RNA synthesis, enabling the creation of diverse RNA primer libraries. These libraries can then be screened using RGEN-seq in high-throughput, automated environments.

Together, the combination of RGEN-seq and RGEN-mod offers a comprehensive and platform-agnostic strategy for identifying and validating the most efficient and specific CRISPR nuclease complexes, thereby advancing the development of safer and more effective gene therapy applications.

Kuzin et al. RGEN-seq for highly sensitive amplification-free screen of off-target sites of gene editors. Sci. Rep. 11, 23600 (2021).

1136 Preclinical Safety Assessment of Anti-Viral Gene Editing in Chronic Hepatitis B Models

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Chronic hepatitis B (CHB) is estimated to affect more than 250 million people globally and there are no FDA-approved curative therapies. Nucleos(t)ide analogues are the standards of care and reduce hepatitis B virus (HBV) load however these therapies do not block low level viral replication which allows for HBV persistence in infected hepatocytes. Multiplex gene editing has been demonstrated to remove viral DNA from chronically infected cells and is being developed as a therapy to cure infectious diseases, including CHB. While nuclease-induced double strand breaks lead to rapid clearance of viral DNA, they could potentially cause genome instability in the forms of HBV DNA integration or chromosomal translocations.

To assess these safety concerns, we performed a series of deep sequencing assays with HBV-infected primary human hepatocytes (PHHs) and two CHB mouse models edited by CRISPR-Cas9 targeting HBV. In animal models, viral replication stems from two distinct sources: episomal and chromosomally integrated constructs carrying the viral genome. We characterized DNA repair outcomes following cleavage of the intended target sites within the two structurally different forms of HBV DNA. To achieve this, we first confirmed that multiplex gene editing with a pair of HBV-targeting guide RNAs substantially reduced the viral DNA in hepatocytes and blocked secretion of viral antigens in vitro and in vivo. Small insertions and deletions (indels) and structural variants such as excisions and inversions were detected on the residual HBV DNA recovered from the edited samples, indicating that our HBV-targeting nuclease efficiently cleaves the viral DNA in vitro and in vivo. Consistent with this finding, the level of HBV DNA integration was suppressed in cells receiving our anti-HBV CRISPR-Cas9, suggesting that nuclease-mediated fragmentation of episomal HBV DNA copies does not promote viral DNA integration. To verify sequence specificity of our editing approach, we analyzed the flanking sequences of the HBV integration sites and found that very few sites shared high sequence identity with the HBV on-target sites. Collectively, these results suggest that HBV-targeting multiplex gene editing inhibits viral replication and integration with minimal risk of chromosomal aberrations. The potential for HBV-associated translocations will be discussed as well.

1137 INDUCE-seq®: Ensuring the Safe Development of Cell and Gene Therapies by Gene Editing

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Advances in genome editing are making it increasingly possible to develop new cell and gene therapies. Synthetic genes and modifications to existing ones can be made in a site-specific manner, while immune-compatible cells created for use in allogeneic patients enable production of ‘off-the-shelf’ cellular therapies.

However, gene editing is a new way of treating disease, requiring new tools to ensure their safe and efficacious use in patients. Genome editing comes with risk, including malignant transformation of target cells caused by culturing cells outside the body. This can introduce mutations that confer growth advantages to cells. Furthermore, gene editing can directly cause genomic instability. In cells harboring pre-existing DNA repair defects, the selection for outgrowth of clones with oncogenic mutations may result.

Finally, the gene editing tools themselves can cause DNA breaks in the genome at sites other than the intended target. Such off-target editing could activate proto-oncogenes, or disrupt tumor suppressors thus driving carcinogenesis. Off-target mutagenesis may also generate neoantigens, triggering autoimmunity, or other types of cellular dysfunction. Furthermore, rare penetrant mutations are now known to confer severe risk of common disease, underscoring the importance of identifying off-target gene editing.

More precise methods are needed for testing off-target editing during all phases of therapeutic development, including treatment follow-up. At present standardised assays to assess the safety of gene editing-based therapies are lacking. Here, we describe the development and characterization of INDUCE-seq to address this. CRISPR-Cas9-based gene editing of five well-studied genetic targets was conducted by two independent industry partners, using two different cells types. On and off-target gene editing was assessed by measuring breaks in the genome using INDUCE-seq. The genetic changes at these locations were subsequently measured using Duplex Sequencing, which allows for sensitive detection of exceptionally rare mutations. The results of the study will be presented.

1138 Evolving Solutions for Effective Genotoxicity Assessment in CRISPR-Cas Gene Editing

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Next-generation CRISPR-based editing systems continue to be developed, bringing more diverse editing capabilities to the 'toolbox'. Sensitive nomination of off-target sites in diverse editing systems (e.g. blunt double-stranded break (DSB), staggered DSB, nicked SSB) is a challenge that needs to be addressed in pre-clinical studies during genotoxicity safety assessments. To this end, we present an improved off-target nomination workflow using a proxy system to sensitively nominate off-target sites across different cell types and editors spanning a variable range of gRNA specificity levels. Using clinically relevant cell types (e.g. iPSCs, Primary T-cells) it is demonstrated that the workflow serves as a sensitive (<0.01% indel editing nominated) and effective (>99% event nomination) method across a range of relevant cell types and gRNAs. By quantifying down to 0.01% indel editing frequencies and 0.5% base editing frequencies in the nominated targets, it is demonstrated that editing with diverse editor types (e.g. ABE, CBE), correlates well to frequencies observed using DSB based editors in HSPCs using mRNA delivery. Furthermore, the method shows evidence of nominating sites uniquely undergoing indel editing only with SSB editing modalities in HSPCs. This set of experiments across a broad range of gRNA specificities demonstrates an end-to-end to process for nominating and confirming bona fide off-target sites using both current and next-generation editors.

1139 Evaluating Optimal Volume and Dose of AAV9-mCherry in Stereotaxic targeting of Putamen in C57BL/6 mice

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Introduction: It is estimated that approximately 3% of the population is affected with some form of monogenic neurological disorder. Adeno-associated virus (AAV) gene therapy has the potential to treat these central nervous system indications, however delivering the viral vector carrying the correct genetic information to the specific neural tissue is a significantly challenging process. Systemic administration of the viral vector results in a greater dose requirement to achieve clinically relevant levels due to difficulty navigating the blood brain barrier. This in turn increases exposure in non-target tissues and the risk of immunotoxicity associated with a broader biodistribution and can result in increased off target expression. A direct, stereotaxic administration of AAV gene therapy can safely and effectively deliver genetic payload into defined structures in the brain, alleviating concerns associated with systemic administration and requires a lower vector dose for clinically relevant transgene expression, poses a lower risk of full immune response and limits unwanted expression in non-target tissues.

Method: In this 4-week study we evaluated the biodistribution and transduction efficiency of AAV9-mCherry via qPCR when administered as a single, bilateral stereotaxic dose in 3 different volumes and dosages to the putamen of female C57BL/6J mice. We used mouse as a model for this study to leverage the availability of a wide variety of established genetic murine models for CNS disease. We assessed the spatial mCherry gene expression within individual cells in neural target tissue sections by RNA In-situ hybridization. The mCherry protein level expression was assessed by immunohistochemistry. In addition, we evaluated for procedure-related traumatic brain injury by immunolabeling for biomarkers of neuro inflammation and trauma using markers for microglia activation (Iba-1), astrogliosis (GFAP) and inflammation (MMP9). We evaluated tissue specific transduction efficiency in the putamen, thoracic dorsal root ganglion, cervical, lumbar and thoracic spinal cord and liver tissues.

Result: A volume/dose dependent increase in the transduction efficiency was noted across all the tissues and the highest biodistribution of AAV9-mCherry was noted in the putamen dosed with 7 µl/7.42 x 10^{10} GC/site, with the liver having approximately 50-fold lower expression. Similar trends in the mCherry expression were noted via the mCherry immunohistochemistry and mCherry RNA in situ hybridization. Minimal expression of Iba-1 and GFAP in control group animals and minimal to moderate expression of Iba-1 and GFAP in animals dosed with 7 µl /7.42 x 10^{10} AAV9-mCherry GC/site indicated the presence of some procedure or volume related neural inflammation. Expression of MMP9 was not observed in either the control or AAV9-mCherry dosed animals.

Conclusion: Taken together, this data demonstrates that efficient neural tissue biodistribution of AAV9 vector can be achieved with limited/minor post-surgery related tissue inflammation in stereotaxic dosed murine models. The volumes and dosages used in this study are a reliable starting point for developing refined genetic murine models for CNS diseases targeting putamen or other neural structures to obtain high transduction with minimal off target expression and lowered risks of neural inflammation. This information can also serve as a guidance for precision targeted CNS dosing in large animal species and humans as well.

1140 Precise Gene Writing System for CAR-T cell therapy generation.

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The development of effective, precise, and safe gene-editing technologies for ex vivo therapies, particularly in hematopoietic stem cells (HSCs) and chimeric antigen receptor T cells (CAR-Ts), posesa critical challenge. Current platforms face technical limitations and safety concerns, impeding the realization of their full therapeutic potential. To address these challenges, FiCAT emerges as a groundbreaking gene-writing platform with the aim of providing a flexible and universal solution for safe editing of small regions and efficient, programmable editing of large genes in clinically relevantprimary cells.

FiCAT is composed of a CRISPR-Cas nuclease and propietary engineered transposase, with outstanding efficiency reached through rational protein engineering and an unsupervised variability generation approach. Over 400,000 variants are tested using a proprietary high-throughput screening system grounded in synthetic biology principles. In practical application, FiCAT RNPs had been produced and validated in T cells and HSCs using nucleofection for targeted integration of a minimal reporter. Our system compared favourably against existing systems like homology-directed repair (HDR) or homology-independent targeted insertion (HITI) using CRISPR-Cas9 alone. To enhance both efficiency and viability, electroporation is coupled with inhibitors targeting toxicity associated with double-stranded exogenous DNA. In addition, FiCAT therapeutic potential has been shown with the successful integration of CAR19 in a relevant locus (TRAC) for T cells . In vitro Functional validation of FiCAT generated CAR-T cells revealed comparable cytotoxicity with LV-generated CAR-T cells.

These results open up new possibilities with enhanced cargo size integration in HSCs and T cells, surpassing current gene editing techniques . This is of critical importance for both HSCs (i.e. correction of monogenic blood diseases) and T cells, since new generation of immunotherapies relies on targeting more than one epitope (Dual CAR) but also benefits from the inclusion of immunomodulators to enhance CAR-T cells efficiency and persistence.

FiCAT is designed to overcome precision and safety limitations in gene editing, streamlining the cell engineering process into a single step. Its potential to reduce costs and eliminate the need for viral vectors promises expanded accessibility to treatment. In summary, FiCAT represents a significant advance in gene editing, poised to enhance precision, safety, and cost-effectiveness in clinical applications.

1141 Development of viral-free FiCAT gene writing platform for liver-directed in vivo application

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FiCAT is a novel genome writing platform developed to achieve on-target integration by coupling DNA scanning and cutting activity of Cas9 with donor DNA processing and transfer activity of piggyBac transposase (Pallares et al., Nat. Comm 2021). In our platform, we use a triple-mutant version of the hyperactive piggyBac transposase (hyPB-t) in which random integration activity is lost while the excision capacity of the transposon is increased,

therefore integration can occur only when hyPB-t is coupled with Cas9. We have previously described that FiCAT allows efficient and precise transposon or donor DNA integration, achieved also with long non-viral donor DNA (>4kb), and we have shown up to 20% on-target integration of therapeutically relevant cargos such as CAR19 in primary human T cells. Here, we aim to further develop our platform to target hepatocytes in vivo, by delivering the integration machinery in mRNA format exploiting lipid nanoparticles (LNP). We are able to efficiently co-encapsulate FiCAT mRNA and gRNA in LNP, and obtain up to 40% of editing in mouse liver, at a level similar to Cas9+gRNA only. As an initial proof of concept, we used AAV8 to deliver donor transposon DNA. Although AAV vectors are known to efficiently target hepatocytes, episomal AAV genome is lost upon cell proliferation leading to reduced expression in the long term, undermining its use in particular into pediatric patients. Co-delivery with FiCAT mediates on-target integration of the AAV genome, overcoming these limitations. We initially tested co-delivery of AAV vector and LNP by random integration of donor DNA carrying luciferase gene mediated by hyPB. We observed up to 20% of hepatocytes expressing luciferase upon liver growth when treating newborn, and >2-log higher luciferase expression when hyPB is co-delivered compared to AAV-only condition, showing that integration leads to higher expression levels, in both adult and newborn-treated mice. In this context, we then validated FiCAT-mediated programmable integration in vivo in newborn and adult mice. AAV vectors present some further important restrictions, such as the limited cargo capacity, reduced but widely described random integration activity and pre-existing immunity in the population that can impair clinical application. Therefore, our aim is to develop a viral-free system, by delivering also the donor DNA with LNP. We optimized LNP formulation conditions to encapsulate DNA with >90% efficiency. In vivo delivery of viral-free DNA is impaired by inefficient nuclear translocation. We observed that hyPB-t can increase 2-fold the nuclear translocation of donor DNA in vitro by binding the transposon in the cytosol before moving into the nucleus. Overall, our results show the potential of FiCAT platform to achieve viral-free on-target integration in vivo.

1142 A Versatile Anti-CRISPR Platform for Opto- and Chemogenetic Control of CRISPR-Cas9 and Cas12a across a Wide Range of Orthologs

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CRISPR-Cas technologies have revolutionized the life sciences by enabling programmable genome editing across diverse organisms. Achieving dynamic and precise control over CRISPR-Cas activity with exogenous triggers, such as light or chemical ligands, remains an important need. Existing tools for CRISPR-Cas control are often limited to specific Cas orthologs or selected applications, restricting their versatility.

Anti-CRISPR (Acr) proteins, natural inhibitors of CRISPR-Cas systems originating from bacteriophages, offer a highly flexible regulatory layer for CRISPR control. A wide range of Acrs have been discovered, targeting class II, type II, V and VI CRISPR effectors. While most Acrs are ortholog-specific, some have been found to exhibit broad-spectrum activity and effectively block various Cas orthologs. Prominent examples include AcrIIA5 and AcrVA1, broad-spectrum inhibitors targeting Cas9 orthologs from the II-A, -B and -C subtypes or CRISPR-Cas12a, a type V effector, respectively.

In this study, we built on our previously reported concept for optogenetic activation of CRISPR-Cas effectors with engineered, light-switchable Acrs and expanded it from ortholog-specific Acrs towards AcrIIA5 and AcrVA1 and thereby towards broad spectrum control. First, hybrids between AcrIIA5/-VA1 and the *Avena sativa* light-oxygen-voltage 2 (LOV2) domain were engineered by inserting the photosensor into allosteric surface sites of these Acrs. The resulting Acr-LOV2 chimeras release Cas9 or Cas12a activity upon stimulation with blue light, thereby activating genome editing in human cells. Beyond employing conventional, strong Polymerase II promoters to drive the CRISPR-Cas effectors, we were able to co-express all required components, i.e. the Acr-LOV2 chimera, Cas effector and RNA guide, from a single H1 promoter (Polymerase III), resulting in a particularly compact transgene

conformation. Next, we investigated whether the system could be modularized in terms of input and mode of action (stimulus activated vs stimulus inactivated). To this end, we generated a set of circularly permuted ligand receptor domains derived from the human estradiol receptor β, thyroid receptor β and glucocorticoid receptor 2 (GR2). Replacing the LOV2 domain with these novel ligand binding domains gave rise to a chemogenetic Acr platform that facilitates ligand-dependent suppression of genome editing with six different, clinically relevant drugs in various human cell lines. Finally, we demonstrated that the circularly permuted GR2 domain can be plug-and-play integrated into other Acrs, CRISPR-Cas effectors, and even unrelated protein species, highlighting the potential for a broad application in synthetic biology.

In sum, this work establishes a versatile platform for opto- and chemogenetic control of CRISPR-Cas systems, with immediate applications in basic research and biotechnology and potential for therapeutic use in the future.

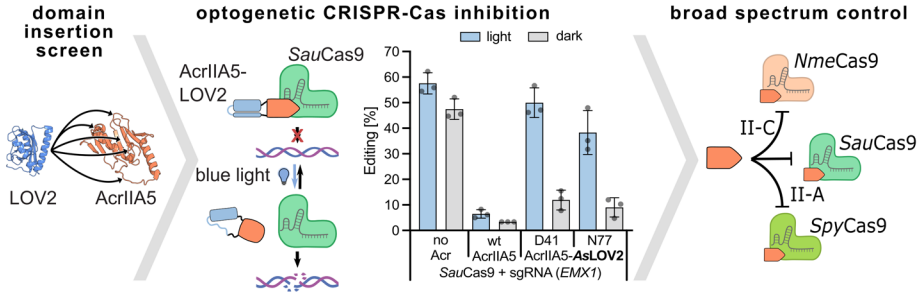

Figure 1: Design of AcrIIA5-LOV2 chimeras for broad spectrum optogenetic CRISPR-Cas9 control. Insertion of LOV2 into surface-exposed loops of AcrIIA5 results in AcrIIA5-LOV2 hybrids (left). These were co-expressed with *S. aureus* Cas9 and an EMX1-targeting sgRNA in HEK293T. Cells were exposed to blue light pulses or kept in the dark for 72 h, followed by assessment of InDels via NGS (center). The resulting AcrIIA5-LOV2 chimeras enable broad-spectrum optogenetic control of type II CRISPR effectors (right).

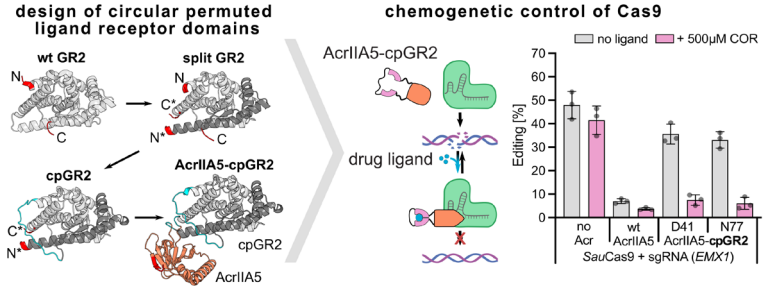

Figure 2: Generation of circularly permuted receptor domains for ligand-mediated suppression of genome editing. The concept for engineering circularly permuted (cp) receptors for allosteric Acr control is illustrated using GR2 as example. Receptor domains were opened at previously identified split-sites (Rithar et al. (2023) *Nat Chem Biol.*), thereby creating new N- and C-termini that are in direct proximity (C*, N*). Former N- and C-termini were connected by fusion via a long GS-linker indicated in blue (left). Schematic of ligand-dependent CasOFF switch.The cpReceptors take on a compact conformation in presence of the ligand, due to proximate N- and C-termini, but are relaxed in absence of the ligand. This results in ligand-dependent disorder of the fused Acr structure (center). Cortisol-dependent genome editing in HEK293T cells using an AcrIIA5-cpGR2 hybrid for CRISPR-Cas9 control (right).

1143 Engineering miniature SpaCas12f genome editing potential

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The miniature (497 a.a.) CRISPR-Cas12f1 nuclease from Syntrophomonas palmitatica (Spa) is triggered by a 5' G(G>W>C)TTC protospacer adjacent motif (PAM) to cleave double-stranded (ds) DNA targets and prefers elevated temperatures, 44-58°C. To broaden its utility as a genome editor, we have simplified its PAM recognition and performed protein and guide RNA (gRNA) engineering to enhance cellular activity and enable robust dsDNA substrate cleavage at temperatures as low as 30°C without measurably impacting its intrinsically high fidelity gRNA target recognition. Our advancements have more than doubled the number of sites that can be targeted and provide around a 10-fold improvement in editing efficiency in human cells at 37°C. Altogether, we have improved SpaCas12f1 cellular activity, shifted its thermal preferences, and expanded its targeting range, paving the way for its use as a compact RNA-guided genome editing tool.

1144 Engineering a potent CRISPR-Cas12l genome editor with C-rich PAM recognition

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Here, we solved the structure of a compact (~850 aa) type V CRISPR effector, Cas12l, at 2.9 Å using cryo-electron microscopy (cryo-EM) and used it to engineer variants capable of editing the human genome. Structural analyses revealed similarities to type V CRISPR nucleases as well as new previously unobserved features. These included: 1) an H-type RNA pseudoknot forming guide RNA (gRNA) scaffold; 2) a unique N-terminal domain that stretches from the beginning to the end of the ribonucleoprotein (RNP) bound DNA target site effectively locking the ternary complex in place. Next, using structure-guided engineering, we improved Cas12l DNA target editing in human cells by approximately 10-fold by fine-tuning its electrostatic binding potential at DNA and gRNA interfaces mapped in our structure. To maximize potency, amino acid substitutions predicted with large language protein models to improve folding and stability were next introduced and evaluated for additional enhancements. In combination with the structure-guided modifications, two of the AI-predicted substitutions enabled efficient Cas12l editing when delivered transiently as mRNA or RNP in human cells. Collectively, this work expands the RNA guided genome editing repertoire to one that is compact, recognizes a simple C-rich PAM, and displays a distinctive DNA target lock and catch mechanism.

1145 A Panel of Novel Engineered Nucleases Unlocks the Full Potential of Genome Editing

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A successful CRISPR based gene editing strategy requires developing an optimized composition of nuclease and gRNA per target gene per organ. Different editing strategies require specific nuclease characteristics, such as nuclease size, diverse PAM recognition, compatibility with delivery systems and high activity and specificity to the target site. A panel of nucleases, the OMNI nuclease series, was developed by EmendoBio to overcome such key challenges in the field. The OMNI nucleases recognize different PAM sequences that cover approx. 80% of the human genome; they vary in size; and they are compatible with all common delivery modalities, including AAV. These nucleases are engineered to be as active as industry gold standard while portraying enhanced

specificity, thus eliminating off-target effects and enabling allele specific editing. EmendoBio's technology enabling the development of tailor-made nucleases by combining a discovery pipeline and cutting-edge protein-engineering capabilities, which is supported by extensive computational tools, including proprietary AI.

While EmendoBio has developed more than 300 OMNI nucleases, this abstract focuses on three OMNI nucleases exceeding promise. OMNI-A1 is an allele specific editor, capable of full discrimination between two alleles that differ even by a single nucleotide. OMNI-A2 is a small nuclease that fits into an AAV; using EmendoBio's engineering technology, the activity of OMNI-A2 was increased by 4-fold. OMNI-A4 is a novel nuclease capable of recognizing a non-NGG PAM sequence. Using a non-NGG PAM enables an extended freedom-to-operate beyond that of SpCas9 and expanded genome coverage. OMNI-A4 is highly active, can achieve high editing efficiency and knock-out of multiple gene targets commonly used for cellular therapy.

EmendoBio's panel of novel nucleases, and its proprietary techniques to customize such nucleases, enables expansion of the existing CRISPR toolbox and overcomes major gene editing challenges, offering solutions for a wide range of diseases.

1146 Systematic Analysis of Oligonucleotide Approvals in the EU and US: Identifying the reasons for Non-Approval Decisions

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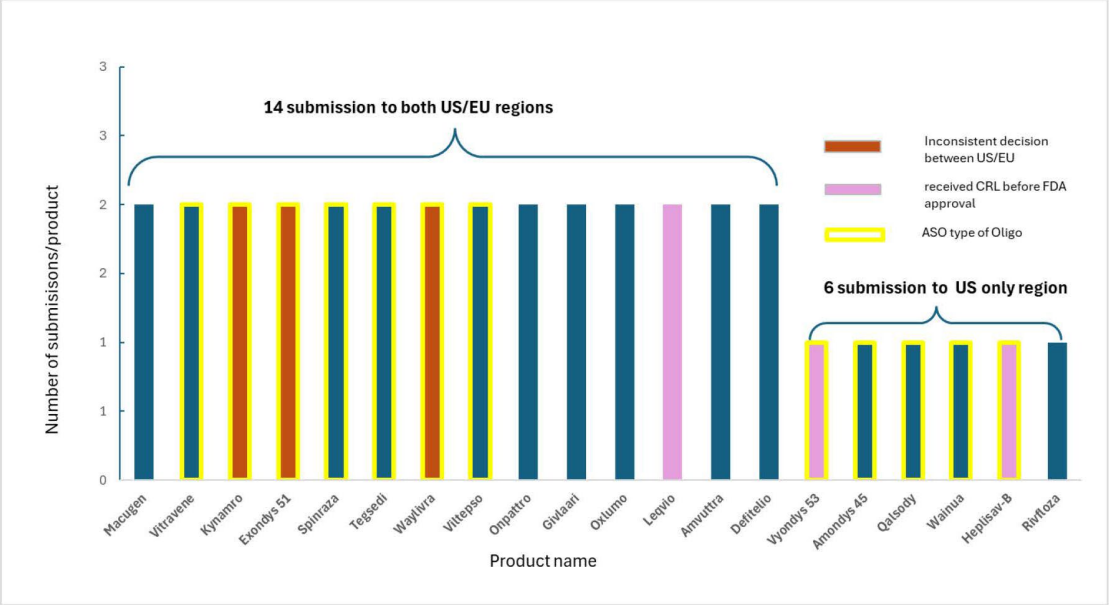
Background Oligonucleotides therapeutics show their potential for treating a wide range of conditions, especially previously untreatable rare diseases. Continue efforts for oligo development are on-going to expedite patient access to life-changing therapies. To date, a total of 20 oligo drugs have been approved in the US and EU. However, an analysis of these approvals reveals inconsistencies in market authorization for certain types of oligo therapies between the two regions. Furthermore, some approved oligo therapies have gone through multiple review cycles from the FDA before receiving approval. Analyzing approval and non-approval data can provide valuable insights for future oligo therapy development, offering a unique perspective to both drug developers and regulators on how to accelerate these often-complex therapeutics to market.

Methods Data were gathered from the regulatory filings publicly disclosed by the FDA and EMA, specifically from the Drugs@FDA database and the EU EPAR reports, respectively. Complete Response Letters (CRLs) are confidential and can only be made public once the drug has been approved by the FDA. Information on CRLs for approved drugs can be found in the drug-specific FDA application review files under "Other Action Letters." Plot and chart were generated with Excel.

Results Of the 20 approved oligonucleotide drugs (Fig.1), 14 (70%) were submitted to both the EU and US agencies for market authorization. These submissions covered various types of oligonucleotides, including aptamers, antisense oligonucleotides (ASOs), ASO vaccine adjuvants, siRNAs, and single-stranded/double-stranded DNA (ss/ds DNA). Among these, 7 out of 14 (50%) submissions were for ASO therapies. Notably, 3 out of these 7 ASO submissions (43%) received inconsistent decisions between the EU and US regulators (approval vs. non-approval). This inconsistency has only been observed in ASO therapies so far. Additionally, among the oligonucleotide drugs approved by the FDA, 3 out of 20 (15%) required more than one review cycle, with deficiencies addressed through FDA-issued Complete Response Letters.

Conclusions It was observed that the three inconsistent decisions between EU and US regulators were centered around the ASO type of oligonucleotide products. In the two non-approval cases from the EU agency, it was concluded that the efficacy and safety of the products were not sufficiently demonstrated. The major reasons included a limited clinical dataset for analysis, such as high withdrawal rates in patients, limited participation of EU patients, or a small number of patients per study arm. In the non-approval case from the FDA, the specifics were not clearly described as the Complete Response Letter (CRL) for a non-approved drug is not disclosed to the public. This non-approval may be due to safety concerns associated with the drug. The discrepancies between EU and US regulators underscore the importance of robust clinical datasets and safety profile in the regulatory approval for

ASO type oligonucleotide products. Among all the approval cases from the FDA, three products underwent more than one review cycle and received CRL before approval. The deficiencies addressed in the CRL covered issues related to clinical benefits, serious safety concerns, inspection issues, and CMC issues. Notably, inspection issues were mentioned in two out of three CRLs, serving as a reminder to the industry to ensure facility readiness for commercial production.



Confidential - Company Proprietary

1147 Targeted Delivery of siRNA to Activated Hepatic Stellate Cells Using Aptamers: A Novel Approach for Fibrotic Liver Treatment

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A lack of effective strategies for targeted delivery of gene therapies to different cell types (beyond hepatocytes) is hindering the development of therapeutic modalities such as RNAi for many diseases. Aptamers offer the potential to selectively deliver RNAi therapies to new biomarkers and cell types.

Activated hepatic stellate cells (HSCs) are a key driver of fibrosis within the liver; thus, targeting this cell type and the fibrotic differentiation process may offer new potential treatments for liver fibrosis. Applying cell-based aptamer selection methods to human primary activated HSCs, with negative selection against hepatocytes, an aptamer was developed that selectivity binds and internalises to activated human HSCs. Targeting of the aptamer to activated HSCs is ideal for targeting the cells actively involved in fibrotic disease without affecting the remaining hepatic cell

population. Further screening with alternative cell types showed the aptamer does not interact with T lymphocytes, renal tubule epithelial cells, prostate, breast, or lung epithelial cells, or other hepatic cell populations, including hepatocytes and Kupffer cells. Multiple cleavable and non-cleavable Optimer-siRNA linker types have been tested for functionality, with the greatest siRNA silencing effect seen with a cathepsin-cleavable linker. siRNA conjugation to the aptamer delivery vehicle did not interfere with aptamer binding or selectivity. Four different siRNA molecules have been conjugated to the aptamer delivery vehicle, including those targeting ColA1 and PDGFR as critical molecular mediators of fibrosis. Each of the tested aptamer-siRNA conjugates resulted in significant silencing of the respective genes in HSCs by flow cytometry and qPCR ($p < 0.0001$), with no observable off-target gene silencing in hepatocyte cultures, indicating that the developed aptamer is selective and can deliver diverse siRNA payloads with sufficient endosomal escape to enable effective gene silencing.

Taken together, these data demonstrate for the first time the development of a delivery vehicle selective for activated HSCs, with the potential to deliver diverse therapeutic cargo to this cell type. Advancing this delivery vehicle into the clinic with a relevant siRNA conjugate opens the potential for new treatment approaches in liver fibrosis.

1148 Multi-Targeted Organ Specific MicroRNA Intervention for Cancer (MOSAIC)

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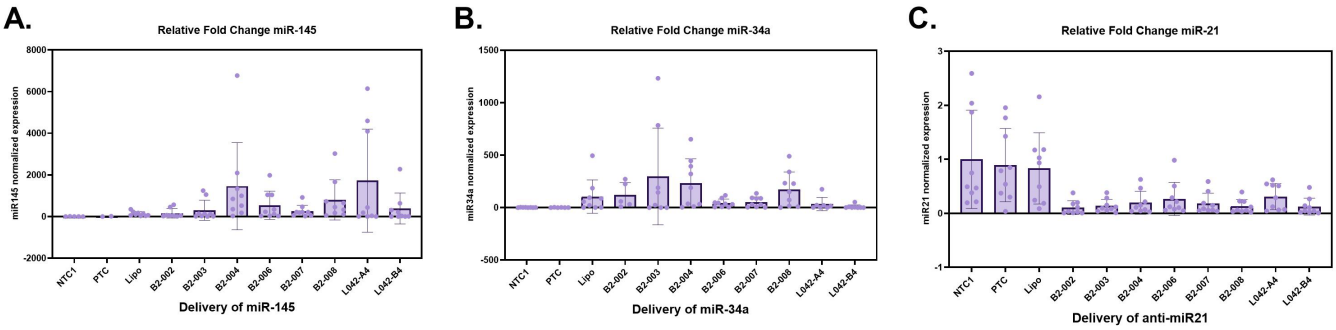
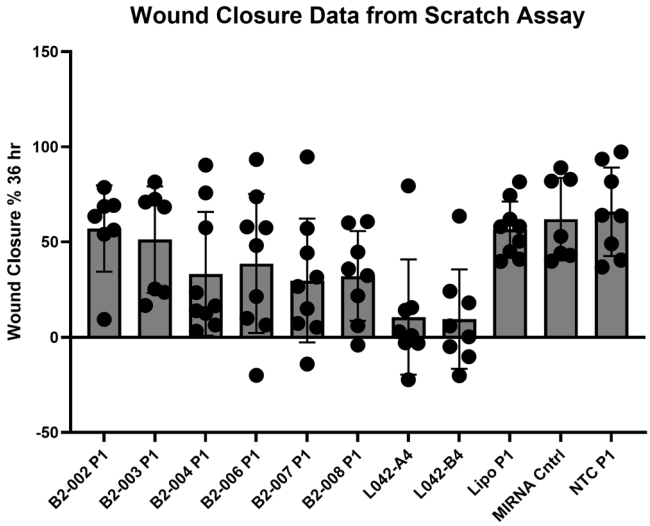
Introduction: Lung cancer is one of the most common and deadly forms of cancer worldwide. Traditional therapies for lung cancer include surgery, radiation therapy, and chemotherapy. However, these methods often come with severe side effects and limited efficacy, particularly in advanced stages of the disease. MicroRNAs (miRNAs) are emerging as a novel approach in lung cancer therapy due to their ability to regulate gene expression. These small, non-coding RNA molecules can inhibit the translation of specific messenger RNAs (mRNAs), thereby controlling the production of proteins that are involved in cell growth, differentiation, and death. In the context of lung cancer, specific miRNAs have been found to be dysregulated, leading to uncontrolled cell proliferation and tumor growth. By introducing or inhibiting these specific miRNAs, researchers can potentially alter the behavior of cancer cells. For instance, miRNAs that act as tumor suppressors can be introduced to inhibit tumor growth, while those that promote cancer can be inhibited. The challenge lies in the delivery of these miRNAs to the target cells. Recent advancements in nanoparticle technology have shown promise in addressing this issue, allowing for targeted delivery of miRNAs to lung cancer cells, thereby enhancing the efficacy of the therapy and minimizing side effects. Nanoparticles have been shown to help shield molecules and offer the ability of targeted delivery with minimal toxicity. In this work we identified two lung cancer tumor suppressors miRNA-34a and miRNA-145 and one oncogenic target, miRNA 21 from literature. A combination of the two mimics was co-delivered with the anti-miRNA oligo (AMO) using polymeric nanoparticles (PNPs). Delivery of miRNAs using these PNPs has demonstrated the ability to increase and decrease the targeted miRNAs and markedly reduce growth in A549 lung cancer cells.

Methods: Six PNPs were designed based on results from previous *in vivo* work and literature to increase lung cell targeting. Polymers were synthesized using reversible addition fragmentation chain transfer (RAFT) polymerization. A549 lung cancer cells were cultured and plated on 48 well plates to analyze the efficiency of PNP delivery. PNPs were loaded with a miRNA cocktail of miRNA-34a, miRNA-145 and AMO-21 at a final concentration of 0.13 µmol/L. Cells were dosed with each loaded PNP in triplicate 24 hours after plating at a PNP concentration of 0.05 mg/mL. The cells were processed and analyzed 48hrs post dose via imaging, flow cytometry and RT-qPCR.

Results and Conclusions: Relative fold change from RT-qPCR data showed an upregulated expression for miRNA 145 and 34a (Fig1A,B), compared to the negative control and a downregulated expression in miRNA 21(Fig1C). These results are consistent with the hypothesis that PNPs successfully delivered the cocktail of miRNAs to lung cancer cells *in vitro*. Moreover, analysis by flow cytometry indicated an increase in apoptotic cells upon delivery with PNPs compared to controls. The wound healing assay (Fig2) also showed decreased cell migration in cells treated with loaded PNPs. The selected polymers prove to be promising vehicles for co-delivery of miRNA mimics

and inhibitors. This data supports a potential for a novel miRNA combinatorial therapeutic for not only lung cancer but any disease with dysfunctional miRNA targets.





1149 Aptamer-Based Discovery of Annexin A2 as a Novel Plasma Biomarker for Early Detection of Hepatocellular Carcinoma

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Introduction: Hepatocellular carcinoma (HCC) is a major global health challenge, with its incidence in the United States having tripled over recent decades. Early detection through effective surveillance and the use of reliable diagnostic biomarkers is critical for improving patient outcomes. Alpha-fetoprotein (AFP), the most commonly utilized serum biomarker, has limited specificity and reliability, particularly in early-stage HCC when tumor sizes are less than 3 cm. Alternative biomarkers, such as Glypican-3 (GPC3), AFP-L3, and Des-γ-carboxyprothrombin (DCP), have been explored but have shown mixed results. Aptamer-based protein biomarker discovery has emerged as an attractive solution due to its rapid selection, target-specific, and versatile modification approaches. Aptamer-based protein biomarker discovery has become a promising approach due to its rapid selection process, target

specificity, and versatile modification capabilities. Notably, modified aptamers achieve enhanced binding specificity and affinity, ranging from nanomolar to picomolar levels, by conjugating drug-like ligands to the aptamer backbone.

Methods: We developed a bead-based modified aptamer (XA) library to identify novel proteomic biomarkers and their corresponding affinity reagents. Pooled plasma samples from HCC patients (n=10) and healthy controls (n=10) were immunodepleted of abundant proteins, labeled with distinct fluorescent dyes, and incubated with the bead-based XA library. Proteins bound to XA beads were sorted via flow cytometry, followed by next-generation sequencing (NGS) to identify high-frequency aptamer sequences. Top XAs were synthesized and incubated with plasma samples to pull down target proteins for mass spectrometry (MS) analysis, with subsequent validation by ELISA and western blot. Functional analysis was conducted using DAVID Bioinformatics Resources and the Human Protein Atlas.

Results: Annexin A2 (ANXA2) was identified as the top target protein in the HCC group, showing significantly elevated levels compared to healthy controls. Validation using ELISA and western blot assays confirmed increased Annexin A2 levels in an independent cohort (HCC: n=39, controls: n=20). Functional analysis revealed Annexin A2 overexpression in HCC and its association with tumor aggressiveness.

Conclusion: Our aptamer-based platform enabled the rapid and specific identification of annexin A2 as a novel plasma biomarker for HCC. Elevated ANXA2 levels, correlated with tumor aggressiveness, may complement AFP, particularly in AFP-negative cases. A diagnostic model integrating ANXA2 and AFP could improve early detection and disease stratification. Future studies incorporating machine learning or statistical modeling may further enhance diagnostic accuracy and clinical utility.

1150 Selective ASO-based JAK2 Inhibitor for the Treatment of Polycythemia Vera

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Introduction: Myeloproliferative disorders (MPD) are clonal hematopoietic stem cell malignancies with cytokine independency or hypersensitivity. Due to dysregulation of the JAK-STAT signaling pathway, MPDs display excessive proliferation of megakaryocytic, erythroid, and granulocytic progenitors, causing patients to develop splenomegaly, thrombosis, and bleeding as an overproduction of blood cells occur. Polycythemia vera (PV) is a chronic MPN characterized by aberrant hematopoiesis of myeloid lineage with exuberant red cell production and increased release of pro-inflammatory cytokines. The increased kinase activity of JAK2 is present in several hematologic malignancies, and the somatic *JAK2* V617F mutation can be found in at least 98% of PV patients. Inhibiting JAK2 acts to suppress hematopoiesis, consequently reducing red blood cell, neutrophil, platelet, and lymphocyte production. We hypothesize that preventing *JAK2* transcription by antisense oligonucleotide (ASO)-mediated exon masking of the *JAK2* intron-exon junction will selectively reduce *JAK2* mRNA and protein by providing nonsense mediated decay (NMD) in the reading frame. This approach aims to mitigate the clinical manifestations of hematologic malignancies, such as PV, by curtailing downstream signaling and $JAK2^{V617F}$-driven autonomous cell proliferation.

Methods: We designed a series of 19mer ASOs targeting *JAK2* exon-intron junctions (providing NMD in the reading frame, steric, non-RNAse H1) and tested these at a range of concentrations. ASOs were designed with a phosphorothioate 2'-O-methoxyethyl (MOE) backbone and were prioritized based on *in silico* binding affinity and limited off-target binding. A human HEL cell line harboring the V617F *JAK2* mutation underwent ASO treatment (1μM) and 72-hour incubation, followed by qPCR and Western blot analyses. Peripheral blood mononuclear cells (PBMCs) obtained from PV patients carrying the V617F *JAK2* mutation were plated in methylcellulose, containing erythropoietin, in the presence or absence of ASO (1 μM, 48-hour incubation).

Results: Following ASO treatment of HEL cells, *JAK2* qPCR results show a ~50% reduction of the target *JAK2* transcript, with a dose-response reduction in mRNA observed. Western blot results reveal a significant JAK2 protein

decrease (~70%) in ASO-treated HEL cells compared to untreated samples. STAT5 phosphorylation status further confirmed this effect, and we report a 35% pSTAT5 reduction. The ASO was shown to be a selective inhibitor of JAK2 with no significant JAK1, JAK3, or TYK2 inhibition. Additional *in silico* analyses demonstrated no off-target effects of the ASO. Evaluation of endogenous erythroid colony (EEC) formation following ASO treatment to PV PBMCs revealed a significant reduction of erythroid colonies. Other non-erythroid colonies were produced in this treatment condition.

Conclusions: Our preclinical data supports this ASO as a highly selective JAK2 agent, affecting direct levels of JAK2 as well as downstream STAT signaling. ASOs have the potential to target *JAK2* mutations with high specificity, effectively decreasing JAK2 protein levels without off-target effects on other kinases. Currently, there is no available therapeutic that selectively inhibits JAK2. We propose that the application of our ASO to selectively reduce JAK2 protein may alleviate the disease burden for PV patients with a decrease in unwanted side effects, ultimately improving both the quality and longevity of their lives.

1151 Targeting Local Inflammation in Human Coronary Arteries *Ex Vivo*

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Atherosclerosis, the leading cause of heart disease worldwide, is driven by chronic inflammation triggered by endothelial damage, which fosters plaque growth. The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) demonstrated that systemic delivery of anti-IL1β monoclonal antibodies reduced adverse cardiac event risks by 15%. However, this approach increased susceptibility to infections and sepsis, highlighting the need for targeted therapies that suppress inflammation exclusively at plaque sites.

Our study aims to identify key inflammatory markers in human atherosclerotic coronary arteries and develop self-assembling peptide-based plaque-targeting nanoparticles for delivering IL1B siRNA therapy. Coronary arteries from male (65.6%, n=21) and female (34.4%, n=11) donor hearts provided by the LifeLink organization were screened for TNF and IL1B by RT-qPCR. Additional arteries were balloon-injured and perfused with pro-inflammatory cytokines for 4 hours, followed by administration of the plaque-targeting nanoparticle, (previously developed by the Totary-Jain lab DOI: 10.1016/j.ymthe.2021.01.032), containing IL1B siRNA for another 4 hours.

Patient histories revealed that 53.1% of cases (n=17) were cardiovascular (CV)- or stroke-related deaths, with 47% of these occurring in individuals aged 40–59 and another 47% in those 60 and older. In male patients with CV-related deaths (n=4), TNF and IL1B expression were significantly elevated compared to controls ($P<0.001$ and $P<0.01$, in figures 1 and 2, respectively). In all patients (male and female) with CV deaths, TNF was significantly elevated ($P<0.01$), while IL1B was not. Statistical significance was determined by one-way ANOVA, no significant elevations in inflammatory markers were observed in other causes of death (e.g., stroke, blunt injury).

Notably, arteries perfused with IL1B siRNA nanoparticles exhibited a 70-fold decrease in IL1B expression compared to the scramble control siRNA. Our findings build on the success of the CANTOS trial, and demonstrates the potential of this plaque-targeting siRNA nano therapy to combat atherosclerosis and potentially mitigate the risks of systemic IL1B inhibition.

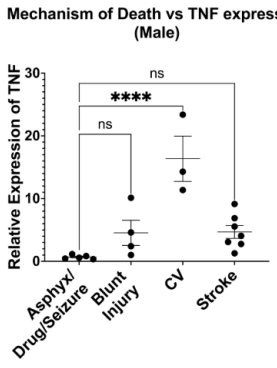

Figure 1. Mechanism of Death vs TNF Expression in Males

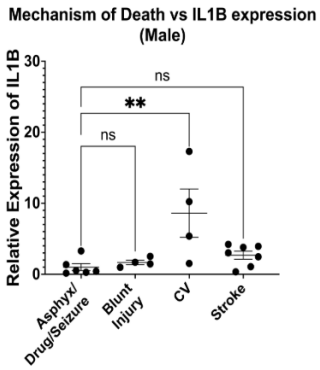

Figure 2. Mechanism of Death vs IL1B Expression in Males

1152 COATSOME® SS Series: Highly Versatile Ionizable Lipid as Vital Component of Lipid Nanoparticle for Gene Vaccine Application

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BACKGROUND: Nucleic acid vaccines, DNA and messenger RNA (mRNA), have emerged as promising modalities for infectious disease and for cancer immunotherapy due, in part, to shortened producting cycles and high potency. Lipid nanoparticles (LNP) composed of ionizable lipids are important components of such vaccines as they can convey and present the nucleic acid effectively to the immune system. However, toxicity of ionizable lipids and the challenges of cold chain logistics of LNP have limited their clinical use. Further development of LNP requires 1) reduced systemic toxicity, 2) improved endosomal escape, 3) potent and cytotoxic T-cell specific adjuvating function, 4) reduced adverse reactions, 5) improved storage stability of LNP.

RESULTS: We have previously demonstrated that LNP composed of our biodegradable ionizable lipids (COATSOME® SS series), can deliver plasmid DNA or mRNA in mice to the liver or the solid tumor via intravenous injection and achieve high levels of expression with less toxicity. We also evaluated the safety of the lipids in mice where total lipid doses of up to 175 mg/kg were well tolerated. Here, we describe their potential applications for mRNA-based vaccines. We finely tuned the LNP formulation for infectious disease. First, subcutaneous administration of hemagglutinin (HA)-encoding mRNA encapsulating LNP composed of ssPalm O-Phe (COATSOME® SS-OP) (HA-LNP$_{SS\text{-}OP}$) and neuraminidase (NA)-encoding mRNA encapsulating LNP composed of SS-OP (NA-LNP$_{SS\text{-}OP}$) induced equal amount of anti-HA antibody production compared with the globally-marketed LNP vaccine. This result implies high vaccination effects for infectious disease. On the other hand, subcutaneous administration of HA-LNP$_{SS\text{-}OP}$ induced no significant differences in the levels of inflammatory cytokines in the plasma compared with PBS. This result suggest that HA-LNP$_{SS\text{-}OP}$ attenuates severe inflammatory responses and adverse reactions. Furthermore, the addition of ssPalm E-P4C2 (COATSOME® SS-EC), another type of our ionizable lipids, significantly improved cytotoxic T-cell (CTL) activity as a cancer vaccine. Next, we demonstrated that LNP containing SS-OP and SS-EC clearly suppressed the tumor growth in a mouse model of disseminated malignant diseases. Thus, SS-EC would be used as a promising material for cancer vaccines.

CONCLUSION: We here demonstrate that LNP containing SS-OP stimulate cellular immune responses, in particular, remarkably high antigen-specific antibody responses and low adverse reactions. Moreover, the addition of SS-EC improved CTL activity and suppressed the tumor growth. Finally, we achieved the development of freezing LNP which can improve storage stability.

1153 Translational Potency of Double Stranded and Single Stranded mRNA In Vitro

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We have previously reported a novel form of double-stranded (ds) mRNA, possessing one million-fold greater metabolic stability compared to single-stranded (ss) mRNA (1). Importantly, ds mRNA was found to be translationally equipotent to ss mRNA in mouse liver when dosed hydrodynamically (1). However, other reports strongly suggest ds mRNA is lower in potency compared to ss mRNA (2,3). To reconcile these differences, we developed an in vitro transfection assay to compare the potency of ds and ss mRNA delivered by Lipofectamine LTX, Lipofectamine MessengerMAX, TransIT-mRNA, jetMessenger, and lipid nanoparticles (LNPs). Transfection of HEK 293T and HepG2 cells with ss and ds mRNA encoding luciferase established that ss mRNA was consistently 1000-100-fold more potent than ds mRNA regardless of transfection agent. The reason for this large discrepancy in transfection potency in vitro versus in vivo was investigated further to rule-out ds mRNA toxicity and ds mRNA self-knockdown by a siRNA mechanism. Since most in vitro transfections rely upon rapidly dividing cells, we investigated the cell seeding density and percent confluency which appears to play an important role in ss versus ds mRNA potency. When cells were seeded and allowed to achieve confluency, ss mRNA transfection potency decreased 1000-100-fold whereas ds mRNA was not affected. The results demonstrate that ds and ss mRNA LNPs are equipotent in non-dividing quiescent cells, which most closely reflects the in vivo environment. The finding that only ss mRNA potency is highly dependent on rapid cell division suggests two different classes of ribosomes may be involved in ss versus ds mRNA translation.

1. Poliskey, J. A., et al. Metabolically stabilized double-stranded mrna polyplexes. Gene Therapy, (2018) 25, 473–484.
2. Uchida S, et al. Designing immunostimulatory double stranded messenger RNA with maintained translational activity through hybridization with poly A sequences for effective vaccination. Biomaterials. (2018) 150, 162-70.
3. Tockary, T. A. et al. Comb-structured mrna vaccine tethered with short double-stranded RNA adjuvants maximizes cellular immunity for cancer treatment. PNAS (2023) 120, 1-11.

1154 Mannose-functionalized PEG-free LNPs targeting antigen-presenting cells for effective RNA delivery

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Lipid nanoparticles (LNPs) have emerged as a promising platform for nucleic acid delivery, particularly for messenger RNA (mRNA). LNPs are composed of ionizable lipids, helper lipids, cholesterol, and polyethylene glycol (PEG)-lipids in specific molar ratios. While PEG-lipids improve LNP stability and reduce immunogenicity, they may also provoke immune responses or hypersensitivity due to the presence of anti-PEG antibodies, highlighting the need for careful evaluation in clinical applications. Antigen-presenting cells (APCs) play a pivotal role in vaccines by processing and presenting antigens to T cells, thereby orchestrating adaptive immune responses. LNPs targeting APCs can significantly enhance vaccine efficacy by efficiently delivering mRNA to these cells. The mannose receptor on APCs is crucial for recognizing and internalizing antigens and thus serves as a target for LNP-based delivery systems. In this study, we introduce mannose-functionalized biodegradable polycarbonate-lipids, where the lipid component is either DTD (two-tailed alkyl group) (DTD-PTMCPMan) or cholesterol (Chol-PMan), as alternatives to PEG-lipids, and offer enhanced targeting capabilities. We synthesized a series of mannose-functionalized polycarbonate-lipids, and after in vitro screening, identified promising candidates, DTD-PTMC6PMan9 and Chol-PMan12. LNPs formulated with DTD-PTMC6PMan9 exhibited hydrodynamic sizes of approximately 170 nm with near-neutral zeta potentials, comparable to those containing ALC-0159 (a commercial PEG-lipid used in Comirnaty). For Chol-PMan12 LNPs, a lipid molar ratio adjustment to 1.0% (from 1.6% in ALC-0159 LNPs and DTD-PTMC6PMan9 LNPs) yielded particles with an average size of about 250 nm and neutral zeta potential. To evaluate their delivery performance, firefly luciferase (Fluc) mRNA was encapsulated in the LNPs and transfected into HEK293T and DC2.4 cells. After 48 hours, DTD-PTMC6PMan9 LNPs demonstrated transfection efficiencies that were 3-fold higher in HEK293T cells and 23-fold higher in DC2.4 cells compared to ALC-0159 LNPs. Similarly, Chol-PMan12 LNPs exhibited 2-fold and 9-fold higher transfection efficiencies in the respective cell lines. Notably, both DTD-PTMC6PMan9 and Chol-PMan12 LNPs showed negligible cytotoxicity, maintaining

cell viability above 80% relative to PBS-treated controls. In summary, we successfully replaced PEG-lipids with mannose-functionalized polycarbonate-lipids in LNP formulations. DTD-PTMC6PMan9 and Chol-PMan12 LNPs exhibited comparable physicochemical properties, significantly enhanced mRNA delivery efficiency, and excellent biocompatibility compared to ALC-0159 LNPs. These mannose-functionalized LNPs represent a promising approach for APC-targeted mRNA delivery in vaccine applications, addressing PEG-associated side effects while improving delivery specificity and efficiency.

1155 Oligo(EG)-functionalized biodegradable polycarbonate-lipids for efficient mRNA delivery with alleviated anti-PEG immunity

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Lipid nanoparticles (LNPs) are highly versatile non-viral delivery systems that can be utilized for encapsulation of messenger RNA (mRNA), with remarkable applications in both pre- and clinical research. Conventional LNP formulations comprise four components: ionizable lipids, helper lipids, cholesterol, and polyethylene glycol (PEG)-lipids. PEG-lipids are amphiphilic lipids, and largely determine the stability of LNPs and prevent aggregation. However, PEG-specific antibodies can be induced or boosted by repeated administration of PEGylated LNPs, leading to hypersensitivity reactions and diminished mRNA delivery efficacy. To mitigate this issue, we have synthesized a series of biodegradable and amphiphilic polycarbonates consisting of polycarbonate functionalized with oligo(ethylene glycol) (EG) and hydrophobic lipids to replace PEG-lipids in LNP formulations. The compositions of hydrophilic polycarbonates were screened, and DTD-P(MTC-MPEG3)$_{13}$, DTD-P(MTC-MPEG3)$_{23}$, DTD-P(MTC-MPEG9)$_4$, and DTD-P(MTC-MPEG9)$_7$, which had similar total EG units and molecular weights to the commercial PEG-lipid ALC-0159, were selected for LNP formulation at various molar ratios. Notably, DTD-P(MTC-MPEG9)$_4$ and DTD-P(MTC-MPEG9)$_7$ LNPs exhibited hydrodynamic diameters between 100 and 150 nm, neutral surface charge, and up to 8-fold improvement in transfection efficiency compared to ALC-0159 LNPs. When reduced the EG units in the side chains from 9 to 3, the hydrodynamic diameters of DTD-P(MTC-MPEG3)$_{13}$ and DTD-P(MTC-MPEG3)$_{23}$ LNPs exceeded 500 nm, accompanied by a sharp decline in encapsulation and transfection efficiency, despite their total EG units being comparable to those of DTD-P(MTC-MPEG9)$_4$ and DTD-P(MTC-MPEG9)$_7$. Further investigation demonstrated that DTD-P(MTC-MPEG9)$_4$ and DTD-P(MTC-MPEG9)$_7$, which exhibited superior performance, could be applied in combination with different ionizable lipids. These include the commercial lipids ALC-0315 and SM-102, as well as the in-house lipid APDEA, to formulate LNPs for mRNA delivery. In vivo studies revealed that DTD-P(MTC-MPEG9)$_7$ LNPs elicited lower levels of anti-PEG antibodies compared to ALC-0159 LNPs. Moreover, LNPs formulated with DTD-P(MTC-MPEG9)$_4$ which had a shorter polycarbonate chain showed even greater reduction in anti-PEG responses. In summary, the polycarbonate lipids functionalized with short EG units display exceptional potential, offering high transfection efficiency coupled with an improved safety profile. These attributes establish them as promising alternatives to the commercial PEG-lipids, with significant potential for efficient mRNA delivery across a range of therapeutic applications and clinical translation.

1156 Synthetic DNA for IVT mRNA production and delivery with Hermes™ lipopolyplex nanoparticles

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The large-scale manufacture of plasmid DNA using traditional fermentation methods has become a major bottleneck in the RNA therapeutics and vaccine industry with a worldwide lack or capacity and long lead times for GMP grade DNA. 4Basebio has developed a novel DNA IVT template, termed opDNA™, a linear molecule, covalently closed at the 5' end with a single-stranded hairpin, but open at the 3' end, allowing for use directly in an IVT reaction without prior linearisation or de-ending. Synthesis of opDNA™ is fully enzymatic, facilitating large-scale

production of linear DNA devoid of bacterial backbone with improved yields over traditional plasmid fermentation processes. The process is optimised to handle complex sequences, such as ITRs or long, continuous polyA tails >120 bp.

Here, IVT synthesis of mRNA using opDNA™ was compared to linearised plasmid across several constructs ranging from 1.5-12kb. mRNA delivery was assessed with Hermes™ nanoparticles, which exploit a cationic scaffold component, combined with traditional lipids, to drive payload condensation and provides the option to include a targeting moiety. The system was validated in a cancer vaccine setting, using mRNA encoding 20 neoantigens in the MC38 tumour model.

Maintaining polyA tail length in conventional plasmid DNA templates poses a major challenge due to instability in bacterial hosts. To confirm polyA tail length was maintained in opDNA™ during enzymatic synthesis, capillary gel electrophoresis and LC-MS were performed. In all constructs, a single polyA tail population of the expected length was identified with both methods. Next, IVT synthesis of mRNA was compared for opDNA™ and plasmid DNA templates. opDNA™ was found to achieve significantly higher yields than linearised plasmid, with plasmid requiring greater mass to achieve the same yield. At equimolar ratios, the kinetics of the IVT reaction were faster with an opDNA™ template compared to plasmid DNA. Capping efficiency, dsRNA impurities and RNA integrity were comparable between opDNA™ and plasmid for all constructs. Finally, when encapsulated in Hermes™ nanoparticles, mRNA encoding firefly luciferase from an opDNA™ template achieved comparable bioluminescence to linearised plasmid in HEK293T cells and in mice following intramuscular delivery.

Next, mRNA synthesized from opDNA™ was tested in a cancer vaccine setting. The rapid manufacturing timescale of synthetic DNA is particularly advantageous in this setting, considering that needle to needle time is paramount to clinical outcome. In a prophylactic protocol, mice were vaccinated intramuscularly at days 0, 14 and 28 with Hermes™ nanoparticles encapsulating mRNA encoding 20 neoantigens, and an MC38 tumour challenge was induced on day 52. The vaccine correlated with a significant reduction in tumour growth with 6/7 animals protected from the challenge and, a significant increase in CD8+IFNγ+ and CD8+TNFα+ T cells was observed. Similarly, in an early tumour treatment model where treatment began 2 days following tumour inoculation, mice treated with the Hermes-mRNA vaccine in combination with the αPD-L1 immune checkpoint inhibitor demonstrated a significant reduction in tumour growth compared to untreated animals or either Hermes-mRNA or αPD-L1 alone.

Therefore, IVT synthesis of mRNA from an opDNA™ template is both efficient and efficacious, lending itself to large-scale gene therapy or vaccine applications.

1157 Optimized prime: a transformative LNP composition for scalable RNA vaccines

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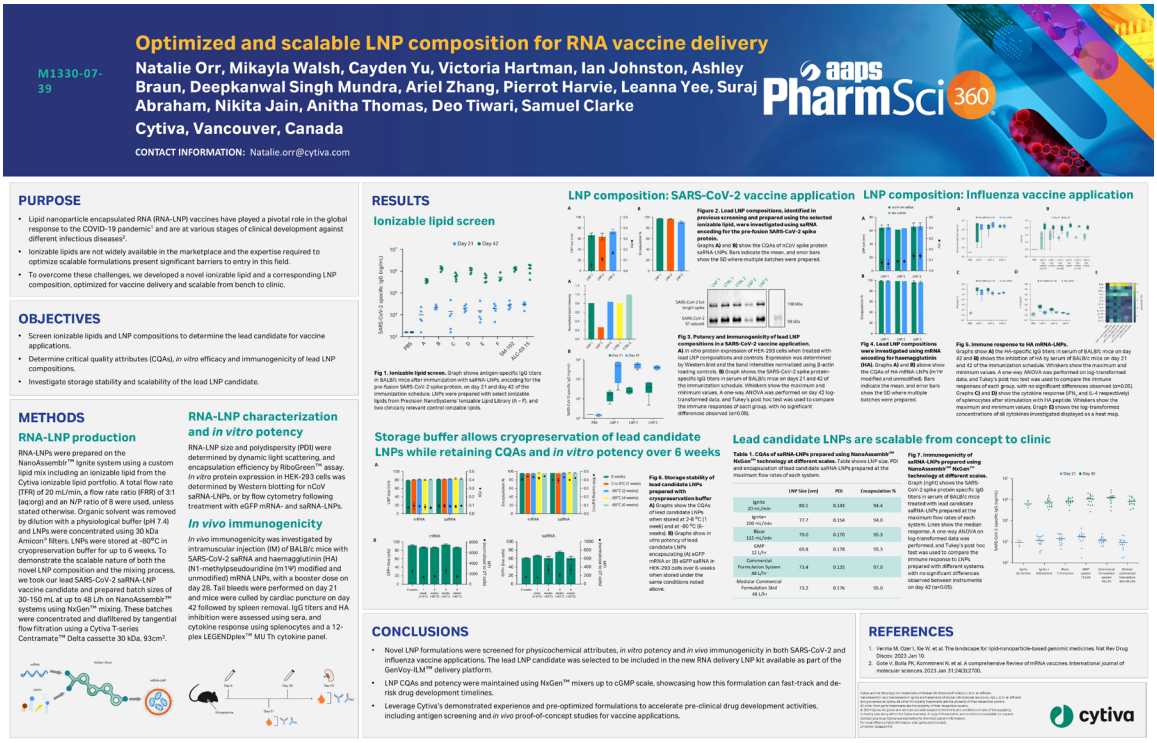
Purpose: Lipid nanoparticle-encapsulated RNA (RNA-LNP) vaccines have played a pivotal role in the global response to the COVID-19 pandemic (1). With proven safety and efficacy, RNA-LNPs are now at various stages of development against a variety of other infectious diseases (2). However, limited access to potent ionizable lipids, a key component in LNPs, and the expertise required to formulate and scale LNPs present real barriers to entry in this field. To overcome these challenges, we developed and optimized a novel ionizable lipid and a corresponding LNP composition for the delivery of RNA in vaccine applications. Using a scalable mixing platform, we formulated vaccine-candidate LNPs with both mRNA and self-amplifying RNA and investigated their physicochemical attributes and *in vivo* immunogenicity.

Methods: RNA-LNPs were prepared on the NanoAssemblr™ Ignite™ system, using a custom lipid mix including an ionizable lipid from our lipid portfolio, at a total flow rate of 20 mL/min. LNPs were diluted with a physiological buffer (pH 7.4), concentrated using 30 kDa Amicon® filters, and stored at -80°C in a cryopreservation buffer. RNA-LNP size and polydispersity were determined by dynamic light scattering and encapsulation efficiency by RiboGreen™ RNA assay. We determined *in vitro* protein expression in HEK293 cells by flow cytometry and we

investigated *in vivo* immunogenicity by intramuscular injection of BALB/c mice with RNA-LNPs, with a booster dose on day 28. Tail bleeds were performed on day 21 and mice were culled by cardiac puncture on day 42 followed by spleen harvesting. To demonstrate the scalable nature of both the novel LNP composition and the mixing process, we took our lead SARS-CoV-2 self- amplifying RNA-LNP vaccine candidate and prepared batch sizes of 100 mL at 48 L/h using the NanoAssemblr™ commercial formulation system.

Results: The novel LNP composition, combined with a scalable mixing process, resulted in high-quality RNA-LNPs with reproducible particle sizes of 60 to 90 nm, polydispersity indices < 0.2, and RNA encapsulation of > 80%. When stored in cryopreservation buffer at -80°C for 6 weeks (wk), we observed no detrimental effects on physicochemical attributes and *in vitro* potency. We completed *in vivo* immunogenicity studies using SARS-CoV-2 self-amplifying RNA and haemagglutinin mRNA-LNPs. A strong immune response was mounted against both antigens, where we observed antigen-specific IgG titers of 10^6 ng/mL, and IFNγ responses > 100-fold higher than naïve mice. Finally, we found that RNA-LNPs prepared on a commercially relevant scale were comparable to those prepared at the benchtop scale with respect to physicochemical attributes and immunogenicity.



1158 Using *in vitro* screening and *in vivo* biodistribution studies to determine endogenous tissue preference of LNP formulations to predict therapeutic potential

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Lipid nanoparticles (LNPs) have emerged as a promising non-viral delivery modality for cell and gene therapies due to their adaptability and chemical customizability. While there have been commercially successful uses of LNPs for vaccines and liver-targeted siRNA drugs, targeted delivery to extrahepatic tissues remains a challenge to expanding the development of this modality, and there is significant interest in understanding the inherent targeting capabilities of different LNP formulations. We screened different LNP formulations *in vitro* on several different cell lines and primary cell types and through iterative rounds of optimization we were able to identify multiple formulations sharing a common cationic lipid that are able to transfect primary T cells *in vitro*. We then performed an *in vivo* pilot

biodistribution study using four different LNP formulations with this cationic lipid and demonstrated that by changing one element in the LNP, in this case the PEG lipid, we can achieve vastly different biodistribution patterns illustrated through IVIS imaging. qRT-PCR analysis revealed that one formulation specifically enabled delivery of luciferase mRNA to the spleen and lymph nodes efficiently while keeping the expression in the liver about 20,000 fold lower, indicating the potential for extrahepatic delivery. Continued profiling of LNP formulations in this manner could lead to discovery of endogenous tissue selection without the necessity of an additional targeting ligand component and could aid in the selection of the most appropriate formulations for specific therapeutic strategies based on the organ or cell type of interest.

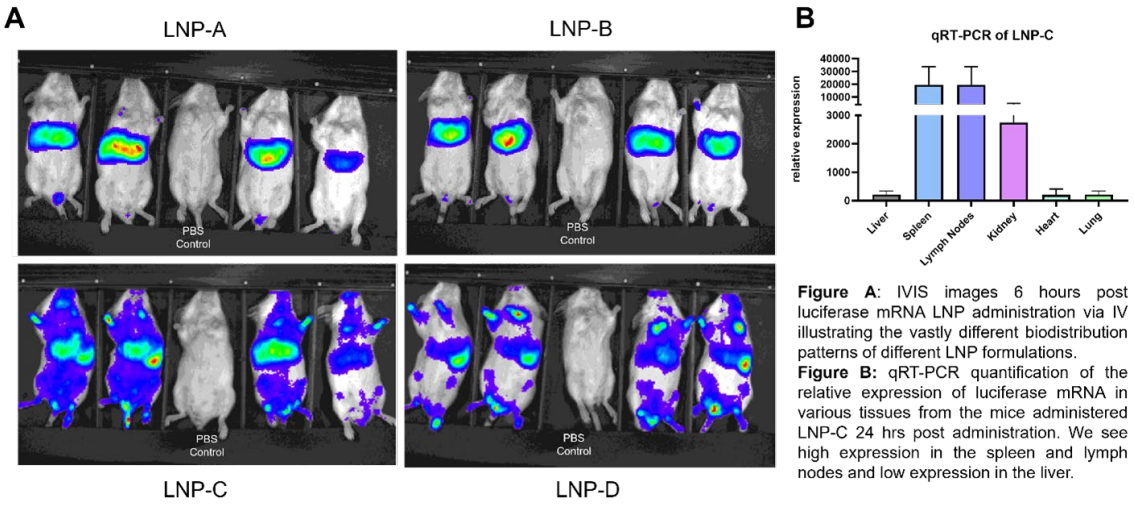

Figure A: IVIS images 6 hours post luciferase mRNA LNP administration via IV illustrating the vastly different biodistribution patterns of different LNP formulations.
Figure B: qRT-PCR quantification of the relative expression of luciferase mRNA in various tissues from the mice administered LNP-C 24 hrs post administration. We see high expression in the spleen and lymph nodes and low expression in the liver.

1159 High-Throughput Screening of Lipid Nanoparticles for Efficient CRISPR RNA Delivery *In Vitro* and *In Vivo*

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Introduction: Efficient and precise delivery of CRISPR/Cas9 components remains one of the critical challenges toward the advancement of gene-editing therapies. Here, we have developed a high-throughput Barcode-Integration Nanoparticle Screen (BINS) method to evaluate a library of 96 lipid nanoparticles (LNPs) for their delivery efficiency across *in vitro* and *in vivo* models.

Methods: For proof of concept, we designed a library of 96 LNPs made from 24 distinct ionizable lipids, without and with one of three different SORT (Selective Organ Targeting) lipids: cationic-SORT, anionic-SORT, and brain-targeting SORT. Each LNP encapsulated CRISPR/Cas9 mRNA, sgRNA, and a unique double-stranded oligonucleotide barcode. The pooled LNPs were delivered to retinal pigment epithelial (RPE) Cells and human skeletal muscle precursor cells (SkMPCs), and mice through *in vivo* systemic intravenous injections. Integrated barcode DNA was analyzed via PCR and Next-Generation Sequencing (NGS) to quantify integrated barcode, which reflects the delivery efficiency of the barcoded LNP.

Results: We observed different barcode integration profiles in different cells and tissues. Negative control LNPs without sgRNA showed no barcode integration in all cell/organs detected. Whereas many LNPs showed low or no barcode integration in all cell/tissues analyzed, LNPs with high barcode integration frequencies were found for RPEs, SkMPCs and different organs. These LNPs will be validated for their delivery efficiency to their respective target cells.

Conclusions: This work highlights the potential of the BINS method in screening for candidate LNPS for targeted RNA delivery. The screening of multiple ionizable lipids and SORT lipids together with barcoding-based tracking offers an opportunity to optimize LNP formulations for specific organ systems. The LNPs found this way will have translational values in precision medicine.

1160 Learn-Design-Make-Generate: ML Platform for Developing Novel LNP Delivery Systems

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Lipid nanoparticles (LNPs) are a preferred delivery vehicle for RNA therapeutics but face challenges in extrahepatic delivery. Companies often under-utilize data and rely on empirical trial-and-error. Machine learning (ML) can analyze these datasets, predict optimal formulations, and accelerate development.

To address these challenges, Mana.bio has developed an ML platform that predicts key LNP features to optimize particle composition and formulation. The platform leverages proprietary ML algorithms to predict key LNP characteristics, including physicochemical properties, cell/tissue targeting, and toxicity. It operates in an iterative Learn-Design-Make-Generate cycle, continuously improving LNP design through data-driven insights.
In the "Learn" phase, Mana.bio collects relevant data from public literature using proprietary software tools to automate data extraction. This dataset forms the foundation for initial prediction models. During the "Design" phase, the platform defines and generates a theoretical LNP space encompassing up to 100s of millions of in-silico compositions. A two-step screening process filters out LNPs that fail predefined criteria (e.g., size below 90 nm, PDI below 0.2, and positive zeta potential), and a multi-objective optimization process, where we optimize LNP predicted performance (e.g., low transfection efficiency in target cell lines, low cytokine levels, or reduced liver toxicity). Mana.bio's Freedom-to-Operate (FTO) tool integrates data from all relevant patents to ensure the selection of novel LNP components. Top-ranked candidates are selected for empirical tests in the lab based on component diversity. In the "Make" phase, LNPs are synthesized and encapsulated. During the "Generate" phase, physicochemical parameters and in-vitro activities are tested. Experimental results are stored in Mana.bio's database to continuously retrain the models, improving performance in each iteration.

A recently developed capability enhances the "Design" phase by enabling autonomous iteration of lipid design. Inspired by evolutionary methods, the model systematically mutates lipid structures, ranks the generated mutants, and advances the most promising candidates to subsequent cycles. This innovation accelerates the discovery of high-performance LNPs tailored for specific therapeutic applications. So far, across 120 cycles and more than 4,300 LNP formulations tested, Mana.bio has collected over 45,000 experimental data points and generated over 80,000 total data points. To evaluate our models, we apply 5-fold cross-validation. As for evaluation metrics, we use MAE and RMSE for regression models and ROC AUC for classification models. Notable examples include 1) PDI (polydispersity index), which achieved a 0.79 ROC AUC with a threshold of 0.2 for "good" LNPs, 2) particle size, where a regression model achieved an MAE of 27.95 and RMSE of 43.79, 3) transfection in non-activated primary T cells, where the model achieved an MAE of 4.32 and RMSE of 6.54, and 4) ALT and AST toxicity models, which achieved 0.8 and 0.82 ROC AUC, respectively with classification thresholds tailored by mouse strain and sex. These results underscore the platform's capacity to generate accurate, data-driven predictions, accelerating the discovery of safe and effective LNPs for diverse therapeutic applications.

1161 LNP-mRNA-Based Therapeutics for Genetic Mitochondrial Hepato-Encephalopathies

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Mitochondrial disorders represent a heterogeneous group of genetic conditions with a broad clinical involvement caused by mutations in mitochondrial DNA or nuclear genes encoding for mitochondrial protein. Among them, MPV17 mutations cause an early onset pathology classified in the subgroup of Mitochondrial DNA Depletion Syndromes, characterized by a significant decrease of the copy number of mitochondrial DNA in the affected tissues, mainly the liver and brain. Current treatments are mainly focused on symptom management. The objective of this study is to develop an mRNA-based therapy with the aid of novel LNP targeting mitochondrial disorders, specifically MPV17-related hepato-cerebral syndrome. This project aims to mitigate liver and central nervous system dysfunction stemming from oxidative phosphorylation assembly disruptions and mitochondrial DNA depletion in in vitro and in vivo models of the disease. Characterization of primary derived MPV17-mutated cell lines confirmed the typical features of the pathology prompting the possibility of testing the delivery of mRNA in vitro, alongside optimization of mRNA strategies through plasmid generation and transfection, evaluating efficiency and viability. In vivo experiments demonstrate the potentiality of lipid-encapsulated mRNA as an effective delivery system, with promising results indicating robust mRNA uptake in both liver and brain tissues of wild-type mice. Different routes of administration and dosage were tested to enhance distribution and expression in the target organs with the aim of shifting the more promising approach to the MPV17-KO mouse model. Overall, this data will offer new insight into the efficacy of a mRNA-LNP non-viral gene therapy, specifically focusing on its application in addressing multisystemic disorders, as the one caused by MPV17 mutations, holding significant promise for advancing therapeutic strategies for mitochondrial disorders.

1162 Conformation-adaptive Lipids for mRNA-Based Gene Editor Delivery via Lipid Nanoparticles (LNPs) in Non-Human Primates: Achieving a Wide Therapeutic Window

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Lipid nanoparticle (LNP)-mRNA therapies have demonstrated proven track record in infectious disease vaccines and are now advancing as a transformative platform for genetic medicine. However, gene editing via intravenous delivery demands substantially higher doses than vaccines, thereby requiring a delivery system with high efficiency, low toxicity, and minimal immunogenicity. Here, we introduce a series of "conformation-adaptive" ionizable lipids designed to enhance endosomal escape through pH-triggered changes in hydrogen bonding and molecular hydration. LNPs formulated with these lipids achieved significant editing of the transthyretin (TTR) gene in the liver of non-human primates following a single intravenous dose, leading to near-complete reduction in serum TTR protein at well-tolerated levels. To optimize tissue specificity, biodegradability, and high transfection efficiency, we screened over 200 preselected ionizable lipids formulated with luciferase mRNA, identifying multiple candidates displaying notably high luminescence intensity both in vitro cell tests and in vivo mice screening. Further in vivo studies in mice and rats confirmed favorable biodistribution and toxicity profiles, with top candidates—CX-322, CX-571, CX-108, and CX-601—showing >99% liver-specific distribution and no significant impact on body weight and liver panel parameters. When co-formulated with Cas9 mRNA and highly modified sgRNA, these LNPs resulted in >65% hepatic TTR gene editing at 0.6 mg/kg in mice, exceeding commercial benchmarks by over fourfold. In cynomolgus macaques, editing efficiencies of liver TTR gene surpassed 75% at 1.5 mg/kg for the CX-108 and CX-601 formulation, with the 3 mg/kg dose being well tolerated and a no-observed-adverse-effect level (NOAEL) established at 1 mg/kg. Stability assessments indicated consistent in vitro characteristics after six months at both –80°C and 4°C. Overall, these conformation-adaptive ionizable LNPs offer a potent, safe approach for in vivo CRISPR/Cas9-based genome editing, underscoring their potential as a clinical platform with a broad therapeutic window.

1163 Evaluation of lipid nanoparticles and polymersomes for gene editing in a mouse model.

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Introduction: Inherited monogenic diseases pose a significant clinical burden, many of which cause irreversible pathology in-utero or perinatally, which is a motivation for development of fetal gene editing therapy. Lipid nanoparticles (LNPs) and polymersomes are advanced nanocarriers that have gained significant attention in the fields of drug delivery and biomedical applications. LNPs are composed of lipid materials that form a spherical structure capable of encapsulating both hydrophilic and hydrophobic drugs, enabling controlled release and enhanced bioavailability. On the other hand, polymersomes are amphiphilic block copolymer-based vesicles that mimic the structure of natural lipid vesicles, offering improved stability and versatility in drug delivery as well as low immunogenicity compared to LNPs. In this study, we are evaluating a number of LNPs and polymersomes for the delivery of eGFP mRNA to hepatocytes after intravenous delivery into adult mice and mouse fetuses.

Methods: LNP were formulated using proprietary lipids, such as MC3 and KC2, as well as non-proprietary lipids, such as DOTAP. LNPs contained eGFP mRNA. Briefly, an ethanol phase (containing the LNP components) and an aqueous phase (containing that mRNA to be packaged) are combined via channels in a microfluidic device and subsequently dialysed and purified against PBS and passage through a 0.2µm filter. Polymersomes were also prepared by solvent dispersion method, followed by extrusion to obtain uniform size distribution. After eGFP mRNA encapsulation, the non-encapsulated mRNA was removed by dialysis. Following formulation, the LNP (n=4) and polymersomes were characterised and encapsulation efficiencies determined prior to use in *in vitro with mouse AML cell lines* and *in vivo* studies with both adult NSG mice and C57BL/6 mouse fetuses. six-weeks old NSG mice were injected intravenously (0.5mg/kg,) via the tail vein with the various nanoparticles. Fetal (E15-16) C57BL/6 mouse were also injected via the intravitelline vein (0.15-1.5mg/kg). After 24h, liver cells were isolated from the animals using collagenase digestion, spleen and bone marrow were analysed for the expression of GFP via flow cytometry. Percentage GFP and MFI in the various tissues were determined and compared.

Results: Four different LNP were compared (LNPA, LNPB, LNPC, LNPD), along with polymersomes. In mouse AML cells, the nanoparticles that were found to be able to transfect more than 50% of the cells (100ng/ml) were selected for further in vivo studies. In adult mice, the highest rate of transduction with GFP was with LNPA (80%, MFI 80k) and the lowest with LNPD (25%, MFI 34k). For comparison, polymersomes achieved 43% transduction, MFI 42k. The GFP transduction is quite specific to hepatocytes as there was <10% transduction in the bone marrow or spleenic CD45+ cells. The results are also similar in the mouse fetus with LNPA (60%, MFI 150k) producing the highest transduction rate in liver cells, and LNPD the lowest (20%, MFI 20k). The polymersomes also managed to transduce of the liver cells in both the adult (40%, MFI 40k) and fetal mice (40%, MFI 175k) with much more specificity to liver cells compared with LNP A (<5%).

Conclusion and future work: We identified two nanoparticles LNP A and polymersome that have the potential to conduct further studies with gene editing in utero. These nanoparticles will be packaged with base editors for future liver gene editing work.

1164 Delivery, Potency and Tolerability of Lipid Nanoparticle Formulations of mRNA in PXB-Mice

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Lipid nanoparticles (LNP) enable effective delivery of RNA-based therapeutics and have been clinically validated in approved intravenous products, including ONPATTRO®. Upon entry into the systemic circulation, LNP bind circulating apolipoprotein E (apoE) and interact with low-density lipoprotein receptors to facilitate their uptake into hepatocytes. Given their efficacy, low immunogenicity relative to other delivery vectors, and versatility in encapsulating a wide range of therapeutic modalities, there has been extensive adoption of LNP technology in the development of nucleic acid-based therapeutics, with increasing application in the areas of gene editing and gene modulation. A major challenge in evaluating the efficacy and tolerability of LNP-based therapeutics is the availability of suitable preclinical models. Notable limitations of traditional mouse models include species-specific differences in physiology, drug metabolism and excretion, and limited utility for therapeutics with human-specific targets or diseases with restricted tropism. More human-relevant preclinical models have emerged, which may help bridge the translational gap. Humanized liver chimeric mice, such as PXB-mice, have livers that are repopulated with functional human hepatocytes. Their livers express key transporters and metabolic enzymes, and unlike conventional mouse models, they have human-like lipoprotein profiles. These, along with other features, better represent key aspects of human liver physiology and function. Here, we evaluated the delivery, distribution, potency and tolerability of three different LNP in PXB-mice to characterize these mice as a model for LNP-mediated delivery and activity. Mice were injected intravenously with different LNP (identified as LNP07, LNP09 and LNP13) encapsulating IgG mRNA, and their relative potency was assessed by serum IgG expression. LNP13 exhibited the greatest activity of the three LNP and an expanded dose titration was performed, with doses up to 5.0 mg/kg being tolerated with no observed clinical signs. Interestingly, immunohistochemistry staining of liver sections from LNP-treated mice revealed earlier IgG protein translation in mouse hepatocytes; however, this differential in expression relative to human hepatocytes was reduced at later time points. To further investigate this, we performed pharmacokinetic analyses and MALDI mass spectrometry imaging (MSI) to visualize and quantify the distribution of LNP-derived ionizable lipid in LNP-treated mouse livers. MSI revealed extensive LNP distribution throughout the liver across both mouse and human compartments. This was corroborated by RNA in situ hybridization, which similarly showed widespread mRNA distribution at early time points post-dose. Altogether, these data suggest a potential delay in the uptake and/or expression of mRNA-LNP in human hepatocytes. Despite this, PXB-mice (and other liver-humanized mice) represent a promising preclinical model for mRNA-LNP testing and may be particularly useful in characterizing the biology of human-specific payloads when using highly active LNP.

1165 *In Vivo* Gene Editing and Disease-Associated Biomarker Reduction for Multiple Liver Targets in Non-human Primate Using AsCas12a Nuclease Delivered by LNP

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CRISPR-Cas gene editing is a transformative technology with the potential to enable durable treatments for many diseases with unmet medical need. Clinical success of this technology has been demonstrated with *ex vivo* and *in vivo* gene therapies. E*x vivo* gene therapies are costly and can have a long and burdensome patient journey and manufacturability challenges, which have hindered their widespread adoption for treating genetic diseases. In recent years, *in vivo* delivery of CRISPR-Cas using lipid nanoparticles (LNPs) has shown promising preclinical and clinical data, but most studies utilize the Cas9 nuclease. AsCas12a is a highly efficient and specific nuclease that targets a different protospacer adjacent motif sequence (TTTN) which could be advantageous for some targets. To date, *in vivo* gene editing for liver targets using AsCas12a has not been demonstrated. Here, we report the proof-of-concept results from the first *in vivo* mouse and non-human primate (NHP) studies showing high levels of target gene editing in the liver and corresponding biomarker response following intravenous administration of AsCas12a

mRNA and chemically modified guide RNAs delivered using LNPs. Using two different LNPs from a highly potent LNP platform, we demonstrate efficient target editing in the liver and corresponding reduction of target biomarker in the serum. Additionally, biodistribution properties of these novel LNPs were assessed using a GFP reporter mRNA. We observed high levels of GFP mRNA and corresponding GFP protein in the liver with minimal to no GFP mRNA and corresponding protein detected in other non-target tissues. Specifically, we detected minimal to no signal for both mRNA and corresponding protein in the ovary. Furthermore, we demonstrate the translatability of *in vivo* gene editing and biomarker knockdown across *in vitro* and *in vivo* preclinical models, including primary human hepatocytes, humanized mice, and NHPs. Our data highlights the ability to achieve high levels of *in vivo* gene editing and disease-associated biomarker knockdown using a 'plug-n-play' strategy that permits cargo components to be easily interchanged when delivered with this proprietary LNP platform. This strategy enables the rapid identification of efficacious combinations to build a preclinical pipeline and advance novel, *in vivo* genetic medicines to address serious genetic diseases.

1166 CRISPR/Cas9 Genome Editing Using Lipid Nanoparticles for Hepatocellular Carcinoma Therapy

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Hepatocellular Carcinoma (HCC) is the 6th most common cancer and one of the most lethal worldwide. The number of cases keep increasing yearly, and current predictions are that HCC mortality will reach 1 million annual deaths by 2030. As of today, HCC remains resistant to most therapies, including new immunotherapy regimens, which often achieve only a few months of progression-free survival. Therefore, new therapeutic approaches are necessary, with gene editing being a promising direction. Gene editing is a method in which engineered nucleases are utilized to induce double-stranded breaks at specific loci to introduce genomic modifications. One of the most common nucleases currently used in gene editing is the CRISPR/Cas9 system. In the case of cancer, CRISPR/Cas9 can be used to knock out a critical gene and lead to cancerous cells` death. In this work, multiple genes were tested as potential targets, with all sgRNAs leading to high levels of editing. The screening process highlighted that PLK1 is the best candidate as it showed ~65% reduction in viability following the editing process. Next, various lipid nanoparticle formulations were tested to determine the best one at delivering mRNA to the tumor cells via GFP measurements. The chosen formulation was then used to co-encapsulate Cas9 mRNA with PLK1-targeting sgRNA, showing above 90% editing and above 90% reduction in viability in HCC cells, highlighting the system's potential. Next, when testing LNPs' biodistribution in an orthotopic xenograft model, the LNPs showed ~12% penetration into the tumor. Finally, we are set to start a final efficacy experiment in an animal model to test both editing of the tumor and the effects on tumor development. It is our belief that this work may open up a new avenue of treatment for HCC, and potentially one day make the leap from the bench to the bedside.

1167 Apolipoprotein Fusion Enables Spontaneous Functionalization of mRNA Lipid Nanoparticles with Antibody for Targeted Cancer Therapy

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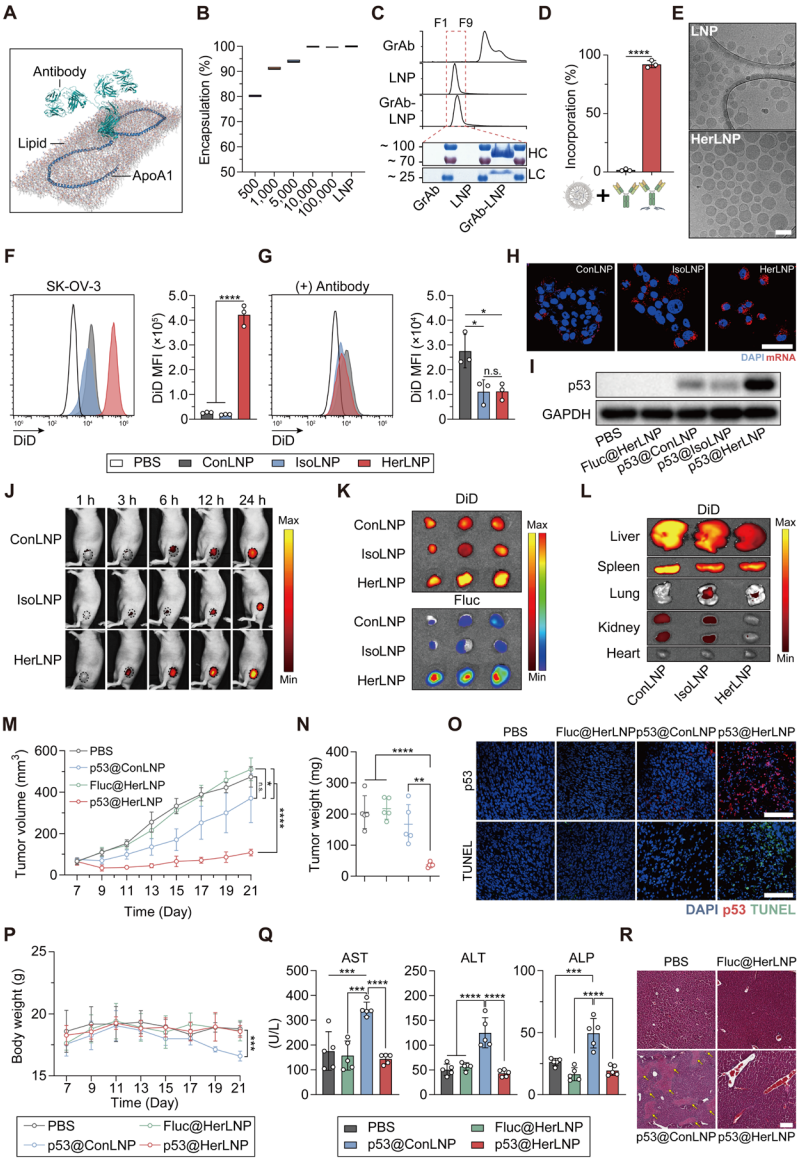
Introduction: The clinical success of mRNA@lipid nanoparticle (LNP) vaccines, especially during the COVID-19 pandemic, has highlighted the broad therapeutic potential of mRNA for various diseases. While LNPs provide effective protection against enzymatic degradation and facilitate cellular uptake of mRNA, limitations such as non-specific tissue distribution and unintended liver accumulation have constrained their clinical utility. Passive targeting approaches often rely on the enhanced permeability and retention (EPR) effect and can fall short in achieving precise cell specificity. Although antibody-mediated active targeting can address this issue, existing

chemical conjugation strategies typically involve multi-step reactions, toxic reagents, and PEG detachment problems, prompting the need for simpler, more stable alternatives. In response, we developed a chemical-free platform by fusing an apolipoprotein (ApoA1) directly to an antibody—termed "Grab Antibody" (GrAb). Owing to the strong lipid-binding affinity of ApoA1, GrAb spontaneously assembles into a stable protein corona on the LNP surface, maintaining appropriate antibody orientation. Using trastuzumab as a model antibody, we demonstrate that HER2-targeted GrAb-LNPs can efficiently deliver p53 mRNA to HER2-overexpressing tumors, yielding significant antitumor effects with minimal off-target toxicity.

Results: A 30-minute incubation at 37°C allowed GrAb to form a robust protein corona on the LNP surface (Fig. 1A). Modulating the lipid-to-protein (LP) molar ratio from 500:1 to 100,000:1 revealed a drop in mRNA encapsulation efficiency at higher GrAb levels, but negligible leakage once the ratio exceeded 10,000:1 (Fig. 1B). Size-exclusion chromatography (SEC) showed that GrAb strictly co-eluted with LNP fractions (Fig. 1C), and fluorescence measurements confirmed high incorporation of Cy5-labeled antibody (Fig. 1D). Cryo-TEM images of nude LNPs and trastuzumab GrAb-conjugated LNPs (HerLNPs) appeared similarly spherical (Fig. 1E). In HER2-overexpressing SK-OV-3 cells, HerLNPs exhibited selective binding and increased DiD fluorescence signals (Fig. 1F), which were abolished when HER2 receptors were pre-blocked (Fig. 1G). Intracellular uptake of Cy5-labeled mRNA was notably higher with HerLNPs (Fig. 1H), leading to greater p53 protein expression *in vitro* (Fig. 1I). *In vivo*, intravenous injection of DiD-labeled Fluc mRNA@HerLNPs led to superior tumor accumulation and reduced liver uptake, as shown by near-infrared fluorescence imaging (Fig. 1J, 1K, 1L). Repeated dosing with p53@HerLNPs significantly suppressed tumor growth (Fig. 1M, 1N) and induced marked apoptosis (Fig. 1O) in an SK-OV-3 xenograft model. Importantly, treated mice maintained stable body weights (Fig. 1P) and showed normal levels of liver enzymes (Fig. 1Q), indicating minimal systemic toxicity. Histological examination confirmed the absence of hepatic damage in HerLNP-treated animals, whereas mice receiving p53@ConLNPs displayed evident liver injury (Fig. 1R). Together, these findings validate GrAb-LNPs as an effective, targeted mRNA delivery platform offering enhanced therapeutic efficacy and reduced off-target effects in cancer treatment.



1168 Innovative Cationic Lipids Fuel Next-Gen LNPs for Targeted Oncology and Advanced Cell Therapies

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Lipid nanoparticles (LNPs) have shown promising potential in organ selective targeted -Oncology and Gene Modified Cell Therapies; however, the critical challenge lies in fine-tuning their formulation to enhance specificity, efficiency, and safety, to maximize therapeutic outcomes.

Here, we present a novel approach through the development of lipid nanoparticles (LNPs) derived from our library of Innovative cationic lipids. These LNPs demonstrate expanded extrahepatic biodistribution, improving drug delivery and cellular targeting. This addresses major challenges in cancer treatment for the development of therapeutic vaccines via targeting antigen presenting cells (APCs) and T cells in the spleen, and organ selective

oncology treatments via local injection. Optimization of LNP formulations around our cationic lipids leads to localized expression without off-target effects unlike commonly used ionizable lipid-based LNPs.

In addition, we investigate ex vivo cell engineering, employing LNPs. We highlight the efficacy and versatility of LNPs based on our library of lipids, which enable the transfection of mRNA in different types of immune cells, leading to transient engineering of CAR-T and NK cells. This data suggests that LNP formulations based on our cationic lipids, with the addition of targeting ligands, could also prove effective for the generation of CAR-Ts and NKs in vivo. The data underpinning these applications were collected through a blend of proof-of-concept studies conducted both in vitro and in vivo, aimed at evaluating the efficacy and safety of imidazolium lipid-based LNP systems. Molecular assays were employed to monitor biodistribution and cellular uptake, offering real-world insights with an emphasis on safety and dosage optimization.

1169 Delivery of synthetic DNA using Lipopolyplex nanoparticles: a thermostable alternative to mRNA-LNPs

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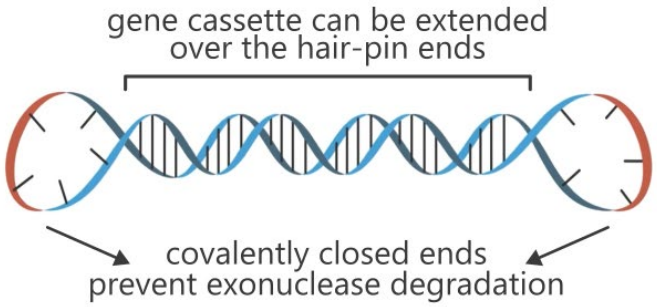
mRNA lipid nanoparticles (LNPs) have been hugely successful, but issues remain with mRNA stability and cold chain storage requirements, as well as bottlenecks in the GMP manufacture of DNA IVT templates. 4basebio has developed a fully enzymatic, GMP-compliant synthesis process of linear DNA via our Trueprime™ amplification technology. DNA yields are >1g/L, several orders of magnitude higher than plasmid fermentation, allowing a small footprint using benchtop equipment. The process utilizes isothermal multiple displacement amplification of circular templates. Uniquely, 4basebio's circular templates do not contain typical plasmid backbone features, including antibiotic resistance genes. Combination of sequence-agnostic proprietary primase and engineered, high-fidelity Phi29 DNA polymerase can amplify DNA with high accuracy ($3.75x10^9$), accommodating construct size up to 60kb. The platform is optimized to handle high complexity sequences including high GC regions (up to 80% GC content across 2kb regions), homopolymer regions including poly-Adenine and poly-Guanosine regions and, palindromic sequences such as ITR and recombinase sites. Unique modifications, including fluorescent tags or functional polymer conjugates, can be incorporated. Here, Hermes™ nanoparticles comprising a cationic ligand, as well as traditional lipids, driving payload encapsulation and formulation stability, were optimised for delivery of 4Basebio's hairpin (hp)DNA™. Interestingly, lipid compositions optimal for mRNA delivery demonstrated poor formulation stability with DNA payloads. Using a DOE approach, hpDNA™ formulations with favourable biophysical characteristics, encapsulation efficiency and in vitro transfection rates were identified. In mice, intramuscular delivery of luciferase hpDNA™ demonstrated >1-log improvement in bioluminescence with the optimised formulation over a conventional LNP formulation. Moreover, expression was greater in the hpDNA™ group, compared to plasmid DNA groups at equimass and equimolar dosing, demonstrating the merit of combining both 4basebio's DNA and Hermes™ platforms. The storage stability of DNA in liquid buffers offers a huge advantage over mRNA. To investigate hpDNA™ thermostability, Hermes™ formulations encapsulating an eGFP hpDNA™ were stored at 4°C or -80°C for 3 months. Biophysical characteristics were maintained for Hermes™ formulations over the duration, which was driven by inclusion of the Hermes™ ligand, and a consistent transfection efficiency of >85% was observed in HEK293T cells. Further, no difference in hpDNA™ integrity was noted between 0 and 3 months, or between 4 or -80°C, as determined by capillary electrophoresis. Finally, to evaluate therapeutic potential, hpDNA™ encoding 20 neoantigens was delivered intramuscularly with the optimised Hermes™ formulation, or with electroporation, in the MC38 tumour model. Three doses of Hermes™ 28-days apart prevented development of tumours in 2/7 mice following a tumour challenge at day 52, which was comparable to the electroporated hpDNA™. Furthermore, survival rate was significantly greater in Hermes™ treated mice compared to untreated mice; 71% of Hermes™ or electroporated hpDNA™ treated mice compared to 0% in the untreated group. Therefore, combining the fast, scalable manufacture of 4Basebio's synthetic DNA with the DNA delivery capabilities of Hermes™ lipopolyplex nanoparticles, can offer an effective, thermostable alternative to mRNA-LNP for gene therapy and vaccines.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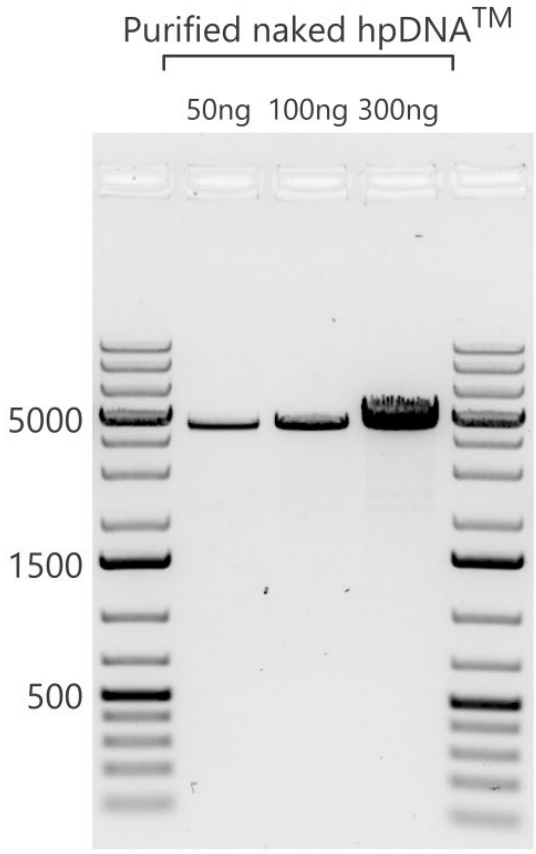
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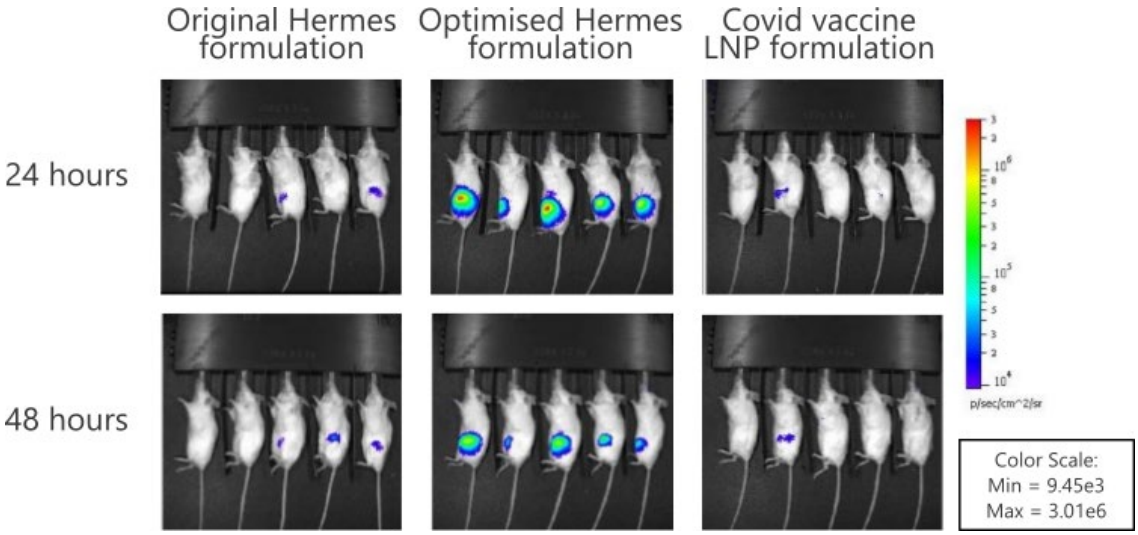

1170 A Non-integrating, Redosable, Non-viral DNA Vector Platform for Highly Potent, Durable, and Non-inflammatory Gene Transfer

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Efficient and safe delivery of genetic material into cells is critical for translating gene therapy into clinical practice. Recent advancements in molecular biology and nanotechnology have significantly contributed to the development of non-viral vector systems and DNA delivery technologies, which serve as promising alternatives to virus-based gene delivery. While non-viral vectors have several advantages, there are critical issues currently limiting their translation into clinical success. Here we describe a novel circular DNA molecule (HALO™ DNA) with unique structural DNA elements designed to favor nuclear uptake, nuclear retention, and immunomodulation, which greatly increases the efficiency, durability and redosability of gene delivery in vivo. Following intravenous delivery to a mouse of lipid nanoparticle (LNP)-formulated HALO DNA encoding cynomolgus macaque tissue non-specific alkaline phosphatase (cmTNALP), a surrogate of a therapeutic transgene for the treatment of hypophosphatasia, we demonstrate greater than 100-fold supraphysiological levels of plasma ALP activity that remains remarkably stable for more than a year. Potency, at least in part, is mediated by increased nuclear uptake as evidenced by quantitative confocal imaging of HALO DNA in primary human hepatocytes. HALO DNA integration site analysis (ISA) performed by target enrichment sequencing (TES) failed to detect evidence of integrated HALO DNA in liver cells even at very high vector copy numbers and over a year following in vivo DNA administration. Surprisingly, the structural elements present within HALO DNA are also able to modulate immune responses to the transgene product and allow redosing. Indeed, expression of cmTNALP in a mouse using a DNA construct lacking these structural elements was limited and highly inflammatory which prevented re-administration. However, repeat administration of LNP-formulated HALO DNA was non-hepatotoxic, as assessed by evaluation of ALT/AST levels, and non-inflammatory, as assessed by evaluation of a panel of pro-inflammatory cytokines, and led to stable gene expression. Importantly, this immunomodulatory effect is irrespective of the presence of pathogen-associated molecular patterns (PAMPs) such as CpG elements, or selection markers, within the DNA. Using an RNA-Seq based transcriptomics approach following delivery of DNA to mouse liver in vivo, we show that standard mini-circle DNA induces changes in the expression of many genes involved in immune responses, cytokine production pathways, and DNA repair. However, HALO DNA is largely silent, impacting only a small number of genes and to a significantly lesser extent. The primary determinant of this immunomodulatory phenotype appears to be the presence of the unique structural elements within HALO DNA. Finally, HALO DNA is easily produced in a scalable, low-cost manner, providing additional promise for the continued development of this differentiated non-viral DNA vector system.

1171 Simplifying Multigene Vector Design and Construction for Streamlined Development of Complex Therapeutics

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Molecular therapeutics have revolutionized personalized medicine by offering long-lasting, low-risk alternatives to traditional treatments. Many fatal diseases, including heart disease, neurodegeneration, and cancer involve multiple, complex regulatory pathways that necessitate multigenic therapeutic approaches rather than conventional monogenic solutions for molecular therapy. However, the development of multigenic vectors is often hindered by high costs, lack of precision, off-target effects, and tedious troubleshooting of vector design and construction. Furthermore, the complexity and size of multigenic vectors is challenging for modern DNA synthesis techniques, so traditional time-consuming and cumbersome cloning techniques are necessary for creating recombinant multigenic vectors. To address these challenges, Osprey Bio's Bird of Prey complex vector system provides a standardized, cost-effective method for tunable multigenic vector assembly using modular genetic units, making multigenic vector design as simple as traditional single gene vector design. In this study, we leveraged the Bird of Prey vector construction system in conjunction with Zymo Research's highly efficient cloning technology to construct a quadgene vector incorporating four distinct reporter genes: *rLUC, fLUC, GFP,* and *RFP*. To optimize vector assembly, a DNA gel extraction system with micro-elution spin columns was employed to easily recover highly concentrated, ultra-pure restriction digested DNA inserts suitable for cloning. The ligated vectors were then transformed into specially prepared competent DH10B *E. coli* cells that do not require heat shock, enabling efficient propagation of the recombinant plasmids. Highly concentrated, endotoxin-free plasmid was quickly purified from the transformed *E. coli* using a novel high plasmid binding spin-column purification chemistry to ensure all recombinant plasmids were suitable for transfection. Finally, to assess vector functionality and subcloning success, individual monogenic, bigenic, and final quadgenic vectors were transfected into HEK293T cells and reporter gene expression was quantified using fluorometric and luciferase assays. Results confirmed successful subcloning of all genes of interest, with no observable decrease in gene expression or transfection efficiency as the number of genes per vector increased. These findings demonstrate the adaptability and efficiency of the Bird of Prey subcloning system for creating multigenes when combined with Zymo Research's robust cloning technologies. By significantly streamlining multigenic vector construction, this approach enables efficient testing and development of multigenic therapeutic solutions, facilitating advancements in gene and cell therapy.

1172 Enhanced Yield and Structural Integrity in Plasmid Biomanufacturing Employing the PowerS-ITR E. coli Platform

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Introduction: As gene therapy and synthetic biology continue to advance, the need for effective plasmid production systems has grown significantly. These systems must address challenges like maintaining high yields and ensuring molecular stability. The PowerS-ITR E. coli cell line emerges as a transformative platform, purpose-engineered to support the propagation of low-copy plasmids with structurally intricate elements such as Inverted Terminal Repeats (ITRs) and some PolyA tails. This investigation delineates the capabilities of PowerS-ITR in optimizing production workflows and achieving consistency in plasmid biogenesis.

Methods:
1. **Cell Line Engineering**: The PowerS-ITR strain was cultivated under rigorously optimized parameters to enhance replication fidelity and expression capacity.
2. **Plasmid Construct**: A prototypical low-copy plasmid harboring a PolyA tail and ITR sequences was synthesized to serve as a benchmark for experimental validation.

3. **Optimization Screening**: High-throughput methodologies were employed to scrutinize plasmid yield variations across a spectrum of growth media and environmental conditions.
4. **Quantitative Metrics**:o Plasmid yield quantified through spectrophotometric analysis and agarose gel electrophoresis. o Structural integrity evaluated via restriction endonuclease profiling and next-generation sequencing.5. **Benchmarking**: Comparative analyses were performed against the widely utilized E. coli Stable and JM108 strains, facilitating a comprehensive assessment of relative performance indices.

Results: Initial findings underscore the PowerS-ITR cell line's superior performance, as evidenced by:

- A statistically significant 2-fold increase in plasmid yield relative to standard strains.
- Exceptional maintenance of plasmid structural fidelity, with negligible recombination or degradation artifacts which may be present in the comparative strains.
- Demonstrated scalability in bioreactor systems, ensuring reproducibility across production batches.

Conclusion: The PowerS-ITR platform offers meaningful improvements in plasmid production, addressing key limitations of traditional methods. By delivering amplified yields and ensuring sequence stability, it meets the stringent demands of modern biopharmaceutical applications. Ongoing research will prioritize refining production conditions, scaling to high-capacity bioreactors, and extending the evaluation to encompass diverse plasmid architectures with therapeutic relevance.

Keywords: PowerS-ITR, E. coli, plasmid biomanufacturing, low-copy plasmid, PolyA tail, ITR sequences, yield optimization, structural fidelity, gene therapy

1173 Incorporation of 5-methoxyuridine Enables Efficient Targeted Transgene Insertion in iPSCs and T Cells

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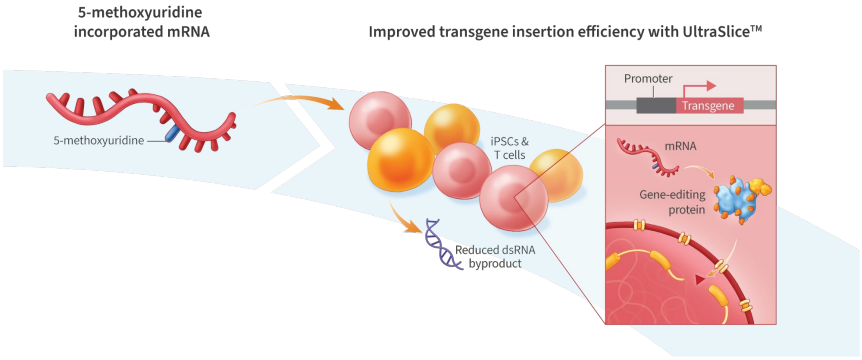
mRNA-based gene editing allows transient expression of editing proteins, enabling efficient on-target editing while minimizing off-target effects. mRNA synthesized by *in vitro* transcription (iVT) can contain immunogenic double-stranded RNA (dsRNA) byproducts, which can trigger a potent innate immune response. Mutant T7 RNA polymerases (mT7) and modified nucleotides (NTs) are being explored as strategies to mitigate dsRNA production during iVT. Since mRNA-mediated gene-editing relies on the cell's DNA-repair machinery to complete the edit, we hypothesized that dsRNA-derived immune responses could limit gene-editing efficiency. We synthesized UltraSlice™ gene-editing mRNA with combinations of mT7s and modified NTs and measured targeted insertion efficiency and immunogenicity in T cells and induced pluripotent stem cells (iPSCs).

We first analyzed dsRNA byproduct, immunogenicity, and protein expression of GFP-encoding mRNA. RT-PCR of innate immune markers (OAS1, OAS2, MDA5) in electroporated iPSCs and iPSC-derived mesenchymal stem cells (iMSCs) showed that the mRNA synthesized with standard conditions (standard T7 and canonical (AUGC) NTs) resulted in the greatest immune response ($p<0.01$ for all markers) compared to samples synthesized with mT7 and/or 5-methoxyuridine (5moU/mT7+5moU). dsRNA dot blot confirmed that the standard synthesis condition also resulted in approximately 10x more dsRNA compared to mT7. When iPSCs and iMSCs were electroporated with GFP mRNA, all synthesis conditions resulted in similar percentages of GFP-expressing cells. However, twice the median fluorescence intensity (MFI) was observed in both cell types for mRNA synthesized using mT7 or 5moU.

To assess the effect of dsRNA on targeted insertion efficiency, UltraSlice™ mRNA targeting the AAVS1 safe harbor and T Cell Receptor Alpha Constant (TRAC) loci were synthesized using standard conditions, mT7, 5moU, and mT7+5moU. PBMC-derived T cells were co-electroporated with TRAC UltraSlice™ mRNA and a linear GFP transgene. 5moU mRNA more than doubled the number of GFP-expressing cells compared to mRNA made with standard conditions (17.6% vs 6.71%), while mT7 and mT7+5moU had less of an effect (12.7% and 8.93%, respectively). These results suggest that incorporation of 5moU into iVT mRNA reduces immunogenic dsRNA

byproducts during iVT and enables efficient targeted insertion of transgene sequences. 5moU-containing mRNA may therefore serve as a useful tool for the development of both *ex vivo* and *in vivo* gene editing therapies.



1174 TREX1 Knockout Does Not Improve Gene-Length Insertions Using Single-Stranded DNA Templates

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CRISPR/Cas9-based genome editing offers a promising approach for treating genetic disorders by modifying DNA sequences or regulating gene expression. Upon cleavage by Cas9, the homology-directed repair (HDR) pathway could be leveraged to generate precise modifications at a target locus in the presence of an exogenously introduced repair template. While adeno-associated virus (AAV) has been the gold-standard template for gene editing, P53 activation by AAV reduces the regenerative potential of some edited stem cells. There is an interest in non-viral single-stranded DNA (ssDNA) templates because they induce less P53 activation. However, the degradation of ssDNA within the cells by intracellular nucleases limits the efficiency of gene insertion using ssDNA templates.

Previous studies found that a single exonuclease, three prime repair exonuclease 1 (TREX1), reduces HDR efficiency using ssDNA. However, the study only included short ssDNA (<200 nt) and it was unclear if blocking the activity of TREX1 is essential for replacing complete genes using ssDNA templates. While short ssDNAs can correct point mutations, gene-length ssDNAs templates needed for whole gene replacement are >2 kb long. Here, we investigate gene insertion using ssDNA >2 kb in wild-type and TREX1 knock-out primary airway basal stem cells (ABCs), human umbilical vein endothelial cells (HUVECs), and induced pluripotent stem cells (iPSCs).

While editing with ssDNA <200 nt, TREX-KO ABCs, and HUVECs show significantly higher gene insertion (knock-in %) compared to WT cells (Figure 1A). This result confirms the findings of previous studies. To evaluate the effect of TREX1 KO on gene insertion by long ssDNA, we designed a 2297 nt template to insert expressing green fluorescent protein (GFP) to Exon1 of the CFTR gene. We then evaluated gene insertion in WT and TREX1 KO ABCs and HUVECs. Surprisingly, no difference in editing efficiency was observed between wild-type and TREX-KO cells (Figure 1B). These results suggest that while TREX1 degrades ssDNA <200 nt and significantly decreases the editing efficiency, gene-length ssDNA templates are not degraded by TREX1. To further verify the role of TREX1 in reducing gene insertion using gene-length ssDNA, we edited iPSCs, which do not express TREX1 naturally. Surprisingly, GFP insertion in WT iPSCs is significantly higher (~ 26% GFP+ cells) than in ABCs and HUVECs (~ 5% GFP+ cells) (Figure 1C). These findings indicate that a factor distinct from TREX1 (present in ABCs and HUVECs and absent in iPSCs) is likely responsible for restricting gene insertion. Future studies will investigate alternative factors that limit gene insertion by ssDNA >2kb in ABCs and HUVECs. We believe this study would help to improve our understanding of CRISPR-mediated gene editing systems using long ssDNAs to replace whole genes.

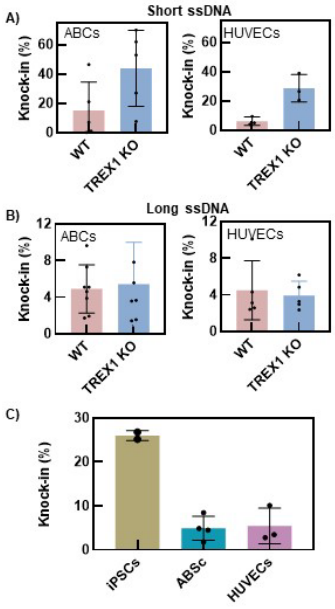

Figure 1. A. Editing wild type and TREX1-KO ABCs and HUVECs using a short ssDNA confirmed the role of TREX1 in degrading the short ssDNAs and decreasing the gene insertion efficiency. B Gene insertion efficiency of wild type versus TREX-KO ABCs and HUVECs using a long ssNDA template. This result showed that TREX1 does not affect gene insertion using a long template. C) Substantial editing efficiency was observed in iPSCs versus ABCs and HUVECs using a long template.

1175 Development of a Novel Full-Length F8 CELiD DNA Vector (ceFVIII) for Hemophilia A Gene Therapy

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Hemophilia A is an X-linked bleeding disorder caused by mutations in the factor VIII (F8) gene, resulting in factor VIII deficiency. While adeno-associated virus (AAV)-based gene therapies have shown promise for treating Hemophilia A, their utility is limited by AAV's packaging capacity (~4.7 kb). The F8 coding sequence (CDS), exceeding 7 kb, poses significant challenges for delivery using AAV. Truncation strategies may compromise F8 protein folding and functionality, while dual-AAV approaches often suffer from low efficiency. To overcome these limitations, we have developed a novel closed-ended linear duplex (CELiD) DNA vector for Hemophilia A. CELiD DNA has previously demonstrated long-lasting expression and stability in vivo, making it an attractive alternative to viral vectors.

The newly designed CELiD DNA vector, ceFVIII, incorporates a full-length FVIII expression cassette (8.3 kb) driven by a ubiquitous promoter. This study outlines the production of ceFVIII and highlights its potential for therapeutic development. The CELiD DNA-based ceFVIII vector represents a novel, non-viral platform that overcomes the size constraints of AAV-based systems. With its potential for durable in vivo expression and scalable production, ceFVIII holds promise as a next-generation gene therapy for Hemophilia A, addressing critical unmet needs in this patient population.

1176 Unlocking Safe and Scalable Engineering: Non-Viral and Non-Integrating SMAR DNA Vectors with Improved Payload Capacity

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The ability to efficiently deliver large DNA sequences is of pivotal importance in the advancement of cell therapies. Conventional methods for cellular engineering face limitations in both safety and payload capacity, associated with the use of viral vectors and gene editing technologies such as the CRISPR/Cas system. These issues are especially problematic for the clinical translation of next-generation cellular therapies employing multiple receptors and complex genetic circuits to enable precise control over gene expression and therapeutic outcomes. To address these challenges, we developed a non-viral non-integrating approach for long-term genetic modification of cells using DNA vectors. At the core of our platform are Scaffold/Matrix Attachment Regions (SMARs), sequences derived from human DNA that allow episomal maintenance and extra-chromosomal vector replication within the host cell nucleus. This means that, unlike integrative technologies, these vectors do not carry the risk of insertional mutagenesis. Additionally, employing DNA vectors has the key advantage of enabling the inclusion of large and/or multiple independent transgenes without size constraints, making this technology highly versatile and simple to use.

We have demonstrated the utility of SMAR vectors across a range of cell types, including primary human T cells: engineered cells exhibit stable and robust expression of transgenes and maintained high cytotoxic activity when equipped with Chimeric Antigen Receptors (CARs) or T Cell Receptors (TCRs). In this study we built on previous work to generate dual-promoter constructs and evaluate the performance of multi-cassette systems in Jurkat T cells. Several iterations of the vectors were assembled, testing various promoter and transgene configurations, with sizes ranging from 5.7kb to 9kb. We successfully expressed multiple proteins in parallel, including fluorescent markers and clinically validated therapeutic receptors such as the anti-CEA CAR and anti-MART-1 TCR, with no adverse effect of larger vectors on cell viability post-delivery. Importantly, the engineered cells were able to successfully initiate intracellular signaling cascades essential for immune responses upon stimulation.

The promising results obtained thus far emphasize how the SMAR vector platform provides a safe, flexible alternative to current cell engineering methods, with the potential to stably deliver larger payloads without compromising genomic stability. Follow-up studies are ongoing and focused on further characterizing vector dynamics in the nucleus, as well as incorporating other molecular designs such as logic-gated CARs. With these advancements, we aim to offer a scalable, safe, clinically compliant solution for the genetic modification of cells used in various applications, including cancer immunotherapy and beyond.

1177 A Next Generation Immune and Stem Cell Engineering Platform to Create Multifunctional Cells, at Clinical Scale, Using a Silicon Membrane

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Despite their unprecedented efficacy in the clinic, cell and gene therapies remain inaccessible to many patients due to their immense cost and complex manufacturing. Currently, delivery of cell modulating materials (e.g. CAR) to cell types of interest is done through viral, lipid, or electroporation technologies which are expensive, can only accommodate specific cargos or cells, and lack scalability - creating a bottleneck. We have developed a cell engineering platform by leveraging mechanoporation, a technique based on rapid mechanical deformation of cell membranes, to achieve universal compatibility across diverse cell types and cargo molecules. This approach enables efficient delivery of a wide range of cargo, including mRNA, CRISPR RNP, circular RNA, siRNAs, and proteins, to various cell types, notably primary immune cells and hematopoietic stem cells, which have traditionally been challenging to modify. For example, we have shown multiplexed delivery of mRNA and CRISPR RNP to naive T cells efficiently engineering more than 85% of total viable cells with both GFP expression and deletion of B2M. In iPSCs, we have used this technique to deliver mRNA encoding the transcription factor Neurogenin 2 (NGN2) to drive the differentiation of iPSCs into neuronal precursors while maintaining a viability of 75% at a RNA expression level of over 70%. The silicon membrane-based delivery system streamlines the cell engineering process, facilitating seamless integration with existing clinical manufacturing equipment and enabling scalable production of cell

therapies. Using our clinical scale integration module, we have the capacity to deliver cargo to more than 1 billion cells per minute. We show high viability and over 80% mRNA expression in activated T cells when integrated into standard GMP manufacturing equipment. This approach is simple, economical, amenable to many cell and cargo types, and scales from research to clinical applications. This technology has the potential to alleviate major bottlenecks enabling broader accessibility of impactful cell therapies.

1178 Computationally Designed Miniproteins Enable Precise And Potent In Vivo Delivery Of Gene Editors To Challenging Tissues

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Gene editing technologies hold extraordinary potential to correct the root causes of genetic diseases by precisely targeting affected tissues in vivo. Despite these exciting prospects, current delivery methods—including lipid nanoparticles (LNPs), viral-like particles (VLPs), and viral vectors—pose significant limitations. Not only do they come with safety concerns, such as the risk of immune responses, but they also lack the characteristics required for diverse tissue accessibility and specificity (i.e. muscle, heart, kidney, etc.).

To overcome these challenges, we have developed an innovative approach centered on protein-based therapeutics. This strategy pairs gene editors with customizable miniprotein targeting moieties that enable precise self-delivery to hard-to-reach tissues through receptor-mediated uptake. Notably, these therapeutics are designed to be modular, potent and transient, offering distinct advantages over traditional delivery methods.

Building on recent breakthroughs in machine learning for protein structure and function, we engineered miniproteins fused to guide gene editors to specific cell surface receptors—targeting muscle cells, neurons, and kidney. By performing self-delivery experiments in cellular models engineered to overexpress the corresponding receptors, we observed that our targeted gene editors achieved a 4,000-fold increase in potency over untargeted editors. When paired with an endosomal escape agent, we achieved potencies between 10 and 100 pM with the targeted gene editors *in vitro*. Furthermore, by chemically engineering both guide RNA and Cas9 protein, we demonstrate enhanced stability of the ribonucleoprotein (RNP) complexes within diverse biological matrices, ensuring greater durability and functionality in complex biological environments.

We use transgenic mouse models to evaluate the translational potential of the targeted gene editors. We perform histological analysis to assess the ability of de novo designed miniproteins to deliver gene editors to muscle and neurons in vivo, focusing on their co-localization with intended receptor targets. Additionally, we utilize localized delivery methods, such as intramuscular and intrastriatal injection, to explore targeted uptake and gene editing, highlighting the platform's modularity and versatility.

These results mark a significant step forward in our ability to precisely and safely deliver gene therapies to difficult-to-drug tissues.

1179 Engineering the CRISPR-Cas9 Ribonucleoprotein Complex for Carrier-Free Gene Editing

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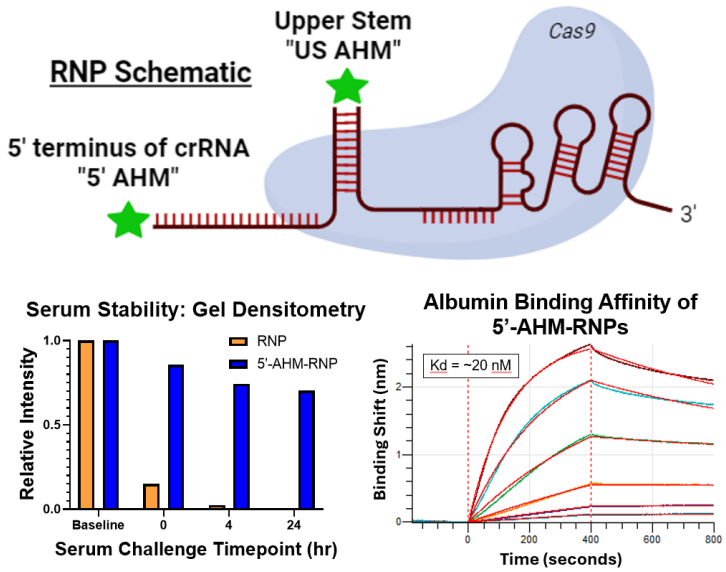
Introduction: Despite the rapid development of CRISPR-Cas9 delivery technology for gene editing, translating these technologies for application outside of liver and eye has proven to be a challenge. Systemic delivery of high doses of viral carriers comes with toxicity risks, motivating nonviral approaches, such as lipid or polymeric nanoparticles. However, ionizable carrier systems come with relatively narrow therapeutic indices due to carrier associated toxicities. Our work focuses on increasing the stability and pharmacokinetics (PK) of the RNP to allow its systemic delivery for in vivo carrier-free editing of muscle. To achieve this goal, we are synthesizing RNPs that bind to albumin through a lipid modification placed on the RNA component. *In situ* albumin hitchhiking is known as a promising strategy for cargo stabilization and is validated clinically (e.g., Ozempic) to extend *in vivo* PK. We seek to optimize an albumin-hitchhiking modification (AHM) in combination with other RNA stabilizing modifications to engineer RNPs that are stable against nuclease degradation, have extended PK to enable delivery to sites of tissue inflammation, and maintain targeted DNA cleavage.

Methods: We use solid-phase synthesis to introduce the AHM with varying linker chemistries as well as protecting 2′ and backbone chemical modifications to the dual guide RNA (dgRNA) system. Plasmid cleavage assays (to assay targeted dsDNA cleavage) and *in vitro* gene editing studies in primary mouse cells are used to assess the activity of the modified RNPs, while biolayer interferometry and serum challenge assays determine the albumin-binding potential and serum/nuclease stability, respectively. We put a special emphasis on exploring the differences in activity and stability introduced through varying AHM locations and linker lengths.

Results and Conclusions: Chemically modified dgRNAs can achieve similar plasmid cleavage efficiency as unmodified dgRNAs, but the cleavage kinetics are slower. Similarly, RNPs with heavily or fully modified dgRNA and added AHM have reduced gene editing activity in primary mouse cells as do RNPs without the AHM, likely due to steric hindrance of DNA targeting or RNP formation. However, we demonstrate that varying AHM linker length and location can be used to somewhat restore the gene editing activity, and an optimal length was determined for one of the AHM locations that enables 3.6-fold increase in activity relative to other linker lengths ($p<.0001$). Importantly, AHM-RNPs all demonstrate albumin-binding affinities of approximately 20 nM and ~70% retained product after 24 hours of incubation in serum compared to 0% non-AHM-RNP product. Finally, all AHM-RNPs were shown to have significantly increased carrier-free cellular uptake ($p<.0001$, ~30% more uptake at 24 hours) in mouse muscle cells relative to normal RNPs. Therefore, we have found that the incorporation of the AHM and chemical modifications to the dgRNA comes at a cost of reducing editing activity but that these modifications greatly improve cell uptake and the stability of the complex, while providing high-affinity albumin-binding. These results motivate future work exploring the *in vivo* PK and gene editing activity of lead modified RNPs vs. unmodified RNPs to dissect the contribution of RNP potency vs. stability. We will further explore new, additional AHM linker chemistries and chemical modification patterns for optimization of the stability and activity of the RNPs.



1180 Microglia-specific miRNA gene therapy with homing peptides for amyotrophic lateral sclerosis in mice model

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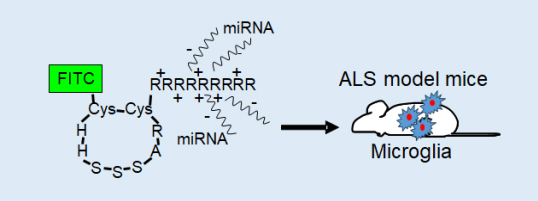
Introduction: Amyotrophic lateral sclerosis (ALS) is an intractable neurodegenerative disease, most of which is sporadic, and no established treatment has yet been developed. Microglia are universally known as immunocompetent cells in the central nervous system and have been related with pathogenesis in ALS, recently. Therefore, microglia is an important target for treatment of ALS, and we decided to develop the selective delivery of therapeutic miRNA to microglia by our previously identified homing peptides for the treatment of ALS. We also screened for the miRNAs to be delivered and developed a novel treatment method using a complex of microglia targeting peptide and therapeutic candidate miRNA.

Material and Methods: (1) Screening the candidates of therapeutic targeting miRNA, miRNA array was performed in spinal cord of SOD1(G93A) transgenic mice (ALS mice) at early, middle, and late stages of the disease. (2) The affinity of the microglia homing peptides with candidate miRNA, and the stability of the complex between microglia homing peptide and miRNA was also evaluated. (3) The gene delivery experiments of miRNA into microglia with homing peptides was performed *in vitro*. Transduction efficiency in the complex including microglia homing peptide was evaluated by comparing with the chemical regents for transfection of genes. (4) miRNA-microglia peptide complexes were administrated into the ALS mice and their therapeutic effects were evaluated by behavioral experiments and survival curve. And mRNA expression levels were analyzed in cytokines and growth factors.

Results: (1) Several miRNAs were identified as therapeutic candidates against microglia in ALS. (2 and 3) The miRNAs with microglia homing peptides specifically targeted to microglia and were successfully delivered into microglia. The complexes showed higher transduction efficiency than lipofection regents. (4) Application of miRNA-microglia peptide complex showed therapeutic effects on motor behavioral tests and survival rate in the ALS mice. And the expression of pro-inflammatory genes was suppressed in treatment group, whereas the expression of neuroprotective genes was increased by miRNA-microglia peptide complex.

Conclusion: A novel gene therapy for ALS with miRNA delivery targeting to microglia was developed, and clinical application will be expected.



1181 Non-Viral Cell Engineering and Gene Delivery Optimization from Discovery to Clinical Applications Across Broad Cell Types

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Cell and gene therapy (CGT) demands workflows that transition seamlessly from research-level optimization to clinical-scale manufacturing. Thermo Fisher Scientific offers a powerful portfolio designed to enable this scalability, including the Neon™ NxT Electroporation System (single- and 8-channel), the CTS™ Xenon™ Electroporation System, and the CTS™ Rotea™ Counterflow Centrifugation System. The Neon NxT system provides a flexible research use platform for rapid, small-scale optimization of electroporation conditions, while the CTS Xenon system allows these optimized protocols to be seamlessly scaled up for high-volume non-viral gene delivery, supporting GMP-compliant cell therapy manufacturing, ensuring consistent performance at all stages. The CTS Rotea system supports efficient upstream preparation, enabling high-quality starting material that enhances the reproducibility of downstream electroporation results. We demonstrate the scalability of this workflow using multiple cell types, showcasing its versatility and reproducibility. In T cells, knock-out (KO) of the TRAC locus and knock-in (KI) of a GFP donor DNA payload yielded comparable editing efficiencies and consistent viability on both the Neon NxT and CTS Xenon system. Similarly, iPSCs edited at the CD38 locus using CTS HiFi Cas9 RNP for KO and a MESO3-V5 dsDNA donor for KI retained their pluripotency markers post-electroporation, confirming their potential for banking or differentiation applications. NK cells, a challenging cell type to engineer, were successfully edited at the B2M locus using CRISPR, achieving equivalent KO efficiencies across both platforms. These results collectively validate the seamless scalability from the Neon NxT to the CTS Xenon system, enabling consistent gene editing outcomes across diverse and demanding cell types. By combining flexibility at the research level with robust performance at manufacturing scale, Thermo Fisher Scientific empowers researchers to efficiently optimize gene editing and delivery workflows for diverse and challenging cell types. This integrated approach accelerates the development of next-generation therapies, driving their successful translation from bench to bedside.

1182 Enhancing CAR NK Cell Production - A Comparative Analysis of Non-Viral Gene Engineering Systems

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Chimeric antigen receptors (CARs) are artificial fusion proteins comprising an extracellular domain, typically a single-chain variable fragment (scFv) derived from a monoclonal antibody, a transmembrane domain, and an intracellular signaling domain. Expressed in immune cells like T or NK cells, CARs enable antigen recognition and activation, leading to the targeted destruction of cells. Despite their efficacy, CAR T-cell therapies can lead to complications such as graft-versus-host disease (GVHD). In contrast, natural killer (NK) cells offer inherent advantages due to their intrinsic ability to kill without prior antigen-stimulation and reduced risk of inducing GVHD. These properties make allogeneic CAR NK cells viable as ready-to-use treatments, eliminating the need for patient-

specific lymphocyte preparation. Non-viral methods of genetic engineering offer advantages over viral transduction, including a better safety profile, lower immunogenicity, cost-effectiveness, and fewer regulatory challenges. Our aim is to refine the non-viral genetic engineering of CAR NK cells using the NK92 line to enhance the speed, efficiency, and simplicity of the process. To achieve this, we aim to enhance the transfection rates by exploring various non-viral gene engineering systems, including diverse electroporation devices and mechanoporation techniques.

We have successfully transfected the NK92 cell line to express CARs using the non-viral Sleeping Beauty (SB) Transposase system equipped with the SB100x hyperreactive transposase. Performing the transfection through electroporation utilizing a Neon Transfection device initially yielded approximately 3 to 14 % CAR-positive cells. The cells expressing the transgene were subsequently selected and further cultivated to establish cell cultures with an average of 95 % CAR-positive cells. Using digital droplet PCR (ddPCR), we quantified the copy-number variation (CNV) of integrated CAR DNA within the NK92 cell genome. Cells electroporated with two distinct CAR constructs, each incorporating different scFvs, displayed CNVs ranging from 3 to 48. These constructs were further modified to include a gene cassette for continuous IL-15 production, increasing their size. Surprisingly, CNVs were 1.6-fold higher for constructs with the IL-15 cassette than those without it, regardless of the scFv type. Additionally, we assessed the surface density of CARs on NK92 cells using flow cytometry. The analyses revealed a significant 1.7-fold increase of the Antibody-Binding Capacity, aligning closely with the ddPCR results. Engineered CAR NK92 cells have demonstrated reactivity to their specific targets, effectively initiating signal transduction. This activation is evidenced by a significant secretion of IFN-γ (up to 1400 ng/ml) with up to 38 % degranulating cells. These properties are currently compared to NK92 cells engineered on various non-viral gene delivery systems.

Increasing transfection efficiency will not only accelerate the production process but also facilitate the adaptation of these methods to primary NK cells, thereby making CAR NK cell therapies a more viable clinical option.

1183 Making Personalized Possible: A Miniaturized, Automation Integrated Nonviral Delivery Platform for High-Throughput Arrayed CRISPR Screening in Rare Patient-Derived Cells

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The development of clinically relevant drug discovery workflows requires scalable and efficient tools to interrogate genetic function in preclinical models. Arrayed CRISPR screening offers a powerful approach to systematically evaluate gene perturbations, enabling multi-parametric high-content assays. However, translating arrayed screening to patient-derived cells remains a challenge due to the high cellular inputs required, the cost of reagents, and the need to pool samples from multiple donors, which can obscure inter-patient variability.

To address these limitations, we have developed an automated microfluidic platform that enables miniaturized, nonviral transfection at scale. This platform leverages a novel droplet-based electroporation system that leverages digital microfluidics for precise micro volume liquid handling with a unique electrode geometry that leverages the electric field to transiently porate cells in a manner that optimizes delivery efficiency while preserving cell viability. By reducing the required cell numbers from millions to just a few thousand per perturbation, our approach makes genome-wide arrayed CRISPR screening feasible in primary and patient-derived cells. We have validated robust delivery of diverse payloads—including mRNA, RNP, and DNA—demonstrating high-efficiency editing with no impact on cell function across multiple primary cell types, including immune cells, stem cells, and 3D organoid structures.

Here, we present a fully automated 48-plex editing cartridge designed for seamless integration with standard laboratory automation. We will detail the workflow adaptations that enable compatibility with SBS-format consumables and acoustic liquid handling technologies, allowing precise deposition of 48 independent guide RNAs across the cartridge. Furthermore, we will showcase our automation efforts, which support robotic loading and unloading of reagents, enabling hands-free execution of thousands of edits per day. Finally, we will highlight our

custom user interface, which facilitates real-time monitoring and parameter customization for each electroporation event.

By enabling high-throughput, cost-effective, and patient-specific genetic screening, our platform has the potential to accelerate drug discovery and improve translational relevance, ultimately advancing precision medicine and the development of more effective therapies.

1184 Development of Novel CaptureSelect Affinity Tools for Extracellular Vesicle Purification and Detection; Enabling a New Class of Gene Therapy Products

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Extracellular vesicles (EV) are small vesicles produced by various cell types in the range of 30-100 nm diameter, and are involved in intercellular communication by delivering proteins, metabolites and nucleic acids into recipient cells that effectively alter their biological response. These natural properties of EVs make them a promising platform for new gene therapy products. The manufacturing process of these type of biopharmaceuticals is challenged by their complexity and heterogeneity, and scalable downstream processes are not available. Currently, most of the purification strategies described in literature are based on time consuming and non-scalable options like density gradient ultracentrifugation.

Using the CaptureSelect ligand technology, based on llama single domain antibodies, we have developed a range of affinity ligands that target different tetraspanin membrane proteins that are present on EVs and selectively bind three different proteins being CD81, CD9, and CD63.

Here we report the development of a novel affinity purification tool for EVs by immobilizing the CaptureSelect CD81 affinity ligand on a magnetic bead platform. EVs containing CD81 protein were specifically captured from cell-line derived and human plasma feed streams and eluted from the magnetic beads with arginine-based buffers. Efficient elution was achieved at mild pH which kept the EVs structurally intact. Due to their complexity, orthogonal analytical methods were implemented to characterize and quantify EVs throughout the workflow.

Sandwich ELISA based on affinity ligand conjugates targeting three distinct tetraspanins (CD81, CD9, and CD63), SDS-PAGE, western blot analysis, and SP-IRIS with immunofluorescence (Leprachaun™) were performed for phenotypic characterization, intactness, sizing, and titer determination.

Analysis demonstrated an effective and efficient purification of CD81 bearing EV particles enabled by this magnetic bead platform. The development of this easy purification method together with ligand-based analytical characterization assays can help advance the development and manufacturing of new EV based therapies.

CaptureSelect ligands and resins: For Research Use or Further Manufacturing. Not for diagnostic use or direct administration in humans or animals.

1185 The anti-inflammatory properties of Mescenchymal Stromal Cell derived Extracellular Vesicles on feline neuroinflammation

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Introduction. Extracellular vesicles (EVs) secreted from mesenchymal stromal cells (MSCs-EVs) are lipid nanoparticles with multiple inherent anti-inflammatory and immunomodulatory properties. Their biological action is dependent on the MSCs' culture conditions, and it is still unclear which cell culture condition produces the most immunosuppressive EV isolates. Moreover, EVs are able to diffuse through the blood-brain-barrier and exhibit low

immunogenicity, so they are promising therapeutic candidates for neuroinflammation. Neuroinflammation is triggered by neuronal death and when persists exacerbates the progression of neurodegenerative diseases. Traditional anti-inflammatory drugs have side effects and offer minimal symptomatic relief. Therefore, there is a critical need to develop novel treatments that dampen neuroinflammation and reverse its role in neurodegenerative disease prognosis. Our goal is to evaluate the anti-inflammatory effects of MSC-EVs in Sandhoff Disease (SD), which is a fatal and aggressive inherited neurodegenerative disease. Our objectives was a) to identify the anti-inflammatory effects of different MSCs-EVs on inflammatory mixed glia, b) to assess their immunomodulatory properties in peripheral mononuclear blood cells (PBMCs) from SD cats c) and to determine their *in vivo* anti-inflammatory effects in SD cats.

Methods. Primary mixed glia were isolated from feline brains and were phenotypically characterized via immunofluorescence. MSC-EVs were isolated under different culture conditions including a serum free environment (SF-EVs) or in the presence of FBS (FBS-EVs) or following priming of MSCs with IFN-γ and TNF-α (IF-EVs). Subsequently, EVs were added to naïve and LPS-stimulated cells for 24 and 48 hours. Cell culture supernatants from mixed glia were collected and analyzed for inflammatory factor expression through ELISA and RTq-PCR. Additionally, PBMCs from SD cats were treated with concanavalin-A for 48h and stained against FOXP3/CD4/CD3 markers to evaluate the effect of IF-EVs on T-regulatory cell proportion via flow cytometry. Potential cytotoxicity of the IF-EVs was assessed via XTT assay. Furthermore, 10^{12} UCMSC-EVs were intravenously administered in SD and normal cats at 4 months old. Brain and peripheral tissues were collected three days post injection. Enzymatic activity assays, immunohistochemistry and gene studies via qPCR were performed for main inflammatory mediators.

Results. Isolated brain cells from normal cats were confirmed as a mixed glia population due to the expression of neuronal (anti-NeuN), astrocytic (GFAP), microglial (Iba-1) and pericyte (PDGFR-β) cell markers. The addition of MSC-EVs into inflammatory feline mixed glia suppressed the pro-inflammatory cytokine production and downregulated factors involved in inflammatory signaling pathways. IF-EVs exerted the best immunomodulatory capacity compared to SF- and FBS-EVs. Also, IF-EVs reduced SD systemic inflammation via increasing the proportion of the T-regulatory cells compared to the control and no cytotoxicity effects were observed. Lastly, the IV administration of MSC-EVs suppressed neuroinflammation by decreasing the expression of Iba-1, GFAP and MHCII and CD4 inflammatory markers in the brains of SD cats compared to the untreated controls.

Conclusions. Our findings reveal that MSC-EVs reduce feline neuroinflammation during neurodegeneration.

1186 Engineering Extracellular Vesicles for Neuroprotective Gene Delivery to the Central Nervous System

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Introduction: Acute central nervous system (CNS) injuries, such as traumatic brain injury (TBI) and stroke, initiate a cascade of neuroinflammatory and degenerative processes that contribute to long-term functional deficits. Gene therapy offers a promising strategy to modulate these pathological responses and promote neuroprotection. However, efficient and minimally invasive delivery of therapeutic genes to the CNS remains a significant challenge. Engineered extracellular vesicles (EVs) have emerged as a versatile and non-immunogenic delivery platform capable of transporting genetic cargo across the blood-brain barrier (BBB) to target neural cells. Here, we present our preliminary work on engineering EVs for the delivery of neuroprotective genes to the CNS following acute injury.

Methods: EVs were isolated from genetically modified producer cells engineered to package neuroprotective genes of interest. The efficiency of gene loading into EVs was assessed using quantitative PCR and fluorescence-based reporter assays. In vitro studies were conducted by exposing naïve cell cultures to engineered EVs to evaluate cellular uptake and transgene expression using fluorescence microscopy and qRT-PCR. For in vivo studies, EVs

were administered intranasally or via direct intracranial injection to the striatum or thalamus in Td-Tomato-reporter mice, followed by histological analysis of brain tissues to assess gene transfer efficiency and cellular targeting.

Results: Our data confirm that engineered EVs successfully incorporate neuroprotective genes and deliver their genetic cargo to recipient cells in vitro, leading to detectable transgene expression in naïve cells. Preliminary in vivo experiments demonstrate that intranasally administered EVs can transduce neurons in the cerebellum of murine brain and directed intracranially injection results on regional specificity depending on the mode of administration. Immunohistochemical analysis reveals sustained transgene expression within neuronal populations, suggesting potential for therapeutic CNS intervention.

Conclusions: These findings support the feasibility of engineered EVs as a non-viral vector for gene delivery to the CNS, highlighting their potential to mitigate neuroinflammation and promote neuronal survival after acute injury. Further studies are underway to optimize EV surface engineering for enhanced brain targeting and therapeutic efficacy in mouse models of traumatic brain injury. This approach holds promise for developing next-generation of transient gene therapies for CNS disorders and injury.

1187 Improving and targeting programmable RNAi enriched Extracellular Vesicle for biomedical applications.

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Extracellular vesicles (EVs) are emerging as important vehicles in biotherapeutic applications. Small EVs (Exosomes) derived from multivesicular bodies can deliver nucleic acid and protein payloads to recipient cells, representing an engineerable non-viral biological nanoparticle for the delivery of biomedicines. EVs are considered non-immunogenic, biocompatible, and non-toxic. Furthermore, targeting of nanoparticles to specific tissues remains a challenge, and EVs are amenable to surface modification with targeting ligands through modified EV-enriched scaffolds. RNA interference (RNAi) is an established therapeutic approach; however, targeted delivery remains a significant barrier to the wider adoption of RNAi-based treatment. Various methodologies to selectivity load RNAi into EVs have been explored, but suffer from low programmability for new targets, complex loading procedures that use immunogenic architecture, cumbersome post-production loading processes, or questionable effective transfer of potent RNAi. Furthermore, strand selectivity can prevent toxic off-target effects through the 'passenger' strand, making it a beneficial feature of a highly on-target RNAi technology

To this end, we have identified an innovative RNAi scaffold termed the Exo-shRNA system that exploits dicer-independent short hairpin RNAs to enrich RNAi within small EVs to transfer functional cargo to recipient cells. The Exo-shRNA's simple architecture is readily programmable and is strand selective because of the dicer-independent maturation process. However, improving potency is essential to ensure competitive applications of the EV-delivered Exo-shRNAs. Exploring different features of the Exo-shRNA, such as the promoters, sequence features, export motifs, and host factors screened using high-throughput assays, we identified features of Exo-shRNA that can enhance knockdown by 2-fold through alternative promoter and shRNA sequences. Furthermore, a host factor, Cx43, can improve RNAi enrichment in EVs by doubling mature RNAi effectors per particle. Furthermore, building on previous work, we modified the EV-enriched CD63 scaffold to target the GP160 receptor of HIV, which resulted in selective reporter knockdown on GP160 expressing cells versus unmodified cells, demonstrating the Exo-shRNA is amenable to targeted delivery. Finally, Human Papillomavirus (HPV) causes a range of epithelial malignancies, and the viral oncogenes (E6 and E7) are critical for tumor transformation and maintenance of cervical cancer, representing ideal targets for therapeutic intervention. As proof-of-concept application of the Exo-shRNAs to a therapeutically relevant cancer target, Exo-shRNAs were designed to the E6/E7 viral oncogene, which reduced expression in reporter models, and when transferred by EVs to an HPV-associated cancer cell line reduced proliferation. Overall, the Exo-shRNA system represents a generalizable, next-generation biocompatible delivery platform for targeted gene knockdown applications for biomedicine.

1188 *Engineered SIRPα-Enriched Extracellular Vesicles Enable Targeted Clearance of CD47+ Pathological Fibroblasts in Fibrotic Disease*

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The persistence of pathological fibroblasts driven by CD47-mediated "don't eat me" immune evasion has shown a critical challenge in addressing fibrotic diseases. Here, we present an innovative therapeutic approach using engineered extracellular vesicles (EVs) enriched with Signal Regulatory Protein Alpha (SIRPα) to block the CD47 signal and promote clearance of diseased fibroblasts through professional phagocytes.

We have stably transduced human bone marrow-derived mesenchymal stem cells (MSCs) using lentiviral vectors to express SIRPα. Furthermore, we have developed a novel three-dimensional large-scale manufacturing process for EV production that achieved over 90% purity and a tenfold increase in yield compared to conventional methods. The therapeutic potential of SIRPα-EVs was evaluated in a bleomycin-induced idiopathic pulmonary fibrosis (IPF) mouse model, supported by single-cell transcriptomic analysis of human IPF samples that confirmed significant CD47 upregulation in disease-associated fibroblasts and macrophages. Biodistribution studies using fluorescently labeled SIRPα-EVs demonstrated preferential accumulation in fibrotic lung tissue. Flow cytometry revealed approximately fivefold higher uptake in pathological fibroblasts compared to normal fibroblasts. In vivo data indicated that SIRPα-EVs exhibited superior therapeutic efficacy compared to conventional treatments (dexamethasone, nintedanib, pirfenidone) at lower doses, evidenced by restored alveolar architecture, reduced fibrotic lesions, and decreased Ashcroft scores. Molecular analyses confirmed the downregulation of key fibrotic markers, including α-SMA, CD47, inflammatory cytokines, and TIMP1.

These findings highlight the transformative potential of SIRPα-EVs as a targeted therapy for fibrotic diseases and establish a foundation for clinical translation in conditions that currently lack effective treatment options.

1189 Development of a Scalable and Reproducible Large-scale Purification Method for Biologically Active Extracellular Vesicles Using Tim4-Phasphatydilserine Affinity Interaction

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Introduction: Extracellular vesicles (EVs) are lipid bilayer membrane structures secreted by cells and contain various bioactive substances such as lipids, nucleic acids, and proteins. Since EVs derived from mesenchymal stem cells (MSCs) have therapeutic potential on various diseases, the use of EVs as therapeutic agents in medicine has been thought to be promising. Consequently, multiple methods have been developed for a larger scale EV purification. Here, we have sought to develop a scalable and reproducible method for affinity purification of EVs by using Tim4 protein, which specifically binds phosphatidylserine (PS) displayed on the surface of EVs. Because the binding is Ca^{2+}-dependent, intact EVs can be easily released from Tim4 by adding Ca^{2+} chelators. This method is capable of purifying EVs derived from cell culture supernatants with high recovery and purity.

Methods: Recombinant Tim4 protein was covalently immobilized on the surface of agarose resin and the Tim4-resin was packed into a 1 mL column. 200 mL of MSC cell culture supernatant was directly applied into the Tim4 column. After washing the column, 4 mL of EDTA solution was applied to elute the captured EVs. The resulted EVs were analyzed by using NTA, ELISA, BCA methods, and cell-based functional reporter assay. In parallel, EVs were also purified from the 200 mL of the same culture supernatant by using tangential flow filtration (TFF), TFF +

size exclusion chromatography (SEC), or TFF + anion exchange chromatography (AEX), and the resulted EVs were analyzed for comparison.

Results: More than 5 X 10^{11} particles of EVs were purified per 1 mL of the Tim4 resin, and it was confirmed that yield of the purified EVs with the PS affinity method was higher (70 – 90%) than the other commonly used methods, such as TFF combined with SEC or AEX method. We used the same Tim4 column repeatedly for 10 times, and the resulted each EV preparation showed a similar tetraspanin protein expression profile to what observed with the EVs purified with the Tim4-immobilized magnet beads-based small scale purification method, indicating that the Tim4 column is scalable and reusable with an appropriate washing procedure. Furthermore, we observed the EVs purified using the Tim4 column showed higher purity and anti-inflammatory effect than the EVs purified with the other conventional methods.

Conclusions: Our affinity purification method using Tim4-immobilized resin can purify EVs with higher recovery, purity and biological activity in fewer steps than competing methods and is also scalable. The method is also cost-effective because of the multiple-use capability. Therefore, we propose that our affinity purification method has potential for use in manufacturing process of therapeutic EVs.

1190 Designing a Plug-and-Play System to Produce Engineered Extracellular Vesicles

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Extracellular vesicles (EVs), including exosomes, are being studied extensively and have emerged as a promising novel technology for the delivery of complex therapeutic payloads such as nucleic acids or proteins. EVs are naturally occurring within the body and have several potential advantages over other delivery vehicles such as LNPs or viral vectors, such as their ability to evade the immune system response and therefore support repeat dosing, as well as their capacity to access biological barriers and deliver a wide range of cargo to specific target tissue types given their tropism profiles. These attributes make EVs favorable candidates to engineer as drug delivery systems.

Here, we discuss a plug-and-play platform approach to express biomolecules using engineered EVs. Therapeutic cargo may be loaded onto or within EVs via identified scaffold proteins using novel plasmid design. This concept was explored in the development of therapeutics such as exoSTING™, exoASO-STAT6™ and exoIL-12™, which have completed Phase 1 clinical studies within oncology applications. Alternatively, small molecules may be linked to EVs using a proprietary linker technology. A dedicated cell line for EV production was also generated, which resulted in a clone capable of high particle titer, integrity, and purity. These technologies will be made available to the wider scientific community under a research license, or via Lonza's CDMO service offerings.

1191 CD47-Targeted Extracellular Vesicles Enable Enhanced Endolysosomal Escape for Oligonucleotide Therapy Delivery

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Despite significant advances in oligonucleotide therapeutics, translating small interfering RNA (siRNA) and antisense oligonucleotides (ASO) into clinical use remains challenging due to inadequate drug delivery systems (DDS). While these therapeutics hold promise for targeting "undruggable" pathogenic genes such as mutant KRAS in cancer and NLRP3 in inflammatory diseases, their efficacy is often hindered by rapid degradation, poor cellular

uptake, and insufficient endosomal escape. The current lipid nanoparticle (LNP) delivery system has notable limitations, including high immunogenicity, predominant accumulation in the liver, and endosomal entrapment.

To address these issues, we developed a CD47-mediated oligonucleotide delivery platform using engineered extracellular vesicles (EVs) expressing SIRPα membrane protein (SIRPα-EVs). By developing optimized lentiviral transduction and isolation protocols, we generated SIRPα-EVs and incorporated therapeutic oligonucleotides through cholesterol-based conjugation. Mechanistic studies revealed that SIRPα-EVs utilize clathrin-independent endocytosis for cellular uptake, allowing for efficient endolysosomal escape and enhanced therapeutic delivery compared to conventional endocytic pathways.

In vivo imaging and flow cytometry confirmed selective accumulation of SIRPα-EVs in CD47-overexpressing pathological tissues. Therapeutic evaluation in two disease models highlighted the versatility as a DDS platform: KRAS siRNA-loaded SIRPα-EVs achieved 60% complete remission in combination with PD-1 blockade in a KRAS-mutant tumor model, while NLRP3 ASO-loaded SIRPα-EVs demonstrated superior anti-inflammatory effects in an LPS-induced inflammation model. Both applications exhibited significantly improved oligonucleotide delivery compared to naive EVs.

This translational study establishes CD47-targeted EVs as a versatile platform for oligonucleotide delivery, demonstrating efficient endolysosomal escape and superior therapeutic outcomes across multiple disease models. Our findings provide compelling evidence for clinical applications in both cancer and inflammatory conditions characterized by CD47 overexpression.

1192 Extracellular Vesicle Delivery of Oligonucleotide Antiviral Therapy Against Rabies Encephalitis

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Introduction: There are many neuropathic viruses that can cause viral encephalitis in humans that are mostly lethal and incurable. Among these viruses, zoonotic rabies virus (RABV) is invariably fatal to humans following the onset of clinical symptoms. Although there are preventable measures for rabies post-exposure prophylaxis, existing interventions are ineffective once the virus reaches the central nervous system and neurologic symptoms become apparent. The primary reason current treatment methods for encephalitis are unsuccessful is the blood brain barrier which limits the delivery of treatments into the brain. Therefore, it is essential to develop novel therapeutic approaches to treat rabies encephalitis. Extracellular vesicles (EVs) derived from plasma membrane presents an innovative platform for developing novel therapeutics with their ability to shuttle molecules between cells and through crossing the blood brain barrier. Moreover, RNA interference (RNAi) using synthetic short double stranded DNA (siRNA) is an innovative approach used to selectively bind targeted viral messenger RNA to induce viral mRNA degradation. In this project our objective is to modify extracellular vesicles to express rabies virus glycoprotein (RVG) derived peptide, which binds to nicotinic acetylcholine receptor, which will target neuronal cells and deliver therapeutic antiviral siRNAs designed to inhibit rabies virus replication into the central nervous system. We hypothesize that engineered extracellular vesicles expressing RVG will increase brain tropism and increase the efficiency of anti-viral siRNA delivery into the central nervous system to rapidly inhibit rabies virus replication. This project aims to implement a novel therapeutic approach and advance the viral suppression therapy for rabies encephalitis.

Methods: We modified EVs derived from HEK293 cells to express RVG by transfecting parent cells with a plasmid encoding Lamp2b-construct fused to the neuron specific RVG. A plasmid consisting of Lamp2b-construct fused to FLAG was used as the control. We used a surrogate siRNA (GAPDH) to evaluate the efficiency of encapsulation into RVG modified EVs and to evaluate the delivery of cargo into neuronal cells. Next, we screened forty-six

antiviral siRNA candidates against rabies N gene for their high binding and gene inhibition *in vitro* using psiCHECK2 luciferase assay system. In addition, we used RT-PCR to evaluate gene expression.

Results: Our preliminary data showed successful modification of EVs with the RVG peptide and loading of a surrogate siRNA (GAPDH). Moreover, we found that RVG modified EVs were able to deliver their load to neuronal cells *in vitro*. In addition, our preliminary data from Luciferase assay shows that six antiviral siRNAs candidates can induce more than 90% N gene interference in HEK293 cells.

Conclusions: We aim to evaluate the therapeutic effect of EVs to deliver antiviral siRNAs in rabies infected neuronal cells and mice via bio distribution and gene expression assays.

1193 Targeted Extrahepatic Gene Delivery and Editing with Non-Viral Fusogenix Proteolipid Vehicles

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Genetic medicines hold great promise to treat a wide array of diseases, yet success in the clinic has been hindered by limitations in the tolerability, biodistribution, scalability, and immunogenicity of current delivery platforms. We developed a novel delivery platform that overcomes these limitations by combining aspects from viral and non-viral platforms. The Fusogenix proteolipid vehicle (PLV) incorporates fusion-associated small transmembrane (FAST) proteins isolated from orthoreovirus into a well-tolerated lipid formulation using a scalable microfluidic mixing approach. Incorporation of FAST protein into an optimized lipid formulation was found to significantly increase the expression of DNA/mRNA in vitro and in vivo, without accompanying increases in toxicity. Systemic administration of PLVs encapsulating DNA-GFP or mRNA-GFP in mice and non-human primates efficiently delivered these nucleic acids to numerous extra-hepatic tissues, primarily muscle, lungs, and kidneys. This led to substantial gene expression, confirmed with histology, ELISA, and PCR. Systemic DNA delivery is a major challenge for non-viral platforms; therefore, to establish a suitable benchmark we compared the systemic delivery of DNA-FLuc to a clinically approved lipid nanoparticle (LNP) formulation. PLVs displayed exceptional tolerability, with no liver toxicity or elevated cytokine levels that were observed with the LNP formulation – all without sacrificing DNA expression. Next, we examined the extrahepatic nature of PLVs and compared the delivery of mRNA-FLuc to three clinically approved LNP formulations across multiple administration routes, including intramuscular, intracerebroventricular, and intravitreal injection. Unlike LNPs, which showed significant off-target liver expression regardless of injection route, PLVs achieved robust localized expression in the intended tissues with no off-target expression. The combination of low immunogenicity, sustained efficacy, and precise delivery highlights the PLV platform's potential for diverse gene therapy applications. Building on these findings, we explored the versatility of the PLV platform for extrahepatic gene editing. Using the Ai9 mouse model, systemic delivery of mRNA-Cre via PLVs induced widespread tdTomato expression across multiple tissues, while intrastriatal (IS) injection localized expression to the striatum, hippocampus, cortex, and thalamus. We then optimized PLVs to co-encapsulate mRNA-Cas9 and sgRNA, ensuring effective colocalization of CRISPR components. In vivo validation in Ai9 mice mirrored the Cre results, with IV delivery achieving gene editing in the same tissues and IS injection facilitating extensive neuronal editing. Translating these results into a therapeutic context, we developed a durable, DNA-based leptin gene therapy (DNA-Lep) for the treatment of obesity. IV injection of leptin-deficient mice with DNA-Lep resulted in a sustained increase in circulating leptin levels, extending beyond a six-month duration. This was associated with significant improvements in body weight and metabolic function relative to control animals. These findings were validated in a diet-induced obesity model, where DNA-Lep treatment increased circulating leptin levels, resulting in a corresponding reduction in food intake and body weight while improving markers of metabolic health. The favorable activity and safety of the PLV platform defines a new standard for redosable gene therapies and genetic medicine.

1194 Streamlined Cell Therapy Manufacturing: Highlighting Workflow Flexibility and Functionality with Advanced Processing and Fill Finish Instrumentation

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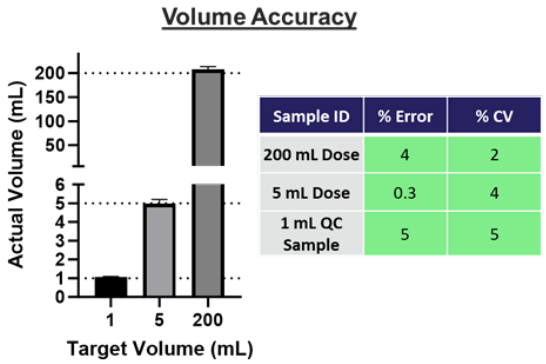
Background and Aim: Advancing cell therapy manufacturing necessitates robust, flexible, and efficient systems to ensure high-quality outcomes for patients. This study evaluates primary T cell recovery, viability, and phenotype in the concentration, formulation, fill and finish steps of the cell therapy workflow using semi-automated instrumentation. Additionally, we investigated the volume accuracy and cell concentration consistency of a new fill finish instrument capable of producing between 1 and 10 outputs with a volume between 750mL and 0.5mL. Both instruments were analyzed independently to understand their capabilities and potential applications in the field as part of the larger workflow.

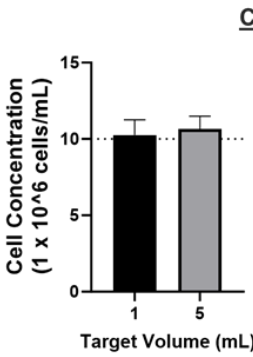
Methods, Results & Conclusion: This work was conducted through a series of workflow-based experiments designed to assess the core functionalities of the two instruments. Key performance metrics included primary T cell recovery, viability, and phenotype for washing and concentration as well as volume accuracy, and cell concentration consistency between final output containers. Data were collected from multiple runs to ensure reproducibility and accuracy.

In cell processing step we saw consistent primary T cell recovery exceeding 80% and little to no change in viability before and after the T cell processing steps. Additionally, the phenotype of the primary T cells remained stable throughout the processing. Within the fill-finish steps, high accuracy, recovery, and viability of primary T cells was achieved. Output samples from 200mL to 1mL showed percent errors below 5% from the target volume, and cell concentration was consistently within 10% between all seven outputs using a starting cell concentration of 10e6.

Both instruments exhibit significant strengths in their respective functionalities, offering valuable tools for cell therapy manufacturing. This evaluation highlights their potential to enhance product yield and consistency in manufacturing. The high viability, recovery, and phenotype of T cells during processing, along with the accuracy, precisions, and high recovery rates during fill-finish operations, underscore their effectiveness in supporting cell therapy manufacturing. Additional work is in progress to explore seamless workflow integration of these systems.



Sample ID	% Error	% CV
200 mL Dose	4	2
5 mL Dose	0.3	4
1 mL QC Sample	5	5



Sample ID	% Error	% CV	Δ % Viability
5 mL Dose	3	8	-1
1 mL QC Sample	1	10	-1

1195 Evaluating CAR T-Cell Potency in 2D and 3D Models Using Fluorescent Live-Cell Imaging

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The development of immunotherapies relies on the use of in vitro potency assays—which are key for understanding complex interactions between immune (effector) cells and cancer (target) cells. Immune effector T-cells, specifically CAR T-cells, show great promise due to their innate cytotoxicity. CAR T-cell therapy utilizes genetically engineered T-cells expressing a chimeric antigen receptor (CAR) that binds to a specific antigen on tumor cells. Assessing the efficacy and potency of such CAR T-cell therapies in vitro is vital for preclinical development. In this study, we describe an in vitro potency assay using the Omni live-cell imaging systems to quantify immune cell-mediated cytotoxicity of fluorescent cancer cells by HER2-targeted CAR T-cells in 2D and 3D cancer cell models. First, a GFP fluorescent HER2-expressing lung cancer cell line (A549) was seeded into 96-well plates, and HER2 CAR T-cells were added at various effector-to-target (E:T) ratios (1:10, 1:5, 1:2, 1:1, and 5:1) at 24 hours after target cell seeding. Fluorescent images of target cells were captured every 5 hours using the Omni Pro 12, an automated, high-throughput live-cell analysis platform that continuously images inside an incubator. Automated image analysis quantified attachment, proliferation, and cell death of the A549 target cells, which demonstrated a dose-dependent decrease in green fluorescence confluency, with near-complete cell lysis at a 5:1 E:T ratio by 160 hours. Next, cancer spheroids were formed in a U-bottom 96-well plate from A549-GFP (low expression of HER2) and SKOV3-GFP (overexpression of HER2) cells. After 48 hours of incubation, HER2 CAR T-cells were added at E:T ratios of 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, and 10:1. CAR T-cells showed antigen-specific killing, with higher E:T ratios (2:1, 5:1, and 10:1) resulting in approximately 75% reduction in green fluorescence intensity in SKOV3 spheroids at 72 hours. In contrast, A549 spheroids exhibited similar killing only at the 10:1 ratio. These findings suggest that the efficacy of CAR T-cell therapies may be influenced by the target antigen expression levels on cancer cells, emphasizing the need for optimized dosing and E:T ratios dependent on tumor characteristics. Real-time monitoring with live-cell imaging tools like Omni provides valuable insights into the dynamics of immune cell interactions, helping to refine and improve CAR T-cell therapies.

1196 Assessing *In Vitro* Cytotoxicity of CAR-T Cells Against Cancer Cells Using Three Assay Platforms

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Introduction. Determining CAR-T cell potency is a critical parameter to measure its efficacy at various stages of development. Comparing different assay platforms is essential in selecting a suitable method based on assay parameters and intended purposes. In this study, the potency of CAR-T cells was evaluated by comparing the cytotoxicity of CAR-T cells against tumor cells using three different assay platforms - Flow cytometry, Maestro Z, and CLARIOstar.

Methods. CAR-T cells recognizing the CD19 molecule were generated by transduction and used as effector cells, and Raji-Luc cells ($CD19^+CD20^+$) were used as target cells. Raji-Luc cells were incubated and re-suspended in complete medium and plated in a 96-well round-bottom plate (25,000 cells/well). In parallel, freshly generated CAR-T cells were washed and re-suspended in a medium supplemented with rhIL-2. The CAR-T cells and un-transduced cells were added to the wells at appropriate dilutions to obtain different effector-to-target (E:T) ratios (1:1 and 5:1). Target and effector cells were co-cultured at 37°C for 24 hours and 48 hours and analyzed by flow cytometry after staining with an antibody cocktail (anti-CD3, -CD20, -CD69, FMC63, and 7-AAD). Raji-Luc cells were tethered to the wells of a CytoView-Z plate using anti-CD40 for 24 hours before co-culture in the Maestro assay for real time monitoring over 48 hours. Luciferase expression of Raji-Luc cells was measured 48 hours post co-culture using CLARIOstar microplate reader.

Results. Flow cytometry data revealed that, compared to un-transduced cells, the percentage of tumor cells ($7\text{-}AAD^-$ $CD20^+$) was significantly lower when co-cultured with CAR-T cells at E:T ratio of 5:1, with 43% and 81% of tumor cells killing observed at 24 hours and 48 hours, respectively. At E:T ratio of 1:1, approximately 25% of tumor cells were killed at both 24-hour and 48-hour time points indicating specificity of effector cells towards tumor targets. CAR-T cells exhibited significant upregulation of CD69, an early activation marker, at both 24-hour and 48-hour time points, while control un-transduced T cells showed lower activation. Real time cytotoxicity determined by Maestro Z showed comparable killing efficiency. At E:T ratio of 5:1, 71% and 85% of Raji-Luc cells were killed at 24 hours and 48 hours post co-culture, respectively. At E:T ratio of 1:1, 33% and 38% were killed at the

corresponding time points. Cytotoxicity assessment using CLARIOstar microplate reader showed 6% and 84% killing at E:T ratios of 1:1 and 5:1, respectively. These results demonstrate comparable cytotoxicity of anti-CD19 CAR-T cells across these three assay platforms, particularly at the high E:T ratio (5:1) within our experiment setup.

Conclusion. Compared to both the Maestro and CLARIOstar assay platforms, flow cytometry provides additional information on the activation and expansion of CAR-T cells, while real-time cytotoxicity can be easily monitored by Maestro Z. Therefore, these assay platforms can be complementary for evaluating the potency of CAR-T cells depending on the purpose.

1197 Real-time, Label-free Assessment of HER2-targeted Antibody-drug Conjugate Therapies

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Antibody-drug conjugates (ADCs) consist of monoclonal antibodies covalently linked to small molecule drugs that, together, can improve both components' anti-tumor effects. Several ADCs are targeted towards human epidermal growth factor receptor 2 (HER2), a cell surface receptor that is overexpressed in many cancers. Its increased expression in tumor cells makes HER2 an attractive target for cytotoxic therapies, allowing for the specific killing of cancer cells while minimizing off-target effects. Here, we developed an in vitro assay to assess the cytotoxic capacity of ADCs that use the HER2-targeted monoclonal antibody trastuzumab. This assay was performed in a real-time, label-free manner using the Maestro Z platform. The Maestro Z measures electrical impedance via electrodes embedded in the surface of the well to monitor cell growth and proliferation. To assess the cytotoxic effects of one HER2-targeted ADC, trastuzumab-deruxtecan (DS-8201a, branded as ENHERTU®), 5,000 HER2-positive SKOV3 cells were plated in CytoView Z plates and monitored for 24 hours before being dosed with increasing amounts of DS-8201a. SKOV3 cells were killed in a dose-dependent manner, and higher doses of DS-8201a led to larger decreases in SKOV3 impedance, indicative of greater SKOV3 death. SKOV3 cytolysis at the highest dose reached 90.9% at ~ 5 days post dose, and the EC50 value was calculated to be 30.7 µg/mL. We also tested the effects of DS-8201a against other cell types with varying degrees of HER2 expression, including A549 (low HER2 expression) and MDA-MB-231 (no HER2 expression) cells. As expected, impedance data showed that DS-8201a had enhanced cytotoxic effects on the high HER2-expressing SKOV3 cells compared to the other two cell types with lower HER2 expression. Finally, we compared the cytotoxic effects of DS-8201a to another trastuzumab-based ADC, trastuzumab-emtasine (T-DM1, branded as Kadcyla®) on the Maestro Z. Taken together, these results show that the cytotoxic capacity of ADC therapies toward HER2 positive cell types can be screened using the Maestro Z. The assay presented here can be used to further develop ADC therapies aimed at HER2-positive cancers as well as ADCs toward other cancer-specific targets.

1198 Development of a Faster Potency Assay for Evaluation of Immune Cell-mediated Cytotoxicity In Vitro

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Evaluating the effectiveness of CAR T cell therapy depends on reliable in vitro potency assays that measure the interaction between CAR T cells and cancer cells. Conventional methods typically require target cells to incubate for over 24 hours, which limits assay throughput and efficiency. In this study, we sought to significantly reduce assay duration by employing an impedance-based system to track the cytotoxic activity of CAR T cells against SKOV3 cancer cells. By comparing the standard protocol with an expedited approach that shortens the target cell resting period, we aim to develop a more time-efficient framework for potency testing—one that preserves accuracy while accelerating advancements in CAR T cell therapies. Beyond reducing overall assay time, introducing CAR T cells earlier in the process ensures that actual Effector to Target (E:T) ratios remain accurate by minimizing the opportunity for target cell proliferation. Here, we describe an in vitro potency assay that reduces the assay duration from over 72 hours to just 24 hours for immune cell-mediated cytotoxicity. SKOV3 target cells were seeded at

10,000 cells per well in a 96-well microplate equipped with embedded electrodes to monitor cell attachment and growth. HER-2 CAR T cells were introduced either 4 or 24 hours after SKOV3 cell seeding at various E:T ratios (1:10, 1:5, 1:2, 1:1, and 5:1). Killing kinetics were assessed by calculating Kill Time 50 (KT50), the time required to eliminate 50% of target cells. For target cells treated at 4 hours post-seeding, KT50 values were 92.5 ± 3.6 (1:10), 55.1 ± 21.9 (1:5), 20.6 ± 1.5 (1:2), 17.1 ± 0.7 (1:1), and 9.8 ± 0.7 (5:1) hours. In contrast, target cells treated at 24 hours post-seeding exhibited KT50 values of 154.2 ± 6.1 (1:10), 116.6 ± 7.2 (1:5), 84.5 ± 4.5 (1:2), 50.1 ± 2.8 (1:1), and 29.8 ± 0.7 (5:1) hours. As expected, earlier CAR T cell addition resulted in faster KT50 times due to reduced SKOV3 proliferation and more precise E:T ratios. At 24 hours post-CAR T addition, a dose-response curve was generated, determining EC50 values of an E:T ratio of 1:2.4 for target cells treated 4 hours post-seeding and 1:1 for those treated at 24 hours. Overall, these findings highlight a label-free, in vitro workflow using the Maestro Z impedance assay, providing a faster and more efficient method for assessing CAR T cell potency.

1199 Identification of Pharmacologically Relevant Species for IND-Enabling Toxicology Studies by Deep Comparative Transcriptomic Profiling of PBMCs Treated With Recombinant Human Payloads

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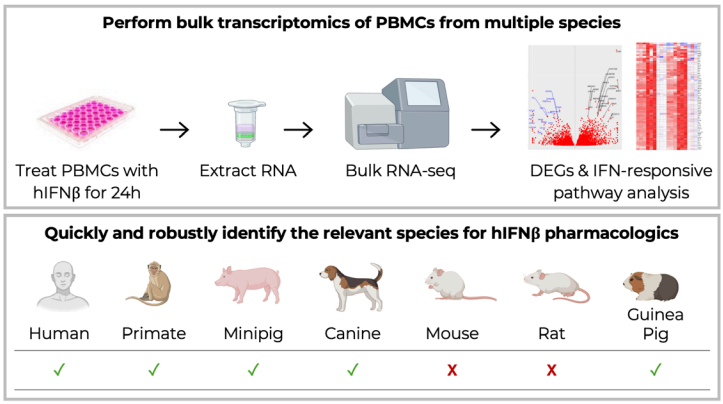
BACKGROUND: Identifying pharmacologically relevant animal species for toxicology assessments ensures study findings translate into safe human clinical trials. Siren Biotechnology has developed AAV vectorized cytokines as a novel modality for the treatment of solid tumors. As our cytokine payloads are predicted to exhibit species specificity, we developed an assay to identify pharmacologically relevant species for IND-enabling toxicology assessments. Here, we describe the methodologies and findings of this assay when implemented for an engineered human interferon beta (hIFNβ) transgene payload.

METHODS: We performed bulk transcriptomics of peripheral blood mononuclear cells (PBMCs) from human, primate, pig, canine, guinea pig, mouse, and rat donors following treatment with recombinant hIFNβ and classified each species as responsive or non-responsive. RNA was purified from PBMCs treated in triplicate with either 1,000 IU/mL (high dose) of hIFNβ, 100 IU/mL (low dose), or media alone, for 24h. Bulk transcriptomic profiling was performed by RNA-sequencing on an Illumina NovaSeq 6000 with 150 bp paired-end reads. Reads were aligned to the host species' genome using STAR v2.7.11b and counted using subread v2.0.6. Differentially expressed genes (DEGs) were identified relative to media alone and were considered significant if pAdj<0.01.

RESULTS: We first defined a target hIFNβ response signature based on the transcriptomic response of human PBMCs to hIFNβ. As expected, the human response was robust and dose-responsive (4,071 and 6,082 DEGs for low and high doses, respectively). Pathway analysis confirmed this response was Type I IFN signaling-specific, with 92% of genes from the 'Hallmark Interferon Alpha response' curated gene list being differentially expressed. The leading genes driving significance included overexpression of 3 key IFN response genes: STAT1, ZBP1, and ISG15. Mouse PBMCs were used as a negative control, as their IFNAR1 receptor (the receptor for IFNβ) is minimally cross-reactive to hIFNβ. Indeed, mouse PBMCs showed no significant treatment response (0 and 13 DEGs for low and high doses, respectively). Primate PBMCs were used to verify the method's sensitivity and accuracy to detect cross-reactivity, as primates are a "gold standard" toxicology species and have been used in nonclinical safety packages for approved hIFNβ drugs. Treated primate PBMCs showed robust and dose-responsive Type I IFN-specific transcriptomic changes, albeit with fewer DEGs than humans (192 and 821 genes for low and high doses, respectively). Excitingly, our screening approach also identified pigs, canines, and guinea pigs as highly cross-reactive alternatives to NHPs based on DEGs, hallmark pathway analysis, and consistent overexpression of STAT1, ZBP1, and ISG15. Like mice, rat PBMCs showed no significant transcriptomic changes in response to hIFNβ treatment.

CONCLUSION: We demonstrated that bulk transcriptomic profiling of cytokine-treated PBMCs can be used to quickly and robustly identify pharmacologically relevant species while bypassing development of costly species-specific reagents, enabling investigators to propose cost-effective and easier to source species alternatives to NHPs

for IND-enabling studies. This approach shows promising applications to guide species selection for other immunotherapeutic modalities or to justify the use of species-specific drug surrogates if a relevant species cannot be found.



1200 Fibroblast Activation Protein (FAP) Directed Bispecific Antibodies Target Pediatric Sarcomas

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**Corresponding Author*

Solid tumors contain a diverse tumor microenvironment (TME) that can drive cancer progression by secreting growth factors and signaling molecules that stimulate tumor proliferation, promote angiogenesis, invasion, and metastasis, remodel extracellular matrix, and drive immunosuppression and chemoresistance. Cancer associated fibroblasts (CAFs) are a critical stromal component of the TME that mediate these processes. CAFs have been well studied in adult tumors, but their prevalence and role in pediatric tumors has been largely unelucidated. A previously studied CAF target in adult tumors, fibroblast activation protein or FAP, is highly expressed on the cell surface of activated CAFs but has little to no expression on tissue fibroblasts and other cells throughout the body making it an ideal target for T-cell based cancer immunotherapy. Using bulk RNA sequencing datasets from Treehouse Childhood Cancer Initiative and internal sequencing of patient samples from the Institute for Genomic Medicine at Nationwide Children's Hospital, we screened a panel of pediatric tumors and controls for FAP expression, stromal score by ESTIMATE, and CAF content by EPIC. We identified that pediatric sarcomas showed the highest expression of FAP and had the highest stromal and CAF scores – with osteosarcoma (OS) leading the sarcomas tested. This is not unexpected as these tumors have previously been identified to have fibroblast-like properties and histology. We developed a bispecific antibody construct that combined the anti-FAP scFv from clone BIBH 1 (sibrotuzumab) with that of the anti-CD3 scFV LK2-07 (blinatumomab). To evaluate binding efficacy, we engineered a human fibrosarcoma cell line, HT1080, to overexpress FAP (HT1080-hFAP) compared to control vector (HT1080-Ctrl) to test FAP domain and human PBMCs to assess CD3 domain. We found that our construct specifically bound the overexpressed FAP on the HT1080-FAP line but not HT1080-Ctrl line in a dose-dependent manner. Similarly, our construct bound CD3 on T cells in a dose-dependent manner. Due to the high expression of FAP in OS, we tested a panel of OS cell lines and PDX models for FAP expression and found variable cell line dependent expression (ranging from no expression to high). To evaluate the killing efficacy of our bispecific antibody construct we performed an in vitro killing assay using the Incucyte system and co-cultures of target cells with human PMBCs. We found that our construct had efficient killing of target cells in a manner that was directly proportional to surface expression of FAP. Overall, we have identified that pediatric sarcomas contain a high proportion of CAFs and furthermore the that the tumor cells can express fibroblast surface markers, including FAP. We have developed a bispecific antibody that in combination with human PBMCs, successfully kills FAP-high expressing cells, while sparing those with no or low expression. Coupled with our lab's novel rAAV expression

system, this approach shows promise as a targeted therapy against CAFs and FAP-expressing sarcomas, though further in vivo studies are needed to assess its anti-tumor efficacy.

1201 Dual Costimulatory Receptors enhance antitumor immunity through cell -extrinsic and -intrinsic signals

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Adoptive cell therapy (ACT) utilizing genetically modified T cells has shown clinical efficacy against some hematological cancers; but ACT success against solid tumors is limited by (1) immune suppressive states of innate immune cells, (2) lack of T cell costimulation in the TME, and (3) tumor heterogeneity. Dendritic cells (DCs) are innate immune cells that act as messengers between the innate and adaptive systems. DCs can infiltrate solid tumors, capture, and process tumor antigens, and catalyze an adaptive immune response. Flt-3 Ligand (FLT3L) can increase the number of immature DCs by facilitating their differentiation from precursor cells. After differentiation, DCs are licensed and activated into mature DCs through CD40 signaling, enhancing antigen-specific T cell priming, epitope spreading, inducing a more robust antitumor immune response. Drugs that systemically activate FLT3 and CD40 have shown clinical efficacy but have caused significant toxicity in non-tumor organs. Here, we propose a novel strategy to locally deliver these important signals - we developed novel engineered fusion proteins, Dual Costimulatory Receptors (DCRs), that are expressed on the surface of cytotoxic, tumor-targeted T cells, which localize to the site(s) of tumor. DCRs combine a FLT3L or CD40L ectodomain with different costimulatory endodomains (e.g. CD40, 4-1BB, OX40), to provide both T cell-extrinsic and -intrinsic costimulatory signals, respectively. We hypothesized that the combination of FLT3L and CD40L DCR-T cells would synergize to further enhance antitumor immunity against solid tumors. Through *in vitro* assays, we showed that DCRs provide cell intrinsic costimulatory signals that enhance T cell function. Additionally, FLT3L and CD40L DCRs functionally signal to increase DC frequency and maturation. In a murine pancreatic cancer model (KPC), CD40L and FLT3L DCR-T cell therapy significantly enhanced tumor control ($p<0.001$). DCR-T cells increased dendritic cell populations, induced maturation, and increased endogenous antitumor T cell responses. These results demonstrate innovative approaches to deliver immune-activating signals to an immunosuppressive TME.

1202 Persistent Genetic Modification of NK Cells Using Non-Integrating SMAR DNA Vectors for Cancer Immunotherapy

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Enhancing the anti-tumour capability of NK cells via genetic modification remains a complex task. Current clinical studies primarily rely on retroviral and lentiviral transduction, which present significant limitations. NK cells are integral to the immune defense against viral infections and exhibit strong reactions to viral vectors, resulting in low transduction efficiencies. Furthermore, risks such as insertional mutagenesis and constraints on transgene size hinder the overall efficacy of viral transduction methods.

To overcome these obstacles, we have developed a DNA vector platform devoid of viral components, featuring a minimal bacterial backbone. These vectors are non-integrative and replicate extrachromosomally in the nuclei of dividing cells through the incorporation of Scaffold/Matrix Attachment Regions (S/MAR). This design offers a

promising alternative for the long-term, persistent genetic modification of NK cells while preserving genomic integrity.

Recognizing the inherent difficulty in transfecting NK cells, we optimized electroporation protocols and refined our SMAR vectors to enhance their performance. By combining a feeder-based expansion system with electroporation, we achieved transfection efficiencies of up to 70% in peripheral blood-derived NK cells with SMAR vectors expressing chimeric antigen receptors (CARs).

Additionally, we evaluated our vectors in the NK-92 cell line, which is widely employed in preclinical and clinical studies for NK cell-based cancer immunotherapies. We successfully established multiple NK-92 cell lines which stably express reporter genes or CARs targeting common tumor antigens such as CD19 and CEA. Transgene expression remained stable for at least six months and the CAR-NK-92 cells demonstrated enhanced and CAR-specific cell killing against target-expressing cancer cell lines, while their molecular phenotype remained unchanged. We found that our vectors typically establish at a single copy in NK92 cells.

During protocol development for NK-92 genetic modification, we observed significant variability among commercially available NK-92 cells from different manufacturers. The cells differ in their response to transfection with our vectors and to DNA sensing inhibitors, their transcriptomic profiles, and their killing ability against various cancer cell lines.

Our study underscores the potential of our non-viral vector system and refined transfection strategies in overcoming current limitations, paving the way for more effective and stable genetic modification of NK cells for cancer immunotherapy.

1203 NK-like Cells (GAIA-102) Induce Antitumor Adaptive Immunity via the Host NK derived IFN-γ

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Background: We have developed a novel cell therapeutic agent, GAIA-102, which exhibits an NK cell-like phenotype characterized by $CD3^-$/CD56bright/$CCR5^+$/$CCR6^+$/$CXCR3^+$/integrin-$\alpha1^+\alpha3^+\beta3^-$ expression and demonstrates significant anti-tumor activity in vitro. In previous studies, we evaluated the potential of GAIA-102 against solid tumors in various mouse models. While a marked reduction in tumor mass was observed in immunodeficient mice, the survival remained low at approximately 30%. By contrast, immunocompetent mice treated with GAIA-102 showed markedly improved survival rates (over 80%). In the subsequent second challenge test, mice that were completely cured were inoculated subcutaneously with the original tumor (CT26-GFP) and a third-party tumor (4T1). Specific rejection to CT26-GFP not 4T1, occurred in all cured individuals, indicating the establishment of tumor-specific adaptive immunity. However, the underlying mechanism remained unclear. IFN-γ, a crucial humoral factor known to activate and program the immune infiltrates in cancer vaccines and other immunotherapies, plays an uncertain role in the GAIA-102-induced immune response. Here, we aimed to evaluate whether IFN-γ is essential for the host immune response induced by GAIA-102.

Methods: We used a peritoneal dissemination model of mouse colon cancer (CT26-GFP) with repeated administration of GAIA-102. Since there is no cross-reactivity between human and mouse IFN-γ, we hypothesized that GAIA-102 would upregulate mouse-derived IFN-γ (mIFN-γ). To confirm this, we measured mIFN-γ levels in tumors, spleens, and lymph nodes on day 7 using ELISA. We then performed in vivo neutralization of mIFN-γ with anti-mIFN-γ antibody to assess its functional significance. Peripheral blood samples taken on day 7 and 17 were analyzed by flow cytometry for Ki67 expression in $CD8^+$T cells and for tumor-specific (tetramer-positive) T cells. Additionally, we investigated the role of host NK cells as potential producers of mIFN-γ by flow cytometry and evaluated the effect of NK cell depletion on $CD8^+$T cell response using GM1-asialo treatment.

Conclusion: GAIA-102 treatment led to a significant increase in mIFN-γ secretion in tumors, whereas no substantial level was detected in other immune organs. Notably, the marked rise in $Ki67^{+}CD8^{+}$T cells observed on day 7 was completely abolished following mIFN-γ blockage. By day 17, tumor-specific tetramer-positive $CD8^{+}$ T cells also diminished, emphasizing the crucial role of mIFN-γ in tumor control. Further investigation revealed that host NK cells infiltrating the tumors were the primary source of mIFN-γ. Consistent with this, GAIA–102–treated mice displayed increased NK cell numbers in both tumors and spleens. Depletion of NK cells suppressed the induction of proliferating and tumor-specific CD8+ T cells, highlighting the importance of the NK-derived IFN-γ (NK-IFN-γ axis) in promoting an effective immune response**.** Our findings demonstrate that the host adaptive immunity induced by GAIA-102 is dependent on the NK-IFN-γ axis and suggest that strengthening the interactions between T cells and NK cells may yield improved antitumor response in solid tumors.

1204 CRISPR-Guided Evolution of Oncolytic Virotherapies for Improved Delivery

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Directed evolution, the iterative process of genetic diversification and selection for genotypes that confer improved function, has been widely used to optimize small genome viral vectors for gene therapy. Nucleocytoplasmic large DNA viruses (NCLDVs) can deliver long genetic payloads, rendering them as promising candidates for vectored vaccines and oncolytic virotherapy. However, technological challenges for targeted diversification of NCLDV's complex genomes limit their amenability to be optimized by directed evolution using conventional DNA synthesis and cloning techniques. To address this challenge, we have developed CytoEvolvR, an RNA-guided, error-prone polymerase that localizes to and continuously diversifies cytoplasmic DNA in cells. Next-generation sequencing and fluorescence conversion assays showed that optimized versions of CytoEvolvR generated tunable, multiplexable, site-specific, and non-PAM-constrained diversification to all four nucleotides within endogenous loci of a double-deleted oncolytic vaccinia vector. CytoEvolvR-mediated diversification of a vaccinia surface protein, A34R, coupled with selections for extracellular enveloped vectors uncovered a novel variant that enabled increased survival and decreased tumor burden in syngeneic mouse models of colorectal cancer. Moreover, CytoEvolvR screening uncovered a previously unknown protein that is expressed from an open reading frame overlapping with the A34R gene and has fitness implications in the viral replication cycle. Accordingly, CytoEvolvR is a powerful tool for rapidly improving the therapeutic properties of oncolytic virotherapies and elucidating new biology.

1205 Molecular modification of viral surface protein enhances targeting ability of oncolytic virus

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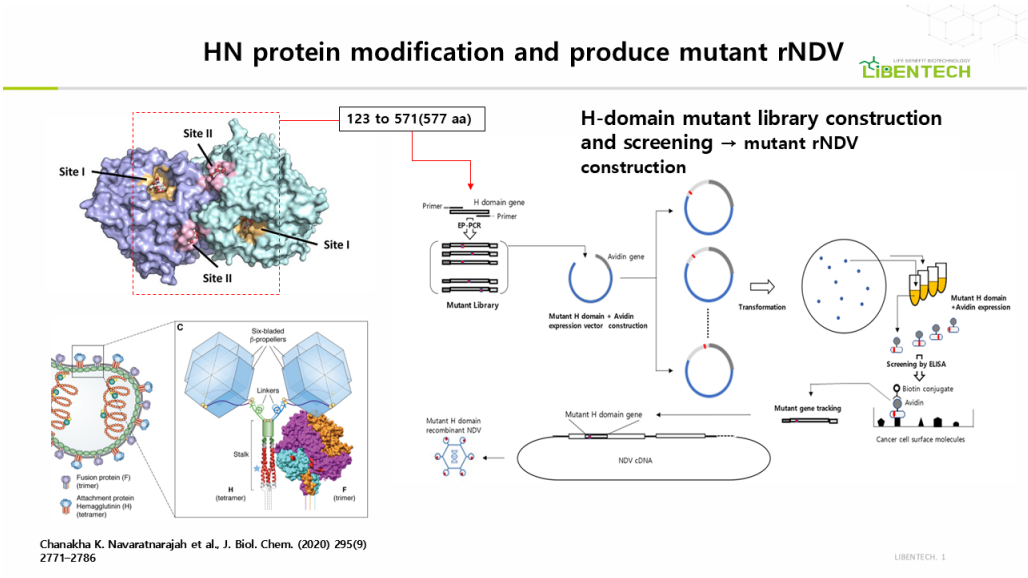
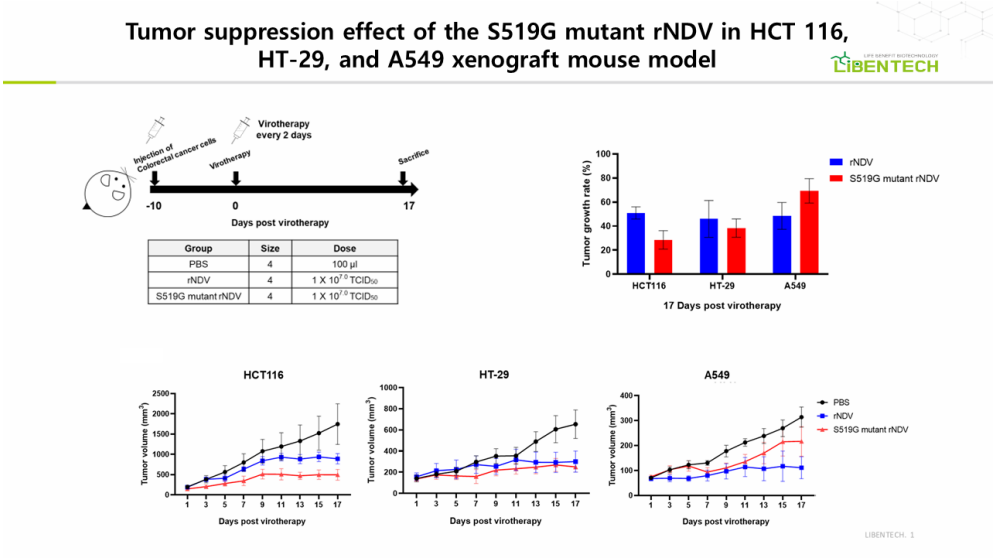
Oncolytic viruses represent a promising therapeutic approach for cancer treatment due to their selective targeting and destruction of cancer cells. However, enhancing their specificity for certain tumor types remains a critical challenge. This study utilized directed evolution to modify the hemagglutinin-neuraminidase (HN) surface protein of Newcastle Disease Virus (NDV), aiming to improve its targeting ability toward colorectal cancer cells. A mutant library of the HN protein was generated using error-prone PCR and screened for affinity to HCT 116 colorectal cancer cell surface proteins. The S519G mutation was identified as significantly increasing specificity and binding affinity for HCT 116 cells. Recombinant NDV (rNDV) incorporating the S519G mutation (S519G rNDV) exhibited enhanced oncolytic effects and higher tumor specificity in vitro and in xenograft mouse models compared to wild-type rNDV. Tumor suppression was most pronounced in HCT 116 xenografts, achieving a 71.6% reduction in tumor volume. Importantly, the mutation did not alter the pathogenicity of NDV, as demonstrated by intracerebral pathogenicity and median death time tests. This research highlights the potential of molecular modification of viral surface proteins to improve the tumor-targeting specificity of oncolytic viruses, paving the way for more effective cancer therapies.





1206 Using primary cell culture models to study adenoviral vectors for virotherapy of head and neck squamous cell carcinoma

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Introduction: Adenoviruses (AdVs) are investigated as oncolytic adenoviral vectors (OAdV) and for tumor gene therapy (TGT). Antitumor effects can be enhanced by expression of effectors such as gene editing tools or immune

modulators. Head and neck squamous cell carcinomas (HNSCC) have a poor prognosis, are difficult to treat or resect and common treatments cause severe side effects. Moreover, a portion of HNSCC is HPV positive. As HPV oncogenes can complement OAdV replication, HPV related cancers potentially support and enable OAdV-mediated tumor lysis. Therefore, HNSCC are interesting targets for OAdVs therapy. As HNSCC are heterogeneous with regard to HPV status and other biomarkers and response to treatment, we aimed to address these individual differences between patients by establishing primary patient derived tumor cell cultures to identify characterize novel improved AdV vectors for HNSCC. AdV vectors are mostly based on AdV5, having limited applicability due to high seroprevalence and strong liver tropism. Alternative AdV serotypes might have beneficial features for the treatment of HNSCC such as transduction efficiency and oncolytic potential and can be used as OAdV or for TGT instead of AdV5.

Methods: Following tumor resection we established a pipeline for primary patient derived tumor cell cultures to study their response to AdVs. Using a library of reporter gene expressing AdV serotypes representing the natural diversity of human AdVs, we investigate the potential of alternative AdVs Serotypes for oncotherapy of HNSCC. Transduction of individual primary HNSCC cell cultures with reporter gene expressing AdV library identified AdV candidates with improved transduction and gene expression as well as inhibitory effects on tumor cell viability. Selected serotypes were vectorized by replacing AdVE1 promotor with hTert Promotor and armed with AdV death protein or P19. Subsequently their oncolytic properties in primary HNSCC cells were compared to their AdV5 based counterpart.

Results: Transduction of individual primary cell cultures with different AdV serotypes and comparison of reporter gene expression and the effects on tumor cell viability identified AdV4, 21, 34, and 35 and 37 as superior to AdV5 in HNSCC even though we observed differences from patient to patient and differences in transduction and cell killing efficiency between the different serotypes. AdV4, 14, 21, 34, and 35 were vectorized and armed. Comparison of their oncolytic potential in established and primary HNSCC cultures revealed 10 to 100 fold improved tumor cell killing in vitro.

Discussion: Our primary tumor cell culture pipeline in combination with our reporter gene expressing AdV library allowed identification and vectorization of improved AdV vectors. Newly developed armed OAdV based on alternative serotypes are promising candidates to improve oncolytic therapy or TGT for HNSCC. Investigation of HPV status and AdV receptor expression of primary tumor cells and cells and collection of disease-relevant patient information will allow to correlate these findings with the outcome of AdV transduction experiments. In the future, this will enable us to predict treatment outcomes and to choose the optimal OAdVs for each individual HNSCC patient. The combination of OAdV treatment with radiation and chemotherapy will give insights on the influence of pretreatments on the potential response towards viral tumor therapy.

1207 CF33-hNIS Alone and Combined with Anti-PD-L1 Eliminates Gastric Cancer Peritoneal Metastasis in an Immunocompetent Mouse Model

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INTRODUCTION: Peritoneal metastasis (PM) from gastric cancer (GC) is fatal. Despite improved survival with the addition of anti-PD-L1 therapy to systemic chemotherapy, majority of GCPM patients have tumors with genomic stability, low tumor mutational burden, and anti-tumor immunogenic inactivity, leading to immunotherapeutic failure. To overcome these barriers, we are developing a peritoneal-directed strategy using oncolytic virus (OV) combined with anti-PD-L1.

METHODS: CF33-hNIS, a novel orthopoxviral chimera was used to treat five human (intestinal-type: AGS and MKN-74; diffuse-type: KATO-III, MKN-45 and SNU-16) and 2 murine (ACKPY3944 and ACKPY4113) GC cell lines at various multiplicities of infection (MOI 0.01, 0.1, 1.0 and 10.0). Standard plaque and cell proliferation assays were performed for viral replication and cytotoxicity. Cell surface and intracellular expression of PD-L1 were

evaluated by flow cytometry. ACKPY3944 cells derived from transgenic mice (KRAS G12D mutant/CDH-1 attenuated) were transfected with ffluc (ACKPY3944-ffluc), and 10^6 cells were injected IP (day 0) to establish a reproducible PM model in C57BL/6 mouse. We separately evaluated immunogenicity and anti-tumor efficacy, and overall survival in these mice. First, we compared 4 different cohorts: (A) IP PBS, (B) IV anti-PD-L1, (C) IP CF33-hNIS ($3x10^7$ pfu, days 3, 5, 7), (D) IP CF33-hNIS ($3x10^7$ pfu, days 3, 5, 7) + IV anti-PDL-1. Peritoneal fluid/washings were collected on days 5, 9, and 21 and analyzed by flow cytometry and immunohistochemistry for immune cell changes. Then, we compared 6 cohorts: (A) IP PBS, (B) IP anti-PD-L1, (C) IP CF33-hNIS ($3x10^7$ pfu, day 3, 5, 7), (D) IP CF33-hNIS ($3x10^7$ pfu, day 3, 5, 7) + IP anti-PDL-1, (E) IP CF33-hNIS (10^8 pfu, once), and (F) IP CF33-hNIS (10^8 pfu, once) + IP anti-PD-L1. Survival was observed daily, and peritoneal tumor burden was monitored weekly using bioluminescence.

RESULTS: CF33-hNIS infected, replicated in, and killed both human and mouse GC cell lines in a time and dose-dependent manner. While no differences in oncolytic viral properties were observed between diffuse- versus intestinal-type human GC cell lines, greater antitumor efficacy was seen against human versus mouse GC cells. We confirmed endogenous PD-L1 expression in ACKPY3944 and ACKPY4113 cells. Compared to PBS control, IP CF33-hNIS transiently but significantly increased the total CD45+ leukocytes, CD3+ T cells, and CD8+ T cells in the peritoneal cavity on day 9. All IP CF33-hNIS cohorts (alone or combined with IP anti-PD-L1) prolonged mouse survival. A one-time high dose of IP CF33-hNIS (10^8 pfu) + IP anti-PD-L1 showed the highest efficacy against peritoneal tumors with 6 of 8 survivals for 112 days (Figure 1). In contrast to PBS (0 survival), IP anti-PD-L1 (0 survival), cohorts treated with 3 doses of $3x10^7$ CF33-hNIS had 3 survivals; 3 doses of $3x10^7$ CF33-hNIS + IP anti-PD-L1 had 4 survivals; and one dose of 10^8 CF33-hNIS had 2 survivals.

CONCLUSION: CF33-hNIS exhibits robust anti-tumor activity against GCPM, transiently upregulating PD-L1 with immune activation. Combining a one-time high dose of IP CF33-hNIS with IP anti-PD-L1 eliminated peritoneal tumors and prolonged survival in an immunocompetent mouse model. These results demonstrate the robust potential of CF33-hNIS against GCPM.

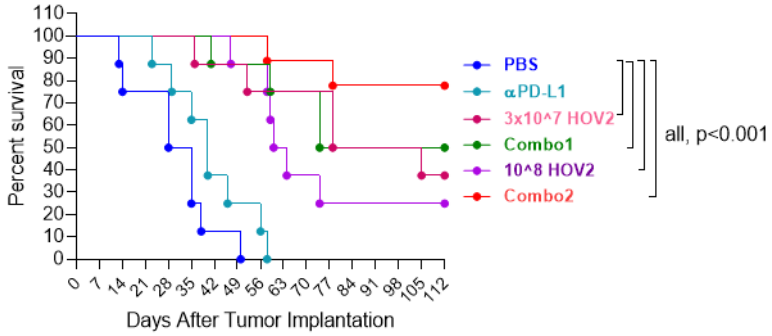

Figure 1. Intraperitoneal (IP) delivery of CF33-hNIS with anti-PD-L1 cure gastric cancer peritoneal metastasis in an immunocompetent mouse model. Kaplan-Meier survival analysis of ACKPY3944 peritoneal tumor-bearing mice following treatment with IP CF33-hNIS with or without IP anti-PD-L1 or PBS control. *** p<0.001. Note: Combo1 = CF33-hNIS ($3x10^7$ pfu, three times) plus anti-PD-L1 antibody, Combo2 = CF33-hNIS (10^8 pfu, one time) plus anti-PD-L1 antibody.

1208 Intraperitoneal Delivery of Chimeric Orthopoxvirus CF17 Improves Survival in a Syngeneic Mouse Model of Gastric Cancer Peritoneal Metastasis.

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[1]City of Hope National Medical Center, Duarte, CA, [2]City of Hope Inc, Duarte, CA, [3]City of Hope, Duarte, CA, [4]City of Hope National Medical Center, [5]Cedars Sinai, Los Angeles, CA, [6]City of Hope Medical Center, Duarte, CA

INTRODUCTION: Peritoneal metastasis (PM) from gastric cancer (GC) remains lethal despite improved systemic therapies. Peritoneal-directed therapies are under investigation to improve the survival of GCPM patients who have a 2% five-year overall survival. CF17 is a novel chimera with a unique genome created by the random recombination of nine orthopoxvirus species. We investigated the antitumor effects of CF17 on mouse GC cell lines in vitro and in a syngeneic mouse model of GCPM.

METHODS: We infected two mouse GC cell lines (ACKPY3944 and ACKPY4113) with CF17 or CF17-GFP and analyzed infection, replication, and cytotoxicity using immunofluorescence microscopy and standard plaque and cell proliferation assays. We further established an immunocompetent GCPM mouse model in C57BL/6 mice by intraperitoneal (IP) inoculation of ACKPY-3944-ffluc cells ($1x10^6$). These mice were intraperitoneally treated with either PBS (n=11) or $3x10^7$ pfu CF17 (n=11) on days 3, 5, and 7. Peritoneal tumor progression and tumor burden were monitored weekly using bioluminescence imaging. Each group was further divided into two groups: one for analyzing immune cell changes by flow cytometry and immunohistochemistry by collecting peritoneal washings and tumor (day 9; n=3); and another for observing survival (n=8).

RESULTS: CF17 infected and replicated in ACKPY3944 and ACKPY4113 cells with GFP expression of CF17-GFP and more than 4-log increase viral titers of CF17 than control after 72-hour infection. Treatment with CF17 showed anti-tumor activity in a dose and time-dependent manner (91% cell killing on day 5 of MOI=1 *vs.* 95% cell killing on day 3 of MOI=10). In the syngeneic GCPM mouse model, IP CF17 continued to decrease tumor burden between days 7 to 21 ($P<0.05$) and prolonged survival ($P<0.001$), with an average survival of 41.5 days (CF17) *vs.* 18.7 days (PBS). IP CF17 also significantly increased leukocyte infiltration into the tumor microenvironment of the peritoneal cavity, which included CD45+ cells (2.5x), CD3+ cells (5.1x), CD8+ cells (10.1x), and into the solid tumors, which included CD8+ cells and CD4+ cells.

CONCLUSION: IP CF17 shows effective anti-cancer potential in an immunocompetent GCPM mouse model with significant antitumor activity and survival benefit. A significant increase of CD8+ T cells in the tumor microenvironment of the peritoneal cavity and tumors after IP CF17 treatment suggests that CF17 may increase the immunogenicity of the peritoneal tumors.

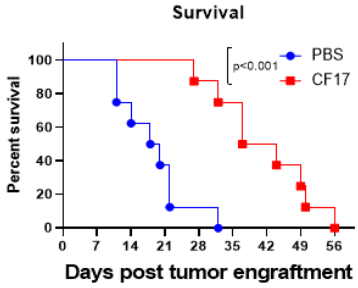

Figure 1. Intraperitoneal (IP) CF17 treatment improves survival. Kaplan-Meier survival analysis of IP ACKPY3944 tumor-bearing mice following treatment with IP CF17 or PBS control. P<0.001

1209 Arming an RNA-Based Oncolytic Picornavirus Platform Using LNPs for Replicon Co-Delivery

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Oncolytic virotherapy is the use of viruses to treat cancer. With their lytic and immunogenic lifecycles, picornaviruses are excellent candidates for oncolytic vector development with several being explored in clinical

settings. However, the strict genome size limitation of these viruses prevents efficient gene insertion for arming. Since picornavirus genomes are inherently infectious, vector constructs can be delivered without a capsid, enabling the use of non-viral carriers like lipid nanoparticles (LNPs) for co-delivery of external RNA expression constructs. One such construct is the replicon, a modified picornavirus genome in which the capsid proteins are replaced with a transgene. Without the structural proteins, they act as self-amplifying expression vectors but cannot spread beyond the initially transfected cell. If the capsid proteins are provided in trans, replicons can be packaged. Thus, co-delivery of virus genome and replicon can facilitate the spread of transgene expression with the virus without directly encoding the gene within the genome. Previous studies, including our own, have demonstrated the feasibility of formulating oncolytic picornaviruses as infectious RNA (iRNA) using Coxsackievirus A21 (CVA21), a clinically developed oncolytic virus. However, preclinical evaluation in immunocompetent models is limited, as mice are not a natural host for CVA21. In contrast, mengovirus can be explored in unmodified immune competent mice. Previously, we showed a significant improvement in overall survival with our optimized mengovirus viral platform, MC_0NC, in an immune competent murine MPC-11 model. Here, we use an attenuated mengovirus construct, MC_0R, optimized for replication as an iRNA platform. MC_0R is attenuated via deletion of the polyC tract, a known virulence factor, and inclusion of microRNA-target sequences corresponding to tissues of pathogenicity. Specific infectivity, translation, and cytotoxicity of the iRNA were evaluated *in vitro*. *In vivo* studies determined the efficacy via disease burden and survival. Mice bearing MPC-11 subcutaneous tumors were treated intratumorally with empty LNPs or LNPs containing MC_0R iRNA or luciferase expressing replicons (MC_0R-fLuc). MC_0R iRNA, *in vitro*, demonstrated specific infectivity, translation, and cytotoxicity similar to unmodified genomes. *In vivo*, the MC_0R-LNP treated groups showed control of tumor growth, improved overall survival and measurable viremia compared to empty and MC_0R-fLuc LNP treated groups. MC_0R-fLuc treatment showed minimal therapeutic efficacy but resulted in consistent intratumoral expression 6hrs following treatment with a small amount of expression maintained out to 24hrs. *In vivo* studies are ongoing to determine the impact of iRNA and replicon co-delivery. Overall, oncolytic picornavirus iRNAs delivered by non-viral vectors can elicit therapeutic efficacy, and self-amplifying RNA alone is not enough to achieve the same effect. Further, replicons can act as short-term expression vectors. This work supports the continued development of mengovirus as an RNA-based oncolytic virus platform and the use of replicons as RNA-based arming factors.

1210 In Vivo Efficacy of a NIS-expressing Oncolytic Adenovirus for Colorectal Cancer Treatment

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**Corresponding Author*

Introduction: Colorectal cancer (CRC) remains a leading cause of cancer-related death throughout the world. Many patients develop metastatic disease, but conventional therapies are often not effective at treating these advanced malignancies. Therefore, novel therapeutics are needed, and our group is investigating oncolytic adenoviruses (OAd) as a novel treatment approach.

Methods: Our research group has designed an oncolytic adenovirus which expresses the sodium iodide symporter (NIS) with each cycle of viral replication (OAd-NIS). Using multiple human CRC cell lines, the viral vectors were evaluated for optimal fiber structure, cytocidal effects, and transgene expression. Murine models were also utilized to test the therapeutic effect of the virus in addition to its ability to enable radiotracer (^{124}I) uptake and imaging in human CRC tumors.

Results: As we have previously described, the adenovirus employs the ΔE3 NIS backbone structure. Our *in vitro* studies in multiple human CRC cell lines demonstrated that the Ad 5/3 chimeric fiber is optimal for virus binding and transduction. Crystal violet cell viability assays demonstrated strong cancer-killing effects across all tested cell lines at early time points with low viral doses. Importantly, *in vitro* immunohistochemical staining demonstrated strong NIS expression. Human CRC xenografts (LoVo cell line) were established in the flanks of nude mice and then treated with a single, intratumoral dose of OAd-NIS. After radiotracer (^{124}I) administration via an intraperitoneal route, small animal PET/CT images were acquired with the Sofie G8 system and demonstrated high

levels of signal within the flank tumors. The avid signal in the tumors confirms the ability of the OAd-NIS virus to facilitate *in vivo* NIS expression to enable radioiodine uptake, and this was correlated with viral copy number via PCR analysis. A separate experiment was conducted with LoVo subcutaneous xenografts in nude mice, where they were treated with multiple injections of virus ($1x10^9$ pfu on Days 0 and 3). The OAd-NIS group demonstrated robust and statistically significant tumor growth suppression when compared to the PBS control group.

Conclusion: Our OAd-NIS virus demonstrates excellent transduction efficiency, transgene expression, and cell-killing effects in CRC cell lines. Strong NIS expression in our murine models enables PET/CT imaging with radioactive iodine and highlights the ability of the virus to support radionuclide-based imaging for CRC. The *in vivo* tumor growth suppression demonstrated by our murine model suggests a high potential for clinical translation of the OAd-NIS virus to be used as part of a novel regimen for CRC treatment. Future studies will test the effects of the virus in combination with radioiodine ^{131}I.

1211 Intraperitoneal Administration of The CD133-targeted Oncolytic Adenovirus Suppresses Peritoneal Metastases of Colon Cancer in Mouse Xenograft Model.

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Introduction: Colorectal cancer (CRC) is the third most common cause of cancer-related death in the US, and about 20% of patients with local advancement develop peritoneal metastasis (PM) during follow-up. The 5-year survival for patients with PM is limited to @14%. The patients with PM who had previously been treated with chemotherapy frequently show resistance to chemotherapy regimens, and their prognosis after acquiring resistance is dismal. It is therefore critically important to develop novel therapeutic approaches for targeting treatment-resistant cells toward improved prognosis and quality of life for CRC patients with PM. Cancer stem cells uniquely contribute to establishment of metastases, recurrence, and treatment resistance. It has been demonstrated that a variety of cancer treatments increase expression of CD133, and these CD133-positive ($CD133^+$) cells contribute to the acquisition of treatment resistance. Interestingly, elimination of $CD133^+$ cells restores the sensitivity to chemotherapy in CRC. We therefore hypothesize that the development of a novel approach eliminating $CD133^+$ cells would lead to a more effective treatment of CRC with PM.

Methods: We used CD133-targeted oncolytic adenovirus to eliminate CD133-positive cells. We generated CD133-targeted OAd (CD133-OAd) by using our adenovirus-based library screening system. We evaluated the target specificityof the virus *in vitro* and *in vivo* analysis. For in vitro analysis, we used binding assay and cell killing assay with CD133-positive or CD133-negative CRC cell lines. As a proof of concept, the *in vivo* theraputic potential of the CD33-OAd was investigated in a mouse peritonerally metastatic colon cancer model with 2 CD133-positive CRC cell lines (HCT116 and LoVo). Luciferase-labeled cells were inoculated intraperitoneally into mice, and treatment started 5 days post cell inoculation. On day 6, mice with tumors were divided into 4 groups: PBS intraperitoneal (i.p.) injection (200ul, 3 times), wild-type Adenovirus type 5 (Ad5-WT), CD133-OAd, and integrin-targeted OAd (RGD-OAd) i.p. injection (1×10^{10}vp/200ul, 3 times). All mice were monitored by IVIS imaging once a week to confirm and track tumor growth.

Results: Binding and cell killing assay confirmed that the CD133-OAd selectively infected CD133-positive cells and lysed them efficiently. *In vivo* anti-tumor assay demonstrated that CD133-OAd significantly suppressed the peritoneal metastasis of CD133-positive CRC cells compared to administration of PBS or RGD-OAd. The survival of mice with PM was significantly increased by treatment with CD133-OAd compares with PBS, Ad5-WT, or RGD-OAd treatment. Interestingly, Adenovirus type 5 (Ad5-WT) showed anti-tumor activity, and its level was comparable with CD133-OAd. However, Ad5-WT injected mice started to show severe toxicity. At necropsy of Ad5-WT injected mice, all of the mice showed abdominal adhesion. While the level of IL-6 and TNF-alpha in serum were significantly high in Ad5-WT injected mice, the level in CD133-OAd injected mice were similar to the control mice.

Conclusions: Intraperitoneal administration of CD133-OAd has therapeutic potential to suppress the peritoneal metastasis in mouse model without significant toxicity, resulting in prolonged survival.

1212 ML-Driven Design of Lipid Nanoparticles for In-Vivo T-Cell Delivery

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T-cell-based immunotherapies like CAR-T have revolutionized cancer treatment, offering life-saving treatment for patients with refractory cancers. However, high manufacturing costs, challenging supply chains, labor-intensive ex-vivo manipulation, and prerequisite lymphodepletion limit scalability. In-vivo mRNA delivery to reprogram T-cells directly within the body offers a potentially transformative alternative by streamlining production, reducing costs, and broadening patient access. However, developing efficient delivery systems to target T-cells remains a critical challenge.

To address the complexity of lipid nanoparticle (LNP) development, Mana.bio has created a machine learning-driven platform to optimize LNPs for T-cell transfection in-vivo and ex-vivo. Our platform integrates over 80,000 data points from proprietary experiments and 45,000 from public literature, using advanced ML algorithms to screen ~50 million formulations. The model demonstrates high performance with predicted T-cell transfection accuracy (MAE: 3.72, Pearson correlation: 0.83) and T-cell viability (MAE: 13.71, Pearson correlation: 0.72), effectively predicting lipids and LNP compositions based on key parameters. These algorithms rapidly identify LNP candidates for therapeutic development based on physicochemical properties, toxicity profiles, and the likelihood of successful T-cell transfection. Initial validation using eGFP mRNA showed over 90% transfection efficiency in activated CD3+ T-cells from human peripheral blood mononuclear cells (hPBMCs) within 24 hours, with >80% cell viability, effectively outperforming an industry-standard immune cell-transfection reagent at the same dose. Notably, our LNPs also maintained high transfection efficiency (70%) in non-activated CD3+ hPBMC T cells. Mana's formulations exhibited enhanced transfection efficacy compared to MC3, a clinically validated LNP, as well as toxicity profiles assessed by measuring TNF-α, IL-1β, and IL-6 levels in both activated and non-activated hPBMCs 24 hours post-treatment. In-vivo activity was validated in NSG mice injected with hPBMCs three days before LNP administration, and eGFP expression was evaluated in blood, spleen, lung, and liver after 24 hours. The results demonstrated successful transfection, with lead LNP candidates yielding ~25% transfection in CD3+ T-cells. Lead formulation preferentially transfected subsets of CD4+ and CD8+ T cells with our leading formulation, achieving 18% and 24% transfection, respectively. Spleen analysis exhibited a 10% transfection rate of CD3+ T-cells. Immuno-histological analysis of liver tissues revealed minimal to no eGFP expression, while flow cytometry analysis of lung sections showed only 1% of lung cells transfected with eGFP; thus demonstrating selective T-cell targeting in-vivo.

Our ML-driven optimization model and custom-designed LNPs enable highly specific T-cell targeting with low toxicity. Beyond CAR-T, this technology has therapeutic potential in autoimmune and infectious diseases, where targeted T-cell reprogramming is crucial. By integrating advanced computational modeling and experimental data, Mana.bio is laying the foundation to unlock the potential of RNA delivery, accelerate therapeutic development, and enable next-gen immunotherapies.

1213 A novel polymeric gene delivery platform to precisely target and reprogram cells *in vivo* for haematological malignancies & beyond

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With seven marketed products and more than 900 ongoing clinical trials, CAR-T cells have revolutionized blood cancer treatment by targeting and eliminating B cells. However, their *ex vivo* manufacturing remains complex, costly and directly impacts the fitness and efficacy of engineered cells. *In situ* CAR-T therapies emerged as promising alternatives but face challenges such as off-targeting, immunogenicity, and viral particle potency.

Alaya.bio is a preclinical-stage biotechnology company developing a scalable gene delivery platform to target and reprogram cells *in situ* using nanoparticles consisting of engineered lentiviral vectors coding for various payloads and coated with cationic and shielding biodegradable polymers. Up to 20 monodispersed nanoparticles were successfully formulated with different surface potentials, excellent batch-to-batch consistency and stable gene delivery in human cell models relevant for gene and cell therapies.

Here we report on nanoparticles consisting of lentiviral vectors lacking the VSV-G immunogenic protein and encoding CD19 CARs or GFP coated with poly(beta-amino ester)s biodegradable polymers with or without a polysarcosine lipid shielding polymer allowing the bioconjugation with CD3-targeting agents. *In vitro*, we achieved long-term, dose-dependent expression and integration after exposure of human cell lines to PBAE-based nanoparticles. Interestingly, cell toxicity was directly linked to the quantity of cationic polymers used in the formulations. In addition, the stability of the polymeric nanoparticles was confirmed biophysically in various cell culture media with or without serum. Importantly, stable delivery of the transgene was achieved in human primary T cells without the need of CD3/CD28 activation addition normally required for transduction with classical lentivectors.

In vivo, the biodistribution of nanoparticles has been investigated in immunocompetent and humanized mice after multiple intravenous administrations or infusions and showed a different profile compared to pseudotyped lentivectors. Long-term expression of GFP or CAR transgenes was detected in blood leukocytes up to 80 days post-treatment. The pronounced tropism for blood cells observed *in vivo* with our polymeric nanoparticles provides an obvious advantage for CAR T-cell therapy of blood malignancies. Repeat administration was safe and well tolerated as no obvious sign of distress, body weight loss, hepatotoxicity, change in blood cell count or cytokine levels were reported. Preliminary efficacy data have shown in healthy Balb/c mice that repeat administration of anti-CD19 CAR encoding nanoparticles induced B cell depletion. In mice with A20-induced lymphoma, repeat treatment cycles showed promising anti-cancer efficacy and reinfusion at relapse was possible.

To conclude, this technology shows great potential for further preclinical and clinical development of *in situ* universal CAR T-cell therapy. In addition, Alaya.bio's versatile platform offers the potential to target and reprogram a broad range of therapeutic cells beyond the immuno-oncology field and treat genetic disorders.

1214 LENTIVIRAL VECTOR ENGINEERING WITH POLYMER SURFACE MODIFICATION FOR CAR-T CELL THERAPY

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CAR-T cells have revolutionized blood cancer treatment by targeting and eliminating B cells. However, their *ex vivo* production remains complex and costly. *In situ* therapies emerged as promising alternatives but face challenges such as off-targeting, immunogenicity, and viral particle potency. Alaya.bio's technology combines biodegradable polymers with lentivectors (LVs). Our VSV-G-deficient LVs lack cell transduction ability until coated with pH-responsive cationic polymers, while modification with shielding polymers enhances stability and allows bioconjugation with targeting agents.

Alaya.bio's LVs lacking VSV-G immunogenic protein are engineered and manufactured through a scalable and reproducible GMP-compatible bioprocess. In addition, we developed high-throughput biophysical read-outs using

among others DLS and interferometry allowing us to screen 40 families of biocompatible cationic and shielding polymers with various shapes (linear, star, dendrimers), nature (polyamino acids, polysaccharides, poly β-amino esters (PBAE), polyethylenimine-derived polymers), molecular weights, and pK_A values. These polymers were used to formulate monodisperse LV-based nanoparticles with controlled biophysical characteristics (Dh<300 nm and PdI<0.3). Two surface modification strategies were compared: layer-by-layer coating with cationic/anionic polymers and self-assembled coating with shielding lipid/cationic polymers. For layer-by-layer coating, key parameters included mixing process, buffer composition, salt concentration, and polymer molecular weight. Coating optimization involved pH and polymer concentration adjustment to achieve surface saturation while minimizing cytotoxicity. Polysarcosine lipid self-assembled coating resulted in monodispersed particles with shielding properties. Subsequent cationic coating produced stable nanoparticles with positive zeta potential requiring lower polymer concentrations.

So far we have developed over 20 polymeric nanoparticles with different surface potentials and confirmed the excellent batch-to-batch consistency according to biophysical and functional characteristics. Each formulation specific parameters were easily transposed to various lentiviral vector batches coding either for GFP and several CD19-CAR constructs, reflecting the robustness of the selected surface modification strategies.

Taking the example of PBAE coating, dose-dependent GFP and CAR transgene expression and integration were achieved in human cells treated with nanoparticles. While single-layer cationic nanoparticles demonstrated higher transduction efficiency at lower concentrations compared to shielded cationic particles, both formulations were functional *in vitro* over 10 days.

These encouraging results prompted us to engineer T-lymphocytes targeting nanoparticles by conjugating anti-CD3 scFv to shielding polymers. The best binders have been screened for affinity, specificity and activation before being added to shielding polymers by amine coupling. The cell-specific gene delivery of modified targeting nanoparticles is currently investigated.

To conclude, Alaya.bio has developed polymeric nanoparticles with different surface potentials and *in vitro* properties. By combining optimized LV bioproduction and formulation screening, Alaya.bio has now access to next-generation nanoparticles for gene delivery. These will be applied to the rapid and universal manufacturing of CAR T-cells with preserved naive and memory phenotypes.

1215 Introduction of Novel *in vivo* Cell Specific Payload Delivery Platform - CARGO

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Chimeric Antigen Receptor-mediated (CAR) cell therapy, such as CAR-T cell therapy, represents a groundbreaking advancement in the field of immunotherapy, offering a novel paradigm for cancer treatment. Despite its potential, current CAR cell therapies face significant challenges, including weak cytotoxicity, cytokine release syndrome (CRS), and limited efficacy, primarily in treating blood cancers rather than solid tumors. While CAR cell therapy focuses on destroying target cells, the adeno-associated virus (AAV) platform has emerged as a widely used tool for delivering genetic material, offering a solution for reprogramming malfunctioning target cells. AAV-mediated *in vivo* gene therapy holds promise as an alternative, enabling the delivery of therapeutic genes for sustained protein expression in target tissues. However, several issues still need to be resolved, such as non-specific targeting, unclear in the factors affecting cellular and whole-body disposition, dose-exposure and exposure-response relationships, as well as the impact of product quality and immunogenicity on these properties.

Recognizing the potential of CAR-mediated cells to be engineered for precise cell targeting and addressing the limitation of AAV in lacking target specificity, we have developed a novel concept called CARGO, which leverages CAR-mediated cell-specific targeting as its core principle.

CARGO is a new strategic platform designed to deliver molecules of interest to target cells by facilitating interactions between CAR-mediated cells and their target cells (e.g., CD19CAR to CD19). Thus, CAR-mediated cells can serve as delivery vehicles to transport molecules or drugs to target cells, enabling specific gene editing or cell killing. As proof-of-concept, The CD19CAR module was used as the targeting segment, and HSVTK with in/out cell transporting sequences was used as the payload package in delivery cells. CD19-expressing cell-specific targeting, expression, and migration of HSVTK were observed in vitro and in vivo, demonstrating that the proof-of-concept CARGO system is effective and provides new insight into innovative drug delivery system based on direct binding between delivery and target cells. Utilizing the CARGO platform, the key limitations of current CAR-mediated cell therapy and AAV-mediated cargo delivery can be improved.

1216 Generation of Human iPSC-Derived Hematopoietic Progenitor Cells and Natural Killer Cells with Anti-Tumor Functionality Using a Feeder-Free Differentiation Platform

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Securing a reliable source of high-quality hematopoietic stem and progenitor cells (HSPCs) is critical for the development of many autologous and allogeneic cell and gene therapies. The therapeutic use of primary HSPCs from sources such as bone marrow or cord blood is constrained by the availability of source materials and HLA-matched donors. Induced pluripotent stem cell (iPSC)-derived HSPCs (iHSPCs) offer a consistent source for generating various clinically relevant cell types, including Natural Killer (NK) and T cells. However, these differentiation workflows typically take weeks or months to complete, and labor- and material-intensive testing during development phases can rapidly increase costs. Implementing an efficient differentiation system early in the process is crucial for the effective development and characterization of both the therapeutic product and the production processes.

Here, we describe a fit-for-purpose feeder-free iHSPC differentiation system suitable for disease modeling and the development of cell-based therapies. The system utilizes cell therapy grade and regulation-compliant reagents and can be initiated from either 2D or 3D iPSC culture. This flexible protocol supports testing throughout process development by enabling cost-effective, small-scale testing that can be scaled up using shake flasks. By avoiding steps that can hinder culture scale-up, like spin embryoid body formation and complicated handling procedures, this protocol facilitates the translation of process development insights to scale-up or scale-out manufacturing.

Using CTS™ StemPro-34™ serum-free xeno-free medium in this differentiation system, we generated iHSPCs that were >65% CD34+CD43+ and >90% viable, in both 2D multi-well plate and 3D shake flask culture formats. To demonstrate their functionality, the iHSPCs were differentiated into iNK cells in 100 mL cultures in shake flasks, followed by two weeks of expansion resulting in $>10^9$ CD56+ CD3- iNK cells, with >90% purity and >80% viability. NK cells have significant potential as “off-the-shelf” allogeneic therapeutic products due to their ability to target cancer cells in an antigen- and HLA-independent manner. We therefore tested the functional ability of the expanded iNK cells to mediate anti-tumor activity against the K562 erythroleukemia cell line and cancer organoids (“tumoroids”) derived from human colorectal adenocarcinoma. The iNK cells displayed significant cytotoxic activity, killing >80% of target K562 cells and adenocarcinoma tumoroids in a dose-dependent manner, without requiring enrichment prior to use.

In conclusion, we demonstrate the use of a streamlined, versatile differentiation workflow to generate clinically relevant cell types, including functional iHSPCs and iNK cells. Utilizing a cost-effective differentiation system like this during process development can help to identify and resolve production pain points, ensure product quality, and facilitate a smooth transition from the research stage to clinical trials and ultimately commercialization.

1217 CRISPR-Cas9-induced Double Strand Breaks Cause Chromosome Catastrophe and Eliminate Pancreatic Cancers

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Pancreatic cancer (PC) is the third leading cause of cancer-related deaths in the U.S. with a 5-year survival rate of just 13%. Most patients are diagnosed at advanced stages when their cancers are unresectable, and current standard of care has shown limited efficacy with most patients eventually developing resistance. We have previously adapted the CRISPR-Cas9 gene editing technology as a cancer-specific treatment modality targeting somatic mutations in PC. However, the tumoricidal potential of CRISPR-Cas9 remains unclear, especially the cell death mechanism and risk of treatment resistance.

Using multi-target sgRNAs containing 2-16 target sites in the noncoding regions of the human genome, we found that targeting more sgRNA target sites correlated with increased γH2A.X foci and decreased clonogenic survival in both Panc10.05 and TS0111 PC cell lines. Multi-target sgRNAs with >10 target sites exhibited >90% clonogenic inhibition. Using sgRNA tag survival (where lethal sgRNAs are eliminated from the pool of sgRNA "tags" over time), we found that multi-target sgRNAs with higher number of target sites demonstrated a greater degree of sgRNA tag loss. Interestingly, most tag reduction occurred between days 7 and 21 post transduction of sgRNAs instead of the first 7 days. We further demonstrated that scission and repair happened within the first few days and peaked 3-4 days post transduction, indicating that CRISPR-Cas9-induced DSBs did not immediately and directly trigger cell death.

Cytogenetic analyses on PC cells transduced with a 14-cutter sgRNA revealed accumulation of karyotypic abnormalities (e.g. chromatid breaks, dicentrics, ring chromosomes) peaking at day 14, polyploidization peaking at day 10, and decreasing by day 21. Whole genome sequencing (WGS) on surviving colonies from cells treated with multitarget sgRNAs also revealed that CRISPR-Cas9-induced structural variants (SVs) increased as a function of the number of sgRNA target sites. Importantly, 87% of SVs detected were novel SVs arising from endogenous DSBs rather than direct CRISPR-Cas9 scission. Our results demonstrated that CRISPR-Cas9 scissions led to ongoing chromosomal rearrangements which peaked before cell death, indicating that CRISPR-Cas9 induces "chromosome catastrophe" that is incompatible with cell survival.

To investigate whether the surviving/resistant colonies from the clonogenicity experiment increased tumor growth, we injected parental and resistant cells into athymic, nude mice and observed tumor growth over 33 days. We found no significant differences in growth with tumors grown from resistant cells compared to the parental/non-transduced controls. We also harvested tumors and found no significant differences in size and weight between resistant tumors and non-transduced controls, except for 5-cutter-resistant tumors that showed decreased growth. Finally, surviving colonies that were resistant to 5-, 8-, and 16-cutter sgRNAs were re-challenged with their original multitarget sgRNA, 12-, and 14-cutter sgRNAs. Re-treating with original sgRNA showed no growth inhibition, while >95% cytotoxicity was observed by re-challenging them with 12- or 14-cutter sgRNAs. Our results demonstrate that cells resistant to one sgRNA retain sensitivity to other sgRNAs and do not induce significantly more aggressive disease.

1218 Personalized CRISPR/Cas9 Cytokine Gene Therapy to Remodel Solid Tumor Microenvironment and Enhance Adoptive T Cell Therapy

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Therapy-resistant solid tumors remain a significant challenge in oncology, requiring innovative therapeutic strategies. Immunotherapy, such as chimeric antigen receptor (CAR) T cell therapy, has demonstrated remarkable

success against hematological malignancies but often fails in solid tumors due to poor T cell infiltration and an immunosuppressive tumor microenvironment. Solid tumors with increased immune cell infiltration and elevated cytokine levels have shown better prognoses and enhanced responses to immunotherapies. To address this challenge, we developed a personalized CRISPR/Cas9-based cytokine gene therapy to remodel the tumor microenvironment and enhance the efficacy of CAR T cell therapy in solid tumors.

Central to this effort is CancerPAM, an automated multi-omics pipeline we developed for identifying tumor-specific CRISPR/Cas9 knock-in target sites. This Python-based tool, analyzes cancer- and healthy tissue genomes to discover novel tumor-specific PAM sites, enabling safe and effective transgene integration. In a cohort of 54 neuroblastoma patients, 8570 potential CRISPR targets were identified in the coding genome, representing 14% of all called single nucleotide variants. The pipeline employs a robust ranking algorithm that integrates factors such as copy number, gene expression, and CRISPR efficiency and specificity scores, ensuring precise target prioritization while minimizing off-target risks. CancerPAM's versatile framework facilitates the discovery of genomic CRISPR targets across various solid tumors, making it a valuable tool for personalized in vivo gene therapy **(Fig.1)**. Our therapeutic strategy employs CRISPR/Cas9 knock-in via homology-directed repair to engineer tumor cells to secrete T cell-attracting chemokines (CXCL10, CXCL11) or the T cell-activating cytokine IFNG. These cytokines enhance CAR T cell infiltration and activation, even in tumors with immune-exclusion signatures. Unlike conventional tumor gene therapy, our augmentative approach does not depend on gene transfer to all tumor cells. Instead, cytokines secreted by the targeted tumor cells modulate the surrounding cells and reshape the tumor microenvironment. Proof-of-concept studies in neuroblastoma cell lines demonstrated precise transgene integration and cytokine expression. Functional validation in vitro revealed significant enhancements in CAR T cell migration in trans well migration assays which was confirmed in a 3D bioprinted tumor model for transgene expressing tumors. Moving in vivo, xenograft-studies with CXCL10-expressing neuroblastoma cells after targeted knock-in, transplanted into immunodeficient mice, revealed early increases in CAR T cell infiltration and an increased remission rate compared to unmodified tumors in a CAR T cell resistant tumor-model. These findings underscore the potential of our approach to overcome the immunosuppressive barriers of solid tumors and enhance the efficacy of adoptive T cell therapies **(Fig. 2)**.

In conclusion, our multi-omics pipeline-based and personalized CRISPR/Cas9 cytokine gene therapy offers a versatile and scalable solution to the challenges of solid tumors. By reprogramming the tumor microenvironment to promote immune cell activation and infiltration, this strategy holds significant promise for improving immunotherapeutic outcomes across diverse solid malignancies, with neuroblastoma serving as a proof-of-concept model.

Figure 1

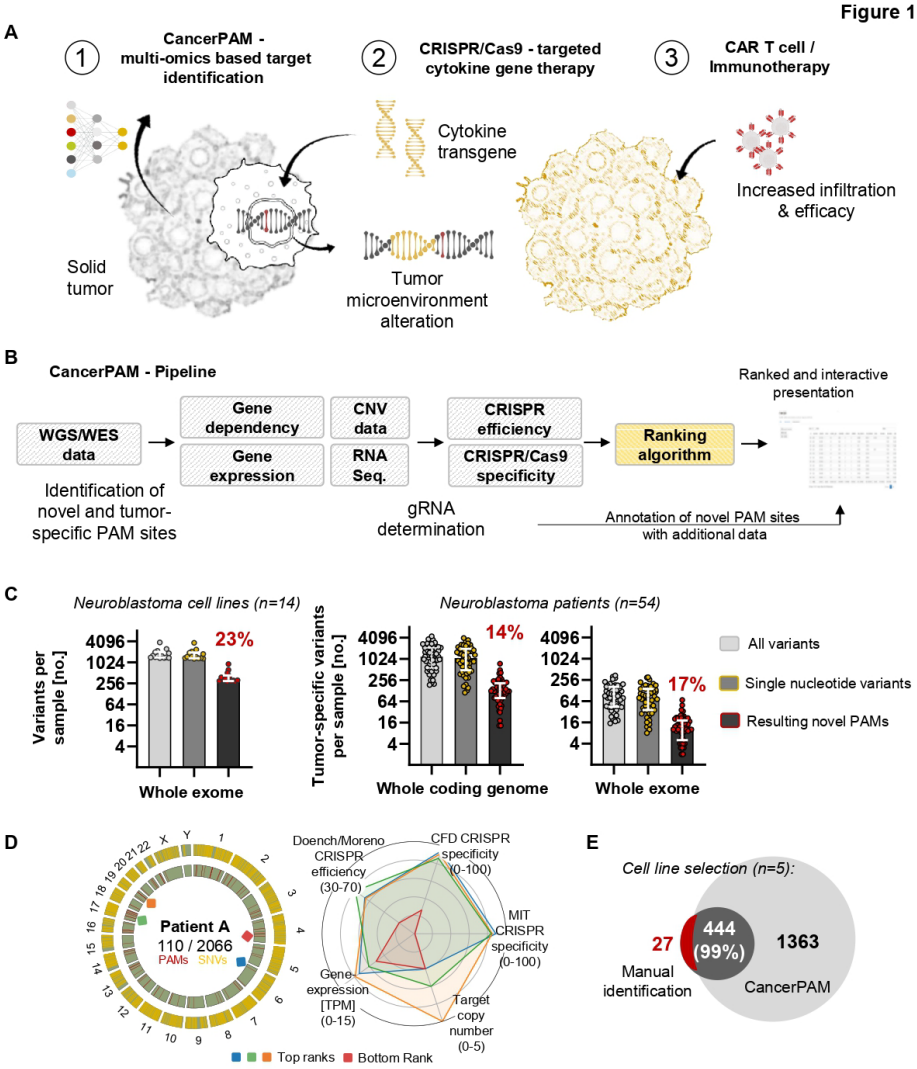

Fig. 1. The CancerPAM multiomics-based automated pipeline identifies and ranks targetable tumor-specific PAM sites in cancer cell lines and patient samples. (A) Schematic representation of the developed CRISPR/Cas9-based gene therapeutic approach, highlighting target identification, cytokine gene integration, tumor microenvironment remodeling, and enhanced CAR T cell infiltration and activation.**(B)** Overview of the CancerPAM pipeline, which integrates whole-genome/whole-exome sequencing (WGS/WES) data analysis, gRNA identification, multiomics feature annotation, to identify and rank tumor-specific novel PAM sites. **(C)** Quantification of identified variants and tumor-specific PAM sites in neuroblastoma cell lines (from WES data) and neuroblastoma patients (from WGS data filtered for coding genome or exonic variants). The *all variants* group includes insertions, deletions, InDels (<300 bp), and single nucleotide variants (SNVs). **(D)** Circos plots showing the chromosomal distribution of SNVs and novel tumor-specific PAM sites and radar plots visualizing annotated feature characteristics for the top three highest- and the lowest-ranked PAM site in two patients (A and B) **(E)** Overlap between novel PAM sites identified via manual screening of five neuroblastoma cell lines and those identified using the CancerPAM pipeline shows a 99% sensitivity of the CancerPAM pipeline. *Data are presented as means ± SD.*

Figure 2

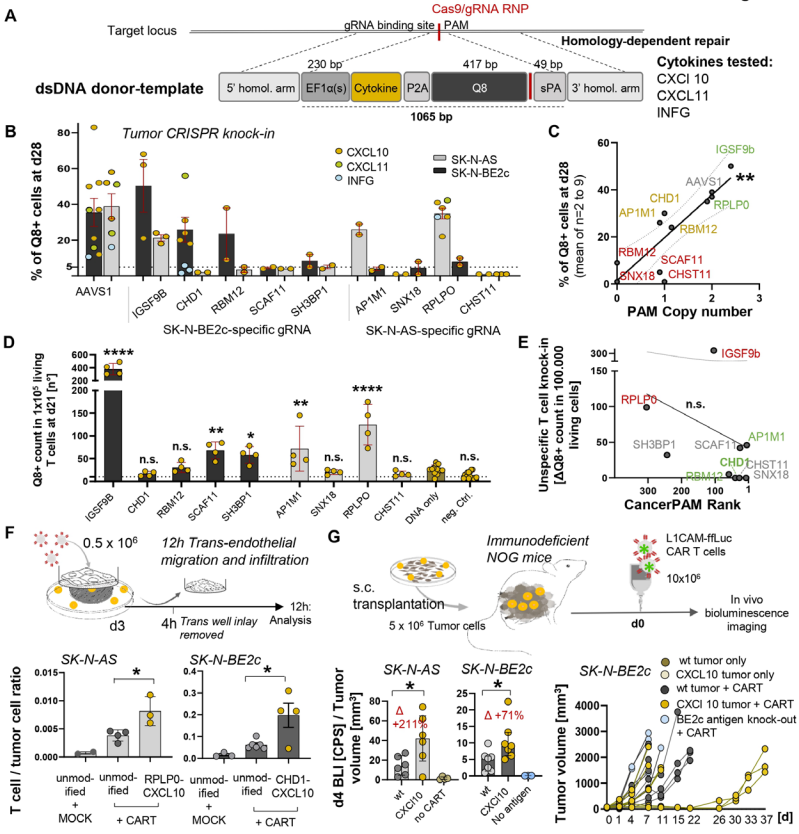

Fig. 2. Gene therapeutic CRISPR knock-in of cytokine transgenes is specific to top-ranked novel PAM targets and enhances CAR T cell infiltration and efficacy in vitro and in vivo. (A) Schematic representation of CRISPR/Cas9-mediated transgene knock-in via homology-directed repair (HDR) following ribonucleoprotein (RNP) electroporation. The linear double-stranded (ds) donor templates consisted of 5' and 3' homologous arms (400bp), a custom-designed EF1a promoter, the cytokine transgene linked via a P2A self-cleaving peptide to Q8 (CD34 epitope and CD8 transmembrane domain), followed by a synthetic poly A (sPA) sequence. **(B)** Knock-in efficiency of three cytokines (CXCL10, CXCL11, IFNG) measured by Q8 antigen expression via flow cytometry at day 28 post-electroporation across different novel PAM sites in two neuroblastoma cell lines. Site-specific knock-in was confirmed by dPCR and Sanger sequencing (data not shown). **(C)** A positive correlation between knock-in efficiency and novel PAM copy number was observed. **(D)** Off-target knock-in in primary T cells from two donors lacking the identified novel PAM sites, assessed by Q8+ expression at day 21 post-RNP electroporation using guide RNAs targeting the novel PAMs identified in (B). **(E)** Correlation of off-target knock-in in T cells with CancerPAM ranking of novel PAM sites showed a good safety profile for highly ranked novel PAM sites (e.g. CHD1 for SK-N-BE2c). **(F)** 3D tumor trans-endothelial migration and infiltration assay using L1CAM-targeting CAR T cells and unmodified or cytokine-expressing neuroblastoma cell lines (SK-N-AS ist L1CAM low, SK-N-BE2c is L1CAM high). T cell infiltration was quantified after 12 hours and represented as the T cell-to-tumor cell ratio. **(G)** Xenograft mouse model using unmodified or cytokine-expressing neuroblastoma cell lines and L1CAM CAR T cells transduced with firefly luciferase (ffLuc). CAR T cell tumor infiltration was assessed by in vivo bioluminescence imaging (BLI), analyzed via the BLI-to-tumor volume ratio at day 4. CXCL10-expressing SK-N-BE2c tumors showed early complete response in 3/7 animals, which was not observed in control groups (0/8). CAR T cells disappeared by d15 followed by relapse in early-response cases (Data not shown). *Data are presented as means ± SD. Statistical analyses: (D, F, G) Kruskal-Wallis with Dunn's post hoc test; (C, E) Spearman correlation. P values: *<0.05, **<0.01, ***<0.001, ****<0.0001; n.s., not significant.*

1219 OUTWIT: An *In Vivo* Approach to Engage Innate Immune Cells with Clinically Approved T Cell Engagers

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T cell engagers (TCEs) activate T cells by linking their T-cell receptors (TCRs) to a target antigen on tumor cells. They have achieved remarkable successes both in liquid and solid tumors. However, they are vulnerable to antigen escape and can lead to exhaustion of T cells and loss of tumor control. Because innate cells can be directly anti-tumorigenic and can also promote epitope spreading, a strategy that endows clinically approved TCEs with the ability to engage innate immune cells in addition to engaging T cells presents an opportunity to improve the durability of TCE-based therapies and, ultimately, prevent tumor antigen escape. Here we describe OUTWIT, a receptor for TCEs that can be delivered to innate immune cells *in vivo* to render them engageable by TCEs.

OUTWIT is comprised of an extracellular domain that all TCEs can bind that is linked to intracellular signaling domains to drive innate cell effector function upon TCE-mediated target binding.

CD3ε is the constant subunit of the TCR that is universally bound by the anti-CD3 binding side of TCEs. Equipping OUTWIT with the ectodomain of CD3ε allowed all anti-CD3 antibody clones tested to bind to OUTWIT-expressing monocytes. Linking these cells to antigen via TCE induced NFκB signaling and cytokine expression. Coculture with target-positive tumor cells resulted in TCE-mediated phagocytosis of tumor cells by OUTWIT-expressing monocytes. Furthermore, we demonstrate efficient *in vivo* delivery of mRNA transcripts to >80% of innate immune cells in circulation following IV injection of loaded lipid nanoparticles (LNPs). Taken together, these data lay the groundwork for a promising strategy to endow any TCE in the market with the ability to enlist innate immune cells to home to tumors and be activated therein. As innate cells have potent cytotoxic and epitope-spreading abilities, this approach could be employed in combination with any current or future approved TCEs to orchestrate a broader and more complete immune rejection of tumors.

1220 Preclinical efficacy of a HER2 synNotch/CEA-CAR combinatorial immunotherapy against colorectal cancer with HER2 amplification

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HER2 amplification occurs in about 5% of colorectal cancer (CRC) cases and is associated only partially with clinical response to combined human epidermal growth factor receptor 2 (HER2)/epidermal growth factor receptor (EGFR)- targeted treatment. An alternative approach based on adoptive cell therapy (ACT) using T-cells engineered with anti-HER2 chimeric antigen receptor (CAR) proved to be toxic due to "on-target off-tumor" activity. Here we describe a combinatorial strategy to safely target HER2 amplification and CEA expression in CRC using a synNotch-CAR based artificial regulatory network. The natural killer cell line NK-92 was engineered with an anti-HER2 synNotch receptor driving the expression of a CAR against CEA only when engaged. After being transduced and sorted for HER2-driven CAR expression, cells were cloned. The clone (5F) with optimal performances in terms of specificity and amplitude of CAR induction demonstrated significant activity in vitro and in vivo specifically against HER2amp/CEA+ CRC models, with no effects on cells with physiological HER2 levels. To further improve survival, tumor penetration and in vivo efficacy of the NK-92.5F clone, a more complex system was built, in which HER2 synNotch engagement drives expression of both the CEA-CAR and different cytokine (5Fck). 5F-IL cells displayed further increased cytotoxicity in vitro. In vivo, 5Fck cells drastically increased survival of mice carrying HER2amp CRC xenografts with respect to the parental 5F clone. In conclusion the HER2-synNotch/CEA-CAR-NK system provides an innovative, scalable and safe off-the shelf cell therapy approach with potential against HER2amp CRC resistant or partially responsive to HER2/EGFR blockade.

1221 *piggyBac* transposon-mediated EPH family protein-specific CAR-T cells engineered through scalable automated manufacturing with optimized T cell metabolic fitness and sustained stemness

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Background: *piggyBac* (PB) transposon-mediated CAR-T cells have emerged as a promising therapeutic platform due to their enhanced T cell stemness, which is associated with long-term antitumor efficacy and sustained clinical responses. This stemness is achieved by directly introducing the PB CAR transgene into resting CD45RA-positive peripheral blood mononuclear cells (PBMCs) and expanding these cells using a feeder cell-based system that preserves physiological T cell activation. Leveraging this platform, we developed dual-targeting PB CAR-T cells which redirected to both EPHB4 and EPHA2 (PB EPH CAR-T cells), two crucial targets in solid tumors.

Methods: A modified Ephrin B2, natural ligand of EPHB4, fused with CD28 and CD3ζ signaling domains, was cloned into a wild-type PB transposon plasmid. Beyond the previously developed manual manufacturing system for PB CAR-T cells, we established a GMP-compliant, semi-automated, and closed manufacturing process for PB EPH CAR-T cells. In this process, autologous PBMCs were collected by leukapheresis and these cells were enriched using the automated centrifugation system, transfected with the PB CAR transgene and transposase plasmid through an optimized electroporation protocol, and expanded over 14 days in a feeder cell-based system within automated cell expansion platform. The system was further optimized by regulating dissolved oxygen levels to enhance cell expansion and functionality. Comprehensive assessments of phenotypic characteristics, metabolic profiles, and antitumor efficacy were performed through in vitro sequential co-culture assays and in vivo xenograft models.

Results: PB EPH CAR-T cells were successfully manufactured with high CAR expression, a dominant population of stem cell-like memory T cells (Tscm), and low PD-1 expression, reflecting a less exhausted phenotype. Compared to manually produced CAR-T cells, the semi-automated process enhanced CAR expression and expansion efficiency while preserving OXPHOS-driven metabolic fitness despite robust proliferation. Functional studies demonstrated that PB EPH CAR-T cells, with its modified ligand-based CAR design, possessed dual specificity for EPHB4 and EPHA2. PB EPH CAR-T cells effectively eliminated EPHB4+ and EPHA2+ solid tumors and exhibited superior, durable antitumor efficacy. Notably, direct comparisons with retrovirally (RV) engineered EPHB4/EPHA2 CAR-T cells revealed the superior performance of PB EPH CAR-T cells, which showed enhanced resistance to immune exhaustion and maintained long-term cytotoxic activity through multiple tumor rechallenges. Specifically, tumor cell lysis rates (%cytolysis) at 100 h after co-culture for PB CAR-T cells surpassed RV CAR-T cells in both the second (92.2 ± 0.63% [PB] vs. 59.7 ± 12.7% [RV]) and third (73.5 ± 3.7 % [PB] vs. 0 ± 11.3 % [RV]) rounds of rechallenge **(Figure 1)**.

Conclusion: PB EPH CAR-T cells demonstrated potent and durable antitumor efficacy by preserving T cell stemness and resisting immune exhaustion, even under chronic antigen stimulation. The semi-automated manufacturing process enhances scalability and ensures optimal metabolic fitness, enabling the production of high-quality CAR-T cells. We have initiated a Phase 1 clinical trial targeting EPHB4-positive Ewing sarcoma and other solid tumors, with plans to expand to global Phase 1/2 trials for colorectal cancer and hepatocellular carcinoma, further extending the therapeutic potential of this platform.

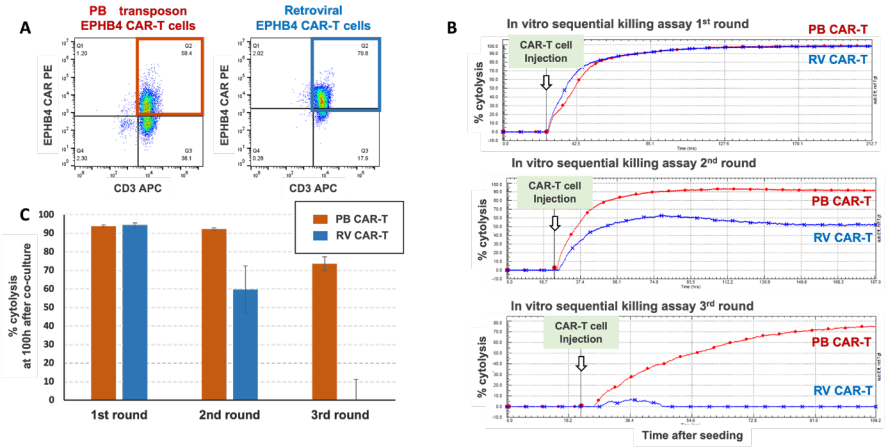

Figure 1 Anti-tumor efficacy of PB- and RV-EPH CAR-T cells
A. CAR positivity of PB- and RV-CAR-T cells. **B.** Anti-tumor efficacy of EPH CAR-T cells during sequential tumor rechallenges. EPH CAR-T cells and Rh30 cells were co-cultured at an E:T ratio of 1:2, and % cytolysis was monitered by real time cell analyser, xCELLigence. **C.** % cytolysis at 100h after co-culture for each tumor rechallenge. Data are presented as mean ± SD.

1222 Induced Pluripotent Stem Cells (iPSCs) Provide an Unlimited, Effective Claudin18.2-targed CAR-NKT cell for Allogeneic and Off-the-Shelf Immunotherapy and gastric and pancreatic cancer

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Background: Invariant natural killer T(iNKT) cells exhibit potent anti-tumor activity through direct killing mechanisms and indirectly through triggering the activation of other anti-tumor immune cells, overcoming limitations of CAR-T therapy in solid tumors challenged by immunosuppressive environments. Compared to classic αβT, iNKT cells are not restricted to HLA and bind exclusively to the monomorphic CD1d molecule, avoiding graft versus host disease. To address the scarcity of iNKT cells in PBMC, we aimed to generate clinically competent NKT cells from induced pluripotent stem cells (iPSCs). It would also be possible to activate the iPSC-iNKT cells by their direct recognition of tumor cells upon expression of chimeric antigen receptors (CARs) in the iPSC-iNKT cells.

Methods: We amplified iNKT cells from PBMC and generated iPSCs through episomal reprogramming, combined with the specific medium and induction method. iNKT-iPSC clone was identified by iNKT specific TCR α chain(Vα24) and TCR β chain (V β 11) gene rearrangements. Good pluripotency iPSCs were re-differentiated into iNKT cells through optimized cytokines and transduced with Caludin18.2-targeted CAR using gene editing. Caludin18.2 antigen is widely expressed in many types of solid tumors including gastric and pancreatic cancer. The function of CAR-iNKT cells was assessed by surface markers, cytotoxicity, and cytokine secretion.

Results: We established a robust protocol for reprogramming human Vα24+ iNKT cells, obtaining different iNKT cell-specific TCR types of iPSCs, and re-differentiating them into functional CAR-iNKT cells. These iPSC-derived CAR-iNKT cells expressed >90% TCRα on CD3+ cells. We evaluated the target-mediated CAR-dependent cytokine production of CAR-iNKT by co-culturing the cells with NUGC4 (gastric cancer line) or ASPC1 (pancreatic cancer line) as a stimulator**.** We also found that the iPSC Vα 24+ iNKT cells can be activated by target cells overexpress CD1d and produce a large amount of interferon-γ and tumor necrosis factor upon activation, and exhibit good cytotoxic activity against other tumor cell lines(K562/Huh7/SKOV3) at ratios of 5:1, the cytotoxicity was about 100%.

Conclusions: iPSCs can provide an unlimited source of allogeneic, off-the-shelf CAR-iNKT cells without GVHD risk. Our findings demonstrate the potential of NKT-TCR carrying CAR-T cells as a universal cancer immunotherapy strategy against gastric and pancreatic cancer.

1223 Preclinical Study of Combination therapy of GUCY2C CAR-T with Immune Checkpoint Inhibitors for Metastatic Colorectal Cancer

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Background: Chimeric antigen receptors-T (CAR-T) cell therapy has shown demonstrable success against hematologic malignancies, but much lower efficacy against solid tumors. One important reason lies in the immunosuppressive microenvironment of solid tumors, leading to exhaustion and insufficient expansion of CAR-T cells in vivo. Previously, we developed a GUCY2C-targeted CAR-T cell and observed over 30% of overall response rate in the 3rd- or late-line therapy of microsatellite stable (MSS) metastatic colorectal cancer (mCRC) patients. Immune checkpoint inhibitors (ICIs) have shown clinical benefits in microsatellite instable (MSI) mCRC, but limited effects on MSS mCRC which accounts for the majority of metastatic colorectal carcinomas. To further improve the clinical response and durable efficacy of GUCY2C CAR-T, we investigated the potential of combination therapy of GUCY2C CAR-T cells with ICIs for MSS mCRC in vitro and in vivo.

Methods: To construct the mouse GUCY2C CAR-T cells, T cells isolated from BALB/c mice were transduced with lentiviral vectors carrying the GUCY2C CAR gene, and the tumor killing efficiency was examined in vitro. We used the murine MSS colorectal cancer cell line CT26 to establish a metastatic CRC model that exhibited disseminated tumor cells in the lung tissues of female BALB/c mice. CT26 cells were transduced with lentiviral vectors with GUCY2C gene to establish the GUCY2C-positive CRC cell line (GUCY2C-CT26). BALB/c mice were inoculated individually with 0.1 million of GUCY2C-CT26 cells via the tail vain injection. After 5 days, each mouse received 4 Gy irradiation and was then intravenously infused with 1 million GUCY2C CAR-T cells. For the combination therapy group, additional administrations of ICIs (αPD-1 or/and αCTLA-4) were conducted for 4 times within 10 days. Monotherapies of CAR-T cells and ICIs were used as control groups. Peripheral CAR-T cell expansion was monitored along with time. The tumor fluorescence intensity and survival of mice were analyzed statistically.

Results: GUCY2C CAR-T cells derived from BALB/c mouse T cells exhibited high killing efficiency and excellent expansion after multi-round chronic stimulation with GUCY2C-CT26 cells in vitro, and significantly suppressed tumor growth in vivo. Combination therapies of CAR-T cells with ICIs including αPD-1, αCTLA-4, or combined αPD-1 and αCTLA-4 demonstrated superior inhibition of tumor relapse compared with CAR-T monotherapy. Among the ICIs, combination therapy of CAR-T with αPD-1 and αCTLA-4 showed the best antitumor efficacy, exhibiting the lowest tumor burden and the longest overall survival. CAR-T cells combined with αCTLA-4 showed higher benefits for mouse survival than CAR-T cells combined with αPD-1. All the ICIs showed limited effects on the amplification of T cells or CAR-T cells in the peripheral blood.

Conclusions: This study demonstrated that combination therapy of GUCY2C CAR-T cells with ICIs has superior efficacy in MSS mCRC, especially the triple combination of CAR-T, αPD-1, and αCTLA-4.

1224 Glypican-3-Specific CAR-T Cells Co-expressing CD40L Exhibit Antitumor Activity Against Osteosarcoma

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Background: Chimeric Antigen Receptor T-cell (CAR-T) therapy for solid tumors often demonstrates limited efficacy, primarily due to the complex tumor microenvironment (TME), which is rich in immunosuppressive cells and molecules. These factors hinder CAR-T cell expansion and persistence. CD40L is a co-stimulatory molecule that is inducibly expressed on activated T cells. CD40L interacts with CD40 on antigen-presenting cells (APCs), promoting their differentiation, survival, cytokine production, and antigen-presenting function. CD40L can also interacts with CD40 on tumor cells, leading to tumor cell apoptosis. In this study, we targeted glypican-3 (GPC3), a protein that is overexpressed in various solid tumors, to develop a next-generation CAR-T therapy: GPC3-CAR-T cells armoring CD40L (named IM83), and assessed the antitumor activity against osteosarcoma both in vitro and in vivo.

Methods: The expression profile of GPC3 in osteosarcoma and normal tissues was confirmed using immunohistochemistry. Lentivirus carrying the IM83 CAR construct was transfected into primary T cells to generate IM83 CAR-T cells. These cells were then co-cultured with osteosarcoma organoids to evaluate the cytotoxic effects of IM83 CAR-T cells in vitro. To study the role of CD40L, a control "unarmored CAR" (lacking CD40L) was constructed. Both IM83 and "unarmored" CAR-T cells were co-cultured with GPC3-CD40+ tumor cells, and tumor cell apoptosis was assessed. Furthermore, to investigate the impact of IM83 on APC activation, both CAR-T cell types were co-cultured with monocyte-derived dendritic cells (DCs). The potential for IM83 to activate bystander T cells was also tested by co-culturing the CAR-T cells with monocyte-induced DCs, tumor antigens, and $GPC3^{-}CD40^{-}$ tumor cells. In vivo tumor suppression was evaluated using a patient-derived xenograft (PDX) model of osteosarcoma. Finally, the clinical efficacy of IM83 was assessed in a patient with osteosarcoma.

Results: In the osteosarcoma organoid model, IM83 induced a high tumor cell death rate (62.27%). Mechanistic studies confirmed that IM83 CAR-T cells, when compared to "unarmored" CAR-T cells, induced higher levels of tumor cell apoptosis when co-cultured with $GPC3^{-}CD40^{+}$ tumor cells. Additionally, IM83 CAR-T cells more effectively activated monocyte-derived DCs than the "unarmored" CAR-T cells, leading to enhanced activation and antitumor activity of bystander T cells. In the PDX model of osteosarcoma, IM83 induced tumor suppression at lower doses compared to "unarmored" CAR-T cells (1×10^6 vs. 5×10^6 cells). To assess the clinical potential of IM83, a 58-year-old patient with osteosarcoma, who had undergone three lines of treatment, received an infusion of IM83 CAR-T cells at a dose of 5×10^8. The patient experienced grade 2 cytokine release syndrome (CRS) on day 2 post-infusion. Neurotoxicity was not observed. By day 12, MRI imaging revealed a liquefied necrosis area in the target lesion, measuring 5.5 cm in length.

Conclusions: This study demonstrates that IM83 CAR-T cells possess significant antitumor activity against osteosarcoma. These findings suggest that IM83 holds strong therapeutic potential, warranting further clinical investigation

1225 Identifying genetic determinants of solid tumor infiltration of T cells using a microscopy-based cell isolation approach.

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A major challenge in immunotherapy has been engineering CAR-T cells capable of infiltrating and effectively targeting solid tumors. The genetic factors that regulate infiltration, targeting, and the persistence of immune responses remain incompletely understood. Unlike in hematologic malignancies, CAR-T cells face multiple barriers in solid tumors, including a hostile tumor microenvironment, physical exclusion from tumor sites, and T-cell exhaustion. Identifying and manipulating key genetic determinants could enhance CAR-T efficacy, providing a path toward more durable and effective treatments for solid tumors. To achieve this, we developed a microscopy workflow that enables isolating tumor-infiltrating immune cells from *in vitro 3D* solid tumor-immune cell co-culture models. These isolated immune cells can then be subjected to transcriptomic and proteomic profiling to identify differentially expressed genes and proteins, providing insights into the genetic and molecular determinants of immune cell infiltration and function.

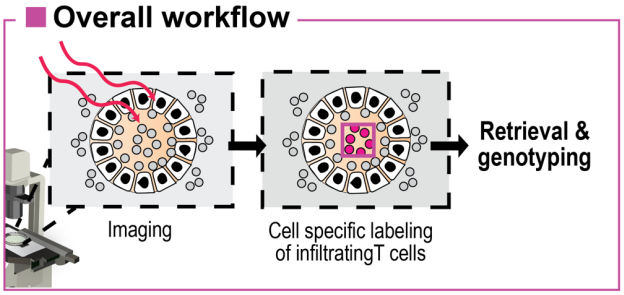

Figure 1. Overall workflow of our method to isolate tumor infiltrating immune cells from spheroid-immune cell co-cultures.

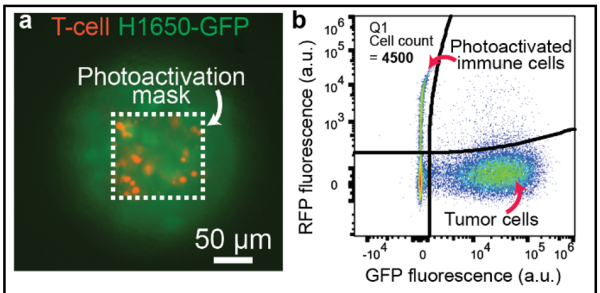

Figure 2. (a) Image of spheroid-T cell co-culture. T-cells expressing PAmCherry1 were optically labeled and isolated (white dashed box). (**b**) 24-wells of co-culture in a 96-well plate were photoactivated, as seen in (a). Cells were dissociated, pooled, and ran under FACS. Around 4500 T-cells were detected.

1226 Expanding the Potential of T-Cell Therapies with the ARTEMIS Cell Receptor Platform

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Conventional chimeric antigen receptor (CAR)-T cells "redirect" T-cell activation by delivering both T-cell receptor (TCR) signals (signal 1) and costimulation (signal 2) through a single chimeric surface receptor. While effective against hematologic malignancies, CAR-T cells can cause unwanted toxicities, have poor persistence, and face efficacy challenges in solid tumors due to limited tumor infiltration in immunosuppressive microenvironments – challenges primarily arising from the inherent design of the CAR construct.

To address these limitations, we developed the ARTEMIS® Cell Receptor Platform, directing the synthesis of two molecules: an antibody-T-cell receptor (AbTCR) and a co-stimulatory molecule. The AbTCR incorporates a target-binding domain fused to an effector domain from human γδ TCR chains, while the co-stimulatory molecule contains a target-binding domain coupled with a co-stimulatory domain from a human co-stimulatory receptor. Both proteins are co-expressed from the same transgene, co-localizing on ARTEMIS T cells, thus mimicking the natural TCR/co-stimulatory receptor system. This arrangement promotes regulated T-cell activation and enhances the destruction of target-positive cancer cells while maintaining a safety feedback mechanism to prevent unwanted toxicity. We also conducted a screening campaign identifying a co-stimulatory domain that significantly enhances solid tumor infiltration by ARTEMIS T cells.

Initially, we compared the AbTCR and CAR constructs by engineering T cells to express CD19-targeting domains. AbTCR-T cells matched CAR-T cell potency while significantly reducing cytokine release during target cell elimination in vitro and in mouse models. To further optimize the anti-tumor immune response, we incorporated a co-expressed, yet separate, co-stimulatory molecule into the platform, designed to bind either the same or a different tumor-specific antigen as the AbTCR. Subsequently, we developed an AFP-targeting ARTEMIS T cell therapy for hepatocellular carcinoma (HCC), where the AbTCR recognizes an AFP peptide/HLA-A2 complex, and the co-stimulatory molecule targets glypican-3 (GPC3). These T cells demonstrated potent anti-tumor activity, significant infiltration, and no adverse reactions in HCC xenografts. These findings suggest that the ARTEMIS Cell Receptor Platform may offer a safer and more effective alternative to conventional CAR-T therapies across various cancers.

To clinically validate the ARTEMIS Cell Receptor Platform, several proof-of-concept studies were conducted. In trial NCT03642496, CD19-targeting ARTEMIS T cells showed efficacy and a favorable safety profile in adults with relapsed/refractory (r/r) B-cell lymphoma. In trial NCT03888859, AFP-targeting ARTEMIS T cells showed significant efficacy in a heavily pre-treated advanced HCC patient, resulting in reduced serum AFP levels and significant shrinkage of both primary hepatic and metastatic lung tumors. Building on these promising results, we initiated U.S. IND trials for both liquid and solid tumors. Current Phase 1/2 trials include STARLIGHT-1

(NCT06343311), evaluating CD19-targeting ARTEMIS T cells in high-risk lymphoma patients, ARYA-2 (NCT04634357) testing AFP-targeting ARTEMIS T cells in pediatric patients with r/r liver cancer, and ARYA-3 (NCT04864054) assessing GPC3-targeting ARTEMIS T cells in adults with advanced HCC.

1227 Antigen-Specific T cells Generated Using Photothermal Nanoparticles Demonstrate Enhanced Function Compared to Alternative Tumor Antigen Release Methods

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Multi-antigen-specific T cells are a promising immunotherapy for solid tumor treatment, offering advantages over current T cell approaches, which target pre-defined subsets of antigens that may lead to tumor antigen escape (PMID: 30135176) and ultimately treatment failure. Our lab has developed a T cell therapy targeting multiple antigens using Prussian blue nanoparticle photothermal therapy (PTT). T cells generated using dendritic cells (DC) primed with PTT-treated tumors display more potent tumor-specific cytotoxicity and function than those generated from tumor lysis alone (PMID: 37278683, 38818122).

We hypothesized that nanoparticle-based antigen capture and immunogenicity of our tumor treatment approach enhances tumor-specific T cell activation at each of the three activation signals—antigen presentation to T cell receptors (TCR), costimulation, and cytokine stimulation—offering an advantage over classical multi-antigen-specific T cell generation methods (heating, freeze-thaw lysis, and oxidation with lysis). Proinflammatory cytokine secretion was higher in PTT compared to other treatments (including IFN-γ, IFN-β, TNF-α, and IL-6) though this difference was not statistically significant across all treatments. Costimulatory marker expression in primed DCs was comparable across treatments. Proteomic analysis revealed unique protein profiles in PTT-treated tumors. MHC-presented peptide analysis identified 55 peptides upregulated in PTT-primed DCs compared to lysate- or un-primed DCs. Of these peptides, 62% overlapped with the tumor peptidome, and two peptides induced an IFN-γ response in PTT-derived T cells but not in heating- or lysate-derived T cells. These findings support our hypothesis that PTT promotes proinflammatory signaling and distinct antigen profiles that amplify tumor-specific T cell activation and proliferation.

We additionally studied whether these differences in proinflammatory signaling, tumor antigens, and TCR-MHC interactions affected tumor-specificity of the T cell product. PTT-derived T cells (PTT-T) had higher tumor-specific cytotoxicity than the other treatments. PTT-T cells also had a higher percentage of CD8 T cells co-expressing markers of activation and degranulation when compared to other treatments, as measured by flow cytometric staining of CD107a and IFN-γ. TCR sequencing revealed an increase in T cell Simpson clonality (from 0.04 to 0.14) and maximum productive frequency (from 2.3% to 9.1%) in the T cell product, suggesting selective expansion of multiple tumor-specific TCR clones. This data further supports our hypothesis that proinflammatory signaling and distinct antigen presentation during PTT-T cell culture stimulates expansion of a more tumor-specific and potent T cell product.

Our findings suggest that differential antigen release, antigen presentation, and proinflammatory cytokine stimulation during PTT-T cell manufacture promote both tumor specificity and T cell activation signaling, yielding a more efficacious product. These findings also highlight the need for further study of the mechanistic differences driving these observations at the antigen release, presentation, cytokine secretion, and T cell recognition stages. Our results demonstrate that PTT expands a multi-targeted yet clonal tumor-specific T cell product, supporting its potential for further development as an adoptive cell therapy for solid tumors alone and in combination with complementary immunotherapeutic modalities.

1228 Metabolic Reprogramming Enhances HER2-CAR T Cell Therapy for HER2-ADC-resistant tumors

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Background: HER2-ADC therapy (e.g., T-DXd) has limited success in HER2-positive tumors, with a response rate of just 29.4% in solid tumors. Resistance is driven by HER2 heterodimerization with HER3 or EGFR. Meanwhile, HER2-CAR T cell therapies struggle in clinical settings due to poor survival in the glucose-deprived tumor microenvironment (TME). Building on our findings that inosine can fuel CAR T cells in glucose-deprived conditions (*Nat Metabolism*, 2020), we recently showed that adenosine deaminase 1 (ADA1) enhances CAR T cell persistence by converting adenosine into inosine (*Cell Rep Med*, 2024). This study evaluates ADA1-based metabolic reprogramming to improve HER2-CAR T cell efficacy in HER2-ADC-resistant tumors.

Methods: Human PBMCs were transduced with retroviral vectors encoding HER2-CAR, ADA1, and CD26 (HER2-MRCAR). Functional assays assessed transgene expression, cytotoxicity, and persistence in vitro. Antitumor activity was evaluated in HER2-positive xenograft mouse models resistant to HER2-ADC therapy.

Results: HER2-MRCAR T cells outperformed conventional HER2-CAR T cells in vitro, showing greater proliferation, reduced exhaustion, and superior targeting of HER2-ADC-resistant cells. CD26 further enhanced T cell trafficking and invasion into tumor tissues. In xenograft models, HER2-MRCAR T cells achieved significant tumor regression compared to standard HER2-CAR T cells, with no additional toxicity observed.

Conclusions: Metabolic reprogramming via ADA1 and CD26 transforms HER2-CAR T cells into a potent therapy for HER2-ADC-resistant tumors. This approach enhances metabolic fitness, persistence, and antitumor efficacy, offering a promising strategy to overcome current therapeutic limitations. Further clinical validation is warranted.

1229 FT836, a Novel MICA/B-targeting CAR T-cell Therapy Engineered to Eliminate the Need for Conditioning Chemotherapy with Broad Activity Across Solid Tumor Indications

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Several challenges hinder the successful application of chimeric antigen receptor (CAR) T-cell therapies in solid tumor settings, including tumor antigen selection, antigen heterogeneity and escape, and inconsistencies in the manufacture of drug product. Furthermore, autologous and allogeneic CAR T-cell therapies have traditionally required administration to patients of lymphodepleting chemotherapy to eliminate the host immune systems and promote product expansion. However, adverse events following lymphodepletion, especially the susceptibility to opportunistic infections remain a significant and ongoing concern. The limited activity of CAR T-cell therapies within the solid tumor space combined with the toxicity risks of lymphodepletion underscores the need for (i) novel antigen targeting solutions and (ii) reduction or elimination of conditioning chemotherapy.

We have developed FT836, an off-the-shelf CAR T cell engineered to broadly target solid tumors without requiring administration of conditioning chemotherapy to patients. Through a novel CAR (3MICA CAR) that recognizes the conserved α3 domain of the inducible and highly polymorphic NKG2D stress ligands MHC class I related proteins A and B (MICA/B), FT836 targets a wide range of tumor indications across varying antigen density levels, reaching 94.5% $\pm$ 5.0% tumor growth inhibition (TGI) in xenograft models of prostate, ovarian, lung, and cervical cancer, comparable to primary CAR T-cells (Fig. 1). Notably, combination with chemotherapy, radiotherapy, or antibody drug conjugates, such as trastuzumab-deruxtecan, increased MICA/B expression on tumor cells and enhanced FT836 CAR-mediated anti-tumor activity. FT836 is also engineered with a high affinity non-cleavable CD16 (hnCD16) that allows for combination with therapeutic antibodies targeting EGFR, cMET, PDL1, or HER2, promoting antibody-dependent cellular cytotoxicity and enabling the elimination of MICA/B+ and MICA/B- target cells.

FT836 has also been specifically engineered to support functional persistence without the need for conditioning chemotherapy. Resistance to host immune cell-mediated rejection is the direct result of: (i) CD58 deletion to unidirectionally limit immune synapse formation by allogeneic immune cells, and (ii) alloimmune defense receptor (ADR) expression to target and eliminate reactive 4-1BB+ allogeneic immune cells. FT836 demonstrated functional persistence over multiple rounds of tumor challenge even with repeat exposure to allogeneic immune cells primed to specifically target FT836 (Fig. 2).

In summary, FT836 is an off-the-shelf, multiplexed-engineered CAR T cell targeting the α3 domain of MICA/B for pan-tumor applicability that demonstrates robust and durable anti-tumor activity across multiple solid tumor indications, including in the presence of primed and potent allogeneic immune cells. As a result, FT836 is uniquely equipped to eliminate the need for conditioning chemotherapy, facilitating broader clinical application both as monotherapy and in combination with standard-of-care agents such as chemotherapy and therapeutic monoclonal antibodies, overcoming multiple challenges that limit adoptive cell therapies in treating solid tumor.

Fig. 1: ***FT836 demonstrates broad and potent anti-tumor activity in several xenograft settings in vivo, uniquely matching primary CAR-T cell activity.***

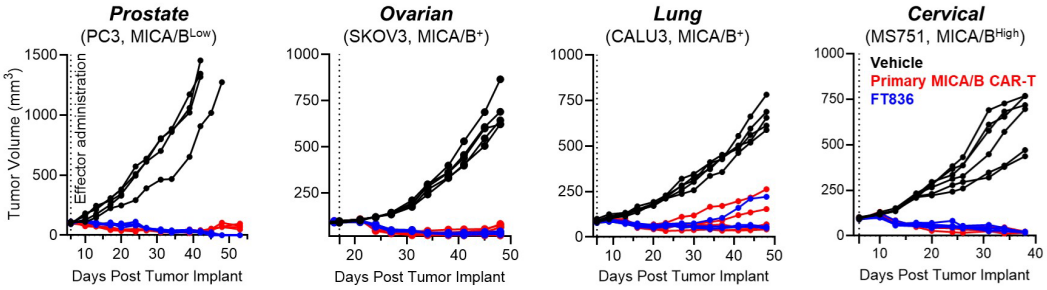
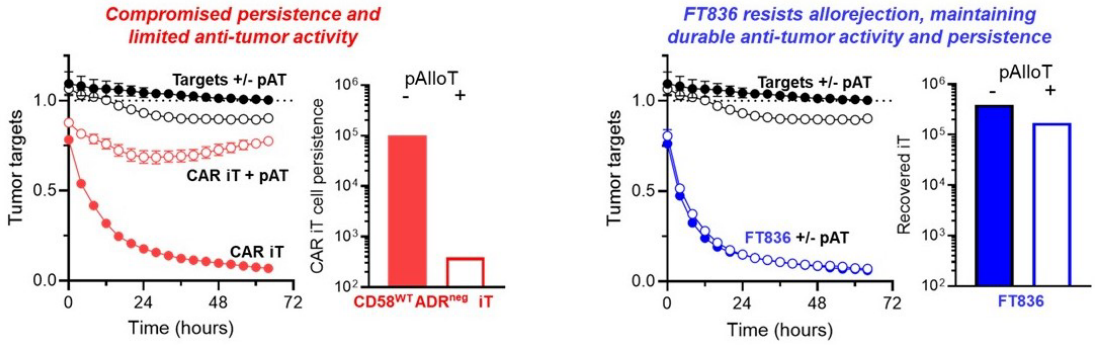


Fig. 2: ***FT836 resists allorejection while maintaining durable anti-tumor activity and persistence.***



1230 Onboard, Membrane-tethered IL-12 Boosts Potency and Maintains Selectivity of a Tmod NOT Gate

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Introduction: Cell therapies for solid tumors are associated with unique challenges compared with liquid tumors, such as antigen tissue-specificity, accessibility to tumor tissues, and problematic tumor microenvironment. Cells engineered with receptors that act as synthetic logic gates promise to address the issue of tumor specificity by

targeting antigen profiles rather than single antigens. Nevertheless, there are limits to the potency benefit that can be achieved at the level of the antigen-targeting receptors. One approach to enhance potency beyond the acute sensitivity of antigen receptor activation is to co-opt a major source of ancillary stimulation in the normal immune response, signal 3 cytokine pathways.

Methods: Enhancing CAR efficacy with engineered onboard cytokines, often referred to as 'armoring', is one such approach to boosting potency. However, such constructs run the risk of overriding tumor selectivity and eroding the therapeutic window. Here we design and test membrane-tethered IL-12 that enhances potency and preserves selectivity of a synthetic NOT logic-gated, dual-receptor CAR-T system called Tmod^{TM}, potentially addressing some of the major challenges in oncology in a single synthetic design[1 2] (Figure 1).

Results: We focused especially on a module that encodes T cell activation-inducible membrane-tethered IL-12 (mem-IL-12) and found that it significantly augments Tmod antigen-dependent cytotoxicity and long-term persistence both *in vitro* and *in vivo*, without compromising the NOT gate tumor selectivity. Notably, 2 substantially different *in vivo* models, including one that employs mouse surrogate antigens[3] (Figure 2), were used to assess preclinical dose-dependent efficacy and safety. In addition, the membrane-tethered format displayed minimal soluble IL-12 release both *in vitro* and *in vivo*, limiting the potential systematic toxicity caused by IL-12.

Conclusions: Together, these studies demonstrated the robustness of the design of membrane-tethered IL-12 as a new module that can be added to the Tmod^{TM} platform and suggested further avenues for improvement of solid tumor cell therapy.

References:

1. DiAndreth B, Hamburger AE, Xu H, et al. The Tmod cellular logic gate as a solution for tumor-selective immunotherapy. *Clin Immunol* 2022;241:109030. doi: 10.1016/j.clim.2022.109030
2. Tokatlian T, Asuelime GE, Mock JY, et al. Mesothelin-specific CAR-T cell therapy that incorporates an HLA-gated safety mechanism selectively kills tumor cells. *J Immunother Cancer* 2022;10(1) doi: 10.1136/jitc-2021-003826
3. Oh J, Kirsh C, Hsin JP, et al. NOT gated T cells that selectively target EGFR and other widely expressed tumor antigens. *iScience* 2024;27(6):109913. doi: 10.1016/j.isci.2024.109913

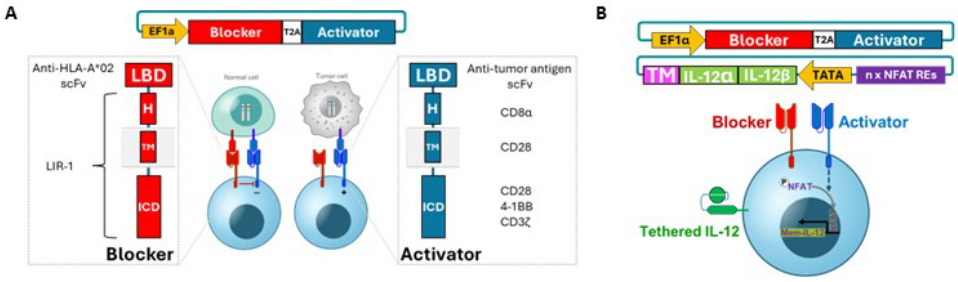

Figure 1 (A) Molecular composition of tumor antigen-targeted Tmod(sh) constructs. Tumor antigen-targeting activator and HLA-A*02-targeting blocker receptors linked by a T2A were co-expressed on a single transcript. The blocker receptor is composed of a LILRB1 (LIR-1) hinge, transmembrane (TM), and intracellular domain (ICD). The activator receptor is a generation 3 (Gen3) CAR composed of a CD8α hinge, CD28 TM, and fused CD28, 4-1BB, and CD3z ICDs. (B) Tmod(sh) and membrane-tethered IL-12 are co-expressed from two separate lentiviral constructs, with one construct encoding HLA-A*02 blocker and activator and the other construct encoding IL-12 heterodimer linked to B7 transmembrane domain (membrane-tethered).

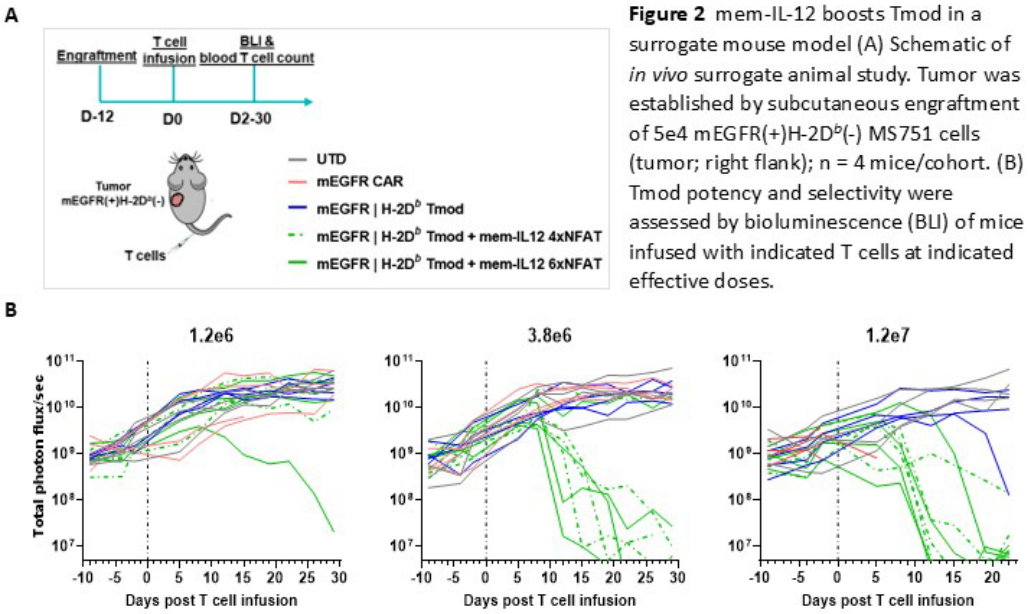

Figure 2 mem-IL-12 boosts Tmod in a surrogate mouse model (A) Schematic of *in vivo* surrogate animal study. Tumor was established by subcutaneous engraftment of 5e4 mEGFR(+)H-2D^b(-) MS751 cells (tumor; right flank); n = 4 mice/cohort. (B) Tmod potency and selectivity were assessed by bioluminescence (BLI) of mice infused with indicated T cells at indicated effective doses.

1231 Enhancing CAR-T Cell Therapy Efficacy Against Solid Tumors Using a Synthetic Pathway Activator (SPA)

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Chimeric antigen receptor T (CAR-T) cell therapy efficacy against solid tumors is hindered by antigen escape, T cell exhaustion, limited persistence, and adverse events (e.g. CRS, IEC-HS and ICANS). We introduce a novel synthetic biology module termed a Synthetic Pathway Activator (SPA) that is designed to overcome these challenges.

To develop the SPA, we screened a library of synthetic cytokine receptors to identify those that increase the potency, expansion and persistence of autologous T cells. The SPA selected for inclusion in our clinical programs significantly enhanced both the short-term and long-term potency of CAR-T cells, demonstrated through cytotoxicity assays in vitro against diverse target cell lines and in vivo via human xenograft models. Specifically, SPA-expressing CAR-T cells showed improved proliferation in response to TCR ligation and CAR antigen stimulation without exhibiting cytokine independent outgrowth. Additionally, expression of the SPA rendered CAR-T cells more sensitive to activation by tumor antigens, enabling effective targeting of low antigen-expressing cells. Furthermore, SPA-expressing CAR-T cells exhibited increased persistence in vitro and in vivo, alongside notable delay or prevention of exhaustion. Importantly, SPA altered the effector gene expression and cytokine secretion profile of CAR-T cells, showing elevated levels of Perforin, Granzyme B, and IL-10.

Ongoing mechanistic investigations reveal that SPA elevates intracellular pSTAT3 and pSTAT1 levels, thereby upregulating downstream gene expression linked to T cell proliferation. We tested the effect of SPA expression on the development of T cell exhaustion in a 14-day repetitive stimulation assay. Temporal single-cell RNA sequencing (scRNA-seq) and pseudotime analysis of SPA-expressing CAR-T cells indicated distinct differentiation trajectories compared to control CAR-T cells lacking SPA, with enhanced retention of memory markers. Importantly, SPA-expressing CAR-T cells did not develop the transcriptional or epigenetic hallmarks of exhaustion, while control CAR-T cells did.

In our ongoing phase I/II clinical trial for clear cell renal carcinoma (ccRCC) using SPA-expressing CAR-T cells, we aim to clinically validate the enhanced anti-tumor activity achieved through this innovative design. Our work continues to deepen our understanding of the mechanisms driving the next generation of CAR-T cell therapies targeting solid tumors.

1232 Label-free, High-throughput Functional Assays for Evaluating the Potency and Safety of Cell Therapies

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Chimeric antigen receptor (CAR) T cells have emerged as a promising treatment for several cancers. Effective CAR T cell products should have cytotoxic effects that are sufficient to eliminate tumor cells and that are specific to cancerous tissue, thereby avoiding off-tumor adverse events. To that end, we developed in vitro assays to assess on-tumor CAR T cell potency using the Maestro TrayZ and off-tumor CAR T cell cardiotoxicity using the Maestro Pro system. Both assays utilize the high-throughput, label-free capabilities of the Maestro platforms. To assess CAR T cell potency, HER2+ ovarian cancer cells (SKOV3s) were treated with HER2-specific CAR T cells at three effector-to-target (E:T) cell ratios (1:5, 1:2, 2:1). Real-time electrical impedance measurements on the Maestro TrayZ detected SKOV3 proliferation and death in four different plates simultaneously. SKOV3s were killed in a CAR T cell dose-dependent manner; that is, higher CAR T cell doses induced larger decreases in SKOV3 impedance, indicative of increased SKOV3 death. At the highest dose (E:T = 2:1), the KT50 was 10.4 hours, with a coefficient of variation of 9.8% across the four plates. Following this assessment of CAR T cell potency, we used induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) to study the same HER2-CAR T cells' possible cardiotoxic side effects. The iPSC-CMs, confirmed to express HER2, were dosed with increasing E:T ratios (1:80 to 1:1) of HER2-CAR T cells. Both MEA viability (resistance) and electrophysiology measurements were taken each day for up to 7 days using the Maestro Pro. We found a dose-dependent decrease in resistance detected from iPSC-CMs dosed with increasing E:T ratios of HER2-CAR T cells and detected significant killing (55.6% decrease in resistance) by the 1:1 CAR T group as early as Day 1 post-dose. By comparison, we observed no difference in resistance between HER2-negative iPSC-derived neuron control wells and those treated with HER2-CAR T cells. Further, we dosed the iPSC-CMs with non-transduced T cells and saw a minimal (5.7%) decrease in iPSC-CM resistance at Day 4 compared to dosing with CAR T cells at the same E:T ratio (71.8% decrease). Finally, we measured the changes in Fridericia-corrected field potential duration (FPDc) of iPSC-CMs dosed with CAR T cells at increasing E:T ratios. Interestingly, large decreases in the FPDc were apparent at 1 hour post-dose in the 1:1 CAR T dose group, illustrating that these electrophysiological changes may signal on-target, off-tumor effects even earlier than cytotoxicity data. In summary, the assays developed here on the Maestro platforms can be used to assess two of the most vital qualities of a given CAR T cell therapy: potency and safety.

1233 High Output, Low-Background Hypoxia Responsive Synthetic Gene Circuits for Improving the Safety and Potency of Solid Tumor CAR T Cell Therapy

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Chimeric antigen receptor (CAR) T cell therapies have demonstrated promise in treating hematological malignancies, but their application in solid tumors remains limited due to challenges including (1) a lack of unique solid tumor antigens, leading to potential off-target effects and safety risks, and (2) the immunosuppressive microenvironment that hinders T cell efficacy. To address these challenges, we engineered synthetic gene circuits that incorporate a hypoxia biosensor (HBS) that enables cells to sense and respond to the hypoxic conditions ubiquitous to solid tumor microenvironments. The biosensor circuit drives expression of CAR along with the native hypoxia-inducible transcription factors HIF1α and HIF2α to enable tumor-specific activation. Expanding on our prior work applying the sensor in an oxygen-degradable CAR expression system, we evaluated biosensor-circuit engineered HEK cells expressing LumiScarlet reporter under normoxic (21% O_2) and hypoxic (5% and 1% O_2)

conditions. This approach identified candidate circuits with high reporter output and low background expression, providing new insights into hypoxia-responsive gene regulation. Additionally, we observed differential CAR transgene expression across HIF1α- and HIF2α-containing circuits, suggesting distinct regulatory mechanisms. We also demonstrated that incorporating synthetic transcription factors and promoters markedly amplifies transgene expression beyond that achievable with HIF1α and HIF2α circuit architectures. Ongoing studies are assessing the function of these constructs in primary T cells, with the goal of enhancing CAR-T cell function in solid tumors. This work establishes a foundation for safer, more effective cell therapies by leveraging synthetic biology to overcome challenges posed by the solid tumor microenvironment.

1234 *In Vivo* Persistence and Clonal Dynamics of Genetically Barcoded IL-15-Armored vs. Unarmored Anti-CD20 CAR-NK Cells in an Autologous Rhesus Macaque Model

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Natural killer (NK) cells can recognize and kill virally-infected or malignant cells. Chimeric antigen receptor (CAR) NK cells combine the inherent immunosurveillance of NK cells with the directed precision of CARs, and are associated with efficient targeting, antitumor activity, and low toxicity. Interleukin-15 (IL-15) is a key cytokine supporting NK homeostasis, proliferation, and survival. Murine xenograft studies suggested that inclusion of the IL-15 cDNA in CARs enhances CAR-NK expansion, persistence, and cytotoxicity *in vivo*, with IL-15-armored CAR-NK showing promise in clinical trials for hematologic cancers. However, the long-term safety, persistence, and clonal dynamics of IL-15-armored versus unarmored CAR-NK remains underexplored.

In this study, we utilized barcoded lentiviral vectors expressing anti-CD20 CAR with or without soluble IL-15 to engineer CAR-NKs, which were administered post-cyclophosphamide conditioning in autologous macaque at a concentration of $1x10^7$ CAR-NK cells/kg. Equal proportions of IL-15-armored (CAR-IL15-Lib21) and unarmored (CAR-EGFRt-Lib19) CAR-NK cells were infused, allowing a detailed comparison of their dynamics, trafficking and clonality in peripheral blood (PB), bone marrow (BM), liver, spleen, lymph nodes (LN) and implanted subcutaneous B cells (Fig. A). The transduction efficiency of NK cells was robust, reaching 50% for CAR-EGFRt-Lib19 and 60% for CAR-IL15-Lib21 vectors, with similar phenotypic profiles at the time of infusion. *In vivo*, CAR-NK expansion in PB peaked on day 12, before declining to low levels by day 28, accompanied by a significant reduction in PB CD20+ B cells as early as day 1, persisting through day 28 (Fig. B). Similarly, tissue resident B cell percentages decreased over time, particularly in BM and liver. By day 7, CAR-NKs were more abundant in tissues compared to PB, gradually diminishing by day 17, with better persistence in LNs (Fig C). Additionally, CAR-NKs were enriched at B cell injection sites compared to control sites 7 days after subcutaneous B cell injections (Fig. D). Interestingly, IL-15-armored CAR-NKs did not demonstrate superior *in vivo* expansion or persistence compared to unarmored CAR-NKs, as revealed by barcode analysis. Notably, IL-15-armored cells exhibited similar clonal diversity up to day 17 (Fig. E), with significantly lower number of clones (Fig. F), without overt oligoclonal expansions. No toxicities related to the CAR-NK infusion was noted.

These findings suggest while anti-CD20 CAR-NKs effectively target B cells and traffic into tissues, inclusion of IL-15 does not significantly enhance short-term expansion or persistence of CAR-NK and may not be necessary in an autologous or non-xenogeneic setting. Ongoing studies will explore the long-term impact of IL-15 on CAR-NK cells in additional animals.

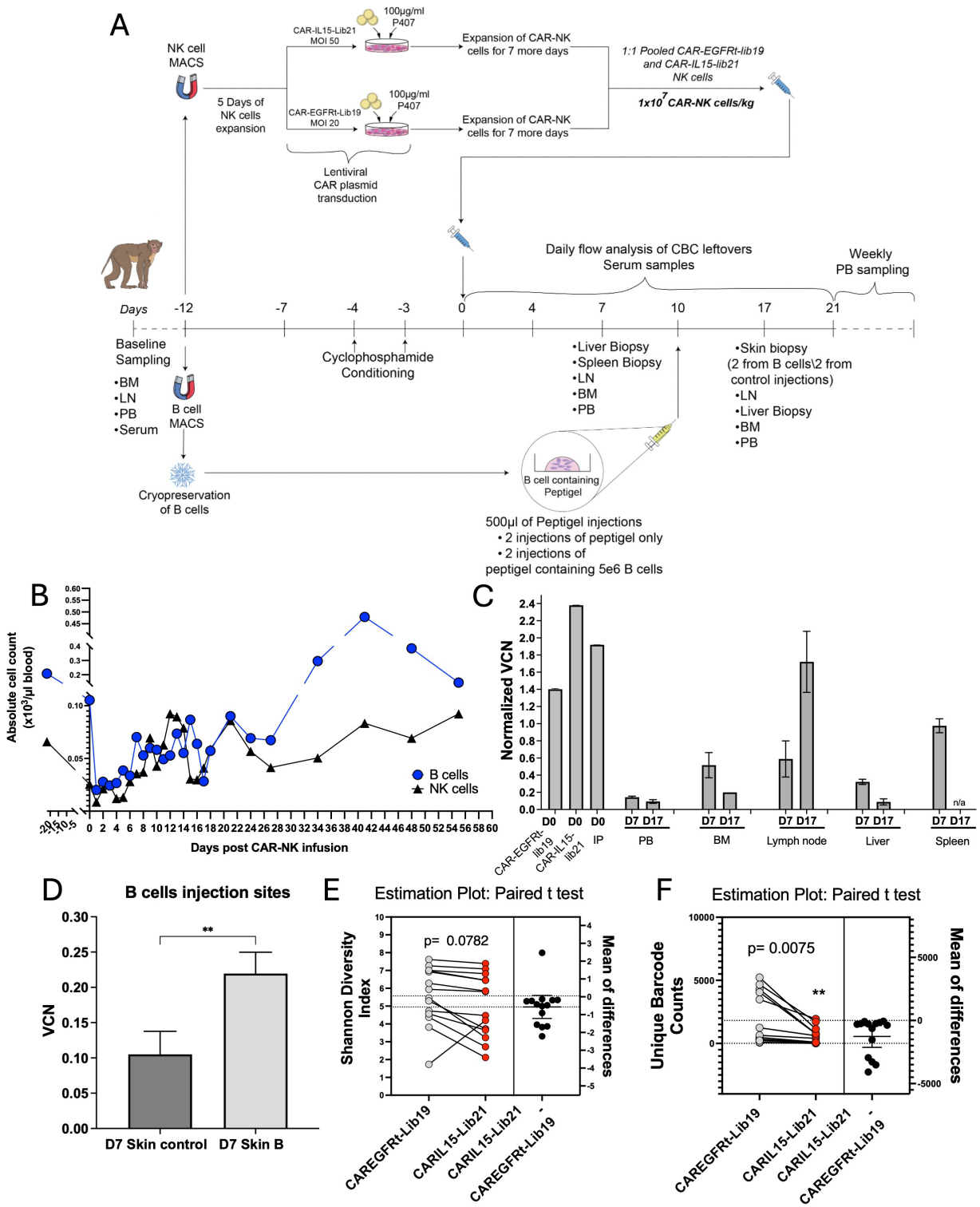

Figure. (A) Experimental design, including the isolation of peripheral blood (PB) NK cells, expansion, transduction of rhesus macaque NK cells using CAR-EGFR-Lib19 and CAR-IL15-Lib21 lentivirus barcode libraries, and the autologous infusion of CAR-NK cells. (B) Absolute counts of B and NK cells over time in PB following CAR-NK infusion. (C) Vector copy number (VCN) of the CAR cassette in NK cells from *in vitro* and *in vivo* samples, detected by ddPCR. "IP" indicates the infusion product. VCN was normalized based on the percentage of NK cells in mononuclear cells isolated from *in vivo* samples. (D) CAR vector copy number (VCN) in cells from skin biopsies at subcutaneous B cell injection sites, collected 7 days after injection, detected by ddPCR. (E) Statistical analysis of barcodes for both the IL-15-armored and unarmored arms in each sample showing that IL-15-armored CAR-NK cells exhibit similar overall diversity to IL-15-unarmored CAR-NK cells in peripheral blood (PB) and tissues *in vivo*. The left panel shows results from a paired t-test for Shannon diversity across all *in vivo* samples, while the right panel displays the mean differences in Shannon diversity indexes. (F) Statistical analysis demonstrating that IL-15-armored CAR-NK cells exhibit fewer clones compared to unarmored CAR-NK cells in both PB and tissues *in vivo*.

1235 Understanding the role of *NEFM* and *UACA* genes in CART cell effector function and dysfunction

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Introduction: Despite the high initial responses to chimeric antigen receptor T (CART) cell therapy, most patients relapse within the first 1-2 years. T cell quality influences the success of CART cell therapies greatly, and T cells from cancer patients are prone to transition into dysfunctional states, such as exhaustion and senescence. Previously, we developed an *in vitro* model for repeated cycles of antigen-specific CART cell activation followed by rest periods, mimicking cancer relapse, to study the development of CART cell dysfunction and identify the mechanisms behind it. We repeatedly activated/rested CART cells generated from three healthy donors. Through RNA-seq analysis of CART cells at different activation/resting stages, we identified Uveal Autoantigen with Coiled-Coil Domains and Ankyrin (*UACA*) and Neurofilament Medium Chain (*NEFM*) genes to be downregulated in recurrently activated/rested CART cells that are costimulated with 4-1BB. To expand on these results, we induced overexpression of *NEFM* and *UACA* in CD19-targeted CART cells with costimulation via 4-1BB (BBζ) or CD28 (28ζ) and examined CART immunophenotype and effector functions.

Methods: Briefly, *UACA* and *NEFM* were ectopically expressed in BBζ or 28ζ CART cells (n=4, healthy donors) through lentiviral integration. *UACA* and *NEFM* gene expression was measured by qPCR. The resulting CART cells were flowed for exhaustion and senescence markers. The amount of cycling CART cells after target cell coculture was measured by 5-Ethylyl-2'-deoxyuridine (EdU) assay. The efficacy of control, *UACA,* and *NEFM* overexpressing BBζ and 28ζ cells was measured by antigen-specific cytotoxicity and proliferation assays.

Results: *UACA* and *NEFM* genes both showed multifold overexpression as compared to controls in both BBζ and 28ζ cells (BBζ: $p=<0.001$; 28ζ: $p=<0.001$). CART cell cycling 24 hours after target cell coculture increased in *UACA*- and *NEFM*-overexpressing BBζ and 28ζ cells as measured by EdU assay (BBζ: $p=<0.001$; 28ζ: $p<0.05$). Cytotoxicity assays after 48 hours of target cell coculture showed increased killing in *UACA*- and *NEFM*-overexpressing BBζ and 28ζ cells as compared to controls across multiple effector-to-target ratios (BBζ: $p<0.01$; 28ζ: $p<0.05$). Finally, when looking at T cell exhaustion markers LAG3 (BBζ: $p<0.001$; 28ζ: $p<0.01$), TIM-3 (BBζ: $p=0.001$; 28ζ: $p<0.01$), and PD1 (BBζ: $p=<0.001$; 28ζ: $p<0.05$) were all significantly downregulated in *UACA*- and *NEFM*- overexpressed CART cells compared to control CART cells.

Conclusion: Our prior RNA-seq analysis indicated downregulation of *UACA* and *NEFM* in recurrently activated/rested BBζ. In the present study, we overexpressed these two genes of interest in CART cells that were costimulated with either 4-1BB or CD28. Ultimately, our study indicates that *UACA* and *NEFM* overexpression appears to confer improved cytotoxicity and proliferative ability in CART cells as compared to control CART cells. The increased expression of these genes also leads to a decreased exhaustive immunophenotype. Further elucidation into the role of these two genes could shed light into important pathways that are key for CART cell dysfunction.

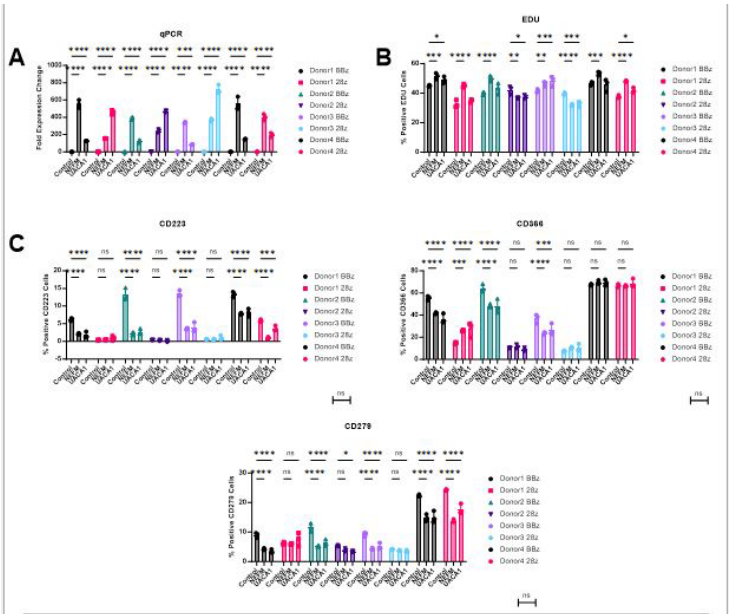

Figure 1: *NEFM* and *UACA* overexpression increases Proliferation and reduces dysfunction. A. UACA and NEFM overexpression was confirmed by qPCR analasys across all 4 healthy donors. **B.** EdU assays displaying percent positive cells after 24 hour of coculture with target cells. **C.** Immunophenotyping across all 4 healthy living donors for LAG3 (CD223), TIM-3 (CD266), and PD1 (CD279)

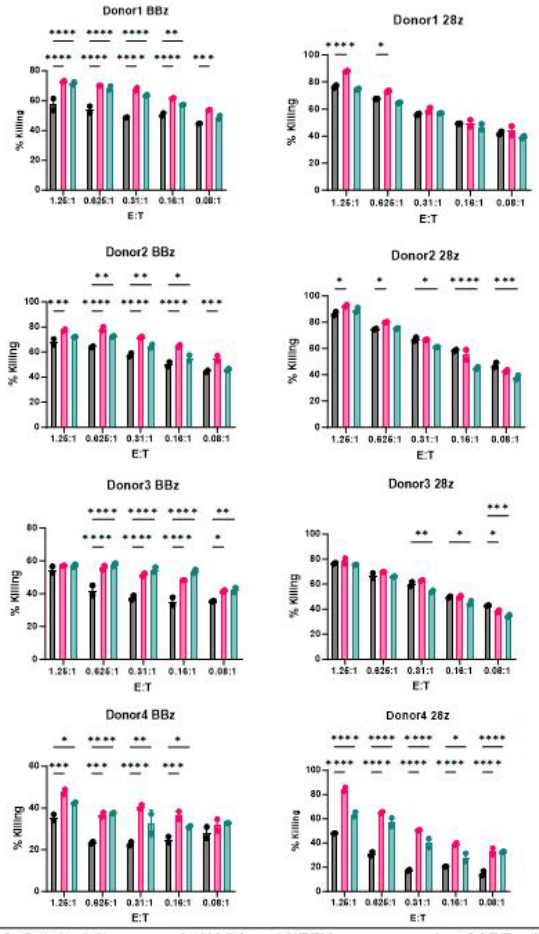

Figure 2: Cytotoxicity assays in UACA and NEFM overexpressing CART cells. Cytotoxicity assay were completed after 48 hours of coculture of CART cells transfected with either a control plasmid, *NEFM*, or *UACA* with luciferase expressing target cells

1236 Gamma Retroviral Vectors Pseudotyped with Either Baboon or RD114 Envelope Achieved High Transduction Efficiency and Improved Cytotoxicity of CAR-CYNK Cells

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Introduction: Celularity CYNK cells are allogenic, off-the-shelf, cryopreserved natural killer (NK) cells differentiated from placental CD34$^+$ cells. CYNK cells are shown to have the potential to be good resources for CAR-NK cell therapy. However, low transduction efficiency and the loss of transgenes in the lentiviral vector-based transduction method present a major hurdle in developing CAR-CYNK cells for cell therapy. To address this, in this study, we compared the transduction efficiency and functional activities of CYNK cells with gamma retroviral vector pseudotyped with either baboon or RD114 envelope glycoprotein, and lentiviral vector pseudotyped with VSV-G envelope glycoprotein.

Methods: The differentiation and expansion of CYNK cells from placental CD34$^+$ cells were achieved following a 35-day cell culture protocol developed in Celularity. CD19-CAR was cloned into lentiviral and the gamma retroviral vector backbone. The lentiviral vector was pseudotyped with VSV-G envelope glycoprotein (VSV-G-LV), and the gamma retroviral vector was pseudotyped with either baboon (BaEV-RV) or RD114 (RD114EV-RV) envelope glycoprotein using 293Vec-RD114 and 293Vec-Baev cell lines from BioVec Pharma. Transduction was performed on Day 5 of the cell differentiation process. The transduction efficiency was determined by flow cytometry using

CAR detection reagents. The CAR-CYNK cell purity, phenotype, and functions were assessed after the differentiation was completed.

Results: Compared to the VSV-G-LV, both BaEV-RV and RD114EV-RV mediated transductions demonstrated significantly higher transduction efficiency. The highest transduction efficiency of CYNK cells after differentiation was observed with BAEV-RV (79.1%) followed by RD114EV-RV (68.9%). VSV-G-LV based transduction efficiency of CYNK cells was only 10.3%. Moreover, unlike lentiviral vector transduction, in which transgene expression is significantly reduced during the differentiation process, retroviral vector-based transduction of CYNK cells showed improved transgene persistency. Due to the higher transduction efficiency and stable CAR expression, the cytotoxic function of CAR-CYNK cells against target cells also improved in retroviral-based transduction method. Both lentiviral and retroviral vector-mediated transduction did not significantly impact CYNK cell phenotype and cell expansion compared to non-transduced cells.

Conclusions: Baboon and RD114 envelope pseudotyped gamma retroviral vector-based transduction method could be a potential gene modification tool for placental CD34+ derived CYNK cells.

1237 Methotrexate-Based Selection of S/MAR DNA Nanovectors Enhances Cytotoxic Activity of CAR-Modified Blood Cells

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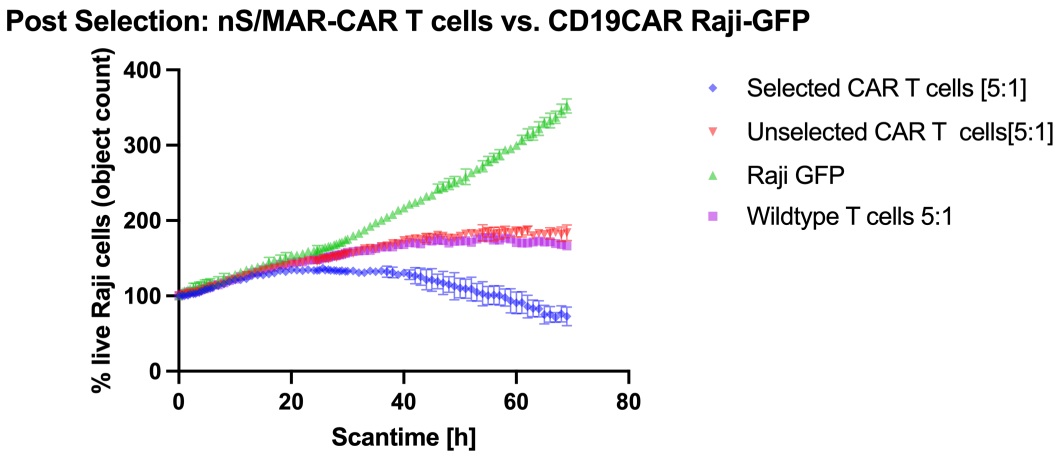
Genetic modification of blood cells such as T cells and NK cells holds significant promise in cancer therapy, enabling targeted and potent immunotherapies. While CRISPR/Cas9 and viral vectors are frequently employed for introducing engineered constructs such as chimeric antigen receptors (CARs), these approaches still involve risks like off-target effects and insertional mutagenesis. To address these limitations, we have developed a Scaffold/Matrix-Associated Regions (S/MAR) based non-viral DNA nanovector platform (nS/MAR). S/MAR elements, derived from chromatin-associated regions, confer episomal persistence to the nanovectors, thereby minimizing the risk of mutagenesis. Unlike viral vectors, nS/MAR does not trigger strong immune responses. Additionally, nS/MAR can carry large therapeutic transgenes or complex genetic constructs and has the potential to reduce manufacturing costs for clinical-scale gene modification of primary blood cells.

In this project, we have developed nS/MAR vectors that carry a double-mutant variant of the human dihydrofolate reductase gene (hDHFR), conferring resistance to the clinically established drug methotrexate (MTX). Using the MaxCyte ExPERT GTx™ electroporation system, we transfected K562 cells, NK92 cells, and primary T cells. Transfection efficiency, cell viability, and transgene persistence were assessed by flow cytometry. Cell killing assays were conducted against Raji-GFP cells using the IncuCyte SX5™ system. We demonstrate that MTX serves as an effective selection agent for nS/MAR vectors across multiple blood cell types. This enabled stable genetic modification of K562 cells. Strikingly, MTX-based selection significantly enhanced the cytotoxic activity of CAR-modified T cells, prolonging their tumor cell-killing function in vitro (Figure 1). Similarly, in NK92 cells, MTX selection achieved higher transgene positivity (97.3%) compared to puromycin (28.9%), with comparable viabilities (~50%).

These results underscore the potential of combining nS/MAR vectors with MTX selection to achieve safe, stable, and functionally enhanced genetic modification of T and NK cells. The capability for in vivo selection using a clinically approved drug like MTX offers a promising pathway to more effective and controlled immunotherapies for hematologic malignancies and solid tumors.



1238 Cytokine Release Syndrome and Cardiovascular Outcomes: A Population-Based Comparison Between Patients Infected With Severe Acute Respiratory Syndrome Coronavirus 2 and Those Who Received Chimeric Antigen Receptor T-Cell Therapy

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Introduction: Cytokine release syndrome (CRS) is common in patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and those receiving chimeric antigen receptor (CAR) T-cell therapy, leading to complications such as cardiovascular collapse and death. However, comparative analyses of CRS-associated cardiovascular outcomes between these groups are limited. This study aims to fill this gap in literature.

Methods: We identified patients from the National Inpatient Sample with SARS-CoV-2 infection and those who received CAR T-cell therapy from 2018 to 2021, using the International Classification of Diseases, 10th Revision. We included those with a concurrent diagnosis of CRS. We compared sociodemographic characteristics and comorbidities between SARS-CoV-2 patients with CRS and CAR T-cell therapy patients with CRS. Our primary outcome was all-cause mortality, and we studied various secondary cardiovascular outcomes. We utilized multivariate regression models to evaluate outcome disparities between the groups, with statistical significance set at $p < 0.05$.

Results: We analyzed 20,785 SARS-CoV-2 patients and 2,150 CAR T-cell therapy patients with CRS. The mortality rate was 19.8% for SARS-CoV-2 patients versus 3.5% for CAR T-cell patients. SARS-CoV-2 patients were younger (mean age 59.9 vs 61.5 years, p=0.03) and had a higher proportion of non-white patients (40.5% vs 23.3%, p<0.001). They had higher odds of all-cause mortality, with an adjusted odds ratio (aOR) of 19.75 (95% confidence interval [CI] 10.34-37.72) as well as acute congestive heart failure (aOR 5.16, 95% CI 2.20-12.08), acute coronary syndrome (aOR 44.92, 95% CI 5.92-341.19), cardiogenic shock (aOR 17.60, 95% CI 1.54-200.74) and ischemic stroke (aOR 11.40, 95% CI 2.92-44.61). Conversely, they had lower hospitalization charges ($167,489 vs $1,279,681, p<0.001).

Conclusion: Patients with CRS related to SARS-CoV-2 experienced higher rates of adverse cardiovascular outcomes and mortality than those with CRS from CAR T-cell therapy. These differences may be due to the greater severity of CRS in SARS-CoV-2 cases, underscoring the need for tailored management strategies for patients with CRS.

1239 Exploring the Impact of Chronic Kidney Disease on In-Hospital Outcomes in Patients Receiving Chimeric Antigen Receptor T-Cell Therapy: Insights From a United States Population-Based Study

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Introduction: Since the FDA approved the first Chimeric Antigen Receptor (CAR) T-cell therapy in 2017, it has revolutionized cancer treatment. Currently, six CAR T-cell therapies are approved for relapsed or refractory non-Hodgkin's lymphoma (NHL), B-cell acute lymphoblastic leukemia (ALL), and multiple myeloma (MM). While effective, CAR T-cell therapy can cause cytokine release syndrome and tumor lysis syndrome, which may contribute to kidney injury. Many patients with hematologic malignancies have preexisting kidney disease (CKD); however, data on CAR T-cell therapy outcomes in CKD patients are limited to single-center studies with inconsistent results. This study aims to assess the impact of CKD on outcomes in NHL, ALL, and MM patients receiving CAR T-cell therapy, using national data.

Method: We used the National Inpatient Sample to identify adults with ALL, NHL, and MM who underwent CAR T-cell therapy between 2018 and 2021, based on relevant ICD-10 codes. After excluding those with end-stage renal disease, we stratified the cohort by the presence or absence of CKD and applied complex sampling weights for national representativeness. We compared sociodemographic characteristics and comorbidities between CKD and non-CKD patients. The primary outcome was all-cause mortality, and secondary outcomes included acute kidney injury, renal replacement therapy, respiratory failure, mechanical ventilation, shock, vasopressor use, tumor lysis syndrome, immune effector cell-associated neurotoxicity, disseminated intravascular coagulation, gastrointestinal hemorrhage, intracerebral hemorrhage, length of stay, and total hospitalization charges. Multivariate regression models assessed outcome disparities between the groups, with statistical significance set at $p < 0.05$.

Results: A total of 4,120 adults with NHL, ALL, and MM received CAR T-cell therapy from 2018 to 2021 (82.5% NHL, 12.6% MM, and 4.9% ALL). Among these, 370 patients (9.0%) had a concurrent CKD (1.4% stage I, 14.9% stage II, 29.7% stage III, 4.1% stage IV, and 50.0% with unspecified stages). Patients with CKD were older (mean age, 64.8 vs. 60.2 years; $p<0.05$) and had a higher proportion of males (74.3% vs. 58.3%, $p<0.05$). A higher proportion of CKD patients had Charlson Comorbidity Index >2 (100.0% vs. 38.9%, $p<0.001$), atrial fibrillation (25.7% vs. 10.4%, $p<0.001$), and hypertension (60.8% vs. 37.8%, $p<0.001$). CKD patients had higher odds of developing acute kidney injury (adjusted OR 2.9, 95% CI 1.5–5.6). No significant differences were observed in the odds of all-cause mortality or other secondary outcomes between CKD and non-CKD patients. The mean hospitalization charge for CAR-T patients was $1,196,413, and the mean length of stay was 17.0 days, with no significant differences between the two groups.

Conclusion: Our study suggests CKD patients undergoing CAR T-cell therapy have a higher risk of acute kidney injury compared to non-CKD counterparts. However, these patients do not have an increased risk of all-cause mortality or other complications. Acute kidney injury in CAR T-cell therapy patients is typically mild and reversible with treatment. This evidence should be considered when developing strategies for CKD patients receiving CAR T-cell therapy, ensuring approaches are tailored to this population.

1240 Pooled CAR-T screening in nonhuman primates identifies architectures with enhanced proliferation, trafficking, and persistence

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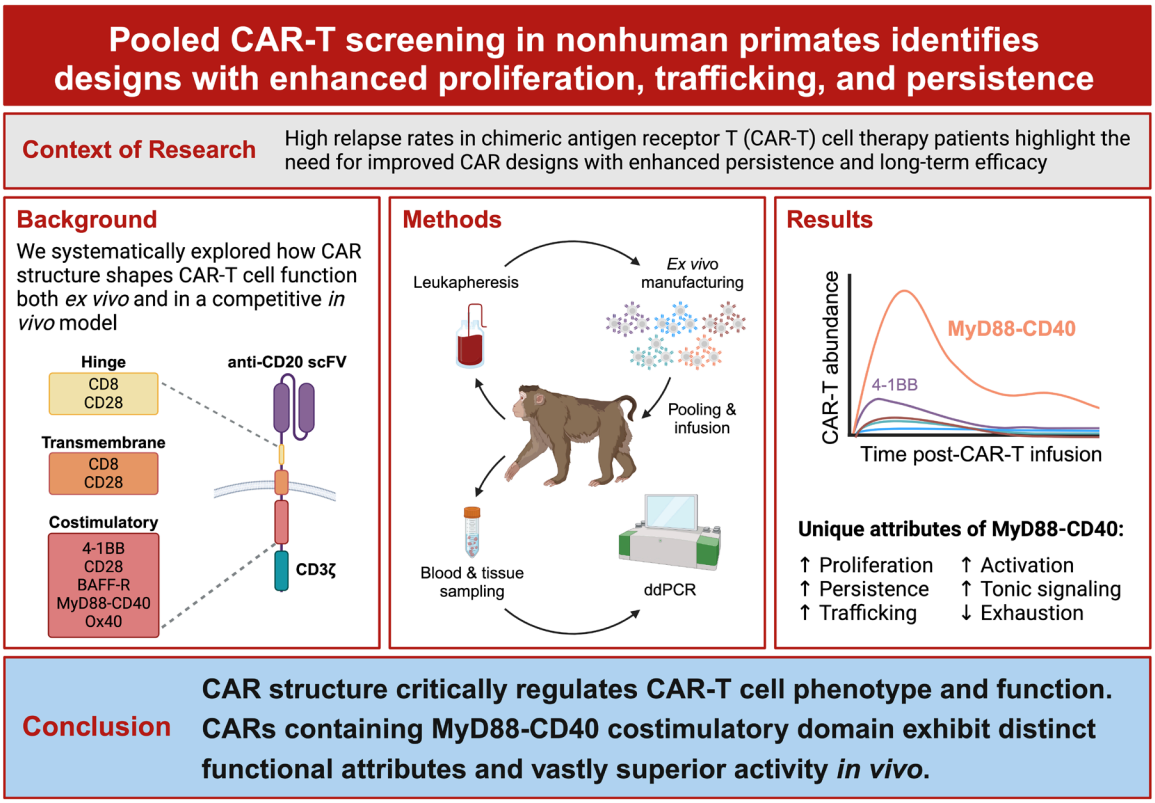
Introduction: Chimeric antigen receptor T (CAR-T) cell therapy has revolutionized treatment for B-cell malignancies, yet over 60% of patients relapse within one year, often due to insufficient CAR-T persistence. While mouse and primary cell models have been instrumental in advancing CAR-T therapy, they frequently fail to predict clinical outcomes, underscoring the need for more translationally relevant models.

Methods: We conducted the first systematic evaluation of CAR structure-function relationships in an immunocompetent nonhuman primate (NHP) model. We engineered an array of 20 CD20-targeted CARs, each containing distinct combinations of hinge, transmembrane, and costimulatory domains. Following rigorous *ex vivo* characterization, we administered pooled autologous CAR-T arrays to three NHPs and tracked CAR abundance longitudinally using a novel digital droplet PCR assay.

Results: *Ex vivo*, CAR-T cells incorporating the MyD88-CD40 costimulatory domain exhibited markedly distinct functional profiles, including increased activation, unique cytokine secretion, tonic signaling, and resistance to exhaustion. *In vivo*, MyD88-CD40 CARs expanded dramatically, comprising up to 100% of peripheral T cells and significantly outperforming canonical CD28- and 4-1BB-based CARs. This expansion was associated with robust B-cell depletion in peripheral blood across all animals. MyD88-CD40 CARs, particularly those with a CD28 hinge and transmembrane domain, demonstrated superior trafficking to secondary lymphoid tissues and persistence through study endpoint, unlike other CARs which waned by day 28.

Conclusions: Our findings highlight the value of immunocompetent NHP models for screening CAR designs and identify MyD88-CD40 CARs as candidates with unmatched potency. The unique functional attributes conferred by this domain, including hyperactivation, tonic signaling and resistance to exhaustion, may provide key insights into features that drive enhanced CAR-T cell activity.



1241 Long-term Dynamics and Integration Site Analysis of CAR-T Cell Therapy in Relapsed/Refractory Lymphoma and Multiple Myeloma: A Retrospective Study

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Background: CAR-T cell therapy has emerged as a promising treatment modality for relapsed/refractory lymphoma and multiple myeloma, achieving notable clinical success. Despite these advancements, several key questions remain unanswered. Specifically, the relationship between the persistence of CAR-T cells in the body and their therapeutic efficacy is still not well understood. Additionally, the impact of integration sites on T cell persistence and the potential for oncogenesis remains an area of investigation. Studies have shown that integration site at *TET2* gene can enhance the persistence of CAR-T cells in both preclinical and clinical settings. highlighting the potential significance of specific genetic modifications in CAR-T treatment. To address these gaps in knowledge, the current study employs a retrospective analysis of 18 patients with relapsed/refractory lymphoma and multiple myeloma who underwent CAR-T therapy. This investigation aims to elucidate the dynamics of CAR-T cells within the body and it potential associations with patient clinical outcomes. Furthermore, the study compares the persistence of different CAR-T cell products and evaluates the risk of secondary malignancies, providing valuable insights for the optimization of CAR-T therapy and informing clinical decision-making

Methods: This retrospective study included 18 patients who received CAR-T therapy for relapsed/refractory lymphoma (n=8) or multiple myeloma (n=10). CAR-T products used included relma-cel (n=5), axi-cel (n=1), humanized CD19 CAR-T (n=2), BCMA single-target (n=5), BCMA/CS1 dual-target (n=4), and BCMA/CD38 dual-target (n=1). During follow-up, digital droplet PCR (ddPCR) was used to monitor CAR-T cell levels by measuring CAR-T copy numbers. LAM-PCR (S-EPTS/LM-PCR) and high-throughput sequencing were employed to analyze integration sites of CAR lentiviral vectors in the host genome during expansion and low-level phases.

Results: Among the 8 patients with relapsed/refractory lymphoma, all currently remain in complete remission. Of the 10 patients with multiple myeloma, 7 are in complete remission, while 3 have relapsed. Digital droplet PCR (ddPCR) monitoring showed that the levels of CAR-T cells in the body did not significantly differ based on the type of CAR-T product infused. However, the 3 relapsed patients exhibited significantly lower CAR-T cell levels at 6 months compared to the other 7 patients (t-test, $p < 0.05$). We show that integration sites in the T cell genome were not randomly distributed, with the chromosome 11q13 region identified as the top integration site-enriched region. Notably, long-term follow-up (over 24 months) using LAM-PCR (S-EPTS/LM-PCR) identified several persistent integration sites near PACS1 gene and RPTOR gene in the 8 patients with relapsed/refractory lymphoma, suggesting these sites may confer selective advantages for T cell evolution and potential long-term efficacy of CAR-T therapy. Furthermore, no dominant clone was observed at integration sites within the previously identified common integration regions during the Long-term follow-up, the risk of secondary malignancies following CAR-T therapy was therefore found to be low, However, further long-term follow-up studies are warranted.

1242 Development of Humanized VHH-Based Bi-paratopic BCMA CAR-Ts for Multiple Myeloma Treatment via a Streamlined High-Throughput CAR^2-pool Screening Platform

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Multiple Myeloma (MM) is a malignancy of plasma B cells characterized by high relapse rates and poor prognosis. B cell maturation antigen (BCMA), a plasma B cell-specific marker, has emerged as a promising target for CAR-T cell therapies. Carvykti, an FDA-approved bi-paratopic CAR-T treatment, demonstrates superior clinical efficacy,

highlighting the advantages of targeting BCMA with dual binding domains. Factors such as antigen escape, immunosuppressive tumor microenvironments, and T cell exhaustion further emphasize the need for innovative CAR-T designs and screening to achieve durable responses. Elevated levels of the BCMA ligand APRIL in MM patients contribute to drug resistance by competing for BCMA binding to CAR-T cells, thusly promoting tumor survival.

To address these challenges, we developed a novel high-throughput functional screening strategy to streamline bi-paratopic CAR development. A BCMA-specific humanized VHH binder pool was enriched through panning against a semi-synthetic humanized VHH yeast display library. The 2,124 BCMA binders were cloned in bulk in a tandem repeat format into a lentiviral vector and transduced into primary T cells, generating a bi-paratopic CAR library designated as the CAR^{2} pool. These CAR-T cells underwent cell-on-cell sorting in the presence of APRIL to enrich for CAR-T bound cancer cells. Sequential multi-round co-culture with BCMA^{+} cancer cells was performed to select for CAR-T expression, cytotoxicity, expansion, and persistence. CAR VHH elements amplified from genomic DNA of CAR-T cells collected from each round of co-culture were subjected to Nanopore sequencing. Millions of CAR sequences emanating from each round of culture of CAR-T cells with cancer cells were processed, generating a ranked list of unique CAR sequences from both CD4^{+} and CD8^{+} CAR-T cell populations detailing the dynamics of the 2,046 bi-paratopic CARs relative to the round of CAR-T killing of cancer cells. A panel of 15 CAR candidates with the highest abundance and/or accumulation during the multiple rounds of co-culture of CAR-T cells with cancers cells was selected and characterized as single-clone bi-paratopic CAR-Ts. 14 out of 15 bi-paratopic CARs identified by Nanopore sequencing promoted potent MM1.S cancer cell killing by CAR-T cells with no off-target killing of BCMA-negative RS4;11 cells. The 4 leading candidates, maintained persistent CAR expression during multi-round co-culture with BCMA^{+} cancer cells, demonstrated robust CAR-T functionality even in the presence of APRIL, exhibited BCMA^{+} target cell dependent cytokine secretion, and facilitated killing of MM1.S or U266 cancer cells during 4 or 3 rounds of co-culture, respectively.

Future work focuses on evaluating these novel CARs in preclinical models and advancing the most promising candidates into clinical development. Overall, our streamlined CAR development platform provides a versatile, library size independent, comprehensive strategy for high-throughput CAR-T screening and engineering, with the potential to enhance CAR-T function and persistence in both *in vitro* and *in vivo* settings.

1243 Development of a Universal, Off-the-Shelf CAR-T Cell Immunotherapy for Multiple Myeloma

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Background: Multiple myeloma (MM) is the second most prevalent hematological malignancy worldwide. It is forecasted that by 2029, the global market of multiple myeloma will reach $28 billion. Chimeric antigen receptor T cells (CAR-T) have shown transformative efficacy for hematologic cancer. However, as an individualized treatment, the huge cost has become the biggest obstacle to the widespread use of autologous CAR-T products. Human pluripotent stem cells (iPSCs) have unlimited expansion potential, are easy to genetically manipulate and can be differentiated into T cells, making them an ideal universal source of immunotherapy. Here we aimed to develop an iPSCs-derived universal CAR-T product for multiple myeloma by targeting BCMA.

Methods: iPSCs were engineered to express tumor specific BCMA CAR from the T cell receptor alpha constant chain (TRAC) locus, controlling the CAR expression timing and eliminating the risk of GvHD. Immunogenecity-related MHC-I, II genes were knocked out as well to avoid HvGD. In serum- and feeder- free cultures, iPSCs were first differentiated into hematopoietic progenitor cells (iHPCs) via the well established embryoid body system and followed by differentiation on the matrix containing immobilized-delta like 4 protein and retronectin to induce the generation of CD8αβ T (iT) cells. Then iT cell maturation was accomplished by BCMA antigen stimulation. Incubation with MM cell line MM1S, H929 and OPM2 cells were used for evaluating the expansion potential, in vitro cytotoxicity capacity and cytokine release level of iT cells upon target-dependent stimulation.

Results: We developed a scalable, serum- and feeder- free differentiation system, which could efficiently induce the engineered iPSCs into iT cells. During the iHPC stage, CD34+ cell percentage could reach more than 90% with high level of CD45 expression. T progenitor stage was predominantly CD7+ (95%) cells, co-expressing CD5 (72%). Late T cells after stimulated with BCMA antigen could reach into 82% CD4-CD8α+, co-expressing 73% CD8β, and showed a low exhaustion profile (0.5% Tim3 and 11% PD-1) and memory T cell type (83% CD45RA–CD45RO+). The iT cells could be activated repeatedly by MM1S cells accompanying on average of 56-fold increase after each stimulation. In vitro killing assay showed that iT cells could efficiently kill target cancer cell line MM1S, H929 and OPM2 cells. In addition, the cytokine IFN-γ, TNF-α and IL-2 were released rapidly in iT cells upon the stimulation

by MM1S cells.

Conclusions: Here we report the development of a highly efficient and scalable method for CAR-T cell generation from engineered human iPSCs in the absence of serum and feeder layers. The resulting iT cells could specifically and efficiently kill the targeted cancer cells for multiple rounds. This system, for the first time, provides a foundation to manufacture universal iPSC-derived CAR-T products for multiple myeloma by targeting BCMA.

1244 BCMA CAR engineering arms T cells with superior functions post-*ex vivo* stimulation by MUC1 peptide-loaded autologous PBMCs

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Chimeric antigen receptor T (CART) cell therapy has shown clinical successes in B cell hematological malignancies. Two B cell maturation antigen (BCMA)-directed CART cell therapies treating multiple myeloma (MM) were approved by FDA, but most patients relapse within 1-2 years. *Ex vivo*-expanded antigen-specific T cells as a cancer treatment rely on TCR-mediated tumor killing. We hypothesized that arming *ex vivo*-enriched tumor-specific T cells with CARs targeting a different antigen improves T cell functions.

First, we expanded mucin 1 (MUC1)-activated T cells from patients with MM through *ex vivo* stimulation by MUC1 peptide-loaded autologous PBMCs for 19 days. Then, we engineered MUC1-activated T cells with 4-1BB-co-stimulated BCMA CAR to generate MUC1 TCR^+ BCMA CAR^+ T cells. Baseline T cells without MUC1 stimulation were engineered with BCMA CAR as control.

Baseline T cells, MUC1 T cells, baseline BCMA CART cells, or MUC1 BCMA CART cells were cocultured with $luciferase^+$ RPMI-8226, a $MUC1^+$ $BCMA^+$ MM cell line, for 3 days. MUC1 BCMA CART cells showed stronger tumor killing than BCMA CART or MUC1 T cells in all 3 patient samples ($p<0.0001$). MUC1 BCMA CART showed higher proliferation than BCMA CART or MUC1 T cells ($p=0.0765$, $p<0.0001$, respectively) after a 5-day stimulation by RPMI-8226. To exclude possibility of metabolic alterations by 19-day PBMC incubation leading to better T cell functions in MUC1 BCMA CART, baseline and MUC1-stimulated T cells from 3 healthy donors underwent BCMA CAR transduction and *ex vivo* expansion for 19 days and compared in the same assays. Day 19 BCMA CART cells showed inferior tumor killing compared to baseline BCMA CART in all 3 donors ($p<0.0001$). Lower T cell proliferation was also observed in Day 19 BCMA CART compared to baseline BCMA CART ($p=0.0838$). Our data indicate that improved T cell functions in MUC1 BCMA CART were unlikely due to metabolic changes but rather were likely through the MUC1 TCR.

Additionally, MUC1 T cells, BCMA CART, or MUC1 BCMA CART cells from 3 tested patients were stimulated by RPMI-8226 for 24 hours and then isolated for bulk RNA sequencing. Both BCMA CART and MUC1 T cells had distinct transcriptomic features from MUC1 BCMA CART cells. Differential gene expression analysis showed upregulation of T cell function-associated genes (*RYR2*, *ITGA1*, *GNLY*, *GZMA*, etc.) in MUC1 BCMA CART vs BCMA CART. Enrichment of T cell function-associated pathways was also observed in MUC1 BCMA CART. Analysis between MUC1 T cell and MUC1 BCMA CART showed upregulation of T cell function-associated genes (*SF2, IL2, IFNG, GZMB*, etc.) and enrichment of immune response pathways in MUC1 BCMA CART. Our findings suggest that equipping *ex vivo*-enriched tumor-specific T cells with CARs targeting a different antigen improves T cell functions *in vitro*. Importantly, this provides a rationale for a therapeutic regimen where patients receive *ex vivo*-enriched T cells followed by CAR engineering. Persistence of the engineered T cells is being investigated.

1245 Pre-Existing Immunity to Adeno-Associated Virus: A Global Meta-Analysis of Anti-AAV Neutralizing Antibodies in 9,887 Patients

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Introduction: Adeno-associated virus (AAV)-based gene therapy has emerged as a promising approach for treating a wide range of genetic disorders. However, pre-existing immunity poses a significant challenge to its clinical success. Anti-AAV neutralizing antibodies (NAbs) can inhibit vector transduction, reducing therapeutic efficacy and potentially increasing the risk of adverse reactions. Consequently, the presence of NAbs often leads to the exclusion of patients from clinical trials. Understanding the global seroprevalence of anti-AAV NAbs is essential for optimizing product development, refining clinical trial design, identifying populations at risk of being ineligible for treatment, and ultimately expand global access to AAV-based gene therapy. This meta-analysis comprehensively investigates the worldwide seroprevalence of anti-AAV NAbs across multiple serotypes in diverse patient populations and geographical regions.

Methods: A systematic search of major electronic databases (PubMed, SciELO, and CNKI) was performed to identify articles reporting data on the seroprevalence of anti-AAV NAbs calculated utilizing cell-based methods with a provided titer definition. Data was extracted and classified according to AAV serotype and NAb titer. Data on geographical region, patient age, and disease status was also extracted for subgroup analyses. Data extraction was supported with PlotDigitizer as appropriate. For data pooling, NAb seropositivity titer cutoffs were classified as low (≤1:10), intermediate (1:16–1:20), and high (≥1:50). Pooled seroprevalence estimates (PSE) with 95% confidence intervals (95% CIs) were calculated using MetaXL v5.3. This study was prospectively registered on PROSPERO (CRD42025636026).

Results: A total of 42 studies (9,887 patients) from all major continents and 28 countries were included in the analysis. Among included studies, 22 (52.3%) reported data at an intermediate titer (1:16-1:20). Subsequent analyses in this meta-analysis utilized this cutoff for consistency in reporting. The highest global PSE was observed for AAV2 (39.0%, 95%CI: 30.1-48.3%), followed by AAV1 (32.2%, 95%CI: 26.5-38.2%), AAV9 (28.9%, 95%CI: 20.8.-35.8%), and AAV3B (28.7%, 95%CI: 9.1-52.9%). The lowest global PSE was observed for AAV5 (19.0%, 95%CI: 11.9-27.4%). In adults, the highest PSE was observed for AAV2 (45.6%, 95%CI: 28.3-63.3) and AAVrh74 (35.2%, 95%CI: 31.5-39.1). The PSE in children was generally lower as compared to adults, with the highest PSE observed for AAV2 (25.7%, 95%CI: 13.2-40.5%), followed by AAV8 (23.5%, 95%CI: 13.2-35.7%) and AAV9 (22.0%, 95%CI: 12.6-33.0), with the lowest PSE observed for AAV5 (6.8%, 95%CI: 3.1-11.6%). Overall, a trend towards higher PSEs were observed across most AAV serotypes (AAV1, 2, 8, and 9) in East Asian and European populations as compared to North American populations. As compared to normal donor samples, no significant differences in PSEs for any serotype were observed among patients with hemophilia A, hemophilia B, or cystic fibrosis.

Conclusions: Our analysis reveals significant variability in the seroprevalence of anti-AAV NAbs across different populations and geographical regions. These findings provide valuable insights into the global epidemiology of pre-

existing immunity to AAV. This knowledge can inform the development and selection of more effective AAV vectors and guide clinical trial design, including patient selection and choice of trial locations.

1246 Depletion of AAV8 Antibodies in Seropositive Individuals Treated with the IgG Cleaving Enzyme Imlifidase

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A proportion of the patients in need of gene therapy has previously been exposed to wild-type adeno associated virus (AAV) and thus has high prevalence of antibodies against AAV. These pre-existing anti-AAV antibodies are a major challenge in AAV-based gene therapy, as they inhibit transduction and may constitute a safety concern[1]. In the first part of this study, serum samples from 200 healthy individuals were screened for anti-AAV8 seropositivity using a total IgG anti-AAV8 antibody (TAb) assay. In the second part, serum samples from 44 clinical trial subjects treated with the IgG cleaving cysteine protease imlifidase were anonymized and analyzed. Out of the 200 screened samples, 106 (53%) were positive for anti-AAV8 in the TAb assay, and 10 (5%) had anti-AAV8 titer >1:2560. Out of the 106 seropositive samples, the majority (70%) were low positive (RECL<25). Out of the 44 screened imlifidase-treated individuals, 11 (25%) were positive for anti-AAV8 in pre-imlifidase treated samples and further analyzed in the TAb assay for post-imlifidase treated samples at different timepoints (6h, 24h, 48h, 72h, day7, day14 and day64). The highest pre-dose titer was 1:2560. Additionally, the seropositive samples from ten imlifidase-treated individuals were analyzed in a functional neutralizing assay. For all subjects, a dramatic decrease in AAV8 antibody titers was observed as early as 6 hours after dosing with imlifidase. The titers remained low for several days, creating a potential time window for gene therapy dosing. The results from the TAb assay confirmed the ability of imlifidase in depleting anti-AAV IgG antibodies, with a similar pattern to that of total IgG depletion following imlifidase dosing. Taken together, the results indicate an anti-AAV IgG free window of 5-7 days post-imlifidase treatment, during which time gene therapy dosing would be most efficient. The potential of imlifidase to remove AAV antibodies has previously been demonstrated [2-4], both *in vivo* and *in vitro* and is under further evaluation in collaboration with our gene therapy partners. The results with serum from imlifidase treated individuals, support the potential use of imlifidase as pre-treatment to AAV-based gene therapy in patients with elevated pre-existing anti-AAV antibodies.

Disclaimer:
Imlifidase is an investigational product and is not approved in the USA for any use.

References:
1. Weber T. Anti-AAV Antibodies in AAV Gene Therapy: Current Challenges and Possible Solutions. Frontiers in Immunology. 2021.
2. Leborgne C. et al. IgG-cleaving endopeptidase enables in vivo gene therapy in the presence of anti-AAV neutralizing antibodies. Nature Medicine. 2020.
3. Elmore ZC. et al. Rescuing AAV gene transfer from neutralizing antibodies with an IgG-degrading enzyme. JCI Insights. 2020.
4. Ros-Ganán I. et al. Optimising the IgG-degrading enzyme treatment regimen for enhanced adeno-associated virus transduction in the presence of neutralising antibodies. Clinical & Translational Immunology. 2022.

1247 Modulation of Adenovirus Vector Immunogenicity by Non-neutralizing Antibodies and Complement C3 Deposition

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Adenovirus vectors are an excellent platform for gene therapy. They have a large payload capacity, are cost-effective to manufacture, and can be administered systemically to target disseminated cells throughout the body.

However, one major concern with using adenovirus vectors is the potential activation of a cytokine storm and the unpredictable responses of individual patients to high-dose infusions.

In our study, we examined the cytokine responses of human PBMCs to human adenovirus 5 in the presence of sera from healthy volunteers. Surprisingly, even non-neutralizing sera caused dramatic variations in cytokine responses. Furthermore, these sera deposited complement C3 on the viral particles, suggesting that antibodies from patients with no prior exposure to Ad5 can facilitate complement deposition and thereby alter the way the virus is presented to cytokine-producing myeloid cells. To investigate this phenomenon further, we conducted a mouse experiment in which we could control the adenovirus species to which the mice were exposed and against which they developed antibodies. Groups of mice were immunized with phylogenetically distant adenoviruses including Ad2, Ad3, Ad6, Ad11, Ad28, and Ad5 and, 35 days post-immunization, were injected intravenously with Ad5. We then analyzed vector distribution at 30 minutes and 6 hours post-injection, along with cytokine production and the capacity of antibodies from pre-immunized mice to deposit C3 on Ad5 particles. We designated the non-neutralizing antibodies capable of C3 fixation as Cryptic Opsonizing Non-neutralizing antibodies (CON-Abs). Unlike neutralizing antibodies, CON-Abs did not prevent virus-mediated membrane rupture or the subsequent activation of cytokine responses in spleen macrophages. In fact, cytokine responses were universally stronger in the presence of CON-Abs compared to those in naïve mice or mice with neutralizing antibodies. Interestingly, IL-12 was the only cytokine that remained at consistent levels in mice immunized with Ad5, and we observed a direct correlation between the amount of IL-12 released and the sera's ability to fixate C3 on the viral particle. To further elucidate the mechanism behind adenovirus-induced cytokine production, we sought to identify the responding cells following adenovirus injection. Given the importance of antibody binding and complement fixation in this process, we evaluated cytokine responses in pre-immunized Fcer1g KO, Trim21 KO, C3 KO, C1q KO, and C4 KO mice. We also performed immunofluorescence staining of mouse spleen tissue to pinpoint the cells responsible for cytokine production.

In conclusion, the severity of immunological responses to adenovirus vectors depends on the individual's antibody repertoire and the capacity of these antibodies to mediate C3 fixation on viral particles. Myeloid cells in the spleen phagocytose the adenovirus vectors and produce cytokines in response to the composition of the opsonized viral particles.

1248 Immunobiology of Proteasome-Targeted Strategies to Prevent Anti-AAV T-Cell Responses

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RATIONALE: Early AAV gene therapy failures linked to anti-capsid CD8+ T-cell responses prompted studies revealing proteasome-dependent cross-presentation as the primary driver. Strategies targeting the ubiquitin-proteasome system, such as capsid Y-F mutations or proteasome inhibitors (bortezomib, doxorubicin), reduced MHC-I antigen presentation and enhanced transgene expression, likely via improved AAV nuclear import. Doxorubicin is now being tested in a cystic fibrosis inhaled gene therapy trial (NCT06526923). While these approaches mitigate T-cell-mediated cytotoxicity, their effects on de novo anti-AAV immune priming and their interactions with antigen-presenting cells remain understudied. This work evaluates their potential to suppress CD8+ T-cell activation and amplify transgene expression in lung-directed AAV therapy.

METHODS: Using intubation, we delivered $1x10^{11}$ vector genomes (vg) of lung-tropic Adeno-Associated Virus serotype 6.2 or 6.2FF (AAV6.2-Luc) with or without doxorubicin to the respiratory tract of the BALB/c mice by intubation. At one month, transgene expression was assessed and mice were euthanized to assess the development of anti-vector immunoglobulins (IgG, IgM, IgA) in bronchoalveolar lavage fluid (BALF) and lung by ELISA and the development of AAV capsid-specific T-cells by MHC-I tetramer staining and flow cytometry. To validate our findings, similar experiments were conducted using AAV6.2-GFP and AAV6.2FF-GFP vectors, and the protein content of our AAV preps was assessed by Bicinchoninic Acid (BCA) Assay. We also repeated the AAV6.2-Luc experiment with or without doxorubicin, tracking transgene expression over time by *in vivo* bioluminescent imaging.

Finally, we evaluated the response of mouse bone-marrow derived dendritic cells (BMDC) to doxorubicin treatment *in vitro*.

RESULTS: Surprisingly, delivery of AAV6.2FF-Luc appeared to result in significantly increased antibody responses relative to AAV6.2-Luc and marginally increased T-cell responses. Inclusion of doxorubicin in viral inoculum resulted in significantly increased capsid-specific T-cell responses (Fig 1A). BCA assay revealed that while AAV6.2FF-Luc had greater protein content per vector genome than AAV6.2-Luc, the opposite was true for AAV6.2FF-GFP and AAV6.2-GFP. When comparing the GFP vectors, there were no differences in T-cell responses and a lower amounts BAL IgA in AAV6.2FF-GFP treated mice (Fig 1B). For doxorubicin, tracking of transgene expression over time revealed doxorubicin significantly increased transgene expression at early timepoints (1d, 3d) but differences converged and were no longer statistically significant after 7d. Interestingly, numbers of transgene-specific T-cells were not increased with doxorubicin treatment. *In vitro* treatment of mouse BMDC with 0.25µM of doxorubicin induced greater expression of costimulatory molecules CD80 and CD1d.

CONCLUSIONS: Our results comparing AAV6.2 with AAV6.2FF highlight the importance of characterizing antigen load or empty/full capsid ratios across AAV preps when assessing the immunological impact of capsid mutations. We further found that while doxorubicin promotes increased transgene expression at early timepoints, it may activate capsid-specific T-cells and result in loss of transgene expression. Our *in vitro* data suggest that doxorubicin may activate dendritic cells; studies are ongoing to validate these results *in vivo*.

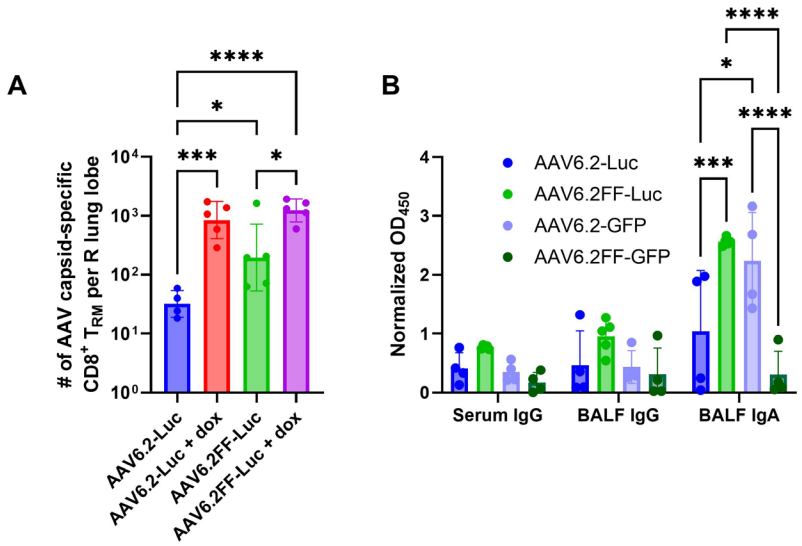

Figure 1. Immune responses to proteasome-targeted strategies. (A) Enumeration of AAV capsid-specific CD8+ T-resident memory cells (T_{RM}, defined as live,$CD45^{iv-}CD3^{+}CD8^{+}CD38^{+}CD69^{+}CD103^{+}$) in the mouse lung 1 month after delivery of AAV6.2/AAV6.2FF-Luciferase with or without 200µM doxorubicin, n=4-5 mice per group. (B) ELISA signal for AAV-specific IgG in serum and BALF, and IgA in BALF, n=4-5 mice per group. OD values are normalized to a control sample. *p<0.05,***p<0.001,****p<0.0001 by ANOVA.

1249 A Novel NK Inhibitor Provides "Best-in-Class" Protection of HLA Knockout Allogeneic Human Engineered Regulatory T Cells to Effectively Evade Immune Rejection

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Allogeneic regulatory T cell therapies offer substantial advantages over autologous therapies, but are limited by persistence for treating chronic indications due to host immune rejection. Knockout (KO) of human leukocyte antigen (HLA) class I and II combined with natural killer (NK) cell inhibition is a promising approach to evade

immune rejection. While molecules such as HLA-E and CD47 have successfully prevented NK rejection in certain contexts, allogeneic cell therapies need more robust approaches which provide protection of HLA KO cells broadly across a heterogeneous population.

Here, we observed the extent of HLA class I KO cell elimination by peripheral blood mononuclear cells (PBMCs) significantly correlated with the frequency of NK cells expressing a particular inhibitory receptor (Receptor X). HLA class I-negative cell rejection did not associate with other NK or major immune subsets in the donor PBMC, suggesting missing-self rejection is driven by Receptor X+ NK cells in humans. Based on this finding, we assembled a panel of inhibitory molecules that bind to Receptor X. When expressed on HLA KO human engineered regulatory T cells (EngTreg), these NK inhibitors demonstrated varied levels of target cell protection from PBMC killing.

We focused on a top performing inhibitor and demonstrated superior protective function over field-standard NK inhibitors such as CD47 and HLA-E across 10 PBMC donors using a 1-day killing assay. The NK inhibitor specifically protected inhibitor-expressing cells and not bystander HLA KO cells lacking the inhibitor in a three-cell co-culture system, demonstrating cell-intrinsic protective effect of the inhibitor. To highlight the applicability of the Receptor X inhibitor approach to our EngTreg platform, we generated an HLA KO and inhibitor-expressing version of our EngTreg asset specific for a pancreatic islet-specific autoantigen to treat type 1 diabetes. Inhibitor expression protected the EngTreg from PBMC killing compared to HLA KO alone and did not interfere with islet antigen-specific EngTreg responses.

Lastly, we explored molecular variants of the inhibitor to optimize its activity. We also developed an aggressive in vitro rejection system that concurrently captures both T and NK mediated rejection over 7 days. Using this rigorous rejection model, we observed that NK inhibitor optimization enabled higher persistence and expansion of HLA KO EngTreg in the presence of allogeneic PBMCs (12 donors tested) compared to the field-standard NK inhibitors CD47 and HLA-E, as well as an emerging CD300a trans-antigen signaling receptor (TASR) NK inhibitor technology. In summary, we have developed a potent immune evasion molecule, Receptor X Inhibitor, that suppresses an NK subset which may be responsible for missing-self rejection in humans. Importantly, Receptor X inhibitor outperforms competing NK inhibitory technologies for immune evasion, and this technology can be readily incorporated in GentiBio's dual AAV manufacturing process to generate future off-the-shelf allogeneic EngTreg products.

1250 Epigenetic targeting of macrophage polarization

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Macrophages are key players in the innate immune system. The two major polarized states, M1 and M2, exhibit distinct phenotypes and specialized functions in immunity and inflammation. M1 macrophages are characterized by enhanced microbicidal activity and the secretion of pro-inflammatory cytokines, while M2 macrophages promote inflammation resolution, and immune regulation. Dysregulation of M1/M2 polarization has been implicated in autoimmune diseases and cancer. Understanding the molecular mechanisms that govern macrophage polarization is essential for developing targeted therapies aimed at modulating immune responses and restoring tissue homeostasis[1]. Epigenetic modifications, particularly DNA methylation, have emerged as key regulators of macrophage plasticity by modulating gene expression patterns that define M1 and M2 phenotypes. Recent studies suggest that DNA methylation influences macrophage fate by repressing or enhancing specific transcriptional programs; however, its precise contribution remains incompletely understood[2]. Here, we addressed the impact of DNA methylation

perturbation on macrophage-like and primary macrophage polarization. We established protocols to differentiate monocytes into macrophage-like cells using both a cell line model and primary human peripheral blood mononuclear cells (PBMCs). Phenotypical differentiation into M1 or M2 macrophages was confirmed by flow cytometry analysis of both primary cells and the cell line, as well as by morphological assessment using Wright-Giemsa staining. To investigate the impact of DNA methylation in macrophage polarization, we treated cells with the hypomethylating agent 5-azacitidine (5-Aza)[3] at different stages of differentiation. Flow cytometry and neighborhood analyses revealed an enrichment of the M2 phenotype over the M1 phenotype in 5-Aza-treated cells. Cytokine secretion and phagocytosis evaluation further demonstrated that treated U937-derived macrophages exhibited lower cytokine production and reduced phagocytic capacity compared to untreated controls. In primary macrophages, we observed increased presence of M2-like upon 5-Aza treatment in M1 stimulated cells and distinct M1 and M2 polarization upon M2 stimuli unlike untreated cells. Notably, IL-6 expression was absent in M1- but present in M2-stimulated cells following 5-Aza treatment, suggesting a DNA methylation-dependency of IL-6 expression. Lastly, we used a DNMT1 selective inhibitor, recently developed in our laboratory[4] – aptaDiR, to evaluate the effect of targeted DNA demethylation on macrophage polarization. Following assessment of cellular uptake of aptaDiR formulated in APC-labeled lipid nanoparticles (LNPs), we did not observe toxicity in peripheral blood stem cells as compared to 5-Aza. Remarkably, when compared to 5-Aza, aptaDiR treated cells exhibited a skewed toward M2 polarization in the tested culture conditions, supporting a potential DNMT1-mediated role in macrophage polarization. Our findings suggest that perturbations of DNA methylation do affect macrophage polarization. The absence of IL-6 in M1-stimulated, 5-Aza-treated cells and its presence in M2-stimulated cells highlight a potential methylation-dependent regulatory mechanism. Furthermore, DNMT1 inhibition via aptaDiR supports the involvement of DNA methylation in shaping macrophage fate. These results provide new insights into the epigenetic regulation of macrophage plasticity and their implications in inflammatory responses.

Bibliography

1. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, Seifi B, Mohammadi A, Afshari JT, Sahebkar A. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 2018 Sep;233(9):6425-6440. doi: 10.1002/jcp.26429. Epub 2018 Mar 1. PMID: 29319160.
2. Ivashkiv LB. Epigenetic regulation of macrophage polarization and function. Trends Immunol. 2013 May;34(5):216-23. doi: 10.1016/j.it.2012.11.001. Epub 2012 Dec 4. PMID: 23218730; PMCID: PMC3647003.
3. Neuendorff NR, Gagelmann N, Singhal S, Meckstroth S, Thibaud V, Zhao Y, Mir N, Shih YY, Amaro DMC, Roy M, Lombardo J, Gjærde LK, Loh KP. Hypomethylating agent-based therapies in older adults with acute myeloid leukemia - A joint review by the Young International Society of Geriatric Oncology and European Society for Blood and Marrow Transplantation Trainee Committee. J Geriatr Oncol. 2023 Apr;14(3):101406.
4. Esposito CL, Autiero I, Sandomenico A, Li H, Bassal MA, Ibba ML, Wang D, Rinaldi L, Ummarino S, Gaggi G, Borchiellini M, Swiderski P, Ruvo M, Catuogno S, Ebralidze AK, Kortylewski M, de Franciscis V, Di Ruscio A. Targeted systematic evolution of an RNA platform neutralizing DNMT1 function and controlling DNA methylation. Nat Commun. 2023 Jan 6;14(1):99. doi: 10.1038/s41467-022-35222-4. PMID: 36609400; PMCID: PMC9823104.

1251 *Targeting immune checkpoint receptor ligands to reactivate T-cell function and eliminate persistent hepatitis B virus infection*

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Introduction: Hepatitis B virus (HBV), a member of the Hepadnaviridae family, primarily infects liver hepatocytes and can cause acute or chronic infection. Chronic hepatitis B (CHB) infection is a major global health problem that can lead to liver cirrhosis and hepatocellular carcinoma. Although therapeutic approaches such as pegylated interferon alpha and nucleoside analogs are available for CHB, they rarely eradicate the virus. Therefore, there is an urgent need for novel therapeutic strategies to achieve a complete cure for persistent HBV infection. A robust T-cell response is essential for viral clearance during acute HBV infection. However, in CHB, virus-specific T cells are scarce and dysfunctional with increased expression of inhibitory molecules like PD-1 and TIM-3. These molecules bind to their ligands, suppressing T-cell activation and weakening the immune response. This study aims to knock down the ligands for PD-1 and TIM-3, specifically PD-L1, PD-L2, and Galectin-9 (Gal-9), which are predominantly expressed on non-parenchymal liver cells, in order to restore T-cell function and potentially cure chronic hepatitis B infection.

Methods: Short hairpin RNAs (shRNAs) were designed and screened for mouse PD-L1, PD-L2, and Gal-9. The knockdown efficiency of the shRNAs was evaluated using a dual-luciferase assay. Continuous expression of shRNAs was achieved using adeno-associated viruses (AAV). This project used a capsid with enhanced tropism for non-parenchymal liver cells, specifically liver-resident macrophages. Selected shRNAs targeting immune checkpoint receptor ligands were cloned in a single self-complementary AAV genome (AAV-ICI). As a control, a scrambled shRNA sequence was cloned into the same backbone as AAV-ICI. The toxicity of AAV-ICI and AAV-Ctrl was tested *ex vivo* in primary mouse hepatocytes. An AAV carrying a 1.2-fold overlength HBV genome was used to establish persistent HBV replication. Additionally, HBV transgenic mice were used to evaluate the therapeutic effect of AAV-ICI.

Results: Selected shRNAs targeting Gal-9, PD-L1, and PD-L2 showed up to 85% knockdown efficiencies. The knockdown efficiency of the final construct was verified by a dual luciferase assay. Knockdown levels varied due to the different promoters: Gal-9 at 90%, PD-L1 at 80%, and PD-L2 at 25%. Neither AAV-ICI nor AAV-Ctrl exhibited toxic effects *ex vivo* in primary mouse hepatocytes. However, *in vivo*, high alanine transaminase (ALT) levels, indicating liver damage, were detected, along with a strong innate immune response induced in both the AAV-ICI and AAV-Ctrl groups. Additionally, no persistent HBV replication was established when mice were co-infected with AAV-HBV and either AAV-ICI or AAV-Ctrl.

Conclusion: Targeting liver macrophages with a self-complementary AAV led to significant liver damage with both AAV-ICI and AAV-Ctrl treatments. Thus, suppression of immune checkpoint ligands has to be tackled using small interfering RNAs with diverse modifications to specifically target non-parenchymal cells.

1252 The Intracellular Fc Receptor TRIM21 Promotes Type I Interferon Responses to Antibody-complexed AAV

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Tripartite motif-containing protein 21 (TRIM21) is an intracellular Fc receptor and E3 ubiquitin ligase that facilitates antiviral immunity by sensing virus-antibody complexes, triggering their proteasomal destruction and mediating downstream immune signaling through NF-kB, AP-1, and IRFs. Non-enveloped viruses, such as adenoviruses, picornaviruses, and rhinoviruses, have been shown to be particularly susceptible to TRIM21 activity due to direct cytoplasmic exposure of antibody-bound capsids during cellular infection. However, whether TRIM21 may also impact the immune response to non-enveloped viral vectors used for gene therapy, such as adeno-associated viral vectors (AAV), remains uncertain.

Here, we hypothesized that TRIM21 may contribute to immunogenicity of AAV in the presence of pre-existing anti-AAV IgG. To test this hypothesis, we isolated human PBMCs from whole blood of healthy volunteers, treated with AAV in the presence or absence of an AAV-specific monoclonal human IgG1 (anti-AAV hIgG1), and analyzed IFN-α secretion ~18hr later. We found that in the absence of anti-AAV IgG1 both AAV8 eGFP and AAV9 eGFP

vectors failed to elicit a detectable IFN-α response, even at a relatively high MOI (1e6). However, in the presence of anti-AAV hIgG1, we observed a dramatic boost in IFN-α secretion, consistent with a potential role for TRIM21. To more directly interrogate the contribution of TRIM21, we introduced a mutation to the antibody Fc domain (of the same AAV mAb clone) that ablates TRIM21 binding, but not FcγR binding (hIgG1 H433A). Unexpectedly, compared to unmodified antibody, co-treatment of AAV8 eGFP with anti-AAV hIgG1 H433A resulted in a substantial reduction in IFN-α secretion, in some cases by >90% (Mean [SD]: 77.1% [15.5]; n=8). Importantly, introduction of the H433A mutation had no measurable impact on antibody binding to AAV8 by ELISA. Taken together, our data suggest TRIM21 may be a previously unappreciated innate immune sensor that can exacerbate immune responses to AAV-antibody immune complexes. Future work will be critical to understand whether TRIM21 impacts AAV transduction and immunogenicity *in vivo* in the context of pre-existing antibodies to AAV.

1253 *In vivo* Engineering of HSPCs for HIV Gene Therapy using Helper-Dependent Adenoviral Vectors expressing HIV Fusion Inhibitors

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HIV fusion inhibitors, which block the viral fusion process, have shown promise as therapeutic agents. Enfuvirtide, a widely used fusion inhibitor peptide, controls HIV replication when administered via subcutaneous injection. However, it requires bi-daily dosing, increasing the risk of injection site reactions, infections, costs, and reducing bioavailability with limited delivery to the HIV reservoirs. We hypothesize that engineering hematopoietic stem and progenitor cells (HSPCs) in vivo would lead i) to the expression of membrane-localized HIV fusion inhibitors in HIV target cells, thereby protecting them from infection, or ii) to the secretion into the parenchymal space, which would also provide bystander protection of non-transduced cells. To test this, we are evaluating three fusion inhibitors expressed from two promoters (human β-Actin and human ubiquitin-C): i) de-immunized C46-v20, ii) stability-enhanced C34 (Sifuvirtide, C34-SFT), and iii) FDA-approved Enfuvirtide (T20). All of them are under the regulation of miRNA126 to suppress expression in HSPCs. We validated the expression of T20 secreted and membrane-anchored forms using ELISA and Flow Cytometry. We used real-time qRT-PCR to measure the expression of C34 SFT and c46v20. Plasmid transfections in TZM-bl cells showed that secreted forms blocked HIV effectively, while membrane-anchored forms were less effective, likely due to limited membrane density of the fusion inhibitors. We produced HDAd5/35++ vectors and transduced them into PM-1 and TZM-bl cells. A single-round HIV infection assay demonstrated up to 90% resistance to HIV in cells expressing fusion inhibitors as well as 60-90% resistance to replication-competent R5, X4, and SIVmac239 strains, depending on the dose tested. In these studies, we observed that prolonged exposure to the secreted form of T20 induced cytotoxicity in various cell lines. Additionally, we transduced HDAd encoding fusion inhibitors along with a Sleeping Beauty transposase vector (for integration) into CD34+ cells, enriched stably transduced cell with O^6BG/BCNU, and differentiated them into macrophages. In vitro HIV challenge assays in macrophages derived from transduced cells showed HIV resistance. Among the fusion inhibitors tested, the secretory version of C46-V20 was selected for in vivo transduction due to its broad efficacy and low immunogenicity. To assess its safety, we performed in vivo HSPC transduction in G-CSF/AMD3100-mobilized human CD46 transgenic mice. One-week post-transduction, we observed severe toxicity with 50% mortality. The administration of dexamethasone, MMF, and rapamycin rescued the remaining mice, suggesting inflammatory responses by/to C46-v20. We also hypothesized that ubiquitous expression of C46-V20 from the UbC promoter might have contributed to in vivo toxicity. Notably, serum collected from in vivo transduced mice (under immunosuppression) neutralized infectious HIV in TZM-bl cells. To fully harness the capabilities of HDAd vectors (which can accommodate transgenes up to 35kb), we are developing a combination vector for CCR5 base editing and lineage-specific expression of anti-HIV genes. These genes include the secreted decoy receptor eCD4Ig expressed from erythroid cells and HIV fusion inhibitors expressed from macrophages, along with modules that allow for the expansion of transduced cells such as tEPOR, tHMGA2, and $MGMT^{P140K}$. We believe that this strategy will improve the safety and efficacy of HIV therapy.

1254 Toward a Prophylaxis of SIV Infection in Rhesus Macaques by in vivo HSC Transduction with HDAd-S-eCD4Ig

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We have developed an *in vivo* HSC transduction approach that requires only intravenous injections and could be provided as outpatient treatment. In this approach, HSCs are mobilized from the bone marrow into the peripheral blood stream and transduced with intravenously injected *in vivo* gene transfer vectors (helper-dependent adenovirus 'HDAd' vectors) that target receptors present on primitive HSCs. HSCs transduced in the periphery return to the bone marrow, persist there long-term, and can be expanded by O^6BG/BCNU treatment to therapeutic levels in mouse models of disease. Here, we used HDAd 6/3+ vectors to transduce HSCs *in vivo* in rhesus macaques to express eCD4-Ig-Emm06, a secreted decoy receptor that targets the primary viral receptors for HIV, CD4 and CCR5. eCD4-Ig-Emm06 expression was driven by the constitutive EF1a-promoter within an integrated cassette that also encoded the sulfation enzyme TPST2, which we demonstrated to sulfate all 7 tyrosine residues in eCD4-Ig-Emm06 for efficient HIV neutralization. Rhesus macaques were assigned to receive either an HDAd vector expressing eCD4-Ig-Emm06 (N=3) or a control HDAd vector (N=3). Animals underwent two rounds of O^6BG/BCNU *in vivo* selection followed by serial escalating-dose challenges of SIVmac239, an HIV-like virus used to assess protection from viral replication and disease progression. Analysis of bone marrow aspirates showed preferential transduction of $CD34^+$ cells with HDAd vector copy numbers in the range of 0.1-1.2 copies per cell. Because of lung toxicity associated with O^6BG/BCNU selection, we were not able to achieve similar VCNs in PBMCs. Nevertheless, in the test animals, stable serum levels of eCD4-Ig-Emm06 were 2.1 µg/mL to 38.3 µg/mL, which is 100-1000-fold higher than the serum IC_{50} concentrations (10.6 to 34.7 ng/mL). One animal exhibited eCD4-Ig-Emm06 levels exceeding therapeutic thresholds, effectively delaying onset to viremia following infection. This was accompanied by lower peak viral loads, lower setpoint viral loads, and protection from $CD4^+CCR5^+$ T-cell depletion, suggesting enhanced protection against viral replication and disease progression. eCD4-Ig-Emm06 was detectable across a broad range of cell subsets and tissues, including T cells, B cells, and monocytes within PBMCs, bone marrow, spleen, lymph nodes, and gut tissues. Tissue viral reservoirs were significantly reduced in tissues with higher VCNs, likely due to greater tissue-specific levels of eCD4-Ig-Emm06. Single-genome sequencing of SIV from serum samples is ongoing to identify routes of viral escape from eCD4-Ig-Emm06, which will inform the development of future therapeutic strategies targeting these escape mechanisms. In summary, this study demonstrates that *in vivo* HDAd transduction efficiently generated long-term levels of eCD4-Ig-Emm06 to protect against HIV/SIV infection. To further enhance the anti-HIV effect, we have developed combinatorial HDAd vectors that express an integrated eCD4-Ig-Emm06 cassette and a transiently expressed base editor to knockout CCR5 ('dual combo') to synergistically target major viral replication mechanisms. To improve safety for otherwise healthy people living with HIV, these new combination vectors incorporate a tHMGA2 auto-expansion approach (PMID: 39282078) to enrich *in vivo* transduced HSCs. These dual combo HDAd vectors are currently being tested in rhesus macaques.

1255 Advancing Precision Immunotherapy for EBV-Associated Malignancies Through HLA-Driven TCR Engineering

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Epstein-Barr virus (EBV)-associated diseases, including post-transplant lymphoproliferative disorder (PTLD), EBV+ lymphomas, nasopharyngeal carcinoma (NPC), and gastric cancer, pose a significant therapeutic challenge

due to their diverse latency patterns (types I, II, and III) and poor clinical outcomes. While EBV-specific adoptive T cell therapy (ACT) has shown remarkable efficacy against PTLD (latency III), limited success in latency I and II malignancies highlights a critical unmet need for more precise and effective therapies.

To address this, we investigated how HLA alleles influence immune responses against specific EBV antigens. Preliminary experiments using T cells from five healthy donors with diverse HLA alleles revealed distinct differences in their ability to control autologous EBV tumor growth *in vitro*, despite successful expansion of antigen-specific T cells. For example, in case of EBNA-1 specific T cells (median 4.22% IFNγ^+ T cells, range 1.16-7.06%, measured by intracellular cytokine staining 6h after EBNA-1 peptide pool stimulation), only two donors exhibited significant tumor control, making them more suitable for the ACT treatment of Burkitt's Lymphoma (latency type I, presenting only EBNA-1 latent protein). Notably, these donors shared a number of HLA alleles, including B35. We hypothesize that these findings underscore the importance of HLA-driven variability in immune responses and suggest a path forward for selecting optimal donors for allogeneic ACT or designing transgene TCR therapies. We are aiming at testing a substantial number of donors and establishing adequate EBV latency type I and II tumor models and assays to further investigate the relevant HLA alleles.

Our ongoing work involves developing personalized models of EBV latency type I and II tumors and systematically identifying high-performing cytotoxic T lymphocyte (CTL) clones from diverse HLA backgrounds. By establishing a comprehensive library of EBV-specific TCRs optimized for HLA profiles, we aim to enhance therapeutic precision and broaden the applicability of cancer immunotherapy. This project promises transformative advancements in targeting EBV-driven malignancies, improving patient outcomes through personalized, HLA-informed T cell engineering.

1256 STING activation, DNA clearance, and autoimmune disease: Investigating pathogenic mechanisms with DNAse dysfunction and novel therapeutics in SLE and AGS models

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Background: DNase1, DNase1L3, and TREX1 enzymes play crucial roles in clearing excess chromatin and DNA in healthy individuals. Mutations or autoantibodies against these enzymes impair DNA clearance, leading to DNA accumulation at inflammatory sites. Systemic lupus erythematosus (SLE) is a leading cause of death among young women and shares features with type I interferonopathies like Aicardi-Goutières syndrome (AGS), a rare autoimmune disorder marked by neuroinflammation, with 30% of AGS cases linked to TREX1 deficiency.

Objective: This study investigates the therapeutic potential of a novel long-acting DNase biologic in autoimmune diseases resulting from TREX1 deficiency, focusing on its effects in AGS.

Methods: We engineered a bioavailable, dual acting DNase1/DNAse1L3 biologic and tested its efficacy in an AGS mouse model (Trex1 knockout, TKO). Survival in treated mice was monitored over 250 days, and a smaller cohort underwent mechanistic analysis of immune biomarkers (cytokines, autoantibodies) and molecular pathways.

Results: DNase biologic-treated mice showed improved survival vs. controls. Treatment also reduced biomarkers, such as anti-dsDNA abs, anti-MYH6 abs, cfDNA, and IFN-γ. Untreated TKO mice experienced significant weight loss and myocarditis, which were alleviated in treated mice.

Conclusions: The dual-acting DNase1/DNAse1L3 biologic significantly improved the survival of TKO mice, demonstrating the role of extracellular free DNA and chromatin in the pathogenesis of AGS. Our observations have important implications for the development of novel therapeutics for autoimmune disorders.

1257 Selective Inhibition of Human Plasmacytoid Dendritic Cells Reduces TLR9-mediated Type I IFN to CpG DNA in AAV Gene Therapy Vectors

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CD8+ T cell immune responses to adeno-associated virus (AAV) capsid protein can contribute to the loss of successfully transduced cells, resulting in toxicity and reducing efficacy. The generation of de novo capsid-specific CD8+ T cells requires Type I interferon (IFN) production from plasmacytoid dendritic cells (pDCs). CpG DNA motifs in AAV gene therapy vectors stimulate pDC production of Type I IFN in a toll-like receptor 9 (TLR9)- and IL-1R associated kinase 4 (IRAK4)-dependent manner. Reduction in CpG DNA content in viral vectors is one proposed approach to reducing pDC Type I IFN in response to AAVs and thus, capsid-specific CD8+ T cells. For internal programs, we tested this hypothesis by establishing a human in vitro assay to measure Type I IFN production in human peripheral blood mononuclear cells (PBMCs) to AAV9, a clinically relevant serotype. Using AAVs with varying CpG amounts, we demonstrate that human Type I IFN induction in PBMCs from various human donors positively correlate with CpG content of the AAV vectors. Furthermore, Type I IFN production in this assay was TLR9 and IL-1R associated kinase 4 (IRAK4) dependent. Given that CpG motif reduction may not be possible for all AAV cargos and current pharmacological modulators of this pathway (e.g. IRAK4 inhibitors) are not specific to pDC-TLR9 signaling, we investigated whether blocking activation of pDCs via a pDC-specific mechanism could reduce TLR9-dependent Type I IFN production against AAV. Previous work established that targeting a pDC-specific receptor blocks TLR9-mediated Type I IFN production to CpG-A, a synthetic TLR9 stimulus, in pDCs. Promisingly, we significantly reduced Type I IFN production in response to an AAV9 vector containing TLR9-stimulatory CpG content in PBMC from multiple donors. Our data suggests we can selectively inhibit pDC mediated Type I IFN following AAV gene therapies while maintaining other immune responses.

1258 Targeting human plasma cells using small molecule regulated BCMA CAR T cells eliminates circulating antibodies in humanized mice

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Pathogenic long-lived plasma cells (LLPCs) secrete autoreactive antibodies, exacerbating autoimmune diseases and complicating solid organ transplantation. Targeted elimination of the autoreactive B-cell pool represents a promising therapeutic strategy, yet current treatment modalities fall short in depleting mature plasma cells. Here, we demonstrate that chimeric antigen receptor (CAR) T cells, targeting plasma cell marker BCMA utilizing a split-receptor design, offer a controlled and effective therapeutic strategy against LLPCs. Dimerizing agent-regulated immune-receptor complex (DARIC) T cells demonstrated robust rapamycin-dependent targeting of BCMA+ tumor cells in-vitro and in-vivo. Using an in-vitro differentiation platform, DARIC T cells also selectively targeted primary human plasma cells from a mixed B cell population. Notably, in humanized mouse models, DARIC-T cells regulated peripheral human immunoglobulin levels through specific elimination of human LLPCs from the bone marrow. Furthermore, DARIC constructs were efficiently integrated into the T-cell receptor α constant (TRAC) locus while maintaining potent antigen-specific cytotoxicity. These findings underscore the potential of split-receptor CAR T cells in autoimmune and transplant medicine, highlighting their versatility in applications beyond oncology.

1259 Next-Generation Off-the-Shelf CAR T-Cell Therapies for Conditioning-Free Treatment of a Broad Spectrum of Autoimmune Diseases and Hematologic Malignancies

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Chimeric antigen receptor (CAR) T-cell therapies have achieved durable remissions in the treatment of hematologic malignancies and continue to present promising clinical outcomes in autoimmune disease (AID) indications, driving the demand to increase patient accessibility and expand applicability to other disease settings. However, approved CAR T-cell therapies face limitations, such as inadequate targeting of disease-causing cells, inconsistencies associated with manufacturing of heterogeneously edited cells, and the need for conditioning chemotherapy (CCT), which mandates hospitalization and treatment at specialized centers to monitor adverse events including the risk of severe infection.

To maximize patient access and expand CAR T-cell therapy to multiple disease indications, we developed a platform that allows for the creation of clonal, multiplexed-engineered induced pluripotent stem cells and their differentiation into next-generation multi-antigen targeting (NxM) CAR T-cell therapies. Off-the-shelf NxM CAR T cells incorporate novel synthetic functional elements designed to effectively eliminate a range of aberrant cells while evading recognition by alloreactive immune cells.

NxM CAR T cells exhibit a CD8αβ T-cell phenotype, lack TCR expression to avoid graft versus host disease, lack CD38 expression for improved fitness and avoidance of fratricide, and express two unique CARs to target pathogenic CD19+ cells and a second antigen to target a broad range of disease-causing cells. NxM CAR T cells targeting cell surface glycoprotein CD38 (NxM 19x38 CAR T cells) specifically eliminated a variety of malignant cell types, including CD19+ lymphoma and CD38+ multiple myeloma cell lines, in several *in vitro* cytotoxicity assays and *in vivo* models. Notably, NxM 19x38 CAR T cells showed enhanced elimination of CD19+CD38+ cells *in vivo* compared to single CAR controls ($p<0.05$) as well as robust eradication of aberrant CD19+ B cells, CD38+ plasma cells, and CD38+ activated T cells from AID patient-derived peripheral blood mononuclear cells (PBMCs). Additional expression of a high-affinity, non-cleavable CD16 supports NxM 19x38 CAR T cell combination with monoclonal antibodies to further enhance antigen targeting and versatility.

To eliminate the need for administration of CCT alongside CAR T-cell therapy, NxM 19x38 CAR T cells also express a novel allo-immune defense receptor (ADR), which promotes CAR T-cell expansion, function, and persistence in an allogeneic setting, and harbor genetic disruption of the immune synapse adhesion ligand CD58 ($CD58^{KO}$) for passive evasion of host immunity. In an *in vitro* allogeneic re-stimulation assay, ADR/$CD58^{KO}$ NxM CAR T cells eliminated target cells upon serial re-challenge in the presence of alloreactive PBMCs, displaying enhanced persistence and cytotoxicity compared to control. Additionally, ADR/$CD58^{KO}$ NxM CAR T cells maintained tumor growth inhibition and persistence *in vivo* in the presence of unmatched alloreactive T cells.

With the potential to specifically target several disease-causing cell types without requiring administration of CCT, NxM 19x38 CAR T cells can be manufactured at scale in a cost-effective manner, administered off-the-shelf to broadly reach patients, and used to target multiple pathogenic cell types for the treatment of autoimmune diseases and hematologic malignancies.

1260 Allogeneic CD19-targeted CAR-T Therapy for refractory Systemic Lupus Erythematosus (SLE)

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BACKGROUND: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease associated with significant morbidity and mortality. It can affect various tissues and organs throughout the body, leading to a broad spectrum of clinical symptoms. The treatment approach for SLE primarily involves immunomodulation and immunosuppression. However, these drugs have considerable toxicity and are not effective in all patients. Conceptually, a deep depletion of $CD19^+$ B cells and plasmablasts could trigger an immune reset in autoimmune diseases. Autologous CD19-targeted CAR-T therapies has been explored in several autoimmune diseases and reported promising efficacy in recent studies. In this study, a healthy donor-derived, multiplex genome-edited allogeneic CD19-targeted CAR-T product (BRL-303) was developed for refractory SLE patients. Here we report effciacy and safety profiles of BRL-303 after treating 3 patients with systemic lupus erythematosus (SLE).

METHODS: This is an investigator-initiated trial evaluated the safety and efficacy of BRL-303 in adult patients with refractory SLE (NCT05988216). Participants were screened and underwent lymphodepletion chemotherapy with cyclophosphamide (300 mg/m², day -5 to -4) and fludarabine (25 mg/m², day -5 to -3), then BRL-303 infusion at day 0 with a dose level of 1×10^6 cells/kg. After BRL-303 infusion, each patient underwent safety and systematic follow-up assessments according to the protocol.

RESULTS: As of June, 13, 2024, 3 patients with refractory systemic lupus erythematosus have been treated. These patients were unable to control disease progression despite treatment with corticosteroids and various immunosuppressants. After BRL-303 infusion, CAR-T cells expanded in vivo in all subjects significantly within 7 days and reached a peak around 14-21 days. Notably, Patient SLE-03 exhibited the most robust CAR-T cell expansion, with peak CAR copy numbers reaching 13,680/μL at day 14 post-infusion. CAR-T cells remained detectable in all patients at 3 months.With expansion of BRL-303, B cells in peripheral blood of patients reached deep and persistent depletion about 1-3 months. After BRL-303 infusion, all patients met the Systemic Lupus International Collaborating Clinics (SLICC) criteria for a sustained response (SRI-4). Moreover, all subjects showed a significant decrease in serum ds-DNA and increase in complements (C3 and C4). Meanwhile, 24-hour proteinuria significantly decreased in 2 subjects with lupus nephritis. Importantly, SLE-01 discontinued all immunomodulatory and immunosuppressive medications, including glucocorticoids, at 3 months, achieving drug-free remission, while other patients require low-dose glucocorticoid maintenance therapy (≤ 5 mg). The most commonly observed grade 3 or 4 adverse events (AEs) within the first few weeks included neutropenia, lymphopenia and fatigue, which were considered relatively to the lymphodepletion conditioning regimen. All of them developed grade 1 CRS for 2 or 3 days, which recovered soon without tocilizumab and steroid usage. No serious infection (Grade 3/4), no ICANS and GvHD observed in all subjects.

CONCLUSIONS: Overall, SLE subjects treated with BRL-303 achieved significant clinical remission, with a controllable safety profile, offering a potential paradigm shift for SLE patients who are refractory to currently available treatments. Further observation of SLE and more autoimmune disorders treated with BRL-303 are ongoing in our studies.

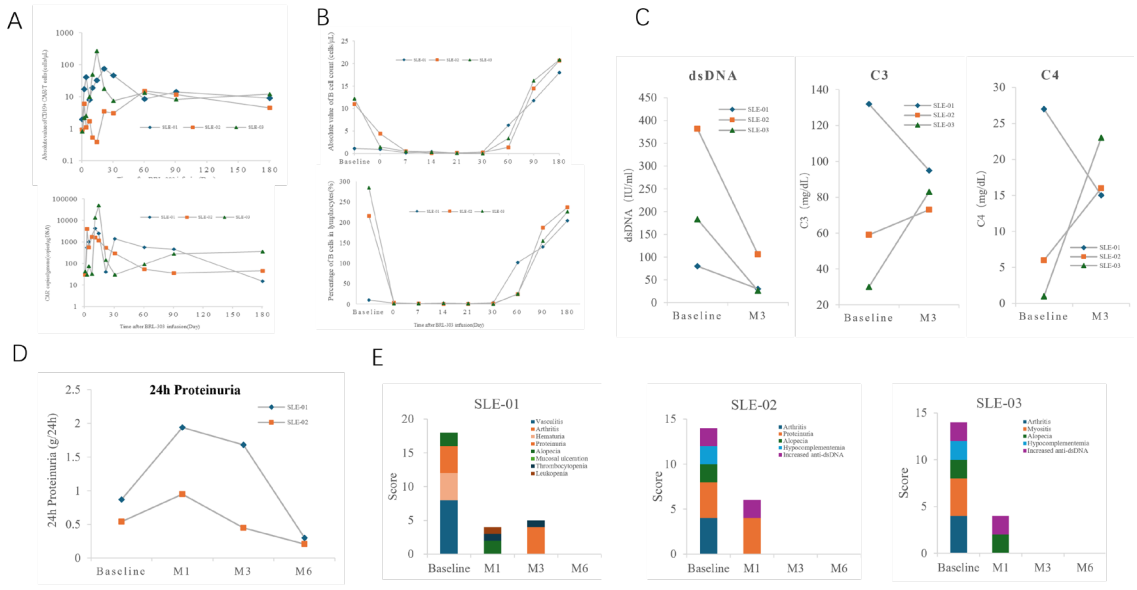

Figure 1 Effects of BRL-303 for SLE patients befor and after BRL-303 treatment (A, BRL-303 detected by flow and qPCR. B, B cell percentage and absolute B cells value. C, ds-DNA, C3 and C4 change. D, 24-hour proteinuira changes. E, SELENA-SLEDAI Score changs);

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Repurposing oncology drugs to treat autoimmune disease

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Introduction: Approved drugs have successfully passed costly toxicological studies and their pharmacokinetics and pharmacodynamic properties are well characterized. Drug repurposing can provide new treatment options for patients with unmet medical needs. Rituximab is the first monoclonal antibody approved for treating lymphoma, becoming one of the most popular and versatile drugs in clinical application and revenue. Rituximab is widely used to treat autoimmune diseases mediated by B cells. In 2014, Blincyto was approved by the FDA for treating acute lymphoma, opening the door to developing T cell engagers (TCE). In addition to treating cancer, new indications for TCE are unlocking, especially autoimmune and inflammatory diseases. SLE is an autoimmune disease caused by the loss of B-cell tolerance leading to auto-antibody production and multi-system damage. Gempharmatech developed two humanized SLE models by transferring peripheral blood mononuclear cells (PBMC) from SLE patients to immunodeficient mice or transferring human hematopoietic stem cells (HSC) to immunodeficient mice followed by TLR7/8 agonist treatment to induced lupus. Humanized SLE models can partially reconstruct the human immune system and mimic the human in vivo microenvironment. It will build a bridge for repurposing oncology drugs to treat autoimmune diseases.

Objective: Develop humanized autoimmune mouse model for oncology drug repurposing

Methodology: PBMC-induced SLE model was constructed by intraperitoneal injection of SLE patient-derived PBMC. Blincyto was administered intravenously 7 days after PBMC inoculation, twice a week for three consecutive weeks. The peripheral blood was collected 14 and 28 days after PBMC inoculation for flow cytometry analysis of plasma B cells. The plasma was separated simultaneously for ELISA test of human anti-dsDNA and total IgG. The kidney was collected and freshly embedded for immunofluorescence staining of human IgG. TLR 7/8 agonist Resiquimod was applied on the ear of immunodeficient mice after 14 weeks of HSC reconstitution, 3 times a week, with a modeling period of 6 weeks. Rituximab was administered intravenously 4 weeks after Resiqiumod induction, 3 times a week for 3 consecutive weeks. The peripheral blood will be collected 10 and 26 days after

Rituximab treatment for flow cytometry analysis of human CD19+ B cells. The plasma was collected 3, 10, 17, and 26 days after the dosing for ELISA test of human anti-dsDNA.

Results: In the SLE patient-derived PBMC-induced model, after 1 week and 3 weeks of Blincyto administration, the level of plasma anti-dsDNA and IgG levels, as well as the number of peripheral plasma B cells, were significantly reduced. IgG deposition in the kidneys was also considerably reduced 3 weeks after the drug dosing. In the TLR 7/8 agonist-induced SLE model based on HSC reconstituted mice, Rituximab treatment could significantly increase mice survival and inhibit the production of human anti-dsDNA. Human CD19+ B cells were effectively depleted after Rituximab treatment.

Conclusion: Developing new drugs requires a significant investment of time and economic costs. The research on drug repurposing has significant advantages over developing new drugs. Humanized SLE models exhibit similar pathological features seen in SLE patients such as high levels of auto-antibodies and pathogenic B cells. It will be an excellent tool for research on the repurposing of B cell-directed oncology drugs.

1262 RESET-SLE^{TM}: Clinical Trial Evaluating Rese-cel (Resecabtagene Autoleucel), A Fully Human, Autologous 4-1BB Anti-CD19 CAR T Cell Therapy in Non-Renal SLE and Lupus Nephritis: Correlative Findings

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Background: Current therapies for systemic lupus erythematosus (SLE) aim to control disease activity, reduce organ damage, and decrease long-term morbidity and mortality. Achieving durable clinical responses without chronic immunosuppressive drugs is desired. CD19-targeting chimeric antigen receptor (CAR) T cells have demonstrated durable, drug-free responses in SLE patients in an academic program. Rese-cel (formerly referred to as CABA-201) is a fully human, autologous 4-1BB anti-CD19-CAR T cell therapy, designed to deeply and transiently deplete CD19 positive B cells following a one-time weight-based infusion of $1x10^6$ CAR T cells/kg. Rese-cel may enable durable responses without chronic immunosuppression. RESET-SLE^{TM} (NCT06121297) is an ongoing Phase 1/2 trial evaluating the safety and efficacy of rese-cel in two cohorts of non-renal SLE and lupus nephritis (LN).

Results: Six patients, 4 in the non-renal SLE cohort (SLE-1, SLE-2, SLE-3, SLE-4) and 2 in the LN cohort (LN-1, LN-2), have been dosed with rese-cel with at least 1 month of follow-up. Rese-cel has been well-tolerated with Grade 1 cytokine release syndrome (CRS) reported in 2 patients, one from each cohort (SLE-2 and LN-1). As reported previously, the LN-1 patient experienced Grade 4 immune effector cell-associated neurotoxicity syndrome (ICANS), linked to a potential occult infection, which resolved rapidly following standard management. No other patient experienced ICANS. Infusion products were predominantly CD4+ CAR T cells and showed *in vitro* cytolytic activity towards a NALM6 target cell line. Leukocyte and lymphocyte counts reduced quickly and transiently after a standard preconditioning regimen of fludarabine ($25mg/m^2$/day x 3) and cyclophosphamide ($1000mg/m^2$) that inversely corresponded with a transient elevation in serum IL-15 levels. Following rese-cel infusion, peak CAR T cell concentration occurred between days 7 and 16 post-infusion (C_{max}), with the LN-1 patient also experiencing a second peak at day 29, followed by contraction. Serum IFNγ levels peak before or in parallel with CAR T C_{max}. In the CAR T population at $C_{max,}$ CD8/CD4 ratios increased in 4 out of 6 subjects (CAR T cells were too low to be

detected by flow cytometry in 2 subjects). Circulating B cells were rapidly reduced within the first 29 days post-infusion. Early-stage transitional B cells (CD19+CD20+CD38++CD24++) were evident in 4 out of 6 patients (SLE-1, SLE-3, SLE-4, LN-2) at 8 weeks post-infusion and at 12 weeks for SLE-2. B cells in LN-1 were detectable but too low to phenotype at 20 and 24 weeks post-infusion. Serum BAFF levels increased during periods of B cell depletion and stabilized in most patients over time. Vaccine related antibodies were stable in most patients, whereas serum levels of lupus-specific and related autoantibodies decreased in 5/6 subjects, as of the most recent follow-up. Clinical improvement has been observed in all 6 patients (follow-up range 1 month to 9 months), with all patients exhibiting substantial reductions in Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores which have deepened with increasing follow-up.

Conclusions: These emerging data detail the pharmacokinetics and pharmacodynamics of rese-cel in patients with SLE and LN and support the potential for rese-cel to lead to durable, drug-free remissions. Evaluation of safety and efficacy of rese-cel continues in the ongoing Phase 1/2 RESET-SLE™ trial.

1263 RESET-SSc™: Clinical Trial Evaluating Rese-cel (Resecabtagene Autoleucel), A Fully Human, Autologous 4-1BB Anti-CD19 CAR T Cell Therapy in Systemic Sclerosis: Correlative Findings

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Background: Treatments for systemic sclerosis (SSc) aim to control disease activity, limit progression of organ damage, and decrease morbidity and mortality but many fail to provide durable clinical responses without requiring chronic immunosuppression. CD19-targeting chimeric antigen receptor (CAR) T cells produced durable, drug-free responses in SSc patients in an academic program. Rese-cel (formerly referred to as CABA-201) is a fully human, autologous 4-1BB anti-CD19-CAR T cell therapy, designed to deeply and transiently deplete CD19 positive B cells following a one-time weight-based infusion of $1x10^6$ CAR T cells/kg. Rese-cel may enable durable responses in patients without the need for chronic immunosuppression. RESET-SSc™ (NCT06328777) is an ongoing Phase 1/2 trial evaluating rese-cel in two cohorts of adults with SSc and either severe skin or organ involvement.

Results: A total of 3 patients have been enrolled and one patient dosed in the RESET-SSc™ trial. The dosed patient had severe skin involvement (SSc-Skin-1) at baseline prior to rese-cel. The patient has completed 3 months of follow-up. The patient tolerated rese-cel with no dose-limiting toxicity or immune effector cell-associated neurotoxicity syndrome (ICANS). The patient experienced grade 2 cytokine release syndrome (CRS), manifesting as fever and hypotension which resolved following intravenous fluids without requiring tocilizumab. Infusion product from the SSc-Skin-1 consisted of predominantly CD4+ CAR T cells and exhibited in vitro cytolytic activity towards a NALM6 target cell line. Leukocyte and lymphocyte counts dropped quickly and transiently after a standard preconditioning regimen of fludarabine (25 mg/m^2/day x 3) and cyclophosphamide (1000 mg/m^2) which inversely corresponded with an elevation in serum IL-15 levels. Following infusion of rese-cel, the patient exhibited CAR T cell expansion that peaked 2 weeks post-infusion (C_{max}) and was undetectable by day 29. CAR T cells exhibited a mainly CD8+ phenotype at C_{max}. Serum IFN-γ levels peaked prior to rese-cel C_{max} and IL-6 peaked in parallel with C_{max}. Peripheral B cells were rapidly reduced following infusion and were undetectable by 2 weeks post-infusion. The patient also exhibited depletion of B cells in lymphoid tissue obtained from ultrasound guided inguinal lymph node biopsies and immunologic staining performed at the University of Michigan in a separate biorepository study designed to support exploratory analyses. Lymph node sections consisted of a mixture of T cells ($CD3^+$) and B cells ($CD19^+$ and $CD20^+$) at baseline. At 21-days post-infusion, lymph node sections contained T cells and were fully devoid of B cells. Peripheral B cell reconstitution was observed at 8 weeks post-infusion with reemergent B cells

presenting with a transitional naïve phenotype ($CD19^+CD20^+CD38^{++}CD24^{++}$). Antibodies associated with vaccinations remained stable, whereas serum levels of SSc-associated autoantibodies decreased over the first 16-weeks. The patient has maintained a drug-free clinical response through 3 months of follow-up.

Conclusions: These emerging data detail the pharmacokinetics and pharmacodynamics of rese-cel in one SSc patient. Rese-cel deeply and transiently depleted B cells in circulation and in lymphoid tissue and support the potential for rese-cel to induce durable, drug-free remission. Evaluation of the safety and efficacy of rese-cel continues in the ongoing Phase 1/2 RESET-SSc™ trial.

1264 Modeling Innate Immune Cell Loss-of-Function in Nonhuman Primates with Anti-*CD16 and Anti-NKG2A CAR T Cells*

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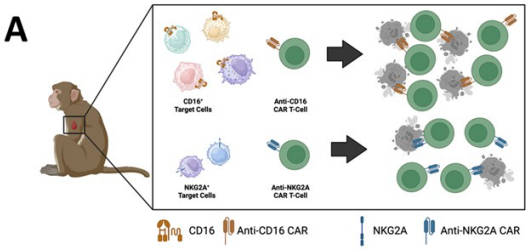
Introduction. Innate immunity is the first line of defense against foreign pathogens. To better understand the interplay between innate and adaptive immune responses, we have applied our nonhuman primate (NHP) model of chimeric antigen receptor (CAR) T-cell gene therapy to deplete broad (CD16) or narrow subsets of innate immune cells (NKG2A). CD16/FcγRIII is a member of the immunoglobulin superfamily, expressed on numerous innate immune subsets including monocytes, macrophages, granulocytes, and natural killer (NK) cells. NKG2A is a member of the C-type lectin-like receptor family. Although primarily known as an inhibitory receptor on NK cells, it also supports the survival of $CD8^+$ T-cells. Our long-term goal is to apply this model to better understand innate immune mechanisms in the pathogenesis of infectious diseases such as HIV and autoimmune diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE).

Methods. We screened an array of NHP-adapted CAR molecules in *ex vivo* primary cell-based assays, then evaluated the most promising anti-CD16 and anti-NKG2A CARs in 6 rhesus macaques. Our pilot study included one animal that received anti-CD16 CAR T-cells, three that received NKG2A targeting CARs, and two controls that received low-dose conditioning but no CAR T-cells (**Figure 1**). In addition to peripheral blood, tissue collections were performed 2 weeks after each infusion to quantify CAR T-cells and target depletion in solid organs. Necropsy was performed 36-65 days after infusion. Total $CD16^+$ cells and NK cells ($CD3^-CD20^-CD8a^+NKG2A^+CD8b^-$) were quantified by flow cytometry and $\%CAR^+$ T cell in PBMCs were quantified by qPCR.

Results. Anti-CD16 CAR T-cells showed limited potency in peripheral blood with a peak of 13% CAR in PBMC at Day 11 with a decrease in %CD16 cells by flow to 0.5% on Day 12, however, the target cells rebounded quickly by Day 17 (**Figure 2A**). Anti-NKG2A CAR T-cells were administered in two doses: the first infusion had limited efficacy whereas the second infusion led to significant and more sustained NK depletion (**Figure 2B, 2C, 2D**). We detected anti-CD16 and anti-NKG2A CAR^+ cells in spleen and PBMCs and at low levels in axillary, mesenteric, and peripheral lymph nodes. All 3 animals displayed decreases in NK cells in the blood around the time of the second transplant that dropped to below 1% total NK. Low levels of NK cells were sustained in animals 32258 and 31530 until necropsy.

Conclusions. Our first NHP anti-CD16 and anti-NKG2A CAR T-cells experiments showed target depletion in blood, with anti-NKG2A CAR activity augmented by a repeat infusion. Modest *in vivo* activity relative to our previously published NHP CD20 CAR T-cell experiments may reflect key roles of $CD16^+$ and $NKG2A^+$ subsets for CAR cytotoxicity. Future work will focus on higher CAR T-cell infusion doses, which may be necessary for potent and sustained ablation. To account for overlap in marker profiles for NK cells, T cells and other innate immune subsets, Boolean-gated CARs could be designed for example to target NKG2A (NK cells) but not CD3 (T-cells). In our NHP model of HIV pathogenesis and cure, optimized anti-CD16 and anti-NKG2A CAR T-cells will be applied to unravel positive and negative impacts of innate immunity on the virus-specific inflammatory response and viral replication both at early and late infection time points.



B

Animal ID	Infusion #	Infusion Dose (cells)	% CAR by EGFRt Flow	CAR+ cells/kg
35898 (anti-CD16 CAR)	Primary	1.2e8	31%	5e6
31530 (anti-NKG2A CAR)	Primary	1.0e8	29%	4e6
	Secondary	2.4e8	29%	1e7
32258 (anti-NKG2A CAR)	Primary	2.4e8	28%	1e7
	Secondary	4.0e8	28%	2e7
35905 (anti-NKG2A CAR)	Primary	1.5e8	41%	1e7
	Secondary	3.0e8	41%	2.1e7

Figure 1. A) Graphical Abstract demonstrating two different CAR T cells targeting CD16 (top) or NKG2A (bottom) to kill innate immune effector cells in a rhesus macaque model. **B)** CAR T cells infusion dose for each of the four animals on study.

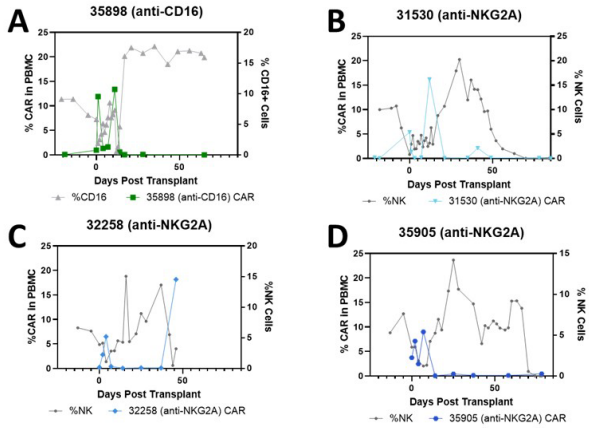


Figure 2. A) %CAR in PBMC by qPCR overlaid with **A)** %CD16+ cells for animal 35898 or % NK+ cells for animal **B)** 31530 **C)** 32258 and **D)** 35905

1265 A model to generate tolerogenic DCs derived from human iPSCs with CD86 biallelic deletion

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Background: Dendritic cells (DCs) are professional antigen-presenting cells involved in the initiation of both immune defense and immune tolerance. Any dysfunction in the frequency or function of the two DC types can lead to onset and progression of autoimmune disorders (AD), infectious diseases, and cancer, among others. Due to their role in maintaining immune tolerance to harmless antigens, tolerogenic DCs (Tol-DCs) hold significant potential for treating various ADs, making their study of utmost importance. While obtaining primary DCs from blood is feasible, their low frequency limits their use in therapeutic vaccine and basic studies. Induced pluripotent stem cells (iPSCs) offer an attractive alternative for differentiating DCs with high efficiency. Traditional methods to generate Tol-DCs from iPSCs risk shifting the Tol-DC phenotype from tolerogenic to stimulatory upon encountering self-antigens,

pro-inflammatory cytokines, or microbial agents which acts as danger signals. The use of CRISPR/Cas9-engineered iPSCs offers an alternative method to maintain the immunotolerant phenotype of Tol-DCs by genetically modifying the cells to preserve this characteristic. Here, we describe the technical flow to generate either stimulatory DCs from human wild type (WT) iPSCs or TOL-DCs from CD86 knockout (KO) iPSCs.

Methods: In this study, we used a fibroblast-derived human iPSC (hiPSC) line (KOLF2-C1) referred to as WT and CD86 KO-derived clones (CD86-KO) generated by partially deleting exon 3 in the CD86 coding region in the WT using the CRISPR/Cas9 system. We identified and selected suitable KO clones by PCR and Sanger sequencing and confirmed the absence of CD86 protein in iPSC-derived DCs through flow cytometry, as shown in Figure 1. We established the culture conditions for obtaining conventional DCs (cDCs) from human WT or CD86-KO iPSCs and evaluated their ability to induce proliferation and differentiation of human naïve $CD4^+$ T cells.

Results: After approximately 35-40 days of differentiation, iPSC-derived DCs exhibited a switch from CD34+ to $CD45^+$, displayed typical DC morphology, and expressed markers such as HLA-DR, CD11C, and CD1C. The partial deletion of exon 3 of CD86 efficiently led to the disruption of this protein, as shown in Figure 1. Functional analysis of cDC2 (a DC subset) in co-culture with naïve $CD4^+$ T cells confirmed their differential efficiency. CD86-KO iPSC-DCs showed dramatically diminished effects on $CD4^+$ T cell differentiation, with reduced cytokine production, particularly interferon gamma, behaving as expected like Tol-DCs (Figure 2).

Conclusion: Our study established the method to generate Tol-cDCs from iPSCs, with potential clinical applications and a valuable tool for studies focused on AD diseases.

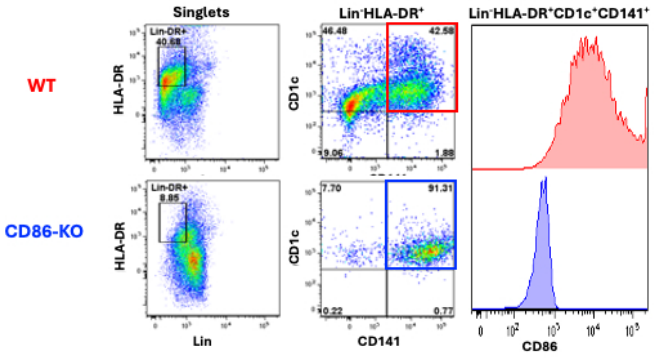

Figure 1: Validation of cDC2 iPSC-derived CD86-KO vs. WT (Day 36 of differentiation). Flow cytometry analysis confirms that CD86-KO cDC2 cells do not express the CD86 protein, as expected, while WT cDC2 cells show normal CD86 expression.

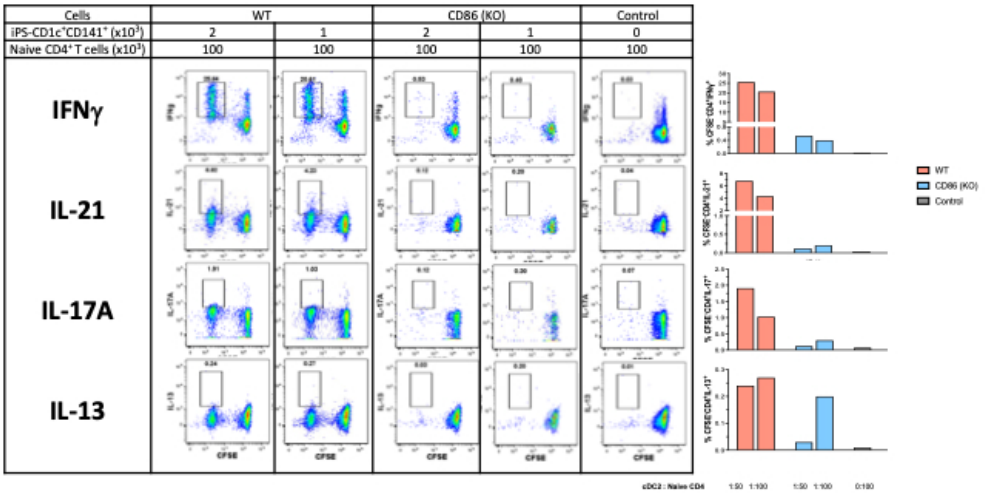

Figure 2.Capacity of cDC2 derived from WT iPSC to induce proliferation and differentiation of naïve CD4$^+$ T cells is abrogated in cDC2 derived from CD86-KO iPSCs.

1266 Neural Induction of Gene-Edited mRNA-Reprogrammed iPSCs Yields Highly Proliferative Neural Stem and Progenitor Cells That Display Persistent Transgene Expression

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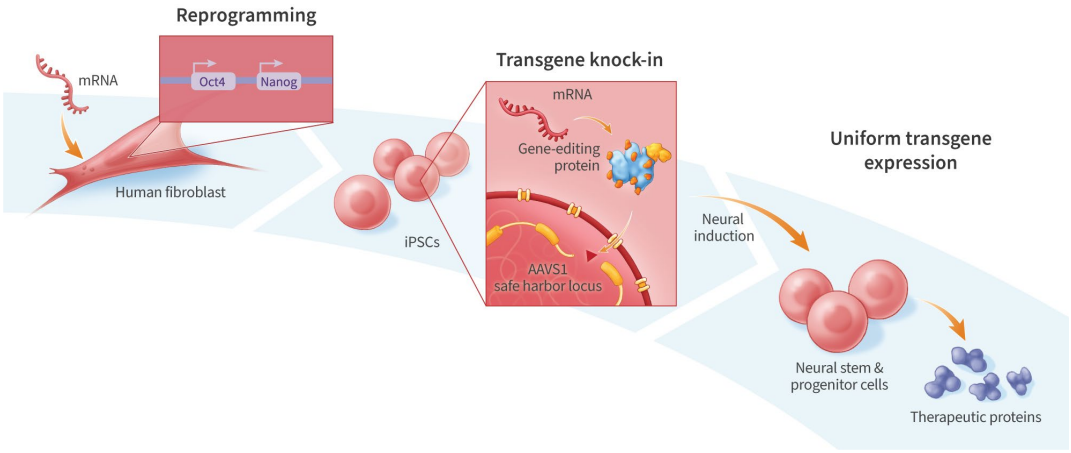
Background. Engineered neural stem and progenitor cells (NSPCs) can provide neuroprotection and modulate inflammation and thus are a promising therapeutic modality for neurodegenerative diseases. However, recent clinical trials for CNS disorders employ virally-transduced cortical-derived NSPCs, introducing risks of insertional mutagenesis and restricted by scarce source material. Here, we detail a virus-free process for scalable neural induction of wildtype or engineered induced pluripotent stem cells (iPSCs) that maintain transgene expression through differentiation.

Methods. mRNA-reprogrammed iPSCs were co-electroporated with single-stranded DNA donors and mRNA encoding gene-editing endonucleases for targeted insertion into the AAVS1 safe harbor locus. Clonal lines were subsequently established via colony selection and Sanger sequencing. iPSCs were then seeded into a 0.1L vertical wheel bioreactor and transitioned to neural induction medium to facilitate embryoid body (EB) formation. EBs were transferred to flasks and developed rosettes, which were selected, replated, and expanded to generate NSPCs. In parallel, a fully static differentiation was performed using microwell plates. Microwell-formed EBs were transferred to flasks and the remainder of induction was carried out identically to the bioreactor differentiation.

Results. Both static- and bioreactor-derived iNSPCs expressed characteristic NSPC markers Sox1, Sox2, Pax6, GAP-43, and Nestin, and did not express pluripotency markers Oct4 and Nanog. After induction, iNSPCs were expanded and harvested every 5-9 days to yield, on average, 3.2 x 10^6 cells/cm^2/harvest (static induction) and 3.8 x 10^6 cells/cm^2/harvest (bioreactor induction). iNSPCs retained characteristic marker expression and morphology for >4 passages and after cryopreservation. We also demonstrated that bioreactor-derived iNSPCs could be engineered to overexpress neurotrophic factors and cytokines, including interleukin-10 (IL-10), an anti-inflammatory protein with therapeutic applications to neurodegenerative disorders. Electroporation of 3.0 x 10^5 iNSPCs with mRNA encoding IL-10 resulted in >3ng/mL of secreted protein, compared to undetectable levels in untransfected iNSPCs. In addition to comparing neural induction of wildtype cells, we evaluated differentiation potential of iPSCs knocked-in with a GFP reporter. Induction efficiency, calculated based on rosette quantity, was 95% in the wildtype cells and 100% in the knock-in cells. Both displayed upregulation of characteristic NSPC markers and

downregulation of pluripotency markers. Notably, transgene expression was retained through differentiation, with >99% of iNSPCs expressing GFP at the end of neural induction.

Conclusions. We demonstrate that neural induction of mRNA-reprogrammed iPSCs can be executed in small bioreactors – with potential for scalability to larger vessels – to generate clinically relevant yields of iNSPCs. Additionally, we show that iNSPCs can be directly engineered to express therapeutic proteins and can be derived from transgenic iPSCs. These results suggest that scalable neural induction of iPSCs engineered to secrete therapeutic proteins could yield iNSPCs with uniform and stable transgene expression in an entirely non-viral process, which could contribute to the development of engineered cell therapies for neurodegenerative diseases.



1267 A Platform for Designing Neuron, Astrocyte and Hepatocyte Specific Promoters for Gene Therapy

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In recent years, gene therapy approaches relying on targeted expression of transgene(s) in specific cell types, organs, or systems have become more prevalent and important in the treatment of various diseases. However, limiting the expression of therapeutic transgenes to the intended ON-target cell types remains a challenge. A contributing factor may be promoter leakiness which leads to undesirable transgene expression in OFF-target cell types. Risks associated with OFF-target cell types include immunogenicity and/or inflammatory responses.

In this work we developed highly specific, potent, and compact synthetic promoters for three clinically relevant cell types -- neurons, astrocytes and hepatocytes -- to address the above challenges. Specifically, neuron specific promoters were designed to be inactive in astrocytes and hepatocytes; astrocyte specific promoters were designed to be inactive in neurons and hepatocytes; and hepatocyte specific promoters were designed to be inactive in Kupffer cells and cardiomyocytes.

In all cases, we utilized two design approaches in parallel: (1) pooled screening of short, fully synthetic promoters, and (2) clonal screening of natural sequences from endogenous promoters and putative enhancers. For the first approach, we designed libraries of arrays of binding sites for transcription factors or short fragments of natural sequences that are differentially regulated in the desired ON / OFF target cell combinations which were then screened in a Massively Parallel Reporter Assay (MPRA). For the second approach, differentially regulated

promoters and enhancers in each of the ON / OFF target cells combinations were cloned and screened individually by transient transfection. Promoters and enhancers displaying the desired expression pattern were functionally dissected by targeted mutagenesis to identify core regulatory elements. Both approaches rely heavily on bioinformatics mining of publicly available as well as de novo generated transcriptomics and chromatin accessibility data. Screening was done using primary cells or surrogate cell lines representative of each ON and OFF target cell type. We developed a powerful computational platform for mining and analyzing the experimental results and built machine learning models aiding the generation of new improved promoter designs. Strong hits from the two approaches were then combined in designs that further improved their strength and specificity. The outlined strategy yielded a wide spectrum of highly compact (less than 1kb) promoters for the three ON target cell types. Our neuron specific promoter designs outperform the widely used hSyn1 promoter by several fold both in terms of strength and specificity. Likewise, we designed several compact astrocyte-specific promoters with superior strength and specificity (several fold in both cases) compared to the GFAP promoter. We were also able to design hepatocyte specific promoters vastly outperforming a well-known hepatocyte-specific promoter with many of them being extremely compact (less than 200bp).

These results validate our strategy and workflow for designing highly compact, specific, and potent clinically relevant synthetic promoters, which can be applied across various cell types and diseases of interest.

1268 Investigation of the Long-Term Effects and Post-Disease Onset Impact of Wild-Type Hematopoietic Stem Cell Transplantation in a Mouse Model of Alzheimer's Disease

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Alzheimer's Disease (AD) is the most common form of dementia and poses a significant global health burden, yet there is currently no effective treatment for AD. We previously showed compete rescue of memory and neurocognitive impairments, microgliosis, neuroinflammation, and decrease of amyloid plaque burden in the 5xFAD model of familial AD after a single systemic infusion of wild-type (WT) hematopoietic stem and progenitor cells (HSPCs) performed at 2-months of age and analyzed 4-months post-transplantation. When evaluating HSPC-derived therapy for Alzheimer's disease, two key questions emerge: the long-term impact of the treatment and whether HSPC therapy could be effective when administered after disease onset, given that most patients are diagnosed at this stage. To assess the long-term effects of WT HSPC transplantation, 2-month old myeloablated 5xFAD mice were transplanted with $Sca1^+$ HSPCs from either WT (AD/WT_{HSPC}) or 5xFAD (AD/AD_{HSPC}) mice. At 8-months post-transplantation, preliminary data showed that long-term AD/WT_{HSPC} mice exhibited significant improvement in the elevated plus maze (EPM), open field (OF), and novel object recognition (NOR) tests compared to untreated 5xFAD mice, while no recovery was observed in long-term AD/AD_{HSPC} mice. Furthermore, immunostaining of the cortex revealed a reduction in amyloid plaque size and number of $IBA1^+$ microglia in long-term AD/WT_{HSPC} mice compared to their untreated littermates, indicating a sustained decrease in plaque burden and neuroinflammation after a single systemic WT HSPC transplantation. Since amyloid plaque accumulation is detected in 5xFAD brains by 4-months and significant neurocognitive deficits observed by 6-months of age, we are further investigating the effects of WT HSPC transplantation after disease onset in 6-month-old mice. Compared to their untreated littermates and after-onset AD/AD_{HSPC} mice, after-onset AD/WT_{HSPC} mice showed significant improvements in the EPM, OF, and NOR tests at 4-months post-transplantation. Sholl analysis of $IBA1^+$ microglia in the hippocampus of AD/WT_{HSPC} mice revealed increased dendritic complexity compared to untreated 5xFAD mice, indicating a shift towards a less activated microglial state. Neuroinflammation and plaque burden status are being currently analyzed. Taken together, this rescue in cognitive performances and neuropathology of 5xFAD mice transplanted with WT HSPCs both long-term and after disease onset demonstrated the potential of WT HSPCs in treating AD despite the severe accumulation of amyloid aggregates.

1269 Hematopoietic Stem Cell Transplantation with Enhanced Brain Conditioning Corrects Progranulin Deficiency in mouse models

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Hematopoietic stem cell transplantation (HSCT) is a therapy for neuronopathic diseases, offering benefits through allogeneic and autologous approaches. Allogeneic HSCT (allo-HSCT) uses donor-derived cells and has been the standard treatment for severe neuronopathic diseases. However, its application is limited by donor scarcity and immunological complications like graft-versus-host disease. Autologous HSCT (auto-HSCT) gene therapy is an emerging approach that genetically modifies a patient's own hematopoietic stem and progenitor cells (HSPCs) *ex vivo*, avoiding these issues and enabling disease-specific cell engineering, such as enhanced therapeutic protein expression.

Our work focuses on progranulin (GRN) deficiency, a lysosomal protein defect causing neurological diseases, including neuronal ceroid lipofuscinosis type 11 (CLN11; bi-allelic loss-of-function) and frontotemporal dementia (FTD-GRN; mono-allelic loss-of-function). These conditions are suited to HSCT as wild-type or gene-modified cells can secrete GRN and cross-correct GRN-deficient cells through receptor-mediated endocytosis and lysosomal trafficking. We are investigating allo- and auto-HSCT in preclinical models. We previously showed that wild-type bone marrow transplantation (BMT) in *Grn -/-* mice treated with enhanced conditioning improves microglia replacement, achieves biochemical correction in the brain and eye, and normalizes brain disease biomarkers compared to standard myeloablative conditioning (Colella et al., Nat Commun. 2024, PMID: 38969669).
This enhanced conditioning combines busulfan with PLX3397, a CNS-penetrant inhibitor of the Colony-stimulating factor 1 receptor (CSF1R). Building on this, we tested allo-HSCT using purified mouse HSPCs expanded in culture. Transplantation of these HSPCs into immunocompetent *Grn-/-* mice resulted in rapid microglia replacement (91 ± 2.6% of brain CD45+ cells), GRN protein reconstitution in circulation and brain (40% of wild-type levels), and correction of brain biomarkers.

For auto-HSCT, we engineered mouse and human HSPCs using genome editing to integrate GRN transgenes at safe harbor loci via homology-directed repair (HDR). Optimized editing achieved 70% and 90% edited alleles in mouse and human HSPCs, respectively, with non-viral promoter-driving GRN expression. Transplantation of GRN-edited mouse HSPCs into immunocompetent *Grn-/-* mice showed full hematopoietic reconstitution, high retention of edited alleles (100 ± 17% vs. cultured HSPCs, n=18), and GRN protein restoration in the periphery and CNS. The therapeutic effects of auto-HSCT on disease biomarkers are being evaluated. Initial xenotransplantation of human GRN-edited HSPCs into immunodeficient *Grn-/-* mice demonstrated long-term engraftment and multilineage differentiation. We are currently assessing the long-term stemness and engraftment of human HSPCs using optimized genome editing protocols. Our work highlights the potential of allo- and auto-HSCT coupled with enhanced microglia replacement to tackle diseases caused by progranulin deficiency. These findings lay the foundation for advancing these approaches toward clinical application.

1270 Lentivirus-based Hematopoietic Stem/Progenitor Cell Therapy Provides Safe and Long-term Treatment in Hypophosphatasia Mouse Model

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Hypophosphatasia (HPP) is a multi-system metabolic disorder characterized by skeletal hypomineralization, rickets, muscle weakness, and premature tooth loss in milder forms and perinatal lethality in severe cases. It is caused by loss-of-function mutations in the gene *ALPL* encoding tissue-nonspecific alkaline phosphatase (TNALP). Currently, enzyme replacement therapy with asfotase alfa (STRENSIQ®) is the only FDA-approved treatment for HPP, though it is only approved for individuals with severe forms of the condition. Asfotase alfa enhances survival and improves bone mineralization but is burdensome because patients must undergo frequent injections for the rest of their lives. Here we report the development of a lentiviral vector (LVV), RMP100-LVV, to stably express a secreted form of TNALP from LVV-transduced hematopoietic stem/progenitor cells (HSPCs) and their progeny. This vector encodes a codon-optimized secreted form of TNALP fused to a human IgG1 Fc domain to prolong serum half-life and a targeting peptide to deliver the soluble TNALP protein to the bone. We show high levels of gene transfer to HSPC, as well as engraftment and differentiation of human RMP100-LVV-modified HSPCs in humanized bone marrow-thymus-liver (BLT) mice. Gene modified cells were detected in the peripheral blood, the bone marrow and spleen as assessed by vector copy number (VCN) analysis. Importantly, we detected multilineage reconstitution of marked huCD45+ cells, including CD3+ T cells, CD19+ B cells and CD11c+ dendritic cells, indicating that RMP100-LVV transduction of human HSPCs is feasible and does not affect immune reconstitution. Finally, we demonstrate that treatment with this cell therapy approach leads to a durable correction of plasma alkaline phosphatase activity, rescues early mortality, and skeletal manifestations of the disease in the severe HPP mouse model ($Akp2^{-/-}$). All untreated $Akp2^{-/-}$ mice, including those exhibiting mild bone phenotype, died by PND30 due to complications related to bone deformity or seizure. However, all mice transplanted with RMP100-HSPCs survived until scheduled termination on PND30 and there was a significant improvement in bone development based on radiographic image analysis of bone length and spacing. Additional analysis with micro-CT further demonstrated improved bone development that was indistinguishable from WT ($Akp2^{+/+}$) or HET ($Akp2^{+/-}$) controls. Restoration of musculoskeletal activity was assessed by open-field testing. We observed no significant difference between WT and transplanted $Akp2^{-/-}$ mice in total distance traveled, maximum speed (Vmax), or other parameters, further demonstrating the curative effects of RMP100-HSPC transplantation. Long-term efficacy was assessed by following a group of transplanted $Akp2^{-/-}$ mice for up to 1.5 years. Remarkably, all transplanted $Akp2^{-/-}$ mice survived and remained as healthy as their WT littermates, with maintenance of high plasma ALP activity and normal skeletal phenotype during this period. Our study provides critical insights into treating metabolic bone disorders with an autologous HSPC-based gene therapy and demonstrates that this approach is a potential safe and one-time treatment for HPP.

1271 Amnion mesenchymal stromal cells have the potential to preserve muscle function in a mouse model of Duchenne muscular dystrophy

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Background: Duchenne muscular dystrophy (DMD) is an incurable genetic disease characterized by degeneration and necrosis of myofibers, chronic inflammation, and progressive muscle weakness resulting in premature mortality. Immunosuppressive multipotent mesenchymal stromal cells (MSCs) therapy is a possible option for DMD patients. We focused on human amnion-derived mesenchymal stromal cells (hAMSCs), a clinically viable cell source owing to their unique characteristics, such as non-invasive isolation, mitotic stability, ethical acceptability, and minimal risk of immune reaction and cancer. We aimed to identify novel immunomodulatory effects after transplantation of hAMSCs for the functional recovery of skeletal and cardiac muscles.

Methods: We analyzed extracellular secretion of cytokines and chemokines in the co-cultured medium of hAMSCs with myocytes. hAMSCs were intravenously injected into DMD model mice (mdx mice) to assess the efficacy of therapeutic interventions. hAMSCs-treated mice were evaluated using myokine expression, histological examinations, spontaneous wheel-running activities, grip strength, and echocardiography.

Results: In vitro co-culture of hAMSCs and dystrophic myocytes showed disease specific activation of hAMSCs through up-regulated cytokine/chemokine expression, indicating the enhanced secretion from hAMSCs in the dystrophic environment of myocytes and the induction of M2 macrophage activation. After repeated systemic hAMSC injections, limited mononuclear cell infiltration and a decreased number of centrally nucleated fibers were indicative of regenerated myofibers following degeneration, revealing an improved histological appearance of the skeletal muscle of hAMSC-treated mdx mice. Upregulated M2 macrophages and altered myokine expressions were observed in the hAMSC-treated muscles. During long-term experiments, a significant decrease in the grip strength in untreated mdx mice significantly improved in the hAMSC-treated mdx mice. hAMSC-treated mdx mice maintained running activity and had an enhanced daily running distance. Notably, the treated mice could run longer distances per minute, indicating high running endurance. Left ventricular function in hAMSC-treated mdx mice improved when compared to untreated DMD mice. In contrast, we did not detect significant differences in the fibrotic area in the diaphragm and cardiac muscle.

Conclusions: Early systemic hAMSCs administration in mdx mice ameliorated progressive phenotypes, including pathological inflammation and motor dysfunction, resulting in the long-term improvement of the skeletal and cardiac muscle function. The therapeutic effects might be associated with the immunosuppressive properties of hAMSCs via M2 macrophage polarization. This treatment strategy could provide therapeutic benefits to DMD patients.

1272 Mesenchymal Cells as a Therapeutic Targets for ex-vivo Gene Writting in MDC1A

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Congenital muscular dystrophy type 1A (MDC1A) is a severe, progressive pediatric disorder caused by recessive mutations in the *LAMA2* gene. The LAMA2 protein trimerizes with LAMB1 and LAMC1 proteins and is fully secreted as laminin 211 into the muscle extracellular matrix, where it interacts with multiple proteins. The absence of LAMA2 or the presence of aberrant forms due to gene mutations results in an unstable extracellular matrix, reduced muscle mass and function, inflammation, and fibrosis. The large size of the *LAMA2* ORF presents a major limitation for gene therapy, and there is currently no effective treatment for MDC1A.

Since laminin 211 can reach the muscle extracellular matrix upon systemic administration, our study aims to use mesenchymal stem cells (MSCs) as laminin 211 protein pumps. Given the immunomodulatory properties of MSCs, we aim to both target the fibrotic component of MDC1A and develop an ex vivo gene editing approach to restore *LAMA2*expression.

MSCs are an optimal target for producing laminin 211 due to their immunomodulatory properties and low immunogenicity. To address their short lifespan in vivo following injection, we propose pre-differentiation into adipose progenitor cells. Although we have found evidence that MSCs naturally produce *LAMA2*, a gene writing strategy is employed to enhance production and ensure therapeutic levels. MSCs are obtained from wild-type mouse adipose tissue.

Our findings confirm the systemic distribution of laminin 211 following recombinant protein injection in the dy2j MDC1A mouse model. A preliminary study assessing *LAMA2*, *LAMB1*, and *LAMC1* production by MSCs and pre-adipocytes provides strong evidence supporting the potential of MSCs as laminin 211 protein pumps. Additionally,

MSCs have been successfully edited to produce laminin 211 and studied in vivo following intramuscular injection. Our approach—targeting MSCs for both fibrosis regulation and ex vivo gene therapy—introduces novel therapeutic opportunities for MDC1A. Given the critical role of fibrosis in MDC1A pathogenesis, we propose that an effective treatment must not only restore *LAMA2* expression but also modulate fibrosis. Our findings represent a step toward addressing the unmet clinical need for effective MDC1A treatments.

1273 Gene Supplementation via *DARS2*-AAV9 Rescues Phenotype in LBSL Patient Cells and *in Vivo* Mouse Model

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Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) is a rare, neurological disorder cause by variants in the *DARS2* gene. *DARS2*, an enzyme that catalyzes the attachment of aspartic acid to its corresponding t-RNA in the mitochondria, is ubiquitously expressed and essential. All people with LBSL possess compound heterozygous variants on *DARS2*, the most common of which results in a frameshift, premature termination, and decreased protein abundance. Upon disruption of *DARS2* production, clinical presentation can take a wide phenotypic spectrum, most commonly manifesting in progressive spasticity, ataxia, and dorsal column dysfunction, characterized as primary axonopathy with secondary demyelination. In this study, we aimed to test the efficacy of gene supplementation via *DARS2*-AAV9 in LBSL patient-derived sensory neurons as well as in an LBSL mouse phenolog in which *Dars2* is deleted in Advillin-expressing neurons of the dorsal root ganglia. Patient neurons treated with *DARS2*-AAV9 showed increased gene expression, which correlated with functional improvements measured by increased mitochondrial function, axonal growth, and decreased lactate production. Further, knock-out mice reflected functional improvements *in vivo*, displaying improved survival and gait following a single intrathecal injection of *DARS2*-AAV9. These data suggest that gene supplementation via AAV9 improves both cell survival and function, reducing phenotypic burden. Combined with the success of existing AAV9s in treating loss of function monogenetic disorders, these data demonstrate promise for *DARS2*-AAV9 as a potential therapeutic for LBSL and compel further investigation.

1274 Long-term Tolerance to Leydig Cell Transplantation with Localized Treg Immunomodulation Using the NanoLymph Platform

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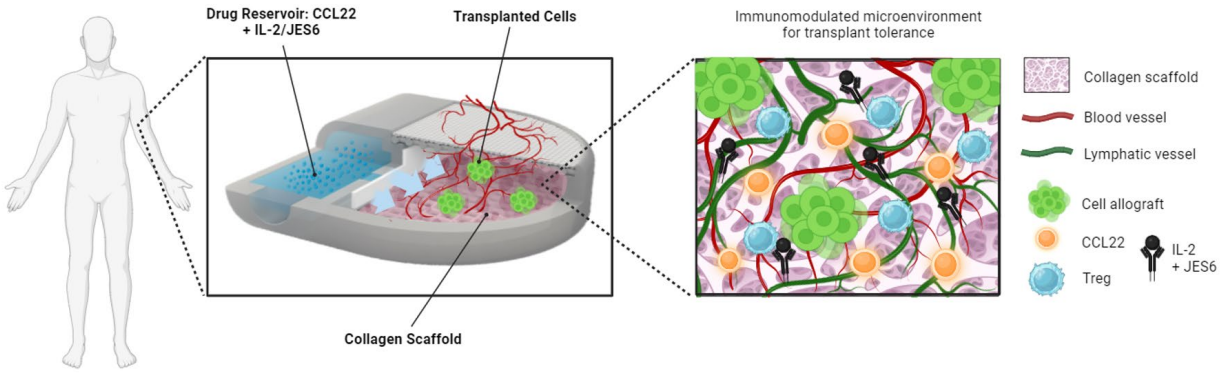
Cellular therapies aim to treat or manage disease by introducing living cells that integrate into the host and restore or eliminate dysfunctional tissues. Despite their clinical success, the host immune response to cellular treatments remains a challenge. Traditional approaches for controlling immune rejection involve systemic immunosuppression, which can result in severe off-target toxicity. An alternative strategy to restrain immune responses involves harnessing the natural immunomodulatory capabilities of Regulatory T-cells (Tregs), a specialized subset of T cells that suppress inflammatory immune responses and promote transplantation tolerance.

To achieve long-term immunological tolerance with fewer systemic side effects, we propose using the NanoLymph platform, an implantable subcutaneous device designed for continuous localized recruitment of Tregs. This device features a dual-reservoir system that supports sustained release of immunomodulatory agents through a nanoporous membrane and a vascularized compartment that facilitates cell homing and allograft integration with the host. The current work presents the development and characterization of the NanoLymph device as a cell transplantation platform supported by Tregs. We show that upon implantation, the NanoLymph platform integrates into the subcutaneous space through vascularization and lymphatic in-growth, creating an environment conducive to long-term allograft integration. We also demonstrate controlled and sustained release of Treg immunomodulatory therapies both in vitro and in vivo. Flow cytometry analysis confirms that these therapies effectively promote Treg

recruitment and expansion at the device site for up to 14 days. Furthermore, using a luc-Treg model, we characterize the kinetics of Treg migration to the device in vivo with high spatial and temporal resolution.

Finally, we assess the long-term efficacy of the NanoLymph platform using a proof-of-concept Akaluc-Leydig cell transplantation model. Longitudinal bioluminescence imaging demonstrates that localized Treg-promoting therapies sustain long-term cell transplant viability compared to systemic immunosuppression controls. Imaging mass cytometry of the local device space reveals interactions between regulatory and effector cells with high spatial accuracy, clarifying the role of these cells in immunomodulation toward tolerogenicity. Additionally, flow cytometry confirms an enriched local ratio of Tregs to CD8+ T cells and cytokine analysis reveals reduced pro-inflammatory cytokine production in the localized Treg-promoting therapy condition, highlighting the platform's capacity to create an immunomodulated environment that supports successful cell transplantation without the need for maintenance immunosuppression.

In summary, the NanoLymph platform supports the sustained release of immunomodulatory agents to create a safe, localized immunosuppressive environment, reducing systemic side effects while maintaining effective immunological tolerance. This innovative strategy holds clinical relevance for advancing cell transplant therapies by minimizing off-target toxicity and promoting long-term success.



1275 CD80/CD86-CD28 Signal Blockade during the Mixed Lymphocyte Reaction Augments the Alloantigen-specific Inhibitory Function of Natural Regulatory T Cells

Kyoko Tsuji-Yogo[1], Kazuyoshi Takeda[2], Ryuichi Murakami[3], Shohei Hori[3], Ko Okumura[4], Koichiro Uchida[2]
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Purpose: Antigen-specific Regulatory T cells (Tregs) therapy is promising approach for transplant rejection and autoimmunity. Our group has reported that co-culture of recipient lymphocytes and irradiated donor lymphocytes (mixed lymphocyte reaction (MLR)) under the condition of CD80/86-CD28 co-stimulation blockade with anti-CD80/CD86 mAbs induced anergic T cells that acquired alloantigen-specific immune suppressive function in mice and humans, successfully inducing operational tolerance in seven out of ten liver transplant patients. Tregs have been suggested to be the major population exerting the alloantigen-specific suppressive function. In this study, we investigated the mechanism by which Tregs augmented donor-specific suppressive function during MLR in the presence of anti-CD80/86 mAbs.

Methods: C57BL/6 *Foxp3*^{hCD2} mice were used to evaluate suppressive function and the expression of function-associated molecules in Tregs before/after MLR with or without anti-CD80/86 mAbs. Mice expressing a single TCRβ chain were used to analyze the TCRα repertoire of Tregs.

Results: The proliferation of Tregs was remarkably inhibited by anti-CD80/86 mAbs. Anti-CD80/86 mAbs significantly enhanced the suppressive function in an alloantigen-specific manner (Figure 1). An increase in the concentration of IL-2 restored the proliferation of Tregs but inhibited the enhanced alloantigen-specific suppressive

function. TCR repertoire analysis showed that several Treg clones were expanded after MLR, but anti-CD80/86 mAbs limited the number of expanded Treg clones (Figure2). Treg clones expanded during MLR with anti-CD80/86 mAbs were different between MLR with BALB/c and that with CBA.

Conclusions: These results suggest that MLR with antagonistic anti-CD80/86 mAbs selectively expanded Tregs expressing high-affinity TCRs for alloantigens.

Figure 1

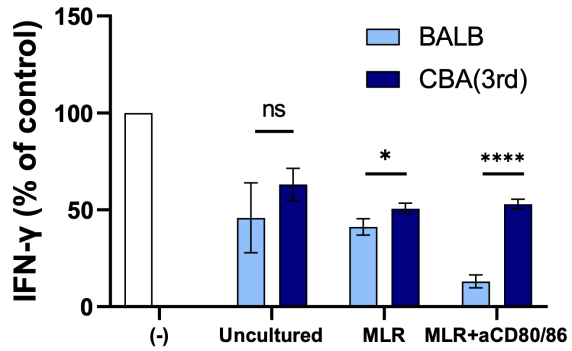
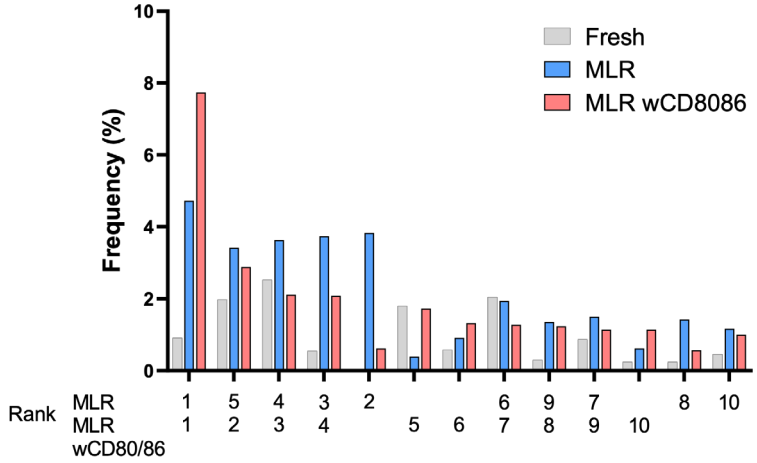


Figure 2



1276 The Therapeutic Effect of Adipose-Derived Stem Cells and Hydrogen Sulfide Donor Sodium Hydrogen Sulfide Combination Treatment on Erectile Dysfunction in a Rat Model Following Radical Prostatectomy

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Introduction: A radical prostatectomy is likely to result in erectile dysfunction (ED) due to cavernous nerve damage. Hydrogen sulfide (H_2S) is an endogenous gasotransmitter produced by cystathionine-β-synthase (CBS),

cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) and involved in penile erectile physiology. Adipose-derived stem cells (ADSCs) provide a significant cellular resource for treating degenerative diseases. We aimed to evaluate the potential therapeutic benefits of the H_2S donor, sodium hydrogen sulfide (NaHS), and the intracavernosal injection of ADSCs, both alone and in combination, on ED induced by bilateral cavernous nerve transection (CNT).

Methods: Sprague-Dawley rats were divided into five groups: Sham-operated, CNT (4-wk), intracavernosal injection) and CNT-treated with NaHS and ADSCs groups. ADSCs were intracavernosally injected after the transection. The *in vivo* erectile response and the *in vitro* relaxant and contractile responses of the corpus cavernosum (CC) were assessed. Protein expression and localization of neuronal nitric oxide synthases (NOS), H_2S synthesis enzymes, TGF-β_1, and HIF-1α, as well as the smooth muscle/collagen ratio were analyzed.

Results: Rats with CNT showed considerable reductions in erectile responses, acetylcholine and electrical field stimulation (EFS)-induced relaxation, as well as phenylephrine and EFS-induced contractile responses of the CC, which were moderately enhanced by NaHS and ADSCs treatments alone. The combined treatment significantly enhanced the decrease. There was a significant decrease in the expression of neuronal NOS (nNOS) and endothelial NOS (eNOS) in the CNT group ($p<0.001$). Furthermore, the protein expression of CSE, CBS ($p<0.001$), and 3-MST ($p<0.05$) in rats with CNT were lower than in control rats. TGF-β_1 and HIF-1α expression, as well as collagen in the penile tissue obtained from the transected group were significantly increased compared to controls ($p<0.001$). The combination therapy elicited more effective alterations in all protein expressions and collagen deposition in rats with CNT.

Conclusions: The combined therapy of NaHS and ADSCs is more efficacious than monotherapies in ameliorating CNT-induced ED. H_2S and cell-based therapy are likely to synergistically recover erectile function via the modulation of molecular processes and alterations in penile structure, which can support to improve clinical outcomes in the treatment of ED after radical prostatectomy.

1277 Grafting Strategies for Transplantation of Organoids into Solid Organs

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Transplantation of organoids is blocked by their inability to engraft into solid organs as well as by immunological barriers. We have overcome the barriers with patch grafting . Successful conditions consist of embedding organoids into Glycosil (thiol-modified hyaluronans), prepared in a defined medium and triggered with poly (ethylene glycol) diacrylate (PEGDA) to form soft hydrogels (less than 100 Pa). The defined medium is designed for stem/progenitors: a basal medium with low calcium, no copper, lipids (free fatty acids and lipid carriers such as albumin and high density lipoprotein), plus insulin and transferrin/Fe but with no or as few as possible other signals that might drive differentiation. Graft conditions enable organoids to express the requisite repertoire of secreted and plasma-membrane-associated forms of matrix metalloproteinases (MMPs), enabling engraftment into solid organs within hours to a few days. Grafting biomaterials are absorbed naturally by host processes leaving donor cells to mature fully amidst host environmental and organ-specific conditions. After transplanting the hydrogel, it is covered with a barrier to tether the graft and to provide protection from abrasion. The barrier can be any of commercially available ones used surgically or can be porous silk impregnated with Glycosil and triggered with PEGDA to form a more rigid hydrogel (~ or >500-600 Pa). The barrier's conditions causes organoids at the barrier interface to mature sufficiently to cease expression of secreted MMPs and so be unable to move. By contrast, organoids placed adjacent to the target organ are able to engraft. During surgery, a coating of the more rigid

hydrogel is applied to the exterior surface of the barrier to minimize adhesions. Patch grafting methods have proven successful to permit engraftment of approximately a gram of donor organoids per patch into every solid organ evaluated (liver, pancreas, intestine, kidneys, large bile ducts) and in the two species tested (mice and pigs). Grafts of donor organoids were able to rescue murine hosts from various disease states such as tyrosinemia (liver) or type I diabetes (pancreas). Efforts are ongoing to learn if the strategies are successful also for organoids of mesodermal and ectodermal epithelial cells and in other host species. **Background References**: Lozoya O, et al. Regulation of hepatic stem/progenitor phenotype by microenvironmental stiffness in hydrogel models of the human liver stem cell niche doi:10.1016/j.biomaterials.2011.06.042. Zhang W, et al. Patch grafting, strategies for transplantation of organoids into solid organs such as liver. doi:10.1016/biomaterials.2021.121067 and . doi: 1016/j.biomaterials.2022.121647.

1278 Development of a mode of action-based testing suite for the screening of regenerative macrophage cell therapy candidates for end-stage liver disease and beyond

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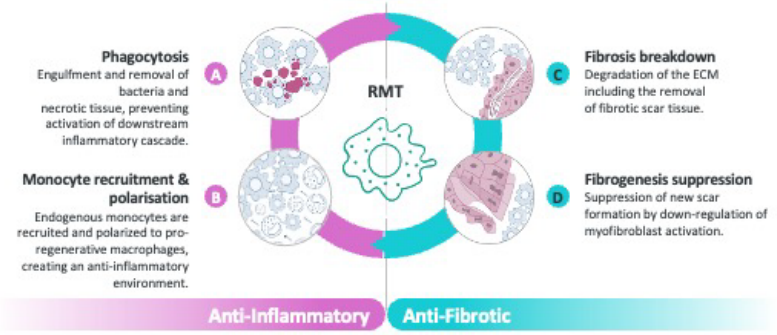
Regenerative macrophages have a well-established role in promoting liver regeneration via control of inflammation and promotion of fibrosis remodelling. Previous data obtained at the University of Edinburgh provided clinical proof of concept of safety and efficacy of autologous regenerative macrophages therapy (RMT) in patients with liver cirrhosis, the final manifestation of chronic liver damage from distinct aetiologies. To transform this academic proof of concept into a cell therapy product with the potential to treat a patient population with decompensated liver disease, Resolution Therapeutics proceeded to engineer the regenerative macrophages with payloads designed to improve its anti-inflammatory and anti-fibrotic potency. To this end, we underwent an extensive screening process to select the most effective payloads. The unique challenge in this context was to design an in vitroscreening suite that could recapitulate the main mechanisms of action (MoA) of the RMT, whilst also allowing benchmarking of the engineered macrophages against the already clinically tested non-engineered cells. Previous in vivo experiments informed us of the key cellular mechanisms underpinning the anti-inflammatory and anti-fibrotic MoA of the regenerative macrophages (Fig.1A). We were able to design a cell-based, research-grade assay for each of the mechanisms. To test the anti-inflammatory MoA, we established assays to measure: phagocytosis, recruitment of monocytes, and polarisation of naïve macrophages to a regenerative phenotype. To test the anti-fibrotic MoA, we set-up assays to measure collagen degradation and de-activation of myofibroblasts, i.e. scar-producing cells. We tested 7payload at 18-24h post-transfection: 5 aimed to improve anti-inflammatory cellular mechanisms, and 2 aimed to improve anti-fibrotic cellular mechanisms. This comprehensive testing suite led to the identification of IL10 and MMP9 as best-in-class candidates for anti-inflammatory and anti-fibrotic MoA, respectively (Fig.1B). Further, we harnessed the same testing suite to compare the combination of IL10+MMP9 payload against IL10-only payload. The combination of IL10+MMP9 offered unique advantages as compared to IL10-only in terms of increased monocyte recruitment (4x enhancement vs non-engineered and 2x enhancement over IL10-only, respectively) and increased suppression of myofibroblasts activity (50% suppression vs 20% suppression, respectively), leading us to select the combination of IL10+MMP9 to engineer our lead asset, RTX001 (Fig.1C). In conclusion, we have established a robust, versatile, research-graded testing suite to benchmark engineered RMTs against the clinically tested, non-engineered RMT. This MoA-based testing suite has allowed the selection of IL10+MMP9 as payloads to increase the anti-inflammatory and anti-fibrotic potency of our lead asset, RTX001, facilitating the treatment of a more severe subset of patients. RTX001 has now progressed to clinical testing in UK and Spain (EMERALD Phase 1/2 study). An important consideration for future applications of the testing suite is that regenerative macrophage phenotype and functions are remarkably conserved amongst distinct organ and tissues. For this reason, we are now investigating the use of RMT in other areas of major unmet medical need, namely lung fibrosis and Graft-versus-Host disease, and it will harness the testing suite presented here to identify the best candidate in these clinical settings.

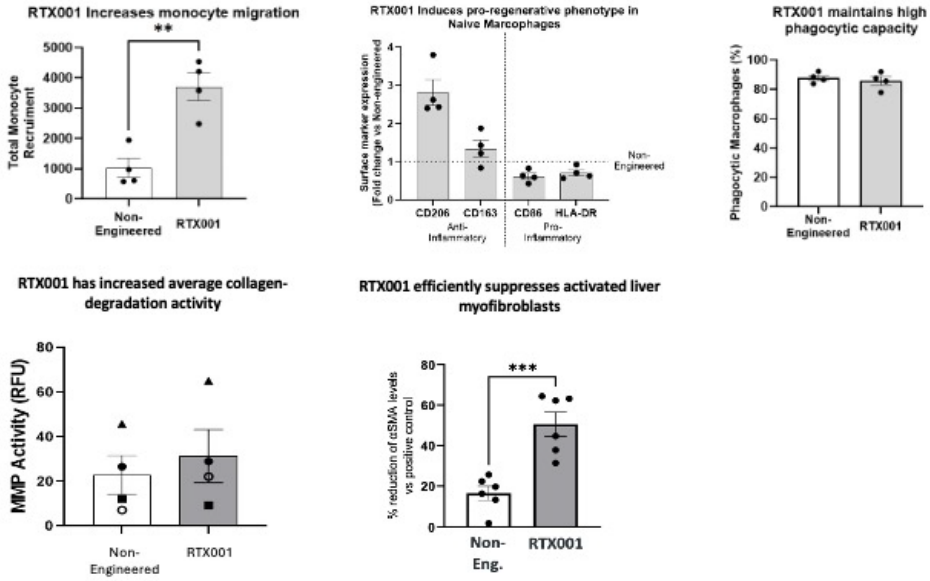
A



B

Payload	Functional category	Phagocytosis	Fibrosis breakdown	Mϕ Polarization	Host monocyte recruitment	RTX001 candidate
IL10	Anti-inflammatory/ monocyte recruitment + Mϕ polarization	-	-	++	+++	Y
CAN2	Anti-inflammatory/ Phagocytosis	±	n.a.	-	-	N
CAN3	Anti-inflammatory/ Phagocytosis	- -	n.a.	-	-	N
CAN4	Anti-inflammatory/ Mϕ polarization	-	n.a.	±	-	N
MMP9	Anti-fibrotic/ Scar Remodelling	±	++	-	-	Y
CAN6	Anti-fibrotic/ Scar Remodelling	-	++	-	-	N
CAN7	Anti-inflammatory/ Mϕ polarization	-	n.a.	±	-	N

C



1279 Development of 3D Printed PLG-Based Liver Tissue Therapeutic for the Treatment of Alcoholic Liver Disease and Liver Failure

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Introduction: Alcoholic liver disease (ALD) is a major cause of liver failure-related deaths globally[CJ1] . Severe forms of ALD, such as alcohol-associated hepatitis (AH) and severe alcohol-associated hepatitis (SAH), have high mortality rates up to 50% at 28 days for AH and up to 70% at six months for SAH. While existing treatment options offer limited benefit, liver transplantation remains the only curative solution. Alternatively, an ectopic implant containing a large number of hepatocytes, capable of providing both immediate and sustained liver function, could be developed. Such an implant could serve as a bridge to liver transplantation or recovery. However, existing strategies that use bioinert hydrogels to deliver therapeutic cells face significant challenges, primarily due to poor tissue integration and inadequate vascularization. In this study, we explore the potential of 3D printed poly lactide-co-glycolide (PLG) scaffolds with an open-pore design as a more effective means of delivering high-density functional hepatocytes through minimally invasive subcutaneous implants, offering a novel solution for liver failure treatment.

Methods: Highly porous PLG scaffolds were 3D-printed in two designs, D1 and D2, with varying porosity. Cryopreserved primary human hepatocytes (PHHs) were first screened for quality via in vitro stability and functionality testing, as well as their ability to assemble into 3D aggregates. PHHs were aggregated into hepatic spheroids and seeded onto 10mm scaffolds at 2×10^6 PHHs per scaffold. In vitro, cell-laden scaffolds were cultured for 2+ weeks and assessed for viability, albumin and urea synthesis, and CYP3A4 activity. In vivo, scaffolds were implanted subcutaneously in NOD SCID mice for one month, with circulating human albumin (hALB) measured and compared to cell-laden hydrogels of similar size and cell density. Explants were analyzed via histology/IHC for tissue integration and vascularization.

Results: In vitro results showed that optimized 3D-printed scaffold designs enabled uniform seeding of hepatic spheroids at densities up to 5×10^6 hepatocytes/ml. Within 24–48 hours, hepatocyte aggregates adhered to the scaffold and organized within the scaffold microporous architecture. Live/dead staining showed high viability after two weeks in culture. In vitro, both D1 and D2 scaffolds maintained stable albumin and urea secretion for over two weeks, with D2 scaffolds exhibiting ~4-fold higher albumin secretion and ~2.8-fold higher CYP3A4 activity than D1, while urea secretion remained comparable. In vivo, steady hALB levels persisted in both scaffold groups for up to four weeks post-implantation. While hydrogel implants initially showed ~2- and ~1.7-fold higher hALB levels than D1 and D2, respectively, levels declined 2 weeks post implantation[RS2] , resulting in ~2- and ~4-fold lower hALB compared to D1 and D2. Histological analysis at one month revealed dense vascular networks in PLG scaffold groups, which weren't observed in[CJ3] the hydrogel conditions.

Conclusions: 3D-printed porous PLG scaffolds provide a promising approach for ectopic liver implants. Our data demonstrates efficient hepatocyte delivery and sustained functional phenotype (synthetic and metabolic), supported by robust vascularization. Future studies will evaluate the efficacy of using PLG scaffolds to deliver hepatocytes for the therapeutic treatment of liver conditions characterized by rapid loss of hepatic function, such as liver

1280 Genome-Orthogonal Gene Circuitry: Improved Synthetic Enhancer-Transcription Factor Systems with Minimal Off-Target Effects

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Background: Cell therapies, which synthesize therapeutics precisely when and where they are needed, hold promise for addressing numerous challenging diseases. However, to fully realize this potential, synthetic regulators are required that can drive therapeutic gene expression without perturbing endogenous transcriptional programs. Zinc finger-based transcription factors (ZFTFs) are highly programmable, human-derived proteins well suited for therapeutic use, given their specificity, small size, and compatibility with small molecule or SynNotch-based control. Building on the recent publication of genome-orthogonal ZFTFs [1], we have developed novel enhancer-ZFTF pairs with improved orthogonality to human and mouse genomes. This advance expands the toolbox for synthetic biology-enabled gene expression in complex cell therapies, enhancing both safety and efficacy.

Methods: To construct genome-orthogonal expression circuits, we identified binding sequences that do not occur in the human genome. Specifically, we screened all possible 19 nucleotide (19mer) sequences composed of six GNN

trinucleotides plus one final N (N= A, T, C, or G). We then computationally filtered these 19mers to remove: 1) sequences that match or nearly match any region of the human genome and 2) sequences that match mammalian transcription factor binding sites. From the remaining 19mers, synthetic enhancers were assembled by placing four repeats of the 19mer in tandem, separated by 10bp spacers devoid of known transcription factor binding sites. Each enhancer was then inserted upstream of a minimal promoter to drive GFP expression. For each synthetic enhancer, two six-finger ZF arrays were designed and fused to a miniVPR transcriptional activator. These enhancer-ZFTF pairs were transfected into HEK293T cells to evaluate their GFP expression strength. Top pairs were subsequently transduced into multiple cell types and analyzed by bulk RNA-seq to assess potential off-target transcriptional effects.

Results: We identified three ZFTFs that outperformed a previously published genome-orthogonal ZFTF [1] by >1.5-fold in transcriptional strength. Bulk RNAseq revealed that these top three ZFTFs drove higher GFP expression per ZFTF RNA molecule than the published ZFTF, while affecting significantly fewer off-target genes (1, 7, or 9 vs. 80). Further studies showed that our most potent ZFTF also exhibited reduced off-target effects in two additional human cell types (T cells and liver cells) and in mouse melanoma cells.

Conclusions: We have designed novel mammalian genome-orthogonal enhancers and ZFTFs that serve as potent transcriptional activators with minimal impact to endogenous gene networks. These tools form the basis of powerful, highly specific gene circuitry that can be incorporated into small molecule or environmentally responsive transcriptional switches. By enabling precise, dynamically controlled therapeutic payload production, our improved genome-orthogonal ZFTFs advance the promise of next-generation cell therapies.

References
Li H, Israni DV, Gagnon KA, et al. Multidimensional control of therapeutic human cell function with synthetic gene circuits. Science. 2022;378:eade0156. doi:10.1126/science.ade0156

1281 Toward Treatment with Gene-Modified B Cells Engineered In Vivo Using iGPS Particles

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Genetically-engineered B-cells are emerging as a powerful platform to secrete therapeutic proteins as treatment for congenital and infectious diseases. Autologous or donor-derived B cells are genetically modified and used to treat patients with or without preparative lymphodepleting chemotherapy. We hypothesize that in vivo modification of B cells could improve cost, patient access and persistence compared to ex vivo manufactured B cell therapies. The in vivo gene placement system (iGPS™), an advanced lentiviral vector (LVV) particle with specific envelope modifications to improve gene transfer efficiency and cell specificity, has shown potent and T cell-specific genetic modification in vivo using CD3 as an entry receptor in preclinical models. Here we tested the feasibility of adapting the iGPS system to target and modify B cells in vivo. Antibodies specific to a variety of B-cell surface molecules were engineered into the iGPS system and screened for potent and B cell specific transduction of primary and established B cell lines. While standard VSV-g pseudotyped LVV are inefficient at B cell transduction, the lead B cell-targeting iGPS molecule caused activation and transduction of ~50% of resting B cells in vitro, including 40% of CD27+ memory B cells. There was no transduction of a Jurkat T cell line demonstrating the transduction was B cell specific. Finally, we tested the transduction of B cells in a humanized mouse model. NSG-DKO mice were injected with 1e7 human PBMCs; 15 days later (when a detectable B cell population is present in this model) mice were dosed i.v. with B-cell targeting-iGPS particles delivering a GFP reporter transgene. Seven days post-iGPS particle injection, we observed dose-dependent GFP+ transduction of up to 4% of all B-cells (CD19+CD3-) in spleen, bone marrow, and blood. Together, these data demonstrate the feasibility of efficiently engineering B cells in vivo toward applications treating congenital and infectious diseases with a single-treatment of B cell-targeted iGPS particles.

1282 In Vivo CAR-T Therapy Powered by Novel CLAMP Technology for T cell-Targeted mRNA Delivery

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Autologous CAR-T cell therapy has demonstrated clinical efficacy in hematologic malignancies and potential in autoimmune diseases (AID) where B cell played a crucial role. However, challenges such as complicated cell manufacturing, lymphodepletion and high costs hinder its use as personalized medicine. An off-the-shelf, cost-effective product with simplified manufacture process could better meet patient needs and democratize genetic medicine. Inspired by the success of COVID-19 mRNA lipid nanoparticle (LNP) vaccine, we developed a T cell-targeted mRNA-LNP (T-LNP) system capable of delivering anti-CD19 CAR to T cells in vivo for AID treatment. T-LNPs were created using Controllable Ligand Attachment Modification and Purification (CLAMP) technology on the Kailash™ platform. CLAMP streamlines surface conjugation and purification of antibody-conjugated LNPs by optimizing components such as chemical linkers and site-specific residues engineered onto antibodies. This platform simplifies conjugation procedures while remaining compatible with scalable LNP production. We evaluated T-LNPs targeting three T cell-specific surface proteins for CAR transfection and B cell cytotoxicity using human peripheral blood mononuclear cells (hPBMCs). Surface protein B emerged as the optimal choice, achieving over 30% CAR expression in total CD3+ T cell population, favoring CD8 subset, and inducing nearly 100% B cell killing at 24 hours. In vivo, T-LNP carrying anti-CD19 CAR mRNA injected into hPBMC-reconstituted NOG mice showed over 95% B cell killing in spleen at 24 hours with a dose of 0.33mg/kg. The therapeutic effect was further evaluated in an aggressive B cell leukemia model, Nalm-6. NOG mice reconstituted with hPBMC were inoculated with Nalm6-luc cells and treated with a single T-LNP dose carrying anti-CD19 CAR mRNA on day 3. Both 0.2 mg/kg and 1 mg/kg T-LNP dose groups achieved complete tumor control, and 1 mg/kg fully eradicated established large Nalm-6 tumors. These data collectively demonstrate that our novel T-LNP delivery platform enables efficient and specific T cell delivery, resulting in sufficient CAR expression and potent B cell killing, highlighting its potential as a universal drug product for AID treatment.

1283 In vivo mRNA CAR-T Cell Engineering Utilizing a Novel CD3/CD5 Peptide Based Targeted Nanoparticles.

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The Chimeric Antigen Receptor (CAR) T cell therapy has emerged as a ground-breaking immunotherapeutic approach in cancer treatment and demonstrated encouraging clinical results in autoimmune disease indications. However, personalized CAR T cell production is a bottleneck due to challenges in cell manufacturing, scalability, side effects and high production costs. In vivo CAR T cell generation has been proposed to make this treatment immediately accessible to patients and applicable for broader use. However, efficient and highly selective gene delivery to target cells in vivo is challenging. To that end, we are developing a new peptide based (non-lipid) delivery platform (PEP-NP) to enable in vivo delivery of mRNA encoding an anti CD19 CAR to reprogram CD3/CD5 expressing T-cells.

The PEP-NP platform is based on short amphipathic peptides that form stable, scalable nanoparticles capable of efficiently packaging and delivering RNA to the target site. The structure of PEP-NPs have been designed to bypass the liver and enhance mRNA expression in the spleen and T-cells in vivo. PEP-NPs were selected based on their efficiency to deliver functional Fluc-mRNA on Jurkat cells and primary human T cells from different donors. Efficiency of PEP-NP to deliver a mRNA encoding for an anti-CD-19 CAR was evaluated on human primary T cells. The cytotoxicity of CAR-PEP-NPs transfected T cells was evaluated by co-cultured with either Nalm6 CD19+ human acute lymphoblastic leukemia cells or K651 (CD19-) cells. In-vivo efficacy of PEP-NP (0.5 and 1.0 mg/kg)

to deliver mRNA in the spleen and specific immune cells, was evaluated in C57BL/C6 mice using luciferase or eGFP encoding mRNA and analyze by bioluminescence or flow cytometry.

PEP-NPs carrying mRNA encoding luciferase or GFP showed a dose responsive transfection starting at 6 hr post treatment with more than 90% of treated T cells expressing Luciferase or GFP after 48 hr. We validated transfection, translation and surface expression of a functional CD19 CAR protein by transfecting human T cells with PEP-NPs mRNA encoding for an anti-CD19 CAR protein. CAR expression was observed in more than 80% of the transfected T cells from different donors. The cytotoxic potential of CAR-PEP-NPs transfected T cells was evaluated by co-cultured with CD19+ Nalm6 cells. CAR-PEP-NPs transfected T Cells showed a robust dose response specific cytotoxic effect, resulting in 65 % to 92% killing of Nalm6 cells, while in contrast, no cytotoxicity was observed on K561 (CD19-) cells or with T cells transfected with PEP-NP eGFP mRNA . We then demonstrated that IV administration of CD3/CD5 T cell targeted PEP-NPs exhibits high Luc or GFP mRNA expression in the spleen. PEP-NPs carrying GFP mRNA, showed a dose-dependent mRNA delivery to T cells in the blood as well as in splenic T cells with 15-18% of T cells positive for GFP after a single injection. No toxicity of PEP-NP was detected after single or repeat dosing. PEP-NP-CAR have been evaluated on NSG-CD45 mice engrafted with human PBMCs and the B cells frequency was monitored over 24 hr.

Our study provides a proof-of-concept that PEP-NP platform can be applied to directly target T cells for in vivo CAR T cell engineering. PEP-NP is a novel strategy for specific CD3/CD5 T cell reprogramming which provide a redosable, off the shelf T cell gene therapies for patients.

1284 A "Plug-and-Play" Erythrocyte-Mediated mRNA Delivery System Enables *In Vivo* Generation of CAR-Myeloid Cells for Cancer Immunotherapy

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Background: Despite the success of COVID-19 vaccines, the targeted delivery of mRNA-based therapies to immune cells remains a significant challenge. Current lipid nanoparticles (LNPs) primarily target the liver when administered systemically, limiting the broader applicability of mRNA for targeting immune cells in lymphoid organs. Additionally, LNPs suffer from low endosomal escape rates and may induce off-target immunotoxicity in healthy tissues. Here, we developed a simple and adaptable erythrocyte-mediated delivery system, which enables the covalent conjugation of mRNA-loaded lipid nanoparticles to erythrocyte membrane proteins in a "plug-and-play" manner, termed mRNA-LNP-Ery. Leveraging the natural splenic homing ability of erythrocytes, mRNA-LNP-Ery selectively targets the spleen, facilitating efficient delivery of mRNA to immune cells for cancer immunotherapy.

Method: We evaluated the *in vivo* gene expression profile of mRNA-LNP-Ery using luciferase-LNP-Ery and Cre recombinase-LNP-mEry in C57BL/6 mice and C57BL/6 Cre-reporter mice, respectively. We further investigated the internalization pathway of mRNA-LNP-Ery in immune cells through co-localization studies. Additionally, we delivered anti-HER2 CAR mRNA via erythrocytes and assessed its anti-tumor efficacy in MC38-hHER2 C57BL/6 tumor models.

Result: Unlike mRNA-LNPs alone, which predominantly accumulate in the liver, mRNA-LNP-Ery exhibited a preferential distribution to the spleen. Over 90% of mRNA expression mediated by mRNA-LNP-Ery localized to splenic CD11b+ myeloid cells, where it was efficiently phagocytosed. *In vitro* co-localization studies revealed that erythrocyte-mediated delivery bypassed endosomal trapping, leading to significantly enhanced mRNA expression compared to conventional mRNA-LNPs. Furthermore, we demonstrated the therapeutic potential of this platform by delivering anti-HER2-specific chimeric antigen receptors (CAR) mRNA via erythrocytes to generate CAR-myeloid cells *in vivo*, which can migrate to the tumor sites. These cells not only directly targeted and killed tumor cells but also reprogrammed the tumor microenvironment, promoting enhanced lymphocyte infiltration. This resulted in more effective tumor suppression in immunocompetent tumor models compared to CAR-LNP treatment. Importantly, CAR-LNP-Ery required only one-tenth of the mRNA dose used by CAR-LNP to generate CAR-myeloid cells *in vivo*, further highlighting the efficiency of spleen-targeted delivery. Additionally, CAR-LNP-Ery caused minimal

side effects, in contrast to CAR-LNP treatment, which induced significant body weight loss and splenomegaly in treated mice, indicating that CAR-LNP-Ery offers enhanced safety and reduced systemic exposure.

Conclusion: Erythrocyte-mediated mRNA delivery enables the generation of CAR-myeloid cells *in vivo* by targeting splenic immune compartments for cancer immunotherapy. This study presents the first cellular-based mRNA delivery platform, offering precise immune cell programming and providing new opportunities for improving cancer therapeutic strategies.

1285 Bioproduction of messenger RNA therapeutics in yeast cells: a proof of concept study.

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Messenger RNA (mRNA) has emerged as a promising biopharmaceutical for a vast array of therapeutic applications. Currently, in vitro transcription (IVT) is the available technology for mRNA production. However, IVT requires a complex supply chain and costly purification processes. Despite the advantage of being cell-free, questions about the reliability and cost of large-scale IVT production arise as therapeutics enter clinical applications, which require high doses.

Our project groundbreaking nature lies in generating a specific mRNA bioproduction process in yeast, integrating extraction and purification techniques—a complete paradigm shift compared to IVT production. To this aim, we built an interdisciplinary consortium coming from the fields of molecular biology, biophysics, biotechnology, material, computational chemistry and chemical engineering working in mRNA therapeutics, yeast biology, engineering and bioproduction as well as purification processes.

By leveraging yeast's established capabilities in industrial biotechnology, our consortium is producing the desired mRNA within yeast cells, followed by packaging into specific cytoplasmic foci, extraction, and purification. Rigorous quality control analyses ensure the structure and efficacy of the yeast-produced mRNA therapeutics.

Our research has the potential to revolutionize the production of mRNA therapeutics and vaccines, making them more affordable and easier to manufacture, thus expanding access to a wider population quickly, safely, and reliably. Furthermore, the development of a yeast-based mRNA bioproduction platform could significantly impact the global response to emerging viral threats.

1286 Overcoming Challenges in mRNA Plasmid Preparation: Advanced Solutions for Poly(A) Tail Integrity and High-Yield Production

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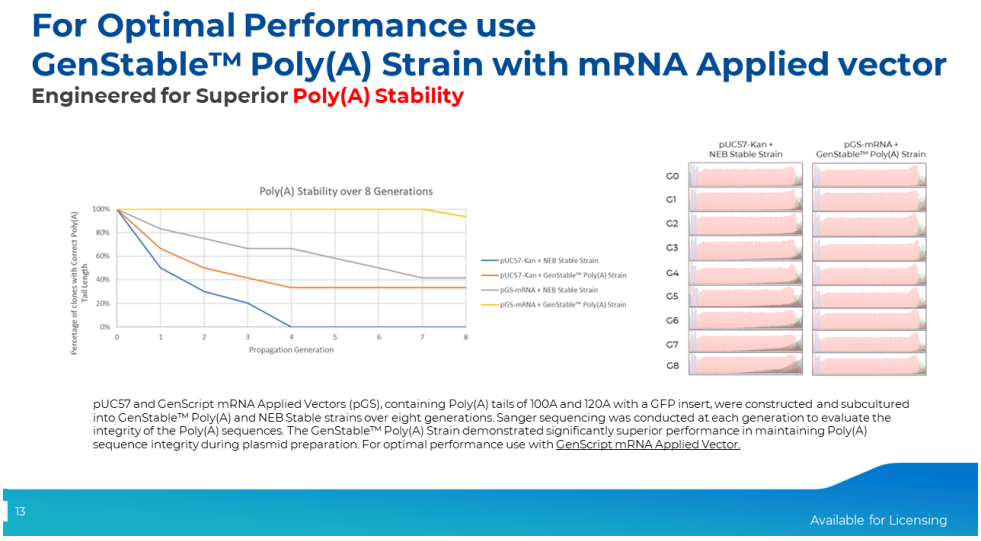
mRNA research has experienced unprecedented growth, driving breakthroughs in fields such as vaccine development, cancer immunotherapy, regenerative medicine, and beyond. As researchers push the boundaries of mRNA-based therapeutics, the demand for efficient, high-quality plasmid DNA templates has surged. These templates are essential for in-vitro transcription (IVT) and play a critical role in ensuring the integrity and effectiveness of mRNA production. However, challenges persist, particularly in propagating plasmids containing Poly(A) tail sequences in *E. coli*. Researchers frequently encounter issues such as low plasmid yields, compromised

Poly(A) tail integrity, and extended development timelines—challenges that significantly hinder therapeutic development and drive-up costs.

The Poly(A) tail is a vital component of plasmid templates for mRNA production, influencing transcription efficiency, mRNA stability, and downstream application success. Recognizing these pain points, GenScript has developed innovative solutions to accelerate mRNA research and streamline plasmid preparation workflows.

GenScript's proprietary mRNA vectors, featuring prebuilt Poly(A) tail sequences, deliver consistent and optimized templates for in vitro transcription (IVT). Paired with our GenStable™ Poly(A) strain—engineered specifically for the propagation of plasmids containing Poly(A) tails—these solutions set a new standard in plasmid stability and productivity. Together, they achieve a remarkable >90% success rate in preparing intact clones with intact Poly(A) tail sequences through 8 generations, representing a 300% improvement over conventional tools, while increasing plasmid yields by over 7-fold, ensuring reliability and efficiency in mRNA production workflows.

GenScript's advanced tools address persistent challenges in mRNA plasmid preparation, dramatically improving yield and intact Poly(A) tail success rates. These enhancements reduce time, resources, and costs, streamlining workflows and accelerating progress in mRNA research and therapeutic development.



1287 A new linear plasmid process platform enabling small scale GMP production with significantly short lead time, high titer, superb polyA stability dedicating to nuclei acid production

Enshi Liu[1], **Jing Zhu[1]***
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mRNA vaccines have been demonstrated as a powerful alternative to traditional conventional vaccines because of their high potency, safety and efficacy, and capacity for rapid clinical development. The overall CMC production process of mRNA vaccines can be divided into three modules: pDNA production, mRNA production, and LNP encapsulation. While tremendous efforts have been dedicated into mRNA and subsequent LNP encapsulation process development, plasmid process has not been improved significantly and still heavily relied on the traditional approaches that was developed for viral vector applications. While it deliveries the DNA template for mRNA production, the tradition plasmid production has become the bottleneck for the mRNA vaccine applications due to its long lead time and high cost for both process development and manufacturing in its supercoil isoform and later linearization and difficulty in maintain the integrity of long coded polyA unit. At ReciBioPharm, we have developed

a new plasmid platform dedicating to nucleic acid application. First, a systematic upstream optimization in cell bank, batch strategy, fed-batch strategy was executed to increase plasmid titer up to 1.5g/L fermentation batch size while maintain the desired full length polyA up to 120A. Second, linearization step was integrated as part of downstream process to reduce 30% unit operation and increase total recovery rate to 40% while reaching all required impurity specifications. This new platform has been well demonstrated with various plasmid sizes from 4000 to 9000bp and different polyA tail length from multiple DNA constructs. Such high titer can offer >500mg final linear plasmid from a 3L GMP batch size, which is enough to meet most of Phase I/II needs for mRNA vaccine clinical trials. Comparing to current 30L batch size based plasmid process in the market, it is no doubt that this new linear-dedicated plasmid process will significantly deployment not only of mRNA vaccines for rapid response against future pandemics but also of novel mRNA therapeutics to patients around the world.

1288 Case Study on an Insect Cell-Produced AAV6 Gene Therapy Vector for Neuromuscular Disease

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Developing AAV gene therapies for systemic delivery presents unique manufacturing challenges, such as producing commercially viable titers, achieving acceptable full capsid ratios and complying with regulatory requirements. These conditions can be met with the use of the inherently safe and scalable insect cell system. This presentation provides a case study of the production and activity of AVGN7, an AAV6-based gene therapeutic for treating rare neuromuscular diseases. The upstream manufacturing process employs a newly introduced expression platform comprised of a rhabdovirus negative clonal Sf9 RV-Free cell line derived from a single cell printing method, as well as a co-developed chemically defined insect cell medium, ESF AdvanCD™. Additional process improvements include formulation of a lysis reagent specific to the baculovirus and insect cell system. Preliminary studies demonstrate the ability to produce high levels of AAV expression using a high cell density, high MOI (multiplicity of infection) methodology followed by increased recovery utilizing the optimized lysis reagent.

RV-Free Sf9 cells were seeded at a density of 3 million per mL in ESF AdvanCD. Culture volumes ranged from 500-2500 mL. Cells were grown overnight to ensure log phase cultures receptive to recombinant baculovirus infection. Cultures were infected at an MOI of 3 for the rep-cap and the hSmad7 constructs and incubated for approx. 72 hours. Whole cell cultures were treated with a novel reagent that simultaneously lysed the insect cells and baculovirus, treated with DNase to remove non-encapsidated DNA, and clarified by depth filtration. AAV6 yields were determined by capsid ELISA and dPCR using primers directed to the gene of interest demonstrating high yields in excess of 10^{14} vg/L (Figure 1).

Downstream processes for purification include conventional affinity chromatography followed by enrichment of full capsids by anion exchange. Clarified lysate was applied to an affinity chromatography column consisting of Poros GoPure AAVX resin. Following two washes with increasing NaCl, capsids were eluted with 75 mM citric acid, 10 mM $MgCl_2$ directly into a 1M tris neutralization buffer. Eluate was diluted 1:20 into an AEX equilibration buffer of 20mM bis-tris propane, 10 mM $MgCl_2$, pH 8. The AEX binding and elution conditions were modified from standard AAV enrichment conditions to reduce the binding affinity of AAV6. The higher binding affinity of AAV6 to the AAVX as compared to other serotypes was correlated with loss of genetic payload. Elution was performed with 20 mM bis-tris propane, 10 mM $MgCl_2$, 250 mM sodium acetate. Eluate was exchanged into PBS containing 2mM $MgCl_2$ and sterile filtered. Efficacy of enrichment was determined by calculating the ratio of viral genomes to intact capsids in following AAVX purification and AEX enrichment followed by buffer exchange. The final product contained greater than 90% full capsids, enriching from 26% full capsids following affinity purification (Figure 2). Finally, potency will be discussed with assessments of bioactivity (data pending).

Here we have presented the application of a novel insect expression platform for the generation of high titers of an AAV6 gene therapy vector for treatment of neuromuscular disease. The combination of an upstream process capitalizing on high cell densities with enrichment procedures specific to the AAV6 serotype results in a high yield of vector exceptionally enriched for full capsids.

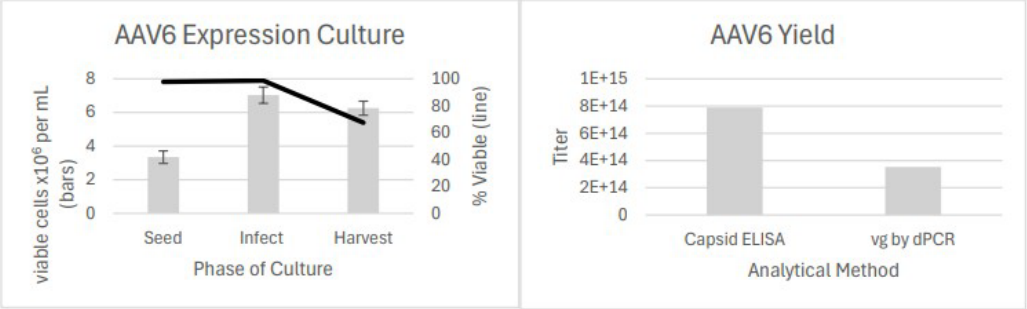

Figure 1. Viable cell density was measured during the course of the production, with harvest at 72 hours post infection and a drop in viability to approximately 65%. Whole culture samples were lysed and assayed for yield as measured by Capsid ELISA and dPCR.

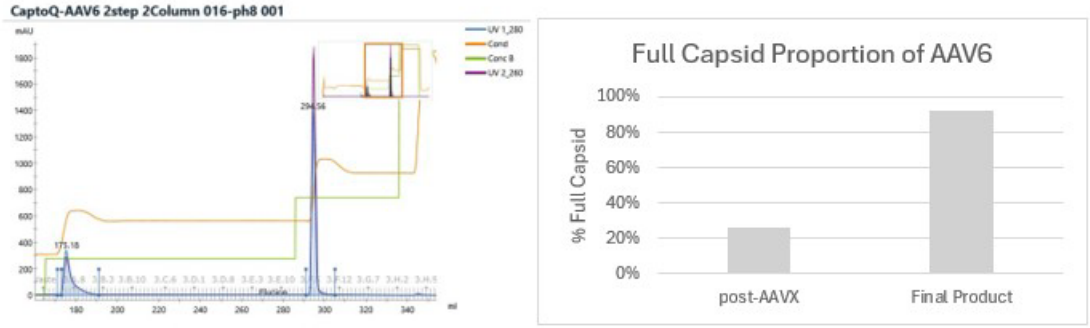

Figure 2. Capto Q AEX enrichment using BTP buffer was set to 15% which favors purity while reducing some yield. Elution was performed using 40% elution buffer which favored capsid stability based on binding strength.

1289 Enhanced Thermostability of a Gorilla-derived Adenoviral (GRAd) vector via E4 region chimerism

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ReiThera has developed a COVID-19 vaccine based on a gorilla adenoviral vector (GRAd-COV2). The vector is replication-defective due to the deletion of the E1 region; two additional deletions have been added in the E3 and E4 regions replacing E4 with human Adenovirus 5's E4 orf6. This backbone is referred to as backbone (c). The GRAd-COV2 (c) vaccine has undergone Phase I and Phase II clinical trials. The GRAd-COV2 (c) vector was initially selected for its high productivity, crucial for rapid vaccine dose production. However, other adenoviral platforms also widely used in clinical settings retain an intact, yet chimeric, E4 region.

Here we show GRAd-COV2 (b2) - that retains GRAd32's E4 orf1, orf2, and orf3, while replacing orfs 4, 6, and 6/7 with those from human Adenovirus 5 - demonstrated superior thermostability, when compared to GRAd-COV2 (c), enabling storage at 2-8°C, which is advantageous for global distribution.

In vitro comparability studies assessed productivity, infectivity, and expression. GRAd-COV2 (b2) exhibited satisfactory productivity, slightly lower than GRAd-COV2 (c) but meeting the necessary criteria for large-scale manufacturing. Infectivity assessments indicated comparable performance between the two vectors, with a marginally better ratio for GRAd-COV2 (b2). Expression analysis via Western blot and FACS showed no significant difference in Spike protein expression levels between the two vectors.

Preclinical studies evaluated immunogenicity in BALB/c mice, with both vectors inducing comparable Spike-binding antibody titers and T cell responses. Toxicology studies in rabbits confirmed that GRAd-COV2 was well-tolerated, with reversible local and systemic effects indicative of an immune response. Biodistribution studies in rats showed that GRAd-COV2 was primarily localized to the injection site and draining lymph nodes, with no significant presence in major organs.

Overall, the GRAd-COV2 (b2) vector demonstrated a good immunogenicity, productivity, and infectivity. In addition, the backbone b2 provide a more thermostable product, a property linked to the possibility of storing formulated vaccine lots at 2-8°C, which is extremely beneficial for transport and storage in different countries worldwide. These important characteristics support the use of this backbone for the Phase I clinical trial for GRAdHIVNE1.

1290 Development of a universal ddPCR based lentivirus infectivity assay

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Introduction: Lentiviral vectors, derived from the human immunodeficiency virus, have been extensively used for gene delivery in eukaryotic cells. In multiple clinical trials, third-generation, self-inactivating lentiviral vectors were used to introduce genes into hematopoietic stem cells to correct primary immunodeficiencies and hemoglobinopathies. Determining the infectious titer of the lentiviral vector samples during upstream and downstream processing is critical for assessing dosing quantities to achieve optimal results. However, several challenges complicate accurate determination of infective titers. These include discrepancies between p24-based viral particle titers and actual infective titers, cell line dependency for performing the assay, sensitivity and variability issues with qPCR-based methods, and inconsistencies in titer measurements caused by variations in multiplicity of infection (MOI). To address these issues, this study aimed to develop a robust, universal droplet digital PCR (ddPCR)-based infectivity assay for reliable lentiviral titer determination, with improved consistency and minimized variability.

Methods: A ddPCR-based infectivity assay was designed using primers and probes targeting the LTR and HIV Ψ regions, with a housekeeping gene as a ploidy control. Although a qPCR method for infectivity was initially developed with the same target, owing to lower sensitivity, data inconsistency, higher reagent cost, and complexity of data analysis, we instead developed a ddPCR based assay using an HT1080 cell line. A control lentivirus from a vendor, with known infectivity titer was used as a reference. Sensitivity, linearity, and range were assessed using serial dilution of a commercial control lentivirus vector (LVV) and compared with internal LVV produced using Catalent off-the-shelf (OTS) packaging, and envelope plasmids, along with model transgene plasmids. A dilution-based variability of infectivity was determined by setting target droplet number cut-off and determining dilutions of the LVV sample and extracted DNA amount. Impact of passage number of HT1080 and source of cell banks was also studied as a variable for controlling the background. The assay was used to evaluate infective titer from several internal model LVV CAR transfer plasmids post LVV production.

Results and Conclusion: The assay dilution range and sensitivity were determined with a commercially obtained lentivirus stock with titer >1e7 TU/ml (Transducing units). The assay performed reliably in the selected dilution range for commercial control, with a %CV of ~20% range as determined from this preliminary study. Infective titer by ddPCR based method was evaluated across multiple batches of upstream, as well as purified lentiviral samples and compared using orthogonal assays (flow cytometry, fluorescence imaging). A Master Cell Bank of HT1080 cell is now in process for use with the assay. The ddPCR-based infectivity assay demonstrated improved reliability and consistency, addressing the previously noted challenges in lentiviral titer determination. The assay has now been successfully adopted at Catalent for routine measurement of lentiviral infective titers during production.

1291 How Far Away are we from Global Regulatory Convergence: Analyzing CAR-T Quality Control Standards Across Six Jurisdictions`

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**Corresponding Author*

Chimeric Antigen Receptor T-cell (CAR-T) therapy represents a breakthrough in cancer treatment prompting regulatory bodies worldwide to establish comprehensive quality control standards safeguarding product safety and efficacy. This analysis compares quality control requirements for CAR-T cell therapies across major regulatory jurisdictions, examining their commonalities and variations, and evaluating potential impacts on patient safety and clinical outcomes. A review of CAR T-cell development and manufacturing documents across six jurisdictions: Canada, Japan, Singapore, Australia, USA and EU were conducted to evaluate commonalities and differences.

This analysis encompassed 12 distinct categories related to CAR T-cell therapy development and manufacturing. Results reveal a complex landscape of regulatory consistency: 41.67% of categories demonstrate high uniformity across agencies, 25% show partial alignment and 33.33% exhibit low harmonization or a lack of comprehensive guidance from some agencies. The FDA CAR-T guidance emerges as the most comprehensive, providing guidance across approximately 90-100% of categories reviewed, while other agencies display varying degrees of coverage with specific focus areas.

Key findings include that 66.67% of agencies recommend site validation and 100% of agencies provide guidance on both autologous and allogeneic products. However, significant inconsistencies exist in areas such as fresh versus cryopreserved products management. Notably, three categories - environmental considerations, Transmissible Spongiform Encephalitis (TSE) risk, and in-vivo versus ex-vivo manufacturing are the least consistently addressed, accounting for 25% of the total categories examined.

This analysis highlights strong alignment in core areas of CAR T-cell development and manufacturing among major regulatory guidance documents. However, it also reveals significant gaps and inconsistencies in newer or more specialized aspects of the field. These findings suggest opportunities for standardization and further guidance development, particularly in emerging areas of CAR T-cell therapy as the science develops.

This analysis emphasizes the critical need for ongoing collaboration among regulatory agencies to address disparities and enhance global consistency in CAR T-cell therapy manufacturing and delivery. Collaboration across agencies will lead to diminishing methodological variability that can impact data quality and comparability across studies and ultimately impact patient safety and CAR-T outcomes. As the field of CAR T-cell therapy rapidly evolves, future efforts should focus on addressing the identified areas of inconsistency and exploring strategies for global regulatory harmonization.

1292 Revolutionizing CAR-T Cell Therapy Manufacturing with AI

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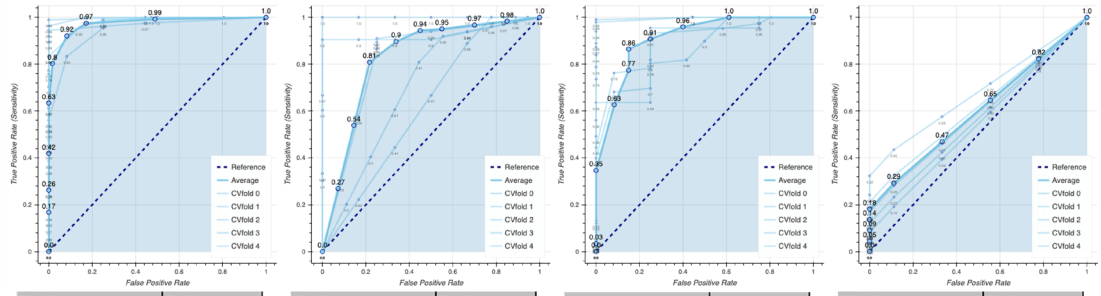
CAR T manufacturing is uniquely complex compared to other treatment modalities. Despite significant advancements in the field, much remains unknown about the intricate processes involved in creating these therapies. Currently, 30% to 60% of patients fail to respond to CAR T therapies, and 30% to 40% of manufacturing runs do not produce enough cells for treatment. We hypothesize that AI-based approaches can offer deeper insights into how manufacturing processes influence cell performance in patients. To address the challenge of predicting cell therapy outcomes, we employed our AI/ML platform, Insights™, which utilizes artificial intelligence and machine learning to optimize and automate key aspects of biomanufacturing. This platform integrates 27 commonly used ML/DL algorithms to build customized models for CAR T therapy. By uncovering critical process parameters, we can better understand how manufacturing impacts patient outcomes. Overall, the Insights™ platform demonstrates significant

potential by efficiently processing large volumes of data generated during development and scaling. This enables faster decision-making and helps identify potential bottlenecks, ultimately advancing the manufacturing process.



Metric	Performance
Accuracy	100%
Balanced Accuracy	100%
ROC AUC	100%
Precision	100%
Sensitivity	100%
Specificity	100%
F1 Score	100%
True Positives	27
True Negatives	5
False Positives	0
False Negatives	0

ADABOOST

Metric	Performance
Accuracy	94%
Balanced Accuracy	88%
ROC AUC	90%
Precision	96%
Sensitivity	96%
Specificity	80%
F1 Score	96%
True Positives	26
True Negatives	4
False Positives	1
False Negatives	1

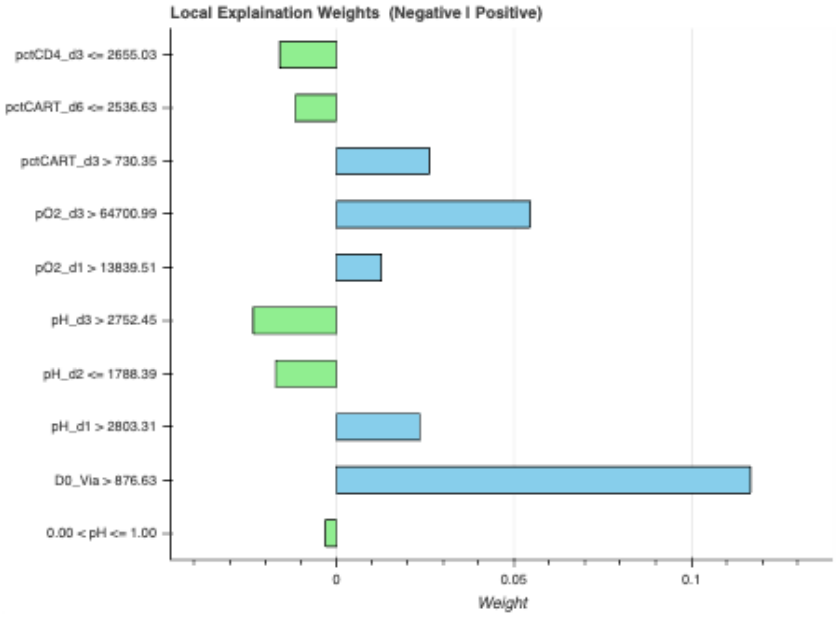
MULTILAYER PERCEPTRON

Metric	Performance
Accuracy	91%
Balanced Accuracy	70%
ROC AUC	88%
Precision	90%
Sensitivity	100%
Specificity	40%
F1 Score	95%
True Positives	27
True Negatives	2
False Positives	3
False Negatives	0

SVC

Metric	Performance
Accuracy	41%
Balanced Accuracy	65%
ROC AUC	65%
Precision	100%
Sensitivity	30%
Specificity	100%
F1 Score	46%
True Positives	8
True Negatives	5
False Positives	0
False Negatives	19

GAUSSIAN PROCESS



1293 Optimizing Stem Cell Collections Using a Hematopoietic Progenitor Cell Apheresis (HPC-A) Collection Calculator Application: Web-App Development and Validation

Claire Shao[1], Maxwell Roth[2]*, Maria Chen[3], Amelia Haj[3], Sanjana Shah[4], Jun Liu[5], Patricia Brunker[3], Kristen Ruby[6]

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Introduction: Hematopoietic progenitor cell apheresis (HPC-A) protocols are designed to minimize the risk of under-collection, resulting in extended collection times, and frequent overcollection. The recent merger of our two academic medical centers (AMCs) presented an opportunity to standardize CD34+ cell collection and potentially address overcollection challenges at both institutions. We developed a HPC-A calculator web-app based on the validated mean collection efficiency (CE2 model), designed to provide patient-specific and standardized estimated blood processing volume recommendations to meet collection goals. In this study, we describe the development of the web-app and validate its performance using simulated data.

Methods: The web-app was developed using the R shiny framework. It is open-access and hosted on the ShinyApps.io platform. Web-app validation was performed using a set of 20 randomly generated simulation patient cases with a range of peripheral CD34+ cell counts (10–200 cells/μL), recipient weights (40–120 kg), target collection doses (2–6 x10^6 cells/recipient kg), and estimated collection efficiency coefficients (0.2, 0.3, or 0.4). To validate the technical accuracy of the web-app, two blinded evaluators calculated estimated processing volumes of the simulated cases using the web-app versus the standard CE2 equation, with >95% concordance set as the passing threshold.

Results: The HPC-A calculator app allows users to input the following parameters: peripheral blood CD34+ cell count (as CD34+ cell percent and WBC count, or direct CD34+ cells/μL), recipient weight (kg), expected CE coefficient, flow rate (mL/min), and y-axis breakpoints for the resultant graphical output. Dynamic input validation ensures adherence to clinical guidelines, such as recipient weight ≥25 kg, CE coefficient between 0 and 1, and flow rates in the expected clinical range, minimizing user errors. The real-time outputs are estimated peripheral blood CD34+ cells per μL, derived from input parameters or provided directly, along with the calculated whole blood volumes (L) required to achieve a specified collection goal under three efficiency scenarios: higher efficiency (HE, +10%), expected efficiency (EE), and lower efficiency (LE, -10%). Additionally, the app generates a plot of CD34+ x10^6 cells collected per recipient kilogram over time given the provided flow rate, plotted across a range of collection durations (0 to 6 hours) and stratified by efficiency scenario. The graphical output features a customizable y-axis (y-axis breakpoints), ensuring that visualization scales are clinically meaningful and adaptable. Simulation validation performed by two blinded evaluators reached 100% concordance, with all web-app predictions matching the manually calculated volumes using the CE2 formula exactly.

Conclusion: We developed and validated a web-based calculator app to standardize CD34+ cell collections at both our AMCs. Our simulation based validation showed perfect concordance between the web-app and the CE2 equations, confirming the technical accuracy of the underlying algorithm. Given the heterogeneity in practice across both AMCs, we hypothesize that strict adherence to the web-app's standardized recommendations will result in decreased over-collection overall, while still meeting collection goals. Future studies will test this hypothesis using both retrospective and prospective cohorts at both AMCs.

1294 Cyclic Olefin Polymer-based Vial Systems for Cell and Gene therapy

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As cell and gene therapy (CGT) drug products are becoming more mainstream, primary packaging consideration for these classes of therapies are evolving and increasingly gaining regulatory attention. When selecting a primary packaging solution, it is imperative to understand the critical quality attributes of an ideal containment system, including low particulate levels, container closure integrity (CCI), ice nucleation, drug-material compatibility, and ease of fill-finish.

Particulates come in many forms. Unlike traditional biologics, the active ingredients of CGT products (e.g., viral vectors, cells) are considered particles themselves. On the other hand, particulates other than the intended active ingredient, which can be of visible and subvisible sizes, or be of intrinsic (e.g., cellular debris) or extrinsic origin

(e.g., environmental contaminants, raw materials, consumables), can accumulate in the drug product from manufacturing through cold chain all the way to the point of care. Using light microscopy and flow imaging microscopy, cyclic olefin polymer (COP) vials were found to have similar or fewer subvisible particles, compared to ISO standard glass vials, polypropylene (PP) screw cap vials and ethylene vinyl acetate (EVA) bags, with or without freeze-thaw, upon ultra-cold (-80°C) or cryogenic (≤ -150°C) storage.

Product sterility depends on the primary container being able to maintain CCI. Containers with proper CCI prevent the ingress of low-temperature gasses, over pressurization of the container, and consequent hazard to its handlers. Oxygen headspace analysis and Helium leak test revealed that COP vials can maintain CCI upon proper crimping after short-term (1 week) or long-term (24 month) cryogenic storage.

Ice nucleation is a critical process parameter for the cryopreservation of biological materials composed of sensitive membrane structure. Controlling ice nucleation within a tolerable range of temperatures and time is important to avoid loss of the high-value material and its potency. Our study presented a new method of measuring ice nucleation that captured a greater extent of critical process deviations in a be packaged aqueous system than the conventional method.

Drug-material compatibility is an important aspect to protect the drug product integrity as any interaction could result in its degradation resulting in reduced potency and/or generation of harmful by-products. Here we show the compatibility of COP vials with different cell types which upon cryo-storage for short-term showed optimal functionality in different tested assays.

Additionally, COP based Daikyo Crystal Zenith(R) (CZ) vials are packaged in a nested tub configuration and designed to meet selected exterior dimensions for ISO glass standard 8362-1, are sterile, ready-to-use offer broad compatibility with automated and flexible fill-finish systems for CGT drug products as well as other advanced therapies.

We hereby emphasize the importance of primary packaging for CGT manufacturing and demonstrate the advantage of COP vials as a primary packaging system in the various technical considerations. The compatibility of CZ vials with different cell types along with its availability in different sizes and packaging formats offers a convenient and efficient containment solution for small batch fill-finish operations for CGT applications.

1295 Autofluorescence Imaging Reveals the Impact of Cryopreservation on T Cell Metabolism and Activation Response

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Introduction: Cryopreservation, which involves using low temperatures (-80°C and -196°C) to slow down cell metabolism and other cellular functions, is central to cell therapies such as Chimeric Antigen Receptor (CAR) T-cell therapy. The complex cell manufacturing process requires specialized facilities and expertise, which necessitates a central manufacturing model that relies on cryopreservation of both the starting materials and final products. However, recovering cell viability and function post-thaw remains challenging. Additionally, the impact of cryopreservation on cell metabolism and function immediately after thawing is not fully understood. Since T cell metabolism is closely linked to function, we used label-free Optical Metabolic Imaging (OMI) of metabolic coenzymes NAD(P)H and FAD to assess the metabolic and functional responses of frozen T cells upon thawing and activation.

Methods: Primary T cells were isolated from three healthy donors and divided into fresh and cryopreserved groups. Fresh T cells were cultured overnight in ImmunoCult XF medium, while frozen T cells were cryopreserved in FBS + 10% DMSO at -80°C for 24 hours before thawing. Donor-matched fresh and frozen T cells were activated with αCD2/αCD3/αCD28 antibodies and imaged with OMI every hour up to 4.5 hours post-activation.

Results and Conclusion: We observed stable metabolism in fresh quiescent T cells throughout 4.5-hour imaging time course (Fig 1A, dashed lines). In contrast, cryopreserved quiescent T cells showed significant increases in NAD(P)H mean lifetime (τm) immediately post-thaw up to 4.5 hours (Fig 1B, dashed lines). Fresh activated T cells demonstrated rapid and significant metabolic changes, characterized by decreased NAD(P)H τm within 30 minutes after activation. However, metabolic differences between cryopreserved quiescent and activated T cells were not observed until 2.5 hours (Fig 1A-B, solid lines). Additionally, Glass's Delta analysis between quiescent and activated groups revealed that frozen T cells exhibited smaller activation responses (D=-0.7 - 0.7) compared to fresh T cells (D=-2.8 - 3.0). UMAP projections based on OMI parameters showed distinct clusters for fresh quiescent and activated T cells, indicating significant metabolic differences post-activation (Fig 1C). Frozen T cells, however, did not cluster by activation status (Fig 1D). We also observed significantly higher levels of IFN-γ secretion by fresh T cells after 4.5 hours of activation compared to donor-matched frozen T cells. Overall, using label-free OMI, we have characterized significant metabolic shifts in T cells immediately post-thaw, along with a delayed and diminished activation response in cryopreserved cells. Our results highlight the need for optimized cryopreservation and thawing protocols to improve T-cell functionality in therapeutic applications.

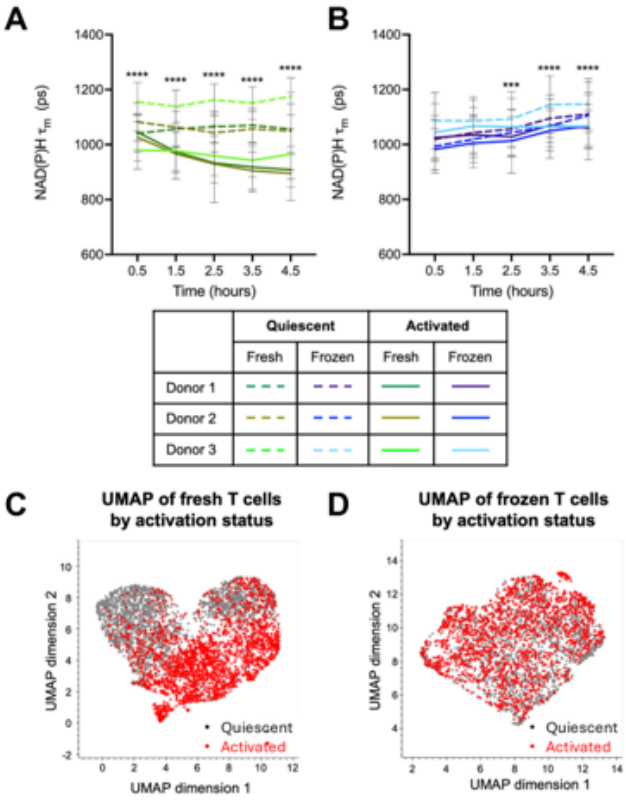

Figure 1. Cryopreservation delayed and diminished activation response in frozen T cells upon thawing. (A-B) Quantification of NAD(P)H τ_m of fresh **(A)** or frozen **(B)** T cells from 3 independent donors. Statistical tests were performed on quiescent versus activated groups. **(C-D)** Uniform Manifold Approximation and Projection (UMAP) of 11 OMI parameters (NAD(P)H and FAD τ_m, τ_1, τ_1, α_1, α_2, and cell size) of **(C)** fresh and **(D)** frozen T cells from 3 donors, color coded by activation status.

1296 Off-the-Shelf GMP-Compliant iPS Cells for Application in Cell Therapy

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**Corresponding Author*

Cell and gene therapy has evolved as an important instrument for the treatment of many life-threatening diseases, including various types of cancer. As a global CDMO, Catalent aims to support cell and gene therapy strategies for the treatment of patients. As such, we enable fully integrated cell therapy services, from manufacturing, banking, and differentiation of induced pluripotent stem cell (iPSC), as well as development and manufacturing expertise, to process and analytical development across various cell types.

To support cell therapy services, we have developed a new process for the banking of GMP-compliant iPSCs that relies on the expansion and cryopreservation of single cells rather than cell clusters, rendering the process significantly more robust and fully controllable. Building on those iPSC lines, we have further developed downstream protocols for the differentiation of iPSCs into multiple cell types, including mesenchymal stem cells (MSCs), retinal pigment epithelium cells (RPEs), cardiomyocytes, and different types of immune cells such as NK cells, T cells and monocytes/macrophages.

Based on our new process we have generated three off-the-shelf iPSC banks of German and American origin, with full compliance with FDA requirements. These iPSC lines are comprehensively tested to ensure their identity, safety, and reliability. Testing protocols included, but were not limited to, comprehensive safety testing involving sterility, mycoplasma, and endotoxin testing according to US Pharmacopoeia, as well as extensive viral screening using various test methods like RNA sequencing and virus-specific PCR. Successful differentiation of our iPSC lines into MSCs, RPEs, cardiomyocytes, and immune cells has demonstrated full pluripotency of the iPSC lines, indicating the great quality and reliability of our innovative iPSC banking process. As a result, these iPSC lines are readily available for use and can be conveniently accessed off-the-shelf, facilitating research and therapeutic applications.

1297 A rapid and cost-effective solution for CAR- T cell manufacturing

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Introduction: The development of rapid and low-cost cell therapy products represents a significant advancement in the field of cell therapy. The rapid CAR- T cell manufacturing process minimizes cell differentiation and exhaustion, thus produces cells with higher potency and higher anti-tumor activity. This innovative process will benefit from streamlined production of therapeutic cells utilizing new technologies and tools, such as automated cell culture systems and efficient cell separation methods and adopting cost-effective materials and scalable production strategies to ensure the economic viability of the cell therapy manufacturing. In this report we demonstrate a novel automated closed cell manufacturing platform, MARS® Atlas, being an end-to-end solution for rapid CAR-T cell manufacturing by completing cell isolation, viral transduction and cell purification/formulation within 72 hours. This solution is intended to revolutionize the traditional CAR-T manufacturing paradigm and enable the delivery of potent, patient-specific therapies at near patient “point-of-care” settings.

Methods and results: Step 1: Fast and economic cell separation. A 1/10 leukopak containing 6E8 cells was incubated with CD4/CD8 magnetic beads at room temperature for 30 minutes. Then, the cell suspension was diluted and pumped through the separation channels on MAG modules for separation. The process resulted in a recovery of 60% T cells with 96% purity. The percentages of CD4 and CD8 cells remained consistent before and after separation. Step 2: Activation and transduction with low-seeding cells. With the advent of the rapid CAR-T process, the clinical dosage for each patient typically ranges from 1E6 to 2E7 CAR-T cells (1.2). Considering the transduction efficiency and QC need, a total of approximately 6E7 cells are needed. In this study, 3E6 T cells were seeded in the "Transduction module," which contained complete T-cell medium supplemented with CD3/CD28 magnetic nanobeads, IL-2, IL-7, and IL-15, and were incubated at 37C with 5% CO2 for 24 hours. The cells were successfully activated, exhibiting high expression of CD25 and CD69. Subsequently, the CD19 CAR lentivirus was added for an additional 24 hours. A phenotype with CCR7 expression reaching 90% is observed. Step 3: Unique Magnetic Harvest. In this method, the final products undergo further purification and formulation through the third MAG module. Notably, our approach demonstrated > 80% cell recovery, >99% virus removal, and completion in 30 minutes.

Function assessment: To assess the functionality of the CAR-T cells produced on MARS Atlas, killing assay and potency assay were performed and results were comparable to CAR T cells manufactured by conventional process. Repeated stimulation experiments demonstrated the long-term proliferation and persistence of rapidly generated CAR-T cells, which effectively exhibited tumor-killing capabilities, evidenced by the progressive reduction in target tumor cells over time. Furthermore, fewer Atlas-harvested cells were required to achieve the same level of tumor cytotoxicity as those produced through the conventional CAR-T process.

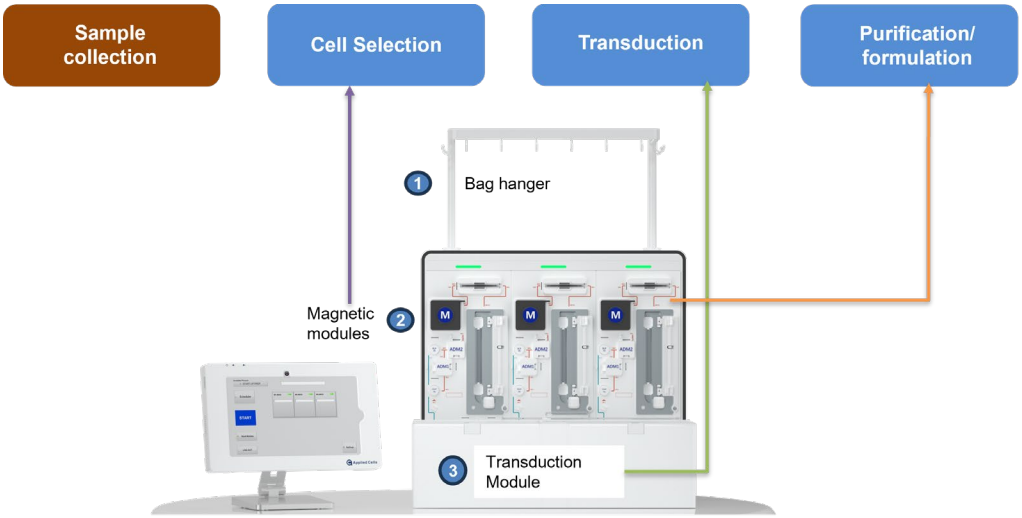

Figure 1: Schematic illustration of the workflow of rapid CAR-T cell manufacturing on Mars® Atlas. The single platform for automated rapid CAR- T manufacturing. Cell selection and purification/formulation are done on the magnetic modules and transduction step is done in the transduction module. All steps are done in closed fluidics and controlled by software.

1298 Development of Low-Cost CAR-T Process Street with Improved Manufacturing Success

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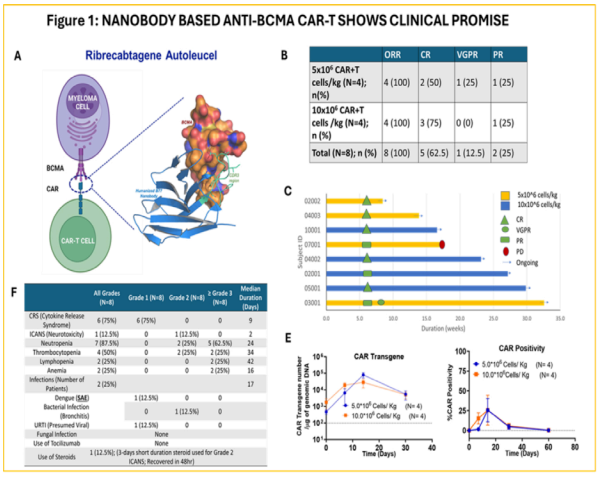
Chimeric antigen receptor (CAR) T-cell therapy utilizes the immune response by modifying T cells in a laboratory setting. These engineered T cells are designed to efficiently locate and destroy cancer cells. CAR T-cells have demonstrated remarkable efficacy in treating multiple hematologic malignancies, including B-cell ALL, multiple subtypes of lymphoma (including the two most common types, diffuse large B-cell lymphoma and follicular

lymphoma), and multiple myeloma. However, any therapy is useful only to those who can access them. The patient-tailored nature of autologous CAR T-cell therapy raises two major concerns, the challenges in manufacturing using highly fragile patient cells and the cost of production per batch. Cellular and gene therapies are among the most expensive therapies on the market, typically over $400,000 USD. Manufacturing constraints have also been well documented for many approved CAR-T therapies, with complaints from doctors about the drug's long waiting list and relatively high rate of production failures.

A Phase 1 clinical trial, SWASTH, (CTRI/2023/11/059795 [Registered on: 14/11/2023]) of an anti-BCMA CAR-T in patients with r/r MM has been completed (n = 8) and the results show that a single dose of DRL-1801 (2.0–10.0 × 10^6 CAR+ T cells/Kg) administered by IV infusion in r/r MM patients is effective and safe, with an overall response rate of 100%, indicating that DRL-1801 may provide significant benefit to patients with r/r MM (Upload 01). However, manufacturing failures were encountered during this Phase I trial, accounting to about 20%. Literature also suggests manufacturing failures in similar range (Blood Adv (2024) 8 (2): 337–342; Meeting Abstract: 2024 ASCO Annual Meeting I). Each manufacturing failure entails cost, longer wait time or no treatment for patients, and a reputational damage for the sponsors. For a developing country like India, where the population is large, cancer burden is surging and CGT therapies are inaccessible and/or unaffordable for many, we decided to have a focussed endeavour at developing cost effective, scalable and robust manufacturing processes for CAR based cell therapies.

The modified manufacturing process for CAR-T cells is a semi-automated, modular Process Street design that involves a manufacturing cost that is significantly lower than in the Western world (Upload 02). We have well defined Manufacturing lines involving Automation for Cell Processing and T cell Purification, G-Rex Bioreactors are used for efficient T cell activation and transduction using an indigenously developed procedure (Patent Application No. 202541001697) and CAR-T cell expansion performed within next 3 days (Upload 02). This process involves minimal intervention with less resource need, reduces the chances of compromised sterility and generates CAR-T cells with better stemness. Also, rapid manufacturing for 5 days helps assess CAR protein expression reliably and does not necessitate additional monitoring for toxicities in clinical practice. Initial data using this new Process reveals successful manufacturing from multiple patient material, with well proven Quality standard of the released batches. Our preliminary data indicate that this robust and cost-effective process can also be implemented across multiple CAR-T products in a “plug and play” mechanism.



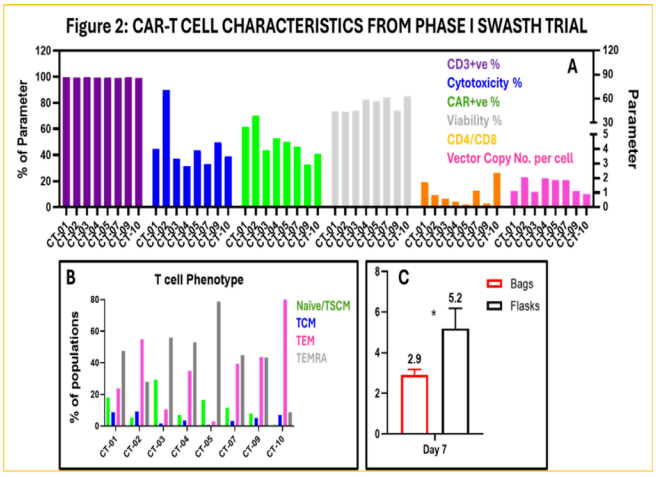


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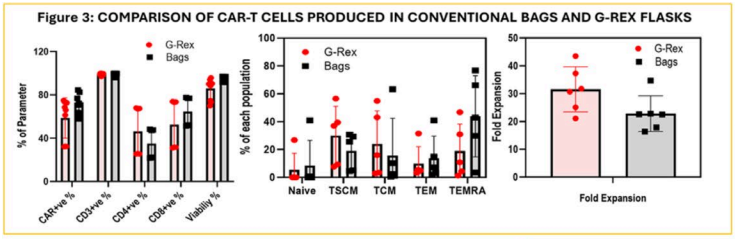
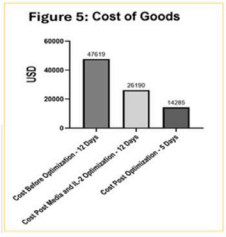
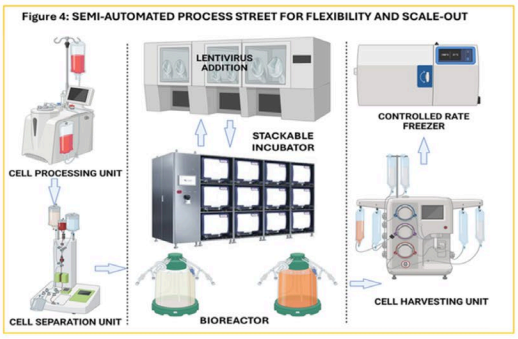
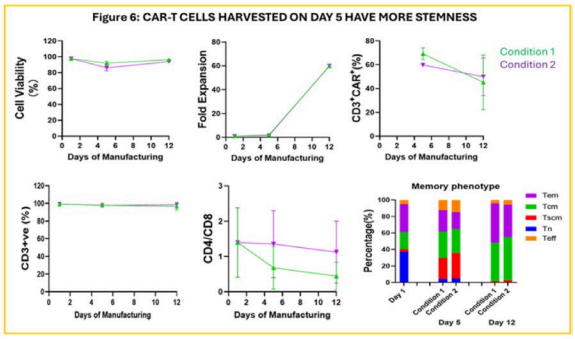
Figure 01. Promising Clinical Data (ORR = 100%) from 8 patients dosed as part of SWASTH Phase – I trial.

Figure 02. A CAR-T cells manufactured in SWASTH Phase – I trial show good purity, CAR+vity and functional potency. B. CAR-T cells manufactured for 12 days show a balance between memory and effector phenotypes. C. Proliferation of patient samples are often compromised in conventional bags when compared to flasks.









Upload. 02
Figure 03. CAR-T cells cultured in G-Rex Flasks and Bags show similar phenotypic characteristics, except for fold expansion, which is significantly higher in G-Rex flasks at 12 days of harvest
Figure 04. Process Street Design using G-Rex flasks stacked in compartmentalized incubators for easy scale-out and minimum human intervention
Figure 05. Cost of manufacturing and release is significantly lowered using optimized process
Figure 06. CAR-T cells on Day 5 have more stem cells

1299 *Challenge to Develop a GMP-compliant Integrated Manufacturing Process for Novel CAR-T Cells Using a Closed and Single-use Automated Culture System at Academic Gene and Cell Processing Facility*

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The effectiveness of gene-modified cell therapy with chimeric antigen receptor T cells (CAR-T) has been widely recognized as a promising modality in clinical applications. The manufacturing process for CAR-T cells is complex and requires advanced technology. A development of manufacturing process with stable quality for clinical trials and establishing infrastructure of manufacturing platform are becoming increasingly important. Therefore, we attempted to develop a manufacturing process for GMP-compliant CAR-T cells using a closed and single-use automatic cell culture system for clinical trials at academic gene and cell processing facility.

As a result, drawing 150 mL of peripheral blood as a raw material, through the main steps of cell separation, gene transduction to the cells, cell expansion, and concentration and filling (packaging), a final 6.5 to 7.5 x E+8 CAR-T cells (survival rate: >95%, transduction efficiency >18.5%) were stably obtained. We also attempted to optimize the process parameters to aim more long-term persistent CAR-T, and as a result, the T cell subsets analysis of Tn, Tscm, Tcm, Tem of the CAR-T products before and after system process optimization showed 0, 2, 75, 21 v.s. 0.05, 6.9, 55.5, 37.5, respectively.

It was demonstrated that GMP-compliant CAR-T cell manufacturing as qualified investigational drug for gene and cell therapy was demonstrated to work effectively in academic CPC. Our findings also reveal that robust CAR-T subsets of Tscm expansion could be obtained, suggesting even higher quality CAR-T cell production was feasible through optimizing the existing system program.

1300 Automating iPSC Processing and Engineering for Allogenic Cell Therapies: The Role of Closed Modular Systems.

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Induced pluripotent stem cell (iPSC)-derived natural killer (iNK) cells have emerged as a promising platform for next-generation immunotherapy, offering a homogeneous, scalable and versatile approach for consistent large-scale manufacturing of off the shelf allogeneic therapies. This approach involves multiple steps including iPSC culturing and banking followed by gene editing and differentiation to tumor specific iNK cells to enhance cytotoxicity, persistence, and tumor-targeting specificity, while minimizing risks of graft-versus-host disease. Building closed modular automated workflows will help minimize risks associated with manual processes associated with these methods. Through this work we built closed automated cell harvest, cell wash and buffer exchange, gene delivery and editing protocols that can enable iNK-based cell therapy manufacturing.

In our current workflow, we cultured and expanded iPSCs up to a billion cells in a 10-layer cell factory system for master cell bank preparation. iPSC manual process and harvest in 10-layer cell factory system is very labor intensive and prone to contamination. Utilizing CTS Rotea counterflow centrifugation system minimized human intervention at multiple stages, including removal of media, washing of cells, addition of cell detachment media, collecting, concentrating iPSCs and delivering cells to collecting bags. Using this protocol, we processed the harvesting of entire iPSC culture in 10-layer cell factory system in a single batch to create a master bank for cell therapy development. The iPSCs prepared using this method retain pluripotency characteristics with good viability and expansion rate. We then successfully used these iPSC banks and carried out CRISPR-based non-viral gene editing using Neon NxT or CTS Xenon closed automated electroporation system for gene delivery, GMP grade CTS™ StemFlex media and high fidelity CRISPR-Cas9 system. Results show successful generation of engineered CAR-iPSC with reproducible KI efficiency of up to 15%. We established the optimal target gene and promoter combination to stabilize transgene expression during the differentiation of iPSCs to iNK cells. With methods developed through this work, we successfully generated potent CAR iNK cells. Together the workflows described here utilizing the clean room compliant closed automated cell processing and gene delivery platforms and the GMP compatibility iPSC and NK media systems enable clinical scale iNK cell therapy manufacturing.

1301 Automated Manufacturing of T cells for Therapy by LNP-Engineering

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Adoptive T cell therapies such as chimeric-antigen receptor (CAR)- or T cell receptor (TCR)-engineered T cells are powerful medical tools, as shown for the treatment of hematological malignancies. However, giving patients in need a widespread access to these technologies is still limited by the complexity of manufacturing and associated costs. Lipid nanoparticles (LNPs) have the potential to be established as pivotal advancement for gene-delivery for adoptive T cell therapy, with a cost-effective, safer and comparable or even better performance compared to lentiviral vectors or electroporation. This study focusses on the specific requirements for scaling the production of LNP-engineered T cells in an automated, closed and Good Manufacturing Practice (GMP)-compliant process using the CliniMACS Prodigy. Based on small scale studies assessing transfection conditions and kinetics, we optimized the transfection protocol reaching up to 98% gene-modified, highly viable (>90%) engineered T cells with a favourable, early memory phenotype. In a second step, we adapted the procedure for a scaled process. Starting from cryopreserved leukapheresis, 1-2 E8 T cells were enriched, subsequently activated and transfected using LNPs. Using an optimized pre-defined activity matrix defining the chronological order of all process steps, we cultured the T cells for 8 days yielding clinical relevant T cell numbers. Transfection efficiency (up to 93%) and viability (>90%) was comparably high as in small scale controls using the same donor. In conclusion, this platform and process

robustly and reproducibly resulted in clinically relevant doses of high-quality cell products and thus have the potential to greatly expand patient access to T cell therapies.

1302 Toward the Development of an Automated Manufacturing Process for Haematopoietic Stem Cell Gene Therapies

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Haematopoietic Stem Cell (HSC) Gene Therapies (HSC-GT) are a powerful technology to address a range of severe genetic diseases. This approach consists of delivering autologous gene-corrected HSCs, following *ex vivo* genetic modification with viral vectors, thus providing potential for long lasting therapeutic benefit with a single administration.

However, challenges in manufacturing could potentially limit the application of HSC-GT to treat more diseases. Manufacture of many cell and gene therapies uses processes that have been established over the last decades, to support investigative treatment in a small number of patients. These historical processes are often highly manual, include multiple open steps, and therefore require highly trained operators and high-grade clean rooms.

Here we aim to address some of these manufacturing limitations by developing a closed and semi-automated manufacturing process for ex vivo HSC-GT. The Miltenyi CliniMACS Prodigy provides an integrated platform for enrichment, viral transduction and culture in a closed system. We have established a flexible process that can reproducibly produce gene-modified HSCs. Most product Critical Quality Attributes were similar to those from a standard manual process, e.g. viability, phenotype and clonogenic potential. Importantly however, measures of potency such as transduction efficiency and vector copy number were improved (by 23% and 86%, respectively), whilst reducing the requirement of vector by 50%.

After transduction, harvested cells are washed, formulated and filled into cryobags. Another cell processing system, the Cue (Fresenius Kabi), has been evaluated to automate these unit operations and again compared to a manual process. The Cue gave similar results to the manual process but with improved operational efficiencies due to automation, such as standardisation, reduced DMSO contact time and electronic records. Furthermore, the Cue was shown to effectively wash cells as demonstrated by the high cell recoveries and viability (>85% and >88%, respectively) and the efficient removal of process residuals, as shown in cytokine spiking studies.

Encouraging results have been obtained with the technologies tested, significantly reducing the hands-on operator and overall processing time, and with comparable or improved product attributes compared to existing processes. Therefore, these cell processing devices could be considered for use in next-generation HSC manufacturing platforms.

1303 Lonza Cocoon® Platform Demonstrate Compatibility with Off-the-shelf Process Analytical Technology (PAT) and Electroporation Systems for Closed and Automated In-process CPP Monitoring and Genetic Modifications.

Peter Szaraz[1], Salvador Flores-Torres[1], Smitha Mathews[1], Ashkan Tabibian[1], Hosna Hadavi[2], Michele Vicentini-Hogan[3], Chad Ibbotson[1], Carlene Petes[1], Nuala Trainor[2]
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Introduction: In-process monitoring of critical process parameters (CPP) is a universal requirement and often cost-driving feature of cell manufacturing. Solutions for closed, automated CPP monitoring are in high demand, yet the

successful integration of analytical capabilities as PATs into the cell production workflow is challenged by the limited versatility of bioreactor platforms. Non-viral genetic modification processes apply external equipment (e.g. Nucleofector® Systems) that requires manual fluid transfers. Ideally a bioreactor platform should provide reliable, closed and automated transfer of target cells.

Methods: 908 Devices MAVEN Systems: Proximal diffusion module and distal sensor/transmitter module were integrated at-line with Cocoon® Platform fluidics. Glucose/lactate readings were obtained from day 1 to day 10 of the Cocoon® Platform cell expansion process. Reference readings were performed by manual sampling and BioProfile® FLEX2 analyzer (NovaBiomedical).

Ovizio at-line prototype system: Quantitative Phase Imaging (QPI), label-free, next gen cell analyzer prototype based on the Ovizio iLine F PRO, connected via reusable connectors to the Cocoon™ platform with a disposable sampling kit containing an optical chamber. Automated cell suspension transfer from bioreactor cultures at day 1, 3, 6 and 9 of the T-cell expansion process. Reference reading were performed by manual sampling and NucleoCounter® NC-200™ (Chemometec).

Lonza Nucleofector® System: Cell suspension transfer of 100 to 500 E6 cells from the Cassette to the proximal LV-reservoir was executed by Cocoon® Platform automated fluidics via reusable sampling connectors. Nucleofector® System closed, controlled fluidics filled LV-cartridge for electroporation and returned the transfected cells to the Cassette. Cell counting was performed by NucleoCounter® NC-200™ (Chemometec). Results: 908 Devices MAVEN systems: At-line glucose readings from culture media were <10% relative error (n=5) compared to reference throughout the entire process. Lactate measurements were <10% relative error (n=5) compared to reference over 1mg/mL media lactate content. Ovizio at-line prototype system: Cell counting relative error was <10% of manual reference when operating in recommended range (≤25x106 cells/ml). Total cell count RE≤8.6%; Viable cell count RE≤6.7% Lonza Nucleofector® System: Viable cell transfer efficiency between Cocoon® Platform and Nucleofector® System unit was 94%+/-3% (n=19)

Conclusions: The presented results demonstrate:
High fidelity, non-invasive in-process sampling of Cocoon® Platform bioreactor and precision delivery of small, representative samples to external devices in a closed and automated manner. Proven compatibility to establish at-line connection with off-the-shelf process ananlytical devices (PTA) to functinally integrate CPP monitoring into the Cocoon® Platform bioreactor process. Precision delivery of cell suspensions formulated in the Cocoon® Platform for genetic modifcation in at-line integrated electroporation unit (Lonza Nucleofector® System).

1304 Perfusion-Powered Production of Over 100 CAR-T Doses in a 2-litre Stirred-Tank Bioreactor With Automation of Downstream Harvest and Concentration Steps

Rukmini Ladi[1], Pierre Springuel[2], Fern Slingsby[3], Nicola Bevan[3], Amanda Frangleton[3], Michal Szelwicki[3], Timo Schmidberger[4], Asma Ahmad[5], Earl Pineda[5], Rachel Legmann[6], Rene Gantier[7], Noushin Dianat[8], Julia Hengst[9], Qasim Rafiq
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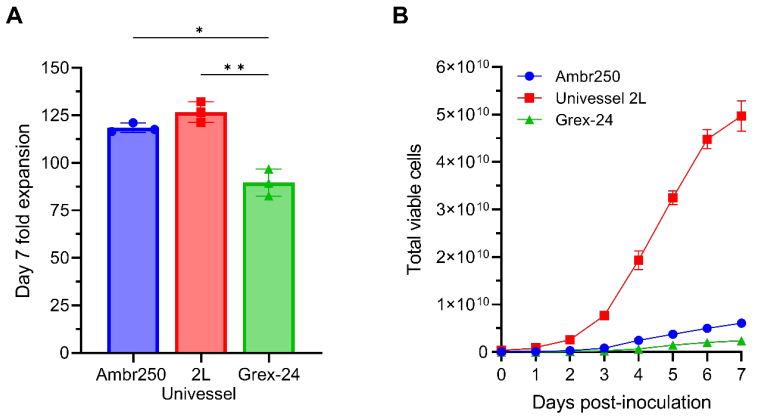
Introduction: Allogeneic CAR-T therapies offers the potential to mass produce "off-the-shelf" doses, addressing the limitations of lengthy manufacturing processes in approved autologous products. To realise this potential, industrially scalable upstream and downstream workflows are required. To this end, we characterised a CAR-T cell perfusion manufacturing process in a 2-litre stirred-tank bioreactor (STR), with a solution to automate the bioreactor harvest, final product wash, and concentration steps.

Methodology: Healthy donor-derived anti-CD19 CAR-T cells were cultured in parallel in the Ambr® 250 High Throughput Perfusion and Univessel® 2-litre single-use STR, the latter coupled with the XCell ATF® 2 cell retention device. CAR-T cells were expanded in 4Cell® Nutri-T serum-free medium in perfusion for seven days,

and performance was benchmarked against a G-Rex® 24 well plate process. The Ksep® 400 was evaluated as a closed, automated solution for the harvesting, concentration, and washing of the final CAR-T product from the 2L STR. Comparability between the 250mL and 2L systems was examined, alongside the impact of automated harvesting on cell yield, viability, activation, maturation, exhaustion, cytotoxicity, and cytokine release.

Results: CAR-T cells were successfully expanded ~125-fold in perfusion in both 250mL and 2L STRs. At the2L scale, final yields of ~50 billion total viable cells were consistently generated, with a CAR transduction efficiency of 34%, corresponding to over 100 representative doses of 150x10 CAR+ T cells. Harvested cells were predominantly in the naive and central memory subset (>95%) and expressed negligible levels of exhaustion markers (<1%). No statistical differences were observed in CAR-T cell yield, viability, surface marker expression, or cytotoxicity between the 250mL and 2L bioreactors. Automated harvesting recovered>90% of the total yields, concentrated cells 8-fold, and had no impact on CAR-T cell phenotype, cytotoxicity, or growth kinetics post-harvest.

Conclusion: These findings demonstrate the feasibility of multi-litre expansion of functional CAR-T cells to generate hundreds of doses in perfusion-based stirred-tank bioreactors. The Ambr® 250 effectively served asa scaled-down model and facilitated seamless scale-up to 2L. Finally, automated harvesting and concentration streamlined the downstream workflow while maintaining CAR-T cell quality. This scalable upstream and downstream workflow is ideally suited for mass production of future allogeneic therapies.



A) CAR-T cell fold expansion in perfusion is comparable between the Ambr® 250 HT Perfusion and Univessel® 2-litre stirred-tank bioreactors, significantly outperforming the G-Rex®24 well plate process. **B)** Perfusion in the 2-litre bioreactor enables final cell yields exceeding 50 billion viable cells. Data represents mean of n=3 replicate runs.

1305 Improving CAR T Cell Production: Comparing Stirred Tank Bioreactors and Static Vessels

Mina Ahmadi[1], Yongchang Ji[2], Max Dotson[3]
[1]Thermofisher Scientific, Carlsbad, CA, [2]Thermofisher, Carlsbad, CA, [3]Thermo Fisher Scientific, Carlsbad, CA

T cell activation and transduction are critical steps in the production of autologous CAR T cells, where primary T cells are genetically modified to express chimeric antigen receptors (CARs) for targeted tumor treatment. Traditionally, these processes have been performed in static culture systems, which require significant manual intervention and are subject to variability in cell expansion and product quality. This has led to challenges in scaling up the production of CAR T cells for clinical applications. Stirred tank bioreactors (STRs) offer a promising solution for automating and streamlining the T cell activation and transduction processes. By integrating both the activation and transduction steps into a single, closed system, STRs minimize the need for manual handling and reduce the risk of contamination. In our study, we compared the simultaneous activation and transduction of primary T cells in STR bioreactors versus G-Rex vessels. Our results demonstrate that activation and transduction levels are similar across both platforms; however, STR bioreactors led to significantly better expansion of CAR-T cells without affecting the

early memory phenotype composition. Our data suggest that, by leveraging STRs in the CAR T cell workflow, the industry can meet the growing demand for personalized immunotherapies while improving efficiency, reducing costs, and advancing automation in cell therapy production.

1306 Scalable and Controlled Expansion of Genetically Engineered CAR-T cells in Stirred-Tank Bioreactors

Max Dotson[1], Mina Ahmadi[2], Yongchang Ji[3]
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Chimeric antigen receptor T cells (CAR T) have emerged as a promising therapy for hematologic malignancies. To achieve successful CAR T manufacturing, automation, closed operation, improved control, and scalability are crucial. Bioreactors provide advantages in cell therapy manufacturing, offering a controlled and optimal environment for cell growth. While rocking motion bioreactors have been widely used for CAR T cell expansion, stirred-tank bioreactors (STRs) have gained attention due to their scalability, precise gassing and agitation control, and smaller footprint. Previous studies have shown enhanced growth of activated T cells in STRs compared to rocking motion or static cultures. However, it remains unclear whether genetically engineered CAR T cells can be cultured in STR platforms and maintain CAR expression and functionality.

In this study, we integrated STR platforms into an end-to-end closed and automated workflow to expand non-virally generated CAR T cells. Our investigation focused on tracking cell growth kinetics and functionality attributes. We observed higher growth and viability of CAR-T cells in STRs compared to static bioreactors; notably, engineered T cells expanded in STRs maintained high expression of the CAR gene and exhibited improved cytolytic function compared to those expanded in static bioreactors.

The findings showcase stirred-tank bioreactors as an improved platform for *ex vivo* culture of engineered T cells, enabling the maintenance of CAR expression and enhanced cytolytic function. This highlights the significance of integrating automation, closed operation, and enhanced control in CAR T cell manufacturing to optimize therapy outcomes and meet the rising demand for effective cell-based immunotherapies.

1307 Scaling CAR-T Manufacturing: Redefining Large Volume Fill-Finish with ARES X20+ Automation

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The final fill-finish step of cell and gene therapies (CGT) manufacturing is critical to ensuring drug product (DP) integrity and consistency. Manual fill-finish of DP is time consuming, requires skilled operators, and introduces considerable risk to meeting regulatory requirements of sterility, uniformity, and efficacy. It also limits DP batches to small volumes, preventing the scaling of DP to larger patient populations. Closed automated fill-finish systems address these risks and scaling issues by decreasing manual process steps, improving process control and the precision of formulation and filling, thereby decreasing process time overall and improving cell viability and DP efficacy. This study assessed the capabilities of the ARES X20+ closed automated fill-finish system to fill both vials and bags and maintain T-cell product viability and efficacy using enriched, CAR-T transduced, CD4+ and CD8+ T cells from n=2 donors. The ARES X20+ system was configured to run a process with specific parameters designed to test various features of the equipment while analyzing the impacts on the health of the CAR-T cell product. For this study, to illustrate the range of fill volumes by the ARES X20+, two disposable manifolds were sequentially used for each donor: Manifold 1 contained 15 bags (30 mL fill vol.) and 5 vials (3 mL fill vol.); Manifold 2 contained 20 vials (10 vials with a 5 mL fill vol. and 10 vials with a 1 mL fill vol.). T-cell product was continuously cooled and mixed during the operation, flow rates were adjusted higher for bag filling, lower for vials with filling, and set to be slightly above target volume to ensure its attainment. The ARES X20+ filling duration and filling

accuracy of bags and vials by the ARES X20+ were compared to manual filling. Manual filling used standard cryo vials and cryo bags. Time assessments of filling included the ARES X20+ line purges and air evacuation of bags before filling and removal of air post fill of bags for both ARES X20+ and manual processes. The ARES X20+ filled Manifold 1 bags and vials 8x faster than manual processes and filled Manifold 2 vials 2x faster. Manual bag filling had an 18-fold higher variance compared with bags filled using the ARES X20+. Manual filling of 1 mL and 3 mL vials had 1.2 -fold and 3.5-fold, respectively, higher variance compared to ARES X20+. For 5 mL, the ARES had a 15.3-fold higher variance compared to manual processes, which reflected a median filling at 5.3 compared to 4.95 for the manual process. To test the health of the T-cell product after ARES X20+ fill-finish, ARES X20+-filled bags and vials were cryopreserved using a controlled-rate freezer. After > than 3 months in LN2 storage, bags and vials were thawed and viability, CAR-T expression, and T cell phenotype were assessed. % viability, TIGIT and PD1 expression were evaluated to assess the health of the T-cell products post-thaw. For both donors, across all bags and vials, TIGIT expression was < 23% on average and PD1 expression was < 8.3 on average with STDEVs less than 2.9 and 2.3, respectively. Viability for both donors was > 90%. CAR-T expression and phenotypic populations (TEM, SCM, CM, and EM) were consistent over bags and vials for each donor. These data show that the ARES X20+ exceeds manual filling at speed and filling precision, while maintaining cell health and phenotype and providing the ability to fill large volumes of DP.

1308 The Simple CAR-T Cell Production: RetroNectin can Promote Simultaneous T-cell Activation & Transduction in G-Rex Bioreactor.

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CAR-T cell therapy has demonstrated high efficacy against hematologic malignancies, achieving partial or complete remission in many patients. A variety of approaches are being developed to target various antigens, making it a promising therapy for the various kind of tumors. However, there are still many challenges in the cell manufacturing process, especially when using autologous T cells. The process is complex and carries the risk of manufacturing failures, leading to high production costs and limiting the number of patients who can receive the therapy. Therefore, there is a need for a simple and efficient method to produce high-quality CAR-T cells. In this study, we have established a simple CAR-T manufacturing method by combining RetroNectin and G-Rex.

RetroNectin is a recombinant human fibronectin fragment containing three functional domains: a cell-binding domain (C-Domain), a heparin-binding domain (H-Domain), and a CS-1 sequence. RetroNectin enhances gene transduction via lentivirus and retrovirus by promoting the co-localization of target cells and viral particles. This is achieved through specific interactions between RetroNectin and various cell surface integrin receptors. The H-Domain of RetroNectin binds to viral particles, while target cells primarily bind to RetroNectin through interactions between their integrin receptors VLA-5 and VLA-4 and the C-Domain and CS-1. By promoting the physical proximity of viral particles and target cells, RetroNectin significantly improves the efficiency of virus-mediated gene transduction. RetroNectin is currently considered the gold standard for enhancing viral transduction in various clinical applications involving genetically modified cells, such as T cells and hematopoietic cells. Furthermore, RetroNectin is known as a T cell activator when used in combination with anti-CD3 antibodies (Abs).

G-Rex is a gas-permeable bioreactor developed by Wilson Wolf Corporation, suitable for high-density culture of immune cells like T cells and NK cells, and it is becoming increasingly utilized in various clinical cell manufacturing processes due to its ease of scalability.

In the conventional CAR-T production process, lentiviral transduction is performed mostly 24-48 hours after T cell activation. By coating the membrane of G-Rex with RetroNectin, we were able to perform simultaneous activation and efficient transduction of T cells with lentiviral vectors. This method is expected to significantly reduce the amount of lentiviral vectors used. Additionally, CAR-T cells cultured in this RetroNectin-coated G-Rex showed higher proliferation rates and a more undifferentiated phenotype compared to uncoated conditions. This effect was consistent with both stimulation methods, including CD3/CD28 activation beads and RetroNectin/anti-CD3 Ab stimulation method, indicating a beneficial effect regardless of the T cell stimulation method.

Moreover, by optimizing the each step in this culture process, we were able to establish a simple manufacturing process requiring only three operation days. This simple CAR-T manufacturing method is expected to be applied to various T cell therapies. The combination of RetroNectin and G-Rex not only simplifies the manufacturing process but also enhances the quality of CAR-T cells and efficiency of CAR-T cell production, making it a promising approach for future clinical applications.

1309 Platforming iPSCs: a modular approach for all differentiation modalities

Adam Janvier, EXmoor Pharma, BRISTOL, South Gloucestershire, United Kingdom

Induced pluripotent stem cells (iPSCs) are mature cells that have been reprogrammed to behave like embryonic stem cells, allowing them to differentiate into any desired cell type. In recent years, iPSCs have become the preferred starting material for cell therapy applications due to their differentiation potential and the absence of ethical concerns associated with embryonic stem cells. However, achieving specific cell types from iPSCs requires distinct culture methods, making it difficult to standardise manufacturing platforms and leading to a variety of novel approaches in the field. While these innovative methods produce high-quality cells at a small scale, they often face challenges when scaling up, particularly in Good Manufacturing Practice (GMP) environments for clinical applications. To address this, we have developed a modular iPSC platform covering expansion, differentiation, and harvest. Our approach integrates commercially available, GMP-grade platforms and consumables, ensuring regulatory compliance and scalability. After assessing your manufacturing process, we select the optimal unit operations for your platform. This may include iPSC suspension culture for cell expansion, adherent culture for differentiation, enzymatic digestion and centrifugation for harvest, and encapsulation ahead of cryopreservation. To ensure quality, we use a standardised analytical panel to assess genetic stability, phenotype, and pluripotency, alongside therapy-specific analytics. Since iPSC cell lines respond differently to environmental and biological factors, we conduct small-scale optimization studies to adapt each platform step. We continuously look to implement the latest advancements in iPSC reprogramming and GMP-ready reagents are enhancing standardisation, enabling scalable, compliant processes that accelerate clinical translation.

1310 Platforming iPSCs: a modular approach for all differentiation modalities.

DAVID VINCENT[1], Adam Janvier[2]
[1]EXmoor Pharma Concepts Ltd, BRISTOL, South Gloucestershire, United Kingdom, [2]EXmoor Pharma, BRISTOL, South Gloucestershire, United Kingdom

Induced pluripotent stem cells (iPSCs) are mature cells that have been reprogrammed to behave like embryonic stem cells, allowing them to differentiate into any desired cell type. In recent years, iPSCs have become the preferred starting material for cell therapy applications due to their differentiation potential and the absence of ethical concerns associated with embryonic stem cells. However, achieving specific cell types from iPSCs requires distinct culture methods, making it difficult to standardise manufacturing platforms and leading to a variety of novel approaches in the field. While these innovative methods produce high-quality cells at a small scale, they often face challenges when scaling up, particularly in Good Manufacturing Practice (GMP) environments for clinical applications. To address this, we have developed a modular iPSC platform covering expansion, differentiation, and harvest. Our approach integrates commercially available, GMP-grade platforms and consumables, ensuring regulatory compliance and scalability. After assessing your manufacturing process, we select the optimal unit operations for your platform. This may include iPSC suspension culture for cell expansion, adherent culture for differentiation, enzymatic digestion and centrifugation for harvest, and encapsulation ahead of cryopreservation. To ensure quality, we use a standardised analytical panel to assess genetic stability, phenotype, and pluripotency, alongside therapy-specific analytics. Since iPSC cell lines respond differently to environmental and biological factors, we conduct small-scale optimization studies to adapt each platform step. We continuously look to implement the latest advancements in iPSC reprogramming and GMP-ready reagents are enhancing standardisation, enabling scalable, compliant processes that accelerate clinical translation.

1311 Revolutionizing Allogeneic Therapies: Unveiling GMP-Grade iPSC Clones for Scalable Functional CD8 αβT and γδT Production

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Autologous cell therapies have demonstrated significant clinical success and sparked a medicinal revolution. However, challenges such as limited cell supply, high costs due to manufacturing complexities, extended patient treatment timelines, and constrained manufacturing and administration sites continue to hinder accessibility and affordability. Developing off-the-shelf, scalable, and standardized cell products offers a potential solution. Allogeneic induced pluripotent stem cell (iPSC)-derived therapies present a transformative approach to overcoming these hurdles, leveraging unlimited cell supply, standardized manufacturing, and low-cost production of homogeneously edited cell products.

To fully utilized potential of iPSC-derived therapies, three critical conditions must be met. First, access to well-characterized, GMP-grade iPSC lines free from pathogenic genetic alterations is essential. Current public or commercial iPSC banks often contain uncharacterized clones, with studies showing that up to 20% harbor cancer-related mutations. Therefore, obtaining iPSC clones with confirmed genomic integrity is crucial. Second, variations in donor genetics and epigenetics mean that not all iPSC clones exhibit equal differentiation or expansion efficiencies for specific cell types. Identifying suitable iPSC clones through rigorous screening is necessary. Third, differentiation and expansion protocols must be optimized to the productivity of the selected clones. The synergy of selecting the right iPSC clone and optimizing differentiation processes will unlock the full potential of iPSC-derived therapies, driving the next wave of innovation in cell therapy.

To address these requirements, a stringent donor recruitment process was implemented, ensuring compliance with regulations across multiple regions. Blood cells from eligible donors were reprogrammed into clinical-grade T cell-derived iPSC lines (αβT- and γδT-derived iPSCs). Rigorous characterization of these T-iPSC clones, including assessments of pluripotency, differentiation capacity, karyotype, and comprehensive genome integrity assessments. Ten clinical-grade T-iPSC lines successfully passed all tests and were found to be free of likely-pathogenic variants present in the OncoKB, ClinVar, and COSMIC databases.

Innovative feeder-free differentiation and expansion platforms were then developed to maximize the potential of GMP-grade T-iPSC clones. These protocols achieved unprecedented efficiency, are capable of generating 95% CD8αβ+ mature T cells and over 99% γδ T cells without enrichment steps. Remarkably, both αβ T and γδ T cells could be expanded more than 10 million-fold from a single iPSC. Functional assays demonstrated robust cytotoxic activity for both cell types, confirming their therapeutic potential.

To enable commercial-scale production, the differentiation and expansion processes were reproduced in a closed system, facilitating large-scale manufacturing in a single batch. These advances represent a competitive edge in scalability and efficiency, significantly improving the accessibility and affordability of iPSC-based therapies.

BeiGene Cell Therapy's iPSC-derived platforms offer a breakthrough in allogeneic T cell therapy, paving the way for scalable, cost-effective, and functional cell products that address current limitations in the field. These advancements hold the promise of transforming cell therapy accessibility and driving future innovations.

1312 Scalable and Consistent Manufacturing of Functional Lung Cells from iPSCs: Ncardia's Advanced Differentiation Protocols for Drug Discovery and Tissue Engineering

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Induced pluripotent stem cells (iPSCs) offer unparalleled potential for drug discovery, disease modelling, and tissue engineering due to their ability to self-renew indefinitely and differentiate into any cell type. However, the manufacturing of functional cells from iPSCs with consistent quality and at scale remains a significant challenge. Variability in iPSC differentiation, influenced by numerous biological factors, complicates standardization, leading to increased risk, cost, and development time. Leveraging over a decade of innovation, Ncardia has developed robust, scalable procedures for differentiating iPSCs into various cell lineages, including the complex endodermal lineage, thereby enabling the production of functional lung cells for multiple applications.

The development process begins with client collaboration to define the target cell quality profile, including parameters such as identity, purity, potency, and scale. Ncardia's expert team then conducts parallel testing of various protocols using scalable platforms to establish proof of concept. This iterative process identifies critical process parameters (CPP), critical material attributes (CMA), and critical quality attributes (CQA), facilitating the selection of optimal protocols for scale-up development. Throughout this process, robust data collection and analysis enable predictive modelling, ensuring consistent and reproducible outcomes.

Ncardia successfully developed scalable differentiation protocols for four main human lung cell types: distal type II alveolar epithelial cells (AT2), proximal lung basal cells, endothelial cells, and stromal cells. The iPSC-derived AT2 cells exhibited characteristic morphology with secretory granules (lamellar bodies) and expressed specific markers (ABCA3, SPC), essential for pulmonary surfactant production. Proximal lung basal cells, crucial for epithelial repair and maintenance, demonstrated typical morphology and expressed TP63 and KRT5. Endothelial cells displayed elongated shapes, formed vessel-like structures, and expressed CD31 and CD144, with functional capabilities such as LDL uptake. Stromal cells showed expression of PDGFRβ and SMA, with assessed growth and contractility, confirming their role in structural support and immune regulation in lung tissue.

Ncardia's approach addresses the challenges of iPSC manufacturing by minimizing variability and enhancing scalability, quality, and consistency. By integrating deep stem cell biology expertise with scalable platforms and data-driven decision-making, Ncardia accelerates process development while reducing risks. The successful differentiation of lung cell types from iPSCs highlights Ncardia's capability to produce high-quality cells in big volumes for drug discovery and regenerative medicine. This comprehensive work highlights Ncardia as a premier partner for advancing therapeutic development through innovative iPSC technologies, offering customized, large-scale manufacturing solutions that enable significant scientific and clinical breakthroughs.

1313 Development of Highly Sensitive Liquid Chromatography Assays to Ensure Excipient Clearance in AAV Products

Micah Witte, Forge Biologics, Grove City, OH

The development of high-performance liquid chromatography (HPLC) assays for the detection and quantitation of residual process-related impurities is essential for the quality control of adeno-associated viral (AAV) vectors used in gene therapy. This work presents the development of HPLC-based analytical methods to detect and measure key residuals commonly used during AAV vector production: Triton X-100, polyethyleneimine (PEI), Poloxamer 188, and RevIT™. These reagents are critical in optimizing transfection efficiency and viral vector purification, but their

residual presence in final products may pose risks to patient safety and product quality. The assays were developed to ensure high sensitivity, specificity, and reproducibility for the accurate quantitation of these impurities at low concentrations. Optimization of chromatographic conditions, including column selection, mobile phase composition, and detection methods, was essential for achieving reliable separation and detection. The methods were validated for parameters such as linearity, accuracy, precision, and limit of quantitation (LOQ), and were shown to provide reproducible results across multiple batches. The lower limit of quantitation (LLOQ) for TritonX-100, PEI, and RevIT™ was determined to be at or nearing 1ppm when achieving sufficient signal: noise ratios on the chromatograms (>20). Poloxamer 188 is often a target value ranging from 10ppm to 100ppm in finished AAV drug substances/products. Therefore, the assay was developed with an LLOQ of 5ppm, also with acceptable S/N ratio >20. The fully developed assays were robust to allow changes in analyst, instrument, and minor errors in sample preparation. These HPLC assays represent a vital tool for routine monitoring of process residuals, ensuring that AAV-based gene therapies meet stringent safety and regulatory standards while minimizing potential risks associated with these residual impurities.

1314 Percent Fulls – What landscape is your data painting?

Clifford Froelich[1], Praveen Kusumanchi[2], Wei Guo[2], Chistopher Aimable[3], Megan Caten[3], Kevin Vera[3], Pouria Motevalian[2]
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Gene therapy manufacturing requires robust recombinant Adeno Associated Virus production, where viral strength (capsid and genome titers) and empty:full purity are critical quality attributes. Traditional "gold standard" methods—sedimentation velocity analytical ultracentrifugation (SV-AUC), size-exclusion chromatography with multi-angle light scattering (SEC-MALS), and charge detection mass spectrometry (CDMS)—provide accurate purity assessments. However, newer technologies such as mass photometry and Bio-Rad's Vericheck Empty:Full kits offer potentially faster and simpler analyses. This study compared these orthogonal approaches across multiple serotypes (AAV2, AAV5, AAV8, AAV9) and different genes of interest to identify optimal strategies for both harvest-stage and purified samples.

Harvest Evaluation –
Compared three strategies to measure viral strength (vg/cp titers) and purity:

- Industry-standard vg:cp ratio.
- Small-scale AAVX affinity purifications on Tecan and KingFisher, followed by mass photometry and A_{260}/A_{280} analysis
- Bio-Rad Vericheck kits for direct vg, cp, and empty:full data.

Orthogonal Testing (Purified Samples)
Analyzed rAAV preparations using SV-AUC, SEC-MALS, mass photometry, and Bio-Rad Vericheck to determine empty:full ratios.

All orthogonal methods produced comparable purity profiles across serotypes, with mass photometry offering rapid results using minimal sample. SEC-MALS enabled quick (~20-minute) titer measurements but required more material. At harvest scale, Tecan and KingFisher workflows yielded similar accuracy, though KingFisher proved more cost-effective. Interestingly, the A_{260}/A_{280} demonstrates great linearity with the mass photometry numbers. Bio-Rad Vericheck required less sample manipulation overall and delivered integrated vg, cp, and empty:full data (~7.5 hours). The traditional vg:cp ratio frequently diverged from the refined orthogonal assays. Orthogonal methods each offer reliable rAAV purity and strength data but differ in cost, sample demands, and turnaround time. Mass photometry and Bio-Rad Vericheck are particularly practical for routine analyses, while SEC-MALS provides rapid titer quantification where enough material is available. Given the limitations observed in vg:cp ratios, re-evaluation of this industry-standard approach is warranted to ensure accurate, cost-effective rAAV characterization.

From the perspective of a CDMO that sees all serotypes of AAVs, human and non-human, with Genes of Interests addressing a broad range of indications, we are finding that being able to “paint the story” with the orthogonal data to drive the needs of the intellectual property and regulatory agencies and how they are homing on a refined image of the Quality of the material.

1315 Accelerating Sterility Testing to <3 Days: Rapid, Real-Time Phenotypic Detection of Microbial Contamination with calScreener+

Frida Svanberg-Frisinger, **Wilhelm Paulander**
Symcel, Stockholms, Sweden

**Accelerating sterility testing is critical for cell and gene therapy products (CGTPs), where rapid and reliable microbial detection ensures compliance with regulatory standards and patient safety. Traditional sterility testing requires up to 14 days, delaying batch release and increasing costs. The Symcel calScreener+ utilizes isothermal microcalorimetry for real-time, phenotypic detection of microbial contaminants in a non-destructive, matrix-independent manner. This study evaluates its 3-day sterility testing performance, focusing on Limit of Detection (LOD), specificity, detection times, and robustness in handling small sample volumes and complex matrices with high eukaryotic cell concentrations.

The calScreener+ detects microbial metabolic activity in sample volumes ranging from 0.05 ml to 0.5 ml. Validation studies determined its LOD, detection times, comparability and specificity across 20 microbial strains aligned with regulatory guidelines. Tests were conducted using TSB and FTM media at 25°C and 35°C, targeting key microorganisms such as Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, Aspergillus brasiliensis, Cutibacterium acnes, and Micrococcus species. Additional evaluations included temperature screening to assess microbial growth dynamics under different incubation conditions, as well as robustness testing under inhibitory conditions, including the presence of cryopreservants commonly used in CGTP formulations. Statistical analyses confirmed reliability and reproducibility.**

Results demonstrated an LOD of <5 CFU and detection within 3 days, even in complex matrices with high eukaryotic cell concentrations (10^6–10^8 cells/ml). Detection times included S. aureus, P. aeruginosa, and C. albicans within 24 hours, A. brasiliensis within 30 hours, Micrococcus within 36 hours, and C. acnes within 60 hours. The system effectively detected a broad range of sterility-relevant microorganisms, including critical contaminants such as Burkholderia complex and Penicillium species, maintaining robust performance across varied conditions. Temperature screening confirmed consistent detection performance at both 25°C and 35°C, while cryopreservants were evaluated for their inhibitory effects, demonstrating that microbial metabolic activity could still be detected under these conditions.

By reducing sterility testing from weeks to days, the calScreener+ offers a rapid, phenotypic solution tailored for CGTPs. Its ability to analyze small sample volumes minimizes material use, making it ideal for limited batch sizes. Eliminating enrichment steps and accelerating detection enhances operational efficiency, streamlining sterility testing workflows and supporting rapid batch release.

1316 The 3 Rs of Replication-Competent Lentivirus (RCL) Formation Risk: Real, Rare or Artificial?

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The development of retroviral and lentiviral vector systems has culminated in the contemporary 3rd generation platforms, which are now being used in the clinic. The chief driver for development has been to minimise the possibility of generating replication-competent virus through recombination events in retroviral/lentiviral vector (RV/LV) production cells. Replication-competent retrovirus (RCR) has been detected in the earlier generations of

RV systems employing: 1] wild type LTRs; 2] homology between genomic, gagpol and envelope sequences, and 3] certain cell lines or packaging cells. To date, there have been no reports of RCL formation in products generated from 3rd generation LV systems. Despite this, RV/LV products still require testing for RCR/RCL prior to batch release, and this activity remains a significant burden, and is often a gating factor to clinical batch release. Whilst the regulatory agencies have recently adjusted these testing requirements, to our knowledge, a fundamental reassessment of the underlying principles and mechanisms of RCR/RCL formation has not been carried out. Moreover, whilst the adjusted guidance enables a degree of reduced testing of RV/LVs intended for *ex vivo* application, these changes have major implications and challenges for high concentration, low doses-per-batch products intended for *in vivo* applications. As LV production scales and yields increase, and the ability to now target LVs, expanded *in vivo* use of LVs will likely soon become a reality.

OXB has been a leader LV platform engineering over three decades. As part of CMC analytical work, this has necessitated RCL assay development across HIV-1, EIAV and SIV-1 based products. Here we provide a summary review of past RCR formation with the publicly available reports, and suggest the likely mechanisms of how these occurred. We'll present mathematical calculations for the (im)probability of RCL formation from 3rd generation LV systems, and propose a roadmap for reduced testing as a product progresses through to commercialization. Our model infers that the probability of generating a single copy of RCL genome (irrespective of replication fitness) for a typical 4-component LV product at 200L bioreactor scale is no greater than 1 in 10 thousand per bioreactor. We propose that the use of packaging cell lines represents an opportunity to lower this risk substantially further, and therefore is part of the proposed roadmap to reduce the burden of testing. This would depend on the approach taken to stably introduce gagpol/rev/envelope components, and places greater emphasis on deeper, 'one-off' characterisation of packaging cell lines (i.e. full NGS) to ensure the absence of 'pre-RCL' intermediate sequences. This argues for reduced testing of LVs produced from these 'clean' packaging cells, since far fewer template molecules are available for recombination compared to the fully transient method. Finally, we provide insights into RCL assay method development, and consider the key aspects of a given assay with respect to choice of amplification cell line, as well as the basis of positive controls and what they are actually controlling/monitoring within the assay. We propose that whilst the end-point assay is typically a pass/fail threshold, the use of a highly sensitive test, such as those based on reverse-transcriptase activity (a necessary property of any RCR/RCL), potentially provides justification for reducing the cell culture (amplification) phase.

1317 Design of Experiment (DoE) Methodology Effectively Reduces Cost and Time to Completion of Complex Analytical Method Development

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Robust assays are a cornerstone of cell therapy program success, inclusive of process development through commercialization and ultimately supportive of product release to the patient population. Thus, assay development underpins a successful cell therapy program as it enables accurate and precise assessment of critical process parameters, ensuring therapeutic product consistency and effectiveness. Assay development, especially for complex cell-based assays, can represent a potentially prohibitive investment of time and money, especially for innovator companies focused on early phase programs. To help address this issue, Kincell Bio has adopted a Design of Experiment (DoE) methodology to optimize complex cell-based assays. This approach accelerates method development by allowing for assessment of more parameters in a single study than traditional one factor at a time experimentation. Moreover, DoEs facilitate a more resource-efficient use of experimental resources and precious raw materials, which ultimately reduces the overall cost of development. Through practical examples using DoE methodology to develop IFN-γ release, target cell killing, and IL-2 independent growth assays, we demonstrate how DoE techniques can lead to significant time savings, cost reductions, and improved decision-making in the development of robust analytical methods for cell therapy programs.

1318 Streamlined Virus Titration Workflow Enables Advancement of Upstream Process Development for AAV Producer Cell Lines

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Introduction: With recent advancements in high-throughput cell culture process development tools, there is an increased demand for high-throughput analytical assays to support process development (PD) for AAV mediated gene therapy to enable the fast pace of gene therapy programs. One measurement of interest for AAV PD is the vector genome titer in cell culture samples, as the productivity measurement. The manufacture of AAV with Producer Cell Lines (PCL) utilizes adenovirus as a helper virus, and it is necessary to know helper Ad byproduct titer for clearance during purification. Ad5 expression competes for resources with AAV affecting AAV productivity and presents challenges to the Ad5 removal steps during purification process at high levels. The ideal workflow for analytical methods supporting PD should be accurate, repeatable, reliable, high throughput, ergonomically friendly, highly flexible and possess a short turnaround time. We report a workflow established within Sanofi's Genomic Medicine upstream PD that exhibits these qualities, enabling rapid advancement of gene therapy programs utilizing PCL processes.

Methods: Cell culture samples were taken from bioreactors and lysed via Triton X-100 detergent based methods. Manual DNAse and proteinase K (PK) and treatment were performed to digest residual host DNA and release AAV DNA, respectively. Using the Lynx LM1200 liquid handler, the process steps after DNAse/PK were automated for ddPCR analysis. The Lynx was programmed using vendor software in two separate methods, post PK dilution and ddPCR setup. The performance of these automation methods was tested by a side-by-side comparison of manually prepared samples and liquid handled samples (n=11), samples were analyzed with two separate dilutions in triplicate. The multiplexing was performed by performing IDT's oligo analysis to determine compatibility of the two assays followed by comparison of single plex vs multiplex on the same set of samples (n=6), samples were analyzed with two separate dilutions in duplicate.

Results: The automated methods vs manual methods performed with an average of 7% difference in titer with the lowest being 2% and highest being 14% amongst the samples tested. In general, the titer was greater in the automation samples and resulted in a coefficient of variation on average of 7% compared to 5% for manual. Multiplexing the AAV and Ad5 assays when compared to their singleplex counterparts had an observed average % difference of 8% in Ad5 and 3% in AAV. As a result of automation, the throughput of plates analyzed by the BioRad QX200 workflow went from 3 to 6 per day. In terms of time saving, the automation reduced the hands-on time it took to perform the post PK dilutions and ddPCR setup for 6 plates from ~4 hours to just 1 hour. By multiplexing the AAV Ad5 assays, not only is the time of running two separate assays cut in half, but also the cost of reagents and plasticware. In addition, utilization of the liquid handler has reduced pipetting steps, resulting in improved ergonomics and the prevention of work-related injuries.

Conclusion: The workflow described has supported PD work for numerous gene therapy programs, enhancing the efficiency of cell culture operations and enabling rapid advancement of programs.

1319 Lentivirus Infectious titer – Scaling up and scaling out on a budget

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Lentiviral vectors (LVs) are widely used in both in vivo and ex vivo gene therapy applications, from direct patient administration to cell-based therapies such as CAR-T. As demand for high-quality, high-titer LV continues to grow, process optimization must be supported by robust analytical assays to ensure efficient viral production and potency. Among these assays, the Infectious Titer assay plays a critical role in decision-making, as it directly quantifies the number of functional viral particles capable of delivering the therapeutic gene. However, traditional infectious titer methodologies are labor-intensive, low-throughput, and lack scalability, limiting their utility in high-throughput process development. A more efficient, high-throughput approach is necessary to support the increasing complexity

of LV process development, where multiple conditions are evaluated in parallel using Design of Experiments (DOE) and automated bioreactor systems such as the Sartorius Ambr15.

To address this challenge, we adapted and scaled the Universal ddPCR-based Infectious Titer assay published by Kandell J, et al. (2023) to streamline the analytical process. Within our lab, the lack of automation in analytical systems created a bottleneck in testing material generated by the automated Sartorius Ambr15 system. We not only needed to implement the titration assay but also scale it up to: (1) handle 48 samples per week, (2) maintain a manageable material and cell load, (3) generate accurate, precise, and robust data to identify optimal conditions in a fully matrixed DOE system, and (4) streamline sample preparation and readout following the four-day infection period.

Here, we present our strategy for optimizing and scaling the assay, demonstrating how we transitioned from a 12-well to a 96-well format. This shift significantly reduced material requirements, ultimately lowering the cost of goods, while maintaining data quality. Additionally, we simplified the sample preparation process for ddPCR readout, improving workflow efficiency. As a result, what previously required five weeks to determine an infectious titer can now be completed in just one week. These advancements enable more agile and data-driven process development, facilitating the rapid optimization of LV manufacturing for gene therapy applications.

1320 Fit-for-Purpose Analytics for Novel Engineered Capsids: Advancing Precision in AAV Characterization

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Introduction: Engineered AAV capsids are designed to enhance tropism, immune evasion, and manufacturability, yet these modifications can introduce unique analytical challenges. Compared to natural serotypes, engineered capsids may exhibit altered physicochemical properties, including reduced thermostability and changes in binding affinity to antibodies used for purification or detection. These differences necessitate the development of fit-for-purpose analytical methods to ensure accurate quantification and characterization of key quality attributes such as genomic titer, capsid titer, and empty/full ratios.

Development of a ddPCR Method Compatible with Both Natural and Engineered Capsids: Engineered capsids may exhibit lower solubility and reduced thermostability compared to natural serotypes, which can introduce challenges in virus dilution and nuclease digestion steps—critical components of ddPCR workflows. To ensure accurate and reproducible genomic titer quantification across both natural and engineered capsids, a refined ddPCR method was developed that accounts for these physicochemical differences. The approach incorporates virus formulation buffer for sample pre-dilution to mitigate aggregation and minimize viral loss while also integrating EDTA treatment to control residual nuclease activity. These optimizations improve assay robustness and reproducibility, enabling precise titer quantification across different capsid variants. The method has been successfully applied to multiple engineered capsids, demonstrating high precision within recommended CV limits and suitability for drug product characterization and release.

Development of a Suitable Capsid Titer Assay: Evaluation of a commercially available AAV9 capsid ELISA kit confirmed its suitability for wild-type AAV9 but showed limitations in detecting certain AAV9-based engineered capsids due to reduced binding efficiency. To address this gap, an assay is being developed using alternative anti-AAV affinity reagents that offer broader applicability and improved binding across a diverse set of engineered capsids. Initial results indicate strong potential for this assay to provide accurate and reproducible capsid quantification.

Mass Photometry for Empty/Full Ratio Analysis: Mass photometry provides an efficient and powerful alternative for measuring empty/full capsid ratios, particularly in cases where full/empty estimations rely on genomic titer in

combination with capsid titer. This single-particle method enables direct measurement of AAV capsid populations without requiring antibodies, making it an effective approach for engineered capsids that may have altered binding properties. Suitability has been confirmed for AAV9, engineered capsids, and capsid libraries, demonstrating high precision and reproducibility across multiple operators and viral lots. Its implementation strengthens analytical workflows by providing a robust, label-free technique for evaluating AAV vector quality and manufacturing consistency.

Conclusion: The development of optimized analytical workflows compatible with both natural serotypes and engineered capsids ensures robust characterization of gene therapy vectors. These refinements align with regulatory expectations and enhance the reliability of engineered AAV drug product characterization, supporting efficient clinical translation and manufacturing scalability.

1321 Development of a ddPCR Assay to Quantify AXV-101 Levels for a Mouse Biodistribution Study

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Introduction: As part of the Preclinical pipeline, a cohort of toxicology study mice were culled 30 days post sub-retinal injection and several tissues harvested post-mortem for analysis of AXV-101(an AAV9 BBS1 gene therapy) [VH1] biodistribution. Prior to sample arrival an in-house assay was developed by Axovia Therapeutics to determine baseline levels in naïve tissues, and upper and lower limits of detection for AXV-101. Tissue samples 'spiked' with ssDNA of AXV-101 were analysed to demonstrate that viral detection is possible from tissue samples, and show that there would be a difference in quantified levels between naïve and 'injected' tissue.

Methods: C57BL/6j mice were culled, tissues excised, snap frozen on dry ice, and stored at -80°C until processing. DNA was extracted using Meridian (feces) and Qiagen (all other tissue) kits. Nucleic acid levels were measured using a spectrophotometer. ddPCR assays followed a previously validated in-house protocol. On all plates positive controls, non-template controls (NTC), and dilution buffer blanks were included, and all test wells run in duplicate. AXV-101 virus stocks were used as positive control reference and spiking material. These were processed through DNase I and Proteinase K ddPCR steps to produce ssDNA ready for droplet generation. Five initial dilutions of spiking material were tested on a plate, starting with stock from the vial (dilution factor from DNase I and proteinase K processing steps) and progressing through a 10-fold serial dilution. The concentration was too high to be within the detection range of the assay until the lowest range, which was selected as the starting concentration for the next test plates, and further serially diluted alternating between 5-fold and 2-fold. Simultaneously, tissue sample was evaluated at different concentrations of DNA to test whether it influenced sample readout, and where a matrix effect might be observed. Eye tissue was selected as the test tissue due to its relevance for the study. A range of 130ng/ul to 0.25ng/ul was tested. After confirming appropriate dilutions for tissues and virus spike material, plates were run with both naïve and spiked tissue samples to compare baseline levels against simulated 'injected' tissues. Samples were all diluted to a concentration of 0.5ng/ul.

Results: AXV-101 levels in naïve tissues were comparable to NTC wells whereas spiked wells of the same tissues had copy numbers comparable to the lowest positive controls. (A,B,C) A viral dilution factor (DF) of 1.76E+07 was the upper limit for detection by ddPCR and sat above the top positive control. DF 8.80E+07 was selected for use as spiking concentration. The lower limits had copies per reaction concentrations comparable with NTC wells at a DF of 8.80E+10 and above. (E) We started to see erroneous results at the lower concentrations of virus in sample wells with DNA concentration 35ng/ul and higher. This increased with concentrations of 60ng/ul and above. (D,F)

Conclusions: The study objective was to develop protocols for ddPCR quantification of AXV-101 in mouse tissues. The data obtained have determined baseline levels in naïve tissues comparable to NTC samples and proven the difference in quantified levels between naïve and 'spiked' tissues with ssDNA of the reference material containing the same AXV-101 gene of interest. This supports the use of the assay in measuring biodistribution of AXV-101 in toxicology study samples.

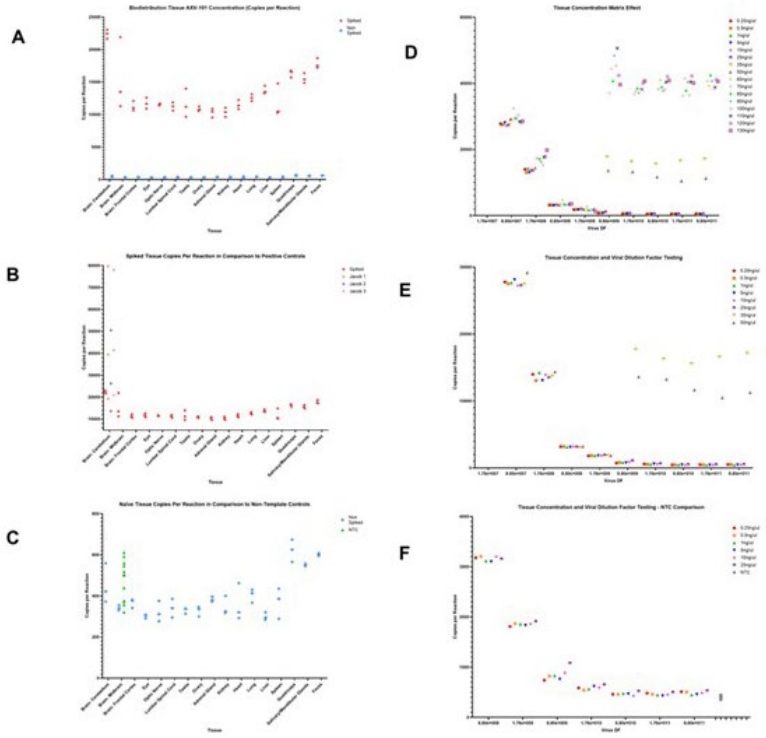

Figure 1. ddPCR quantification of AXV-101 in multiple mouse tissues. A-C. Biodistribution of Naive versus Spiked Tissues C-D Biodistribution of Tissue and Virus Concentration

1322 Validation of Real-Time qPCR Method for Vector DNA Quantitation from rAAV2-shmTOR in Rat and Monkey Tissues Samples

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Recently numerous gene therapies have been already approved and launched for clinical use to treat rare and genetic diseases. Especially, viral vector-based gene therapy such as AAV have emerged as a promising and effective tool for gene transfer in treating neurological, ocular, and hepatic disorders. Preclinical safety evaluation of biodistribution/persistence for novel gene therapies is required for IND application with regulatory authorities. In general, the quantitative analytical method by polymerase chain reaction (qPCR) or droplet digital PCR (ddPCR) is highly suggested to evaluate biodistribution of viral vector gene therapies. In the present study, the objective was to validate a real-time QPCR method for detection of vector DNA from a recombinant AAV2 expressing an mTOR-inhibiting siRNA (rAAV2-shmTOR) in rat and monkey tissue samples. The validation was conducted to evaluate for specificity, linearity, accuracy, and precision. There were no interfering reacts with the targeted sequence between true positive and negative control (no template control) samples. The correlation coefficient of vector DNA were 0.999 and 1.000 ($1/x^2$ weighted) in rat and monkey tissue genomic DNA (gDNA), respectively. The limit of detection for vector DNA was also 50 and 25 copies/500ng of tissue gDNA, respectively. Furthermore, the accuracy and precision were verified < 20% CV in intra- and inter-assay analysis. Therefore, the analytical method was

quantitative with good linearity, accuracy and precision for vector DNA. In conclusion, the highly specific and sensitive qPCR analytical method for vector DNA from rAAV2-shmTOR was well-established in this study and could be also used in biodistribution/persistence studies in animals.

1323 The Fab Four -- Validated GMP QC Assays to More Reliably Assess DNA Impurities for rAAV Batch Release

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During recombinant adeno-associated virus (rAAV) production, the majority of capsids efficiently encapsidate the intended full-length transgene cassette flanked by AAV inverted terminal repeats (ITRs). However, a small proportion of the packaged DNA consists of undesirable fragments which mainly originate from residual plasmid DNA or host cell genomic DNA. These impurities are an unavoidable byproduct of vector manufacturing, and regulatory authorities mandate their rigorous characterization and quantification to ensure that they do not compromise the safety profile and overall efficacy of the final therapeutic product.

In response to these regulatory requirements, we present a comprehensive panel of GMP-compliant QC assays specifically designed to assess DNA impurities in rAAV products. Developed in accordance with ICH Q14 and validated following ICH Q2(R2), our QC assay panel ensures reliable and accurate impurity profiling for batch release while reducing sample volume and costs for release testing.

The four GMP QC droplet digital PCR (ddPCR) assays include quantification of host cell-derived DNA (HCD) via the 18S rRNA gene (202 bp and 482 bp, with sizing) and plasmid-derived DNA impurities including Adenovirus E4orf6, AAV cap, and bacterial kanamycin resistance (kanR) gene sequences. The cap and kanR sequences are measured using a duplex ddPCR approach, allowing simultaneous detection of both targets in a single reaction, thereby saving time and sample material.

Unlike currently used qPCR methods for the assessment of host cell-derived impurities which often measure only a small fragment of the DNA to be quantified, our ddPCR sizing assay targets two distinct fragments of the 18S rRNA gene and allows sizing of DNA fragments up to approximately 500 bp in length, thus meeting recent demands of regulators. Additionally, our ddPCR assays do not require a standard curve for accurate quantification. This eliminates the need for generating and bridging GMP-grade standard material, simplifies the assay process and saves costs. Compared to traditional qPCR methods, ddPCR also offers superior resistance to matrix interference, enabling more reliable impurity quantification in complex rAAV product formulations.

We also highlight strategies to overcome key technical challenges in impurity testing, including the establishment of appropriate assay acceptance criteria.

This work underscores the critical importance of robust impurity profiling for rAAV gene therapy products and complements our EpyQ platform, which has been developed to minimize DNA impurities in AAV clinical batches. It provides a practical framework for developers and manufacturers seeking to enhance the quality, safety, and regulatory compliance of their therapeutic candidates, ultimately facilitating their journey toward clinical and commercial success.

1324 Consistent rAAV In-Process Monitoring with PEA-dPCR Technology

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Recombinant adeno-associated viruses (rAAV) are widely utilized vectors for gene and cell therapies, requiring precise and consistent characterization of key attributes, including genomic titer, viral particle titer, and full/empty capsid ratio. The field currently lacks a single platform capable of consistently and accurately measuring these three attributes across various stages of rAAV manufacturing. To address these limitations, we developed a robust workflow utilizing Proximity Extension Assay (PEA) technology on the QuantStudio™ Absolute Q™ digital PCR (dPCR) platform, delivering consistent data across various stages of rAAV production.

rAAV6 was systematically tested across the entire production process using PEA-dPCR at the following stages: cell culture, tangential flow filtration, affinity chromatography, anion-exchange chromatography, and the final product. By quantifying three genome targets and viral particles collocated within dPCR microchambers, we calculated the capsid content ratio (Poster: Precise rAAV Capsid Characterization via Digital PCR and Statistical Modeling). This approach leverages the dPCR platform's resistance to inhibition from complex sample matrices, providing stable and reproducible measurements. Compared to other methods in the field, including ELISA, ddPCR, and ultracentrifugation, the PEA-dPCR workflow offers improved consistency and reliability across diverse process stages.

This consistency throughout the process allows for accurate tracking of AAV quality attributes, enabling better control and optimization during manufacturing.

In summary, our PEA-dPCR workflow provides a reliable and consistent method for monitoring rAAV production in less than one work shift, offering a significant advancement in characterizing viral capsid content ratio across diverse process samples. The PEA-dPCR workflow enables precise quantification of %full, %partial, and %empty capsids, offering researchers and manufacturers an advanced tool for rAAV sample characterization. This innovative approach ensures robust and consistent results, addressing critical challenges in AAV analytics and supporting the development of high-quality gene therapy products.

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1325 Determination of sgRNA and mRNA Sequence Identity by Ion-Pair Reversed-Phase Chromatography Coupled With High-Resolution Tandem Mass Spectrometry

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RNA vaccines and CRISPR-based gene editing are two breakthroughs used to fight SARS CoV-2 and numerous genetic diseases. The safety and efficacy of RNA vaccines and CRISPR-based gene editing therapeutics highly depend on the stability and purity of the RNA final product. Drug developers are often challenged with accurately characterizing these nucleic acid modalities and finding comprehensive solutions remains a gap. IP-RP chromatography is the current method of choice, but conventional approaches are not often sufficient for longer RNAs such as sgRNA and mRNA. Here, a sgRNA targeting the BRAC1 and Cas9-mRNA were used as model systems and enzymatically digested to be analyzed by IP-RP chromatography coupled with HRMS. This workflow shows full sequence identification and chromatographic resolution of impurities and Phosphorothioate (PS) diastereomers of sgRNA. Similarly, Cas9-mRNA sequence identification was achieved in 5'-UTR / 3'-UTR regions, 5'Cap, and Poly-A tail, all while revealing partially capped species, deletions, and Poly-A heterogeneity.

1326 Dual-Platform NGS for Comprehensive Characterization of Engineered rAAV Vector Integrity

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Recombinant adeno-associated virus (rAAV) vectors represent one of the most promising delivery systems for gene therapy. The demand for engineered rAAV capsids with improved target tissue tropism and reduced toxicity is rapidly growing as clinical pipelines expand to treat both rare and common diseases. As such, the need for precise, robust, and reproducible analytical methods is essential for characterizing viral vectors, accurately detecting full-length viral genomes, and identifying residual DNA species in vector drug products. Short-read sequencing delivers high-resolution and high-throughput data, providing accurate quantification of genetic heterogeneity. However, it struggles to resolve structural variations, repetitive elements, and homopolymeric regions, which limits its ability to fully characterize genomic complexity. Long-read sequencing technologies, such as PacBio and Oxford Nanopore, enable comprehensive characterization of viral genome structure and length but lack the throughput and quantitative precision of short-read sequencing.

We have developed a dual-platform sequencing strategy that integrates long-read and short-read technologies for orthogonal analysis of novel engineered rAAV vectors with a focus on packaging efficiency and analytical performance. This integrative approach facilitates robust assessment of vector genome integrity by evaluating ITR states, alignment terminal hotspots, vector-size distributions, structural variants, and residual quantifications, all of which can critically influence potency, purity, and safety of gene therapy products. Evaluation of results is compared with standard analytical methods, including PCR-based quantifications, gel electrophoresis, and analytical ultracentrifugation (AUC). This hybrid sequencing approach, integrated with our data analysis framework, enhances sequence resolution and enables comprehensive viral payload characterization, offering greater detail and higher throughput than single target assays. It delivers precise encapsidated DNA measurements, streamlines quality testing, and enhances efficiency in process development and manufacturing workflows.

1327 Adapting a Replication Competent Lentivirus (rcLVV) Assay for Commercial Manufacturing

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A universally applicable assay to monitor the emergence and estimate the frequency of recombination events during commercial manufacturing is required. Herein, we report on the development and application a cGMP-compliant, dual cell-based PCR and p24 antigen ELISA assays to detect the emergence of replication competent lentiviruses (RCLs) in manufactured viral vectors and gene-mediated cell therapy products. Lentiviral vectors, derived from HIV-1, are widely used for gene therapy due to their ability to deliver genetic material efficiently. While these viral vectors are designed to be replication-deficient, there still poses a risk that recombination events during vector production could generate RCLs. Thus, even a trace presence of RCLs can pose significant safety risks when administered to patients including insertional mutagenesis, immune responses, and chronic infections. This assay incorporates a dual detection method using droplet digital PCR (ddPCR) and p24 antigen ELISA to ensure high sensitivity and specificity. The validation process confirmed the absence of RCL in tested samples across multiple amplification and indicator phases, demonstrating the assay's robustness. This comprehensive detection strategy meets regulatory standards and enhances the safety profile of lentiviral vector-based therapies for clinical applications.

1328 Rapid Cell-Specificity and Integration Safety Profiling of AAVs

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With more than 200 AAV (Adeno-Associated Viruses) vectors in clinical trials (1), AAV gene therapies will soon become the predominant approach for delivery of gene therapies. However, demonstrating specificity and safety, in the form of cell type specific targeting/binding and lack of random genomic integration of the AAV, remains a laborious and complex challenge. Here we describe a novel combinatory process using cell microarray, digital droplet PCR (ddPCR), linear amplification mediated (LAM) PCR technologies to rapidly screen and validate AAV cell specificity and genomic safety, thus facilitating the identification of lead AAV candidate(s) for gene therapy payload.

First, a functional binding screen is performed using Charles River's Retrogenix® Cell Microarray technology, consisting of >6,500 full length human cell surface secreted and heterodimeric proteins over-expressed in HEK293 cells (2), to identify cell surface receptors with which AAV variants bind, thus identifying the on- and off-targets of the AAV variants compared to wild-type. Second, the therapeutic target patient-derived cells are treated with the 5-10 hit AAV variants and the viral vector transduction efficiency and functional transgene expression of each is quantified in a single ddPCR assay by measuring both AAV vector DNA and functional transgene mRNA. Relevant other cell types expressing the off-target cell surface receptors identified from the Retrogenix® Platform are included to compare the levels of viral vector transduction efficiency and transgene expression that occur. In the final step, the lead AAV variant candidate, selected on highest on-target viral vector transduction efficiency, transgene expression, and selectivity, is assessed for potential genomic integration using NGS of LAM-PCR products and integration site analysis (ISA) (3).

This streamlined process helps to de-risk therapeutic candidates by providing a rapid and cost-effective way to produce the key supporting safety data required for investigational new drug submissions. This accelerates the safe development of personalized AAV therapies for n-of-1 rare disease studies and makes them more accessible.

(1) Goswami R, et al. Gene Therapy Leaves a Viscious Cycle. Front Oncol. 2019; 9:297.
(2) Pillay S, et al. An Essential Receptor for Adeno-Associated Virus Infection. Nature. 2016. 530; 7588.
(3) Gil-Farina I, et al. Improving AAV Gene Therapy Safety Analysis: Multiplex LAM-PCR Provide New Insights into AAV Vector Integration. Molecular Therapy, 2015. Volume 23, S264

1329 Gene Therapy Studies and Germline Integration Assessment Considerations in Preclinical Research

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Introduction: Gene therapies targeting rare genetic conditions and disorders are on the rise. While somatic or tumor cell targets are often the aim of these therapies, risk of germline integration of the vector target sequence in gametic cells is of recent safety and regulatory awareness. With the intent to confirm zero target germline integration, *in vivo* sample collection must be performed with precision and optimal nucleic acid isolations are required.

Methods: Preclinical gene therapy studies are conducted on sexually mature laboratory animals, including nonhuman primates (NHPs), canines, and minipigs. A 6-12 month observation period follows gene therapy administration. Semen is collected at various study timepoints for downstream nucleic acid isolations. Two sperm

cell separation techniques were compared for feasibility and preservation of nucleic acids present: (1) whole cryopreservation method, where whole semen samples were collected, flash frozen, and then thawed prior to washes and nucleic acid isolation; and the (2) swim-up method, where fresh semen was incubated with a 'swim-up' buffer where the sperm cells are separated from the extraneous cells contained in semen; the sperm fraction is then flash frozen and preserved for nucleic acid isolations. For females, ovaries are collected at necropsy and the tissue is manually disrupted under a microscope to isolate individual oocytes; extraneous cellular/tissue debris is removed and 'clean' oocytes are collected and flash frozen for downstream isolation procedures. DNA and RNA are isolated from both frozen sperm and oocytes.

Results: Semen yielded an average of 10^8 purified sperm cells per collection, which yielded between 120 and 4404 ng of DNA. Of the two methods evaluated, the whole cryopreservation method resulted in higher sperm cell isolation and subsequent DNA yields as compared to the swim-up method. Notably fewer gametic cells were obtained from female ovaries; averaging 72 oocytes per ovary. Accordingly, lower DNA yields of 140-892 ng and RNA yields of 72-800 ng were obtained.

Conclusions: Successful and meticulous collection of gametes from *in vivo* test models will be applied to ongoing and future gene therapy studies to assess potential vector target germline integration. These collections and isolations procedures pave the way for and impact preclinical industry standards for sufficient assessments of gene therapies with regard to risk of potential germline integration.

1330 Interlaboratory Assessment of Candidate Reference Materials for Lentiviral Vector Copy Number and Integration Site Measurements

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While lentiviral vectors have played a critical role in the emergence of gene-modified cell therapies, safety concerns remain regarding potential insertional mutagenesis. Regulatory authorities strongly recommend risk assessment and management of vector copy numbers (VCN), integration profiles, and integration sites in the lentivirus-based cell and gene therapy products. However, accurately measuring these parameters remains a significant challenge due to the lack of standardized methodologies and VCN reference materials (RMs). Toward this challenge, we conducted an interlaboratory study on NIST candidate RMs for VCN measurements. The candidate RMs comprise of five human genomic DNA samples or fixed cells from clonal Jurkat cell lines with defined VCNs ranging from 0 to 4. All 12 study participants were able to identify the VCN in the five blinded samples using quantitative real-time PCR (qPCR), digital PCR (dPCR) or next generation sequencing (NGS) assays. Consensus value of VCN and integration sites in these candidate RMs were achieved. The fixed clonal VCN cells were also used to evaluate an emerging imaging-based technology called molecular combing. This interlaboratory assessment demonstrated the utility,

commutability, and suitability of the NIST VCN candidate RMs for quality assurance and improved confidence in VCN, integration profile, and integration site measurements.

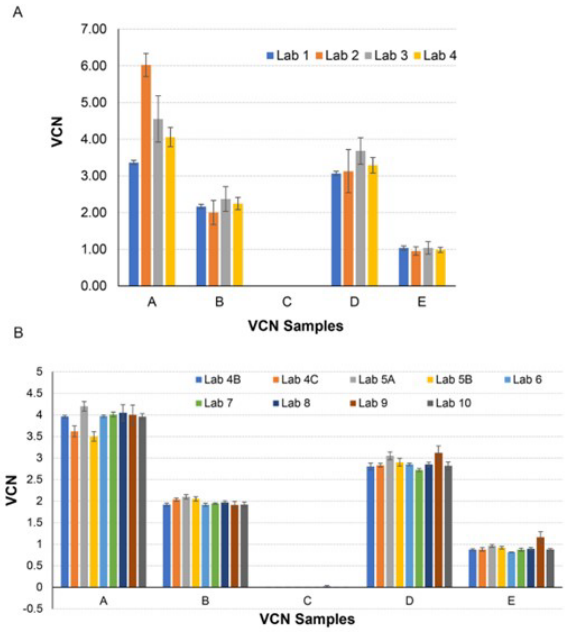

Figure 1. qPCR and dPCR Interlaboratory study results of NIST candidate reference materials.

A) For qPCR, the copy concentration of target and reference genes were analyzed by using individual laboratories' standard curve and raw Cq data. The VCN values were calculated based on the ratio of copy concentration of target and reference genes. The error bar is the standard deviation (N>=6)

B) For dPCR estimates calculated using individual laboratories' protocol, such as provirus targets and reference gene selection. The error bar is the standard deviation (N>=3)

5 samples: A (VCN4), B (VCN2), C (VCN0), D (VCN3), and E (VCN1).

Table 1: Interlaboratory study determine consensus provirus integration sites in NIST candidate reference materials

Samples	Sci Rep Paper* (hg38)	Lab 9 (hg38)	Lab 10 (hg38)	Lab 11 (hg38)	Duplication sequence	Gene	Region
A	chr1:45,634,499	chr1:45,634,495(+) - 45,634,499(-)	chr1:45,634,499-45,634,495	chr1:45,634,495(+)	GTTTG	GPBP1L1	Intron
	chr1:160,440,748	chr1:160,440,776(+) - 160,440,780(-)	chr1:160,440,776-160,440,780	chr1:160,440,780(-)	TGTAC	non-coding	intergenic
	chr4: 5,612,738	chr16:2,259,698(+) - 2,259,702(-)	chr16:2,259,702-2,259,698	chr16:2,259,702(-)	TTAGC	RNPS1	Intron
	chr22:42,082,676	chr22:42,082,672(+) - 42,082,676(-)	chr22:42,082,676-42,082,672	chr22:42,082,672(+)	GTTTT	SMDT1	Intron
B	chr6:45,210,754	chr6:45,210,831(+) - 45,210,835(-)	chr6:45,210,831-45,210,835	chr6:45,210,835(-)	CAGAC	SUPT3H	Intron
	chr22:40,844,112	chr22:40,844,212(+) - 40,844,216(-)	Chr22: 40,844,216-40,844,212	chr22:40,844,212(+)	GATAC	ST13	Intron
C	not detected	not detected	not detected	not detected	NA	NA	NA
D	chr3:130,934,986	chr3:130,934,987(+) - 130,934,991(-)	chr3:130,934,991-130,934,987	chr3:130,934,987(+)	CACCC	ATP2C1	Intron
	chr14:22,564,447	chr14:22,564,447(+) - 22,564,451(-)	chr14:22,564,447-22,564,451	chr14:22,564,451(-)	CAAAT	non-coding	intergenic
	chr15:25,124,969	chr15:25,124,969(+) - 25,124,973(-)	chr15:25,124,969-25,124,973	chr15:25,124,973(-)	CATAT	SNHG14	3'UTR
E	chr3:35,632,784	chr3:35,632,785(+) - 35,632,789(-)	chr3:35,632,785-35,632,789	chr3:35,632,789(-)	ATAAC	non-coding	intergenic

Three laboratories evaluated LV integration sites for candidate materials using NGS based methods following individual laboratories' in-house protocols. Table 1 shows identical integration sites were detected by all three laboratories for 10 integration sites in 5 samples from Sample A (VCN4), B (VCN2), C (VCN0), D (VCN3), to E (VCN1). Surprisingly, the initial results from all three laboratories only reported partially identical integration sites. Strikingly, all three labs reported a site in chr16: 2,259,702 instead of the expected chr4: 5,612,738 for Sample A (or VCN4), that was published previously when these clonal cell lines were established (* Paugh, B. S. et al. Reference standards for accurate validation and optimization of assays that determine integrated lentiviral vector copy number in transduced cells. *Scientific Reports* 11 (2021). https://doi.org/10.1038/s41598-020-79698-w)

1331 Development and Validation of a Replication Competent Virus Safety assay for Adenoviral products

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Recombinant Adenoviruses are powerful vectors for gene therapy and vaccines, offering efficient gene delivery. However, the generation of replication-competent adenoviruses (RCAs) during vector production poses a significant safety risk. RCAs can replicate autonomously, leading to potential adverse events such as viral infection, unintended replication, and host inflammatory responses in patients. Therefore, sensitive and robust RCA detection assays are crucial for ensuring the safety and efficacy of adenovirus-based therapies. We developed and validated a cell-based RCA assay to detect replication-competent adenoviruses in viral vector preparations. This assay utilizes A549 cells and incorporates two culture formats: roller bottles and flasks. The assay involves infecting cells with the test article, a wild-type Ad5 positive control, and a negative control. During 28-day incubation period, cytopathic effects (CPE) are monitored and confirmed using immunofluorescence. Prior to assay initiation, test articles are assessed for non-toxic Multiplicity of Infection (MOI) to avoid cytotoxicity. The assay demonstrated high sensitivity and specificity in detecting the presence of RCAs. The validation process confirmed the absence of RCA in the tested samples. This validated method is GMP-compliant and meets regulatory requirements for ensuring the safety of adenovirus-based gene therapy products. This robust and reliable assay plays a critical role in the quality control of adenovirus-based therapeutics, minimizing the risk of adverse events and ensuring patient safety.

1332 Automation of AAV Capsid ELISA on Tecan Fluent

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Utilizing robust analytical methods for product characterization is of paramount importance in AAV gene therapy development. A widely employed analytical method for measuring a recombinant AAV product's capsid titer is an enzyme-linked immunosorbent assay, or ELISA. Capsid titer results, particularly its role in determining empty/full capsid ratios, help to optimize upstream and downstream processes, and may be informative for maintaining consistency in manufacturing lots used for clinical administration.

Due to the nature of the method, capsid ELISA is a difficult assay to perform correctly. Because of its time sensitive incubation periods, precise pipetting to avoid binding disruption, and repetitive wash steps, the method requires long periods of intensive labor to produce accurate data. Furthermore, the assay has a low sample throughput with a high demand for results. It takes a minimum of 4 hours to run one plate of capsid ELISA, with much of this time spent diluting reagents or samples for the next step of the assay. Samples have a failure rate of approximately 30%, either because the capsid ELISA plate fails system suitability criteria, or the sample does not pass acceptance criteria for reporting due to high % CVs. Despite increasing requests for capsid data to aid process development (oftentimes upwards of 48 samples per week), the current throughput is only a maximum of 8 samples per plate at a time, and analysts can rarely run more than one plate at a time. The high failure rate and the expensive reagents make capsid ELISA an extremely costly assay to re-run.

Automation of capsid ELISA on a liquid handler can readily address some of the method's main complications. Automated liquid handlers mitigate human error, allow scientists to redirect their focus and time towards other tasks, and enhance sample throughput efficiency. In this study, an in-house AAV capsid ELISA method was programmed onto a Tecan Fluent automated liquid handler. 24 samples from varying stages in the production process and with a range of expected titers from e10 to e14 capsids/mL were run on the Tecan. Results demonstrated automated AAV

capsid ELISA performed superior to manual AAV capsid ELISA, resulting in an increase in sample throughput and consistency. The Tecan Fluent increased the method's sample throughput by up to 300% because of its capacity to run plates in parallel. Additionally, there was an average capsid titer variability of 4% amongst samples that were re-run on the Tecan to test consistency, and all samples passed acceptance criteria for reporting.

AAV capsid ELISA, along with other types of ELISAs, are highly sensitive and repetitive methods that see extensive usage, thereby making them strong candidates for automation on a liquid handler. The successful transfer of a manual AAV capsid ELISA method to the Tecan Fluent automated liquid handler supports utilizing preexisting workflows and technical concepts to automate other types of ELISAs in a product characterization panel, as well. The automation of ELISAs may lower costs, generate more sample data faster, and increase productivity for scientists, ultimately improving the AAV gene therapy development process.

1333 *Comparative Analysis of Total Antibody Titer and Neutralizing Antibody Titer: Correlation and Implications for AAV-Based Gene Therapies*

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Today, AAV-based gene therapy still remains one of the most promising and fastest growing branches of modern medicine. However, the prevalence of anti-AAV antibodies in patients limits the efficacy and safety of gene transfer using rAAV vectors. Therefore, it is indispensable to test patients eligible for AAV gene therapy for preexisting AAV antibodies. Two types of assays can be used for this purpose, (1) binding assays that measure total antibodies, including both neutralizing (NAbs) and non-neutralizing antibodies (nNAbs) and (2) neutralization assays that specifically detect neutralizing antibodies.

In this study, AAV antibody responses were analyzed by (i) an AAV serology ELISA for determination of total IgG and (ii) an neutralization assay for the most commonly used AAV serotypes (AAV2, AAV6, AAV8 and AAV9). AAV seroprevalences were calculated and the correlation was compared.

For the seroprevalence study, a cohort of 100 human serum samples from healthy donors were tested for pre-existing anti-AAV antibodies. 18% of the samples were tested IgG seropositive for all tested AAV serotypes 2, 6, 8 and 9. Among all AAV serotypes included in this study, AAV2 was the predominant serotype with 45% seropositive individuals.

Out of this cohort, ten serum samples, including positive, negative and borderline samples, were additionally tested for neutralizing antibodies for AAV2, 6, 8 and 9, using AAV-NanoLuc® viral particles. The EC_{50} value, which indicated the existence of neutralizing antibodies, was then compared to the results gained with the total antibody (TAb) ELISA.

Across all serotypes 92% of TAb positive samples exhibited neutralizing activity. Only two serum samples that tested positive in the total IgG ELISA showed no detectable neutralizing titer for AAV2 and AAV8. This suggests that the antibodies present in these samples are non-neutralizing. Thus, TAb ELISA and the NAb assay showed a strong correlation in this experimental set up. This observation correlates well with percentages reported in the literature, as the prevalence of TAbs is generally considered to be higher since a moderate percentage of TAb positive samples contain low to undetectable NAb titers. However, more research is needed to understand the correlation of TAb levels and their clinical impact in AAV based gene therapies.

1334 *AAV Serology ELISA for Testing Pre-Existing Immunity to AAV*

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Recombinant adeno-associated virus (rAAV) vectors are leading tools for *in vivo* gene therapy. Depending on socio-demographic factors and geographic region, a high proportion of the general population has developed antibodies against various AAV capsids as a result of naturally acquired infections. However, presence of these antibodies can directly affect efficacy and safety of gene transfer using rAAV vectors. Furthermore, antibody levels after rAAV mediated gene therapy might differ compared to those after naturally acquired infections. Thus, testing for pre-existing AAV antibodies in sera is of significant importance for ensuring patient safety and evaluating the efficacy of gene therapy. Monitoring antibody levels after gene transfer and comparing with AAV antibody levels in the healthy population will provide comprehensive data on the natural history of AAV infections as well as immunogenicity in patients after gene therapy.

Currently, PROGEN is developing AAV serology ELISAs for the most commonly used AAV serotypes (AAV2, AAV5, AAV6, AAV8 and AAV9). These antigen-capture assays offer a fast, sensitive and reproducible method for the quantification of total IgG antibodies against AAV in human sera. The assays contain recombinant human chimeric antibodies to generate a standard curve, making them reliable and reproducible. As the kits have a quantitative read-out [in units, (U)], they are useful for patient pre-screening, comparing antibody levels between individuals, and determining antibody kinetics during the course of infection or after systemic gene therapy administration. Additionally, commercially available assays may offer a means to harmonize results between clinical trials, and streamline regulatory hurdles associated with development of individual assays for each gene therapy product.

Here, we show assay data on the detection range, linearity, inter-operator variability, and inter-lab variances for our newly developed AAV2, 6, 8 and 9 serology ELISAs.

1335 *Addressing Pre-Existing Immunity in AAV Gene Therapy: Automation of Serology ELISAs*

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The increasing prevalence of gene therapies based on adeno-associated virus (AAV) vectors highlights their potential in modern medicine. However, naturally acquired infections have led to the development of AAV-specific antibodies in a significant portion of the population, with variations influenced by socio-demographic factors and geographic regions. The presence of these pre-existing antibodies poses challenges for the efficacy and safety of rAAV-mediated gene transfer, making it essential to assess patient sera for AAV antibodies prior to therapy.

To address this need, PROGEN developed AAV serology ELISAs targeting the most commonly used AAV serotypes, including AAV2, AAV5, AAV6, AAV8, and AAV9. These antigen-capture assays provide a rapid, sensitive, and reproducible platform for quantifying total IgG antibodies against AAV in human serum samples. Given the increasing demand for high-throughput analysis due to the growing number of samples and limited processing time, automation of these assays offers a practical solution. Automated systems can reduce human error, variability, and hands-on time, enabling more efficient large-scale sample characterization.

To demonstrate the suitability of our AAV serology ELISAs for high-throughput applications, we tested the AAV2 and AAV9 assays exemplarily on an automated platform. Key performance parameters such as plate homogeneity, intra- and inter-assay variability, and recovery rates were assessed for the automated method and compared with results from manual processing.

The automation of our AAV serology ELISA has been demonstrated as an effective solution for high-throughput analysis of AAV antibodies in human serum samples. The results confirm that the automated system preserves accuracy and reproducibility while significantly improving analytical efficiency.

1336 Screening for Pre-Existing Immunity Against AAV Vectors: A Multipronged Approach

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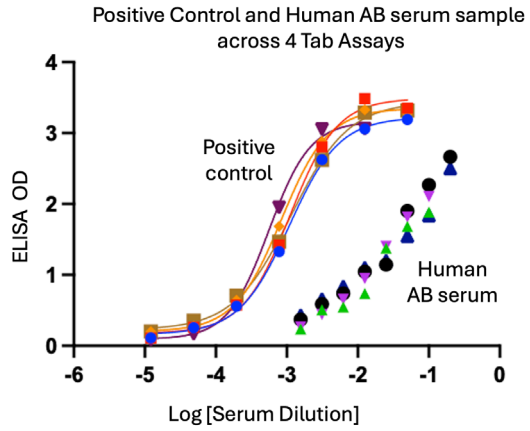
The high prevalence of pre-existing immunity to adeno-associated virus (AAV) in the general population presents a significant challenge for AAV-mediated gene therapy. A robust screening tool is needed to assess neutralizing activity in patients prior to treatment. Cellentia has developed a multi-tiered strategy to evaluate AAV immunity, beginning with the measurement of total antibodies (Tab) against AAV, followed by a neutralizing antibody (Nab) assay that assesses inhibition of 293T cell transduction. An in vivo confirmatory assay is then used to establish an appropriate cutoff for pre-existing immunity.

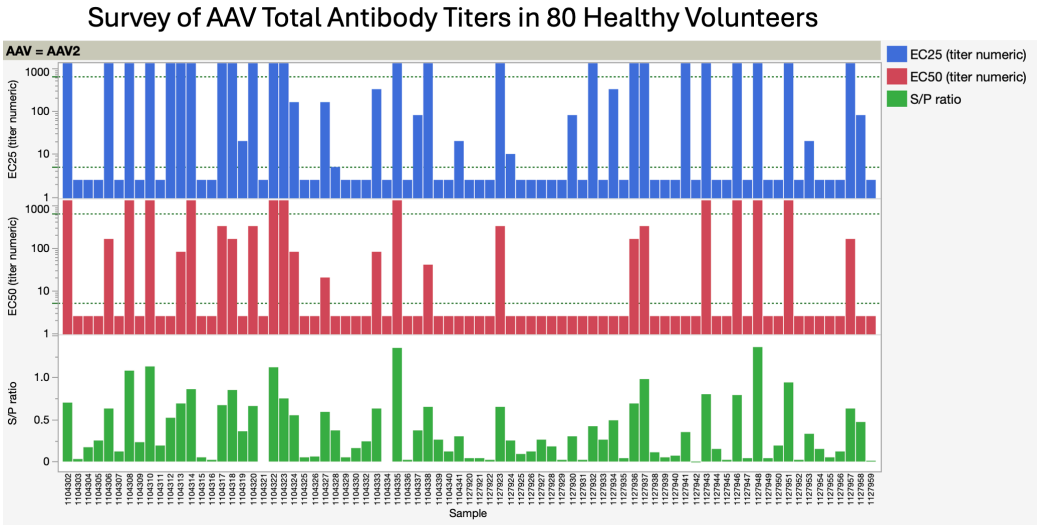
To ensure accurate Tab detection, we developed an in-house ELISA with direct AAV particle immobilization. Specificity, reproducibility, and assay performance were validated, with response curves optimized using a four-parameter model. To enhance data reliability, we implemented algorithms to correct the hook effects at high concentrations and detect statistical outliers in triplicate wells per titration.

We analyzed 80 serum samples from healthy donors for AAV2, AAV9, and other serotypes using our direct sandwich ELISA. Samples that consistently exhibited strong signals across tested serotypes were further evaluated, and one sample was selected as the positive control, while those with negligible or no response were also screened to identify one negative control. The final assay format incorporates both controls in Tab assays for AAV1, 2, 5, 6, 8, and 9, ensuring standardization across assays. Positive control response curves were further utilized to estimate sample titers.

To facilitate comparison, we reported antibody prevalence using three readouts: titers based on two pre-set cutoff values and the sample-to-positive control (S/P) ratio based on the positive control analyzed in the same assay. The prevalence of total antibodies against AAV2 ranged from 30-50%, while AAV9 exhibited a lower prevalence of 20-40% in the tested population, depending on the titer reporting method used. Serum samples with negative or borderline results were further assessed using a 293T cell-based neutralization assay, confirming negativity in most cases, with one sample showing modest inhibition.

These samples are planned for in vivo neutralization testing in mice to evaluate transduction efficiency in the target organ and determine the final acceptance criteria for AAV vector administration. Once a definitive cutoff is established, we aim to refine our ELISA into a streamlined assay tailored for rapid preclinical and clinical screening, facilitating rapid evaluation of pre-existing immunity to novel AAVs in both animal models and target patient populations.





1337 Molecular characterization of acute lymphoblastic leukemia in Tanzania

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Introduction: Acute lymphoblastic leukemia (ALL) cancer diagnosis may be improved by using targeted therapies of tyrosine kinase inhibitor (TKI) management for gene translocation for p190 Philadelphia (BCR-ABL) positive and somatic mutations referred to as single nucleotide variants (SNVs). Molecular diagnosis has led to targeted therapies of specific molecular profiles. The frequency of somatic pathogenic mutations related to ALL in adult and pediatric patients, the uniformity of p190 Philadelphia q-PCR methods with Next Generation Sequencing (NGS), and the clinical presentation and behaviour of ALL patients with the noticed mutations.

Methods: A cross-sectional study from Muhimbili National Hospital and Bugando Medical Centre used samples that were initially suspected of being ALL cancers based on cellular morphology. The study was carried out at the Muhimbili University of Health and Allied Sciences. The two-year study conducted from August 2023 until June 2025. Using a panel of monoclonal antibodies conjugated to fluorochromes (FITC, PerCP, and PE) and the proper isotype controls in accordance with the Euroflow panel guidelines, practice flow cytometry. Library preparation

performed using the AmpliSeq for Illumina Myeloid Panel sequencing. To filter out variants with a higher likelihood of being common benign variants, performing exclude variants with an allele frequency greater than 0.1 in the African population from the Genome Aggregation Database (gnomAD) database. This filtering step helps prioritize potentially pathogenic variants for analysis.

Results: Preliminary findings 21(48%) ALL patients currently recruited, 17(39%) ALL samples currently sequenced. Translocations identified in 5(11%) samples BCR - ABL1 rearrangement from the NGS against 3(7%) from q-PCR of the same group patients. Target ID T00653 breakpoint A chr22:23524426, breakpoint B chr9:133729451, patient currently on going with TKI management. 10(22.7%) had pathogenic mutation deletion variant out of 17 sequenced; the somatic variants where JAK2, and CREBBP.

Conclusion: Describe the most common somatic pathogenic mutations in acute lymphoblastic leukemia that are presently unidentified in Tanzania and the molecular profile that determines whether or not these mutations are associated with poor or favourable outcomes for targeted therapy in ALL.

1338 Deploying Fourier-transform infrared spectral signatures and machine learning to standard cell identities and ages (SCIAGE)

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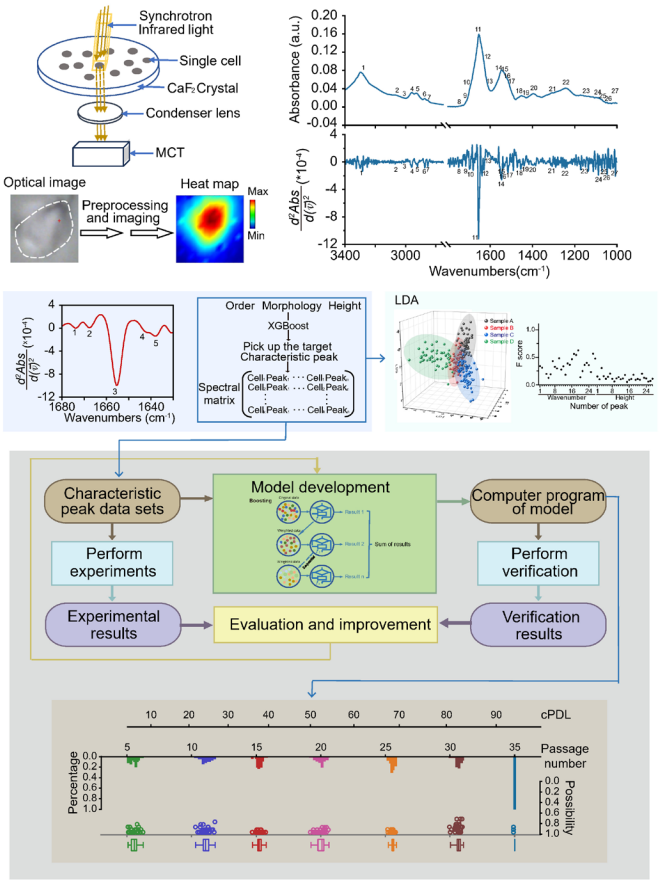
Translating cell-based therapies from laboratory to clinic faces significant challenges, particularly in maintaining cellular integrity during the culturing process and large-scale expansion. Current characterization methods lack standardized "snapshot" measurements of cellular biological states, hindering optimization for therapeutic efficacy. Here, we reported SCIAGE (Standard Cell Identities and Ages), a framework integrating transmission Fourier-transform infrared (FTIR) single-cell microspectroscopy and machine-learning techniques. Firstly, an automatic data-capturing program was developed for extracting the intrinsic FTIR spectral variables corresponding to cellular biomolecular components. Linear discriminant analysis revealed distinct segregation among different cell types, cells from different donors, and cells at various culture cycles, indicating FTIR spectra could serve as label-free cellular molecular fingerprints. We then developed SCIAGE using the eXtreme Gradient Boosting algorithm, based on FTIR spectral variables of neural progenitor cells (NPCs) at different population doubling levels (PDLs). SCIAGE demonstrates highly accurate quantitative characterization of NPCs across various PDLs and effectively characterizes aging states of NPCs subjected to obscured senescence-induction or anti-aging treatments. Together, SCIAGE provides a phenotyping technique for robust cell characterization, sensitive and accurate cell aging state detection and comparison, thereby addressing the critical gap in current cell characterization methods, and potentially accelerating the development and optimization of cell-based therapies.

Graphical abstract



1339 Application of Mass Photometry to Characterize Adeno-associated Virus Particle Content

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Mass Photometry (MP) is a label-free bioanalytical technique utilizing interference reflection microscopy and interferometric scattering microscopy to measure single molecules in solution. This study demonstrates the application of MP for the characterization of adeno-associated virus (AAV) vectors, specifically quantifying empty, partially filled, and full capsids. Compared to traditional methods like analytical ultracentrifugation (AUC) and electron microscopy (EM), MP offers a rapid, cost-effective, and high-throughput platform with minimal sample volume requirements, enabling faster process development and product release decisions. We developed and qualified an MP method using Refeyn's Mass Photometer to determine the percentage of empty, partially filled, and full AAV capsids within a GMP environment. Method qualification encompassed the evaluation of precision, accuracy, linearity, limit of quantitation (LOQ), and specificity. The method demonstrated high intra-day precision, with %CV values of 4% and 10% for empty and full capsids, respectively. Further, the inter-day precision, as measured by the %CV, were 12% and 8% for Empty and Full capsids, respectively. Accuracy was assessed by spiking known amounts of empty capsids into AAV samples, with recoveries ranging from 92-105% for empty capsids and 92-111% for full capsids. A strong linear relationship was observed, with R^2 values of 0.9927 and 0.9990 for empty and full capsids, respectively. The LOQ was determined to be 4.00E+11 VP/mL. In conclusion, the qualified MP method provides a GMP-compliant, label-free, rapid, user-friendly, and orthogonal approach for

quantifying AAV particle content. This method is applicable for characterizing both in-process and final drug substance/product AAV viral vectors.

1340 Use of Statistical Analyses for Evaluating Large Historical Datasets of AAV Productions to Assess Process Parameters – A Case Study.

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**Corresponding Author*

Introduction: Andelyn Biosciences, Inc., a CDMO launched out of Nationwide Children's Hospital in 2020, has developed Curator™ Platforms for adherent and suspension AAV productions. Using the Curator™ adherent platform, we have manufactured more than 450 Phase I and II clinical batches over 17 years, covering a variety of serotypes and indications. To assess the strength of the Curator™ adherent platform and identify potential critical process parameters, historical data generated from each process step of GMP productions-including 44 sublots of Serotype A and 74 sublots of Serotype B, were compiled and analyzed.

Methods: Process parameters (e.g., titer, volume, yield, percent recoveries, confluency at cell growth, pressures during processing, BSA levels, etc.) were compared using a matrix correlation to identify positive and negative correlations (Figure 1). Specific parameters (e.g., cell culture times, concentration factor, pressures, BSA levels, etc.), were then analyzed along with the viral yields and percent recoveries at each stage of the process. To simplify the analysis, *p*-values (for significance) corresponding to the Pearson correlation of the matrix were analyzed along with the *r*-values (for correlation). The matrices were used as a tool to compare parameters with strong or weak interactions. If two parameters had a low *p*-value (0 – 0.05) and/or high *r*-value (0.7 – 1.0), linear regression was used to assess the relationship.

Results: Percent recoveries of the viral yield for each serotype were calculated across each step of the process (clarification, TFF, IDX, etc.). IDX had the lowest recovery with minimal variability (Serotype A: 48.6± 12.1%; Serotype B: 51.3% ± 11.0%). Throughout the analysis, few trends showed strong correlation (r = 0.7-1) or weak correlations (r = 0.5-0.7). Most trends had relatively low r values ($r < 0.5$) with strong or weak significance. Overall, the trends identify steps incurring maximum loss, those requiring close monitoring, and potential process improvement strategies. Notably, Trans Membrane Pressure (TMP) during TFF and concentration factor showed significant correlations with certain parameters. TMP correlated with several parameters including large harvest volumes and long processing times. Though the TMP was targeted in these productions, close monitoring of the TMP throughout the TFF is recommended. Additionally, an increase in the concentration factor correlated with recovery losses for both serotypes across IDX, necessitating process improvements to mitigate the losses. The data suggest that the product yield is mostly unaffected by the different parameters throughout processing. This is also reflected in the lack of correlation trends in chromatography. The overall recoveries compared to harvest were 24% for serotype A and 38% for serotype B. The process thus appears largely optimized.

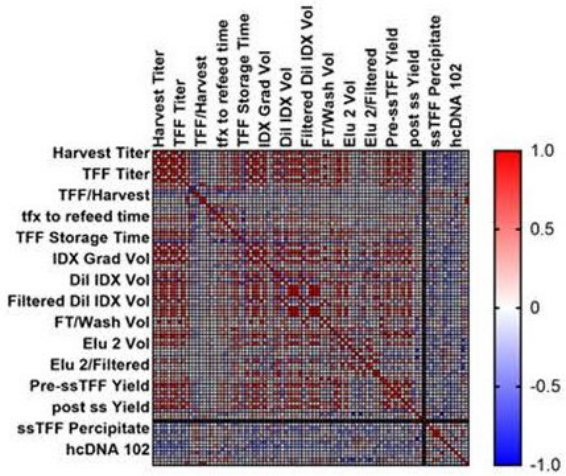

Figure 1. Pearson Correlation Matrix: Initial visual representation of the data. Each square represents a comparison between two parameters; red squares represent positive correlations while blue squares represent negative correlations. Due to the complexity of the figure, only a few parameters can be represented on each axis. For simpler interpretation, individual correlations were analyzed.

1341 As new gene and cell therapies emerge from academia, we must RISE to the opportunity

Nandhitha Uma Naresh[1], Dana Hammill[2], Mark Kessel[3], Roger Hajjar[4], Nathan Yozwiak[5]*
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With over 30 FDA approved products in the United States, the cell and gene therapy (CGT) sector has flourished, as evidenced by the surge in clinical trials and the broadening scope of pre-clinical research. Academic medical centers (AMCs) are critical to CGT developments, serving as a wellspring of early-stage innovation and the sites for clinical trials. Yet reaching the clinic is a lengthy development path, and new CGTs must overcome numerous challenges, including navigating the translational 'valley of death' and balancing the interests of various stakeholders. Unlike traditional small molecule or biologics development, CGTs are further complicated by high costs and complex regulatory requirements. Some federal funding initiatives are now focused on late pre-clinical stage CGTs to accelerate their translation from bench to clinic. Fueling these efforts, AMCs must play a crucial role in overcoming CGT development hurdles by establishing institutional frameworks that offer comprehensive support, extending beyond financial assistance. We propose the adoption of RISE, an institutional model currently implemented by Mass General Brigham's Gene and Cell Therapy Institute (MGB - GCTI) to foster development of CGT innovations that originate in academia. The **RISE** model exemplifies the following: – **R**esource sharing to minimize costs and infrastructure required for CGT manufacturing, **I**nterdisciplinary collaboration by accessing key opinion leaders (KOLs) and patients that intersect at AMCs, **S**ustainable funding by investing into pre-clinical research with long-term commercial viability, and **E**ducational efforts that can engage the research, clinical and patient community in discussions on bridging knowledge gaps. We argue that the promising pipeline of CGT innovations may languish without a concerted focus and investment within AMCs, and the RISE model can serve a win-win approach for both innovators and patients by providing a well-supported path to accelerate the development of new CGTs to the patients in need.

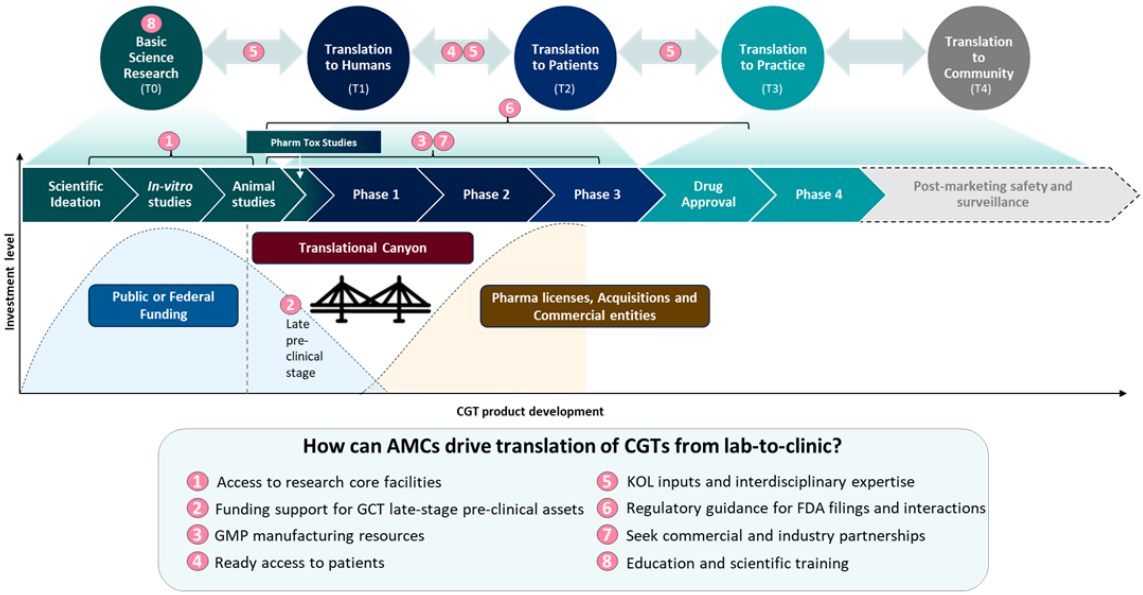

Figure 1: Different ways in which AMCs can drive translation of GCTs. GCTs from lab-to-clinic traverse a long and arduous path which begins with scientific ideation in academic labs. A translational canyon, also called the 'valley of death' develops between late-stage basic science research and clinical applications where the investment needs of GCT product development and type of investors involved shift. AMCs can offer support in multiple ways across all stages of CGT development to bridge existing gaps in clinical translation such as by providing access to shared resources (core facilities, GMP manufacturing, patients), leveraging interdisciplinary expertise, educating scientific community.

1342 *PPQ Ready Risk-Based Identity Testing for Ancillary Raw Materials in Gene Therapy Manufacturing*

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Introduction: Ancillary raw materials play a critical role in gene therapy manufacturing by enabling key processes without becoming part of the final product. Ensuring the identity of these materials is essential to maintain product quality, safety, and compliance. The variety of these materials, their often non-compendial nature, and the complexity of gene therapy workflows present challenges for qualification. A risk-based approach to identity testing provides a balance between compliance and efficiency. This study aims to outline a risk-based framework for identity testing and qualification of ancillary raw materials that focuses on integrating regulatory expectations and widely used analytical techniques to establish robust yet practical strategies for testing.

Methods: Regulatory guidance, including USP <1043> and ICH Q9, were used to develop a framework for assessing risk and determining appropriate identity testing. The risk-based tiers outlined in USP <1043> are critical to assess materials and determine required testing. Per USP <1043> there may be instances where performance testing of materials may serve as identity testing. Ultimately, formal identity testing is required to ensure compliance, but performance testing may bolster material qualification or serve as part of required identity tests. The risk-based framework for test selection incorporates material grade, vendor specified intended use, intended use in manufacturing, critical specifications for performance, vendor qualification, reliance on Certificates of Analysis (CoA's), and risk-based thresholds to guide testing intensity.

Results: A risk-based identity testing approach was designed, prioritizing comprehensive testing for high-risk ancillary materials and reduced testing for lower-risk materials. Purchased custom-prepared solutions proved challenging, as components may be identical at varied concentrations. Critical analytes were identified in the solution through analytical testing (e.g., Fourier Transform Infrared Spectroscopy, Nuclear Magnetic Resonance). Additional characterization tests (e.g., pH, conductivity) were added to distinguish solutions with similar components. The combination of critical analyte detection and characterization tests allowed for solution

identification. Amino acid analysis for complex formulations (e.g., Dulbecco's Modified Eagle Medium) and electrophoretic profiling of materials (e.g., bovine serum) were utilized. For lower-risk materials, simpler techniques or CoA verification testing were deemed sufficient. This approach aligns with regulatory guidance and minimizes redundant testing.

Conclusion: A risk-based approach to identity testing for ancillary raw materials can streamline qualification processes while ensuring regulatory compliance. The framework presented here highlights the importance of integrating robust analytical methods alongside specialized tests to address the unique challenges of gene therapy manufacturing. The outlined approach can lead to substantial cost savings, particularly for prepared solutions where identification of each analyte would be costly. This represents up to 75% savings compared to the total cost in prepared solutions. Other material testing costs were similarly reduced with the risk-based approach. The approach supports consistent product quality, efficient workflows, and a scalable model for managing ancillary raw materials in a cost-efficient manner.

1343 Minimizing Volume Requirements and Turnaround Time for Compendial Testing of Cell and Gene Therapy Products

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Cell and gene therapy (CGT) products have unique needs principally driven by the scarcity of material (i.e. limited volume availability for testing) and the desire for fast turnaround times (TAT), especially for autologous cell therapy products. Compendial methods for critical quality attributes such as Visible Particulates, Color, Clarity, Extractable Volume, Osmolality, and pH often require volumes well above those available for most CGT products, especially when performed in sequence on different samples. We have optimized standard compendial offerings for the specific needs of CGT products. We have developed and a platform method that satisfies these needs. The method is adaptable in terms of manufacturers' preferred testing order and modifiable with respect to total volumes tested by authoring tailored method guidance documents. This process addresses the full range of testing options covering both United States Pharmacopeia (USP) and European Pharmacopoeia (EP) guidelines. Testing offerings include Visible Particulates, Color, Clarity (visual and instrumental), Extractable Volume, Osmolality, and pH. The goal is to highlight the various strategies used to satisfy USP and EP guidelines while minimizing volume and accelerating TAT.

1344 Upstream Process intensification for a Gorilla derived Adenoviral (GRAd) vector by using a Perfusion System

Michela Gentile, ReiThera, Rome, Italy

Recombinant adenovirus vectors are effective gene transfer tools used both in gene therapy, cancer therapy and infectious diseases.

Current processes for manufacturing Adenoviral vectors (AdV) involve the use of serum free media and stirred tank bioreactors by employing HEK293 cells as packaging cell line. Cells are amplified in shake flask, wave and stirred tank bioreactors where viral production is achieved by infecting the cells with the AdV at a specific Multiplicity of Infection.

Developing a process capable of producing large quantities of doses both for clinical and commercial phases has become crucial for the biotech market in recent years.

Viral productivity as well as bioreactors size determine the number of doses that can be produced for each batch. Although large scale stirred tank bioreactors are available for cell cultivation and production phases, huge amount of

cell biomass is needed for the inoculation, thus increasing both the timing for cell amplification and the overall manufacturing costs.

One strategy to boost viral productivity is to increase the concentration of the cells at the infection phase thus improving the total volumetric productivity. However, a challenge with this strategy is the cell density effect, where higher cell densities often lead to lower per cell productivity due to lack of nutrients and toxic cell metabolites accumulation. The perfusion process, which involves continuously replacing exhausted medium with fresh medium, can be a successful solution to achieve higher cell density during the infection phase.

In this study, we present an intensified upstream process based on proprietary HEK293 cell line adapted to suspension growth in a small-scale 2L Stirred Tank Bioreactor for the production of Gorilla-derived Adenoviral vector (GRAd). Through the use of a Perfusion system, we achieved a high cell density culture of approximately 1E+07 cells/mL. Furthermore, infecting the cells at a density of 2E+06 cells/mL after perfusion led to a threefold increase in the total volumetric productivity of the vector compared to standard processes, without affecting vector quality. Cell growth, viability, and metabolite profiles were monitored throughout the process to characterize cell culture, demonstrating that medium exchange can sustain exponential cell growth maintaining high cell viability.

This study establishes that a successful and reproducible intensified process can be applied for the production of GRAd vector in HEK293-derived cell line. This could lead to a reduction in vaccine manufacturing time and an increase in the number of doses produced per batch at the manufacturing scale.

1345 Dissecting the effect of shear during rAAV production using perfusion bioreactors in PCLs

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Recombinant Adeno Associated Virus (rAAV) has recently become one of the most common vectors in gene therapy, with several products already approved by the FDA. While approval is based on therapeutic efficacy, market success in the long term also requires robust quality and cost effectiveness. One way to achieve these goals is through implementation of intensified perfusion-based processes using producer cell lines (PCLs) in combination with a helper virus. While perfusion has proven beneficial in rAAV production, it requires a cell retention device that could lead to unfavorable shear stress on cells. This is relevant especially after virus infection when shear sensitivity increases. This entails a comprehensive understanding of perfusion related process parameters optimization. Using a PCL platform in bench-top bioreactors we examined various operating parameters such as recirculation pump type, recirculation flux, permeate flux, inner diameter of hollow fiber filter (HFF) lumens and total filtration area. The experimental results indicate that shear forces within HFF lumens and the recirculation flow path, influenced by factors like length and inner diameter, can adversely impact cell culture viability and rAAV titers. Additionally, we demonstrate that usage of levitating pumps, that reduce pinch points in the recirculation flow path, can potentially facilitate better productivity and AAV capsid quality. Overall, our studies revealed that shear is an important parameter for rAAV manufacturing that can significantly impact cell physiology and should be carefully examined for perfusion process during process intensification and scale-up.

1346 Establishing a Scalable rAAV Production Platform for Research-Grade Vector Supply within a Pharmaceutical Setting for rAAV Gene Therapy Early Research and Development

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Development, Penzberg, Germany, [4]Roche, Munich, Bayern, Germany, [5]Roche - Basel, Basel, N/A, Switzerland, [6]F. Hoffmann-La Roche Ltd., Basel, Switzerland, Switzerland

Introduction: The increasing demand for research-grade recombinant adeno-associated virus (rAAV) vectors necessitates the establishment of robust, scalable and fast-pace production platforms within pharmaceutical research environments. Here, we describe the evolution and implementation of such a platform, encompassing standardized plasmid design, upstream processing (USP), downstream processing (DSP), analytical characterization, and FAIR data capture to support early-stage rAAV-based gene therapy research and development.

Methods: We formed a dedicated rAAV production team, integrating expertise in molecular biology, cell culture, purification, and analytical development. For plasmid design and production, a standardized workflow was implemented for generating rAAV expression constructs. While initially adherent HEK293 cultures were employed, we early-on transitioned to suspension HEK293 cell cultures for scalable rAAV production using the triple transfection method using different scale formats from deep-well and shake flask up to 1 L scale. Similarly, increased demands required the implementation of additional DSP methods based on affinity and ion-exchange chromatography, which allow for a more scalable, high-yield rAAV purification. Therefore, the adoption of several platforms allows flexibility to cater to various internal programs that require different virus quantities and/or higher construct numbers for screening purposes. A comprehensive analytical panel was established, including assays for titer determination, capsid purity, genome integrity and identity, aggregation, and endotoxin levels. Finally, a standardized IT system for FAIR (Findable, Accessible, Interoperable, Reusable) data storage and management system was implemented to enable data-driven decision making.

Results: The established platform enables the routine production of research-grade recombinant rAAV vectors of various serotypes at scales ranging from milliliters to liters, capable of supporting early-research projects for fast screening in cellular models to supplying rAAV material for NHP studies. We describe here how 1) optimized plasmid designs and 2) improved USP production processes resulted in high-titer AAV production, and 3) the implemented DSP strategy achieved high-quality vector recovery with minimal empty capsid contamination. Furthermore, 4) the implemented rigorous analytical testing suite confirmed the quality and consistency of the produced rAAV batches, meeting pre-defined acceptance criteria. As an example, we present here a research-grade rAAV supply (3 vectors), including extensive analytical panel and CoA criteria, for an ocular NHP study. In addition, we provide comparisons and general learnings–from over a 100 different rAAV batches–on rAAV productivity between vector genome designs, capsid serotypes, and batch-to-batch variability.

Conclusions: This integrated rAAV production platform provides a reliable and scalable source of research-grade rAAV vectors, streamlining early-stage gene therapy research within a pharmaceutical company. The standardized workflows and rigorous quality control measures ensure consistent vector quality and facilitate the rapid progression of promising gene therapy candidates from concept to preclinical development.

1347 ReiCell-AAV: A Fully Documented High-Yield Suspension Cell Line for Scalable AAV Vector Manufacturing

Michela Gentile, ReiThera, Rome, Italy

Adeno-associated virus (AAV) has become a pivotal tool in gene therapy, driving an increasing demand for large-scale production of diverse AAV serotypes, particularly for pre-clinical and clinical applications. Addressing this demand requires enhancing the packaging capacity of producer cells to optimize AAV vector productivity. This study investigates the growth characteristics and AAV packaging efficiency of a high-yield monoclonal cell line derived from HEK293 cells. Starting with a vial of adherent Frank Graham HEK293 cell line frozen at passage 7, the cells were put in culture and successfully adapted to grow at high cell density under animal component-free suspension conditions in shake flasks and bioreactors. To improve AAV productivity, the suspension-adapted cells underwent two rounds of single-cell cloning and screening using the triple-plasmid transfection platform. ReiCell-AAV was further evaluated for production efficiency across multiple AAV serotypes, including AAV DJ, AAV2, AAV8, and AAV9, using shake flasks and 2 L stirred bioreactors. These tests demonstrated stable production capacities, with lysate genome titers ranging from 1E+14 vg/L to 5E+14 vg/L. Host cell DNA (HCD) and empty

capsid ratio in DS were further evaluated. For the AAV8 capsid, preliminary results showed 30–40% full particles at harvest, enabling chromatographic polishing that achieved high enrichment (>90% full capsids) without the need for ultracentrifugation.

In summary, we developed a suspension single-clone cell line, ReiCell-AAV, with fully detailed history starting from the origin of 293 cells and high production capacity, stability, and quality. This platform is well-suited for industrial-scale AAV manufacturing across a range of serotypes, payloads, and production scales, meeting the evolving requirements of gene therapy applications

1348 Demonstrating Key Processes of the T Cell Therapy Workflow in Chemically Defined Media

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[1]FUJIFILM Irvine Scientific, Santa Ana, CA, [2]FUJIFILM Irvine Scientific - Santa Ana, CA, Santa Ana, CA

Although technologies used in the generation of immunotherapies are diverse and rapidly evolving, they share the goal of efficiently generating sufficient cell numbers with high viability and consistent clinical quality. The elimination of undefined media components, such as serum-derived proteins, reduces variability to facilitate safe and efficacious translation for clinical use. Chemically defined media are uniquely amenable to consistent and reproducible scale-up due to the lack of undefined media components, enabling manufacturers to reliably make high-quality products when transitioning from preclinical development to GMP manufacturing of cell-based therapies.

In this study, thawed human PBMCs were activated with αCD3/αCD28 beads, expanded in G-Rex cell culture vessels and Quantum hollow fiber bioreactors in PRIME-XV T Cell CDM with Shenandoah CTGrade GMP rh IL-2_{C126S}, and assessed for functionality. CT Grade GMP IL-2_{C126S}-independent expansion, expansion post-thaw from DMSO-free media without an initial wash step, and lentiviral transduction were likewise performed in G-Rex vessels in chemically defined conditions.

Join us to discover how to:

Achieve scalable expansion, activation, lentiviral transduction of Human PBMC-derived T cells in G-Rex Cell Culture Systems and Quantum hollow fiber bioreactors

Omit the post-thaw wash step to simplify processes and expand cells while maintaining robust functionality in response to antigenic stimulation

Benefit from a powerful toolset for T cell therapy manufacturing with PRIME-XV FreezIS DMSO-Free, PRIME-XV T Cell CDM, and Shenandoah CTGrade GMP Recombinant Proteins working together across a variety of cell sources and culture/activation methods

1349 High-Performing GMP CRISPR Cas9 Nuclease for Cell and Gene Therapy

Sarah Rains, KACTUS, Silver Spring, MD

Introduction: CRISPR/Cas9 technology has been widely used in cell therapy. To address the need for high-quality and high-performing Cas9 nuclease, KACTUS developed a GMP-manufactured Cas9 nuclease and implemented a comprehensive quality control process to make it suitable as an ancillary material for cell therapy applications. The results showed that KACTUS GMP-grade Cas9 enzyme had a good batch-to-batch consistency and long-term

stability. Compared to a leading commercially Cas9 enzyme, KACTUS GMP-grade Cas9 had a comparable gene editing efficiency in both in vitro cleavage assay and in multiple cellular gene editing. Currently, KACTUS GMP-grade Cas9 enzyme has supported multiple cell and gene therapy developments and clinical IND submissions.

Objective: The primary objective focused on achieving stability and high yield, alongside ensuring high activity and consistent quality across batches. This goal was underpinned by the need to minimize risks and align with compliance standards. Achieving these targets was essential for maintaining the reliability and effectiveness of the processes involved, providing a solid foundation for operational excellence.

Methodology: KACTUS addressed these objectives through a multi-faceted approach. They designed the nuclear localization signal (NLS) and optimized the expression and purification process to ensure high yield and activity. The use of animal origin-free materials and the
implementation of a closed process under a Good Manufacturing Practice (GMP) environment were critical steps towards minimizing risks and aligning with compliance. Quality control measures were rigorously applied, including testing for bioburden, endotoxin, residual host nucleic acids, and protein, ensuring the safety and purity of the products. Additionally, both the process and analytical methods underwent thorough validation, further reinforcing the reliability and consistency of the outcomes.

Results: High In Vitro Cleavage Activity
Results: High gene editing efficiency across multiple cell types

Conclusion:
→ High quality and gene editing performance
based on KACTUS GMP manufacturing process
→ Consistent batch-to-batch performance
→ Validated on both T cell and HSC applications
→ Animal origin-free processes and materials

Results: Robust & High-Quality Production

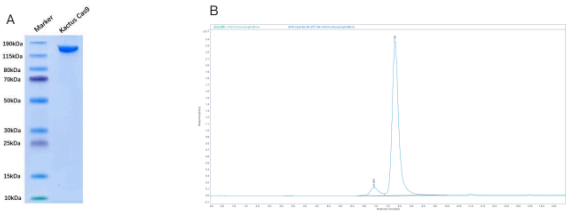

Figure 1. A high purity Cas9 protein was obtained based on the KACTUS GMP production process. (A) detected by Tris-Bis-PAGE.(B)analysis by SEC-HPLC(Agilent). Results show the purity is greater than 95%.

Results: Excellent Long-Term Stability and Batch-to-Batch Consistency

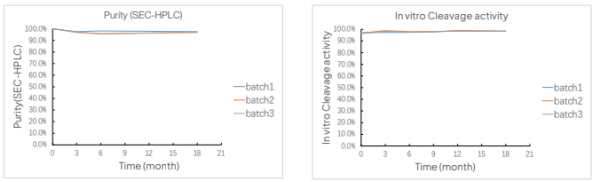

Figure 2. Consistent purity and activity performance observed across three manufacturing batches of KACTUS GMP-grade Cas9. Three batches of KACTUS GMP-grade Cas9 were stored at -20°C for long-term stability test, analyze the purity by SEC-HPLC and in vitro cleavage activity at 3, 6, 9, 12, 15, 18 months, respectively. The results showed KACTUS GMP-grade Cas9 has a good stability and consistent among different manufacturing batches.

Results: High In Vitro Cleavage Activity

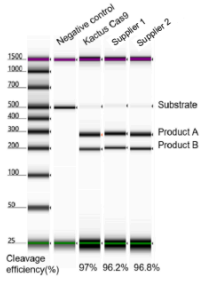

Figure 3. KACTUS GMP-grade Cas9 shows an comparable in vitro cleavage activity with leading suppliers Cas9. 2μL 0.1μg/μL Cas9 protein with 2μL 2μM sgRNA cuts 300ng substrate DNA for 15min at 37°C, then using 4150 TapeStation to analysis the cleavage efficiency. KACTUS GMP-grade Cas9 achieved cleavage efficiency of 97% vs 96.2% of supplier 1 and 96.8% of supplier 2.

Results: High gene editing efficiency across multiple cell types

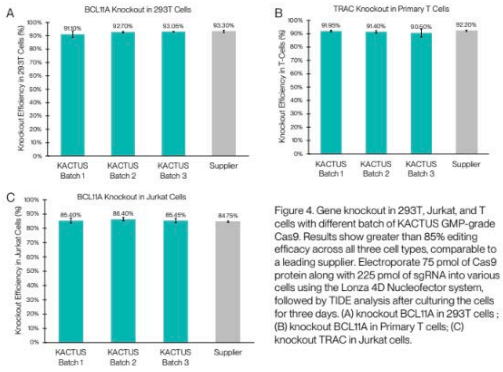

Figure 4. Gene knockout in 293T, Jurkat, and T cells with different batch of KACTUS GMP-grade Cas9. Results show greater than 85% editing efficacy across all three cell types, comparable to a leading supplier. Electroporate 75 pmol of Cas9 protein along with 225 pmol of sgRNA into various cells using the Lonza 4D Nucleofector system, followed by TIDE analysis after culturing the cells for three days. (A) knockout BCL11A in 293T cells ; (B) knockout BCL11A in Primary T cells; (C) knockout TRAC in Jurkat cells.

Case Study 1: Gene editing in primary T cells for UCAR-T

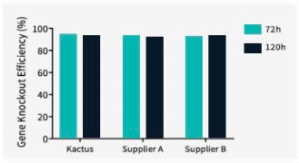

Figure 5. Gene knockout of the HLA gene for UCAR-T application. KACTUS GMP-grade Cas9 demonstrates efficient gene editing activity, comparable to two other suppliers in the market.

Case Study 2: Gene editing in Human Stem Cells (HSCs) for β-thalassemia

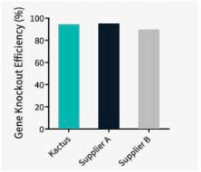

Figure 6. KACTUS GMP-grade Cas9 shows efficient BCL11A knockout activity in gene editing HSCs for the treatment of β-thalassemia, comparable to two other suppliers in the market.

Universal High Sensitivity ELISA Kit for Quantitative Detection of Cas9 Residues

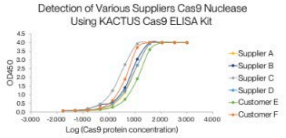

Figure 7. KACTUS has developed a Cas9 residue detection kit (sandwich ELISA method) and tested it with Cas9 products from other commercial suppliers on the market. After testing, the kit has proven to be effective in identifying and quantitatively detecting Cas9 products from various suppliers, demonstrating its potential as a universal Cas9 detection kit.

1350 3-Year Functional Outcomes of Patients With Duchenne Muscular Dystrophy: Pooled Delandistrogene Moxeparvovec Clinical Trial Data vs External Controls

Jerry Mendell[1], Anne Connolly[2], John Day[3], Craig McDonald[4], Crystal Proud[5], Perry Shieh[6], Craig Zaidman[7], Matthew Furgerson[1], **Sravya Ennamuri[1]**, Kai Ding[1], Carol Reid[8], Alex Murphy[9], Jake Elkins[1], Louise Rodino-Klapac[1]

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Delandistrogene moxeparvovec is an rAAVrh74 vector-based gene therapy for Duchenne muscular dystrophy encoding an engineered, functional form of dystrophin shown to stabilize or slow disease progression. It is approved in the US and in other select countries. Here we compare 3-year functional outcomes of ambulatory patients aged 4.0-8.9 y treated with delandistrogene moxeparvovec (treatment group) with those of external controls (ECs), well matched using propensity-score weighting of key baseline prognostic factors.

Delandistrogene moxeparvovec data (n=50) were pooled from studies 101 (NCT03375164, n=4), 102 (NCT03769116, n=26), and ENDEAVOR Cohort 1 (NCT04626674, n=20) and compared with an EC group (n=73) using a propensity-score–weighted median regression model and a mixed-effects model for repeated measures (MMRM). Changes from baseline (CFBLs) to year 3 in the North Star Ambulatory Assessment (NSAA) total score and timed function tests (TFTs) of time to rise (TTR) from floor and 10-meter walk/run (10MWR) were assessed.

At baseline, treatment and EC groups had similar median ages (6.3 y vs 6.5 y), weights (22.0 kg vs 22.6 kg), NSAA total scores (22.0 points vs 21.0 points), TTRs (3.9 s vs 4.3 s), and 10MWR times (4.9 s vs 5.1 s). Median regression analyses showed clinically meaningful stabilization of disease over 3 years in the treatment group vs the EC group. The 3-year CFBL (SE) in NSAA total score for the treatment group vs the ECs was −2.55 (0.76) points vs −5.55 (0.77) points (between-group difference [SE]: 3.00 [0.80] points, *P*=0.0003). MMRM analyses yielded similar results and will also be presented alongside TTR and 10MWR assessments.

Our findings show that treatment with delandistrogene moxeparvovec results in long-term stabilization or slowing of disease progression compared with a rigorously matched EC group, with an increase in between-group divergence over time.

1351 Brogidirsen -The First Dual-Targeting Exon 44 Skipping Drug for Duchenne Muscular Dystrophy Safety, Tolerability, and High Dystrophin Restoration in Long-Term Clinical Studies

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Introduction: Duchenne muscular dystrophy (DMD) is a severe X-linked muscle disorder caused by mutations in the DMD gene, which encodes dystrophin, a crucial protein found at the muscle cell membrane. NS-089/NCNP-02 (Brogidirsen) is the first drug designed for dual-targeting exon 44 skipping and was developed by the National Center of Neurology and Psychiatry (NCNP) in collaboration with Nippon Shinyaku Co., Ltd. Preclinical studies have shown highly effective skipping of exon 44 and restoration of dystrophin in cells derived from DMD patients with mutations suitable for this approach, suggesting the drug's potential to slow disease progression.

Methods: Six participants were assigned into two cohorts in the previous Phase 1/2 study (NCT04129294) for safety and efficacy investigations. Brogidirsen was administered intravenously once weekly at therapeutic doses of 40 mg/kg once a week (low dose, Cohort 1) or 80 mg/kg (high dose, Cohort 2) for 24 weeks in Part 2 of the study. Ongoing extension study (NCT05135663) is evaluating Brogidirsen's safety, tolerability, and efficacy in patients with DMD over 2 years and is ongoing. The study includes molecular and biochemical analyses, pulmonary function tests, and assessments of motor function. In vitro assays conducted with MYOD1-converted urine-derived cells (MYOD1-UDCs), myoblasts, and MYOD1-converted fibroblasts from the same patients demonstrated a dose-dependent efficiency in exon skipping and dystrophin restoration.

Results: No severe adverse events or anaphylaxis related to Brogidirsen were reported, and there were no treatment discontinuations. The efficacy of exon 44 skipping and dystrophin levels in biopsied muscles significantly improved, with the high-dose Brogidirsen achieving 24.47% on average of normal dystrophin levels in 24 weeks. Additionally, plasma biomarkers associated with DMD (such as PADI2, TTN, MYOM2, MYLPF) showed reductions, correlated with improved clinical profiles. After 99 weeks, the high-dose Brogidirsen achieved 18.17% on average of normal dystrophin levels. Four out of six participants maintained or improved their motor function, as measured by the North Star Ambulatory Assessment. In vitro assays showed effective exon skipping and

dystrophin expression in a dose-dependent manner in MYOD1-UDCs, myotubes, and MYOD1-transduced fibroblasts from six patients, supporting the case for human trials.

Conclusion: These findings underscore Brogidirsen's safety and potential to modify the progression of DMD. The extension study is ongoing, and a Phase 2 trial in the USA (NCT05996003), sponsored by NS Pharma, Inc., is also in progress to further evaluate its safety and efficacy.

1352 Design of the Phase 1 ASCEND Study to Investigate the Safety and Biodistribution of INS1201 Gene Therapy in Males With Duchenne Muscular Dystrophy

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Background: INS1201 is a recombinant adeno-associated virus serotype 9 carrying a micro-dystrophin gene expression construct currently in development for the treatment of Duchenne muscular dystrophy (DMD). INS1201, designed to express a micro-dystrophin protein in muscle, has demonstrated histopathological improvement and functional efficacy in the *mdx* mouse and high levels of muscle fiber expression in both mouse and nonhuman primates. INS1201 is delivered via a single intrathecal administration, which has been shown in mice and nonhuman primates to efficiently target skeletal and cardiac muscles at relatively low vector doses compared to those required for intravenous administration.

Objective: To present the study design for the phase 1, first-in-human, open-label, 2-part, dose escalation ASCEND study assessing the safety and biodistribution of INS1201 in ambulatory males aged 3 to <5 years (Part 1) and 2 to <3 years (Part 2) with genetically confirmed DMD.

Design/Methods: The study will evaluate up to 12 participants to determine the recommended phase 2 dose (RP2D). Each study part consists of 2 dose cohorts (n=3 each), with a starting dose of 5.0E+14 vector genomes (vg) in the first cohort and a higher dose of 1.0E+15 vg in the second. The study will open with the starting dose in Part 1 only; enrollment into each dose cohort will be staggered with a 30-day safety observation period between each participant. The study will be monitored by an independent safety monitoring committee, which will review cumulative safety data throughout the study and prior to the opening of Part 2 and all dose level increases. The primary endpoint is the incidence and severity of treatment-emergent adverse events (AEs), AEs of special interest, and serious AEs. The RP2D will be identified based on safety, tolerability, and change from baseline to week 16 in quantity of INS1201 micro-dystrophin protein expression from biopsied muscle tissue. Additional exploratory endpoints will evaluate micro-dystrophin transgene levels, multiple measures of motor function, developmental milestones, and caregiver-reported outcomes. Participants will be followed for 96 weeks after INS1201 administration, after which all participants will be enrolled in a 3-year extension study to assess long-term safety.

Conclusion: The ASCEND study will assess the safety and biodistribution of INS1201 to inform RP2D selection.

1353 Assessment of Cardiac Outcomes in Delandistrogene Moxeparvovec Clinical Trials for Duchenne Muscular Dystrophy

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Delandistrogene moxeparvovec, an rAAVrh74 gene therapy, delivers a transgene encoding a functional form of dystrophin shown to stabilize or slow the progression of Duchenne muscular dystrophy (DMD). Here we assess pooled cardiac safety outcomes from delandistrogene moxeparvovec studies with 1-5 years of follow-up.

Data from 156 patients (mean age [range], 6.7 [3.2-20.2] years; baseline left ventricular ejection fraction [LVEF] range, 48.9%-77.0%; ambulatory, 148 [95%]) were collected from 4 studies (101 [NCT03375164; n=4], 102 [NCT03769116; n=41], ENDEAVOR Cohorts 1-5b [NCT04626674; n=48], and EMBARK Part 1 at 52 weeks [NCT05096221; n=63]); all excluded patients with LVEF <40%. Troponin-I (TnI) was assessed regularly in ENDEAVOR and EMBARK.

Two cases of myocarditis were reported within days of delandistrogene moxeparvovec infusion; both resolved within 3 weeks (1 with sequelae). Except in the 2 myocarditis cases, TnI fluctuations were asymptomatic and compatible with fluctuations observed in DMD. In ENDEAVOR Cohorts 1-3 (n=32), there was no apparent relationship between total capsid load and peak TnI levels 1-2 weeks post-infusion. At week 52, cardiac MRI performed in a subset of patients from EMBARK revealed no relevant differences in cardiac function (including LVEF), volume, or mass between patients treated with delandistrogene moxeparvovec or placebo. Overall, LVEF values were stable across all studies.

Results of delandistrogene moxeparvovec studies to date, with 1-5 years of follow-up, indicate a manageable cardiac safety profile in this population of patients with DMD. Therapy is generally well tolerated and safe, with no signs of persistent treatment-related cardiac injury.

1354 Innovative Intrathecal AAV9-mediated Gene Therapy for Spinal Muscular Atrophy: Phase I/II Safety and Efficacy Topline Results

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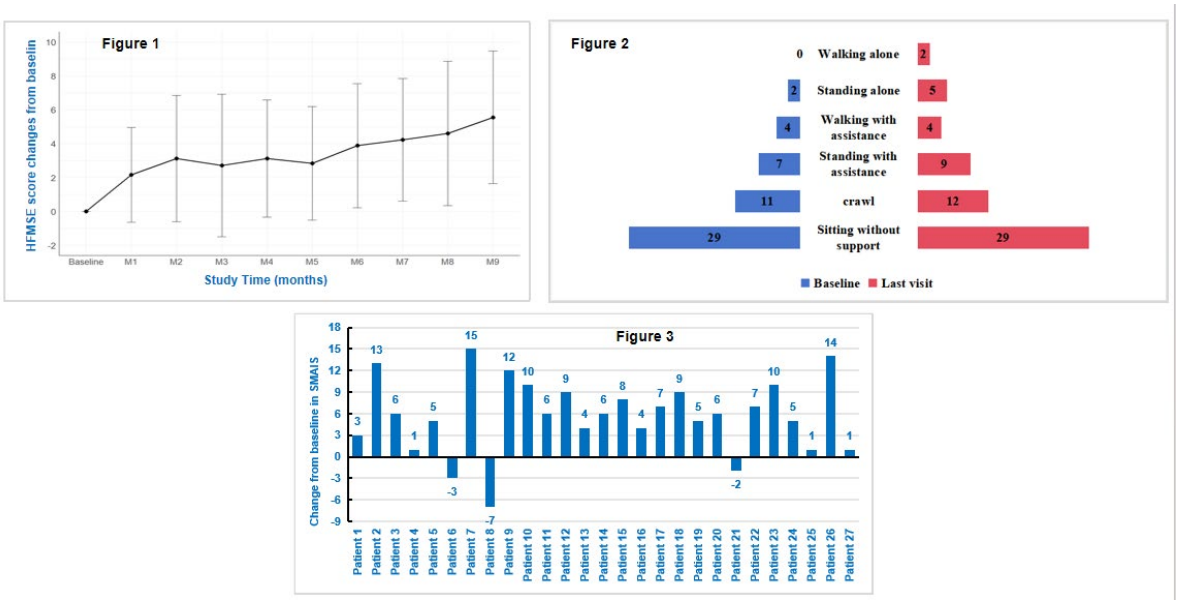
Background: Spinal Muscular Atrophy (SMA) is a severe neuromuscular disorder caused by homozygous mutations in the SMN1 gene, leading to motor neuron degeneration and muscle weakness. Intravenous AAV-mediated gene therapy has shown promise in treating Type 1 SMA but raises safety concerns, including immune responses indicated by elevated liver enzymes. This study evaluates an innovative AAV9-mediated gene therapy (GC101) delivered via intrathecal injection in patients with late-onset SMA, focusing on safety, tolerability, and efficacy.

Methods: A Phase I/II single-arm, single-dose, open-label clinical trial assessed GC101's intrathecal administration in pediatric patients aged 2 to 12 years with genetically confirmed Type 2 SMA, capable of sitting independently but unable to walk. Before GC101, patients completed a washout period, discontinuing Nusinersen and/or Risdiplam. Post-administration, all prior treatments were ceased. Safety assessments monitored adverse events, immune responses, and viral shedding. Efficacy was evaluated using the WHO-MGRS, HFMSE, and RULM scales, with measurements at baseline and predefined intervals over 12 months. Quality of life was assessed using the SMAIS.

Results: Twenty-nine patients received GC101, showing good tolerability with no severe therapy-related adverse events. All serious adverse events were respiratory infections resolved with standard care. Mild immune responses in a few patients resolved with standard management. Efficacy data revealed significant motor function improvements. HFMSE scores increased by 3.85 points on average at 6 months, with 58.6% achieving ≥3-point improvement. By 9 months, HFMSE scores rose by 4.89 points, with 69% showing ≥3-point improvement. Among 13 patients completing 12-month follow-up, HFMSE scores improved by 4.77 points, with 61.5% demonstrating ≥3-point improvement. RULM scores showed a similar trend. Treatment led to significant motor milestone improvements, with 24.1% achieving at least one new milestone, including two gaining independent walking ability and five gaining independent standing ability. SMAIS scores increased by 5.65 points on average at 9 months, indicating enhanced daily functioning and quality of life.

Conclusions: GC101, an intrathecal AAV9-mediated gene therapy, is well-tolerated and shows promising efficacy in Type 2 SMA patients. Patients previously treated with Nusinersen and/or Risdiplam can safely benefit from GC101. These results support GC101's ongoing development as a potential Type 2 SMA treatment, with further studies needed to assess long-term safety and efficacy. The study highlights intrathecal AAV9-mediated gene therapy's potential to provide significant clinical benefits for SMA patients.



1355 *Advances in AAV9 Gene Therapy for CLN3 – Update on the Phase I/II Clinical Trial Indicating Lasting Efficacy at 5 Years Post Dosing and Next Steps in Program Development*

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Juvenile neuronal ceroid lipofuscinosis (JNCL), also called CLN3 Batten Disease, is a debilitating autosomal recessive neurodegenerative disorder affecting children with onset around 4-10 years of age. The lack of functional CLN3 protein causes accumulation of cellular waste (storage material) due to malfunction of the lysosomes, as well as other intracellular defects including mitochondrial dysfunction. Affected children display rapid vision loss in most cases, followed by decline in motor function, behavior and cognition accompanied by development of seizures. While a few treatments are currently advancing through various stages of clinical trials, there is no cure or effective treatment for CLN3 Batten Disease that could change the course of this degenerative disorder. Thus, current

treatment focuses on symptom management. Natural history studies indicate worsening of individuals by 2.86 points/year as measured by the Unified Batten Disease Rating Scale (UBDRS). Investigators at Nationwide Children's Hospital, Columbus, Ohio developed the first gene replacement therapy for CLN3 Batten Disease and in 2018 initiated an open label, dose escalation phase I/II clinical study using lumbar intrathecal delivery of adeno-associated viral vector serotype 9 (AAV9) allowing expression of a functional CLN3 coding sequence in the central nervous system. Four patients were treated with the initial clinical vector lot. Treatment was well tolerated with only a few adverse events recorded, one involving liver enzyme elevation to more than 3-fold normal levels. Evaluation of disease progression indicates stabilization of the disease as measured by UBDRS (-0.13 points/year). Importantly, long-term efficacy data from over 5 years post-treatment demonstrate that 3 out of 4 patients maintain mobility (measured by the 50 meter walk test). We will also present long-term cognitive and adaptive functioning measures. To prepare for a potential dose escalation in clinic, additional high dose large animal studies were also conducted, further underlining the safety of this treatment. Taken together, the highly promising clinical data demonstrates the safety and suggests long-lasting efficacy of a classic gene replacement strategy for CLN3 Batten's Disease. Additional preclinical efforts have positioned this program optimally for further development to bring this therapy to patients with urgent need.

1356 *Completed Phase 1/2 Trial of Dual-Vector rAAVrh8 Gene Therapy for GM2 Gangliosidosis Administered by Bilateral Intrathalamic, Cisterna Magna and Intrathecal Delivery*

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We previously developed a dual rAAVrh8 vector encoding HEXA and HEXB which can restore central nervous system hexosaminidase (Hex) enzyme activity, decrease GM2 levels in cerebrospinal fluid (CSF), and rescue most or all the phenotypic consequences of GM 2 gangliosidosis Tay-Sachs and Sandhoff diseases (TSD and SD) in animal models following simultaneous bilateral injection into the thalami. Following up on a small (N of 2) expanded access (EA) trial, we initiated a Phase 1/2, single-dose, dose-escalation of combined bi-thalamic (BiT), intra-cisterna magna, and intrathecal infusion of rAAVrh8-HexA/HexB in children affected by GM2 gangliosidosis with four cohorts at dosage levels ranging from 1.42E+14 to 3.56E+14 vg per patient (5.87E+12–4.08E+13 vg/kg per patient). The BiT injection volume and vector dose was doubled between each cohort with the lowest dose matching the dose used in the prior EA trial. Six infantile and three juvenile patients were enrolled, and all patients were immune suppressed with rituximab, sirolimus, and corticosteroids prior to being dosed and continued with sirolimus for approximately 7 months and steroids for 3 months post-dosing. CSF HexA enzyme activity, serum total Hex activity, and GM2 levels showed a dose-dependent biochemical correction of the disease [Figure 1]. Importantly, serum Hex activity surpassed 40 nmol/hr/ml, which is 2× the lower limit of normal and neuroimaging demonstrated an increase in the net fiber tract number [Figure 2]. Enzyme activity and fiber tract increases were greatest at 12 weeks, but in decline by 24 weeks post-dosing. Infantile patients experienced global clinical stabilization and prolonged oral feeding without aspiration until 3 to 3.5 years. Adverse events were rare in infantile patients, but worsening dystonia was observed in juvenile patients, and so juveniles were excluded from ongoing enrollment in the study. These data on safety and dose-response effects on GM2 biomarkers and clinical outcomes informs route of delivery and patient selection in the next phase of clinical development for gene therapy treatment of TSD and SD.

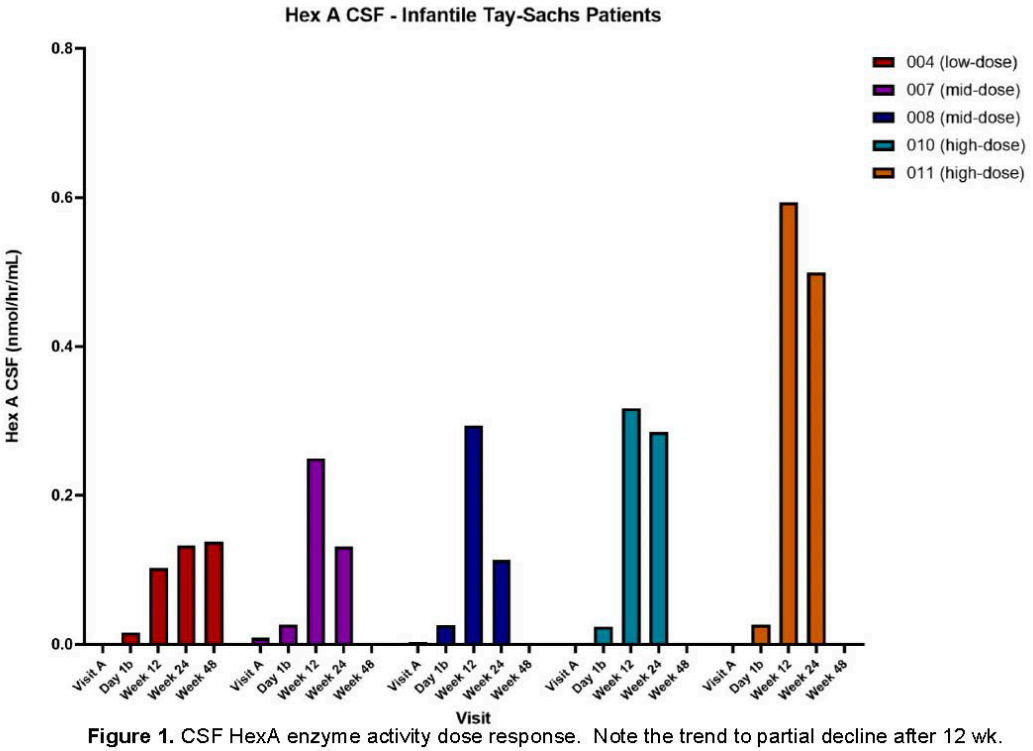

Figure 1. CSF HexA enzyme activity dose response. Note the trend to partial decline after 12 wk.

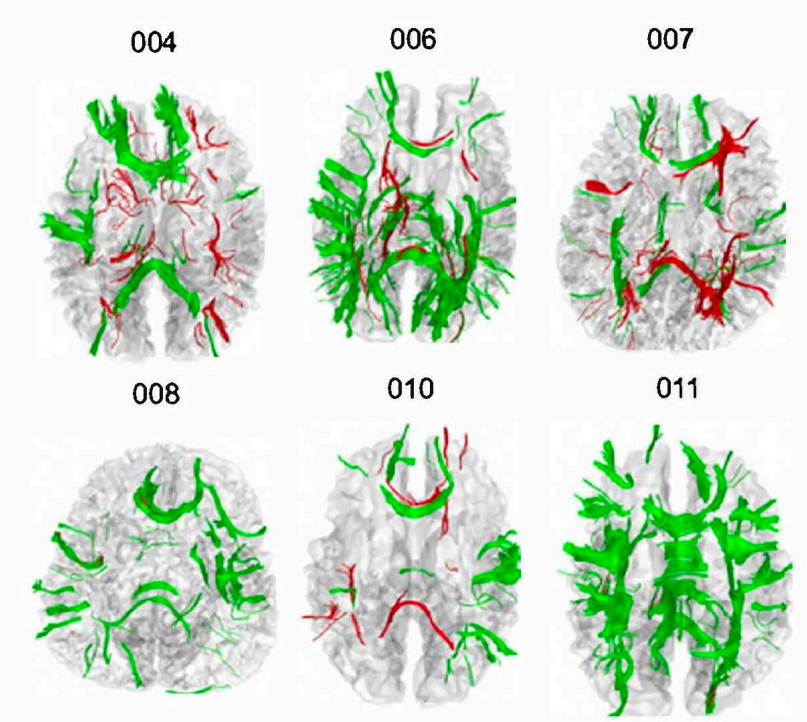

Figure 2. Diffusion Tensor Imaging analysis from scans done prior to vector delivery and at 24 weeks after vector delivery showing areas of increased (green) or decreased (red) fiber track development

1357 Urine NAA Results from the CVN-101 Natural History Study for Canavan Disease: Correlation with Phenotype

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Gene therapies offer an extraordinary promise for rare and ultra-rare diseases. For many reasons, including but not limited to very small sample sizes, placebo-controlled trials are not practical. Therefore, identifying an easily sampled biomarker on the causal disease pathway that is associated with clinical phenotype would be of tremendous value. Here we demonstrate the feasibility of such an approach in Canavan disease (CD), leveraging the power of an independent, prospective longitudinal natural history study.

CD is an autosomal recessive, early-onset fatal spongiform leukoencephalopathy caused by loss- of-function mutations in the ASPA gene. Deficient ASPA enzymatic activity leads to elevated levels of its substrate, N-acetylaspartate (NAA), and profoundly impaired psychomotor development, with typically affected patients failing to gain even the most basic milestones. Currently, there is an actively enrolling trial investigating the impact of restoring ASPA activity via systemically administered AAV gene therapy on CD. Although sampling ASPA activity in the clinic is challenging, urine NAA represents an easily sampled biomarker that directly reflects underlying ASPA activity. Within CD, a subset of patients exhibits a milder phenotype with more milestone acquisition, including the ability to walk independently or with support. The milder phenotype is possibly related to greater residual ASPA activity. As NAA levels reflect residual ASPA activity, we hypothesized that there may be an association between urine NAA levels and clinical phenotype within the CD spectrum.

CANinform (CVN-101; NCT04126005) is an ongoing natural history study evaluating urine NAA levels prospectively and developmental /motor skills assessments, retrospectively and prospectively, in CD patients receiving standard supportive care. To date, 66 participants have been enrolled in this study globally. Prospective urine NAA data were available from 27 participants, 20 with a typical phenotype and 7 with a mild phenotype. These data were leveraged to compare urine NAA levels and associated genotypes in patients classified with mild or typical CD, with the hypothesis that urine NAA levels reflect ASPA activity and therefore can distinguish between the two phenotypes. Urine NAA levels were lower, on average ($p = <0.0001$), in participants with mild (mean 578, range 249.5 – 767.1 mmol/mol Cr) compared to typical CD (mean 1429, range 426.2 – 2440 mmol/mol Cr). In agreement with the literature, mutations R71H, Y288C, and G274R (variants that may harbor residual ASPA activity) were unique to the mild phenotype population (71%, 5/7) and not found in individuals with a typical phenotype in CVN-101 (0%, 0/20). Additionally, collection of prospective assessments of clinical functional tests in this study lends itself to understanding the potential relationship between the course of the psychomotor development impairment and urine NAA levels in CD patients, which is important for determining potential efficacy in gene therapy trials in CD.

Conclusion: In aggregate, the CANinform natural history data suggest urine NAA, by reflecting underlying ASPA activity, is an easily sampled biomarker on the causal pathway of CD and, importantly, can distinguish between mild and typical Canavan disease phenotypes. The Sponsor aims to fully investigate potential correlation between urine NAA and functional status of CD patients in this study.

1358 Volumetric Magnetic Resonance Imaging, Diffusion Tensor Imaging, and Differential Tractography Metrics Correlate with Clinical Outcomes Following Gene Therapy in GM1 Gangliosidosis Patients

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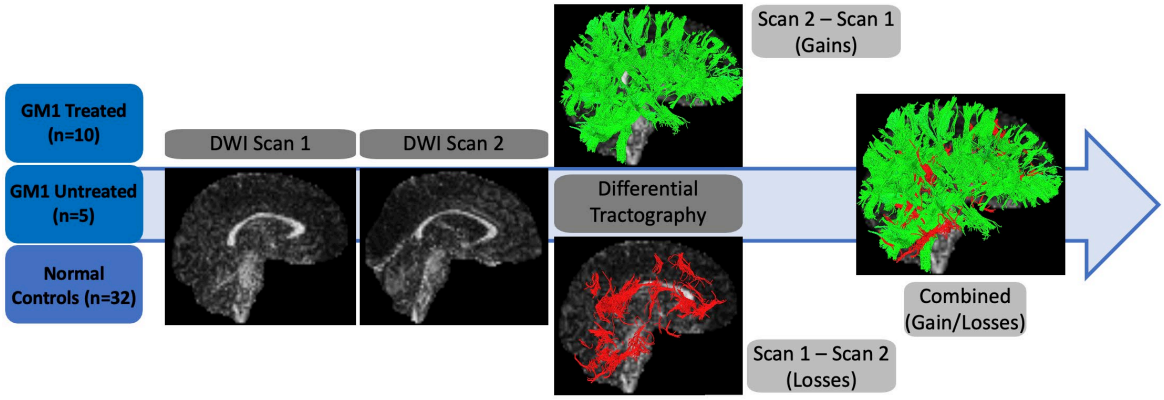
Introduction: GM1 gangliosidosis is a progressive neurodegenerative lysosomal storage disorder caused by biallelic variants in *GLB1*. There are no approved therapies, however AAV9 gene therapy trials are underway. Previous studies have documented imaging findings associated with GM1; however, no study has shown changes associated with an intervention in this cohort.

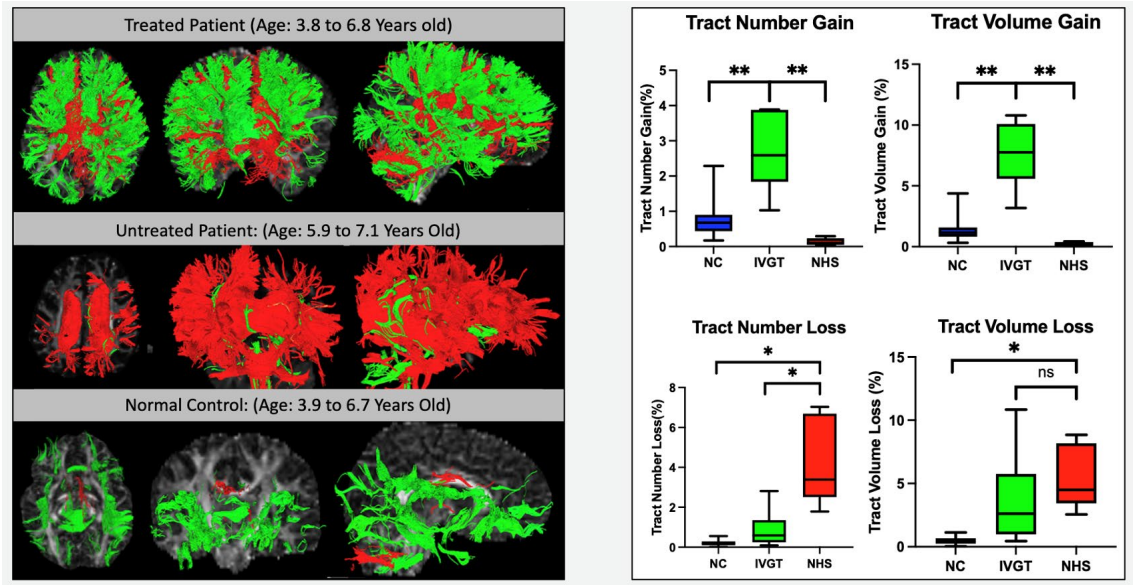
Methods: We compared volumetric magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), and differential tractography between untreated GM1 patients (NHS, n=5, NCT00029965), gene therapy treated patients (IVGT, n=10, NCT03952637), and neurotypical controls (NC, n=32). Gene therapy treated GM1 patients were treated as a part of a Phase 1/2 open-label, dose-ranging, intravenous administration of AAV9-GLB1. GM1 patients from both cohorts underwent sedated MRI using a Phillips Achieva 3T system. Age-matched NC were obtained from open-source data repositories. Volumetric MRI analysis was performed using Freesurfer's *recon-all* pipeline. Atlas-based fiber tractography was performed using DSI Studio to calculate fractional anisotropy (FA) and mean diffusivity (MD) in the whole brain, corpus callosum, cerebellum, cingulum, and corticospinal tracts. Differential tractography (DT) is a subject level DTI analysis technique enabling longitudinal comparisons in white matter fiber tracts within a patient over time. A one-way ANOVA was used to analyze volumetric data, Welch's two sample t-tests to evaluate DTI and DT parameters between NHS and IVGT patients, and linear mixed effects models to correlate DTI and DT metrics with Vineland adaptive behavior scores and CGI-C change in clinical global impressions scores.

Results: Treated patients showed significant reduction in the rate of ventricular enlargement ($p = 0.0297$) compared to NHS patients. Treated patients also showed significantly increased changes in FA in the whole brain ($p = 0.0006$), corpus callosum ($p = 0.0382$), cerebellum ($p = 0.0007$), cingulum ($p = 0.0002$), and corticospinal tracts ($p=0.0411$). Untreated patients showed significantly increased changes in MD in the whole brain ($p=0.0049$), cerebellum ($p < 0.0001$), cingulum ($p=0.0020$), and corticospinal tracts ($p = 0.0424$). Whole brain FA ($\mathbb{R}?^2 = 9.41$, $p = 0.002159$, R^2

= 0.4104) and MD ($\mathbb{R}?^2 = 10.83$, p=0.001001, $R^2 = 0.4434$) correlated with Vineland-3 scores. DT showed increases change in fiber tract density ($p = 0.0047$), and volume ($p = 0.0016$) in the treated cohort compared to untreated GM1 patients. Untreated NHS patients showed decreases in fiber tract density ($p = 0.0116$) compared to treated patients. Net fiber tract density ($p = 0.001373$) and volume were also higher in IVGT than untreated NHS patients. There was no statistical difference in net fiber tract density or net fiber tract volume between IVGT and NC. DT assessed net fiber tract number ($\mathbb{R}?^2 = 31.49$, $p < 0.001$, $R^2 = 0.3764$) and net fiber tract number ($\mathbb{R}?^2 = 36.58$, $p < 0.001$, $R^2 = 0.4176$) both correlated with longitudinal change in patients' clinical presentation as assessed by CGI-C.

Conclusion: This is the first study demonstrating the utility of DTI and DT in evaluating longitudinal changes following therapeutic intervention in GM1 patients. We found improvements resulting from gene therapy as assessed by volumetric MRI, DTI, and DT. We also found our MRI derived metrics correlated with clinical outcomes indicating potential as a robust biomarker.





1359 What is the Formula for Commercial Success of a Gene Therapy? Reflections on Approved GTx

Simone Seiter[1], Christian Hinneburg[1]
[1]*Simon-Kucher, Frankfurt, Hessen, Germany*

Introduction: As of today, fewer than 20 gene therapies (GTx) have been approved in the US and Europe, yet only one has reached blockbuster status, with two more expected to follow. Despite their transformative potential, the majority of gene therapies fail to achieve widespread commercial success. One of the most decisive factors influencing a GTx's market trajectory is the selection of the right indication. This analysis seeks to identify the key characteristics of commercially successful indications and how they drive adoption, pricing potential, and long-term viability.

Methods: A retrospective analysis of approved gene therapies was conducted, focusing on the indications selected by manufacturers. Key variables analyzed include disease severity, prevalence, life expectancy with standard of care (SoC), quality of life (QoL) impairment, the competitive landscape, and clinical development feasibility. Structured expert interviews with industry leaders and real-world case studies were incorporated to validate insights. The session will be presented by Simone Seiter (Senior Partner, Simon-Kucher), leveraging expertise in gene therapy commercialization.

Results: Preliminary findings reveal that the selection of the right indication is one of the most critical determinants of commercial success. The following five key criteria emerged as pivotal:

- Life Expectancy & Disease Severity: Gene therapies targeting life-threatening diseases with limited SoC options tend to achieve higher adoption, as the urgency for a curative treatment is greater.
- Quality of Life (QoL) Impairment: Indications with severe, progressive QoL decline create a stronger value proposition, making them more attractive for both payers and patients.
- Patient Population & Age: The age of the target population plays a critical role—pediatric indications may benefit from longer lifetime value, whereas adult indications often require more established treatment paradigms for adoption.
- Future Treatment Alternatives: The expected evolution of the competitive landscape is a key consideration, as some indications may see disruptive alternative therapies that limit the long-term market opportunity for a GTx.
- Technical & Clinical Feasibility: Indications with clear, clinically validated biomarkers and regulatory precedent for approval tend to have a smoother development and commercialization path.

Conclusions: The success of a gene therapy is highly dependent on indication selection—more than any other commercial factor. An indication that aligns with high unmet medical need, strong patient and provider demand, and regulatory feasibility has a significantly greater chance of commercial success. This session will provide industry leaders with a structured framework for making strategic indication selection decisions, ensuring the long-term viability of their gene therapy assets. The success of a gene therapy is **highly dependent on indication selection**—more than any other commercial factor. An indication that aligns with **high unmet medical need, strong patient and provider demand, and regulatory feasibility** has a significantly greater chance of commercial success. This session will provide industry leaders with a structured framework for making strategic indication selection decisions, ensuring the **long-term viability** of their gene therapy assets.

1360 Characteristics of Long-Term Follow-Up Studies for Gene Therapies registered in ClinicalTrials.gov

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Guidance from the FDA and other regulatory agencies recommends long-term follow-up (LTFU) studies of gene therapy recipients. The primary objective of LTFU studies is to understand the long-term safety of gene therapy

products; the evaluation of efficacy outcomes may be a secondary goal. To learn more about LTFU study design and conduct, we conducted a descriptive study of key characteristics of LTFU gene therapy study protocols registered in ClinicalTrials.gov, including data about the status and duration of the studies, funding source, enrollment, number of clinical trial sites per study, eligibility criteria, geographic location, and intent to monitor and report adverse events (AE). The characteristics of 103 "stand-alone" LTFU studies, as well as eight other records describing GT interventional clinical trials mentioning or incorporating an LTFU component, are described and analyzed. This analysis of registered LTFU studies enabled a better understanding of how these studies are currently designed and stimulated ideas for improvement, which are discussed. Most notably, our results suggest that there is a lack of harmonization in how safety outcomes are monitored and reported across LTFU studies. Standardization and/or harmonization of adverse event and outcome reporting for LTFU studies of GTs may increase their scientific and social value. The development of better guidance and innovative approaches for LTFU study design and conduct may support best practices and the fulfillment of LTFU commitments to understand the overall long-term benefit-risk profile of GT products.

1361 Safety and efficacy of the therapy with cd4+cd25highcd127- t regulatory cells – 12 years after administration

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OBJECTIVE: CD4+CD25highCD127- T regulatory cells (Tregs) remain a drug candidate for immunotherapy of type 1 diabetes (T1DM). We completed three trials testing Tregs in T1DM in recently diagnosed (stage 3) children. Here, we looked for long-term safety and efficacy aspects which is a key to the authorisation of this therapy.

RESEARCH DESIGN AND METHODS: We re-examined 51 T1DM patients tested with Tregs in our previous trials. At the time of assessment, these patients were 7,5 to 12 years after the therapy. They were assigned to the groups treated with Tregs and anti-CD20 antibody (Tregs+antiCD20, N=9), with 1 or 2 doses of Tregs only (Tregs 1 dose, N=10 and Tregs 2 doses, N=8) and standard of care group (SoC control, N=24). The patients underwent diabetes-oriented tests as well as non- to minimal invasive evaluation of the anatomy and function of the eye, micro- and microcirculation, endocrine system, genitourinary tract and fertility, kidney and liver profiles and detailed biochemistry lab tests. Particular attention was paid to any safety issues reported by the patients. Altogether, over 700 variables were analysed.

RESULTS: Tregs+antiCD20 group was superior to SoC control group in terms of C-peptide secretion in mixed-meal tolerance test (MMTT), the time of insulin independence and the time of remission. No clinically relevant between-groups differences were found in other performed examinations. Importantly, no severe adverse effects including tumours or deaths were reported during entire posttreatment time in any of the groups.

CONCLUSIONS: We confirmed long-term safety and efficacy of the therapy with Tregs. Due to superior efficacy profile, the combined treatment with Tregs and antiCD20 antibody should be considered as a routine therapy for recent onset T1DM patients.

1362 Reviving Hearts, Restoring Lives: Long-term Clinical Outcomes of Allogeneic iPSC-derived Cardiomyocytes Transplantation for Advanced Heart Failure Patients

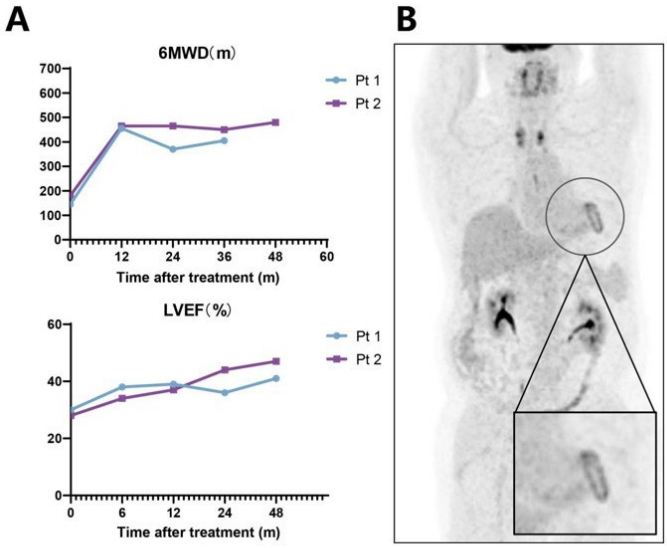
Alex Zhang, OneTwenty Therapeutics, Burlingame, CA

Heart failure is the leading cause of morbidity and mortality worldwide. Standard treatments are often insufficient to reverse heart failure especially in advanced heart failure patients (NYHA III-IV). Heart transplantation is considered the one last choice, limited by the shortage of donor organs. Cell-based therapies emerge as a promising alternative to restore cardiac function. Human induced pluripotent stem cells (hiPSCs) have the capacity for ex vivo differentiation into highly purified cardiomyocytes, thus making them an ideal source for heart regenerative medicine aimed at restoring lost myocardium1,2. However, there are still multiple challenges and technical hurdles

that need to be overcome, such as large-scale differentiation, optimal delivery route, management of the immune response, long-term safety and patient stratification.

Here, we report the First-in-Human study of direct epicardial transplantation of allogeneic iPSC-derived cardiomyocytes concomitant with open-chest coronary artery bypass grafting (CABG) surgery. The study protocol was approved by the Institutional Ethical Committee of Nanjing Drum Tower Hospital (SC2019008). Two patients with advanced heart failure (NYHA Class IV) were enrolled and received intramyocardial injections of allogeneic iPSC-derived cardiomyocytes in May 2019. On completion of the CABG anastomoses, 100 million cardiomyocytes were evenly injected into the peri-infarct tissue at 10 injection sites in the left anterior wall and heart apex. One-month immunosuppressant regimen was used, including methylprednisolone, basiliximab, tacrolimus and mycophenolate mofetil. Both patients successfully recovered from surgery, and completed 12-, 24-, 36-, and 48-month follow-ups.

The current first-in-human study of allogeneic iPSC-CMs transplantation provides the longest safety data in cardiac regenerative therapy and also demonstrates an improvement in heart function. In particular, improvements in exercise capacity, QoL, and NYHA class were observed in both patients by 12-month follow-up. No tumors, off-target effects or severe adverse events were recorded. A striking finding is that the improved cardiac enhancement segment was spatially related to the sites of cell implantation, even at 48-month (Patient 2). While the study design, primarily poised to assess feasibility and safety and which thus entailed a concomitant CABG, prevents to distinguish the effects of cells from those of myocardial revascularization, the clear documentation of safety is a strong incentive to further explore iPSC-derived cardiomyocytes as a new treatment modality for severe heart failure. Further trials, including those under way[5], should more specifically aim at addressing the key clinically-relevant roadblocks that still need to be overcome: the optimal management of immunosuppression; the efficacy of less invasive routes of cell administration and the potential benefits of repetitive dosing.



(A) Exercise capacity assessments by 6MWD and changes in LVEF; (B) 18F-FDG-PET at 48 months after HiCM-188 treatment in Pt 2.

1363 A Scalable Retroviral Vector Production platform for T-cell Therapy

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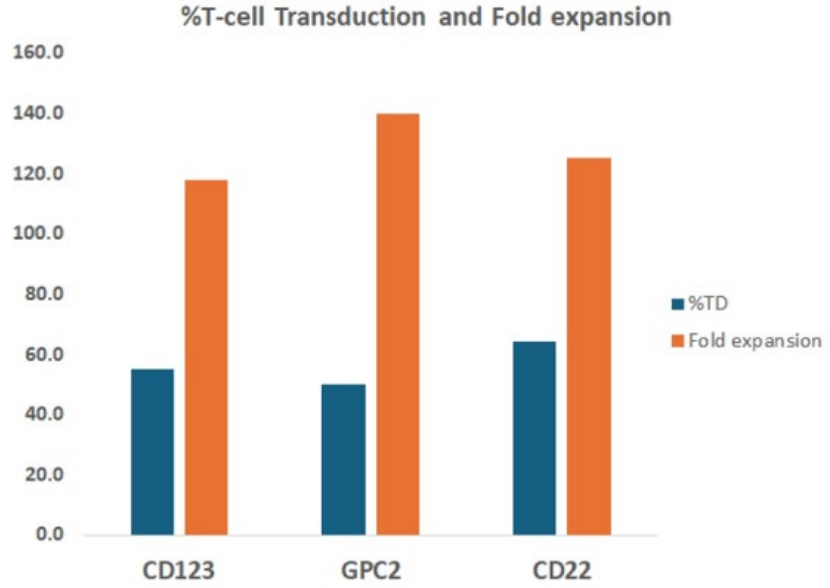
Introduction: T-cell based therapy is one of the most promising approaches to treat liquid and solid tumors as evidenced by numerous successful clinical trials. In many cases, T-cell engineering is performed by using gammaretroviral vectors for gene delivery. Significant advancements have been made to viral vector manufacturing processes which have lowered the time and cost to develop and manufacture viral vectors suitable for early clinical evaluation. We developed a gammaretroviral vector production platform that requires only one gene-transfer plasmid. Using our production platform, we successfully manufactured multiple GMP-grade viral vector lots for use in generating T-cell patient products to support planned early-stage clinical trials that include B-cell leukemia (CD22TCR), acute lymphoblastic leukemia (CD123), and neuroblastoma (GPC2).

Methods: Using a multifactor design of experiment strategy, the transfection conditions for the gene transfer plasmid into the producer cell line (293VecRD114 obtained from BioVec Pharma Inc.) to generate RD114 envelope pseudotyped gammaretroviral vector were optimized and translated successfully to a 10L cell-culture process. Subsequently, the post-transfection harvest was concentrated by an optimized Tangential Flow Filtration (TFF) process into a vector stabilizing diafiltration buffer containing trehalose as the stabilizer. The concentration step resulted in a 3-4-fold titer increase. Finally, the TFF concentrate was sterile filtered. This manufacturing process routinely generated approximately 2L of concentrated, filtered retroviral vector. The transduction efficiency of the retroviral vector batches was tested by an optimized process using G-Rex technology.

Results: The 3-day retroviral vector manufacturing process generated a harvest with an infectious titer of 1-2x10^7 TU/mL in Jurkat cells that was concentrated and had a titer of 3-4x10^7 TU/mL post-sterile filtration. Approximately 30-35% of the total post-transfection virus measured by infectious titer was recovered following the manufacturing process. Transduced T-cells were expanded in the G-Rex resulting in a total of 30-40 million transduced cells. Transduction efficiency of CD4/CD8 T-cells was 40-60% across all retroviral vector batches, and no replication-competent retrovirus (RCR) was detected.

Conclusion: We developed a novel GMP-compliant retroviral manufacturing process that requires only one plasmid. This transient transfection process generates high-titer vector (>1x10^7 TU/mL) in the harvest that can be concentrated to 3-4x10^7 TU/mL. Using this manufacturing process, we were able to produce the required quantities of retroviral vector for generating engineered T-cells to treat patients with different clinical indications.



1364 Enhancing titres of therapeutic lentiviral vectors using PKC agonists

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Achieving high upstream lentiviral vector (LV) titres is fundamental to the development and manufacture of a commercially viable gene therapy product. Product titres can vary considerably with different therapeutic transgene sequences despite being produced in the same mammalian cell system. Implementing new technologies able to recover low titres or improve high titres further is invaluable.

Here, we identify protein kinase C (PKC) agonists as robust enhancers of LV production. Activation of PKC rapidly increased transcription of LV genomic RNA and release of vector particles. This enhanced induction method resulted in a ~3-fold improvement in chimeric antigen receptor (CAR)-LV titre, and a ~9-fold improvement was achieved when combined with co-expression of modified U1 snRNA molecule ('256U1') that binds to and stabilises LV genomic RNA. Importantly, LV produced using PKC agonists had comparable or superior particle-to-infectivity ratios and preserved an equivalent T-cell transduction profile. An in-house HPLC assay has demonstrated that PKC agonists effectively cleared from product following anion exchange chromatography.

Our investigation highlighted that PKC treatment during LV production strongly activated AP-1 transcription factors, whereas enhancement of LV titre was found to be independent of NF-κB activation. This enhanced induction method increases upstream productivity and can be easily implemented within GMP manufacturing schema and therefore offers commercial scope to reduce the cost per patient dose of new gene therapies.

1365 Large-Scale, High Titer Lentiviral Production Using Suspension Transient Transfection Platform

Tsai-Yu Chen[1], Khanh Ho[2], Shuo Lu[2], David Chiu[2], Chen Wei Su[1], **Mariya Viskovska[1]**, Kate Shen[2], Michael Haller[1], Bingnan Gu[3], Wenling Dong[4]
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Lentivirus vector (LVV) is a major player in cell and gene therapies. It efficiently transduces both dividing and non-dividing cells and is able to incorporate large transgenes, making it a versatile viral vector for a number of applications where gene delivery is required. Given the growing demand for the LVV-based therapies, Lonza developed an Xcite® LVV suspension transient transfection platform using a proprietary clonal HEK293T cell line. The platform utilizes a transient transfection with a plasmid expressing the gene of interest (GOI) and three packaging plasmids, and can be scaled up to 250L production and transferred to GMP manufacturing.

The platform as tested with two client-specific GOIs at a shake flask and 3L scale, and then successfully scaled up to 50L production. First, the optimal transfection parameters for each GOI were determined via the design of the experiment (DoE) performed in shake flasks. Conditions with varying plasmid ratios, cell densities at transfection, and a total plasmid DNA concentration were evaluated to determine the highest producing condition for each respective GOI. Second, the highest producing conditions were confirmed at a 3L scale, using the platform upstream and downstream processes. At 3L scale an average titer of 4.6E07 TU/mL was achieved for GOI 1 and 6.1E07 TU/mL was achieved for GOI 2 at harvest, as measured by the infectious titer assay (ITA), with the overall recovery of 23-30% for both GOIs.

Production for both GOIs were successfully scaled up to 50L. GOI 1 production resulted in 2.2E07 TU/mL of LVV at harvest and 2.2E08 TU/mL of bulk drug substance (BDS). GOI 2 production resulted in 1.1E08 TU/mL at harvest and 3.1E08 TU/mL BDS. The overall recovery at 50L was 15.3% and 39% for GOI 1 and GOI 2, respectively. BDS generated from the 50L runs was formulated into a formulation buffer and the final drug product (DP) was tested for stability at room temperature and at 2-8°C. DP for both vectors was stable when held at 2-8°C up to 72 hrs (the last time point tested). When held at room temperature GOI 1 DP showed no significant titer reduction up to 48hrs (last

time point tested). GOI 2 DP showed a 30% titer reduction after 12 hrs and 50% titer reduction at 72 hrs (last time point tested). DP for both vectors were stable for 5 freeze/thaw cycles. The long-term -80°C storage stability studies are currently ongoing.

In summary, Lonza developed a robust, scalable suspension transient transfection platform for LVV production. The platform was successfully applied for two client-specific GOIs at shake flask, 3L, and 50L scale.

1366 Lentiviral Vector Production Process Intensification: High-Yield and Cost-Efficient Manufacturing Solutions

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Introduction: Process intensification for lentiviral vector (LVV) production involves innovative approaches to improve productivity, scalability, robustness, and product quality while reducing overall costs. This study evaluated the impact of two key factors on LVV production in suspension cell culture: (i) transfection at high cell density and (ii) enhancer addition. Tangential flow depth filtration technology (TFDF, Repligen) was used to perform transfections at high cell density, maintain perfusion cell culture, and harvest viral vector material. Sodium butyrate was added as an LVV production booster to enhance the yield.

Methods: Batch and perfusion modes of LVV production were compared in 2-L Univessel™ Single-Use bioreactors (Sartorius) using the Catalent® clonal HEK293 cell line (CAT293), Catalent® Off-the-Shelf (OTS) 3rd-generation lentivirus packaging plasmids, and a transfer plasmid encoding the EGFP transgene. CTS™ Viral Production Medium (Thermo Fisher) was used for cell amplification and LVV production. The concentration of plasmid DNA at transfection was normalized to cell density at the time of transfection. FectoVIR®-AAV (Polyplus) was used as a transfection reagent. Batch mode bioreactors were transfected at 2.0-3.0´10^6 viable cell (vc)/mL density and harvested on day 3 post-transfection by centrifugation. Perfusion mode bioreactors were equipped with 30 cm^2 or 50 cm^2 TFDF filters. Perfusion was performed at 0.75-3´ vessel volumes per day to achieve a 4-7-fold higher cell density at the time of transfection compared to batch mode. The LVV-containing conditioned medium was continuously collected at 2-8°C as a TFDF permeate fraction for 3 days after the transfection.

Results: High cell density transfection in perfusion mode, combined with sodium butyrate addition, significantly increased LVV yields. At 4-fold higher cell density, transgene and capsid production increased by 4.8-7.5-fold and 8.0-9.8-fold, respectively, compared to batch mode. At ~7-fold higher cell density and 4 mM sodium butyrate, a 10-10.5-fold increase in transgene and capsid production was observed. Batch mode processes enhanced by 2-4 mM sodium butyrate achieved only a 1.5-1.9-fold increase in LVV production. The combined effects of the transfection at 4 to 7 times higher cell density and sodium butyrate addition resulted in a 12- and 32-fold increase in transgene production, respectively. Capsid yields were increased by 19 and 22 times under the same conditions. The highest LVV yield was achieved in perfusion culture transfected at 7 times higher cell density with 4 mM sodium butyrate, reaching 1.01´10^{14} transgene/bioreactor and 2.78´10^{14} capsid/bioreactor. In contrast, batch mode production without enhancer yielded 3.15´10^{12} transgene/bioreactor and 1.25´10^{13} capsid/bioreactor.

Conclusion: A comparison of the LVV-intensified and typical batch productions indicated that a single intensified perfusion process could generate viral vector amounts comparable to 32 batch productions. Thus, a single 50-L perfusion run could yield the same quantity of LVV as 1500-L batch production. The perfusion process will require 52% less production medium and 77% less plasmids and transfection reagent.

1367 Exploring the potential of different point-mutated HIV-1 protease variants for continuous lentiviral vector production

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Gene therapy has transformed modern medicine by addressing previously untreatable conditions. Lentiviral vectors (LV) play an important role in the gene therapy field, both on clinical trials and market-approved products. Traditionally, LV production relies on transient transfection, a method limited by scalability constraints, high costs of GMP-compliant plasmids, and a narrow production window due to the cytotoxicity of key LV components, namely the viral protease and the vesicular stomatitis virus G glycoprotein (VSV-G). Alternatively, LV production can be performed in stable platforms such as constitutive producer cell lines which offer a more cost-effective, scalable, and standardized alternative to transient systems. However, their development is hindered by the toxicity of the LV components. Strategies to mitigate this toxicity include the use of gamma-retroviral envelope glycoproteins (e.g., Gibbon ape leukemia virus, GaLV, or amphotropic murine leukemia virus, 4070A) or mutated protease variants like the T26S protease. However, T26S has shown limitations, particularly in supporting infectious titers with clinically relevant pseudotypes such as GaLV, highlighting the need for novel protease variants with reduced cytotoxicity.

Here, we assess the potential of a newly engineered protease variant (mutation X) for LV production and compare it to other existing protease variants. Mutation X reduces the autoproteolytic activity of the protease without a major impact on K_{cat}/K_m when compared to the wild-type (WT) protease. However, its effects on LV production and cytotoxicity remain uncharacterized. To investigate this, we introduced mutation X into the LV packaging plasmid and evaluated its performance in transient and semi-stable LV production using VSV-G, 4070A, and GaLV pseudotypes. The WT gag-pro-pol (GPP), GP(D25N)P (inactive protease), and GP(T26S)P (reduced-activity protease) were used for comparison.

Transient LV production revealed that GP(X)P supports total particle (T.P.) and transducing unit (T.U.) titers comparable to WT GPP, regardless of the pseudotype, suggesting that mutation X does not impair LV proteolytic processing or maturation. Most importantly, it rescued the titers lost with GP(T26S)P, in GaLV-pseudotyped LV. To assess its potential for stable production, cells were stably transfected with either GP(X)P or the control GPP variants, followed by REV stable transfection, in 3 biological replicates. Semi-stable LV production, where the transgene and envelopes were delivered transiently, showed that cells expressing GP(X)P supported T.P. and T.U. titers similar to the WT GPP, at least for VSV-G. These cells also exhibited a higher growth rate than WT GPP-expressing cells. Gag gene expression analysis by qPCR showed that, despite variability across replicates, cells expressing GP(T26S)P or GP(X)P generally supported higher gene expression levels than the WT GPP. Overall, these findings suggest that mutation X results in a less cytotoxic GPP variant, making it a promising candidate for stable cell line development. Further characterization, including cell proliferation assays, are currently ongoing to evaluate the impact of these protease variants on cell viability.

In conclusion, this study demonstrates the potential of a novel GPP variant for LV production, contributing with a new strategy to advance the development of continuous LV producer cell lines with clinically relevant pseudotypes.

1368 Chemically Defined BalanCD HEK293 Viral Feed Boosts Viral Vector Production

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**Corresponding Author*

With a range of applications in cell and gene therapy, the most commonly used cells to produce viral vectors for gene modification are HEK293 cells (typically using standard batch mode for viral vector production). Approaches to increase viral genome titers include engineered cell lines, use of transfection reagents and enhancer supplements, and optimization of viral plasmid systems, all of which require costly process optimization. In this study, we describe a simplified approach to enhance viral vector production by utilizing a chemically defined feed medium for fed-batch production of adeno-associated virus (AAV). Developed using Rational Culture Media Design, Design of Experiment (DoE), and Multivariate Analysis methods, the feed medium, BalanCD HEK293 Viral Feed, was shown

to increase AAV titers 3-fold to 10-fold in multiple AAV serotypes (AAV2, AAV5, and AAV9) when applied at 12% (v/v), 24 hours post-transfection. Performance was further demonstrated in shake flask and small-scale bioreactors, in suspension HEK293 and adherent HEK293T cell lines, and compatibility with various basal growth media and different transfection methods. By adapting the use of this viral feed medium in fed-batch HEK293 cultures, we show increased AAV titers and improved VG/capsid ratios can be achieved with minimal process optimization.

In this presentation, you will learn about how HEK293 Viral Feed can:

- Increase AAV titer 3-fold to 10-fold across various AAV serotypes (AAV2, AAV5, and AAV9) and improve LV genome and functional titer by 3-fold
- Easily convert to fed-batch with minimal disruption to the workflow
- Scale-up viral vector production from shake flasks to bioreactors

1369 Increasing Lentivirus Production and T-cell Transduction Efficiency: Discovery and Validation of Next Generation Viral Sensitizer (VSE™) Compounds

Katelyn Badham[1], Keara Sutherland[2], Abigail Fernandes[1], Glib Maznyi[1], Jondavid De Jong[1], Oliver Varette[3], **Andrea Vervoort[1]**
[1]Virica Biotech, Ottawa, ON, [2]Virica Biotech Inc., Ottawa, Ontario, [3]Virica Biotech, Ottawa

Biomanufacturing of cell and gene therapies is a highly intricate process, often challenged by variability in yield, product quality, and scalability of manufacturing platforms. A critical yet frequently overlooked barrier to efficient viral vector and cell therapy production is cellular innate antiviral defenses. When foreign particles or nucleic acids are introduced into living cells, they activate robust antiviral responses, which can inhibit viral transfection/transduction, assembly, and replication. Virica's Viral Sensitizers (VSEs™) offer an innovative solution: a proprietary collection of small molecules which transiently and effectively suppress these cellular antiviral defenses, thereby enhancing viral production and transduction.

Virica leverages our high-throughput virology capabilities to screen and identify small molecule enhancers of viral vector production and transduction to increase the efficiency of cell and gene therapy manufacturing processes. We provide evidence supporting the use of VSEs in lentivirus-based therapeutics manufacturing, targeting 2 distinct and complementary aspects of the manufacturing process:

1. more efficient production of lentiviral vectors across adherent and suspension-based platforms

2. more efficient T-cell lentiviral transduction processes

We utilized our 96-well high-throughput transient transfection-based production assay to screen a library of 15,000 bioactive compounds to identify several new candidate VSE molecules for enhanced production of lentivirus in adherent HEK293T cells. Upon additional screening iterations, ten candidate molecules that met pre-defined screening criteria were then further validated at 96-well scale in both adherent HEK293T/17 and suspension VPC1.0 cells. Increases in functional lentivirus titer, quantified by a luciferase-based transduction assay, ranged between 1.5-3-fold (adherent) and 2-4-fold (suspension) across VSE candidates. Top VSE candidates from small-scale screening in both adherent and suspension-based platforms were then scaled to T-75 flask and 125mL shake-flask format, respectively, where consistent enhancement in functional lentivirus yield previously demonstrated at small scale was achieved.

Additionally, screening of VSEs for increased transduction efficiency revealed several molecules which enhanced the transduction of T-cells with lentiviral vectors. Further validation of candidate molecules demonstrated repeatable multi-fold increases in transduction efficiency across multiple T-cell donors, with little to no impact on cell viability post-transduction.

Together, this data demonstrates the applicability of next-generation small molecule VSEs for enhanced production of lentivirus-based cell and gene therapies.

1370 Novel nanofiber-based microcarriers designed for improved upstream productivity of viral vector biomanufacturing

Geddy Hamblen, Cellevate, College Station, TX

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1 Cellevate AB, Lund, Sweden, 2. Cellular Medicine, University of Dundee, Dundee, Scotland, 3. UCL Division of Surgery and Interventional Science, London's Global University, London, UK, 4. Redoxis AB, Lund, Sweden.

Viral vector manufacturing needs to ensure scalability while upholding high product yield, quality, and process robustness. Adherent cell cultures systems known for their ability to produce high yields of viral vectors are associated with scalability challenges. Nanofiber-based microcarriers, a novel microcarrier format, providing three-dimensional (3D) cell culturing of adherent cells readily scalable in stirred tank (suspension) bioreactor systems, offers promising avenues for scale up of adherent cell cultures. Data showed that HEK293T cells adhering to Cellevat3d® nanofiber microcarriers formed homogeneous 3D cultures, generating cell-dense spheroids with high cell viability. The scalability of the microcarrier cultures was demonstrated by successful scale-up from spinner flasks up to a 10L SU stirred bioreactor using an enzyme-free passing process and maintaining consistent cell growth curves and viability above 95% throughout the culture. Transient plasmid transfection of HEK293T cells on Cellevat3d® nanofiber microcarriers showed high transfection efficiency (88–94%) for both adeno-associated virus serotype 2 (AAV2) and 9 (AAV9). Importantly, AAV2 vectors yielded high volumetric productivity (1014 vg/L) with higher titers compared to parallel 2D. Benchmarking results conducted by UCL in London demonstrated a 3.1-fold significant increase in AAV9 vector titer using Cellevat3d® nanofiber microcarriers compared to current standard microcarriers. These findings demonstrate the potential of the nanofiber-based microcarriers as a scalable platform for adherent cell cultures in gene therapy applications, advancing bioprocessing strategies for enhanced yield, reduced time and costs leading to improved upstream bioprocessing productivity.

1371 Using High-Throughput Genetic Screening to Improve Stable AAV Producer Cell Lines and to enhance viral vector production

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[1]Cytiva, Köln, Nordrhein-Westfalen, Germany

Adeno-associated virus (AAV) vectors are key materials in many gene therapies, yet how recombinant AAV production is regulated within producer cells—including vector genome packaging efficiency and yield—still isn't well understood. To address this challenge, we developed a high-content, modular genetic screening platform that can evaluate hundreds of plasmid designs in a few weeks.

Our approach pairs combinatorial design with advanced analytics to evaluate how diverse genetic elements influence AAV production. Using this platform, we identified several design combinations that enhance vector genome titer and packaging efficiency, leading to marked increases in vector titers and fullness. These findings provide helpful insights into the molecular determinants of how AAV vectors are regulated and offer a useful tool to optimize AAV production processes to meet the growing demands of gene therapy candidates in the clinic.

1372 Optimising Production of 4th Generation Lentiviral Vectors (LV) of the TetraVecta™ System: Towards Plug-and-Play LV Manufacturing

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HIV-1 based lentiviral vectors (LVs) are a popular choice for the delivery of gene therapies. LV architecture has not markedly changed over the past 20 years, with most therapies utilising 3rd-generation vectors. As the complexities of therapies increase, the LV platform will need to evolve accordingly. LV attributes such as purity and *in vivo* efficacy can be improved, for example, through elimination of transgene expression during production, reducing contamination of the final product and increasing batch consistency.

OXB have previously described a 4th-generation LV platform – called TetraVecta™ - with several improvements over the current 3rd-generation. Firstly, promiscuous splicing from the HIV-1 Major Splice Donor (MSD) during LV production has been ablated through inactivation of the donor sequence (2KO), leading to an increased pool of unspliced, intact vector genome RNA. 2KO-LVs exhibit improved repression of transgene expression during production when employed with the TRiP System™, further reducing contamination of the final drug product. Third-generation LVs utilise the native HIV-1 polyadenylation site for both genomic and transgene RNAs. However, the SIN-LTR safety modification has weakened this polyadenylation activity. We have therefore developed novel SIN LTRs – termed supA-LTR™ - with improved polyadenylation activity of the transgene mRNA in target cells. SupA-LTR™ reduces both transcriptional read-in/out of the integrated LV and generally improves transcriptional insulation without affecting the integration site profile. Rev-dependent LVs of the TetraVecta™ system are called SupA2KO-LVs.

Here we compare the production of LVs encoding a Chimeric Antigen Receptor (CAR) using 3rd-generation or Tetravecta™ vector platforms. LVs were made at 5L bioreactor scale in both 293T and 293T-TRiP cells before purification by anion exchange chromatography and hollow fibre filtration. Purified vectors were then used to transduce primary T cells and CAR-T cell activity was measured by killing assay. SupA2KO-LVs made in both 293T and 293T-TRiP cells attained comparable titres to the 3rd-generation control but displayed a greatly improved vector RNA profile due to the 2KO feature, with reduced levels of aberrantly-spliced transcripts detected in both production cells and LV particles. Furthermore, we observed reduced CAR expression in 293T-TRiP production cells when SupA2KO-LVs were used. Additionally, we demonstrate optimised implementation of the TRiP System™ during use of plasmid-encoded TRAP (the translational repressor) in standard 293T cells. SupA2KO-LVs display altered kinetics of production compared to 3rd-generation LVs. We will discuss our efforts to explore and further improve the production of TetraVecta™ LVs through optimisation of various parameters, including the use of the small-molecule enhancer Ingenol-3-Angelate (I3A).

We show that SupA2KO-LVs transduced primary T-cells with similar efficiency to the 3rd-generation control. Modestly improved expression of the CAR was detected on the target cells, most likely due to improved expression from the transgene cassette as a function of the supA-LTR™. The killing activity of CAR T-cells was comparable between both platforms, confirming that TetraVecta™ modifications do not adversely affect therapeutic LV function. Taken together, these data emphasise the potential of TetraVecta™ technology to improve overall LV quality without affecting function.

1373 A HTPD Approach for Rapid AAV Process Optimisation & Scale-Up

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Pharmaron have successfully designed a platform process which can be tailored to a specific AAV gene therapy product. Through a manufacturing feasibility assessment, a rapid small-scale evaluation of a new product's 'fit' to the multi-serotype platform can be performed in as little as 6 weeks. This provides information on a product's performance using HEK293 cells in Ambr250® mini-bioreactors, before small scale chromatography-based

downstream purification. Targeted areas for process optimisation are rapidly identified, rather than a requirement for full process development.

Cost effective high-throughput development (HTPD) automated robotics platforms, alongside Design of Experiment (DoE), allows multiple conditions to be assessed at micro-scale determining the optimal parameters to meet the product's critical quality attributes (CQA's). HTPD, alongside a tiered approach to analytical testing, gives rapid, targeted results and allows data-driven decisions to be made on the next steps. Once, optimisation is complete, results are confirmed via lab-scale studies and exemplified at pilot scale. This demonstrates consistency across scales and confidence in the process before transferring it from development laboratories into 'at-scale' GMP production.

Pharmaron's reproducible platform process at increasing scales alongside their world-class analytics, provides fast confirmation that a product is ready for GMP manufacture. From a novel transgene plasmid to full GMP production, Pharmaron can accelerate from lab to full product release in as little as 6 months.

1374 A Novel Lentiviral Vector Cargo Gene Silencing System to Benefit Manufacturing of Cell Therapies

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Lentiviral vector (LVV) is the most used delivery tool for genetic modification in ex vivo cell therapies and has also been used recently in in vivo therapy clinical trials. However, cargo gene expression during lentiviral vector (LVV) production can reduce titres and disrupt production cell growth and viability. In addition, the consequent byproducts of production cell death can complicate downstream processing. Ideally, cargo gene expression would be controlled. However, because the cargo gene is nested within the LVV genome, cargo gene knockdown also impairs LVV production, creating a significant challenge to the control of manufacturing processes. To address this issue, we engineered the vector RNA and the cargo gene mRNA to be distinct, enabling cargo gene silencing by an shRNA, the target of which remains unchanged regardless of the cargo gene. For optimal production, we also included an shRNA against an antiviral gene. We tested this system using XOFLX™ Packaging and Producer cell line platforms with anti-CD19 chimeric antigen receptor and pro-apoptotic *BAX* driven by a human promoter. This resulted in up to 95% gene silencing during manufacturing, and up to a four-fold increase in LVV production yield. Importantly, this system enabled the recovery of otherwise unrecoverable producer cell lines. We anticipate that the use of this system will de-risk development of novel LVV-based therapeutics and reduce the requirement to customise downstream processes with novel cargo genes. Process standardisation and timeline reduction is likely to result in decreased costs, therefore improving patient access to LVV-based therapies.

1375 Accelerating the AAV Path to Clinic through Targeted CMC Development

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Pharmaron have successfully developed a manufacturing feasibility assessment as a fast, small-scale method of evaluating their platform's suitability of fit for any new product. This assessment can be completed in as little as 6 weeks from obtaining a novel transgene. The short study provides valuable product quality insight and allows rapid diagnosis of areas for process optimisation.

A triple transfection approach using Pharmaron's suspension HEK293 cell line, AAV Rep/Cap and Helper plasmids and either supplied gene of interest (GOI) plasmid or GOI plasmid produced by Pharmaron, can be used to produce

different AAV serotypes/products in Sartorius Ambr®250 bioreactor vessels. Consistent viral titres, genome titres and transfection efficiencies are observed across all 8 Ambr®250 vessels. Material from the Ambr®250 vessels are processed through a small-scale downstream version of the large-scale manufacturing process. Purification focuses on chromatography, including the challenging separation of empty capsids from full capsids, as a highly scalable solution and a toolbox approach based on the specific AAV serotype. Product performance over clarification, affinity and polishing steps are benchmarked against Pharmaron platform data where they consistently observe greater than 70% recovery for different AAV serotypes. Ion Exchange Chromatography (IEX) is used for removal of 'empty' AAV capsids from 'full' product containing capsids. Data analysis and benchmarking against the Pharmaron platform database facilitates a targeted development pathway.

Manufacturing feasibility, in combination with world-class analytical solutions, gives an early indication of product performance including yield and quality. Once complete, the manufacturing feasibility assessment provides an invaluable data set identifying any areas requiring targeted process optimisation rather than full process development, fast-tracking the product into scale-up and GMP manufacture.

1376 Unveiling the Secrets: Pathway Analysis Provides Molecular Insights into Stable Lentiviral Packaging and Producer Cell Lines (GPRTG)

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Lentiviral vectors (LVVs) are widely used in gene therapy due to their ability to infect both dividing and non-dividing cells, permanently transform target cell genome and allow stable transgene expression. For LVV production, the creation of stable packaging cell lines with integrated genes necessary for viral replication offer a more consistent and scalable alternative to transient plasmid transfection approach.

Although the development of such stable LVV packaging cell lines has been reported[1], the molecular changes induced by stable and inducible viral gene expression and the impact of genome integrated viral genes on cellular pathways remain poorly characterized. For better insight, we investigated the molecular characteristics of a stable LVV packaging cell line and its host cell line (HEK293T/17) by comparing differential expressed genes.

The performed pathway analysis revealed significant changes in pathway usage between packaging and host cell lines, influenced by different viral transgenes. Gag-pol expression was found to suppress host translational processes and nonsense mediated decay, with the majority of ribosomal assembly genes being downregulated. Rev and VSV-G expression modulated mitochondrial pathways, including oxidative phosphorylation and mitochondrial dysfunction, while HIV-1 tat expression activated histone-related genes. These regulatory shifts suggested a strategic reprogramming of host cellular states to favour viral replication, curbing protein synthesis and energy production to levels that support viral assembly, but impair the host's immune defence and the production of immune-related proteins.

Our findings provide a deeper understanding of the molecular changes associated with stable viral gene expression, which can inform the optimization of LVV production in gene therapy applications.

Literature
[1]Throm RE, Ouma AA, Zhou S, Chandrasekaran A, Lockey T, Greene M, De Ravin SS, Moayeri M, Malech HL, Sorrentino BP, Gray JT. Efficient construction of producer cell lines for a SIN lentiviral vector for SCID-X1 gene therapy by concatemeric array transfection. Blood. 2009 May 21;113(21):5104-10. doi: 10.1182/blood-2008-11-191049. Epub 2009 Mar 13. PMID: 19286997; PMCID: PMC2686181.

1377 Improving the safety and performance for ATMP developers using next-generation vectors

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Miniaturized plasmids have been developed to resolve safety issues with gene therapy production, however these typically rely on alternative methods, e.g. supplement-based selection. These plasmids have significant disadvantages including compounding variables and associated cost. We've designed a novel miniaturized plasmid which bypasses the need for either antibiotic or supplement-based selection, providing improved efficacy, safety, and manufacturability for cell and gene therapies. Compared to traditional plasmids, we show that our miniVec plasmid has higher plasmid manufacturing and virus packaging yields, improved transgene expression, and an enhanced safety profile. The miniVec backbone supports a novel combination of antibiotic-free and supplement-free selection and can be applied across diverse viral and non-viral expression systems offering enhanced flexibility and ease of production from development through GMP manufacturing.

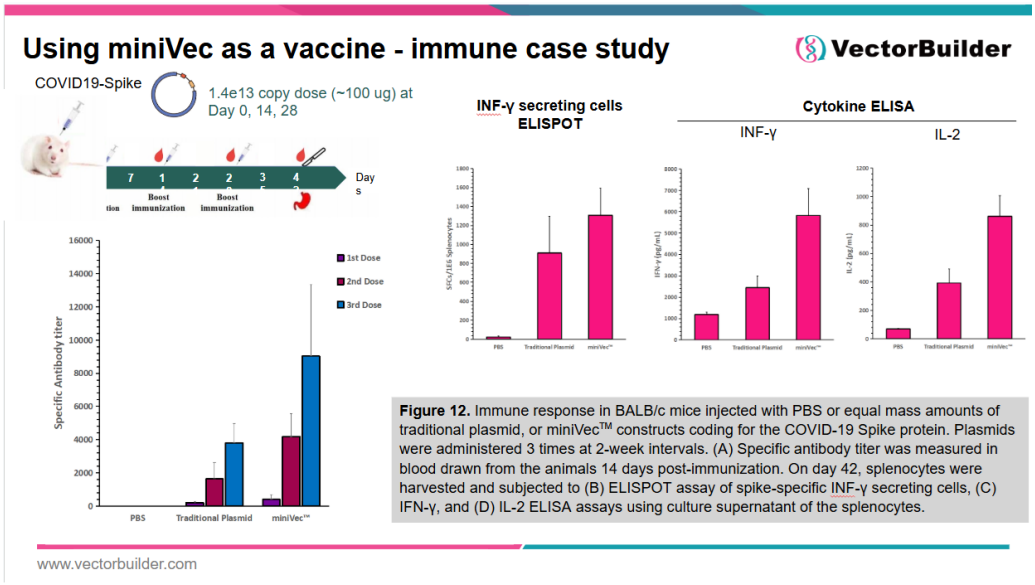

Figure 12. Immune response in BALB/c mice injected with PBS or equal mass amounts of traditional plasmid, or miniVec™ constructs coding for the COVID-19 Spike protein. Plasmids were administered 3 times at 2-week intervals. (A) Specific antibody titer was measured in blood drawn from the animals 14 days post-immunization. On day 42, splenocytes were harvested and subjected to (B) ELISPOT assay of spike-specific INF-γ secreting cells, (C) IFN-γ, and (D) IL-2 ELISA assays using culture supernatant of the splenocytes.

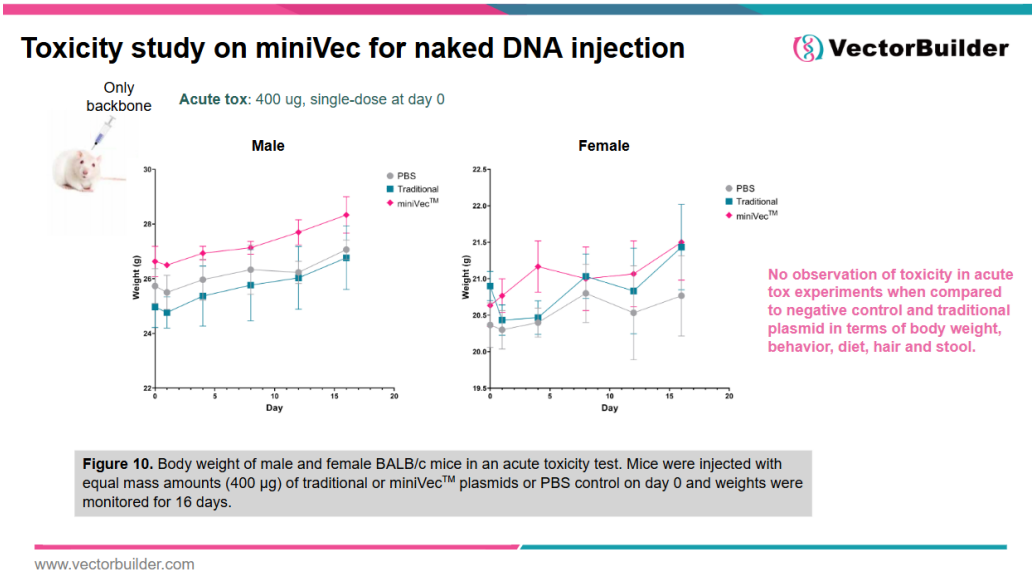

Figure 10. Body weight of male and female BALB/c mice in an acute toxicity test. Mice were injected with equal mass amounts (400 μg) of traditional or miniVec™ plasmids or PBS control on day 0 and weights were monitored for 16 days.

1378 Accelerating and Advancing the Pinnacle PCL™ Platform

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Ultragenyx has developed the Pinnacle producer cell line (PCL) manufacturing platform for production of recombinant AAV (rAAV) gene therapy products, which has demonstrated scalable 2000L production in an industrial setting to support ongoing clinical trials. The process of stably transfecting a large Triple Play plasmid (pTP), containing the therapeutic vector construct and the AAV Rep and Cap genes, into HeLa S3 cells, to isolating a number of high rAAV-producing monoclonal cell lines, involves a series of screening steps and quality assessments. By employing specialized instrumentation, optimized methodology and capitalizing on PCL tractability in culture, the path to banked polyclonal PCLs was reduced by 4 weeks. First, we improved stable transfection efficiency of the pTP plasmid yielding a larger number of potential polyclonal candidates for screening, thereby increasing the probability of isolating high producing, high quality monoclonal PCLs. To support evaluation of the higher numbers of polyclonal candidates we added additional microbioreactor systems to more than double our screening capabilities, which affords us the capability to improve clone screening throughput in an optimized scaled-down process. The implementation of multiple automated and quantitative instruments, such as CE-SDS to evaluate capsid ratios, replaced legacy qualitative assays, further facilitating faster and more comprehensive generation of PCL quality profiles. Finally, next-generation sequencing tools, such as TLA integration analysis and Nanopore sequencing, offer deeper insights into the PCL's genomic landscape and the viral vector produced from the PCL. The cumulation of all these improvements not only decreases the timeline to a monoclonal lineage but allows us to make more informed candidate selection based on desired quality attributes.

1379 A Comprehensive Approach to Adeno-Associated Virus Upstream Process Development

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The development of an efficient and scalable manufacturing platform for adeno-associated virus (AAV) production is critical to meet the growing demand for gene therapies. Our process workflow leverages a robust design of experiments (DoE) framework, utilizing both mixture designs to optimize plasmid DNA ratios, as well as A-optimal designs to optimize other transfection and bioreactor parameters. High-throughput batch purification of upstream samples enables quantitation of a critical quality attribute that requires a purified sample matrix, such as empty, full and partial capsid quantitation using mass photometry. Dynamic Light Scattering (DLS) technology is used to characterize the transfection complexation process, which is a valuable tool for both optimizing the transfection parameters as well as ensuring consistent performance at scale. This process workflow demonstrates seamless scalability from bench-scale production to the 200L single-use bioreactor (SUB) scale and is conducive to a Quality by Design (QbD) approach to process design.

1380 Forced Degradation Studies of Multi-Characteristic Opsin Gene Therapy Drug Product

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Multi-characteristic Opsin (MCO) gene therapy has demonstrated durable efficacy in treating advanced retinitis pigmentosa (RP) patients with severe vision loss. MCO-010 is a trimeric fusion protein with high sensitivity and fast

kinetics to light across the visible spectrum. Since MCO-010 is delivered via adeno-associated virus serotype-2, it is susceptible to environmental conditions such as temperature excursions and freeze-thaw during storage, shipment, and use in analytical labs as well as clinical settings. Hence, we conducted forced degradation studies, along with imposed freeze-thaw cycles, on MCO-010 to evaluate its possible degradation and to gain insights into the formation of impurities that may reduce its potency. In addition, the sensitivity of various analytic methods and stability-indicating critical quality attributes were identified using the force-degraded MCO-010 samples.

Specifically, we examined the impact of temperature excursion at 40°C for up to 4 weeks and 10 freeze-thaw cycles on the integrity and functionality of the MCO-010 drug product. The study tested the detection limits and performance of analytical assays, including ELISA for capsid integrity assessment, SDS-PAGE silver staining for detecting degradation-induced structural changes, qPCR for genomic titer quantification, and flow-based transgene expression for relative potency measurements. The incubation of MCO-010 drug product at 40°C for 1 week resulted in significant decreases in capsid titter (50%), genomic titter (35%), and relative potency (31%). These effects became more prominent in week 2 and week 4, indicating a time-dependent degradation. Additionally, the 10 freeze-thaw cycles caused a moderate decrease in capsid titter (15%), genomic titter (17 %), and relative potency (6%).

The study confirmed the sensitivity of the analytical methods, demonstrating their ability to detect subtle changes in MCO-010 drug product quality under forced degradation conditions. These findings support the use of these validated assays for quality control and stability monitoring during MCO-010 drug product storage and handling for future clinical use.

1381 In Vivo Selection of Randomly Integrated rAAV Does Not Cause an Increased Risk of Tumorigenesis

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Liver-targeted recombinant adeno associated viruses (rAAVs) typically have only a short therapeutic benefit when administered to pediatric patients due to loss of episomes during liver growth. While rAAVs can persist through multiple rounds of cell division by rare random integration into the genome, these integration events are too infrequent to reach a therapeutic threshold. In vivo selection and expansion of targeted hepatocytes results in therapeutic levels of these integration events. However, it remains unknown whether multiple rounds of cell division and selection will increase the likelihood of rAAV mediated tumorigenesis and oncogenesis in the liver.

We have previously developed a pharmacologically selectable shRNA that can allow expansion of a small population of gene edited hepatocytes in vivo. This shRNA functions by blocking expression of Cytochrome p450 reductase (Cypor), which is required for the metabolism of the drug acetaminophen (APAP) to a hepatotoxic byproduct. Accumulation of this hepatotoxic byproduct leads to cell death in hepatocytes. By creating a loss of Cypor linked to the desired gene edits, therapeutic cells can no longer metabolize acetaminophen and are protected from its toxicity.

rAAVs containing a U6-driven shRNA targeting Cypor in cis with a CAG promoter driving a human Factor IX (hFIX) transgene were delivered into wild-type neonatal mice. APAP was then administered to select for hepatocytes with integrated rAAV vectors. Blood serum hFIX levels and immunofluorescence staining of the liver confirmed the >20x expansion of Cypor negative, hFIX positive hepatocytes to a highly therapeutic level. To assess increased oncogenic risk, mice with expanded rAAV integrations were then placed on either a Western diet or regular mouse diet for 6 months. The Western diet, consisting of high fructose and high cholesterol, has been shown to induce tumorigenesis in mice even in the absence of rAAV or APAP treatment. At termination of the experiment, mice on the Western diet did have increased tumor frequency compared to mice on a regular diet as expected. However, there were no significant differences in the frequency of tumor occurrence, tumor size or tumor volume of the tumors between rAAV-treated mice that had been acetaminophen selected as compared to mice that were unselected. These results demonstrate that APAP selection in the liver does not lead to an increased risk of

oncogenesis in the liver. We conclude that APAP selection can be safely used to expand hepatocytes with therapeutic rAAV transgene integrations.

1382 Integration Frequency of Full and Truncated AAV Genomes in Cas9-Induced DSB Sites Is Determined by DNA Repair Pathways

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The unintended integration of adeno-associated virus (AAV) vector sequences at CRISPR-induced double-strand breaks (DSBs) poses significant concerns for AAV-mediated gene editing. Despite reports describing AAV integrations in various *in vivo* settings, the DNA repair mechanisms involved are not fully understood. In a prior study, we delivered CRISPR-Cas9 systems into the mouse liver using recombinant AAVs to disrupt the *Hao1* gene. Using short-read amplicon sequencing, we observed that paired Cas9 nickase editing resulted in fewer AAV sequence insertions on target compared to Cas9 nucleases, despite demonstrating comparable editing efficiencies. Paired nicks were preferentially repaired by microhomology (MH)-dependent pathways. Nevertheless, the widely used amplicon sequencing approach limits the full characterization of inserted AAV sequences. In this work, we further characterize the full range of AAV insertions after paired Cas9 nickase and Cas9 nuclease editing with targeted long-read sequencing and we explore the relationship between AAV integration frequency and DSB repair pathway choice.

We used long-read nanopore Cas9-targeted sequencing (nCATS). This PCR-free method was critical in overcoming the limits of short-read sequencing. Overall, paired nickase editing produced much fewer AAV integrations than paired nuclease editing, consistent with our earlier findings. In both cases, insertion breakpoints were predominantly located on the inverted terminal repeats (ITRs.) We categorized AAV insertions into three types: short insertions (<200 nucleotides) of ITR sequences, full-length AAV genome insertions, and truncated AAV genome insertions. Short ITR insertion frequency was 1.15% with paired nickases compared to 11% with paired nucleases (similar to amplicon sequencing data). Full and truncated AAV insertion frequency was 3.95% with paired nickases compared to 12.5% with paired nucleases. This meant that not only AAV insertion frequencies changed between both approaches, but also the structure of inserted sequences.

To investigate the factors underlying the reduced AAV insertions observed with paired nickase editing, we reanalyzed short-read amplicon sequencing data from multiple studies that used various CRISPR-Cas systems delivered by AAVs. We identified a correlation between increased MH-mediated repair and decreased AAV insertions. Remarkably, the greatest MH scores and the lowest AAV insertion rates were produced by nucleases that generate staggered ends, like Cas12 family nucleases. Since the reanalyzed samples originated from very heterogeneous experiments in terms of AAV dose, tissue, animal age etc., we designed and tested four gRNAs *in vivo* with the same experimental conditions. Using amplicon sequencing, the two gRNAs with the highest MH-mediated repair showed lower editing efficiency-normalized AAV insertions (4% and 9%) compared with the other two (12% and 16%).

These results suggest that the nature of the DSB and the involved repair mechanism significantly impact the extent and type of AAV insertions into CRISPR-induced DSBs. This implies that rational CRISPR system design and target site selection may improve the safety profile of AAV-delivered gene editing treatments by intentionally directing DSB repair toward MH-dependent pathways.

1383 mRNA-loaded AAV Vectors Enable Safer CRISPR Gene Editing for Neurological Diseases

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Background: We developed a novel mRNA-loaded AAV (RAAV) system by incorporating RNA-packaging components into the AAV production process and engineering Rep proteins. This RAAV vector combines the transient mRNA expression with the broad tissue tropism of AAV capsids, addressing critical *in vivo* gene editing safety concerns such as nuclease immunogenicity, possible off-target effects, and genomic integrity. Unlike existing liver-targeted mRNA delivery methods, RAAV enables mRNA-based therapies in non-hepatic tissues, including the brain. We applied RAAV with AAV for a dual-virus delivery system, enabling gene editing in neurological diseases.
Methods: We used RAAV for Cas12Max expression and AAV for gRNA delivery to deliver the CRISPR system. Gene editing efficiency and safety were assessed in HEK293T cells and mouse brains. For therapeutic evaluation in an ALS model, TDP-43 transgenic mice received intracerebroventricular (ICV) injections of RAAV-hfCas12Max and AAV-ATXN2-gRNA on postnatal day 0, P0 (**Fig. 2A**). On-target/off-target editing, ATXN2 protein levels, and toxic TDP-43 aggregation were analyzed 3 weeks post-injection (**Fig. 2B**). To explore broader applications, RAAV was used to deliver ZFcharm, an epigenetic editor targeting Prnp transcription for prion disease treatment. RAAV-ZFcharm was applied to cultured cells and injected into mouse brains, and DNA methylation, Prnp transcription and Prp expression were analyzed.

Results: Co-infection with RAAV-hfCas12Max and AAV-gRNA efficiently edited the hTTR locus in HEK293T cells and ATXN2 locus in mouse hippocampus (**Fig. 1A, 1C**). Off-target editing was measured in the cells/tissues treated with AAV delivery (**Fig. 1B, 1C**), but cells/tissues receiving RAAV had background low levels comparible to mock-treated cells (**Fig. 1B, 1C**).
In the ALS model, RAAV-hfCas12Max with AAV-ATXN2-gRNA achieved 3.8% on-target gene editing of *ATXN2* in the cortex with no significant off-target effects (**Fig. 2C**). Off-target editing rates with RAAV-Cas12Max-infected cells and brains were comparable to those of uninfected controls (Mock) and significantly lower than those observed with conventional AAV-Cas12Max (**Fig. 2C**), highlighting that RAAV-mediated mRNA delivery provides a safer approach for gene editing. Western blot showed a 47.4% reduction in cortical ATXN2 protein levels of treated TDP-43 mice compared to controls (**Fig. 2D**). Toxic TDP-43 aggregates were reduced by 25.3% (**Fig. 2E**). While untreated TDP-43 mice had a median lifespan of ~25 days, RAAV-hfCas12Max-treated mice showed a significant extension of medical lifespan (**Fig. 2F**). The RAAV-ZFcharm treatment effectively induced targeted DNA methylation at the Prnp promoter for prion disease. This led to a significant reduction in Prnp RNA and protein levels in cultured cells and mouse brains, confirming the potential of RAAV for epigenetic editing(Not Shown).

Conclusions: We developed a dual-virus delivery system utilizing RAAV for Cas12Max mRNA expression and AAV for gRNA delivery. This system demonstrated effective gene editing with significantly reduced off-target effects compared to conventional AAV vectors *in vitro* and *in vivo.* RAAV-mediated gene editing showed therapeutic efficacy in ALS mouse model and was successfully adapted for epigenetic editing to treat prion disease. These findings establish RAAV as a versatile platform for safe and effective mRNA-based gene therapies with broad applications in neurodegenerative and other genetic disorders.

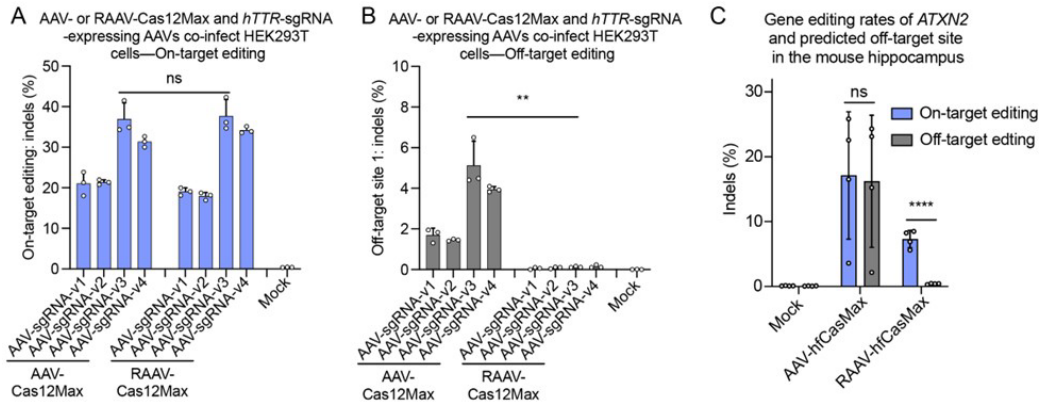

Fig. 1. RAAV-mediated delivery of CRISPR-Cas12Max mRNA reduced off-target effects. (A) and (B) Indels at the *hTTR* locus (A) and the predicted off-target site (B) in HEK293T cells co-infected with RAAV- or AAV-Cas12Max and *hTTR*-gRNA-expressing AAVs. Mock: uninfected control. (C) Indels at the *ATXN2* locus and the predicted off-target site in the hippocampi of mice 4 weeks after stereotaxic injection with the virus mixture of RAAV-hfCas12Max and AAV *ATXN2*-gRNA. Data are mean values ± SD with n = 3 biological replicates, unpaired, two-tailed t-test; **P < 0.01, ****P < 0.0001; ns, not significant.

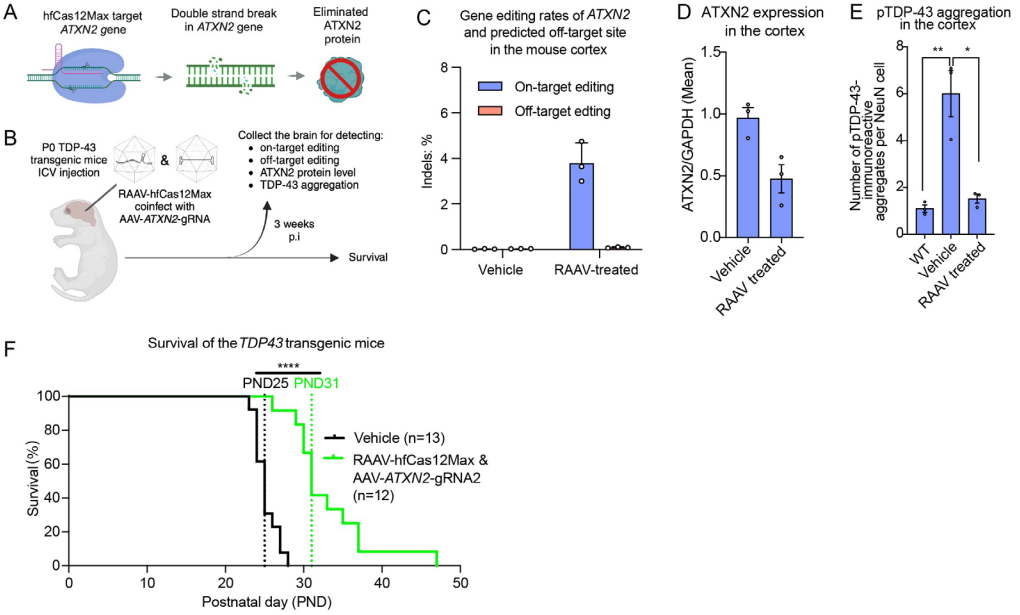

Fig.2. ALS treatment *in vivo* with RAAV-hfCas12Max. (A) The strategy to treat ALS. (B) Workflow for the *in vivo* ALS treatment experiment. (C) Indels at the *ATXN2* locus and the predicted off-target site in the mouse cortex infected by the mixture of RAAV-hfCas12Max and AAV-*ATXN2*-gRNA2. (D) Western blot analysis of ATXN2 protein amount in virus-infected and uninfected mouse cortex. GAPDH is used as a loading control. The intensity of proteins was determined using the ImageJ software analysis system, and ATXN2 proteins were quantitated as a ratio to GAPDH. (E) Immunohistochemistry analysis of the aggregation of TDP-43 in the cortex from virus-treated and untreated *TDP-43* transgenic mice. The pTDP-43 aggregates were quantified and statistically analyzed. Data are shown as individual data points and mean values ± SD for n = 3 mice. Unpaired, two-tailed t-test; *P < 0.05, **P < 0.01. (F) Kaplan-Meier curve in RAAV-hfCas12Max mixture treated and untreated *TDP-43* transgenic mice. ****P ≤ 0.001.

1384 Identification and validation of an optimal stuffer sequence for AAV gene therapy applications using AI/ML

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Recombinant adeno-associated virus (rAAV) vectors have emerged as a promising delivery vehicle for gene therapy, but their efficacy can be significantly influenced by the size of the packaged genome. Presented at ASGCT 2024 were results showing that different AAV serotypes exhibit varying packaging limits (1). This variability underscores the critical role of stuffer sequences in rAAV vectors, particularly when the transgene is small and fails to meet the optimal packaging limit. Stuffer sequences offer a versatile solution to this challenge, allowing researchers to fine-tune vector size, potentially enhancing transgene expression and vector performance. However, the design and implementation of these sequences requires careful consideration to avoid unintended biological effects and ensure optimal vector functionality, especially when rAAV vectors are developed for clinical purposes.

Here, we evaluated multiple stuffer sequences in silico using FORMsightAI, a proprietary artificial intelligence (AI) technology engineered to optimize nucleic acid therapeutics for safety and manufacturability using rational design and AI-Augmented CDS optimization. In silico modeling revealed that the truncation risk associated with a 4340bp sequence from the 3'UTR of human bone morphogenic protein 10 (BMP10) transcript stuffer was low, with few predicted secondary and tertiary structures, no significant CpG islands and nominal %GC content. Supported by these data, different portions of the BMP10 stuffer sequence were strategically selected (lack of start codon & splice sites etc) for synthesis and leveraged for two different gene therapy applications, gene augmentation and gene silencing. This approach allowed for tailoring the stuffer length to meet specific packaging requirements for each application, while maintaining optimal vector performance. The resulting vectors were packaged into AAV8 capsids. Vector genome integrity was evaluated using alkaline gel electrophoresis and empty/full capsid ratio using analytical ultracentrifugation (AUC). For both applications, alkaline gel electrophoresis showed homogenous product at the expected vector size, with AUC demonstrating >75% full capsids, indicating high-quality vector preparation with promising packaging efficiency and genome integrity. Taken together, this study supports the use of in silico tools to identify viable stuffer sequences prior to in vitro and in vivo testing. This approach leads to vector designs with significantly higher manufacturability (>E14vg/L; ~80% full) and improved safety while reducing the time and costs associated with empirical methods and trial-and-error testing for gene therapy applications.

References
Qing et al., Molecular Therapy Vol 32 No 4S1, April 2024 (Pg 141, https://www.cell.com/action/showPdf?pii=S1525-0016%2824%2900237-5)

1385 Comparison of AAV Expression Cassettes Modestly Above and Below AAV Packaging Capacity for Hemophilia A Gene Therapy

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Introduction: Given the size of select transgenes being pursued for therapeutic development, oversized AAV vector cassettes are used widely in clinical AAV programs. This includes all hemophilia A vectors with either regulatory approval or in late-stage clinical development. During manufacturing, the 5' end of the cassette is inserted last with

resultant cleavage of unpackaged DNA. In turn, this may lead to incomplete virion packaging, virion heterogeneity and reduced expression of AAV vectors manufactured from oversized cassettes. Following gene transfer, species lacking complete ITRs will be preferentially degraded. We hypothesized that even modestly oversized cassettes may impact AAV manufacturing yields, virion heterogeneity and transgene expression.

Methods: Two separate 4.65 kb cassettes between the ITRs (*F8*-1 or *F8*-2) were generated. Each cassette was codon optimized, CpG reduced, to encode *F8* and express coagulation factor VIII (FVIII) under a liver specific promoter. Cassettes were then modified to include an intronic sequence, increasing the cassette size to 4.78 kb between the ITRs. The 4.65 kb or short *F8*-1 and *F8*-2 cassettes were compared to the corresponding 4.78 kb or long cassettes. AAV8 vectors (AAV-*F8*-1short, AAV-*F8*-1long, AAV-*F8*-2short and AAV-*F8*-2long) were manufactured from each of the four cassettes and evaluated for manufacturing yield, virion heterogeneity as well as in vitro and in vivo expression.

Results: Preliminary analysis of manufactured AAV vectors by alkaline gel and TapeStation DNA electrophoresis demonstrated greater virion heterogeneity of AAV-*F8*-1long and AAV-*F8*-2 long relative to AAV-*F8*-1short and AAV-*F8*-2short, respectively; long-read sequencing analysis of packaged virion DNA is ongoing. As expected, cassette size did not impact FVIII expression from plasmid DNA *in vitro*. Specifically, transient transfection of Huh7 cells of plasmid DNA of the long versus short cassettes demonstrated no significant difference in FVIII expression measured by one-stage FVIII activity assay. In contrast, transduction of Huh7 cells by AAV vectors demonstrated significantly greater FVIII expression measured by one-stage FVIII activity assay of AAV-*F8*1short relative to AAV-*F8*-1long at all MOIs evaluated. Analogous trends were observed with AAV-*F8*-2short versus AAV-*F8*-2long. Next, 8–12-week-old male hemophilia A CD4 knockout mice (HA/CD4KO) mice were treated with equal AAV vector doses and plasma FVIII antigen was determined by ELISA. Preliminarily, 4- and 6-week expression data demonstrated significantly greater FVIII expression from AAV vectors generated from short versus long cassettes (Fig. 1).

Conclusion: Preliminary data support that even modestly oversized AAV expression cassettes markedly impact virion heterogeneity and diminish transgene expression relative to cassettes under 4.7 kb. Studies are ongoing to investigate the impact on manufacturing yields and further interrogate preliminary expression observations.

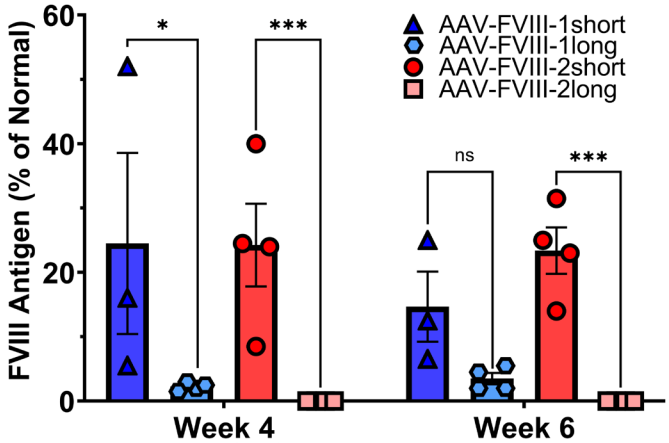

Figure 1. *In vivo* factor VIII (FVIII) expression was significantly greater for FVIII-short constructs relative to FVIII-long. Data are mean ± SEM (n = 4 mice) with 2-way ANOVA, 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***).

1386 MAR Sequences Enhance AAV Transduction and Promote Weight Gain in a Lethal Mouse Model of Methylmalonic Acidemia

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Methylmalonic acidemia (MMA) is a severe and potentially life-threatening autosomal recessive metabolic disorder, primarily caused by mutations in the methylmalonyl-CoA mutase (MUT) gene. MUT deficiency leads to the failure of converting methylmalonyl-CoA to succinyl-CoA in the propionate pathway, resulting in the accumulation of toxic metabolites such as methylmalonic acid in tissues and circulation. Patients with a complete loss of MUT enzyme activity face high morbidity and mortality at an early age. Restoration of MUT activity by AAV gene therapy is a promising approach. However, rapid hepatic growth during the neonatal/pediatric period results in dilution of the episomal DNA (such as AAV genome), consequently leading to loss of therapeutic efficacy. Scaffold/matrix-attachment region (MAR) sequences have been shown to increase plasmid DNA maintenance in the extrachromosomal space and duplicate when cell divides, which could potentially be used to enhance long-term transgene expression for cell and gene therapies. Here we evaluated whether MAR could improve AAV gene therapy in a lethal MMA mouse model.

Two MAR sequences (MAR-A: INFB1 MAR and MAR-B: APOB3'MAR) were tested in AAV GFP vectors and MUT vectors. We found that MARs could improve GFP+ cell percentage (~4X) in 293T cells after 2-3 months of long-term culture. After intravenously injecting these GFP vectors in WT mice on postnatal day 0 (P0), MAR-A vector gave higher GFP expression (~1.5X) in the liver than the control vector (no MAR) at 6 weeks post injection of 1E11 vg/g dose, and MAR-B gave higher GFP expression (~2X) than the control vector in the liver at 18 weeks post injection at 5E9 vg/g dose. When injecting MUT vector with or without a MAR sequence into the MMA mice at 1E10 vg/g dose on P0, all vectors had similar rescue rates for survival (~70%), while all the MMA mice injected with vehicle died within 2-3 days after birth. However, MMA mice injected with MAR vectors had higher body weight than MMA mice injected with the control vector when normalized to their corresponding littermate WT animals (10-20% higher from day 30 through day 160). Liver vector genome copy analysis of 4 mice per group at day 150 showed that MAR vectors gave a trend of higher vector genome copies than the control vector. Episomal over total vector genome quantification using exonuclease V digestion followed by qPCR showed that AAV genome copies in the episomal form were less than 5% of total vector genome for all three vectors, and MAR vectors did not give higher episomal copy ratio.

The data showed that MARs could improve vector copies and body weight gain when injected into neonatal mice with lethal MMA, but did not enhance long-term episomal vector genome maintenance based on the method of episomal and linear genome quantification used. We speculate that MARs did improve AAV genome episomal maintenance early after transduction, and higher vector copies promoted higher body weight gain in the beginning. During liver expansion, the hepatocytes having integrated functional AAV genome were quickly enriched due to growth advantage conferred by the MUT protein, resulting in enrichment of integrated AAV genomes and loss of episomal AAV genomes. The role of MARs could be model-specific and context-dependent. The exact underlying mechanisms of MAR in regulating AAV vector episomal maintenance and integration and protein expression remain to be carefully explored.

1387 *In Vitro* and *In Vivo* Characterization of Oversized AAV Vectors With High Genome Integrity That Encode Microdystrophins With Extended C Terminal Sequences

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Gene replacement strategies utilizing adeno-associated virus (AAV) to deliver truncated versions of the full-length dystrophin gene have emerged as a therapeutic option for DMD patients. Several "microdystrophins" with AAV genome-compatible sequence lengths have been designed and shown to retain many, though not all, functional features of the full-length protein. Incorporation of an extended, but incomplete, fragment of the highly conserved dystrophin C-Terminal domain (CT) into microdystrophin (μDysCT194) provides functional and histopathological benefits in dystrophin deficient mice. However, it is unclear whether incorporation of the remaining portion of the

CT into a microdystrophin could provide additional functional benefits, as it typically pushes the genome size beyond the 4.7kb packaging limit of AAV, leading to genome integrity and potency issues.

To study this, we compared three oversized AAV8 vectors which encode the same base microdystrophin with increasing amounts of the CT domain for productivity, genome integrity, and functionality. The vectors, termed µDysCT243 (4834bp), µDysCT278 (4939bp), and µDysCT325 (5080bp), utilize a newly designed compact muscle promoter. All three vectors had similarly high harvest titers comparable with µDysCT194 (4676bp). Surprisingly, the genome integrity of the oversized vectors was remarkably good with a vast majority of reads (82-88%) corresponding to full-length AAV genomes, as analyzed by long read NGS. However, we observed a slight reduction for *in vitro* potency of the vectors in both differentiated mouse and human myoblasts when compared to µDysCT194, indicating something other than genome integrity was impacting transgene expression. This was not explained by construct design or protein stability as all tested microdystrophins accumulate at similar levels following plasmid transfection. Visualizing AAV particles with negative stain electron microscopy (nsEM) revealed an increased presence of "collapsed" AAV particles when comparing µDysCT194 (3%) to µDysCT243 (23%) and µDysCT325 (29%). This suggests that the integrity of the AAV capsid is diminished when packaging a genome larger than 4.7kb and could additionally explain why the potency of the vector is reduced *in vitro* even when genome integrity is excellent. Interestingly, dystrophin-deficient mice injected with µDysCT243, µDysCT278, and µDysCT325 all showed improvements in eccentric contraction, maximal force, and treadmill assays over untreated controls, suggesting the loss of potency observed *in vitro* may not hamper the functionality of these vectors *in vivo*. This data will provide insights into our understanding of how AAV transgene size affects genome/capsid integrity and potency, and enable the development of functional and manufacturable oversized vectors.

1388 Cell-line and Antibody Epitope-dependent Expression Models Inform Selection of AAV-GNE Epimerase/Kinase Transgenes to Treat GNE Myopathy

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Background/ Rationale: FDA approved AAV gene therapies and promising new AAV investigational products offer new hope to families enduring neuromuscular diseases. Due to AAV packaging limitations, groups developing new therapeutics must often modify endogenous genes. Further, the reduction of CpG dinucleotides in AAV expression cassettes is proposed to increase safety, but must be balanced with transgene expression. As a result, antibodies against etiologic genes do not always recognize transgene protein products, and methods to evaluate novel transgene expression must be carefully considered. Here, we interrogated three open reading frames, with synonymous codon substitutions that varied their CpG content, encoding identical glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase (GNE) protein products. GNE1 contained moderate CpG density near wildtype levels, GNE2 had high CpG content due to traditional codon optimization for human expression, and GNE4 had significantly reduced CpGs. Five GNE splice variants are found ubiquitously in human tissue, potentially confounding cell line-based strategies to measure relative transgene expression. We therefore evaluated novel transgene driven protein expression in human embryonic kidney (HEK293T), cervical cancer (HeLa), and chronic myeloid leukemia (K562) cell lines, using three monoclonal and polyclonal antibodies.

Methods: We transfected cells with 0.5ug to 2.5ug of AAV2-GNE plasmids or GFP control plasmids and incubated under standard conditions. Four days later we observed GFP transgene expression by fluorescence microscopy. Protein was harvested, quantified, and subjected to western blot analyses using 1) rabbit polyclonal (Invitrogen Cat# PA5-55559), 2) mouse monoclonal 2A11E10 (Proteintech Cat# 67649-1-Ig), or 3) rabbit polyclonal (Proteintech Cat# 25079-1-AP) anti-GNE antibody probes.

Results: Consistent with expectations, we recovered the highest protein concentrations from HEK293T, followed by HeLa and then K562. The first polyclonal showed variable GNE expression between cell lines and was excluded from further analyses. The K562 cell banding pattern was non-specific and could not be distinguished from controls. Surprisingly, monoclonal antibodies raised against immunogen Ag18702 and recognizing an epitope between amino acids 376 and 722 bound non-specifically to lysate from all tested cells, and banding patterns from experimental conditions could not be distinguished from controls. The second rabbit polyclonal, raised against immunogen

Ag18689 also bound non-specifically in HeLa cell lysate, but band intensity at the molecular weight for GNE trended with DNA input levels below 2.0ug, suggesting these cell lines do not tolerate higher plasmid concentrations. Importantly, the second polyclonal bound specifically to exogenous GNE expressed in HEK293T cells, and far less to endogenous GNE, suggesting pooled antibodies recognizing variable epitopes may be a valuable tool for verifying expression in the early stages of novel transgene characterization.

Conclusions: We found K562 cells were not a viable method for evaluation GNE transgene expression, HeLa did not tolerate high levels of protein expression, and HEK293T cells consistently showed increasing band intensity corresponding to higher plasmid inputs, likely due to efficient interactions between plasmid SV40 origin of replication and cellular SV40 large T antigen. Future studies will evaluate transgene product function.

1389 AAV-SLB101, a Novel Muscle-Tropic Capsid, Increases Gene Delivery and Expression Versus AAV9 and AAVrh74, in Mouse Models of DMD and FSHD Muscle Disease

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AAV-SLB101, a rationally designed capsid with the goal of increasing muscle tropism, is used in Solid Biosciences' next generation gene therapy SGT-003, which is currently in a clinical study for the treatment of Duchenne muscular dystrophy (DMD) (NCT06138639). To explore its potential for broader indications, we demonstrated markedly increased AAV-SLB101 transduction and expression of multiple reporter and therapeutic transgenes, when compared with first generation vectors, *in vivo* in wild type (WT) mice and mouse models of muscle disease.

In WT mice administered AAV-SLB101, AAV9 or AAVrh74, luciferase expression as measured by whole body IVIS was detected as early as 2 days post-dose for AAV-SLB101. On Day 7, expression for AAV-SLB101 remained substantially higher than for AAV9 and AAVrh74. In a 30-day study, whole body luciferase expression plateaued for all 3 capsids by Day 15, with significantly higher expression levels maintained at Day 30 in AAV-SLB101- versus AAV9- and AAVrh74-treated mice. Increased AAV-SLB101-driven expression was also reflected in *ex vivo* luciferase assays in heart and quad tissues.

Studies comparing AAV-SLB101 versus AAVrh74 delivery of a microdystrophin transgene in the *mdx* mouse model of DMD revealed higher biodistribution of vector genomes in disease-relevant quadriceps, gastrocnemius, diaphragm, and heart of mice treated with AAV-SLB101 versus AAVrh74. Increased AAV-SLB101 biodistribution translated into increased expression of microdystrophin in quadricep tissue as measured by immunofluorescence across all dose levels. Higher microdystrophin expression from AAV-SLB101 versus AAVrh74 resulted in a greater impact on serum biomarkers of muscle health; more reduction was observed in the AAV-SLB101-treated *mdx* mice, reflecting a more profound impact on the disease than in mice treated with AAVrh74.

In wild type mice, AAV-SLB101 and second-generation capsid MyoAAV3a achieved similar levels of muscle biodistribution of mi405, the therapeutic microRNA developed for treatment of Facioscapulohumeral muscular dystrophy (FSHD). Comparable levels of mi405 expression achieved by AAV-SLB101 and MyoAAV3a were also observed in the TIC-DUX4 mouse model of FSHD disease, which expresses elevated levels of the toxic *DUX4* gene. AAV-SLB101-mediated delivery of mi405 also resulted in significantly more muscle expression than for AAV9 in the disease model. Higher mi405 expression translated into protection from DUX4-induced histological damage and substantially lower levels of expression of the DUX4-activated biomarker *Wfdc3*, at low dose levels.

Together, these data highlight that AAV-SLB101 provided enhanced muscle delivery and expression of reporter and therapeutic transgenes versus first generation capsids and comparable levels compared to a second-generation capsid. In mouse models of DMD and FSHD muscle diseases, increased muscle expression of therapeutic transgenes

enabled disease modulating intervention at lower doses, highlighting the superiority of AAV-SLB101 over AAV9 and AAVrh74.

1390 Enhancing AAV gene therapy targeting retinal diseases with AI-guided rational design

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Gene therapy using adeno-associated viral vectors (AAVs) has been proven to be clinically effective for the treatment of retinal dystrophies, with most mutations residing in the outermost retinal cells. To target these cells, viral vectors need to cross the inner limiting membrane, and inner retinal neurons making them more difficult to target via this injection route. Current viral vectors are mostly injected into the subretinal space to target these cells, albeit with the efficacy being limited to the bleb region.

To address these challenges, we developed AI-guided, rationally designed approaches to engineer AAV2-derived vectors for ocular gene therapy, enabling low dose wider spread human retinal transduction via subretinal injection. We applied machine learning approaches to single-cell RNA-sequencing data and protein databases to identify receptors of interest for AAV targeting into human photoreceptors.

We started with the identification of human photoreceptor-specific markers using our proprietary single-cell Atlas and analytical platform. To quantify their specificity to retina, we computed the average expression levels of these markers as well as their percentage of expression at the photoreceptors surface compared to other retinal cell types grouped together. We also computed key metrics such as localization information about associated proteins. Additionally, we evaluated their involvement in both immune and endocytosis pathways. To ensure cross-species relevance in experimental models for pre-clinical studies, we curated single-cell RNA-seq data from pig, performing quality control and cell annotation using automated and manual approaches to identify cell-specific markers in the pig retina. We then explored the conservation of these markers with human.

Once markers expressed at the surface membrane were identified, computational structural biology approaches were performed for target validation. We assessed both the presence of already existing 3D structures and the capabilities of prediction tools to accurately reconstruct them in 3D when none was available. The presence of an interactor in the resolved structure was of importance to consider a marker as a potential candidate using our rational-guided approach. For the final step of our target validation approach, we performed analysis of structural conservation of the interacting site, when known, between human and pig. This allowed for a more effective cross-species AAV design and led us to the selection of one promising target, maximizing all our criteria for the design of novel viral vectors against retinal diseases. The next step will be the creation of optimized AAV libraries by inserting peptides targeting the receptor in the AAV2 vector sequence.

Efficient delivery remains a major challenge in the development of gene therapies for retinal diseases. Our comprehensive approach integrating AI-driven rational design, cross-species marker validation, and computational structural biology helps to overcome this current limitation. By identifying conserved, surface-exposed targets, we have laid the foundation for the development of optimized AAV vectors with enhanced retinal transduction capabilities. These advancements bring us closer to addressing a critical unmet need for more effective and targeted treatments for hereditary retinal diseases.

1391 A Single Suppressor-tRNA Restores Clinically Meaningful Protein Levels in Multiple Disease Models with Premature Termination Codons *In Vivo*

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Premature stop codon (PTC) diseases encompass thousands of genetic disorders caused by PTC, where a single base pair mutation converts an amino acid codon into a stop codon, leading to mRNA degradation and the production of truncated polypeptides with null, reduced or detrimental biological activity. Approximately 11% of all genetic diseases, affecting around 30 million people worldwide, are due to PTCs, of which 40% is the UGA mutation. Over half of these are CGA to UGA mutations, converting an arginine codon to a stop codon (Arg-UGA). We have engineered a series of Suppressor-tRNAs targeting Arg-UGA mutations and tested one of them, CDN001, in various cellular and animal models, delivered through adeno-associated viruses (AAV).

Mucopolysaccharidosis type II (MPS II) is a genetic disease caused by mutations in the iduronate 2-sulfatase (IDS) gene, leading to glycosaminoglycan (GAG) accumulation. In a transgenic mouse model with a PTC in the IDS gene, CDN001 packaged in AAV9 was administered through tail vein and through intracerebral ventricular injection. At Week four, IDS enzyme activity was restored to up to 90% in peripheral organs and 17% in the brain, significantly above clinically meaningful levels. Concurrently, glycosaminoglycan levels were significantly reduced in clinically relevant tissues.

Rett Syndrome is a genetic disease caused by mutations in the methyl-CpG binding protein 2 (MECP2) gene, leading to neurodevelopmental deficits. In a transgenic mouse model with a PTC in the MECP2 gene, CDN001 packaged in AAV9 was administered through intracerebral ventricular injection. At Week four, Full-length MECP2 protein level in brain tissue was restored up to 14% of the levels seen in wild-type mice. Together with the results of the MPSII model, these demonstrated the potential to treat multiple genetic disorders in the same organ with the same suppressor tRNA and the same AAV delivery system.

Stargardt Disease is a genetic disease caused by mutations in the ATP Binding Cassette Subfamily A Member 4 (ABCA4) gene, leading to progressive loss of central vision. In a transgenic mouse model with a PTC in the ABCA4 gene, CDN001 packaged in AAV2-7m8 virus was administered through subretinal injection. Full-length ABCA4 protein level was restored to 10-35% of wild-type levels, which are believed to be clinically meaningful for Stargardt Disease treatment.

Altogether, our data support the hypothesis that a single Suppressor-tRNA product could treat multiple diseases caused by the same premature stop codon mutations across different genes and organs.

1392 Expanding AAV vector delivery capacity: improving efficiency and vector genome cargo size

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Background and novelty: Advanced therapy medicinal products are transforming medicine. Gene therapies are providing treatments to previously unmet medical needs. To be effective, gene therapy requires vectors that deliver the therapeutic material to the affected cells efficiently and safely. Adeno-associated virus (AAV) vectors represent one of the most versatile clinical platforms for either localized or body-wide gene delivery. However, AAV vectors have a small packaging capacity, delivering small therapeutic genes up to 3.5 kb. This limits their use in treating numerous genetic diseases. Additionally, AAV vectors transduce cells fairly poorly, resulting in the administration of exceedingly high doses in the clinic, increasing the risk of adverse events. Herein, we describe vector engineering strategies, at the level of AAV vector transgene cassette and capsid, to enable extended and improved cargo delivery.

Experimental approach: To address the vector limitations in delivering large genes we are using dual AAV vectors enabling the delivery of therapeutic transgenes that exceed their natural packaging capacity. Dual AAV vectors have their cargo split in two AAV vectors. Current state-of-the-art strategies provide low efficiency of full-therapeutic protein reconstitution. To overcome this, we are developing novel molecular technologies for reconstituting the therapeutic full protein in the target cell through DNA recombination or protein trans-splicing. Furthermore, we are exploring rational capsid engineering strategies. Through peptide and protein insertion in specific VP proteins and locations of the capsid we aim to deliver more cargo at lower vector doses. The impact of genome and capsid engineering is being evaluated both at vector production yields and quality of AAV vector particles (i.e. particle stability, percentage of full genome particles, transduction efficiency).

Results and discussion: Naturally discovered split-intein sequence from Nostoc Punctiforme (Npu DnaE) has been extensively studied. In the AAV dual vectors we are assessing several novel split-inteins systems, such as engineered-consensus (Cfa) and cyanophage-like (Gp41-1). Using a split-GFP we observed consistently higher efficiencies of protein reconstitution provided by GP41-1 followed by Cfa. GP41-1 reconstitution rates were over two-fold higher than Npu DnaE and 100% of cells presented protein reconstitution after AAV vector dual co-infection. Targeting the treatment of Duchenne Muscular Dystrophies we developed novel therapeutic mini-dystrophins of about 220 KDa. The superior performance of GP41-1 was again observed for the reconstitution of this bigger therapeutic protein. In parallel we are developing methodologies based on DNA recombination. DNA-level reconstitution approach relies on occasional DNA recombination events occurring between AAV genomes after internalization. We are developing enzyme assisted DNA recombination which shows to have higher reconstitution efficiencies.

To improve vector cargo delivery capacity and transduction efficiency, we are rationally designing novel capsids. We assessed and identified different sites for capsid engineering without impacting vector yields. Studies performing peptide insertion are ongoing showing transduction efficiencies improvements of about 2-fold. This work demonstrates how AAV vector engineering drives the development of higher-performing vectors, ultimately reducing vector doses and costs.

1393 A System for Distinguishing Protein and Gene Delivery Elucidates Mechanisms of EV-AAV Vehicles

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The clinical success of adeno-associated virus (AAV)-based therapies has established a new paradigm in disease treatment. However, challenges such as preexisting humoral immunity and off-target tissue transduction necessitate higher vector doses, which are associated with adverse effects. Encapsulation of AAV within engineered extracellular vesicles (EVs) is a promising strategy to address these barriers by shielding the capsid from antibody neutralization and enabling tissue-specific targeting. A key challenge in evaluating engineered EV-AAVs is differentiating protein delivery, originating from transgene expression in producer cells, from true transgene expression in recipient cells. This challenge is analogous to the phenomenon of pseudotransduction with lentiviral vectors, but the scale of the challenge is heightened for EV-AAV since greater particle (vesicle) numbers are generally required to achieve a given rate of transduction. Developing methods to better separate signal from noise could facilitate characterization and improvement of EV-AAV vectors.

We first investigated whether choice of EV purification method (i.e., size exclusion chromatography, density gradient centrifugation) impacts the separation between gene and protein delivery. We found that no rigorous EV purification method substantially reduced protein delivery to a degree that mock delivery (e.g., EV-AAV produced without the Rep and Cap genes) could be distinguished from true EV-AAV transduction.

Thus, we next developed a new reporter system to rigorously distinguish true transduction from protein delivery by EV-AAVs. We designed a Cre-responsive AAV “stoplight” reporter vector that initially encodes for expression of an mCherry fluorescent protein, but upon Cre-mediated recombination, mCherry is excised such that the vector

drives mNeonGreen expression. By delivering such an EV-AAV vector to Cre-expressing cells, observation of mNeonGreen+ cells unambiguously confirms transduction, while allowing for simultaneous quantification of protein (mCherry) delivery. This validated assay provides a new, sensitive, and specific reporter of EV-AAV transduction.

Using this tool, we investigated surface functionalization strategies to probe how the mechanism of EV-AAV uptake impacts the rate of functional delivery on several target cell types. Interestingly, we found that enhancing EV-AAV uptake by HEK293FT recipient cells increased protein delivery, as expected, but not gene delivery; this was also observed when EVs were functionalized with VSVG to increase fusion as well as uptake. In contrast, when targeting Jurkat T cells, increasing uptake and membrane fusion via VSVG functionalization increased both protein and gene delivery. These results provide a basis for new mechanistic investigations. These insights were uniquely enabled by the discrimination between false and true delivery conferred by this novel EV-AAV characterization tool.

1394 Development of heart tropism AAV capsid by directed evolution

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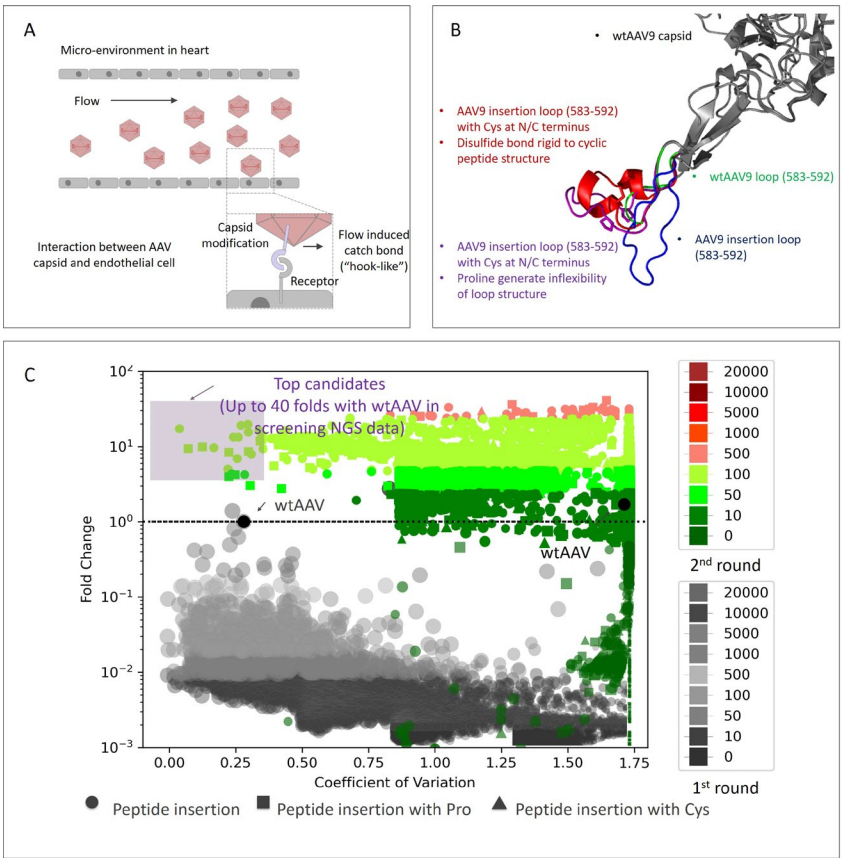
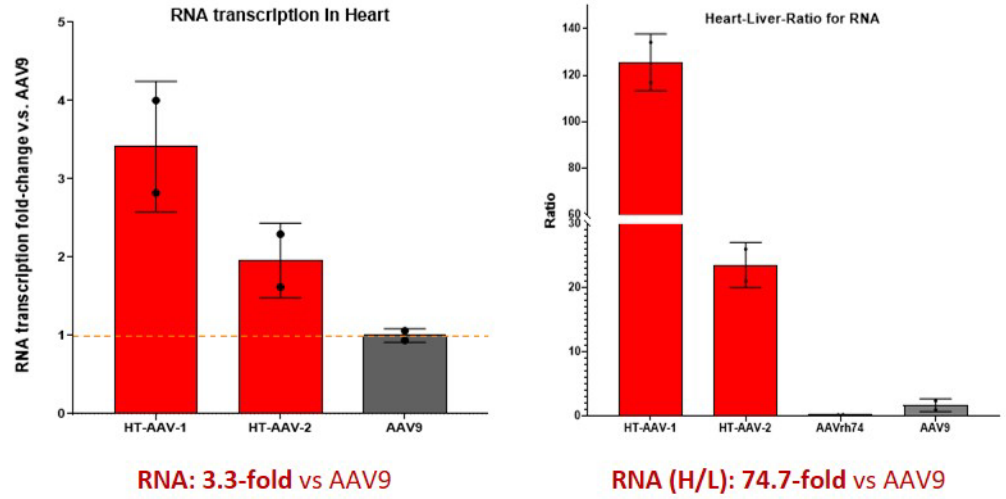
Adeno-associated virus (AAV) is one of the best viral-vector in gene therapies due to the long-last expression and the relatively low immunogenicity. However, the side effects from innate immune response by high dosed AAV vector and the toxicity of liver still remains the limitation for AAV based therapy. Thus, novel AAV capsid with specific organ tropism, which represents not only higher GOI expression in non-liver organs but also low liver target effects, plays important roles in development of innovated AAV gene therapies which would be potentially required by common diseases. Herein, we developed a novel AAV capsid for cardiac tropism with superior liver de-targeting effect.

To obtain the heart specific AAV capsid, directed evolution is the way to generate the heart-directed property. The challenge of cardio tropism screening would be 1) how to maintain the relatively high concentration of AAV in the microenvironment of cardio vascular in a long time period with higher shear force of blood flow by systematic administration and 2) how to increase the transduction efficiency in cardiomyocytes. Those problems may cause the failure of the evolution since the beginning of library design. Therefore, we designed our cardio tropism AAV library with 1) the special peptide insertion sequence, which including the peptide loops located within 2 prolines or 2 cysteines of the N-/C- terminus, to generate rigid loop or cyclic loop respectively which forms “catch-bond” under particular force dependent manner; and 2) the unique DNA shuffling library with wtAAVs especially AAV9, AAVrh74 and AAVrh10 which are the high cardio tropism AAVs so far to increase the transduction potency.

After screening in NHP for 2 rounds. We observed an obvious enrichment of AAV capsid with rigid “catch-bond” design, especially with the insertion loop modified by prolines in N-/C- terminus. The probable reason could be the expectation of di-sulfate bond formed by cysteines modification generate a low yield during the AAV packaging step. Moreover, the top candidates from DNA shuffling library represented better enrichment as well in compare with wtAAV after 2 rounds screening. Therefore, the top capsid candidates were then move forward to single serotype validation in NHPs.

During the single serotype validation, we identified the top cardio tropism AAV capsid named HT-AAV-1 and HT-AAV-2. The HT-AAV-1 represents high heart mRNA transduction efficiency which is 3.3-fold over AAV9 (8-fold over AAVrh74), indicating that the HT-AAV-1 shows better potential GOI expression in heart compared with wtAAVs. Moreover, HT-AAV-1 represents superior liver de-targeting effect than AAV9 and AAVrh74. It represented 74.7-fold heart-liver ratio of mRNA transduction vs. AAV9. The high cardio tropism and super low liver target properties may generate an ideal viral vector in the future of gene therapies for heart diseases.





1395 In vivo evolution of heart-derived AAV capsids: Development of novel vectors with enhanced cardiac tropism

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Heart failure (HF) continues to be a leading global cause of mortality, highlighting the critical need for innovative therapeutic strategies. Gene therapy has the potential to revolutionize HF treatment by targeting its molecular underpinnings, but its success relies on efficient and selective delivery of therapeutic genes to the heart. The commonly utilized AAV9 serotype shows a strong affinity for the liver, often resulting in severe hepatotoxicity and limiting its clinical applicability. To address these challenges, this study focuses on developing novel cardiac-specific AAV capsids through an innovative combination of experimental and computational approaches.

Novel heart-derived AAVs (HDVs) isolated from failing human myocardium were used alongside AAV6 and AAV9 to construct highly diverse libraries through DNA shuffling and peptide display techniques. The libraries underwent two rounds of in vivo evolution after systemic injection into small (mice) and large (pigs) animal models, followed by recovery of AAV capsids efficiently transducing cardiac tissues. Advanced bioinformatic analyses using both short- and long-read sequencing were employed to evaluate biodistribution and transduction efficiencies across cardiac and non-cardiac tissues. Starting with a library of 1E8 variants in the first round, the selection process narrowed this down to 1.5E5 variants in the second library, achieving a 1E6-fold enrichment of cardiac-targeting AAV variants. Across the two screening rounds, the newly created variants exhibited a 9E-2 reduction in liver tropism and demonstrated a twofold increase in the heart-to-liver targeting ratio. After the selection process, 50 capsids demonstrating enhanced cardiac tropism were identified.

Parental sequence analysis of the top 10 capsids variants revealed high shuffling diversity and a predominant contribution from our novel HDV vectors compared to AAV6 and AAV9. In addition, they exhibit distinct transduction profiles and superior cardiac transduction efficiency, with heart-to-organ ratios exceeding 1. Several of our novel capsids demonstrated specificity for different cardiac regions. One left ventricle (LV)-targeted variant achieved 71% transduction in the LV, compared to 5% in the right ventricle (RV), 10% in the atria, and 14% in other organs. A right ventricle (RV)-directed variant demonstrated 30% transduction in the RV, 24% in the atria, and lower levels in the LV (6%) and other tissues (40%). Similarly, an atrial-specific variant achieved 24% transduction in the atria, with minimal transduction in the RV (7%) and LV (4%). Notably, one candidate exhibited a balanced distribution between the heart (36%) and brain (35%), with the remaining 29% in other organs, offering potential utility for treating conditions affecting both the cardiovascular and central nervous systems.

In summary, this study establishes a versatile platform for engineering cardiac-specific AAV capsids tailored for cardiac gene therapy. When paired with optimized promoters and therapeutic transgenes, these novel vectors hold great potential to address both rare and common cardiac disorders, paving the way for transformative advancements in HF treatment.

1396 Engineering Novel AAV Capsids for Cardiac Gene Delivery

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Gene therapy is an emerging treatment option for both acquired and inherited cardiac disorders. Targeting cardiomyocytes following systemic delivery is critical to enable gene therapy treatments for many cardiac conditions. While several natural adeno-associated virus (AAV) serotypes, including AAV9, AAVrh.10, and AAVrh.74, can transduce cardiomyocytes *in vivo* and are being advanced in cardiac gene therapy programs, the requirement of high doses and the substantial viral load to the liver or off-target cell types raise the critical need for novel AAV capsids with enhanced cardiomyocyte targeting efficiency. Our previous work demonstrates that AAV9 more efficiently expresses transgene in cardiomyocytes following systemic administration compared to AAVrh.10 and AAVrh.74 in murine and non-human primate models. Starting from wildtype AAV9, we introduced various modifications to the capsid protein and collected *in vivo* cardiomyocyte transgene expression data for thousands of capsid variants. With these datasets as input, we developed multiple *in silico* methods, including human-directed design approaches and machine-learning based approaches, to extract sequence-to-fitness information, model the functional landscape, and design thousands of new variants that may outperform wildtype, as well as the best-

performing input variant. Next, we screened these *in silico* designed variants in mice and observed >60 newly designed variants showing higher cardiomyocyte transgene expression than the best-performing input variant. Individual validation of selected variants confirmed the library-based screen results. We further characterized a subset of *in silico* designed variants in cynomolgus monkeys and identified multiple candidates with greater cardiomyocyte transgene expression than AAV9, the best-performing input variant, and an industrial benchmark muscle-tropic capsid. Finally, our novel capsid candidates mediate significantly higher *in vivo* cardiac gene editing efficiency compared to AAV9, highlighting their potential therapeutics advantage. Our state-of-the-art capsid engineering platform that combines high throughput *in vivo* experimental screening and various *in silico* design methods has led to the identification of multiple novel capsid candidates with enhanced cardiomyocyte targeting efficiency following systemic delivery in both murine and non-human primate models, which may enable more efficacious and safer next generation gene therapies for cardiac disorders.

1397 An Improved Approach Enables Efficient and Cost-effective Directed Evolution of AAV Capsids in Non-human Primates via Different Delivery Routes

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Introduction: Non-human primates (NHPs) are ideal and valuable models for the directed evolution of Adeno-associated virus (AAV) capsids. It is attractive to use fewer NHPs to obtain more experimental data. Here, we developed a modified platform called DynEvoLib (Dynamic Evolution Library) for simultaneous directed evolution of capsids targeted at multiple tissues via different delivery routes in NHPs.

Methods: DynEvoLib contained three types of libraries and pools (Fig. 1A). The first-round screening was conducted using high-throughput capsid mutation libraries, in which random peptides or peptides derived from known proteins were inserted into the AAV9 capsid. Degenerate code mutations were introduced to flank the insertion site and used as degenerate barcodes (dBCs) to distinguish different libraries. Degenerate oligonucleotides generated peptide inserts (peptide-derived degenerate barcodes, pdBCs) for capsid libraries of subsequent screening rounds. mRNA expression of variants was then quantified by deep sequencing of barcoded AAV pools. Finally, AAVs containing multiple peptide tags were used to characterize variants at DNA, RNA, and protein levels. Libraries containing different dBCs/pdBCs, barcoded pools, and/or pools of peptide tags were administered to a single animal simultaneously via multiple delivery routes (Fig. 1A). Specific oligo-based magnetic beads were used for the enrichment of viral mRNA from total RNA to improve the acquisition of viral transcripts (Fig. 1A).

Results: DynEvoLib was optimized in a mouse model. It showed that dBCs/pdBCs combinations not only facilitated distinguishing between libraries used for multiple delivery routes but also improved the accuracy of peptide transcription analysis based on unique barcode counts compared with the analysis based on mRNA expression levels alone. Up to 576 variants showed more efficiency in brain transduction compared to AAV-F (Fig. 1B). There was good consistency between capsid libraries, barcoded pools, and peptide tags, including enhanced brain transduction and liver de-targeting (Fig. 1B, C, D). DynEvoLib was applied to rhesus macaques via multiple delivery routes. It showed that the mRNA enrichment strategy processed up to 10g of single tissue sample, resulting in a 151-fold increase in the collection of viral transcripts over the regular single-tube RT-PCR strategy. Enrichments of variants were first seen in the heart, muscle, kidney, and bone by intravenous administration, and in the central nervous system (CNS) by intrathecal administration. Characterizations using peptide tags identified several myotropic variants in rhesus macaques via intravenous injection (Fig. 1E). The optimal variant BY087 transduced skeletal muscle 21-fold and cardiac muscle 13-fold more effectively than AAV9 and performed cross-species muscle tropisms in NHPs, mice and rats (Fig. 1F). CNS tropic variants were characterized using the barcoded pool in rhesus macaques via intrathecal injection. BY253 showed 30-fold higher transduction in the spinal cord than AAV9 and transported more efficiently than AAV-DJ in the spinal cord, brainstem, cortex, and cerebellum (Fig. 1G).

Conclusions: DynEvoLib enables efficient and cost-effective simultaneous directed evolution of AAV capsids, simultaneously targeting multiple tissues through different delivery routes in NHPs.

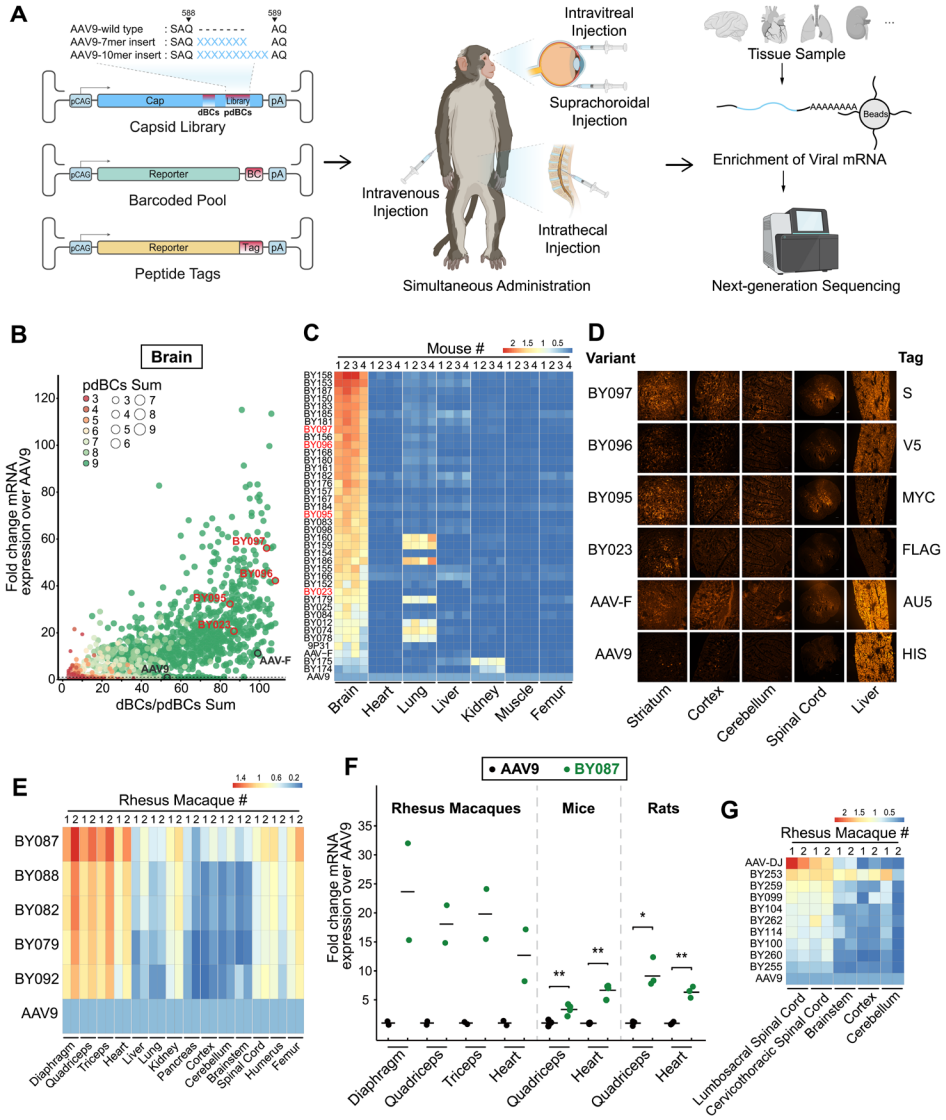

Figure 1. (A) Schematic of the libraries (or pools) design used in DynEvoLib, simultaneous administration via multiple delivery routes, and enrichment of viral mRNA from tissues for RNA-seq. (B) Fold change in mRNA expression of variants over AAV9, the total number (Sum) of dBCs/pdBCs combinations and pdBCs in the brain of BALB/c mice (n = 3) 3 weeks (3w) after intravenous (i.v.) administration of the capsid library containing 30,182 variants selected from a variety of tissue types. (C) Heatmap showing Log_{10} fold of relative mRNA enrichment (normalized to AAV9 control) of variants in brain and peripheral tissues of BALB/c mice (n = 4) 3w after i.v. injection of the barcoded pool. (D) Peptide tags in cryosections of different parts of CNS and liver from BALB/c mice (n = 3) were detected by immunofluorescence 3w after i.v. injection of the pool of peptide tags associated with CNS tropic variants and AAV9, AAV-F controls. Scale bar, 100 µm. (E) Heatmap showing Log_{10} fold of relative mRNA enrichment (normalized to AAV9 control) of myotropic variants in skeletal muscles, heart, and other tissues of rhesus macaques (n = 2) 3w after i.v. injection of the pool of peptide tags. (F) Fold change in mRNA expression of BY087 compared to AAV9 in skeletal muscles and heart of species 3w after i.v. injection of the same pool used in (E) into BALB/c mice (n = 4) and Sprague Dawley rats (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$. (G) Heatmap showing Log_{10} fold of relative mRNA enrichment (normalized to AAV9 control) in the CNS of rhesus macaques (n = 2) 3w after intrathecal injection of the barcoded pool associated with CNS tropic variants and AAV9, AAV-DJ controls.

1398 *In Silico* and *In Vitro* Analysis Suggests Translational Potential of the Smooth Muscle-Tropic Engineered Capsid, AAV-PR

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Introduction: We have recently developed a new AAV9 derived capsid called AAV-PR, displaying the 7-mer peptide, PRPPSTH. AAV-PR mediates unprecedented transduction of brain vascular smooth muscle cells (SMCs) and pericytes as well as SMC in the aorta in mice after systemic delivery. We set out to investigate the translational potential of this new capsid using a variety of methods.

Methods: Database searches were performed to identify potential peptide motifs which could indicate the mechanism of action of the capsid's binding partners. *In silico* modeling was performed to predict peptide confirmations in the VR-VIII loop of the capsid, as well as to predict interactions with AAV-R and T-cell epitopes. We assessed whether AAV-PR could transduce primary human adult aortic SMCs in culture. Cells were transduced with $2x10^5$ vg/cell of either AAV-PR or AAV9 (two independent batches tested for each capsid), both delivering an AAV-CBA-GFP cassette. Seventy-two hours later, cells were imaged for GFP fluorescence (vector transduction), followed by quantitation of GFP concentrations by ELISA. We also assessed the ability of AAV-PR to retain AAV9's galactose binding by comparing binding and transduction on Pro5 and Lec2 cells.

Results: A database search revealed that the AAV-PR peptide had a 100% match to rat FERM domain-containing protein 4A (FRMD4A). Interestingly this motif was conserved in 5-6 out of 7 residues in mice, old world monkeys, and humans. *In silico* modeling of the PR peptide in the VR-VIII loop of the AAV9 capsid revealed that the insertion of PR does not alter the overall structure of the VP3 domain as expected given the insertion position and the short length of the peptide. Interaction energy for the capsid with the AAV receptor's (AAVR) PKD1 and PKD2 domains were performed. Neither AAV-PR or AAV9 interact with AAVR's domain PKD1, (AAV5 is the only natural serotype known to bind PKD1). Strikingly, the calculation predicts that AAV-PR binds stronger to AAVR's domain PKD2 than AAV9, in all three analyzed species (mouse, human, orangutan), with a strength of interaction similar to AAV2. T cell epitopes were predicted to be comparable to AAV9, which may indicate a similar immunological profile. AAV-PR showed a remarkable 16-fold increase ($p<0.012$) in GFP concentration in primary human VSMCs compared to AAV9. Galactose binding was increased with AAV-PR compared to AAV9 when comparing transduction increases on N-terminal galactose deficient Lec2 cells compared to galactose expressing Pro5 cells.

Conclusions: The identify of the multi-species conserved, putative FRMD4A motif may provide directions towards a mechanism of action of AAV-PR's robust transduction of SMCs. Future studies validating the predictions of AAV-R binding affinity as well as the increased binding to galactose may also provide insight into its transduction properties. These findings, combined with the transduction of human VSMCs suggest AAV-PR has potential as a clinically relevant gene delivery vehicle to treat diseases of the vasculature.

1399 Artifical Modification of Adeno-Associated Virus Type 2 for Direction to Vascular Smooth Muscle Cells

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Introduction: Adeno-associated virus (AAV) vectors are widely used to transduce genes of interest safely and effectively. AAV is a small non-enveloped virus with a diameter of 25 nm. The transduction efficiency, called tropism, is known to be determined by amino acid sequence of the protruded region of capsid protein. Especially, variable region VIII (VR VIII) has been focused on to change tropism. In this study, we inserted random sequences of 7 amino acids into VR VIII of AAV2 to develop suitable AAV vectors for smooth muscle cells.

Methods: We cloned a reporter and capsid protein-expressing plasmid which contains two main elements flanked by AAV2 inverted terminal repeats (ITRs), including (a) the mCherry cDNA driven by human UBC promoter and followed by a synthetic polyadenylation sequence, and (b) the AAV5 p41 promoter sequence and splicing sequences taken from the AAV2 *rep* gene followed by AAV2 *cap* gene which contains insertion of 21-mer randomized nucleotide sequence in frame after amino acid 587 of VP1. Recombinant AAVs were generated by triple transfection of 293T cells with the above-mentioned plasmid, adenoviral helper plasmid pHelper (Agilent Technologies), and assembly-activating protein helper plasmid, shown in the previous study by Deverman et al. (Nat Biotech, 2016). Seventy-two hrs after transfection, viral particles were harvested from both the cells and media, and AAVs were purified using chloroform technique (Negrini et al., Curr Protocol Neurosci, 2020). Purified AAVs were transduced into cultured smooth muscle cells of human or mouse, which were sorted using fluorescence activated cell sorter (FACS). The viral DNA of mCherry-expressing cells was extracted and amplified by PCR using primers interposing randomized nucleotide sequence. The PCR-generated sequence was cloned back into the above-mentioned reporter and capsid protein-expressing plasmid. Using this plasmid, recombinant AAVs were again generated and transduced to smooth muscle cells. Seventy-two hrs after transfection, the viral DNA of mCherry-expressing cells was again extracted in the same way as the first round. The nucleotide sequences of *cap* gene in the first and second rounds were identified and the frequency was quantified using the next generation sequencer.

Results: Analysis by the next generation sequencer showed that NRPETNP and SHDPSKE identified in the human brain vascular smooth muscle cells and RENKLGE and KDTVPRE identified in the mouse aorta smooth muscle cells were the two of the most frequent amino acids sequences in frame after amino acid 587 of AAV2 VP1, which showed more than half of the recovered library sequences.

Conclusions: Using the *in vitro* assay system, we have identified smooth muscle cell-directed variant of AAV2. In the next step, we're going to inject above-mentioned variants of AAVs into nonhuman primates and mice to confirm that their tropism is directed to smooth muscle cells *in vivo*.

1400 Development of muscle tropism AAV capsid by directed evolution

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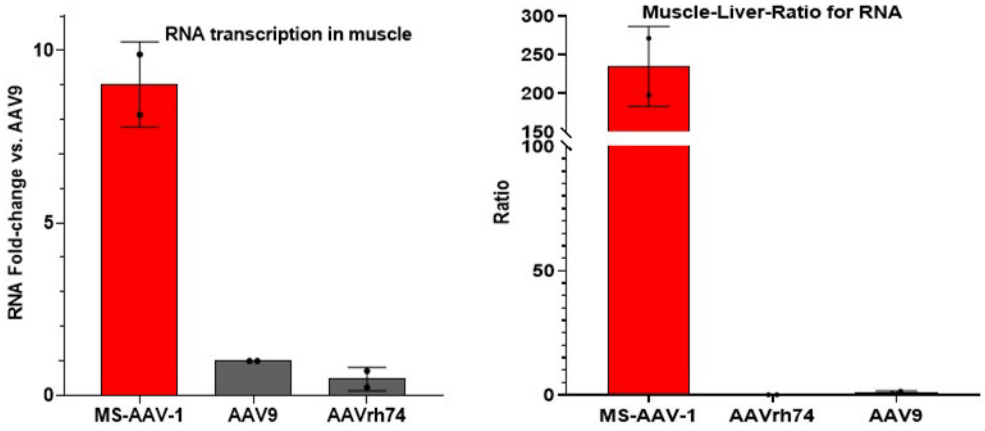
Single gene disorders related to the muscular tissue plays important role in development of gene therapies for rare diseases such as Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). Adeno-associated virus (AAV) gene therapy is one of the ideal therapy for single gene disorders such as FDA proved Elevidys for DMD. However, the side effects from innate immune response by high dosed AAV vector and the toxicity of liver still remains the limitation for AAV gene therapy. Thus, novel AAV capsid with specific muscle tropism such as myo-AAV, an AAV9 based peptide insertion AAV capsid showed 6-fold increasing of mRNA transduction and around 0.5-fold liver targeting effect than AAV9 in NHP, plays important role in development of innovated AAV gene therapies. Herein, we developed a novel AAV capsid for muscle tropism with high muscular mRNA transduction efficiency and superior liver de-targeting effect.

To obtain the muscular specific AAV capsid, directed evolution is the way to generate the muscle-directed property. The challenge of muscle tropism screening would be how to increase the transduction efficiency in myocytes. Thus, we designed our muscle tropism AAV library with the unique DNA shuffling library with wtAAVs especially AAV9, AAVrh74 and AAVrh10 which are the high muscle tropism AAVs so far to increase the transduction potency.

After screening in NHP for 2 rounds. We observed an obvious enrichment of AAV capsid with in muscle, we then validated the top capsid candidates with single serotype validation in NHPs. We identified the top muscle tropism AAV capsid named MS-AAV-1. The MS-AAV-1 represents high muscle mRNA transduction efficiency which is 9-fold over AAV9 (19-fold over AAVrh74), indicating that the MS-AAV-1 shows better potential GOI expression in muscle compared with wtAAVs. Moreover, MS-AAV-1 represents superior liver de-targeting effect than AAV9 and AAVrh74. It represented 204-fold muscle-liver ratio of mRNA transduction vs. AAV9 (1416-fold muscle-liver ratio

over AAVrh74). The high muscle tropism and super low liver target properties may generate an ideal viral vector in the future of gene therapies for muscular single gene disordered diseases.



1401 Identifying Muscle Stem Cell Tropic AAV Variants Through Directed Evolution

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Duchenne Muscular Dystrophy (DMD) is a rare genetic disease caused by frameshifting mutations in *DMD*, resulting in lack of dystrophin protein. CRISPR-based gene editing can restore the *DMD* reading frame and restore an internally deleted dystrophin; however, studies suggest that the restored dystrophin protein diminishes over time. Loss of dystrophin is likely due to the dilution and replacement of edited nuclei by unedited nuclei that derive from muscle stem cells that fuse to muscle fibers. One strategy to retain durable dystrophin is to target Muscle Stem Cells (MuSCs) with CRISPR/Cas9 to correct genomic DNA in the proliferating cell population. We previously conducted an in vivo screen of an AAV capsid library that contains a random 7 amino acid sequences inserted between amino acids 589A and 590Q in the surface region VIII (VR-VIII) of AAV9. Five variants isolated from either muscle stem cells (MuSCs) and five from skeletal muscle fibers (SkMu) were identified and validated in vitro against both mouse and human myoblasts and myotubes. Several variants performed nearly 40x better than wtAAV9 and 15x better than MyoAAV1A in transducing MuSC, while retaining their tropism for myotubes. In addition, in vivo studies in mice have demonstrated a similar enhanced tropism compared to wtAAV9. These data have been confirmed through flow cytometry, luciferase, and qPCR of viral genomes. While further characterization must be done, these new variants provide a potential new platform for achieving long-term dystrophin restoration and durability when AAV is used to deliver CRISPR components in DMD and other myogenic diseases that involve muscle degeneration and regeneration.

1402 Rational Design of AAV9 Capsids for Targeted Delivery to Muscle and Heart and Detargeting of the Liver

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Adeno-associated viruses (AAVs) are widely recognized as efficient vectors for in vivo therapeutic gene transfer. However, a key challenge in using AAVs as gene therapy vectors is to achieve high transduction efficiency while maintaining tissue and cell type specificity. Extensive research has focused on engineering the AAV protein capsid to fine-tune its in vivo tropism and biodistribution. These efforts have yielded numerous functional motifs that target or detarget diverse tissues and cells. Some functional motifs exhibit modularity, retaining functionality when transferred across AAV serotypes or incorporated into different surface-exposed variable regions. In contrast, others may depend on surrounding amino acid sequences. Harnessing these motifs collectively could enable the design of synthetic AAV capsids with versatile functions. In this study, we combined two functional motifs to create a synthetic AAV9 capsid, termed AAV9-GAST-MyoAAV. The first motif mediates liver detargeting, which is achieved by substituting GGSS (residues 266-269) in AAV9 with GAST residues. The second motif mediates muscle targeting, by MyoAAV peptides introduced as 7-10 amino acid insertions or replacements at variable region (VR) VIII of AAV9. Both motifs have demonstrated efficacy in non-human primates. The resulting synthetic capsid exhibited desirable properties. Compared to AAV9, AAV9-GAST-MyoAAV4E demonstrated a 61-fold increase in mRNA expression in the heart, a 34-fold increase in the quadriceps, and a 25-fold decrease in liver expression at a dose of 1E13 vg/kg in adult mice. Superior transduction efficiency was observed in the heart and diaphragm across most doses and in the quadriceps at doses exceeding 1E12 vg/kg. These observations were the same in both sexes. In neonatal mice, the synthetic capsid outperformed AAV9 in transducing the quadriceps and diaphragm but exhibited limited heart transduction, likely due to differential receptor expression levels in neonatal tissues. This study demonstrates the feasibility of combining functional motifs on a single AAV capsid to achieve tailored transduction profiles. If extended to other motifs, this rational "plug-and-play" approach to synthetic AAV capsid design could streamline the development of novel capsids with multifunctional capabilities.

1403 AI-guided Vector Engineering for Enhanced ALS Therapy

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Gene therapy has significant potential for treating a broad range of genetic and non-genetic diseases, but one of the major hurdles is achieving efficient and targeted delivery. Recombinant adeno-associated viruses (rAAVs) are commonly used as vectors for gene therapy, yet their ability to effectively target specific cells or tissues is often limited. This challenge underscores the urgent need for more efficient and safer rAAV vectors. Recent advances have focused on developing next-generation AAV vectors that offer improved specificity and delivery capabilities. One of the approaches is the use of machine learning to enhance rAAV transduction. In this study, we developed InsightMiner^{TM}, an AI that learns the characteristics of viruses that infect humans and suggests the sequence of coat proteins to improve the properties of virus vectors such as AAV. By analyzing a variety of human-targeting virus species and applying machine learning algorithms, we identified 7-mer amino acids that improve BBB penetration and central nervous system (CNS) delivery. Consequently, these 7-mer amino acids were inserted into rAAV9, generating GCAPs that show enhanced delivery both in vitro and in vivo compared to wild-type AAV9. GCAPs were then applied to neurodegenerative disease, amyotrophic lateral sclerosis (ALS). The GCAP001 and GCAP002 efficiently delivered the hSTMN2 gene into cultured motor neurons of STMN2 knockout mice, promoting axon regeneration more effectively than wild-type AAV9. Furthermore, intrathecal delivery of hSTMN2 via the GCAP002 significantly improved motor function at 3 months post-injection. In summary, this study demonstrates the power of combining capsid engineering, human virus data, and machine learning to enhance the effectiveness of gene therapy, especially for ALS.

1404 AAVrh32.33: A Promising Gene Delivery Vector for Skin Disorders

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Skin disorders, affecting millions worldwide, include over 3,000 conditions, with 700 having a genetic basis. These disorders can severely impact physical and mental health, often leading to long-term symptoms. Gene therapy for skin disorders is promising due to the skin's accessibility and ease of monitoring treatment outcomes. However, gene delivery remains challenging, with both viral and non-viral methods having limitations. Adeno-associated viruses (AAVs) are considered a promising option due to their low immunogenicity and non-pathogenic nature, but past studies have struggled to show their effectiveness in targeting skin cells, prompting further exploration of less common AAV serotypes for improved results.

In our study, we evaluated the efficiency of five AAV serotypes—AAVrh32.33, AAV1, AAV2, AAV5, and AAV6—in C57BL/6 mice, all expressing GFP. While AAV2 and AAV5 failed to express GFP, the other three vectors effectively targeted similar cell types, with transgene expression predominantly observed in the dermis. Interestingly, AAVrh32.33 demonstrated the highest transduction efficiency compared to AAV1 and AAV6, despite its known immunogenicity in mice. Flow cytometry analysis revealed that dermal fibroblasts were the majority of transduced cells, followed by bulge and isthmus cells. Some immune cells, including monocytes and macrophages, were also labeled with GFP. Histological and intravital microscopy confirmed that dermal fibroblasts were the predominant transduced cell type. Importantly, GFP-labeled cells exhibited sustained expression for up to two years, despite previous studies reporting CD8 T cell responses and transgene loss with rh32.33. Vector genome analysis showed no significant reduction in the skin over the course of one year. However, flow cytometry data revealed a decrease in GFP-labeled cells among bulge cells, monocytes, and macrophages after one year. Additionally, intradermal injections into human skin biopsies confirmed that AAVrh32.33 is capable of targeting human dermal cells in addition to mouse cells.

To address immunogenicity of AAVrh32.33, we modified the capsid by replacing the previously reported immunodominant peptide with one from AAV6. This modification preserved transduction efficiency similar to the wild-type AAVrh32.33, but notably, there was a statistically significant increase in transduction of dermal fibroblasts compared to the unmodified version. Additionally, fewer monocytes were labeled with GFP using the modified vector. Flow cytometry analysis indicated no significant T-cell response specific to the immunodominant peptide. These findings suggest that modifying the AAVrh32.33 capsid reduces its immunogenicity without compromising transduction efficiency.

In conclusion, our study highlights the potential of AAVrh32.33 as a highly efficient gene delivery vector for skin gene therapy. Its ability to target dermal fibroblasts and hair follicle cells makes it a promising candidate for treating a variety of skin disorders, including hair-related diseases. Furthermore, the modified capsid version enhances its safety profile by reducing immunogenicity, making it an even more attractive option for clinical applications in skin and hair gene therapies.

1405 Engineering the AAV capsid to evade neutralizing antibody-mediated immune responses

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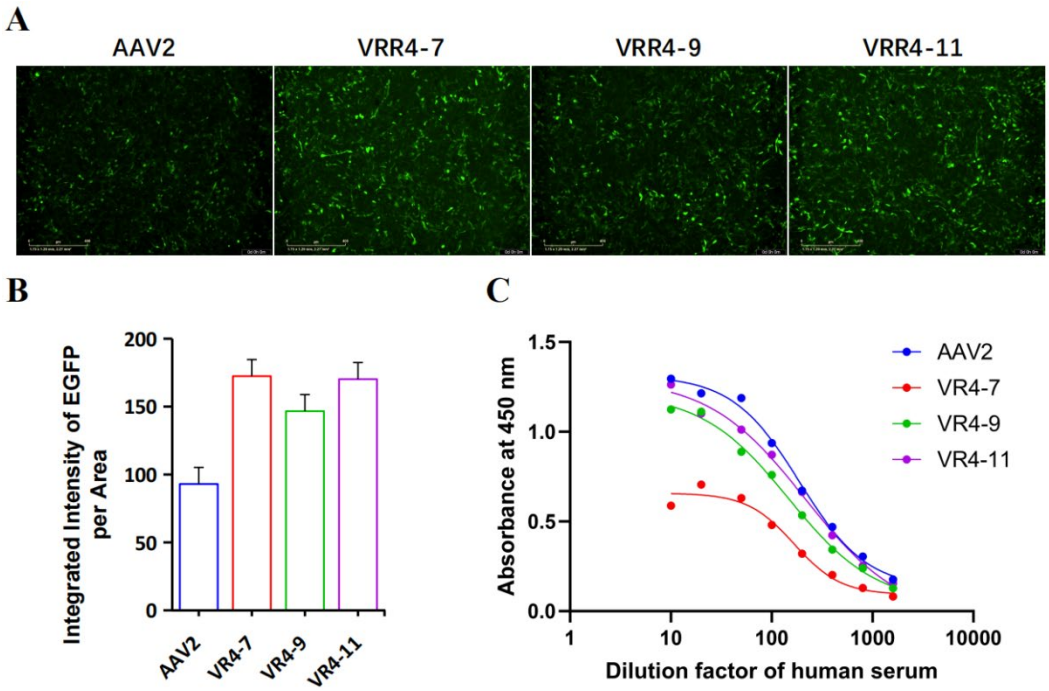
Purpose: AAV2 is one of the most used vector in ocular gene therapy. However, following intravitreal injection, AAV2 frequently elicits an immune response, posing a risk of retinal inflammation, which may restrict its clinical application. Part of the reason for this phenomenon is the presence of pre-existing antibodies (Nabs) within the host. Therefore, modifying viral capsids to evade immune recognition is crucial for safe and effective vector delivery. The current study reported strategies for developing AAV capsids to evade Nabs.

Methods: We employed a domain-swapping strategy, involving the exchange of VRs among different AAV serotypes. Specifically, we substituted the variable region IV (VR IV) of AAV2 with the corresponding VR IV from AAV7, AAV9, and AAV11, generated a series of chimeric capsids include VR4-7, VR4-9, and VR4-11.

Results: The VRR4-7, VRR4-9 and VRR4-11 recombinants AAV variants exhibited significantly improved transduction efficiency in HEK293 cells compared to the wild-type AAV2. Notably, the AAV2-7 variant demonstrated reduced binding affinity to human serum AAV2 NAbs, achieving superior immune evasion ability compared to the parental wild-type AAV2.

Conclusions: Our findings underscore the potential of rational capsid engineering to enhance AAV-mediated gene delivery by improving transduction efficiency and mitigating susceptibility to pre-existing Nabs, thereby presenting a promising strategy for the development of safer and more effective gene therapy vectors.

Fig.1 (A) EGFP reporter gene was packaged into different AAV2 variants. Fluorescent microscopy images showing GFP expression in HEK293 cells transduced with different AAV variants. Transduction efficiencies were assessed at 48h post infection. Scale bar = 400μm. (**B**) Quantitative data of transduction efficiencies for each AAV variant relative to the wild-type AAV2 capsid from (A). (**C**) The binding affinity of different chimeric AAV variants for AAV2 neutralizing antibodies was tested using the ELISA method. Briefly, AAV2, VR4-7, VR4-9 and VR4-11 capsids were coated at an equal concentration in a PVDF membrane. The membrane was incubated with serial dilutions of AAV2-positive human serum, followed by detection of Nabs using an anti-IgG Fc antibody.



1406 Neo-antigen tumor vaccination depends on CD4-licensing conveyed by adeno-associated virus like particles.

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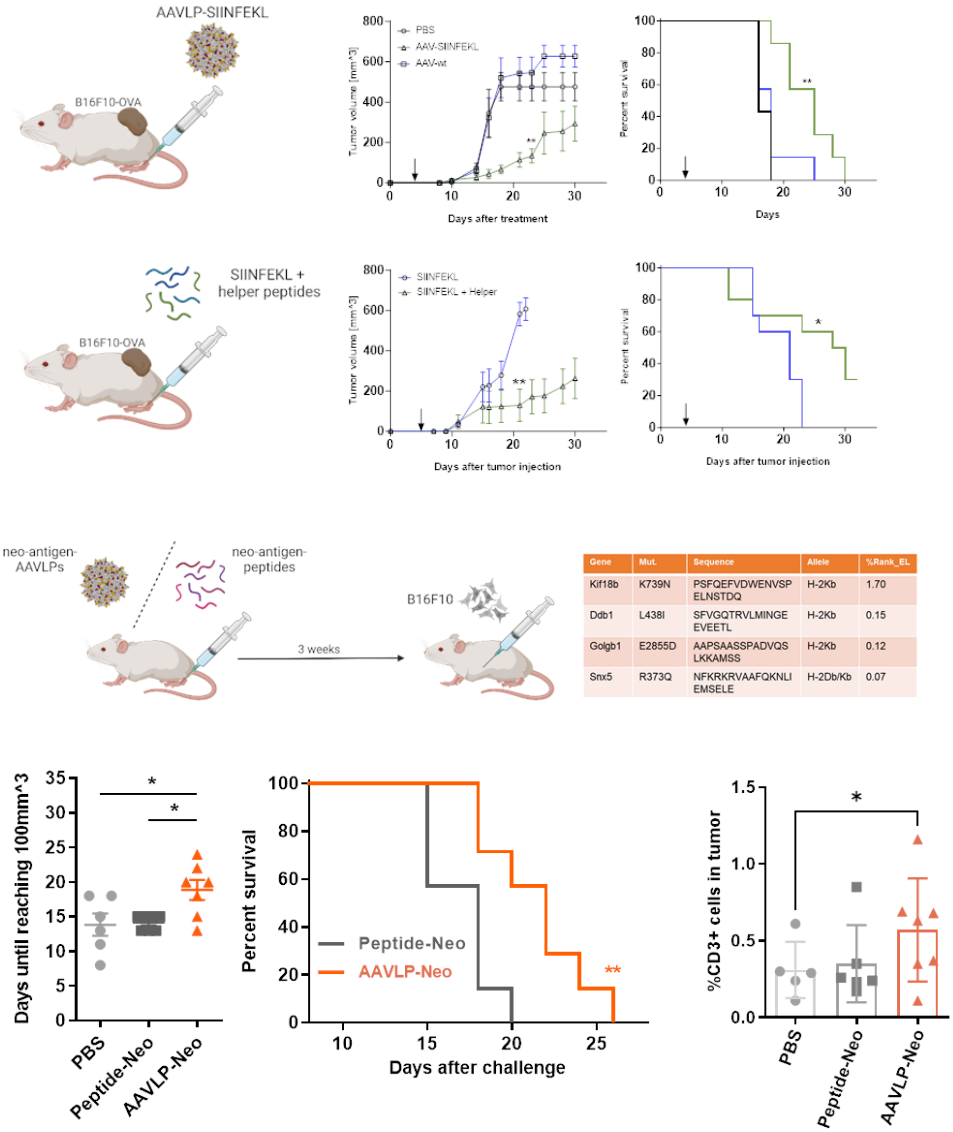
Personalized treatment has become a realistic option for tumor patients, accelerated by significantly reduced sequencing costs of tumor genomes and advances in vaccine formulations. The druggability of cancer neo-antigens caused by individual mutations is centered in this effort. We here use an AAV-based VLP platform to compose a neo-antigen specific protein vaccine that is effective in a murine prevention and treatment setting. Furthermore, we show that CD4+ T cell responses that are provided by the AAV capsid are crucial for an effective murine melanoma treatment. To uncover the optimal composition of a peptide vaccine we de-linked MHC-II helper peptides from the capsid and formulated an efficient neo-antigen specific vaccine, which showed the independence of CD4+ T cell response from tumor sequences. The findings are supported by clinical data of neo-antigen vaccinated tumor patients. Our results punctuate on the significance of MHC-II epitopes for CD8+ T cell responses and suggest a future use of AAVLPs as neo-epitope vaccines in personalized cancer treatments.



1407 Common AAV Gene Therapy Vectors Show Indiscriminate Transduction of Living Human Brain Cell Types

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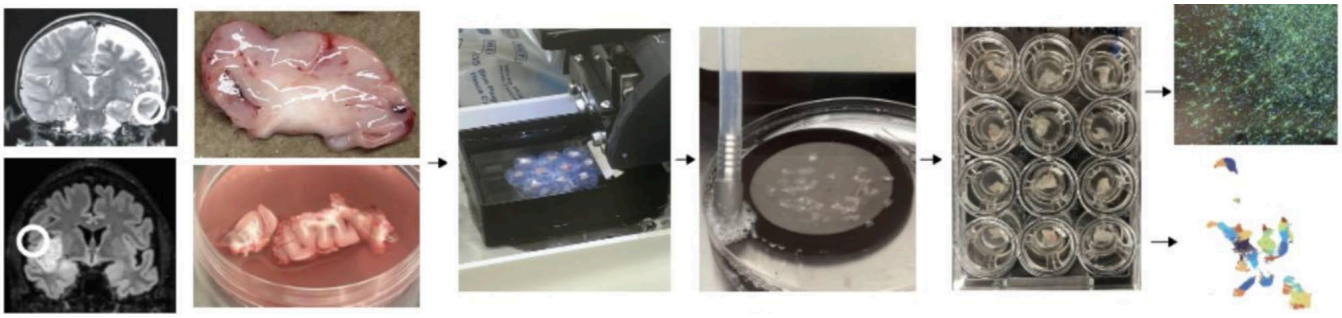
Introduction: The development of cell-type-specific gene therapy vectors for treating neurological diseases holds great promise, and there is growing recognition that the field of medicine will soon see widespread use of molecular tools, including CRISPR-based gene editing, optogenetics and other DNA- and RNA-based therapies. However, in many cases, the safety and efficacy of these tools will depend on our ability to deliver these therapeutics precisely to target cell types while avoiding neighboring but uninvolved cells. Viral vectors, including adeno-associated viruses (AAVs)—the most common vectors in ongoing clinical trials for neurological diseases, rely on their surface proteins' recognition of cellular membrane components to gain entry to a cell. For this reason, modifying AAV capsids to target specific cell types has been an area of broad interest. To date, AAV tropism has been tested mainly in animal models, primarily mice with some non-human primates, and it has been shown that AAV tropism does not usefully translate across species and sometimes even across strains of mice.

Materials and methods: For this study, we have adapted a human brain organotypic slice model using resected neurosurgical specimens not needed for pathologic diagnosis and maintained them in a living state for two weeks in vitro. We determined the tropism profiles of fourteen common AAV vectors via a combination of immunofluorescence and single-nucleus RNA sequencing using eight natural AAV variants (AAV1, 2, 5, 6, 7, 8, 9, rh10), and six engineered AAV variants (DJ8, DJ, AAV2-retro, PHP.S, PHP.eB, Sch9). All serotypes carried eGFP constructs driven by the ubiquitous CAG promoter (ITR-CAG-eGFP-WPRE-hGH polyA signal-ITR). We used brain tissue from eight patients that included temporal lobe cortex and other lobectomy specimens resected due to intractable epilepsies, as well as normal cortex resected en route to deeper tumors.

Results: Of the fourteen variants tested by immunofluorescence, both PHP.eB (60%) and PHP.S (55%)showed the most significant overall transduction rates, whereas AAV5, DJ, and rh10 demonstrated the lowest transduction rates, with all three averaging less than 25% of the total cells in the quantified area. In comparison, AAV2 and AAV9—the most common AAV variants used in neurological trials—both exhibited moderate transduction rates, infecting approximately 30-40% of cells within the analyzed area. Regarding general neuronal transduction, PHP.eB and PHP.S again demonstrated the highest transduction rates (~55%), while AAV9 and AAV2 transduced approximately 40% and 30% of NeuN+ neurons, respectively. Notably, astrocytes (GFAP+ cells) emerged as the most highly transduced cell type for the majority of capsid variants tested. Specifically, AAV6, AAV7, PHP.S, and PHP.eB all transduced ~75% or more of astrocytes within the analyzed areas. In contrast, microglia (Iba1+ cells) showed variable transduction levels, ranging from as low as ~3-15% with AAV5, AAV8, and DJ, to as high as 45% with PHP.S and PHP.eB.

Conclusions: Using immunostaining and single-nucleus RNA sequencing, we found that common AAV vectors show astrocyte preferences but have generally indiscriminate transduction of normal cell types.



1408 Determinants of AAV9 capsid mediated transgene suppression in the rat CNS

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We recently reported that AAV exhibits a capsid-promoter interaction that alters *in vivo* cellular tropism/transduction within the rat and NHP Central Nervous System (CNS). This interaction is seen in AAV9 with an insertion of six glutamates (EU) at aa 139 of the capsid (VP1 numbering) which results in significantly reduced rat striatal transduction that is limited to mostly oligodendrocytes, not neurons. We sought to determine the transduction phenotypes of other CNS relevant AAV capsids (AAV1, AAV2, and rh10) with six glutamate insertions (AAV1EU, AAV2EU, and rh10EU) in both the rat striatum and hippocampus. Transduction is reduced with AAV1EU and AAV2EU like AAV9EU relative to their parental serotype. However, rh10EU retains robust transduction in cells morphologically resembling neurons and colocalizes with rh10 transduction in 76% of cells in both the striatum and hippocampus. Further analysis revealed that AAV9EU has a reduction in both nuclear transgene copies (~ 10- fold less) and relative transgene mRNA levels (~2000-fold less) compared to AAV9, while rh10EU exhibits values comparable to rh10. To identify determinants of the AAV9EU phenotype, thirteen vectors containing different regions of AAV9EU and rh10EU were cloned and assessed for transduction within the rat striatum. Preliminary studies revealed that two mutants mut8 and mut11 exhibited robust transduction similar to rh10EU. Further, sequence analysis identified VP1 capsid residues within rh10EU that contribute to its robust neuronal transgene expression over AAV9EU. Together, these results suggest that capsid-promoter interactions arise from VP1 residues that appear to underly alterations in AAV transduction within the CNS.

1409 Bioconjugated AAV vectors with enhanced functionality and tropism

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Enhancing AAV functionality through capsid engineering has become an area of enormous growth for gene therapy. The most common method to achieve this is to use genetic engineering, whereby sequences encoding short peptides are inserted into exposed loops of the capsid proteins. While many new AAV genetic variants have been described with potential for clinical applications, this approach brings certain shortcomings, most notably the necessity to operate within the constraints of preserving the capsid integrity and vector yield, and the lack of knowledge of the underlying biological mechanism that endowed the selected new capsid variant with the new desired tropism.

Here we describe an alternative approach to capsid engineering based upon bioconjugation of targeting ligands onto exposed residues of an AAV capsid using biorthogonal chemistry. By implementing a post-manufacturing functionalization step, we can make virtually any AAV vector amenable to accept a ligand that will re-direct, modify or enhance its tropism. We show that our approach can accommodate multiple classes of ligands, from active peptides to full-length protein domains, allowing pre-determined human validated targets to be used as binding receptors, and resulting in enhanced AAV vector potency with no impact on yield. Our approach does not require extensive screening of capsids or targeting ligands, since it works from knowledge of the receptor-ligand expression profile and function to the desired tissue tropism. Depending on the ligand chosen we can add new functionalities to AAV, such as improving transduction across a variety of cell types, achieving cell-specific targeting, or increasing transport into the CNS.

By applying optimized chemistry to clinically relevant AAV capsids, performance and specificity of AAV mediated gene therapy can be enhanced across highly coveted target tissues.

1410 Direct Comparison of the Engineered Capsid AAV-DB-3 and Wildtype AAV5 in Nonhuman Primate Basal Ganglia and Cerebral Cortex

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Introduction: Latus Bio is developing an adeno associated virus (AAV) gene therapy as a potential treatment for Huntington's Disease (HD). A major challenge for HD gene therapy is delivery of gene therapies to the most severely affected cell type in HD, medium spiny neurons of the caudate nucleus and putamen. The current state of the art requires multiple intraparenchymal injections per hemisphere under convection enhanced delivery, with surgery times in excess of 10 hours. We previously identified an engineered AAV capsid, AAV-DB-3, in an in vivo screen in nonhuman primates (NHPs), that broadly transduced medium spiny neurons following a single injection per hemisphere in the globus pallidus (GP). Here we compared AAV-DB-3 to AAV5 in a head-to-head experiment in NHPs and evaluated caudate nucleus and thalamus delivery of AAV-DB-3 as alternatives to the GP.

Results: AAV-DB-3 and AAV5 vectors with fluorophore transgenes were delivered to the left and right GP, respectively, at the same dose and volume in two adult rhesus macaques. In a third rhesus macaque, AAV-DB-3 was delivered to the caudate nucleus and thalamus. Following necropsy, tissue was processed for histology and digital droplet PCR (ddPCR) analysis of AAV biodistribution and transgene expression. Following GP delivery, AAV-DB-3 expression was dramatically higher than AAV5 in caudate nucleus, putamen, and motor cortex: 92X in globus pallidus, 531X in caudate nucleus, 184X in putamen and 271X in motor cortex. AAV-DB-3 viral genome biodistribution was also higher than AAV5, but to a lesser degree: 1.2X in the GP, 1.5X in the caudate nucleus, 4.6X in the putamen, and 2.5X in the motor cortex. Fluorescent microscopy in the AAV-DB-3-injected hemisphere revealed broad transgene expression in the GP, caudate nucleus, putamen, and motor cortex. In contrast, AAV5 transgene expression was much lower and largely limited to the injection site in the GP. Neither caudate nucleus nor thalamus delivery resulted in the same broad distribution of AAV-DB-3 in the caudate nucleus, putamen, and motor cortex as revealed by microscopy and ddPCR.

Conclusion: AAV-DB-3 dramatically outperformed AAV5 for transgene expression in key brain areas and cell types for HD gene therapy. In addition, GP delivery led to broader AAV-DB-3 biodistribution in the caudate nucleus, putamen, and motor cortex than caudate nucleus or thalamus delivery. Together, the data support GP delivery of the engineered capsid AAV-DB-3 as a promising approach for the delivery of therapeutic payloads for HD and other disorders of the basal ganglia.

1411 Next Generation Whole Capsid Engineering Yields Translational AAV Capsids for Neurological, Neuromuscular. and Muscular Gene Delivery

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Adeno-associated viral (AAV) vectors show great promise as gene delivery vehicles. In recent years, laboratory engineered capsids have emerged with promising results, but AAV9 continues to be the vector of choice in clinical applications aiming to ameliorate neurological, neuromuscular, and muscular diseases. While clinical trials continue to show success with AAV mediated gene therapies, efficiency and specificity remain as a major bottleneck. Furthermore, AAV mediated gene therapies are among the most expensive treatment options for genetic diseases. Many of these issues can be addressed with improved AAV capsid technology. Previously, we presented work on a novel AAV9-based library generated through controlled randomization to multiple specific regions across the entire capsid gene. To identify species cross-compatible capsids amenable for translational studies, parallel screens were performed in mice and non-human primates (NHPs) using intrathecal (IT) or intravenous (IV) administration of AAV libraries. To overcome previous technological limitations of capsid identification strategies, we employed PacBio's SMRT sequencing of capsid amplicons derived out of isolated nuclei from several tissues of the CNS and peripheral organs. We developed and used a custom bioinformatics pipeline to analyze our datasets and coupled our hit identification with computational protein design tools, Rosetta and AlphaFold. Our lead candidate, "AAV9fyhr," harbors two amino acid alterations across two distinct variable regions with respect to AAV9's CAP gene. These two mutations result in AAV9fyhr's 4-fold higher production yields compared to AAV9 and enhances transduction.

Additional mutations from our screens are currently under assessment for our second generation AAV9fyhr variants. To validate our transduction hits, we developed a multiplexed reporter system to evaluate up to 4 novel AAV capsids compared to AAV9 within the same animal. DNA biodistribution to CNS tissues within NHPs demonstrates 2- to 4- fold higher vector genome delivery of AAV9fyhr. RNA expression in tissue sections via RNA in situ hybridization reveals comparable increased expression levels in the CNS to that of the DNA vector genome biodistribution (i.e. a higher number of transduced cells). In mice, comparable DNA biodistribution is observed to that of the NHP, validating species cross-compatability from small rodent models to larger NHP models. Likewise, we observe comparable RNA expression compared to that of the DNA biodistribution. Capsid systemic circulation in mice reveals that AAV9fyhr has decreased systemic circulation compared to AAV9 up to 72 hours post injection, suggesting that vector is binding more cells compared to AAV9. The additional advantage of our novel multiplexed reporter system is the unique vector design to implement single cell spatial transcriptomics at nanometer resolutions. Assessments of the highest single cell spatial resolution of AAV9's, as well as our engineered capsids, tropism using Vizgen's MERFISH technology are ongoing. Lastly, we shed light on structural rearrangements of AAV9fyhr using Cryo-EM with the highest resolution of an AAV9 derived capsid currently available (~1.86 A). Our findings support translational use of AAV9fyhr in platform gene therapy programs and pave the way towards the generation of novel AAV variants through whole capsid engineering.

1412 Common AAV Capsid Serotypes Better Transduce Resected Glial-type Tumor Tissue, with Feasibility for Engineering Tumor-targeted Gene Therapy Vectors

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Glial-type tumors originate from glial cells within the central nervous system and represent a diverse group of primary brain tumors ranging from low-grade gliomas to one of the most aggressive and malignant brain cancers, glioblastoma multiforme. These tumors account for a significant proportion of brain cancers and current treatment approaches include a combination of surgical resection, radiation therapy, and chemotherapy. However, despite these interventions, the prognosis for glial-type tumors remains poor, particularly for glioblastoma, due to the tumors' infiltrative growth and resistance to standard therapies, highlighting the urgent need for novel therapeutic strategies. The emerging field of gene therapy holds promise as a viable treatment with adeno-associated viruses (AAVs) being the most promising vector candidate. AAVs offer a safe and efficient platform with minimal immunogenicity while having the potential capacity for cell-type-specific targeted delivery of therapeutic genes. However, we still lack the ability to target specific cell types in the human brain, including pathogenic tumor cells. To develop viable AAV gene therapy vectors that efficiently yet selectively target human brain tumor cells, we have adapted a human organotypic tissue culture model to culture resected human brain tumor tissues for two weeks. During this time, we transduce the tissue with 14 common AAV capsid serotypes (AAV1, AAV2, AAV5, AAV6, AAV7, AAV8, AAV9, DJ8, DJ, AAV2-retro, PHP.s, PHP.eB, sch9, and rh10) and allow time for transgene expression to occur. The in vivo transduction profile of these serotypes has been well established within various animal models (including mice, rats, dogs, and non-human primates) but it has been noted that these transduction profiles often poorly correlate between species. Using our model, we can generate the transduction profiles using the cells we care most about for therapeutics: human brain tumor cells. We use immunostaining methods alongside single nuclei RNA sequencing to establish the cell types each AAV capsid serotype is able to transduce. Our findings indicate that most of the 14 common AAV capsid serotypes are able to transduce glial-type tumor tissue (including ependymomas, oligodendrogliomas, astrocytomas, low-grade gliomas, and glioblastomas) better than non-glial-type tumors like medulloblastomas and atypical teratoid rhabdoid tumors (ATRTs), as well as normal-like resected brain tissue. In glioblastoma tissues, using immunofluorescence, capsid serotypes PHP.s (49%), AAV2-retro (37.6%), and PHP.eB (37%) show the highest transduction rates on average, though this varies by patient sample. Single-nucleus RNA sequencing of a patient glioblastoma sample transduced with a combined equimolar AAV library shows the greatest number of cells with identified transcripts from DJ, AAV6, AAV2-retro, and PHP.s. Having shown that the organotypic tissue culture model is a viable assay of AAV transduction of tumor tissue, we will assess the transduction of novel AAV capsid variants in order to identify highly efficient and tumor-targeted variants.

1413 Shuffling of HVR in AAV capsid reveals a context-dependent role for HVR5 in peripheral tissue detargeting while ensuring CNS targeting

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Adeno-associated virus (AAV) vectors have revolutionized gene therapy, providing an effective platform to treat a wide range of genetic disorders. Their versatility stems from the high capsid diversity of wild type (wt) AAVs and engineered variants. Their ability to transduce non-dividing cells, along with a favorable safety profile, has led to the approval of seven AAV-based drugs. However, challenges remain in optimizing AAV gene therapy, particularly vector distribution, safety, and efficacy. A major challenge of AAV use is the broad vector biodistribution with preferential liver and heart targeting. Achieving therapeutic effects in other organs, such as muscle or central nervous system (CNS), often requires high vector doses increasing the risk of tissue overload. Capsid engineering is key to overcoming such a challenge by improving target specificity and enhancing therapeutic outcomes. In this study, we developed a novel capsid engineering method which uses regions derived from wtAAVs (La Bella et al, 2020) to modify the tropism of an acceptor capsid yielding advantageous biodistribution profile, while maintaining low seroprevalence and high production yield. Acceptor capsids were modified in the hypervariable regions (HVR) corresponding to the looped-out domains of the capsid and thus interacting with host-cell receptors (Gao et al, 2003). The biodistribution analysis of 31 wtAAV2-13 variants in mice revealed a strong muscle and CNS tropism with liver detargeting. However, their high seroprevalence in humans (~70%) limits their use. We hypothesized that transferring AAV2-13 HVRs to a low-seroprevalent capsid, such as AAV8, might enable the transfer of AAV2-13 biodistribution properties while preserving the low acceptor capsid seroprevalence. A structural analysis was performed to compare the VP3 of AAV2-13 variants to AAV8 and the local structural similarity of the HVRs. Despite sequence diversity, all HVRs of AAV2-13 showed high structural homology to AAV8, except for HVR5, which displayed a different loop orientation. Interestingly, HVR5 also emerged as the most variable region among AAV2-13 variants, suggesting a key role for this domain. We generated a collection of 49 shuffled AAV8 mutants confirming that the transfer of selected AAV2-13 HVRs on AAV8 led to increased muscle and CNS transduction with reduced liver targeting. Notably, no impact on AAV8 seroprevalence and production was observed in the mutants. The rational shuffling method was also validated using AAV9 as acceptor capsid showing similar results. Four rationally shuffled capsids were then tested in non-human primates (NHPs) using a novel barcoding method. We identified one HVR5-mutated AAV9 being able to target the NHP brain with efficiency comparable to AAV9, the gold standard for CNS delivery, while significantly reducing the off-target of liver (15x), heart (10x), dorsal root ganglion (3x) and muscles (11x). Notably, transferring the same HVR into AAV8 capsid had no effect on the engineered capsid biodistribution. The promising primate data demonstrated that shuffling of wtAAV2-13 HVRs is an effective approach to engineer capsids with high translational potential, unveiling the role of the AAV9 HVR5 in peripheral organ detargeting and CNS targeting. These advancements offer the potential to accelerate the development of more effective and safer AAV-based gene therapies, particularly for treating severe neurological disorders through systemic administration.

1414 A Rationally Designed AAV9 Capsid with Minimal Liver Tropism

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Recombinant adeno-associated virus (AAV) vectors are small, non-enveloped viruses that are unable to replicate in vivo due to the removal of the rep and cap genes. In place of rep and cap, therapeutic transgene sequences can be

loaded into the vector. Thus, AAV vectors represent an ideal gene delivery platform. Further, AAV vectors are generally considered to be safe and minimally immunogenic while providing stable, long-term transgene expression. For these reasons, amongst others, AAV vectors are amongst the most popular gene therapy platforms worldwide. Several AAV serotypes have shown clinical efficacy and are being utilized in over 300 clinical trials that are either recruiting, underway, soon to start recruiting, or completed (ClinicalTrials.gov). This has led to several AAV therapies gaining Food and Drug Administration (FDA) approval. One caveat to systemically administered AAV is that the liver often acts as a 'sponge' for AAV, resulting in high levels of liver transduction. When treating diseases such as Hemophilia, this characteristic is highly desirable as the liver is the target tissue. When treating diseases where extrahepatic tissue is targeted, high levels of liver transduction can result in low therapeutic indices in the target tissues. To combat this, high doses of AAVs are delivered. However, the use of high doses of AAV has resulted in severe adverse side-effects and has even been implicated as playing a possible role in the death of twelve patients in clinical trials (*Hum Gene Ther*. Jul 2020;31, *Hum Gene Ther*. Aug 2020;31, *Hum Gene Ther*. Jul 2020;31, *Human Gene Therapy*. 2022;33). We have argued that a better approach would be to develop AAV capsids with reduced liver tropism. In this work, we have developed a novel AAV9 capsid that shows significant reduction in liver expression as compared to wild-type AAV9 (Fig 1). Further, our new capsid presents lower liver transduction as compared to AAV9-16 or AAV9-HR, the two 'parent' capsids off which our double mutant was designed (Fig 1). Importantly, we show that the reduced liver tropism is mainteined for 5 weeks post vector administration (Fig1). We believe this new capsid will result in delivery of transgene products to extrahepatic tissue, at reduced doses, resulting in improved therapeutic indices.

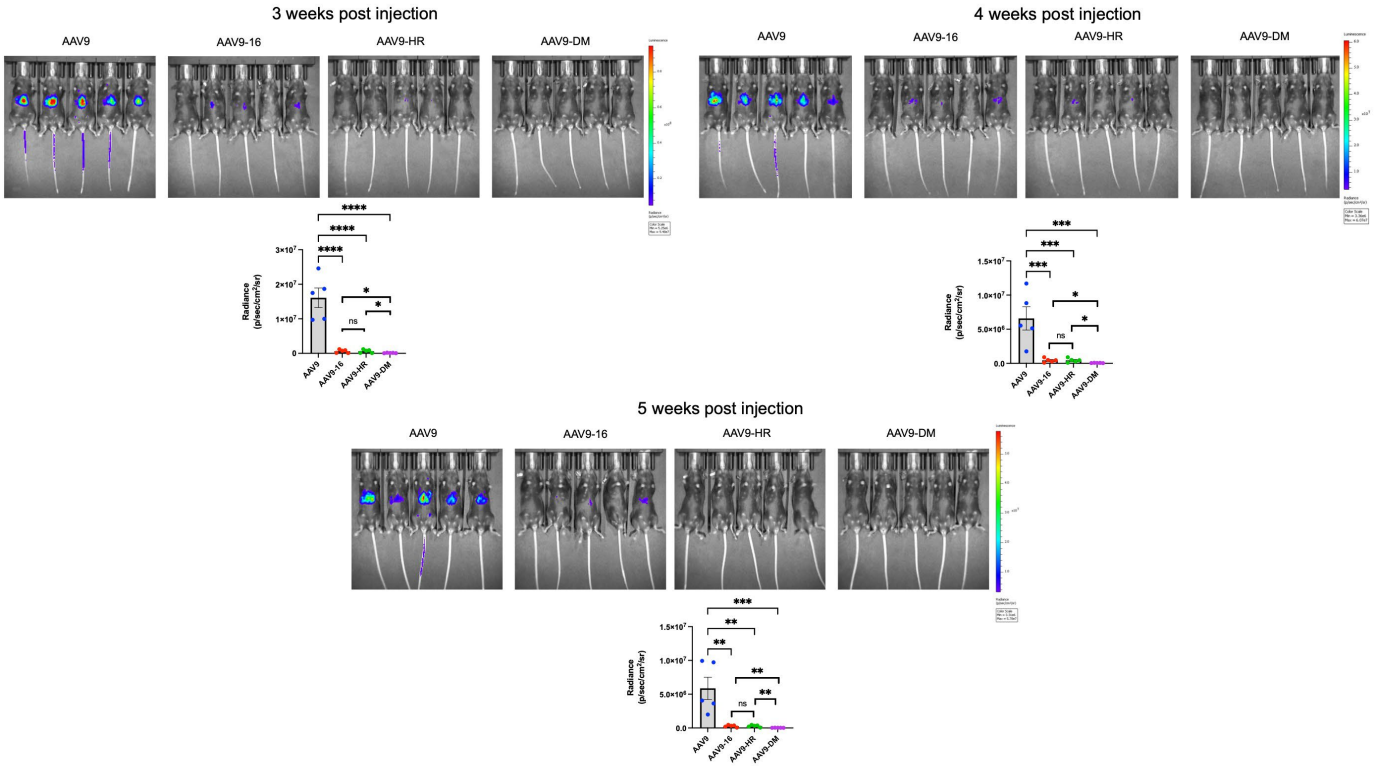

Figure 1: Whole body imaging of AAV capsids. Animals received 1E11vg of either AAV9, AAV9-16, AAV9 HR, or AAV9 DM with firefly luciferase under the control of a CBA promoter. Luciferin was injected subcutaneously, images taken, and bioluminescence quantified at 3, 4, and 5 weeks post AAV administration. The AAV9 DM shows significantly less liver tropism than any of the other capsids tested at all three timepoints.

1415 A single amino acid variant in the variable region I of AAV capsid confers liver detargeting

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AAV capsid serotypes isolated from nature have been widely used in gene delivery and gene therapy. Recently, more than 1,000 distinct AAV capsids were identified from human clinical samples by high-throughput, long-read sequencing (Hsu HL et al. *Nature Communications* 2020). In this study, we tap into this broad natural biodiversity of AAV capsids to develop liver-tropic AAV capsids. We initially screened a subset of variants derived from AAV8 (n=159) for packaging efficiency. The top 30% of these variants were individually used to generate vectors carrying a barcoded transgene. These vectors were then pooled according to their titers to ensure comparable representation in the final vector library. This approach minimized gene delivery bias caused by drastic differences in vector abundance. Following systemic delivery of the vector library to wildtype mice, we evaluated vector DNA abundance and transgene expression in the liver, heart, and tibialis anterior (TA) muscle using next-generation sequencing (NGS). Although no variant surpassed AAV8 for liver targeting, several exhibited a liver detargeting phenotype. Among these, we focused on the N271D variant (AAV8 VP1 numbering), located in the variable region I (VR-1), which has been previously implicated in influencing liver tropism (Cabanes-Creus M et al. *Molecular Therapy Methods & Clinical Development* 2021; Zinn E et al. *Cell Reports Medicine* 2022). To further validate capsid tropism, we generated AAV8 and AAV8.N271D vectors, both packaging the same *EGFP* transgene, and compared their gene delivery and transduction in mice side-by-side. This experiment confirmed that the N271D variant confers liver detargeting: vector DNA and mRNA levels in the liver were reduced by 33-fold and 108-fold, respectively. In contrast, vector DNA and mRNA in the heart were moderately reduced by approximately 3-fold, with no significant changes observed in the TA muscle. We also grafted the N271D variant onto AAV9 and MyoAAV capsids (N270D by AAV9 VP1 numbering), which showed a similar liver-detargeting phenotype, although muscle targeting was moderately reduced. This study reinforces the important role of VR-1 in determining liver tropism and highlights the potential of AAV capsid amino acid variants in modulating tissue-specific delivery for gene therapy. (MX and RX: co-first authors; DW and GG: co-corresponding authors)

1416 Relatively Low Doses of ASP2957 AAV Gene Therapy Demonstrate Efficacy and Safety in a Mouse Model of XLMTM

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X-linked myotubular myopathy (XLMTM) is a congenital disease caused by mutations in the *MTM1* gene that presents with muscle weakness, hypotonia, feeding and breathing difficulties, and in some instances, death. ASP2957 is an investigational gene therapy for the treatment of XLMTM, which has the coding sequence of the *MTM1* gene contained in an engineered muscle-trophic MyoAAV3.8 capsid (Tabebordbar et al., 2021). In this construct, myotubularin is under the control of the muscle-specific promoter MHCK7 and has a transcription enhancing synthetic intron.

ASP2957 was evaluated in a mouse model of XLMTM (*Mtm1* knock-out [KO]) following a single injection at doses of 0 (vehicle), 1E11, 3E11, 2E12, 6E12 and 1.3E13 vg/kg. The observation period was 5 or 10 weeks post-dosing, and experimental endpoints included vector transduction, protein expression, functional efficacy, and safety. There was a dose dependent increase in muscle tissue VCN and myotubularin protein between the 2E12 and 1.3E13 vg/kg doses. Off-target tissue (liver, spleen) protein expression was not observed, suggesting muscle specific expression

via the MHCK7 promoter. After 5 or 10 weeks, 95% (40 of 42) *Mtm1* KO mice treated with vehicle or low doses of ASP2957 (1E11 or 3E11 vg/kg) were found dead or euthanized, likely because of the severe XLMTM-like phenotype, including inability to move, that develops in these mice. Survival rate increased in a dose-dependent manner of ASP2957, as 88% (37 of 42) *Mtm1* KO mice that received ASP2957 at doses of 2E12, 6E12, or 1.3E13 vg/kg survived until their scheduled euthanasia. Consistent with the survival results, *Mtm1* KO mice treated with ASP2957 had body weight gain improvements in a dose-dependent manner, with statistically significant improvements at ≥2E12 vg/kg. The motor function assessment (rotarod test) also showed a significant increase in latency to falling in *Mtm1* KO mice that received 2E12, 6E12 and 1.3E13 vg/kg ASP2957 compared with the few surviving vehicle-treated KO mice. In the open-field test, ASP2957-dosed *Mtm1* KO mice showed a trend towards increased overall activity at doses of 2E12, 6E12 and 1.3E13 vg/kg. Histopathological evaluation of skeletal muscles in vehicle or ASP2957-treated *Mtm1* KO mice revealed various degrees of myofiber hypotrophy, vacuolation and nuclear displacement associated with adipose infiltration, which are hallmarks of myotubular myopathy. The incidence and/or severity of these microscopic findings were generally inversely correlated with ASP2957 dose increases, suggesting improvements or reversal of the *Mtm1* KO phenotype. There were no adverse findings in clinical pathology or histopathology associated with ASP2957, however, mononuclear cell infiltrations in the heart and skeletal muscle were observed, which suggest an immune response to ASP2957.

A dose-response analysis of the rotarod data from *Mtm1* KO mice that received ASP2957, enabled an estimation of a 50% maximal response (ED50) of 2.06E12 vg/kg. This dose range in which robust efficacy was achieved, is several-fold lower compared to previously published reports that utilized AAV vectors to transfer the *MTM1* gene in the same *Mtm1* KO mouse strain (Buj-Bello et al., 2008; Childers et al., 2014). These results demonstrating high potency of ASP2957 in *Mtm1* KO mice are promising in the context of potentially treating XLMTM patients with ASP2957 at a relatively low dose that could achieve efficacy while also reducing safety risks.

1417 Lowering CpG content in rAAV genomes reduces DRG toxicity without compromising therapeutic benefits in mice

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For CNS gene therapy, one major toxicity observed is DRG degeneration in preclinical models. DRG toxicity has been documented for many rAAV platforms, irrespective of the AAV serotype, the therapeutic cassette, or the vector manufacturing method. The root cause of DRG toxicity is not entirely understood. An accumulation of transgene protein causing endoplasmic reticulum stress in rAAV-transduced cells has been suggested as a mechanism. A strong immune response is likely a key driver of DRG toxicity but is regarded as a secondary response to cell damage. CpG depletion or incorporation of TLR-9 inhibitors in the AAV genome can obviate the immune response in animals and enable sustainable transgene expression. Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by progressive degeneration of α-motor neurons, resulting in muscle weakness and atrophy. We previously developed a 2nd-generation AAV9 (2nd-gen) gene therapy expressing a codon-optimized hSMN1 transgene, driven by a native hSMN1 promoter outperforms a benchmark vector that is identical in design to Zolgensma. The 2nd-gen vector utilized the SMN1 promoter, which enables exogenous SMN expression at levels similar to those of the endogenous SMN gene throughout the body. A head-to-head comparison between this 2nd-gen vector and the benchmark vector in SMA mice showed that the 2nd-gen vector had a better safety profile (no increase in liver transaminase levels) and significantly improved efficacy (e.g., lower effective dose, broader therapeutic window, longer life span, and increases in cardiac and motor functions). However, we found hindlimb clasping phenotypes in the wildtype C57/B6 mice injected with the 2nd-generation vector by facial vein at a high dose 3.3E14 vg/kg at P0, but not in the benchmark vector-treated mice. However, H&E staining revealed increased satellite glia cells in DRGs in the 2nd-gen vector-treated mice compared to the PBS-treated animal. Based on the 2nd-gen vector genome, we re-optimized the SMN1 coding sequence to make it CpG-free or depleted of CpGs (CpG-low). Strikingly, no mice (C57/B6 or FVB) that received the CpG-modified vectors showed the hindlimb clasping phenotype after one month of treatment. After 5 months, 10-50% of the animals from these two groups started to show hindlimb clasping phenotype but with less severity. In contrast to the difference in

phenotype from the original 2nd-gen vector and CpG-modified vectors, SMN expression in DRG is abundant and comparable among these three groups, indicating that CpG modification is beneficial for ameliorating toxicity. We injected these vectors into SMA mice at P1 by ICV injection at 3.3E13 or 1.1E13 vg/kg. The CpG-low vector treatment improved the lifespan of SMA mice as effectively as the 2nd-gen vectors at both doses and more efficiently than the benchmark group. The CpG-low vector performed the best in gaining body weight at the 3.3E13 vg/kg dose and was like the 2nd-gen vector at 1.1E13 vg/kg. The CpG-low vector group also performed the best in the grid test among all the groups, including the 2nd-gen vector. In a 90-day study period, no hindlimb clasping phenotype was observed in the treated SMA mice. Taking together, we conclude that the reduction of CpGs ameliorates DRG toxicity without compromising its therapeutic benefits in SMA gene therapy, holding potential for overcoming DRG toxicity in other AAV-mediated CNS gene therapies. **G.Gao, J.Xie.** Co-corresponding authors.

1418 Comparative Analysis of Transgene Expression of Neurotropic AAV Serotypes and Their Potential in Combination Treatments

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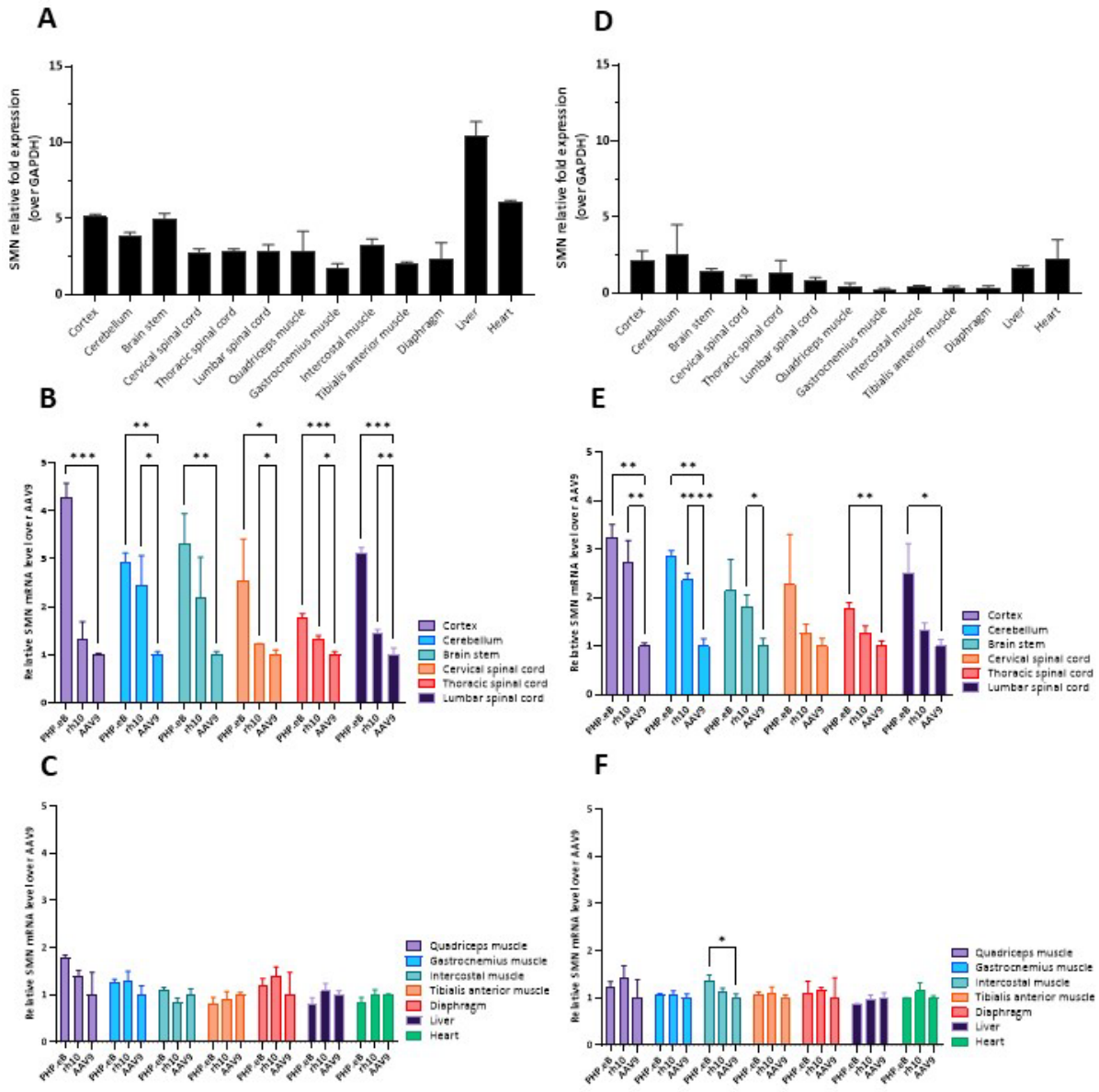
SMN protein have been shown to have a vital role in several cellular pathways. Accordingly, therapeutic approaches should restore the SMN expression not only in central nervous system (CNS) tissues but also in peripheral tissues. Adeno-associated virus (AAV) vectors have revolutionized gene therapy due to their ability to efficiently transduce cells in both the CNS and peripheral tissues. Among the diverse AAV serotypes, AAV9, AAVrh10, and AAV-PHP.eB exhibit considerable tropism toward CNS tissues, making them promising for treating neuromuscular diseases such as spinal muscular atrophy (SMA), however, nonspecific off-target transduction of these vectors results in their side effects. Combinitional therapies using multiple AAV serotypes through different administration routes, while reducing their doses might be an innovative strategy to mitigate their toxicities meanwhile improving the specific on-target therapeutic effects. A comparative analysis of these serotypes can guide the AAV combinational therapy for SMA.

Newborn C57BL/6J mice received intravenous injections of self-complementary AAV (scAAV) vectors (AAV9, AAVrh10, and AAV-PHP.eB) encoding codon-optimized survival motor neuron (coSMN) gene at a dose of 5×10^{11} vector genomes per mouse at P1 (n= 8 per group). Tissue-specific transgene expression was assessed at both 4 and 24 weeks post-injection across various organs, including the brain, spinal cord, skeletal muscles, liver, and heart. SMN mRNA levels were analyzed using qPCR. At 4 weeks post-injection, mice treated with scAAV9-hcoSMN showed the highest SMN mRNA expression in the liver and heart, followed by CNS tissues and muscles (Figure 1A). RT-PCR analysis revealed significantly higher SMN mRNA levels in the cortex, cerebellum, and spinal cord of mice treated with scAAV-PHP.eB and scAAVrh10 compared to scAAV9 (Figure 1B). Results showed no significant differences in peripheral tissues across groups (Figure 1C). At 24 weeks post-injection, SMN mRNA expression remained high in the CNS, liver, and heart (Figure 1D). However, some aforementioned differences diminished over time (Figure 1E). AAV PHP.eB-treated mice exhibited higher SMN mRNA levels in the intercostal muscle compared to AAV9-treated mice, while no significant differences were found in other peripheral tissues (Figure 1F). AAV-PHP.eB demonstrated superior transduction efficiency within CNS tissues, achieving significantly higher SMN expression in the brain and spinal cord compared to AAV9 and AAVrh10. This serotype achieved widespread neuronal transduction, highlighting its potential for CNS-specific applications. AAV9 and AAVrh10 displayed an intermediate transduction pattern, with moderate efficiency across both CNS and peripheral tissues.

AAV-PHP.eB is the most promising vector for CNS-targeted gene therapy due to its robust and sustained transgene expression in the brain and spinal cord. Given the inability of PHP.eB for passing BBB in NHPs (and possibly in humans), using this serotype (with lower doses) through intrathecal or intracerebroventricular routes, would provide the adequate expression in CNS tissues. In combination, lower doses of other serotypes can be used for necessary systemic gene expression, as needed in SMA. This combinational approach helps minimize the toxicity of viral gene therapies in comparison with the intravenous injection that needs administration of high doses of vectors to achieve sufficient CNS expression.



1419 Short-Term Hurdles, Long-Term Promise: AAV9-SMN1 Gene Therapy for SMA

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Introduction: Spinal muscular atrophy (SMA) is a severe neuromuscular disorder caused by mutations in the SMN1 gene, leading to motor neuron degeneration and progressive muscle atrophy. Gene therapy using adeno-associated virus serotype 9 (AAV9) has shown promise in restoring SMN protein expression and improving clinical outcomes. However, concerns about hepatotoxicity and cardiotoxicity following vector administration necessitate comprehensive long-term safety and efficacy evaluations. We hypothesize that overcoming the acute toxicity phase is critical and that, post-recovery, the long-term safety and sustained therapeutic effects of AAV9-based therapies

warrants thorough assessment. This study investigates the safety and efficacy of an AAV9 vector expressing codon-optimized human SMN1 (AAV9-coSMN) in neonatal mice to 24 weeks.

Methods: Neonatal mice were randomly assigned to treatment (n=10) and control (n=10) groups. The treatment group received a single intravenous injection of AAV9-coSMN at 5×10^{11} vector genomes (vg) per mouse at P1, while controls received phosphate-buffered saline (PBS). Clinical parameters, including body weight, well-being (general appearance, skin and fur, eyes, nose, mouth, respiration, urine, feces, and locomotor symptoms), and survival were monitored. Safety assessments included hematological and biochemical analyses, necropsy, and histopathological examination of major organs. Efficacy was evaluated via western blotting to measure SMN expression in key tissues, including the central nervous system (CNS), skeletal muscles, heart, and liver.

Results: AAV9-hcoSMN treatment resulted in a 100% survival rate with no observable adverse clinical signs to 24 weeks. Treated mice exhibited normal growth trajectories, with no significant differences in weight gain compared to controls (Figure 1A). Hematological and biochemical findings remained within normal ranges. In particular, platelet count, ALT, AST, and CK-MB showed no significant differences with the control group (Figure 1B). Necropsy and histopathological examination of the brain, spinal cord, liver, heart, lungs, and skeletal muscles showed no signs of inflammation, fibrosis, or pathological changes. Efficacy analysis demonstrated robust sustained SMN expression, with the highest levels observed in CNS tissues, followed by the heart, liver, and skeletal muscles. (Figure 1C).

Conclusion: This study provides strong preclinical evidence supporting the long-term safety and efficacy of AAV9-coSMN gene therapy for SMA. This 6-month period in mice lifespan is equivalent to approximately 30 years in humans, which could be consider a long-term evaluation. The absence of adverse clinical and pathological findings, combined with sustained transgene expression in target tissues, highlights the potential of AAV9-mediated SMN1 delivery as a viable treatment option.

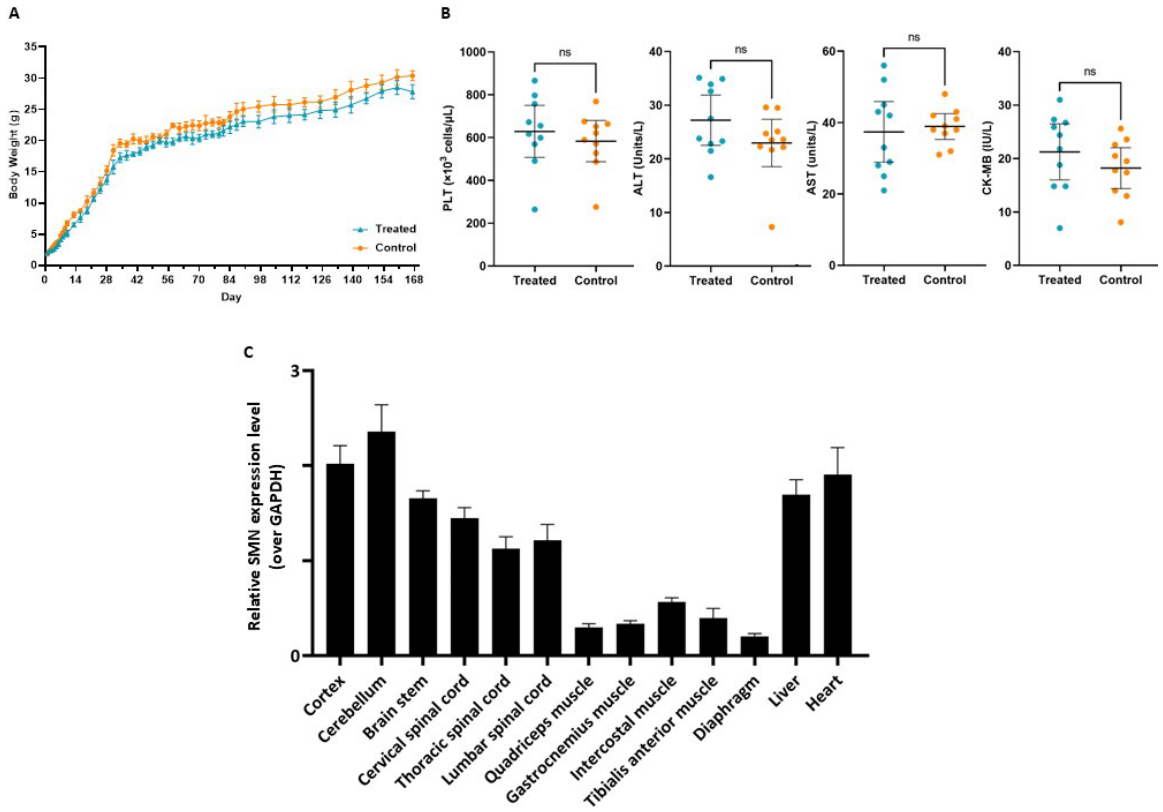

Figure 1- Long-term safety and efficacy of AAV9-coSMN (A) Trend of weight changes over a 24-week follow-up period in two groups. (B) Comparison of laboratory findings between two groups at 24 weeks post-injection. (C) Relative SMN expression (over GAPDH) in various tissues at 24 weeks post-injection.

1420 Evaluating a "silence and replace" approach for development of an AAV gene therapy for Spastic Paraplegia type 4

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Hereditary spastic paraplegia (HSP) is an umbrella of neurodegenerative disorders characterized by weakness and stiffness of muscles in the lower extremities along with other central nervous system impairments. There are more than 80 types of HSPs each caused by mutations in different genes. Spastic Paraplegia type 4 (SPG4) is the most common form of HSP and it is caused by mutations in the *SPAST* gene encoding for spastin. Spastin is a microtubule severing enzyme that is crucial in cytoskeletal regulation, its loss of function can lead to the degeneration of axons and dysfunction in neuronal maintenance throughout the corticospinal tract. This leads to a spectrum of disease phenotypes, termed pure and complex SPG4. Pure SPG4 is more common and presents with lower limb weakness and minimal neurological impairments. Complex SPG4 affects the overall central nervous system more severely, presenting with ataxia, spasticity, epilepsy and other neurological phenotypes. Onset of SPG4 varies by age, childhood onset symptoms can be seen early, progressing to a more severe form of the disease, or late adulthood presenting with slower progression and milder symptoms. Updated prevalence and incidence data remains largely incomplete as HSP is considered a rare condition, affecting around 3.6 per 100,000 cases worldwide. While SPG4 is not considered a fatal diagnosis, its gradual advancement significantly impacts patients' quality of life.

Currently, there is no available treatment for SPG4. Our lab has designed a variety of dual function AAV vectors to silence the endogenous Spastin alleles using an artificial microRNA, while simultaneously expressing normal Spastin from a cDNA where codons in the seed sequence of the miRNA target are modified to make it resistant to silencing. To ensure that expression levels of Spastin remain close to standard physiological levels, we are testing numerous promoters to drive transgene expression. We have achieved successful knockdown of endogenous Spastin and expression of our transgene at both transcript and protein levels. AAV vectors are currently being validated in patient fibroblasts prior to initiating efficacy studies in SPG4 animal models.

1421 Validation of a Göttingen Minipig Gene Therapy Biodistribution Assay for AVGN7 Using Droplet Digital Polymerase Chain Reaction

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Göttingen Minipigs are rapidly gaining popularity as a large animal non-rodent model in preclinical gene therapy studies. Regulatory agencies require biodistribution and pharmacokinetic studies to support IND enabling studies. Molecular biology techniques are utilized to quantitate gene therapy products where traditionally "real-time" qPCR platforms have been used. To increase sensitivity and accuracy, we developed and validated a droplet digital PCR (ddPCR) duplex assay to quantify AVGN7, an AAV gene therapeutic featuring a codon-optimized human *Smad7* gene, and *RPP30*, a reference gene, in Göttingen minipig tissues and blood. The validation parameters included precision and accuracy, limit of detection (LOD), ruggedness/robustness, dilutional linearity, stability, selectivity and recovery. Accuracy and precision were demonstrated across the dynamic range of 5-25,000 cp/µL, producing an inter-assay total error of ≤35.5% for *Smad7* and ≤2.6% for *RPP30*. The assay was sensitive to 10 cp/µg DNA with an LOD of 0.125 cp/µl. Ruggedness was assessed over time using multiple analysts, instruments and reagent lots, demonstrating high reliability and reproducibility. Linearity was established across 1,000-fold dilutions and stability was demonstrated up to 6 freeze-thaw cycles at -20°C and 24 hours at room temperature. Selectivity and recovery were demonstrated by spiking samples after or before nucleic acid extraction, respectively, using multiple lots of different tissue matrices and whole blood. These data not only validate the assay for tracking and quantifying AVGN7 in preclinical studies with Göttingen minipigs, but additionally address challenges of using pig models in general while providing a procedural road map for developing other AAV biodistribution assays.

1422 In Situ Biodistribution and Localization of Bidridistrogene Xeboparvovec (SRP-9003) in LGMD2E/R4 Mice After 1 Year of Follow-up

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Introduction: Limb-girdle muscular dystrophy type 2E/R4 (LGMD2E/R4) is caused by mutations in the β-sarcoglycan (*SGCB*) gene, resulting in loss of functional SGCB protein, progressive muscle degeneration, and shortened lifespan. Bidridistrogene xeboparvovec (SRP-9003, rAAVrh74.MHCK7.hSGCB) is an investigational adeno-associated virus (AAV)-based gene therapy designed to restore functional SGCB expression in muscle and improve clinical outcomes in patients with LGMD2E/R4. RNAscope™ has emerged as a technology capable of determining tropism, transduction efficiency, and in situ localization of targeted gene therapies. We report the use of RNAscope™ to elucidate the in situ spatial biodistribution, cellular tropism, transgene expression, and durability of SRP-9003 following intravenous (IV) administration in LGMD2E/R4 mice.

Methods: Mouse tissues were assessed on necropsy at day (D) 1, 14, 28, 60, 120, 240, and 365 after single IV administration of SRP-9003 (7.41 × 1013 vg per kilogram [vg/kg]). Two RNAscope™ in situ hybridization probes were designed to detect either the AAVrh74-vector DNA or hSGCB transgene messenger RNA (mRNA). Digital droplet polymerase chain reaction (ddPCR) (copies per nucleus) was used to quantify vector DNA, and hSGCB

mRNA was measured using reverse transcription (RT)-ddPCR. Immunofluorescence (IF) was used to assess hSGCB protein expression (as percent beta-sarcoglycan–positive fibers [%PβPF] quantified with HALO® Muscle Fiber FL algorithm).

Results: We previously demonstrated maintenance of SRP-9003 vector DNA and transgene mRNA up to 120 days following systemic administration in LGMD2E mice. Here, we determine SRP-9003 vector durability up to 1 year. Following IV administration, SRP-9003 vector DNA levels (ddPCR) and mRNA expression (RT-ddPCR) were maintained through D365 in the heart and skeletal muscle, indicating vector durability over extended periods in the LGMD2E/R4 mice. In off-target organs such as liver and kidney, detectable SRP-9003 vector DNA and RNA were also present up to D365. In skeletal muscle (tibialis anterior [TA]), vector DNA signal (RNAscope™) gradually declined from D1 to D240, but transgene mRNA signal was stable between D14 and D240. In the heart, vector DNA signal gradually declined from D1 to D120 but stabilized at D240 and D365. A durable transgene mRNA signal was achieved in the heart by D14 and was maintained to D365 post injection. No RNAscope™ mRNA signal was detected in the kidney over the course of the study. In congruence with durable transgene mRNA signal, SGCB protein expression (%PβPF by IF) was maximized at D240 and remained elevated up to D365 in the TA. These data demonstrate targeted SGCB protein restitution via SRP-9003 is maintained up to 1 year to provide long-term efficacy in LGMD2E/R4 mice.

Conclusions: This study demonstrates the applicability of RNAscope™ to determine spatial, cellular organization, and molecular trafficking of SRP-9003. The results indicate the durability of SRP-9003 to generate stable *SGCB* mRNA and protein expression over extended periods of up to 1 year in a mouse model of LGMD2E/R4. These data may be useful to inform future application of RNAscope™ for biodistribution evaluation of AAVrh74 gene therapy.

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1423 CSF Delivery of AAV9/SLC52A2 is Effective for the Treatment of RTD2 in a Novel Mouse Model

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Riboflavin transporter deficiency type 2 (RTD2, OMIM 614707) is a rare neurodegenerative disorder, caused by biallelic pathogenic variants in the *SLC52A2* gene, which encodes the riboflavin transporter RFVT2. It is characterized by muscle weakness, vision loss, deafness, sensory impairments, and respiratory failure. High-dose riboflavin supplementation slows the progression of functional disability and stabilizes or improves function in certain domains. Since 2011, more than 200 patients have been reported.

We have developed new mouse models for RTD2, carrying the p.Leu317Pro and p.Leu344Pro alleles (corresponding to human clinical variants p.Leu312Pro and p.Leu339Pro, respectively). Among reported RTD2 patients, 32% have at least one of these corresponding mutations. Compound heterozygous ($Slc52a^{(L317P/L344P)}$) mice are late embryonic lethal under standard husbandry breeding conditions. Water supplementation with high dose flavin mononucleotide during gestation allows $Slc52a^{(L317P/L344P)}$ pups to be born and survive into adulthood. $Slc52a^{(L317P/L344P)}$ adults exhibit growth impairment, progressive muscle weakness, motor coordination deficits, and 40-50% mortality by 8 weeks of age, thus providing a good time window for intervention.

To assess whether gene augmentation therapy is a potential treatment for RTD2, we developed a rAAV9 vector carrying a codon-optimized human SLC52A2 cDNA. Neonatal administration via intrathecal injection of rAAV9/SLC52A resulted in improved survival, with 100 % of $Slc52a^{(L317P/L344P)}$ mice alive more than 26 weeks post-injection. Additionally, the treatment rescued the growth defects. The $Slc52a^{(L317P/L344P)}$ mice receiving the gene therapy performed indistinguishable from wild type mice on motor and muscle strength tests. The preliminary data

from this proof-of-concept study supports the use of gene augmentation therapy for the treatment of RTD2 and sets the base for further preclinical studies.

1424 AAV-Delivered Anti-PC-OxPL Antibody Fragments as a Novel Therapeutic Approach to Target ALS

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Amyotrophic lateral sclerosis (ALS) is characterized by the progressive loss of motor neurons and premature death. The limited understanding of the mechanisms underlying selective motor neuron death has significantly hindered the development of disease-modifying treatments. Ferroptosis, a form of cell death dependent on iron accumulation, has been implicated in the selective degeneration of motor neurons in ALS. Oxidized phosphatidylcholines (PC-OxPL) have been identified as key effectors in the pathophysiological processes associated with this pathway. Preclinical observations revealed a distinct PC-OxPL profile in the cerebrospinal fluid (CSF) of sporadic ALS (sALS) patients and identified apolipoprotein E (APOE) particles as the primary carriers of PC-OxPL in the CSF. Furthermore, in ALS brain and spinal cord tissue sections, PC-OxPL was found to be predominantly associated with motor neurons. Exposure of iPSC-derived motor neurons to PC-OxPL led to transcriptomic changes in genes known to be linked to ALS, as well as the induction of significant TDP-43 pathology and motor neuron death. To counter this, we developed a single-chain antibody fragment (scFv) encoded by an AAV-delivered DNA construct that specifically targets PC-OxPL neoepitopes (PC-OxPL-VecTab). PC-OxPL-VecTab effectively neutralized PC-OxPL-induced neurotoxicity and TDP-43 aggregation in motor neurons, while preventing motor neuron death and deficits in a sALS CSF mouse model. When administered intrathecally to minipigs, PC-OxPL-VecTab was distributed to both upper and lower motor neurons and expressed at levels predicted to be therapeutically effective. Our work identifies PC-OxPL as a critical pathological factor and a key inducer of TDP-43 pathology in ALS, providing the foundation for a novel therapeutic intervention modality for patients with sALS. Furthermore, it offers the exciting potential to be expanded to diseases characterized by PC-OxPL neurotoxicity.

1425 Immunomodulation Approaches to Improve Safety and Efficiency of Gene Therapy in Friedreich's Ataxia

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Friedreich's Ataxia (FA) is the most common form of hereditary ataxia. It is caused by an autosomal recessive inheritance of GAA (acidalpha-glucosidase) repeat expansions in the first intron of the Frataxin (FXN) gene, resulting in reduced levels of mitochondrial protein. This mutation leads to accumulated iron and decreased activity of iron-sulfur cluster enzymes necessary for energy production. Deficient Frataxin causes cell dysfunction, leading to eventual cell death in cardiac cells and the central nervous system. FA is a systemic disorder with hallmark features including cardiomyopathy (the primary cause of mortality), gait ataxia and other central nervous system manifestations. FXN gene replacement is a promising therapeutic strategy across disease-relevant tissues, preventing progression or reversing the course of the disease. However, host immune response to the viral vector remains a critical challenge. The goal of this study is to evaluate the safest and most effective immunomodulation (IM) strategies in animals with pre-existing immunity to AAV to enable safe and effective dosing with AAV. In this study, six-week-old C57BL6 mice were pre-immunized with a 25 µg systemic dose of AAV9 empty capsid to induce a 5,000U/mL response. Four weeks after eliciting an immune response, different IM regimens were

administered to reduce humoral and cellular responses to AAV9. Six groups receiving different IM regimens were compared to a no-IM control. The groups comprised of 1) rituximab + rapamycin, 2) rituximab + bortezomib, 3) bortezomib, 4) rituximab + daratumumab, 5) daratumumab and 6) Vyvgart. Rituximab was administered biweekly at an 8mg/kg dose through intraperitoneal (IP) injection, rapamycin daily at 4mg/kg orally, bortezomib twice weekly at a 0.75mg/kg IP dose and daratumumab weekly at a 16mg/kg IP dose. Serum and plasma was collected from bleeds performed before AAV9 empty capsid administration (day 0) and before immunomodulation (day 28). Sera was used to quantify circulating anti-AAV9 Tab through ELISA, and plasma was used for flow cytometry analysis using a panel consisting of anti-CD3, CD19 and CD45 antibodies to quantify B and T cells. At day 70, mice were euthanized and the serum and spleen were collected for quantification of circulating anti-AAV9 TAb through ELISA, and quantification of B and T cells through flow cytometry, respectively. ELISA data shows a significant decrease in circulating anti-AAV9 Tab in groups 1 ($p = 0.0111$), 2 ($p = 0.0006$), and 6 ($p = 0.0260$). These groups had a decrease of at least 60% between IM and endpoint compared to the no-IM group, which showed an average decrease of 20%. Flow cytometry data shows a complete depletion of CD19+ cells in groups receiving rituximab, a B-cell depletor, compared to groups receiving bortezomib, daratumumab or Vyvgart alone and the no-IM control, which showed no change in CD19+ cells between days 28 and 70. This study achieves the reversal of AAV pre-immunity using different IM regimens including the anti-CD20 antibody, rituximab, as seen by a decrease in circulating AAV9 Tab and B cells. This could expand AAV-based gene therapy clinical trial inclusion to FA patients currently non-eligible due to pre-existing immunity. Future efforts will aim to identify the most effective IM regimen that leads to higher FXN expression after a systemic dose of AAV. This phase will follow the same layout as described above, receiving a selected dose of AAV9-Des-Endo7-FXN at day 70.

1426 Intracisternal AAV9-MAG-hABCD1 Vector Reverses Motor Deficits in Adult Adrenomyeloneuropathy Mice.

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Thousands of male patients who carry *ABCD1* mutations develop adrenomyeloneuropathy (AMN) in mid adulthood, a debilitating axonopathy affecting ascending and descending tracts of the spinal cord. *Abcd1-/-* mice mimic human AMN. In gene therapy experiments using AAV vectors, the choice of the promoter determines which cells will express the therapeutic transgene. Our strategy against AMN was to use the MAG promoter to target oligodendrocytes (OL) in spinal cord, brainstem and cerebellum. We selected OL as target cells because they provide myelin and energetic fuels to the long axons of the ascending and descending tracts. We previously showed that an AAV9-MAG-*hABCD1* vector injected iv to p10 *ABCD1-/-* mice was able to prevent the disease up to 24 mo of age. In the current study, the same vector was injected intracisternally to *Abcd1-/-* mice at 18 mos of age, when about half of these mice start showing motor deficits. As soon as 1-3 mo after vector injection, motor tests evolved differently in treated and untreated mice, so that at 24 mo of age, motor performances deteriorated in 34 untreated mice were near normal in 24 matched treated mice ($p \leq 0.001$). In the white matter of the cervical spinal cord, expression of hALDP coded by the *hABCD1* transgene at 24 mo of age was still present in 22% (18-27) of OL and 22% of astrocytes (A). hALDP expression was also abundant in OL and A in brainstem or cerebellum, less abundant in lower spinal cord, absent from microglia or neurons in DRG or motor cortex. The fact that motor deficits were rapidly stabilized or reversed despite a long course of the disease suggests that the expression of MAG-driven h*ALDP* in OL peroxisomes could durably improve metabolic exchanges with long spinal cord axons. The current results pave a novel OL-targeting way for the AAV gene therapy of AMN myelopathy in its early clinical stages.

1427 Leveraging host cell modulators of adeno-associated vectors transduction across serotypes to detarget liver

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Recent clinical successes in gene therapy using adeno-associated vectors (AAV) are offering a hopeful path towards the correction of several monogenic disorders. Those achievements fundamentally depend on the efficient delivery of the vector genome and expression of a therapeutic gene into target cells, a complex process that remains incompletely understood. At its core, AAV gene delivery relies on multiple interactions between the capsid and host cell factors, from the initial docking of viral particles onto the cell membrane to the uncoating and viral genome stabilization within the nucleus. Whether different serotypes necessarily hijack the intracellular machinery in a similar manner to transduce a specific cell type and whether those processes are conserved across species for a specific serotype is not known. To identify key host factors either enabling or preventing transduction, whole-genome CRISPR/Cas9 screens were undertaken in mouse (AML12) and human (Huh-7) cell lines using either AAV serotype 2 or 9. While those results certainly confirmed previous reports in identifying key common entry factors such as AAVR or GPR108, our dataset also highlights the potential role of other host cell modulators. Interestingly, while serotype-specific differences were observed for the host cell factors mediating AAV2 or AAV9 transduction in a specific cell line, species-specific divergence was even more striking for each serotype. Leveraging those data, we developed a novel approach to detarget liver transduction by transiently downregulating the expression of key entry factor in this tissue using GalNac-siRNAs prior AAV9 administration. Such manipulation not only blunted hepatic transduction but also re-directed the vector to other transduction-permissive tissues.

1428 Immunocompetent Liver-on-a-Chip Model for Preclinical Evaluation of AAV-Based Gene Therapies

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Gene therapies using recombinant adeno-associated virus (rAAV) vectors require comprehensive preclinical models to assess safety, efficacy, and immunogenicity. Current in vitro systems often lack the complexity to replicate the liver's microenvironment, including vascular structures and innate immune components, critical for accurate toxicological evaluation. The purpose of this study was to develop a scalable, immunocompetent 3D liver sinusoid model in the OrganoPlate(r) Graft platform that enables detailed analyses of rAAV transduction, tropism, and immune response. The OrganoPlate Graft, a 64-chip microfluidic platform in a 384-well plate format, was used to develop a liver-on-a-chip model. The culture chamber was seeded with liver endothelial cells, hepatic stellate cells, and iPSC-derived or primary hepatocytes in a fibrin hydrogel. Gravity-driven perfusion supported vascular bed formation, while monocyte-derived macrophages enabled Kupffer-like cell differentiation. To test AAV tropism, rAAV serotypes with fluorescent reporters were applied at 400K GC/cell for 72 hours, with observations over 8 days. The impact of rAAV9-mCherry was assessed for toxicity (LDH release) and inflammation (IL8 release). Embedding of liver bulk non-parenchymal cells (NPCs) in the hydrogel inside of the OrganoPlate yielded extensive vascular networks visible by VE-Cadherin staining after 10 days. Confocal microscopy confirmed the presence of lumenized microvasculature supported by stellate cells. Tricultures involving hepatocytes, LDECs, and HSCs formed organized liver microstructures after 7 days, with CD31 and LYVE-1 staining indicating vascular development. Proximity between hepatocytes and vascular structures was validated through live Calcein-AM imaging, and hepatocyte polarization was confirmed by MRP-2 staining. Kupffer-like cells were successfully incorporated, maintaining a macrophage phenotype across 11 days, aligning with liver endothelium and distributing uniformly through the Z plane of the culture. TThe vascular networks allowed perfusion, enabling rAAV introduction via simulated bloodstream and assessment of transduction. Differential reporter expression was noted between vascular and direct application. This dual delivery method offers insights into delivery route effects. Serotype screening revealed specific transduction efficiencies, with rAAV9-mCherry showing dose-dependent responses quantified using an ImageJ workflow. Toxicity assays revealed transient LDH release at 72 hours post-exposure to rAAV, which normalized after removal of rAAVs. Notably, IL8 release increased significantly in cultures containing Kupffer-like cells, indicating an immune response enhanced by the presence of immune cells. The data underscored the relevance of including macrophage components to capture immune-mediated effects accurately. The immunocompetent liver-on-a-chip offers a scalable platform for rAAV gene therapy studies,

replicating liver architecture and enabling evaluation of tropism, transduction, and immunogenicity. Macrophage-like cells enhance its ability to simulate immune responses, supporting toxicology and preclinical optimization, thus improving the translation of gene therapies to clinical use.

1429 AAV Gene Therapy for LMNA Associated Laminopathies

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Background: Laminopathies are a phenotypically diverse group of diseases caused by dominantly inherited mutations in the *LMNA* gene. *LMNA* encodes Lamin A and C, two intermediate filament proteins of the nuclear lamina formed through alternative splicing. Laminopathies are a broad group of 13 identified diseases that present with a range of clinical phenotypes, severity, and age of onset. Our focus is a severe *LMNA*-associated congenital muscular dystrophy (L-CMD). The challenges in developing an effective AAV gene therapy for L-CMD are the need to silence the dominant negative endogenous alleles and the need for balanced expression of both Lamin A/C isoforms from a single vector. To achieve this, we used a "silence and replace" approach with a dual-function AAV that encodes an artificial microRNA (miRNA) to target endogenous *LMNA* for silencing, and a miRNA-resistant *LMNA* transgene that mimics the natural alternative splicing mechanism to generate both Lamin A/C.

Methods: The miRNAs were designed to target homologous sequences in the mouse and human *LMNA* gene and screened in human HEK293T and mouse Neuro2A cells for silencing efficiency. The transgene was made miRNA-resistant through alternative codon usage in the miRNA target seed sequence. The AAV-LMNA-miR^{LMNA} vector is driven by a muscle specific promoter (MCK7), given that skeletal and cardiac muscle are the target tissues for L-CMD. We tested different vector designs, where the miRNA position within the vector genome (intron or 3'UTR) and the scaffold used was varied, as these factors can impact silencing efficacy and transgene splicing. The goal is to identify an AAV vector design with the most potent silencing of endogenous *LMNA* mRNA, and balanced expression of both Lamin A and C.

Results: We identified a top miRNA that silenced *LMNA* by ~50% in both human and mouse cells. We cloned this miRNA into the AAV-LMNA-miR^{LMNA} in different locations in the vector genome and within the context of different miRNA scaffolds. We tested the plasmids in human and mouse cells to measure silencing of the endogenous *LMNA* allele, and expression of Lamin A/C. We identified AAV candidates that overexpressed both Lamin A/C isoforms compared to naïve and silenced the endogenous allele by ~50% in human cells.

Conclusions: We have identified 4 top vector designs that will be packaged into a muscle specific capsid (Myo4A) for testing in the LMNA$^{R249W/R249W}$ mouse model of L-CMD. This model has a severe phenotype with a shortened lifespan (~6 weeks), reduced body weight and muscle weakness allowing for rapid testing of therapeutic efficacy. The top vector will be chosen based on the most potent silencing of endogenous mouse *Lmna* mRNA and expression of Lamin A/C in skeletal and cardiac muscle. Successful outcomes will be significant extension of lifespan and improved phenotype.

1430 Micro-utrophin Redesign Improves Physiological and Metabolomic Outcomes in Dystrophic *mdx^{4cv}* Mice.

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Introduction: As the most common lethal genetic muscular disorder of children, Duchenne muscular dystrophy (DMD), is caused by mutations in the 2.2 MB DMD gene. Absence of the dystrophin protein from the dystrophin-glycoprotein complex, leads to myofibers being highly susceptible to contraction injury, leading to progressive rounds of degeneration and regeneration. There is no cure, and DMD patients ultimately succumb to the disease via cardiorespiratory failure. Utrophin (Utr), a dystrophin paralog, has long been suggested to be a potential therapeutic for DMD. We previously demonstrated morphological and functional deficits of a 1st generation μUtr (ΔR4-R21/ΔCT, here defined (μUtr1) to protect from contractile injury and resultant degeneration in the *mdx*^{4cv} mouse model of DMD. The μUtr redesigns included spectrin-like repeat (R) variation, a utrophin-syntrophin binding domain, plus endowing unique dystrophin functionality via neuronal nitric oxide synthase sarcolemma localization.

Method: We evaluated 4 novel micro-utrophins, with the original μUtr1, and micro-dystrophin (μDys5) as controls. Two-week-old *mdx*^{4cv} mice were intravenously administered 5x10^{11} vector genomes of recombinant adeno-associated viral vector (rAAV2/9myo1) harboring human codon-optimized micro-transgene(s), driven by the creatine kinase regulatory cassette (CK8e). At ~8 months of age, lower limb muscles were assayed *in situ* and *in vivo* for protection from contraction-induced injury. After functional testing, animals were sacrificed, and tissues were processed for histology, molecular assays, and metabolomics.

Results: Following rAAV2/9myo1 vector delivery, body-wide striated muscle transduction was revealed by immunofluorescence microscopy. A leading 6-R μUtr variant emerged (μUtr2) based on physiological improvements in resistance to contraction-induced injury and various pathological indices. As evidence, following an *in situ* lengthening-contraction protocol for tibialis anterior, net-injury recovery force yielded 83.34% in wt, 25.94% in *mdx*^{4cv}, and 63.91% in μUtr2 treated *mdx*^{4cv} mice while μUtr1 and μDys5 were 43.39% and 59.87%, respectively. In the contralateral limb we performed *in vivo* (foot pedal) experiments to monitor contractile performance with fatigue, outcomes were similar with μUtr2 showing the most significant improvement. We also performed univariate and pathway analysis of ~200 targeted metabolites. Interestingly, relative to wt, the *mdx*^{4cv} demonstrated 156.97 and 221.14 fold change increase(s) in hippuric acid, for heart and gastrocnemius respectively, which were significantly reversed with either μDys5 or μUtr2 treatment. Tricarboxylic acid (TCA) cycle intermediates were also significantly decreased in *mdx*^{4cv} hearts compared to wt, which were increased in μUtr2 and μDys5 treated *mdx*^{4cv} along with several genes controlling glucose metabolism. Lastly, volcano analysis showed no significant differences in metabolites for μDys5-vs-μUtr2 treated cohorts.

Conclusion: The μUtr2 variant resulted in metabolic and physiological gains towards alleviating symptoms associated with disease progression in the *mdx*^{4cv} mouse model, and may hold promise as a treatment for DMD. Gene delivery of functional utrophin-centric proteins could avoid adverse events that recently occurred in human clinical trials against dystrophin sequences, potentially providing an alternative therapy for some patients not meeting exclusion criteria due to these risks.

1431 Hepatic-Restricted Microdystrophin Expression Induces Immune Tolerance and Enhances Transgene Expression in Dystrophin-Deficient *Mdx* Mice

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Objective: This study evaluates the efficacy and immunological response of hepatic-restricted AAV9-LSP-μDys9.2 and non-hepatic AAV9-Des-μDys9.2 microdystrophin vectors in *mdx* mice under immunosuppressive treatment.

Background: Duchenne muscular dystrophy (DMD) is a rare X-linked disorder caused by DMD gene mutations, leading to dystrophin deficiency, muscle damage, weakness, and eventual cardiac and respiratory failure. Gene therapy involving AAV-mediated delivery of microdystrophin shows promise, but is hindered by adverse immune responses to the vector and/or transgene. This study evaluates whether hepatic-restricted microdystrophin expression (AAV-LSP-μDys9.2), combined with immunosuppression (IMS), enhances immune tolerance and therapeutic outcomes in mdx mice. μDys9.2 differs from PF-06939926 and SRP-9001 by including the nNOS binding domain, and from SGT-001 by including R3 and excluding R23.

Materials and Methods: We synthesized a novel microdystrophin construct (µDys9.2) harboring the desmin (Des) promoter and incorporated it into a single-stranded AAV9 plasmid. µDys9.2 is a 3.7-kb transgene containing key domains from full-length dystrophin, including the actin-binding domain 1 (ABD1), hinge 1, and spectrin repeats R1, R3, R16, and R17, with R16 and R17 forming the nNOS binding domain, critical for muscle physiology. We utilized two vectors: LSP-driven (AAV-LSP-µDys9.2) and desmin-driven (AAV-Des-µDys9.2) microdystrophin. To assess the efficacy and immunological response of these vectors, we used five-week-old male *mdx* mice (C57BL/10ScSn-Dmdmdx/J), divided into four groups of five mice each. The IMS regimen involved three weekly pre-doses of anti-CD20 antibody at 10 mg/kg and oral gavaging with sirolimus at 4 mg/kg three days per week, until week 12. Group 1 received the anti-CD20 regimen, followed by AAV-Des-µDys9.2 i.v. administration (1×10^{14} vg/kg) at eight weeks of age, along with continuous sirolimus dosing until week 12. Group 2 received anti-CD20 before administration of the hepatic-restricted vector AAV-LSP-µDys9.2 (2×10^{13} vg/kg) at eight weeks, followed by AAV-Des-µDys9.2 (1×10^{14} vg/kg) at twelve weeks, with ongoing sirolimus dosing. Group 3 received AAV-Des-µDys9.2 (1×10^{14} vg/kg) at eight weeks without IMS. Group 4 served as a control, receiving no vectors or IMS. We assessed microdystrophin protein expression in the heart, skeletal muscles, brain and liver. Immune responses were evaluated through anti-AAV9 ELISA to measure total antibodies in the serum, and flow cytometry was performed to quantify B cells and T cells across the different groups.

Results: Hepatic-restricted µDys9.2 combined with desmin-driven µDys9.2 delivery under IMS (Group 2) showed superior outcomes compared to AAV-Des-µDys9 alone (Group 3) or with IMS (Group 1). Group 2 exhibited a three-fold increase in µDys9.2 expression in the heart, and a two-fold increase in skeletal muscle compared to Group 3. Additionally, it showed a significant decrease in T and B cell populations at day 22, and an increase in Treg activation as measured by FoxP3+CD25+ cells in the spleen and lymphocytes (Figure 1). Cytotoxic and helper T cells were also reduced in this group. IgG antibody formation against AAV9 was reduced by half at day 22 in Group 2 compared to Group 3.

Conclusions: Hepatic-restricted and nonhepatic µDys9.2 delivery, combined with IMS, enhances transgene expression and immune tolerance in *mdx* mice, improving efficacy and safety.

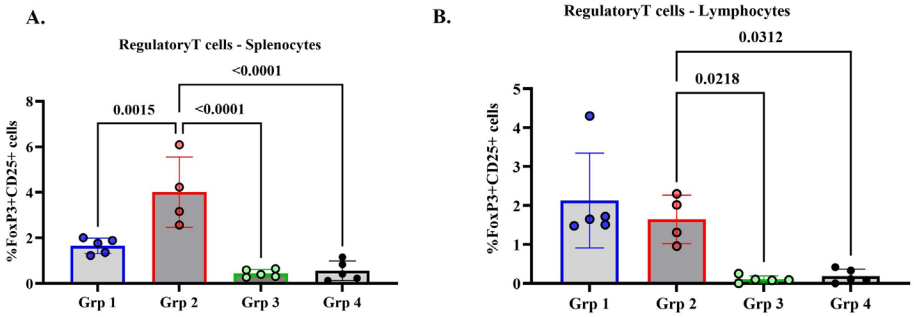

Figure 1: AAV-LSP-µDys9.2 induces systemic tolerance via increased FoxP3+ Tregs. Splenocytes and lymphocytes were stained with anti-CD25–BV421, permeabilized, fixed, and then stained with anti-FoxP3–PE. (A) Percentage of FoxP3+ CD25+ T cells in total splenocytes. (B) Percentage of FoxP3+ CD25+ T cells among lymphocytes isolated from blood. Means and standard error are shown after one-way ANOVA and Tukey's multiple comparison test. p values are shown.

1432 Evaluation of a Novel AAV Capsid with Widespread CNS Biodistribution Following Intra-CSF Dosing in Nonhuman Primates

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Adeno-associated viruses (AAV) have emerged as the preferred delivery vector for neurological gene therapy due to their favorable safety profile and longevity of transgene expression in post-mitotic cells. However, clinical translation of AAV-based gene therapies for neurologic disease has been limited due to the inability of naturally occurring AAVs to broadly transduce the central nervous system (CNS). AAV capsid engineering has enabled the generation of AAV variants with enhanced CNS tropism, transduction efficiency and safety versus naturally occurring AAVs. In this study we report the evaluation of novel capsids administered by minimally invasive intra-CSF infusion in non-human primates (NHPs). In a pooled capsid screen, we identified three capsids, AAV.GMU05, AAV.GMU06, and AAV.GMU09, that demonstrated 1-3 log increases in biodistribution across brain regions compared to benchmark capsid AAV9. Notably, this improvement was CNS-specific, as peripheral tissues including liver and spleen did not show increased vector biodistribution compared to AAV9. In an unpooled validation study, AAV.GMU05 showed superior CNS biodistribution and transgene expression compared to AAV.GMU06 and AAV.GMU09. Of note, AAV.GMU05 showed comparatively low vector genomes in circulating blood immediately post-dosing, suggesting that the increased CNS tropism of AAV.GMU05 may arise from reduced peripheral leakage from the CSF. We further evaluated the spatial distribution and identity of AAV.GMU05-transduced cells across cortical and subcortical brain regions. Transgene expression was observed to follow a largely homogenous distribution pattern, and was detected in neurons, astrocytes, oligodendrocytes, and microglia. Collectively, these data support the continued development of novel capsid AAV.GMU05 for neurological indications. The superior CNS targeting of AAV.GMU05 compared to first- and second-generation capsids can enable efficacious therapeutic delivery to affected regions in neurologic diseases with high unmet need.

1433 Intrathecal delivery of INS1202 AAV9-SOD1-shRNA rescues hallmarks of neurodegeneration in a disease model of ALS and targets glia and neurons in the spinal cord and brain of non-human primates

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INS1202 is an AAV9 shRNA construct targeting human SOD1 that is being evaluated for the potential treatment of amyotrophic lateral sclerosis (ALS). The nonclinical program for INS1202 comprises proof of concept intrathecal route of administration and AAV9 biodistribution in mice and nonhuman primates (NHPs), in-life assessment of muscle function, translational biomarkers and histological evaluation of motor neuron survival and gliosis in the $SOD1^{G93A}$ mouse model of ALS, as well as an ongoing 3-month GLP-toxicology study in NHPs.

Pharmacology: $SOD1^{G93A}$ mice were treated with INS1202 or vehicle via intracerebroventricular (ICV) delivery at postnatal day (p)1 and assessed for disease onset and survival, motor skills and muscle force. Plasma neurofilament levels, a validated predictive biomarker of efficacy, were evaluated at pre-symptomatic and end-stage time points; SOD1 levels, motor neuron numbers and glial activation were analyzed in spinal cords collected at disease end-stage. Treatment with INS1202 in $SOD1^{G93A}$ mice significantly delayed disease onset, as seen by consistent gain and maintenance of body weight throughout the study, compared to the early weight loss seen in mice treated with vehicle control. INS1202 treatment resulted in a significant increase in muscle function in both the rotarod test, which measures motor coordination and endurance, and hind-limb muscle force testing by the *in vivo* Aurora Scientific system. Additionally, $SOD1^{G93A}$ mice treated with INS1202 as compared to those treated with vehicle exhibited significantly reduced levels of plasma neurofilaments, which were similar to levels detected in wildtype mice at both pre-symptomatic (p50) and end stage (p120±10). When vehicle-treated $SOD1^{G93A}$ mice were euthanized at humane end-stage, mice treated with INS1202 were also sacrificed, despite lack of disease symptoms, for histological and protein analyses. INS1202 treatment reduced the levels of both total human SOD1 and misfolded SOD1 by 50-60% in the spinal cord, which resulted in significant rescue of motor neuron numbers, choline acetyltransferase (ChAT) protein expression, ionized calcium-binding adaptor molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP) staining intensity to levels observed in wildtype mice, indicating a complete rescue

of neurodegeneration and glial activation. Taken together, treatment with INS1202 resulted in delayed disease onset, improved muscle function, and amelioration of hallmarks of neurodegeneration in $SOD1^{G93A}$ mice, thus supporting the functional efficacy of INS1202 in a mouse model of ALS.

Pharmacokinetics: Biodistribution studies using ddPCR in both mice and NHPs demonstrated significant vector genome delivery to all segments of the spinal cord, brainstem and motor cortex with limited distribution to other peripheral organs.

Toxicology: A one-month non-GLP toxicology study evaluating in-life parameters, as well as histopathological findings, indicated that INS1202 was generally well tolerated at all doses when administered via intrathecal delivery in NHPs. A 3-month GLP NHP toxicology study is nearing completion to evaluate safety in a larger number of animals, which will provide additional safety and tolerability data.

1434 Assessing the impact of age on AAV9 biodistribution and safety following intravenous administration in infant and juvenile cynomolgus macaques

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AAV9 is used for a number of clinical and commercial stage AAV-based gene therapy products, as well as extensively for preclinical research. Previous studies have shown that AAV9 administrated intravenously can cross the blood brain barrier (BBB) and transduce cells in the brain of non-human primates (Bevan, Mol Ther. 2011). Other studies have also indicated there may be an age-related difference in BBB permeability to AAV9 vectors as well as cell tropism differences, with younger age at dosing associated with a higher degree of neuronal transduction (Foust, Nat Biotechnol. 2009). For an internal program, we tested the hypothesis that the BBB is more permissive to AAV9 at infant age. We performed a direct age comparison by administering 1.1E+14 VG/kg of the AAV9 vector intravenously to approximately 3-month-old (N=3, infant) and 10-month-old (N=2, juvenile) cynomolgus macaques. Animals were selected for dosing based on negative or low seropositivity for anti-AAV9 neutralizing antibodies (NAbs). NAbs analysis was also performed on serum from blood collected just before dosing and at necropsy. After a 6-week in-life period, tissues from multiple brain regions of interest, spinal cord, liver, and muscle were harvested for DNA biodistribution and mRNA expression analysis. Brain, spinal cord, dorsal root ganglia (DRGs), and liver tissue sections were processed for histopathology. *In situ* hybridization (ISH) analysis was also performed to determine % neuronal transduction in various brain regions. DNA biodistribution analysis shows no significant differences between the two age groups for any of the tissues analyzed. Liver shows the highest level of exposure, followed by muscle and spinal cord. The lowest levels of DNA biodistribution were observed in the brain regions tested. Similarly, liver shows the highest transgene mRNA expression levels followed by spinal cord. The lowest levels are observed in the brain. Transgene mRNA expression is nominally higher in CNS and muscle tissues of the infant animals compared to the juvenile animals but is not statistically significant. The ISH analysis shows an overall low neuronal transduction in the brain for all animals, with cortical and subcortical regions of interest showing only 1-6% neuronal transduction. Highest % neuronal transduction are seen in the thalamus. No associations were found between % neuronal transduction and age at dosing. These data do not support the hypothesis that the BBB is more permissive to AAV9 in the 3-month-old infant cynomolgus monkeys compared to 10-month-old juvenile animals tested. This suggests that any changes in AAV9 cell tropism, vector biodistribution, and transduction are minimal during the age range tested. Histopathology analysis shows findings in the liver and DRGs in some animals. The liver histopathology findings consist of single hepatocyte necrosis, bile duct hyperplasia/hypertrophy, mononuclear cell infiltrate within portal areas, and increased cellularity within sinusoids. Interestingly, juveniles were more prone to develop liver lesions, compared to infants, based on severity and incidence of liver findings. In the sacral DRG, there is minimal and focal neuronal necrosis and focal to multifocal mononuclear cell infiltrate in one juvenile and one infant. The incidence and severity of liver findings are higher in

juvenile NHPs compared to those observed in infants. There are no AAV9-related histopathology findings in the brain or spinal cord.

1435 CAP-003, a CNS-Targeted IV-delivered AAV Gene Therapy, Safely Increases Brain GCase in NHPs to Level Supporting Potential Normalization of Activity in PD-GBA Patients

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Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States with no approved disease modifying treatments to date. *GBA1* gene mutations are the most significant risk factor for PD, with current evidence suggesting up to 15% of PD patients may carry mutations. *GBA1* encodes a lysosomal hydrolase enzyme, glucocerebrosidase (GCase), the reduced levels or aberrant function of which is associated with accumulation of alpha synuclein, loss of nigrostriatal dopaminergic neurons and development and spread of aggregated protein inclusions known as Lewy bodies, the pathological hallmarks of PD. CAP-003 is a next-generation gene therapy candidate, comprised of a novel capsid engineered through use of directed evolution and *hGBA1* cargo, that was developed for administration as a single intravenous (IV) infusion to Parkinson's disease patients with *GBA1* mutations (PD-GBA). The CNS-targeting and liver/DRG de-targeting profile of CAP-003 offers the potential to safely and stably supplement wild-type GCase protein throughout the brain, enabling long-term disease modification that could slow or stop the progression of PD-GBA with limited treatment burden.

In a mouse model that exhibits both reduced GCase activity and overexpression of alpha-synuclein in the brain, systemic AAV administration with *hGBA1* cargo resulted in significant and dose-dependent increases in brain GCase protein and activity. This restoration of GCase activity also resulted in significant and dose-dependent decreases in glucosylsphingosine (GluSph), a key GCase substrate shown to contribute to alpha-synuclein pathology. In non-human primates (NHPs), systemic administration of CAP-003 resulted in widespread neuronal transduction of disease relevant brain regions, including the substantia nigra, caudate, and putamen. These levels of transduction show marked improvement over wildtype AAV serotypes and resulted in significantly increased GCase activity that is expected to normalize GCase activity in the PD-GBA patient population. Analysis of clinically relevant biomarkers in treated NHPs showed increases in GCase protein and activity and reductions in GluSph levels. CAP-003 was found to be well tolerated, de-targeted from the liver and DRGs, and lacking adverse clinical pathology, immunogenicity or histopathology findings associated with higher-dose systemic gene therapies. In addition, a novel human receptor for CAP-003 was identified with complete homology between humans and macaques in the predicted binding pocket, thus supporting clinical translatability of CAP-003 performance from NHP to humans. Targeting a 1H 2025 IND filing, CAP-003 is being advanced with the goal of achieving disease modifying clinical benefit for patients with PD-GBA through a convenient single dose intravenous administration.

1436 Focused ultrasound-mediated delivery of a novel engineered AAV capsid to the rat and non-human primate brain using a human clinical FUS system

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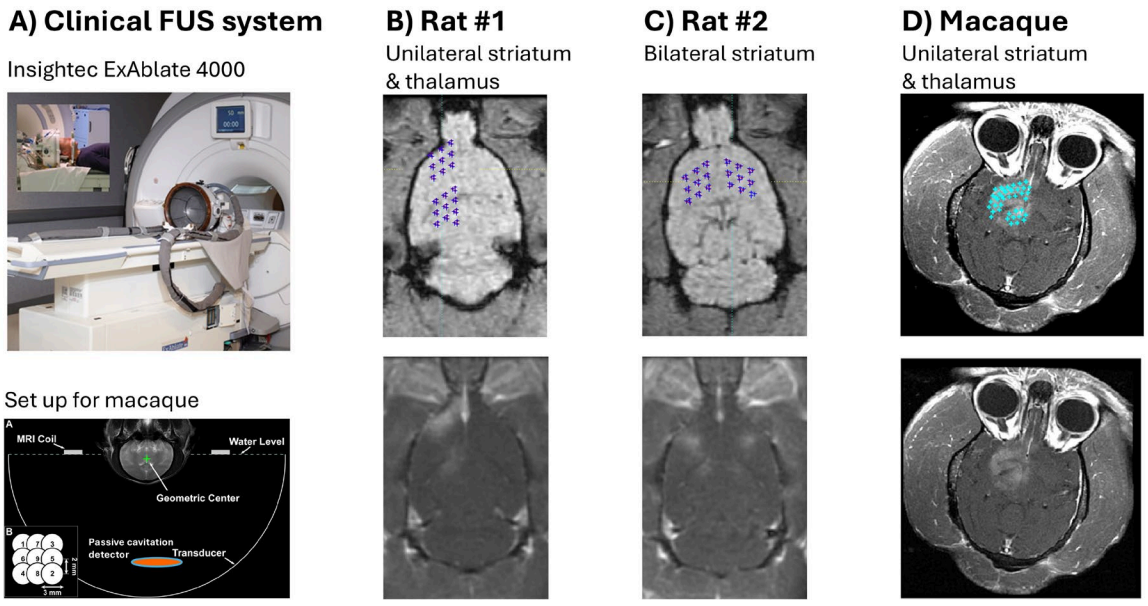
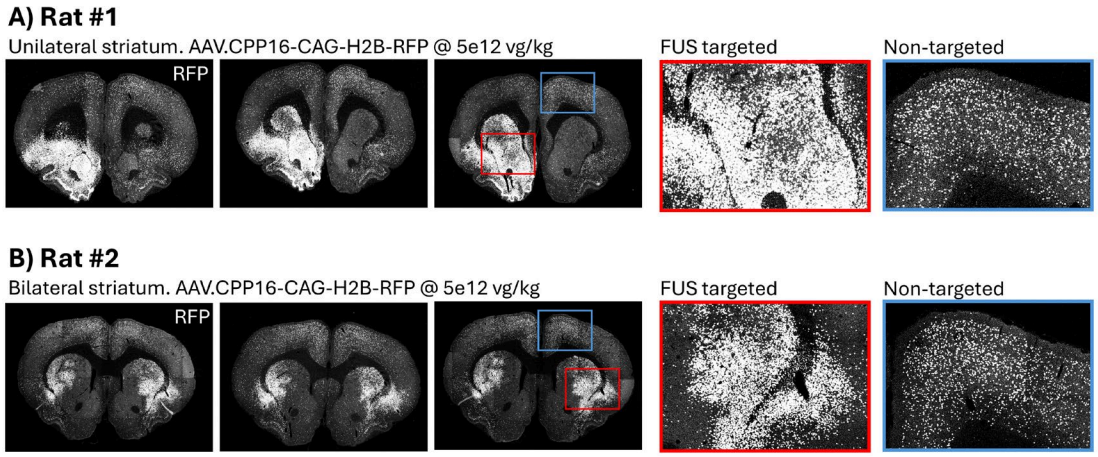
Background: We have previously presented our work at ASGCT showing significantly improved gene therapy delivery to the mouse brain using a genetically engineered adeno-associated virus (AAV) combined with the technology of focused ultrasound (FUS)-mediated disruption of the blood-brain barrier (BBB). The AAV capsid, AAV.CPP16, was engineered for BBB penetrance by inserting a cell penetrating peptide into the receptor-binding domain of the AAV9 capsid. FUS is applied in combination with systemically injected microbubbles to exert mechanical forces on vessel walls that act to increase BBB permeability. Under this approach, we achieved >70% neuronal transduction in FUS-targeted brain regions at a systemic AAV dose of 2e12 vg/kg in mice, 55x lower than the dose for the FDA-approved AAV9 based Zolgensma. The goal now is to validate this FUS + AAV.CPP16 approach in larger animals under a clinically feasible treatment paradigm. Translation challenges include focusing through a thicker skull, electronic beam steering to open the BBB over a larger volume, real-time feedback control based on passive cavitation detection, and regulatory limits on microbubble dose. Here we use a human clinical FUS system for BBB disruption and delivery of AAV.CPP16 in two rats and one macaque non-human primate.

Methods: Two Sprague-Dawley rats (280 g, 258 g) and one macaque non-human primate (10.9 kg) were treated on the Insightec ExAblate 220 kHz FUS system. FUS targeting was done based on MRI imaging and designed to target unilateral or bilateral deep brain structures. 10 ms FUS sonication bursts were applied at 1 Hz repetition frequency over 120 seconds. The FUS focus was electronically steered over up to 32 target locations per sonication set. The FUS intensity was adjusted for each target separately under real-time feedback control based on the strength of the first harmonic signal and an empirically determined goal. After FUS treatment, susceptibility-weighted MRI images were acquired to look for signs of hemorrhage and T1-FSE images were acquired pre- and post-injection of gadolinium to assess BBB opening. AAV.CPP16 with a CAG promoter and H2B for nuclear localization of an RFP reporter gene was injected IV at a dose of 5e12 vg/kg. Rats were sacrificed at three weeks post-treatment for histology; the macaque was just treated and will be sacrificed at three weeks post-treatment for histology.

Results: Figure 1A shows the MRI-compatible clinical FUS system used in this study. Figures 1B – D show the sonication targets and the post-treatment contrast MRI images for the two rats and one macaque. In Rat #1, the right striatum and thalamus were targeted; in Rat #2, bilateral striatum was targeted; in the macaque, the right striatum and thalamus were targeted. BBB opening based on contrast MRI was observed in all three cases, without signs of hemorrhage (not shown). The BBB opening in the rats was more anterior than intended. Figure 2 shows fluorescent imaging of three brain sections for Rat #1 and Rat #2. The nuclear-targeted RFP signal is shown in white. Zoomed in regions show examples of the FUS-targeted striatum and non-targeted cortex. At this low dose, the AAV.CPP16 capsid still shows strong transduction in non FUS-targeted regions. In FUS-targeted areas, the transduction is particularly strong. Next immediate steps will be to perform histology on the recently treated macaque and quantify transduction efficiency in terms of percent neurons transdu





1437 Parkin Gene Therapy for Parkinson's Disease

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Disease-modifying therapies for Parkinson's Disease (PD) are desperately needed. PD patients with a validated genetic cause offer promise for application of precision medicine therapeutics directly targeting the genetic defect. Autosomal recessive PD caused by biallelic loss of the Parkin gene (Parkin-PD) is 100% penetrant and strikes patients as early as the teenage years, yielding life-long disability impacting employment and family-life. NysnoBio is advancing NB001, a recombinant adeno-associated virus (rAAV) gene therapy designed to deliver a functional Parkin gene directly to the substantia nigra of Parkin-PD patients, restoring the missing protein that caused the disease. We have completed nonclinical studies assessing NB001 in human patient fibroblasts, Parkin knock-out (pKO) rats, and non-human primates (NHP). Human patient fibroblasts were used to determine the level of NB001-mediated Parkin expression required to restore Parkin enzymatic activity in cells obtained from Parkin-PD patients. Intra-nigral injection of NB001 in pKO rats revealed the minimum dose required to restore Parkin expression in the substantia nigra to wild-type levels and significantly increase Parkin enzymatic activity. We have also completed non-GLP studies in NHPs to assess biodistribution, target area coverage, and safety following MRI-guided, convection-enhanced delivery of NB001 directly into the substantia nigra. Intra-parenchymal injection of an allometrically scaled dose resulted in NB001 biodistribution that was largely confined to the brain with minimal peripheral exposure. The dose levels tested achieved supraphysiological Parkin expression in the primate substantia nigra that was safe and well tolerated for the entire length of both the 6-week and 12-week studies. The completed nonclinical studies successfully identified the doses, drug delivery devices, and methods for the upcoming GLP-compliant toxicology study. Taken together, these results provide strong rationale for the future clinical translation of NB001 to treat Parkin-PD patients.

1438 Direct Administration of AAV2-Derived Vector Library to the Putamen of Non-Human Primates Identifies Capsid Variants with Enhanced Retrograde Transport Properties

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Adeno-associated virus (AAV) has become a leading vector for gene therapy in the central nervous system due to its ability to transduce brain cells, including mature differentiated neurons, efficiently, durably, and safely. Direct AAV

injection into the brain parenchyma bypasses the blood brain barrier, minimizes transgene expression and immune response in the periphery and results in high neuronal transduction rates at doses that are substantially lower than those required for an intravenous injection. Previous studies reported that AAV2-derived capsids developed using the BRAVE (Barcoded Rational AAV Vector Evolution) approach have enhanced retrograde transport properties in the rodent brain, compared to the parental AAV2 capsid. Retrograde transport enables coverage of connected brain regions after a single focal injection and is therefore a powerful tool for delivery of vectors to distant sites with potentially higher specificity, transduction efficacy and safety. Because transport properties can vary among species, we further characterized a barcoded library of 25 BRAVE-derived AAV2 capsid variants in brains of adult cynomolgus monkeys after intra-putaminal dosing to determine whether these novel capsids retained their properties. Based on RNA and DNA amplicon sequencing, single-nucleus RNA sequencing, and histological assessment, we report here capsid variants with enhanced retrograde transport and biodistribution in the non-human primate brain, which makes them potentially relevant for indications that warrant broader brain coverage beyond the injection site.

1439 Translational Considerations When Comparing Brain Structures in Humans, Cynomolgus Macaques and Adolescent Baboons: Implications for Anatomical Targeting and Drug Delivery

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When developing novel therapeutics for the treatment of neurological disorders, *in vivo* animal studies are essential to assess safety and efficacy before advancing to human clinical trials. Nonhuman primates (NHPs) are the primary models for such studies, with cynomolgus macaques and rhesus macaques being the most commonly used species. However, limitations on the importation and availability of rhesus macaques have led to increased use of cynomolgus macaques, including those as small as 2 kilograms in body weight. These smaller cynomolgus macaques have considerably smaller brains than those of humans, which poses challenges for dose scaling studies and estimations of drug volumes deliverable into brain structures. Using magnetic resonance imaging (MRI), the work herein provides data on the volumetric differences between the brains of cynomolgus macaques and humans. Additionally, it explores the potential of adolescent baboons as an alternative NHP model that addresses the availability issues affecting rhesus and cynomolgus macaques. Several anatomical brain regions were examined including the putamen, thalamus, caudate, substantia nigra, dentate nucleus, and the lateral ventricles. Our findings demonstrate that adolescent baboons offer a closer weight-to-brain size match to humans compared to macaques. This similarity enhances the translatability of surgical and therapeutic interventions, while volumetric data show that the brain structures of adolescent baboons are more comparable to those of humans, which is crucial for accurate dose scaling and effective drug delivery. While cynomolgus macaques remain valuable for neurological research, adolescent baboons present significant advantages in anatomical comparability and practical application for clinical translation of novel therapeutics delivered to the brain. This study supports the consideration of adolescent baboons as a viable alternative model for preclinical neurological studies, which could lead to more accurate and effective therapeutic development.

1440 Potent and Specific AAV9-miRNA Candidates Demonstrate Robust Reduction of Microtubule-associated Protein Tau (*MAPT*) in Non-Human Primates

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Tauopathies, including Alzheimer's disease (AD) and frontotemporal dementia (FTD), are characterized by abnormal Tau protein accumulation, which disrupts neuronal function and contributes to disease progression. Variants in microtubule-associated protein Tau (*MAPT*), the gene encoding Tau, are directly implicated in tauopathies. Tau reduction has emerged as a promising therapeutic strategy for slowing the progression of cognitive decline and is in clinical trials for AD and other Tauopathies. Leveraging Encoded's vector engineering platform, we explored the use of adeno-associated virus 9 (AAV9)-mediated microRNA (miRNA) delivery to knock down *MAPT* expression with a durable, potential one-time therapy. Here, we present the optimization and validation of miRNA candidates to reduce Tau in non-human primates (NHPs). We computationally designed over 7,000 miRNA candidates targeting *MAPT*. Top candidates were selected for further evaluation based on cross-species homology, predicted specificity, and stability. Top miRNAs were evaluated in vitro using human iPSC-derived neurons and in vivo in mice and NHPs. NHPs received a single intracerebroventricular (ICV) injection of AAV9-vectorized miRNA candidates, and *MAPT* knockdown was evaluated via transcript and protein quantification across multiple brain regions. In vitro miRNA screening identified various candidates that achieved up to 96% *MAPT* knockdown in human iPSC-derived GABAergic neurons. The top 4 candidates in vitro were administered to wild-type (WT) mice at postnatal day 1 (PND1) and demonstrated significant, dose-dependent *MAPT* knockdown —achieving over 50% reduction in both transcript and protein levels —with minimal off-target effects, as confirmed by RNA sequencing (RNAseq). In NHPs, a single ICV administration of 3 lead AAV9-miRNA candidates achieved robust *MAPT* reduction across multiple disease-relevant brain regions, with transcript levels decreasing by up to 34% in the cortex and 32% in the hippocampus. These findings provide the first evidence of AAV9-mediated Tau knockdown across large regions of the NHP brain. This study highlights the potential of Encoded's vector engineering platform to develop potent, specific, and durable miRNA-based therapies for central nervous system (CNS) disorders. Robust *MAPT* knockdown achieved broadly in the NHP brain underscores the translational promise of this approach for treating tauopathies such as AD and FTD.

1441 Alternate Reporter Genes for Safe & Durable CNS Delivery of AAV Vectors in Non-Human Primates

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Fluorescent reporter genes are a valuable tool for analyzing adeno-associated virus (AAV) capsid transduction *in vivo*; however, they can result in toxicities that lead to undesired in-life takedown timepoints and potentially underrepresent capsid capabilities. Toxicities are most notable when fluorescent reporter genes, such as green fluorescence protein (GFP), are delivered to the central nervous system (CNS). A head-to-head comparison of a fluorescent reporter gene to novel, less toxic reporter genes is a clear gap within the field. Identification of less toxic reporter genes for the evaluation of AAV and other modalities in CNS would allow full characterization of emerging therapies *in vivo* while maximizing animal use.

This study compares three reporter genes, enhanced green fluorescent protein (EGFP), cynomolgus macaque sodium iodide symporter (CynoNIS), and human alpha galactosidase (hAGA) delivered by a proprietary AAV capsid to cynomolgus monkeys via the intracisternal magna (ICM) route of administration at a single dose. An empty capsid and vehicle arm were included as controls. Animals (n=3 per treatment group) were monitored for 56 days, followed by necropsy and tissue harvesting and processing. Toxicity was measured by clinical observations, clinical and histopathology, anti-drug antibody responses, and cerebrospinal fluid cytokine and biomarker profiling. DNA and RNA were assessed by digital droplet PCR and *in situ* hybridization, and protein was assayed by ELISA and immunofluorescence.

All animals in the EGFP cohort showed neurological toxicities on day 11, were found moribund on day 12 and euthanized. Animals in the other cohorts, CynoNIS, hAGA, and controls exhibited no lasting clinical observations of note, and survived to the scheduled end point of 56 days. This study identifies NIS and AGA as safe and durable reporter gene options to use in the CNS to assess transduction of AAV and support product development.

1442 Rationally designed chimeric AAV capsids demonstrate reduced liver tropism and enhanced muscle transduction in both rodents and non-human primates

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Recombinant adeno-associated viral (rAAV) vectors have emerged as essential tools for gene delivery in therapeutic applications. However, their clinical efficacy is often hindered by insufficient tissue-specific transduction and undesirable off-target effects, particularly liver toxicity. To address these issues, we developed a two-step AAV capsid engineering strategy aimed at reducing liver tropism and enhancing target-specific transduction.

Initially, we engineered AAV2 by loop swapping with AAV9, yielding three novel capsid variants: AAV.Zero1, AAV.Zero2, and AAV.Zero3. Among them, AAV.Zero1 and AAV.Zero3 exhibited a significant reduction in liver transduction while maintaining a low transduction background in various rodent tissues. Building on AAV.Zero3, we further optimized muscle tropism by inserting an RGD-containing peptide, creating PG007, a capsid with enhanced muscle-specific targeting in both rodents and non-human primates.

To evaluate PG007's therapeutic potential, we compared its performance to AAV9, which is under clinical investigation for Duchenne muscular dystrophy (DMD). Systemic administration of a micro-dystrophin gene (2E+13 vg/kg) in dystrophin-deficient (DMD-KO) mice showed that PG007 significantly improved muscle strength, reduced CK and AST levels, and lowered liver and muscle damage compared to AAV9. PG007-treated mice also exhibited broader micro-dystrophin expression in muscles and lower off-target transduction in the liver and kidney.

Immunofluorescence confirmed enhanced dystrophin restoration in skeletal and cardiac muscles of PG007-treated mice. Over a 10-month period, PG007 increased survival rates significantly compared to both AAV9 and vehicle-treated controls.

Our study underscores PG007's therapeutic efficacy and its potential as a next-generation capsid for muscle-targeted gene therapy. Additionally, AAV.Zero1 and AAV.Zero3 serve as versatile backbones for tissue-specific targeting. The π-Icosa serotype screening platform provides a robust pipeline for the rapid development of optimized AAV vectors across various tissues and species, accelerating human gene therapy advancements.

1443 A Comparative Study of Full Adeno-Associated Virus 8 (AAV) Capsid Enrichment Platforms

Shaofeng Wang[1], Jack Ren[2], Mukesh Mayani[3], Patrick Hossler[1], James Warren[4]
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Introduction: The production of therapeutic AAV vectors typically results in a mixture of full, partially full (partial), and empty capsids, with the latter two forms potentially reducing product efficacy and posing safety risks to patients. Minimizing these unwanted species remains a key challenge in AAV-based gene therapy production. Ultragenyx is a gene therapy company with both early- and late-stage assets, spanning various serotypes, administration routes, and target tissues/organs. With over a decade of research in AAV purification, we have evaluated the strengths and limitations of various capsid enrichment technologies including Anion Exchange Chromatography (AEX), iodixanol-based step density gradient ultracentrifugation (IDXUC), and CsCl-based density gradient equilibration (DGEUC). This work will further explore these insights using AAV8 vectors designed for the treatment of a rare genetic disorder.

Methods: The AAV8 affinity pool material was generated in-house and processed through AEX chromatography, IDXUC, and DGEUC, respectively (**Figure 1**). Buffer exchange was performed four times against formulation buffer using a spin column with a 100 kDa cutoff for the AEX eluate, IDXUC harvest, and CsCl DGEUC harvest. Genomic copies (GC) of the gene of interest (GOI) in the load and the three processed harvests were quantified

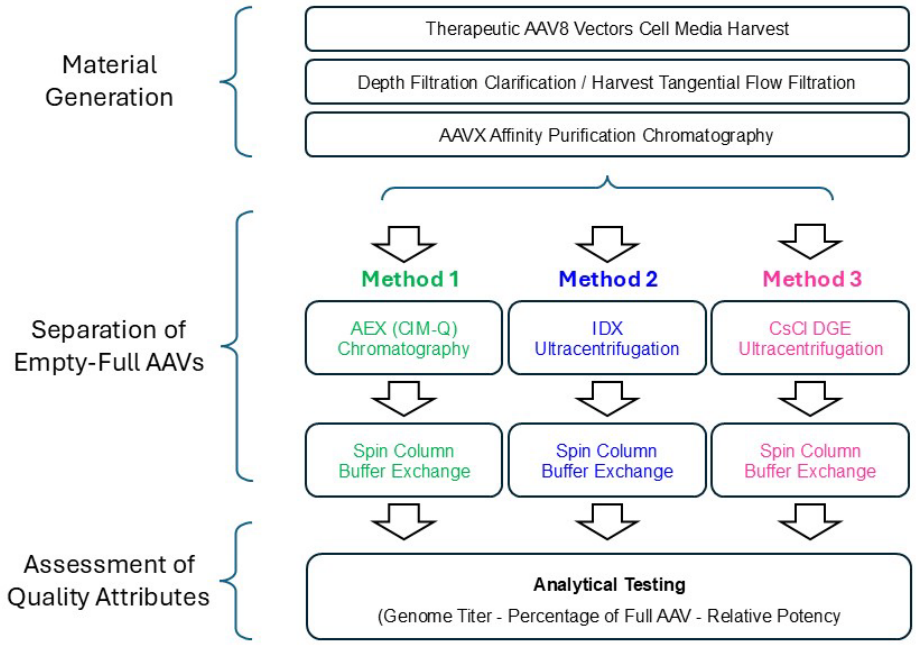
using quantitative PCR. Full, partial, and empty AAV species were assessed by analytical ultracentrifugation (AUC). The relative potency of the AAV vectors was determined through a cell-based assay by measuring the expression of the protein encoded by GOI using a fluorescence-labeled specific antibody.

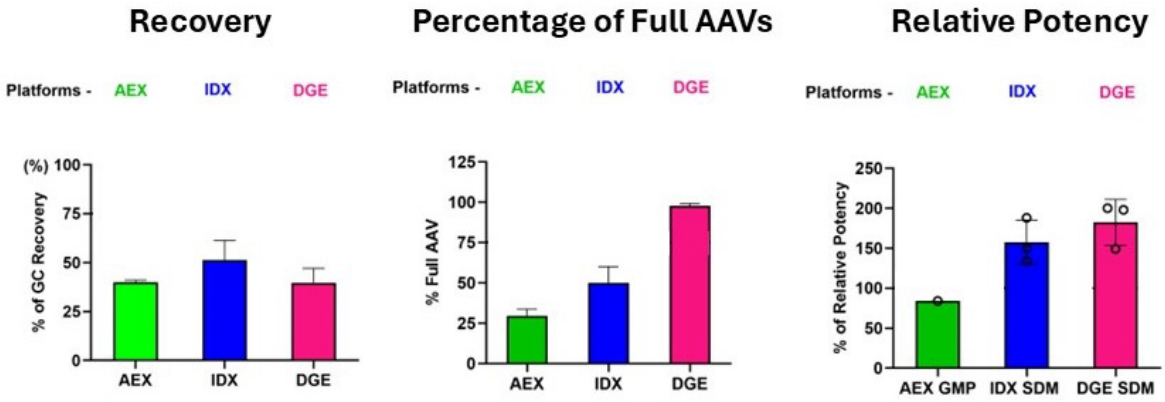
Results: Quantitative PCR analysis in this study revealed comparable GC recovery across all three platforms, with recovery rates ranging from 40% to 50%. DGEUC achieved the highest percentage of full AAV in the harvest (96 - 99%), followed by IDXUC (55 - 65%), and AEX Chromatography (25-32%). The relative potency of harvests from the three platforms were normalized to a reference standard sample by comparing protein expression levels in cells. DGEUC showed a relative potency of 175%, IDXUC exhibited 160%, and AEX demonstrated 87%, respectively (**Figure 2**).

Conclusions: In this study, AEX, IDXUC, and DGEUC demonstrated comparable GC recoveries. The IDXUC and DGEUC showed a better capability in separating empty, partial, and full AAV8 capsids. The observed differences in relative potency are likely attributed to the distinct empty-partial-full profiles of AAV vectors produced by each separation method. Since these platforms rely on different principles for AAV species separation, they may recover varying populations of AAV vectors, which in turn impacts the relative potency of the final harvests.

References

Adeno-associated virus vector as a platform for gene therapy delivery. Dan Wang, et. al, Nat Rev Drug Discovery. 2019 May.





1444 Ensure Accurate Density Gradients Through a Novel Automation Platform – the OptiMATE Gradient Maker

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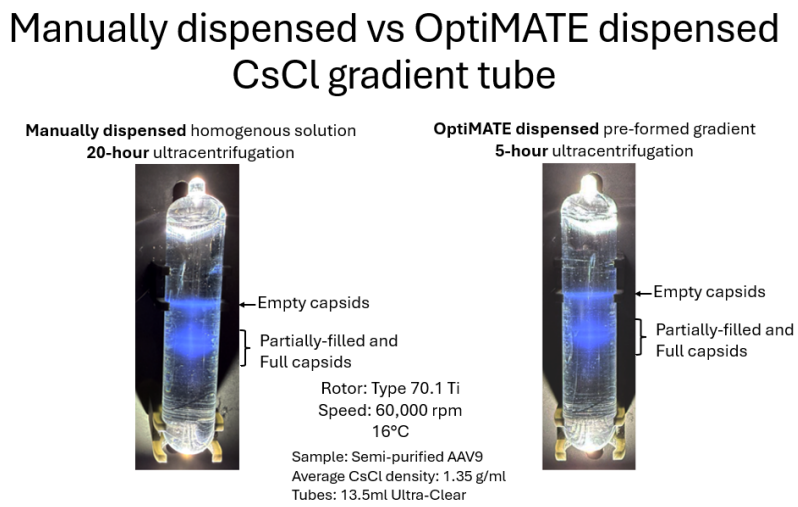
Density gradient ultracentrifugation (DGUC) allows for high-quality separation of empty, partial, and filled Adeno-Associated Virus (AAV) capsids. While DGUC protocols are effective across serotypes and engineered variants of AAV, filling the tubes can be labor-intensive and user-dependent, while the centrifuge run time can be lengthy (>16 hours for self-forming cesium chloride (CsCl) density gradients). The OptiMATE Gradient Maker* from Beckman Coulter Life Sciences allows for automated dispensing of easy-to-program density gradients (step, linear, or a combination of both). It is compatible with a variety of gradient forming materials, rotors and tube types. The OptiMATE Gradient Maker also provides automated sealing of Quick-Seal tubes. This significantly minimizes labor and variability without compromising purification efficiency. Furthermore, the OptiMATE Gradient Maker can dispense accurate linear gradients with AAV sample incorporation. By dispensing a pre-formed gradient with AAV, CsCl separations can now be completed in 5 hours or less (a run-time reduction of 70% or more compared to current protocols). Overall, the OptiMATE Gradient Maker provides plug-and-play operation to generate ultracentrifuge-ready tubes, making high purity AAV purification simpler and more accessible.

*Product in development. Performance characteristics have not been validated.



1445 Large-scale purification of highly effective recombinant adeno-associated virus with a zonal ultracentrifugation

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Background: Recombinant adeno-associated viruses (rAAV) are powerful tools for gene therapy. However, systemic high-dose injections of rAAV have caused severe adverse events (SAEs), including acute liver and kidney injury, cardiopulmonary insufficiency, and death. To decrease these risk, empty and incomplete genome rAAV should be removed as much as possible by purification, and input samples should include as many full capsids as possible. We developed a method to purify rAAV in a short time (4 hours), on a large scale (900 mL), and with high purity (>90% full capsids) by using zonal ultracentrifugation with cesium chloride (CsCl). To increase the ratio of full/empty capsids and decrease the capsids containing, only incomplete genomes or plasmid backbones (including drug resistant gene), we also developed a gene of interest (GOI) plasmid for rAAV that includes a stuffer sequence. The size of the backbone including stuffer sequence is over the limit size for packaging of rAAV (4.7 kb).

Methods: The rAAV-ZsGreen1 was harvested from culture supernatant without cell lysis 3 to 10 days after plasmid transfection. Large-volume rAAV-containing culture media (up to 900 mL) and escalating densities (2 or 4 steps) of CsCl solutions were sequentially added to the zonal rotor. Following large-scale density-gradient ultracentrifugation, the samples were automatically fractionated. The samples were then evaluated for rAAV genome copies by quantitative PCR, for rAAV capsid protein amounts by western blotting, and for ZsGreen1 transduction efficiency by flow cytometry. The ratio of full/empty capsids was measured by mass photometry, analytical ultracentrifugation (AUC), and transmission electron microscopy (TEM) with phosphotungstic acid stain. The genomic DNA regions packaged in rAAV particles were detected by digital PCR (dPCR) using primers and probes targeting the whole vector genome.

Results: We improved 2-step CsCl density-gradient ultracentrifugation with a zonal rotor, allowing for a large volume (900 mL) of rAAV purification as well as a shorter (4 hours) ultracentrifugation with a steeper density-gradient in the range of the rAAV fractions, as compared to the conventional method of 4-step density-gradient (300 mL) for 10 hours. High-purity separation of full and empty fractions was demonstrated with genome, capsids, and biological titrations. These full and empty fractions were also analyzed by mass photometry, AUC, and TEM, demonstrating a high rate (>90%) of full capsids that could be separated with our upgraded method. The full rAAV ratio produced by transfection with plasmids including stuffer sequences increased compared to the one with conventional plasmids. In rAAV produced with stuffer plasmids, the mispackaging ratio of the backbone sequence of plasmid, including the drug-resistant gene, decreased, indicating that the risk of rAAV toxicity was reduced.

Conclusion: We upgraded a 2-step density-gradient ultracentrifugation method with a zonal rotor to 4 hours, allowing for the large-scale purification of functional full-genome rAAV. We also improved the plasmids, including stuffer sequences to increase the ratio of full capsids. Our large-scale rAAV purification method would apply to in vivo experimentation and clinical investigation. Furthermore, we designed a safety cabinet for zonal ultracentrifugation, a seamless multiple-closed bag to produce viral vectors, and plan to prepare cleaning validation and documentation.

1446 Development of a Full Capsid Enrichment Polishing Step in AAV8 Purification Process With CIMmultus® PrimaT® Monolithic Column

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Gene therapies treatments based on AAV vectors are promising for rare diseases. However, while producing AAV vectors for these gene therapies, empty capsids are also produced in a certain amount. Clinical effect of empty capsids is unclear. Empty capsids are not the intended therapeutic product and could include a toxicological effect. Thus, process development teams need to reduce their presence. This objective could be achieved by designing new capsids and transgenes, by changing cell culture conditions or by implementing a polishing step on current purification process. The latter approach is evaluated using the CIMmultus® PrimaT® monolithic column developed by Sartorius. Parameters including viral loading, residence time, conductivity of loading and elution are investigated. Based on the generated data, optimal conditions are determined to enrich the product in full capsids while maintained an acceptable viral genome yield. These conditions are subsequently applied up to 200 L scale. Results obtained confirm the performance, repeatability, and scalability of CIMmultus® PrimaT® monolithic column to remove empty capsids and some product-related impurities.

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Development of a Full Capsid Enrichment Step in the Purification Process of an AAV8 Product by the CIMmultus® PrimaT Monolith

R Rojas Gonzalez[1], D Dulac[1], C Luc[2], G Livet[2], R Fragnoud[1], G Rouby[2], P Santambien[1,2], E Attebi[1], F GASTON[1]
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Background and Objectives

Gene therapy treatments are promising for treating rare genetic diseases, particularly at Genethon, with a focus on muscular dystrophies and metabolic disorders. However, during the production of AAV vectors for these therapies, a certain amount of empty capsids is also generated, and their clinical impact remains unclear. CIMmultus® PrimaT monolithic column was evaluated for its ability to enrich the product in full capsids. Key parameters such as viral loading, residence time, and loading/elution conductivity were tested on an AAV8 product. Optimal conditions for full capsid enrichment and viral genome recovery were identified and successfully scaled up to 200L.

1. Introduction - CIMmultus® PrimaT monolithic column technology

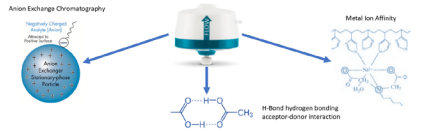

Fig. 1: CIMmultus® PrimaT technology overview.

CIMmultus® PrimaT is a mixed-mode monolithic column based on anion exchange, hydrogen bonding interactions, and metal ion affinity. It uses the physical differences between full and empty capsids to separate the two populations.

2. Method

The AAV8 polishing step was developed using a 1 mL PrimaT column on an AKTA pure 150 system. The product, previously clarified and purified by an affinity chromatography, was diluted X-fold before achieving low conductivity to ensure optimal capsid binding. Elution was performed using a conductivity gradient with a divalent salt. The resulting profile is as follows:

A first peak is obtained, containing primarily full capsids, followed by a second peak with mainly empty capsids and other contaminants. To optimize this separation, various elution volumes, residence times, dilutions, and loadings were tested.

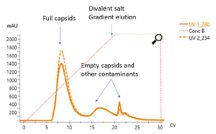

Fig. 2: Chromatogram profile of the PrimaT monolith

The viral genome yield is measured by qPCR and HPLC, while the ratio of full capsids is determined using HPLC (SEC-MALS) and AUC.

3.1 Results - Impact of viral loading

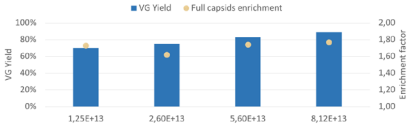

Fig. 3: Viral loading (VG/mL of monolith) evaluated on a 1 mL PrimaT

All viral loadings resulted in similar viral genome (VG) yields, ranging from 70% to 89%, and full capsid enrichment, ranging from 1.62 to 1.77. The selected loading corresponds to a 400L AAV8 production on an 80mL PrimaT monolith.

3.2 Impact of elution volume

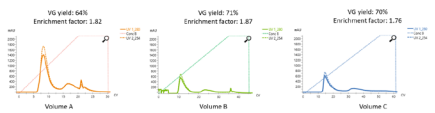

Fig. 4: Chromatography profiles obtained on a 1 mL PrimaT with different elution volumes

Increasing the elution volume further separates the different populations; however, there is no improvement in either the viral genome yield or the enrichment factor.

3.3 Impact of residence time and product dilution

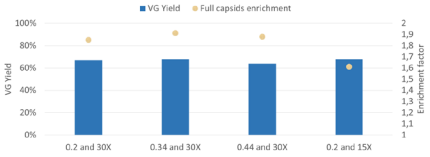

Fig.5: Residence time (min) and dilution impact on a 1mL PrimaT

There is no impact of residence time on the values tested. However, dilution appears to slightly affect the full capsid enrichment. Nevertheless, a 1.6 enrichment factor is still considered as correct.

3.4 Inter and intra batch repeatability

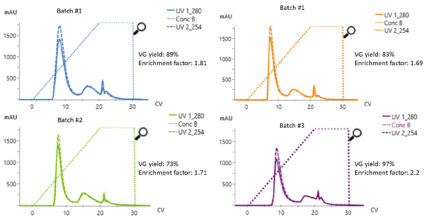

Fig.6: Chromatography profiles obtained on PrimaT 1 mL for the inter and intra batch repeatability

On the 1 mL PrimaT, similar chromatography profiles were obtained across four tests. The viral genome (VG) yield is approximately 80%, with a full capsid enrichment of around 1.8.

3.5 Scale up: 50L and 200L

Conditions developed at a scale of 1 mL were tested on 8 mL and 40 mL monolith scales.

Prima T volume	VG yield[1]	Full capsides enrichment[2]
1mL	80%	1.8
8mL	81%	1.7
40mL	110%	1.8

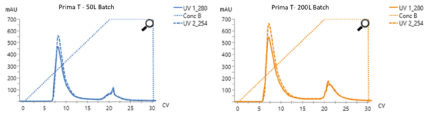

Fig.7: Scale-up results on PrimaT and chromatogram profiles

Conditions developed at a small scale are scalable to 50L and 200L production scales, achieving similar viral genome (VG) yield and full capsid enrichment.

Conclusion

Based on the data generated at multiple scales, conditions were established on the CIMmultus® PrimaT monolith for the industrial purification of a AAV8 product. The process achieves a full capsid enrichment of approximately 1.8 and a viral genome (VG) yield of 80%. Additionally, the PrimaT column effectively removes impurities, including some residual DNA and mainly host cell proteins, thereby improving the overall quality of the final product. These findings underscore the potential of the PrimaT monolith for large-scale AAV production in gene therapy applications.

1447 Development of a Novel Flow-Through Polishing Approach for Simplified Full/Empty Separation of Adeno-associated Viruses

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Adeno-associated viruses (AAVs) are extensively utilized as viral vectors for in vivo gene therapy. Purifying AAVs, particularly separating genome-containing capsids from empty ones, poses major challenges. Traditional chromatographic methods for this separation typically depend on linear salt gradients, leading to high buffer consumption and demanding high resolution from chromatographic columns. In this study, we introduce an innovative anion exchange flow-through polishing method that separates full and empty AAV particles using a single isocratic elution step (**Figure 1 A**).

Our experimental investigations revealed that using the divalent salt $MgCl_2$ as an eluent provides good separtion performance between empty and filled AAV particles (**Figure 1 B**). This method was successfully applied to three different AAV serotypes (AAV8, AAV5, and AAV2) across two types of stationary phases: an anion-exchange membrane adsorber and a multimodal monolithic column. This approach resulted in a 4 to 7.5-fold enrichment of full AAV particles. Importantly, the enriched filled AAV particles maintained their functional potency and structural integrity.

The flow-through polishing method offers several advantages for the AAV purification process. The use of convective matrices, such as monolithic columns and chromatographic membranes, enables higher flow rates compared to traditional chromatographic resins. Additionally, this method allows users to avoid buffer-intensive linear elution gradients and mitigates the risk of overlapping elution peaks, which can lead to poor enrichment of filled AAV particles. A further benefit of this method is that the binding capacity of a column can be increased since empty particles that appear in the flow-through fraction are not competing for binding sites on the column with filled particles.

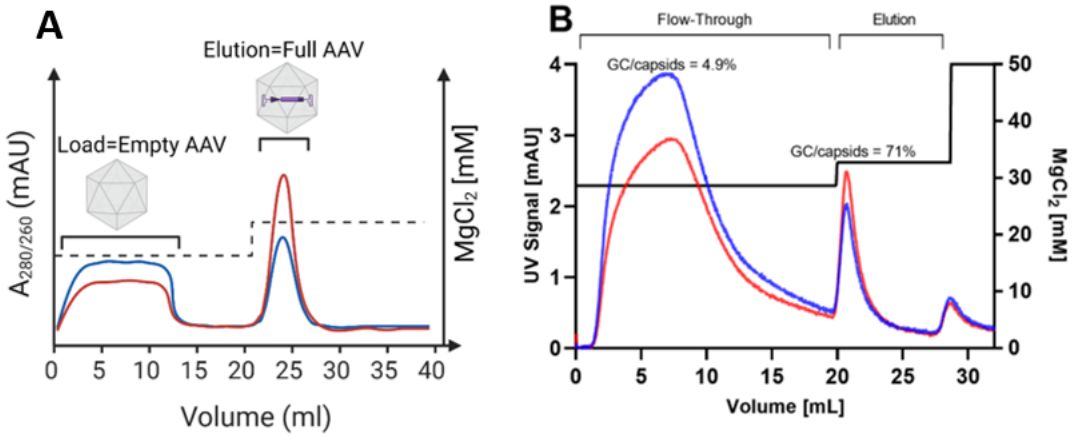

Figure 1: **A**) Exemplary illustration of flow-through chromatogram for empty/full AAV separation. During the sample loading empty AAV particles do not bind to the stationary phase. An increase of the ionic strength with a single isocratic step leads to elution of filled AAV particles. **B**) Flow-through chromatogram of a AAV5 empty/full separation. Using $MgCl_2$ the ionic strength of the AAV solution is adjusted in a way that empty particles appear in the flow-through fraction (4.9% filled particles) whereas the filled particles elute after a single isocratic elution step (71% filled particles).

1448 Purification Process Toolbox Development for Gene Therapy Vector AAV

Lihua Yang[1], Xue Mi[1]
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Gene therapy viral vector, adeno-associated virus (AAV) is becoming a viable and popular option for gene modification. Different serotypes have different tissue tropisms, enabling a broad application for these vectors. However, the current AAV packing technique at genome level is very inefficient, up to 80% of the formed AAV capsids may not contain the gene of interest (GOI), resulting in a mixture of significant amount of empty AAV capsids. In addition, the cell culture process is typically required to lyse the cells to maximize harvest of viral particles, but also release tremendous host cell impurities such as host cell proteins (HCP) and host DNAs. Moreover, AAV engineering is becoming popular to improve their therapeutic efficacy, but engineered AAVs can be more unstable. All these challenges create more complications for downstream purification. In our study, new filtration technologies, resins, chromatography, and operation modes are being explored to improve purification processes with high throughput, yield, full/empty capsid ratio and impurity removal for AAV vectors. We aim to develop a toolbox that can be suitable for different AAV vectors to streamline and speed up the purification process development.

Herein, a downstream purification process for several serotypes of AAV viral vectors are being explored for harvest clarification, tangential flow filtration (TFF), affinity chromatography and anion exchange (AEX) chromatography unit operations. An integrated AEX-based depth filter and filter train of several traditional polypropylene, cellulose, and diatomaceous earth-based, as well as synthetic depth filter with minimal leachable were screened to reduce cells, cellular debris, cell host and process impurities. The traditional depth filter train showed better throughput and a higher degree of reduction of host cell impurities and turbidity than the integrated filter. A large molecular weight cut-off (30, 100 and 300 kD MWCO) tangential flow filtration (TFF) cassette (mPES) and TFF capsule (regenerated cellulose) were compared to reduce the affinity load volume, reduce small-sized impurities and buffer exchange into the desired affinity column load buffer.

In addition to filtration steps, two chromatography downstream unit operations are being developed. A large pore-sized affinity POROS AAVX resin was selected to capture the AAV to reduce the majority of impurity burden, while lowering the mass transfer diffusion hindrance caused by the large viral particles. A high virus recovery rate of over 90% was achieved for each serotype with optimized low pH step elution. A new modality monolith AEX was compared with traditional resin based AEX for evaluation of full/empty capsid separation and further impurity clearance. As high as over 40% increase of full capsids were achieved using the optimal column and operating conditions. With these two steps of chromatography, impurities were greatly reduced to either below assay detection limit or significant low levels.

In conclusion, several purification unit operations were developed for different AAV serotypes to establish purification toolbox, including filtration and chromatography separation, and optimized process parameters for effective manufacture of best quality AAV products. Our next step would include model construction to continuously develop a platform process with high efficiency and performance.

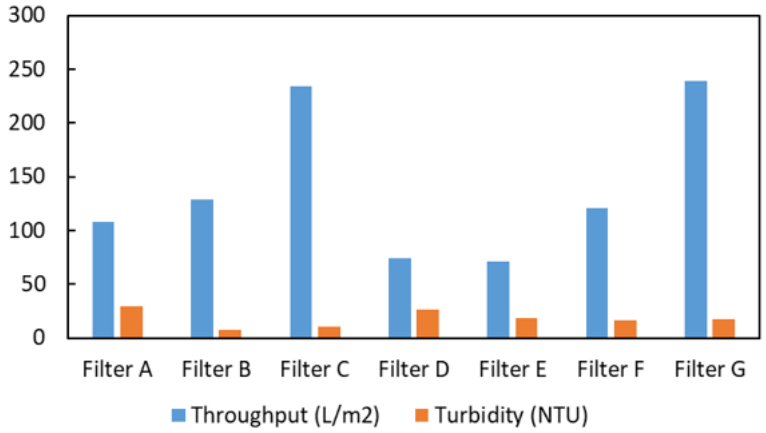

Figure 1. Clarification depth filter performance. Filters C and G show better throughput.

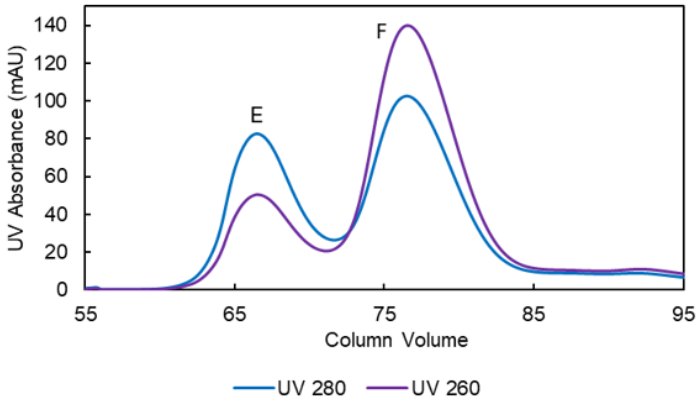

Figure 2. AEX chromatogram. Full (F) & empty (E) capsid ratio increased by over 40%.

1449 Critical Considerations For Full/Empty AAV Separation

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The inefficiency of AAV to package therapeutic genes leads to the production of a substantial amount of empty or partially filled capsids, which can impact the efficacy and safety of AAV-based therapies. Developing a robust and scalable purification method to separate full and empty AAV particles at large scale remains challenging due to the similar physicochemical properties of these two populations.

In this study, we developed a streamline downstream process including, capture and full capsid enrichment. Capture using POROS™ CaptureSelect™ AAVX resulted in 95% recovery of AAV2-GFP functional particles. For the polishing step, we introduce a conductivity-based strategy that combines linear and step gradient elution using

POROS™ XQ resin to enhance the separation of full and empty capsids. This approach demonstrated a 5-fold enrichment of full AAV2-GFP particles, while empty capsids were reduced by 4-fold.

Thus, the removal of empty capsid offers the possibility to further concentrate full capsids carrying the therapeutic gene while minimizing the risk of aggregation. Furthermore, as total capsid dose appears to be responsible of immune-mediated toxicities, eliminating empty capsid with no therapeutic benefit is crucial. These developments offer a scalable and robust solution for AAV manufacturing, which is essential for producing high-quality gene therapies.

1450 Utilization of Real-time Multi-angle Light Scattering (RT-MALS) for Accelearted AAV Chromatography Development

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The use of process analytical technology (PAT) to measure product quality attributes during process development can reduce the analytical workload and enable faster development timelines. Real-time multi-angle light scattering (RT-MALS) is an example of PAT that can be utilized in downstream process development for the measurement of titer, aggregation, and full capsid ratio. The work summarized describes our use of RT-MALS for accelerated AAV chromatography development.

1451 A Novel Harvest-Capture Process for Rapid Clarification and Recovery of rAAV9 from Cell Lysate Using a Macroporous Filtration Device and AVIPure AAV9 Affinity Resin

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Recombinant adeno-associated virus (rAAV) vectors are a leading platform for gene delivery for a variety of human diseases and have been the focus of hundreds of clinical trials over the past several decades. The manufacturing of rAAV therapies at the industrial scale involves a number of labor intensive and expensive steps, such as clarification by depth filters or centrifugation, UF/DF by tangential flow filtration, and chromatography. To optimize the manufacturing of rAAV, Repligen has developed a novel process combining the clarification of rAAV9 from cell lysate and affinity chromatography using a macroporous filtration device and AVIPure affinity resin in a single step. In roughly 3 hours, up to 90% of affinity purified rAAV9 capsids were recovered directly from 1-L of cell lysate. Scale down experiments have also been shown to reduce volumes by >50x from initial cell lysate to final product recovery. The benefits of this process include elimination of depth filters or centrifugation for clarification, removal of the initial TFF concentration step and high clearance of host cell proteins and host cell DNA. The process has been designed to be fully scalable so that a single filter in the 3-hour timeframe can be used for manufacturing runs of up to 2000-L, requiring ~10-L of AVIPure AAV9 affinity resin. The novel harvest-capture methodology will provide value to the rAAV vector space by reducing the number of unit operations, time, and cost of the manufacturing process without negatively impacting product yield and purity to ultimately help bring down the price of these transformative therapies.

1452 Optimization of AAV6 Production at Benchtop Scale: The Role of Affinity Chromatography in Overcoming Aggregation

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Adeno-Associated Virus (AAV) serotypes exhibit distinct characteristics, including tissue tropism and production efficiency. We found that, among AAV serotypes with cardiovascular tropism, AAV6 yields higher transduction efficiency than others when delivered via direct intramyocardial injection. However, standard lab-scale purification methods, comprising of collection and concentration of production media, cell lysis by repeated freeze-thaw cycles, clarification by centrifugation, purification and enrichment by iodixanol density gradient ultracentrifugation, and buffer exchange, are ineffective for purifying AAV6, as anecdotally reported. In contrast, larger batches of AAV6 can be sourced from contract manufacturers, indicating that purification at a larger scale is possible. Interestingly, AAV1, whose capsid protein differs from that of AAV6 by only six amino acids, does not present these purification issues.

To investigate the critical step where AAV6 purification fails, we produced AAV1, AAV6, and AAV9 at a benchtop scale (30×15 cm plates, 0.435 m^2). Quantitative PCR (qPCR) analysis of the clarified lysate confirmed similar yields for AAV1 (1.43×10^{13} vg) and AAV6 (0.99×10^{13} vg), with AAV9 (6.25×10^{13} vg) outperforming both. This suggested that the issue lies in downstream processing. We then performed iodixanol gradient ultracentrifugation and analyzed DNA isolated from all fractions using agarose gel electrophoresis. The full AAV particles of AAV1 and AAV9 were present in the expected fraction, while AAV6 genomes were only detected in fractions corresponding to the protein disc, a finding further confirmed by qPCR.

In large-scale AAV production, affinity chromatography is often employed before iodixanol gradient ultracentrifugation to purify vectors and prevent overloading of the gradient. We hypothesized that this additional step, which is not typically necessary for other serotypes at benchtop scale, might be required for AAV6. Furthermore, it has been reported that human serum protein galectin-3 binding protein, which is also secreted by the production cells, can bind to AAV6, leading to µm scale aggregates. Importantly, this interaction relies on the E531K substitution between AAV1 and AAV6. To test this, we used the AAVx affinity chromatography resin to purify AAV1 and AAV6 from both cell lysates and production media, all clarified using a 0.22 µm filter, following the manufacturer's protocol. Both AAV1 and AAV6 were successfully purified from the cell lysates, whereas only AAV1 was purified from the production medium, pointing to an aggregation problem with AAV6 in the production medium.

To further explore purification strategies for AAV6, we compared iodixanol gradient ultracentrifugation with or without prior affinity chromatography. We found that successful purification and enrichment of AAV6 only occurred when affinity chromatography was included in the protocol.

In conclusion, our study reveals that AAV6 presents unique purification challenges at the benchtop scale despite similar yields to AAV1. These issues likely stem from aggregation, mediated by interactions with galectin-3 binding protein, and are not overcome by conventional purification methods alone. Affinity chromatography is essential for the successful purification of AAV6, providing critical insights into improving its production efficiency and utility for gene therapy applications.

1453 Affinity purification analysis: a comparison of AAV2 and AAV5 serotypes

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Viral vector-mediated gene therapies are among the most efficient approaches for correcting genetic disorders. Adeno-associated viruses (AAV) are acknowledged as potent vectors for in vivo gene delivery. The production of AAV viral vector necessitates a downstream process that encompasses the capture of viral vectors and a full-capsid polishing enrichment step. Antibody-based affinity resins selectively capture vectors while effectively removing most host cell proteins and DNA contaminants. The study evaluates capture chromatography, including elution and wash buffers for AAV2 and AAV5, with the objective of establishing a manufacturing friendly AAV affinity purification process.
In the work, we used Tris-based equilibration and wash buffers for AAV2 and AAV5 maintained at a mildly alkaline pH (approximately 7.5) and a salt concentration 250-500 mM to eliminate non-specifically bound proteins.

In contrast, we utilized low pH elution buffers (approximately 2.5) to effectively elute the bound AAV capsids from the affinity resin. A capture chromatography method was developed specifically for the AAV2 serotype using a low pH glycine elution buffer. The method was subsequently utilized for the AAV5 serotype; however, the lack of AAV5 in the elution pool necessitated a change in the salt used for AAV5 elution. Citric acid which corresponds to different proton state at low pH, resulted in effective elution of AAV5. We evaluated multiple concentrations of citric acid and determined the optimal concentration for the elution. The optimal concentration not only enhanced the effectiveness of AAV5 elution but also reduced the elution volume. We illustrate the similarities of alkaline pH in wash buffers, differences of salts in elution buffers, and efficiencies associated in capturing AAV2 and AAV5 serotypes in downstream vector processing. Our work highlights the significance of tailored elution salts for each serotype. Additionally, it underscores the relatively minor role of the buffers employed during the equilibration and washing processes. The study develops a method that can effectively capture both serotypes by merely adjusting the elution buffer.

1454 Performance of Alkali-Tolerant Affinity Chromatography Resins Immobilized with AAV Receptor and Full/Empty Separation of AAV1

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Adeno-associated virus (AAV) is the most popular vector for in vivo gene therapy. To purify AAV more specifically, we focused on the AAV receptor AAVR (KIAA0319L), which is important for the intracellular entry of various AAV serotypes. So far, we have developed an affinity chromatography resin with immobilized modified AAVR and have reported on its alkali resistance and the purification of AAV8.

Concretely, first, we developed alkaline-tolerant AAVR. The stability in alkaline condition is desired for alkaline cleaning-in-place (CIP), which is conducted to wash out strongly bound host-cell proteins and AAV remaining in resin after use of column. Our AAVR mutant exhibited high stability in acidic and alkalic buffer. Next, we developed the resin immobilized by the AAVR mutant (AAVR resin). And the affinity column packed with this resin have been able to purify AAV8 vector from culture with a recovery yield of more than 90%.

In this presentation, we report on the performance of the AAVR resin for serotypes other than AAV8. Since there are various serotypes of AAV, we confirmed the ability to purify representative serotypes. First, AAV1, AAV2, and AAV9 were cultured and we confirmed that these AAV serotypes can purify with AAVR resin at least.

On the other hand, Tosoh bioscience LLC has presented in papers1) and conferences that the Full rate of AAV2, AAV5, AAV6, and AAV8 can be analyzed using anion exchange chromatography.

By using TSKgel Q-STAT for analysis and TOYOPEARL GigaCap Q-650S for preparative chromatography, and adding choline chloride to the buffer, the Full rate can be analyzed and AAV solutions with high Full rates have been obtained.

We therefore investigated the possibility of purifying AAV1 after purified by AAVR resin in a similar manner, using a GigaCap Q-650S 5-mL column with 20 mM Tris pH 9.0 and 20 mM Tris, 300mM choline chloride, pH 9.0

as the buffers. Although it should vary depending on the specifications of the equipment and the purity of the choline chloride, the results we examined with AKTA showed that a gradient to 66% was performed, washed to remove empty AAV, and then eluted stepwise to 100% to obtain the full AAV fraction. Full AAV fraction was analyzed using the "Refeyn" equipment. Analysis showed that the full AAV fraction, which was 53% after affinity purification, was enriched to 87%.

In summary, we have developed affinity chromatography resin with high durability in both acidic and basic conditions and process of purification of high quality Full AAV. It is founded that AAVR resin can AAV1, AAV2, AAV8 and AAV9. Additionally, we confirmed that AEX can remove the empty capsids from the mixture of full and empty particles not only AAV8 but also AAV1. We believe that the two-step purification process with affinity purification and anion exchange chromatography purification provides AAV with high purity in a simple manner.

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1455 Enabling Large Scale Implementation of Anion Exchange Chromatography for Full Capsid Enrichment of a Novel Adeno-Associated Viral Vector

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During the production of adeno-associated virus (AAV) gene therapies, a significant percentage of the viral capsids generated do not contain the packaged transgene of interest. These empty capsids are considered product-related impurities and must be reduced to the extent possible during downstream purification. Anion exchange (AEX) chromatography has emerged as a viable approach for separating empty and full capsids by exploiting slight differences in surface charge properties. Compared to alternative full capsid enrichment methods such as preparative ultracentrifugation, AEX chromatography is attractive for its linear scalability and ability to automate product collection using standard manufacturing equipment. However, while AEX is amenable to implementation at large scale, a development team must account for operational differences between laboratory-scale and manufacturing-scale equipment to ensure smooth tech transfer. System and scale dependent factors such as pump capacity, peak collection strategy, column sizing, and product stability during processing should be carefully considered for successful scale-up. Here, we share case study data which supports more than 100-fold scale-up of an AEX monolith chromatography operation to enrich full capsid content for a novel AAV feed. An analysis of development scale AEX trends is presented to inform strategies for large-scale implementation.

1456 Efficient Separation of Empty and Full AAV Capsids with Choline Chloride in Preparative Anion Exchange Chromatography

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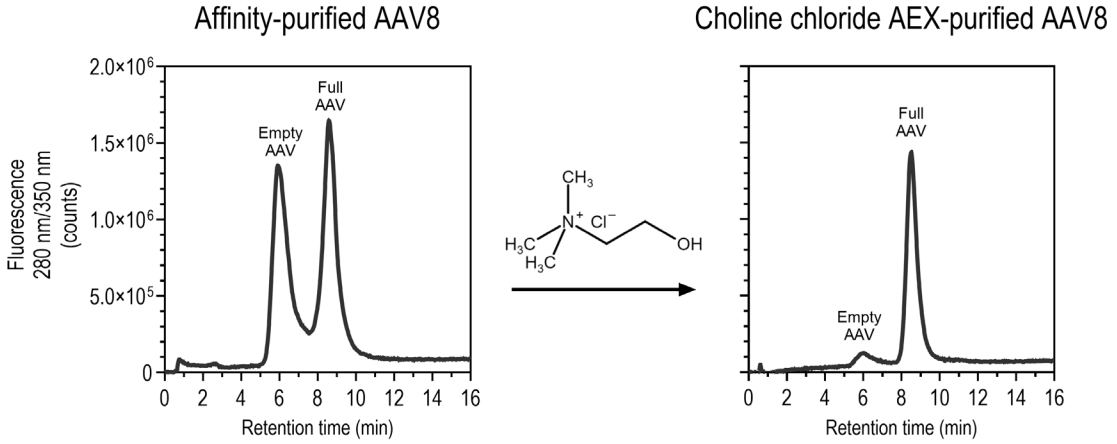
Adeno-associated virus (AAV) vectors are a widely pursued platform to deliver genetic medicine via *in vivo* direct administration. However, in upstream manufacturing, AAV genome packaging is an inefficient process, resulting in a heterogenous population of empty capsids, capsids with partial genomic material, and functional viral vectors containing the full genomic payload. Empty AAV capsids are considered a product-related impurity and may increase the risk of immunogenic adverse events. Empty capsids also may compete with full capsids for AAV receptors, thereby reducing the transduction potential of the full virion. Therefore, there is a strong motivation to

pursue the highest possible full AAV content in a finished drug product to ensure acceptable drug potency and safety.

The small net charge difference between empty (pI ≈6.2) and full (pI ≈5.9) AAV particles due to the encapsidated DNA payload can be exploited as a means of separating empty and full capsids in anion exchange (AEX) chromatography. However, conventional AEX purification schemes often do not result in sufficient empty and full capsid separation. Here, we describe a novel polishing AEX purification method using choline-Cl as the gradient salt on columns prepacked with TOYOPEARL GigaCap Q-650S, a resin functionalized with quaternary amines (Q-type resin ligand). Choline-Cl is a quaternary ammonium salt with a chemical structure similar to that of Q-type resin ligands. Elution with a choline-Cl linear gradient more than doubled the resolution between empty and full AAV8 capsid peaks as compared to ordinary monovalent salts such as sodium chloride. During further optimization, the linear gradient was modified and the method fully optimized using a series of elution steps specific to a particular AAV serotype. Starting from 40-50% full AAV8 content in an affinity-purified fraction, we were able to achieve ≈95% full AAV8 content in a single AEX polishing run. This level of full content is comparable to that of commercially sourced AAV samples purified with preparative ultracentrifugation.

This rapid, scalable, customizable, and adaptable choline-based process for Q-type AEX resins is an efficient chromatographic polishing method to remove empty particles and to enrich full AAV content. This leads to greater cost savings to produce AAV gene therapies by reducing the number of chromatography steps, avoiding loss of full AAV capsids contaminated with empty capsids, and fully eliminating the need for preparative ultracentrifugation. Implementing this choline-based AEX process supports the unmet need for potent, safe, and cost-effective AAV products.



1457 High Yield and Full-Empty Ratios Achieved with a Scalable GMP-Ready AEX Chromatography

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A minimum of 70% full particles is typically recommended for the majority of clinical AAV applications since an excess of non-functional or empty capsids can impact both, therapeutic efficacy and safety. The design of plasmids and overall AAV upstream manufacturing platform significantly impacts the level of full-length AAV packaging.

Technologies such as our EpyQTM can make further enrichment of full particles dispensable, resulting in higher AAV vector titers and overall process recoveries. When a full capsid enrichment step is required in downstream processing, it is very challenging to develop a robust and scalable step to significantly reduce empty capsids. Here we describe a systematic process development strategy to optimize full/empty separation using anion exchange chromatography (AEX), with a focus on methodical screening and stepwise refinement.

Development began with a screening phase to identify a suitable AEX resin to separate full and empty AAV9 capsids. Three commercially available AEX resins were evaluated under linear gradient elution conditions. Following resin selection, the buffer system was optimized by testing various salt compositions and dual salt combinations to ensure operational simplicity and compatibility with previous and following downstream processing steps. Linear gradient experiments with different salt types provided insights into the optimal elution profiles for full capsid enrichment.

Based on these findings, the chosen resin and buffer system were further evaluated to develop a step gradient protocol, replacing the linear gradient for enhanced process reproducibility and scalability. The step gradient resulted in an enrichment to ≥70% full of the already high % full starting material (~50% full for AAV9 & 3kb GOI) with a step recovery of more than 60 %, sufficient to include it in the purification process. Mass photometry was employed as a reliable analytical tool to determine the enrichment factor for full capsids. Concurrently, droplet digital PCR (ddPCR) was applied to quantify vector genome (vg) titers, enabling accurate calculation of step recovery. These two analytical approaches ensured a comprehensive evaluation of process performance.

This study highlights the critical role of robust and qualified analytical tools and a stepwise, well-structured process development for establishing an efficient method to separate full and empty capsids. A scalable, GMP-ready AEX step was developed first for AAV9, and is now available for the serotypes AAV2 and 8 as well. These studies offer a good basis for future projects with different serotypes and gene-of interests.

1458 Maintaining Superior Viral Vector Recovery in Cell and Gene Therapy Applications by using Daikyo Crystal Zenith® Vials

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This study aims to evaluate the impact of vial material of construction on the stability of two adeno-associated virus (AAV) serotypes under varying stress and storage conditions. AAV8 and AAV9 were chosen as representative AAV serotypes, with respective titers in a common buffer formulation, and evaluated in different 2mL vial types: Daikyo Crystal Zenith® (CZ) vial, and two commercially available borosilicate vials. To simulate viral vector recovery in cell and gene therapy applications, the vial combinations were tested across five challenge conditions:

- 3 months at -80°C, recommended condition for long term storage
- 2 months at 5°C, recommended condition for temporary refrigerated storage
- 3 weeks at 25°C, simulating room temp clinical ambient conditions
- 72 hours of 250 RPM agitation, to mimic stress environment during shipping and handling
- 5 cycles of Freeze/Thaw, to mimic handling during manufacturing and the potential for structural damage to proteins or DNA

In all conditions, the Daikyo CZ vial demonstrated improved viral vector recovery compared to borosilicate glass vials. The data shows that there were no significant changes in product quality due to container storage in the Daikyo CZ vials, and that the CZ vials may comparatively improve viral vector recovery across common storage and handling conditions.

1459 Benefits of High-Throughput Sequencing-Based Methods for In-Depth Characterization of Recombinant AAV Products

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With the success of clinical trials, gene therapy today may represent hope for patients suffering from genetic diseases. Recombinant Adeno-associated virus (rAAV) products are extensively characterized prior to release. Among the Critical Quality Attributes (CQAs) used for release testing, those related to the identity and purity of rAAV products are scrutinized to ensure patient safety and treatment efficacy. Although quantitative polymerase chain reaction (qPCR) PCR and Sanger sequencing are currently among the gold standard techniques, the rapid development of high-throughput sequencing (HTS) technologies has paved the way for a much more exhaustive characterization of rAAV product's DNA content.

It has been just over ten years since rAAV products began to be closely studied by high-throughput sequencing, initially using Next Generation Sequencing (NGS) with short-reads (e.g., Illumina) and then using Third-generation sequencing, also known as long-read sequencing (e.g., Pacific Biosciences and Oxford Nanopore Technologies). Studies carried out so far show that both short-read and long-read sequencing technologies are of importance for the characterization of DNA, each with its own strengths. Next-generation sequencing offers HTS depth and a low sequencing error rate, enabling exhaustive identification and quantification of contaminating DNA, and detection of mutations at unprecedented levels. On the other hand, long-read sequencing technologies offer the opportunity to sequence DNA at single-molecule level, allowing the identification and quantification of truncation events in rAAV genomes as well as determining the size of contaminating DNA. There is currently no consensus on the method of choice, and a combination of different HTS technologies is needed to obtain the most exhaustive view of the DNA present in rAAV products.

In this study, both Illumina and Oxford Nanopore Technologies were used to characterize AAV preparations. All the experiments were carried out using laboratory methods established internally, from sample and library preparation, to sequencing and bioinformatics analyses, to guarantee the quality of the results in particular with the sequencing of controls, the introduction of acceptance criteria and determining the limit of quantification of each method. The results provide information on 3 CQAs: (1) payload sequence identity, (2) payload sequence integrity, and (3) residual DNA sequence quantification. The presence of Single Nucleotide Polymorphisms, indels and truncation hotspots were assessed; and DNA contaminants were identified and quantified.

We believe our HTS-based methods can be applied to the exhaustive characterization of both DNA starting materials (e.g. plasmids) and rAAV gene therapy products and could be validated in the near future.

1460 Determination and Characterization of Capsid Full Ratio of the Clincal Grade rAAV Product by Transmission Electron Microscopy, Analytical Ultracentrifugation, Digital Droplet PCR, and Capsid ELISA

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Adeno-associated (AAV) vector is the main *in vivo* gene delivery method, widely used in over 250 clinical trials for various indications. Empty and partial capsids that do not contain full-length genome sequences are by-products of AAV production and significantly affect the clinical safety and efficacy of AAV gene therapy. Therefore, determining the full capsid ratio of AAV products is essential before clinical application. Thus, three orthogonal methods, transmission electron microscopy (TEM), ELISA coupled with ddPCR, and analytic ultracentrifugation (AUC) were comparatively studied. TEM showed homogenous AAV morphology in spherical structure and identified 98.0% full, 0.7% partial, and 1.3% empty capsids. Then, ELISA coupled with ddPCR was performed to measure total capsid concentration and AAV genome copy, respectively. ELISA coupled with ddPCR identified 64.6% full capsids without distinguishing empty and partial capsids. Finally, AUC was performed based on

estimated AAV molecular weight and sedimental velocity analysis. The results showed 60.4% full capsids (101S peak), 37.0% partial (81 and 91S), and 1.1% empty (72S), with a good correlation with ELISA/ddPCR results. In summary, AUC is the most reliable test for determining full, partial, and empty capsids, while ELISA coupled with ddPCR could be a fast, convenient, and cost-effective alternative for in-process measurement.

1462 Methods to Improve the Clarification Process of AAV Therapy Manufacturing

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The high cost of manufacturing for therapeutic grade AAV is a significant barrier to these life-saving therapies. To this end, focus was placed on designing an efficient AAV purification process. This work explores opportunities to optimize the clarification process for an AAV5 vector produced in HEK-293 cells.

Two different approaches to clarification (flocculation and precipitation) were explored together with a screen of depth filtration media. An optimal process was identified based on product recovery, depth filter capacity as determined by differential pressure and filtrate turbidity, sterilizing grade membrane filter capacity, and residual levels of common impurities like host cell DNA and host cell proteins in the filtrate. Maximal particulate retention and impurity clearance were achieved by implementing lysate pretreatment coupled with an appropriately selected depth filtration media. The filter capacity exceeded 250 L/m^2, filtrate turbidity stayed below 5 NTU, and sterilizing grade membrane area was significantly reduced. Residual hcDNA, a key impurity, was reduced by >3 logs and vector recovery was >90%. The superior filtrate quality from this optimized clarification process results in less purification burden for the costlier subsequent affinity chromatographic separation technique.

1463 Optimizing AAV8 Capsid Purification with Oversized GOI: Efficient Separation of Empty, Partial, and Full Capsids Using Anion Exchange Chromatography

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Adeno-associated virus (AAV) vectors are widely used in gene therapy, but the production process often yields heterogeneous populations of viral particles, including empty capsids (lacking a genome), partially filled capsids (containing fragmented DNA), and full capsids (encapsulating an intact genome). Empty capsids, classified as product-related impurities, lack the therapeutic gene of interest and are devoid of functional relevance. While their precise role remains unclear, studies suggest they may influence therapeutic efficacy and safety profiles or act as decoys for neutralizing antibodies. Consequently, effective separation of empty capsids is critical to ensure a high-quality AAV product (Zhu et al., 2025)

Anion exchange chromatography (AEX) has emerged as the preferred method for large-scale separation of AAV capsids due to its scalability, reproducibility, and ability to maintain vector integrity. ReciBioPharm's platform AEX process demonstrated high-resolution separation, achieving greater than 90% full capsid purity with minimal loss of vector potency. In this study, we aim to achieve the same with large sized GOI (>4.9 kb) by systematically evaluating AEX as a platform for oversized AAV8 capsid separation.

Critical factors, including buffer composition, were systematically investigated to achieve optimal separation. Trivalent sodium phosphate (PO_4^{3-}) was selected over monovalent and divalent salts, such as sodium chloride and sodium sulfate, due to its superior resolution power. This advantage is likely attributed to its higher ionic and displacement strength, enabling more precise differentiation between empty, partial, and full AAV8 capsids (Joshi et al., 2021). Analytical methods such as mass photometry, Analytical ultracentrifugation (AUC), and droplet digital PCR (ddPCR) were employed to assess capsid content and DNA encapsulation efficiency. The study also compared partial capsid separation across different conditions to ensure accurate quantification and removal of intermediates.

These findings provide a robust framework for refining AAV manufacturing processes, ensuring improved therapeutic efficacy and compliance with regulatory standards. By leveraging AEX for AAV8 capsid purification, this work contributes to advancing the production of high-quality AAV-based gene therapies.

Reference
1. Joshi, P. R. H., Bernier, A., Chahal, P. S., & Kamen, A. (2021). Development and Validation of an Anion Exchange High-Performance Liquid Chromatography Method for Analysis of Empty Capsids and Capsids Encapsidating Genetic Material in a Purified Preparation of Recombinant Adeno-Associated Virus Serotype 5. Human gene therapy, 32(21-22), 1390–1402. https://doi.org/10.1089/hum.2020.317
2. Zhu, J., Brown, C., Wu, H., & Liu, X. (2025). Advanced Strategies for AAV8 Empty/Partial/Full Capsid Separation Using Anion Exchange Chromatography (AEX). Journal of Chromatography A, 1680, 123456.

1464 Title: The Impact of Empty Capsids on AAV Manufacturing and Strategies for Enhancing Yield, Purity, and Stability in the Production of a Novel Blood-Brain Barrier Penetrant AAV Capsid

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Adeno-associated virus (AAV) is a highly promising gene therapy vector, widely used in clinical applications due to its ability to deliver genetic material to target cells. A challenge in AAV manufacturing is the occurrence of empty capsids in the final product, which can reduce the efficacy and/or impact the safety profile of AAV-based therapies. These empty particles, which lack the therapeutic transgene, can accumulate during the production process, leading to lower viral vector purity and reduced yield of full, functional virions. Additionally, the presence of empty capsids complicates downstream processing, demanding more extensive purification steps, and increases the risk of immunogenic responses upon administration. Overcoming this challenge is crucial to improving the quality, consistency, and scalability of AAV production. In this study, we examine the impact and implications of varying levels of empty capsids on AAV vector production during manufacturing of Sangamo's novel blood-brain barrier penetrant AAV capsid (STAC-BBB) and present strategies for improving yield and purity. Additionally, a stabile formulation is required in order to administer these AAV treatments. We developed a stable formulation buffer for STAC-BBB by evaluating aggregation and capsid stability profiles. With optimized process parameters, we have established a robust, scalable manufacturing process and formulation that ensures critical product quality attributes meet requirements, advancing the development of novel viral vectors and genomic medicines for the treatment of neurological disorders.

1465 Accelerating AAV Processing: Scalable, Serotype-Agnostic Process Eliminating 2-steps (DNase and TFF) and Optimized for Physiological-Like Conditions

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Adeno-associated virus (AAV) production for gene therapy applications has become increasingly important, yet the downstream processing of AAV remains a complex and cumbersome series of steps, each contributing to reduced overall yield and extended processing time. Current methodologies generally consist of seven or more individual steps: DNase treatment>Clarification>TFF1>Capture step>TFF2>Polishing step>Formulation and filtration. The resulting low yields and prolonged timelines hinder the scalability and efficiency of AAV production, posing significant challenges to manufacturing.

This study proposes a streamlined alternative to traditional AAV downstream processing, focusing on reducing the number of steps while simultaneously improving both recovery and process efficiency. We demonstrate a novel approach comprising just five integrated steps shown to work on various serotypes (AAV8, AAV9, AAV2).

The first step involves a direct lysate treatment using CIMasphere, media designed to effectively reduce both host cell DNA and host cell protein levels, thus eliminating the need for an additional DNase digestion step. Next, the lysate undergoes clarification through depth filtration, a well-established method that aids in the removal of large debris and clarifies the sample. The third step incorporates a capture process using novel CIMmultus IEX based column, that operates under near-physiological conditions for both binding and elution steps, specifically with pH and salt concentrations optimized for AAV stability. The capture step eluate seamlessly transitions without the need of TFF into the final polishing phase, which ensures high enrichment of the therapeutic AAV species. Finaly a formulation step using TFF is performed.

The innovative process presented in this study significantly reduces the number of individual steps required for AAV downstream processing. By optimizing the treatment conditions and eliminating redundant steps such as DNase digestion, we achieve higher viral yields and reduce the overall time for processing. This approach not only improves the scalability of AAV production but also offers a more efficient and cost-effective pathway for the production of AAV-based gene therapies, potentially accelerating the development of clinical applications.

1466 Next-Generation Sequencing for AAV Characterization

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Viral vectors are valuable vehicles for the delivery of gene therapies. Adeno-associated viruses (AAVs) are among the most promising and versatile viral vectors, as AAVs are non-pathogenic and lowly immunogenic, and may deliver targets to a range of tissue types. Moreover, several AAV-based therapies have already received regulatory approvals, demonstrating their safety and efficacy. For gene therapies, AAV contains a modified genome that consists of viral inverted terminal repeat (ITR) sequences flanking the target therapeutic gene. An important characterization assay of AAV products is the confirmation of the sequence of the gene being delivered, also known as identity testing. Identity testing of AAV products is critical to verify that the encapsidated genome is accurate, and that no unwanted genetic variants are present which could lead to nonfunctional gene products or detrimental effects in patients. We have applied complementary next-generation sequencing (NGS) technologies, to confirm the sequence identity of the viral encapsidated genome from several AAV serotypes. We have also been able to identify process-related impurities in AAV preparations, including residual host cell genomic DNA, residual plasmid DNA, and genome truncations or partial genomes. Finally, because plasmids are critical raw materials in the production and manufacturing of AAVs, we have used long-read sequencing for the confirmation of plasmid sequence identity. NGS is an important approach for the characterization of viral vectors, thus we have integrated short-read and long-read sequencing platforms as routine steps in our AAV process development and characterization workflow.

1467 Multiplex ddPCR Approach as Proof-of-Principle for Accurate AAV Genome Integrity Assessment and Enhanced Vector Production

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Adeno-associated viruses (AAVs) are widely used in gene therapy due to their ability to achieve targeted expression with minimal immunogenicity. While their small genome size (4–5 kB) limits gene transfer capacity, they provide long-term therapeutic expression, making them valuable for clinical applications. Recombinant AAVs (rAAVs)

replace viral genes with therapeutic transgenes while retaining inverted terminal repeats (ITRs) for efficient gene delivery.

A key challenge in large-scale rAAV production is genome heterogeneity, where capsids may contain truncated or incomplete genomes, affecting efficacy and safety. To address this, we developed a multiplex droplet digital PCR (ddPCR) assay to assess genome integrity, utilizing hydrolysis probes targeting regions near the 5' and 3' ITRs for precise genome quantification.

Our feasibility study designed primers targeting the CMV promoter (FAM-labeled) near the 5' ITR and the TK promoter (HEX-labeled) near the 3' ITR. The assay demonstrated high specificity, accurate quantification across multiple runs, and no off-target amplification. Comparative analysis with mass photometry confirmed strong alignment in detecting full and partial AAV genomes.

This approach offers strong linearity and reproducibility, making it a reliable tool for genome quantification across AAV production stages. By integrating ddPCR with complementary biophysical techniques, we enhance quality control, supporting the production of safe and effective gene therapy vectors.

1468 Evaluating the Performance of Nanoneedle Assays for Robust Quantification and Determination of AAV Genome species packaged and Capsid Titers during Manufacturing Process

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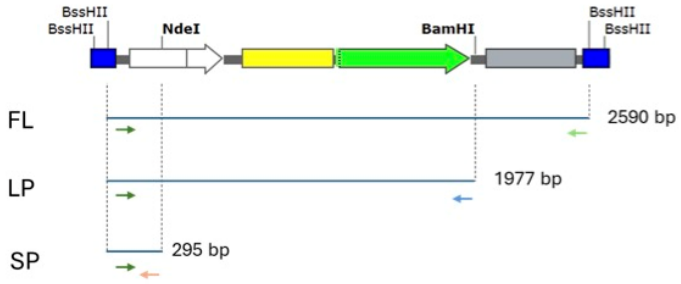
[1]Umass Chan Medical School, Worcester, MA, [2]University of Massachusetts Chan Medical School, Worcester, MA

Adeno-associated virus (AAV) vectors are a leading platform for gene therapy, but robust quality control (QC) measures are critical for their safe and effective clinical application. A major challenge in AAV production is ensuring complete viral genome packaging, as incomplete genomes can significantly impact therapeutic potency and increase the risk of immunogenicity. While next-generation sequencing (NGS) provides valuable information regarding sequence quality and can identify sequence alterations within the AAV preparation, it is not quantitative and offers only a general overview of the DNA fragments present. Current methods for quantifying full-length genomes are limited. Some lack resolution on the completeness of the packaged genome (e.g., AUC, mass photometry), while others, like qPCR and dPCR, only quantify a short fragment, providing limited information on the quantity of intact genomes. Therefore, a fast, accurate, and quantitative method for assessing full-length transgene packaging is crucial for quality control (QC) of the manufacturing process.

Here, we evaluate a novel suite of nanoneedle assays (Tessie nanoneedle technology) designed to quantify various aspects of AAV samples, including full-length AAV genomes, shorter viral-DNA fragments, and capsid titer. To assess the accuracy of the viral-genome assays, we measured a defined mixture of plasmid DNA fragments using three distinct assays targeting: 1) the full-length transgene, 2) a long-truncated fragment, and 3) a short-truncated fragment (see Figure). We further evaluated the repeatability and reproducibility of both the full-length viral genome assay and the capsid titer assay. Finally, we applied these assays to comprehensively characterize AAV samples across various manufacturing stages, from crude material to ultracentrifugation-purified fractions, and compared the results to those obtained using ddPCR.

Our findings demonstrate that the nanoneedle assays are robust, with measurements ranging between 96% and 124% of the known DNA titers. Furthermore, both the capsid and viral-genome quantification assays exhibit high repeatability (intra-plate) and reproducibility (inter-plate), with coefficient of variation (CV) of 6% (intra-CV) and 14% (inter-CV) for the capsid assay and 3% (intra-CV) and 7% (inter-CV) for the viral-genome assay, respectively. This novel suite of assays offers a robust and reliable approach for AAV QC by providing a more accurate and comprehensive assessment of genome packaging, ultimately enhancing the safety and efficacy of AAV-based gene therapies. In addition, the rapid and precise quantification enables efficient evaluation at the early stages of vector design toward the development of more potent and targeted gene therapies.

A



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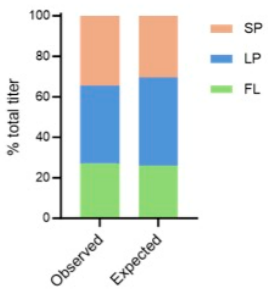

C

	Repeat 1	Repeat 2	Repeat 3	Average	CV	Expected	Observed / Expected
Full-length (FL)	1.30E+09	1.44E+09	1.56E+09	**1.43E+09**	**9%**	**1.26E+09**	114%
Long Partial (LP)	1.76E+09	1.63E+09	2.09E+09	**1.83E+09**	**13%**	**1.47E+09**	124%
Short Partial (SP)	1.86E+09	2.32E+09	1.88E+09	**2.02E+09**	**13%**	**2.10E+09**	96%

Quantification of a known DNA mixture using Tessie nanoneedle Viral-Genome quantification assays. A. *the DNA mixture created for assessment. Top: schematic showing the positions of the indicated restriction enzymes across the transgene used to generate the following DNA fragments that are quantified by the Tessie nanoneedle assays - FL: Full-length fragment (BssHII digest), LP: Long partial fragment (BssHII and BamHI digest), SP: Short partial fragment (BssHII and NdeI digest). Arrows indicate the positions of the affinity primers for quantifying each of the respective fragments.* ***B.*** *The proportion of the total titer for each fragment is shown as stacked bars comparing the measured values to that of expected values. The proportions of the measured titers were ±5% of the expected.* ***C.*** *NanoMosaic titers from the 3 independent repeats, their averages and CVs for the 3 assays: FL, LP, and SP, are shown. Expected titers are based on the DNA mixture of restriction digested plasmid DNA as measured by Qubit HS DNA assay.*

1469 AAV Genome Integrity: Full and Partial Genome Characterization Utilizing Digital PCR and Long Read Sequencing

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The rapid growth of the cell and gene therapy field presents a need for improved product-specific analytics. Critical quality attributes, such as viral genome (VG) or viral particle (VP) titer, do not consistently correlate with drug potency or safety, demonstrating a lack of genome-specific analyses. It is hypothesized that the heterogeneity of packaged genomes is a potential cause for this inconsistency. Therefore, there is a pressing need to improve characterization of packaged genomes for their heterogeneity and integrity. Our approach employs quadplex digital Polymerase Chain Reaction (dPCR), utilizing four primer probe sets that span the critical elements of the AAV cassette. Powerful genome integrity analysis calculates both the absolute number and distribution (heterogeneity) of the target based AAV genome variants. These results are compared and contrasted with Long Read Sequencing

results of the same drug product lots. The results presented here demonstrate correlation of genome integrity measurements across dPCR and NGS for multiple serotypes. These methods have complementary strengths and may provide impactful insights into variable potencies and packaging efficiencies of gene therapy products. dPCR provides absolute quantification, has low sample requirements and samples can be analyzed quickly. NGS provides a platform approach with deeper characterization and is better suited to detect insertions and deletions. Thus, results from these genome integrity analyses may be utilized throughout the drug development pipeline, informing research and development all the way through patient dosing.

1470 Characterization of High and Low rAAV Producer Cell Populations in Pinnacle PCL using Single Cell RNA Sequencing

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Over the last two decades, single cell sequencing techniques have allowed researchers to capture the heterogeneity of their cell populations by revealing gene expression differences on an individual level. Utilizing 10X Genomics single cell RNA sequencing (scRNA-seq) technology, full length mRNA from individual cells was isolated and then barcoded, which was then converted into cDNA and processed through Illumina-based workflows to achieve gene expression profiling at a single cell level. The Pinnacle Producer Cell Line (PCL) system is capable of supporting scalable 2000L clinical grade rAAV production and has been used to generate material for the ongoing UX701 clinical trial for Wilson Disease. By applying scRNA-seq methodology to our cells, we are gaining critical insights into sub-populations that may be influencing productivity and quality within the population and how each producer cell is interacting with helper and production viruses. Here, we present the potential of this technology to identify significant gene expression differences within our polyclonal and monoclonal cell lines during viral production. Experiments were designed to simultaneously examine the profiles of the PCL host cells, the Adenovirus 5 helper virus, and rAAV. Time course experiments enabled us to interrogate the infectious cycle of the helper Adenovirus and determine key events required to provide optimal helper function. Finally, vector constructs with engineered secondary structure have been analyzed to understand key differences between our high and low producing PCL clones.

1471 Quantification of Adeno-Associated Viral Genomes Using a Newly Developed qPCR Assay

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Recombinant adeno-associated viruses (AAV) are widely used as gene therapy vectors in emerging field of *in vivo* gene therapies. Measuring the vector genome copy (GC) titer is crucial for the development of AAV-mediated gene delivery research and AAV gene therapy products. Reliable, accurate, and rapid quantification of the AAV genome titer is essential for precise dosing, which is critical for patient safety. In this context, we introduce the AAV qPCR Fast Titer Kit, a newly developed assay for quantifying AAV genomes in purified vector samples. The core component of the Universal SYBR qPCR master mix is Taq Pro DNA Polymerase, a new generation hot-start polymerase modified through an antibody method. This polymerase offers several advantages, including improved specificity, increased detection sensitivity, and higher amplification yield. Additionally, we have incorporated a specific enhancer and optimized the qPCR buffer to further enhance specificity and sensitivity during the qPCR

reaction. This kit also includes a Specific ROX Reference Dye, which is compatible with all qPCR instruments, eliminating the need to adjust the ROX concentration on different devices. To simplify sample processing, we developed an accelerator buffer that can lyse AAV capsids in just 5 minutes at room temperature. With the AAV qPCR Fast Titer Kit, it is possible to quantitate AAV genome titer in under 2 hours. In summary, our AAV qPCR Fast Titer Kit provides a reliable, accurate, and efficient assay for quantifying AAV genome titer, making it widely applicable in AAV manufacturing and beneficial for AAV gene therapy and AAV-mediated gene delivery research.

1472 Precise rAAV Capsid Characterization via Digital PCR and Statistical Modeling

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Precise quantification of recombinant adeno-associated virus (rAAV) subpopulations—particularly distinguishing capsids containing whole genomes, partially fragmented genomes, or fully fragmented sequences—is critical for advancing gene therapy development and manufacturing for research purposes. Traditional methods often fail to resolve these subpopulations accurately, especially when accounting for linkage between genomic fragments.

To address these challenges, we developed a novel PEA-dPCR workflow leveraging the QuantStudio™ Absolute Q™ digital PCR (dPCR) platform, Poisson-based statistical modeling, linkage analysis, and multiplexed-multiomic detection within dPCR partitions. The dPCR partitions samples into thousands of independent reactions, enabling simultaneous detection of multiple target sequences in each partition. By statistically unwinding the multiplexed results with Poisson-based modeling, we precisely determine the presence and proportions of all genomic subpopulations, including discrete fragments, partially linked genomes, and fully intact genomes. This approach not only resolves the capsid content but also calculates confidence intervals for each measurement, a robust assessment of accuracy and reliability.

Initial validation was conducted using synthetic wet-lab constructs with predefined linkage patterns, simulated datasets spanning a range of packaging profiles, and select rAAV samples. The workflow demonstrated high precision and reproducibility, accurately identifying and quantifying even complex subpopulations while offering statistical confidence in the results.

In summary, this PEA-Absolute Q™–based workflow integrates digital PCR, multiplexed-multiomic detection, statistical modeling, and linkage analysis to precisely characterize rAAV capsid subpopulations. By providing exact quantification of capsid species, their linkage states, and associated confidence intervals, this approach offers a powerful research tool for optimizing rAAV production and the quality of gene therapy vectors.

1473 GeneSwift: a PCR-Free, Hybydization Method to Determine Gene Therapy Vector Titer

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Background: Adeno-Associated Virus (AAV) is one of the most widely used viral vectors for gene therapy. Standard methods for determining AAV titers, such as qPCR and dPCR/ddPCR, are time-consuming, labor-intensive, and require multiple serial dilutions. These methods are also prone to interference from PCR inhibitors. Additionally, mRNA-based gene therapy presents challenges that PCR cannot adequately address, particularly in distinguishing between single-stranded (ss) and double-stranded (ds) RNA, the latter of which can trigger cytotoxic responses. To overcome these limitations, we developed GeneSwift, a PCR-free assay that integrates DNA hybridization, immunochemistry, and biolayer interferometry (BLI) for rapid and accurate gene titer quantification.

Methods: Figure 1A shows the GeneSwift workflow. AAV samples are mixed with two gene-specific DNA oligos (fluorescein-labeled F*Oligo and biotin-labeled B*Oligo) and lysed by heating in Hybridization Buffer. Upon

cooling, the oligos hybridize to the gene of interest (GoI) to form the hybridized complexes. Adding an anti-Fluorescien coated BLI Biosensor captures the hybridized complexes, followed by a Biotin Detection Solution to generate BLI signals. Use of a calibration curve with known GoI levels enables precise titer measurement. Total assay time is 30 minutes, and the oligos can be formatted to hybridize with either ssRNA or dsRNA. To evaluate GeneSwift's performance, we tested AAV8-GFP, ssRNA-GFP, and dsRNA-GFP as model analytes.

Results: The GeneSwift data (Figure 1B) indicated that BLI signals increased correspondingly with the titers of AAV standards. Based on the data testing AAV8-GFP, the assay showed a dynamic range from 4E+9 up to 1E+12 vg/ml, covering typical AAV production levels. Similar results were observed with AAV2 and other serotypes. We tested the performance of GeneSwift in various upstream (cell growing medium or cell crude lysate) and downstream AAV media (anion exchange or in-process buffers). The results (Figure 1C) showed robust signals and dose response curves in all tested AAV manufacturing media, demonstrating a broad compatibility of the assay with various matrices. A comparison using AAV8-GFP spiked into crude insect cell lysates showed excellent correlation with ddPCR ($R^2 = 0.99$, slope = 1.05) (Figure 2A). Both GeneSwift and ddPCR assays exhibited comparable precision with ~10% of CV in crude cell lysate samples (Figure 2B). However, GeneSwift delivered results in just 30 minutes with undiluted samples, whereas ddPCR required ~5 hours and a 10,000-fold dilution, making GeneSwift a much more efficient method for titer determination. To extend GeneSwift's applications to genome integrity assessment, we designed an oligo pair targeting widely separated regions (CMV enhancer and SV40 polyA). The results (Figure 2C) produced a robust dose-response curve, confirming the assay's potential to evaluate genome integrity. Finally, we tested GeneSwift's ability to detect ssRNA and dsRNA. Detection of dsRNA entailed selection of the oligo pair to hybridize to the opposite strands. The results (Figure 2D) still showed very good dose response curves, demonstrating the assay's versatility in measuring both ssRNA and dsRNA titers.

Conclusion: GeneSwift is a rapid, PCR-free assay for quantifying AAV and mRNA vectors. Its speed, accuracy, and broad applicability make it a powerful tool for advancing gene therapy research and bioprocessing.

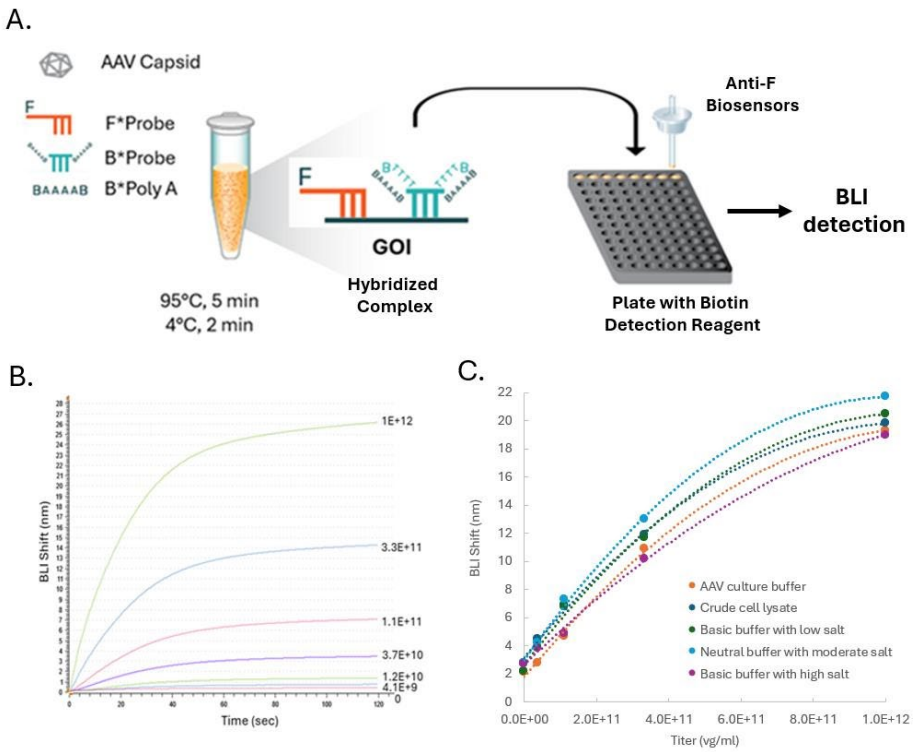

Figure 1. (A) GeneSwift workflow. (B) GeneSwift data showing detection range from 4E+9 – 1E+12 vg/ml. (C) Dose response curve generated in different upstream and downstream AAV manufacturing media.

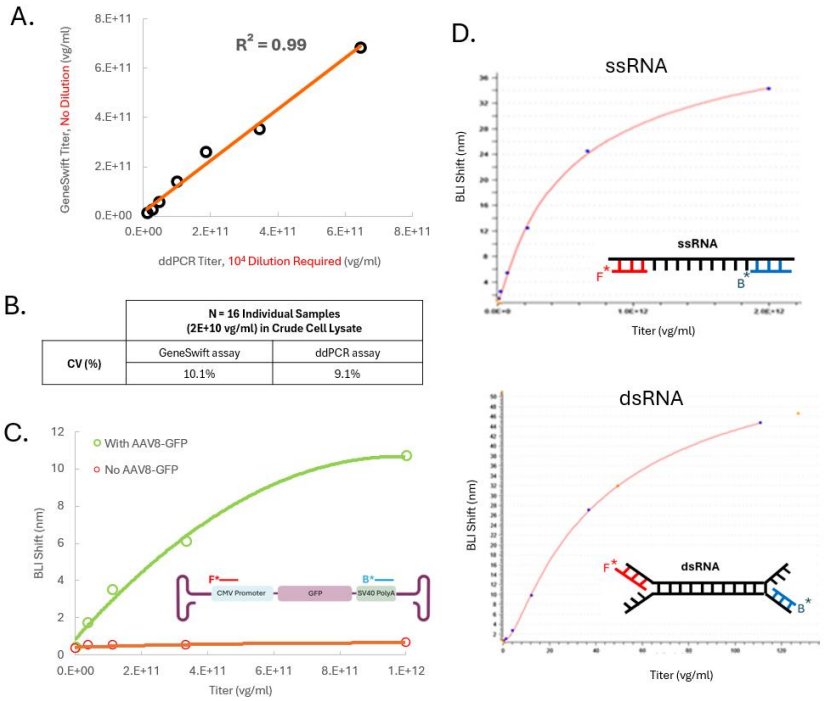

Figure 2. (A) Correlation data between GeneSwift and ddPCR assays in crude cell lysate. (B) GeneSwift and ddPCR precision data. (C) Demonstration of the GeneSwift assay for genome integrity assessment. (D) Demonstration of the GeneSwift assay for ssRNA and dsRNA titer detection.

1474 *Reducing Host-Cell Impurities and Increasing Yield In AAV Manufacturing Using the Novel Technology Photochemical Lysis (PCL)*

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**Corresponding Author*

Adeno-associated virus (AAV) gene therapies hold great promise for curing genetic disorders, but their high cost—often exceeding $2 million per treatment—limits patient access. Lowering production expenses is essential to increase affordability and expand the footprint of this modality. During production, AAV particles accumulate inside the cytoplasm of producer cells. Extracting these particles via cell lysis is a key step before purification. Conventional lysis methods, such as polysorbate 20-based surfactant lysis, indiscriminately break down cell membranes, releasing both AAV and unwanted host-cell contaminants. A significant challenge in downstream processing (DSP) is the loss of up to 70% of viral vectors, with host-cell impurities like residual DNA being an important cause of reduced yield. PCI Biotech's objective is to develop a more selective lysis approach that simplifies DSP, enhances overall AAV recovery, and lowers the manufacturing cost per dose.

Photochemical lysis (PCL) is a novel, light-activated technology that selectively targets the plasma membrane, releasing AAV with minimal host-cell-derived contaminations, especially DNA. PCL uses a GMP-grade amphiphilic photosensitiser that anchors in the plasma membrane. When added at the end of AAV production, it is activated by blue or red light, initiating a localised photochemical cascade that disrupts the plasma membrane while leaving cellular structures like the nucleus intact. This selective action reduces host-cell impurities by over 50% compared to surfactant-based lysis. Here we report the scale-up from HEK293 AAV production in shake-flasks to a benchtop bioreactor using red light illumination, representing a 500x scale-up from initial proof of concept with

adherent HEK293 cells. In this model, photosensitiser removal during DSP and preservation of AAV potency after photosensitiser exposure have been validated. Despite assumptions that some AAV serotypes (e.g., AAV8) are predominantly secreted, we show that PCL improves yield across multiple serotypes, with AAV8 showing close to 100% increase in USP yield in benchtop bioreactor following PCL, strongly outperforming Tween 20. Current efforts focus on optimising PCL for improved net yield in benchtop bioreactors. Success at this scale will pave the way for deploying PCL for research and development purposes. Moreover, we present a compelling case for further scaling PCL to commercial manufacturing using off-the-shelf LED bioreactors, enabling its use to boost AAV yields and reduce manufacturing costs per dose.

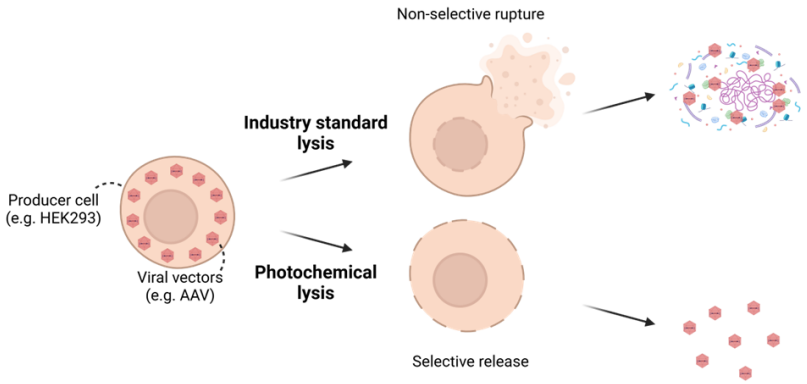

Figure: Cell lysis is an essential step in extracting AAV from producer cells before purification. The industry standard for cell lysis involves the use of chemicals (e.g., polysorbate 20), which non-selectively break open producer cells, releasing AAV alongside host-cell impurities that complicate downstream processing (DSP) — a significant cause of viral vector loss. Photochemical lysis (PCL) is a novel technology that selectively disrupts the plasma membrane of producer cells. The purpose of PCL is to enhance viral vector yield by more targeted and efficient AAV extraction, while simultaneously reducing host-cell impurities and, hence, manufacturing costs per dose.

1475 Strategies for residual DNA Clearance in Adeno associated Virus (AAV) purification process.

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[1]MilliporeSigma, Burlington, MA, [2]MilliporeSigma, Burlington

Host cell Deoxyribonucleic acid (DNA) is a critical process impurity which requires removal during an Adeno-associated purification to mitigate potential oncogenicity and infectivity concerns. In an AAV purification process multiple unit operations can be leveraged either independently or in combination to achieve desired DNA clearance. These unit operations typically include enzymatic digestion, clarification using depth filters and affinity capture chromatography. In addition, pretreatment technologies can also be leveraged as a method to achieve clearance on residual DNA.

In this work, we demonstrated clearance of host DNA using three approaches. In the first approach, we leverage enzymatic digestion as single step for DNA clearance. In second approach clearance levels using an absorptive depth media were established with reduced endonuclease and in third approach we demonstrate leveraging two pretreatment methods to establish achievable DNA clearance.

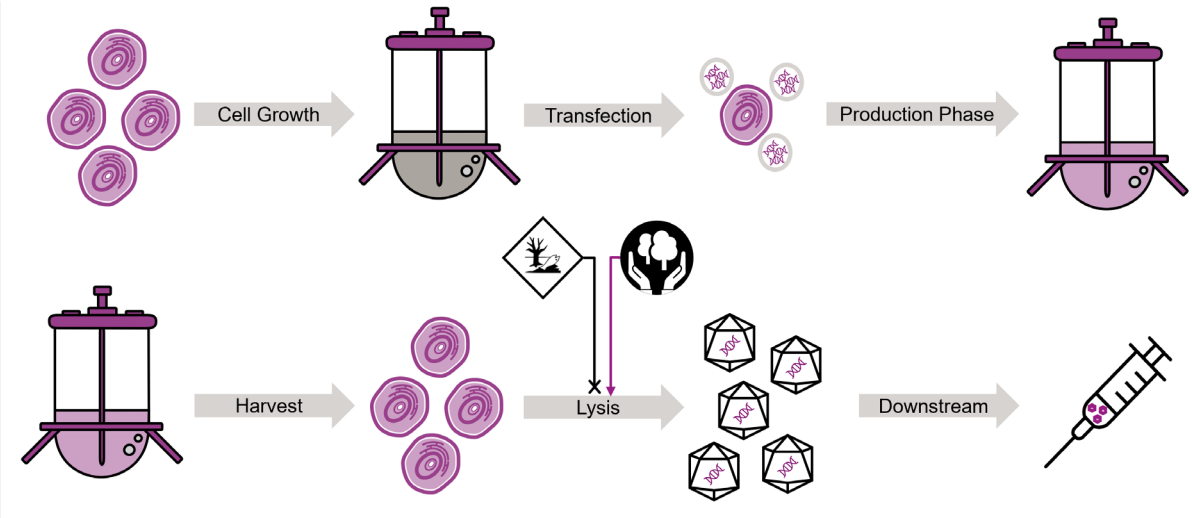
Impact on process, development considerations and Cost of Goods will be provided for the three approaches.

1476 A Functional Alternative to Triton™ X-100 for Cell Lysis in Therapeutic Virus Harvesting

Ewan Stephenson[1], Hwee Sze Ng[2], Abirramee E[1], Xavier Tan[1], Palanivel Balaji[1], Shubham Purwar[1], Johanna Peters[1], Eva Loo[1]
[1]Evonik, Singapore, Singapore, [2]Evonik (SEA) Pte Ltd, Singapore, Singapore

A lysis step is often necessary in the manufacture of cell and gene therapies. Notably for AAV-based therapeutics this is very commonly required, due to the propensity of AAVs to remain intracellular (Figure 1). At the scales necessitated by biopharmaceutical production, the only practical method for this lysis is chemical cell membrane disruption. The most common detergent for this purpose is octyl phenol ethoxylate, known more commonly as Triton™ X-100. Triton™ X-100 is near perfect for this purpose as it is efficient at disrupting membranes to release intracellular contents and yet is mild to proteins and therefore does not disrupt the viral capsid. However, it has become clear that Triton™ X-100 has a significant negative environmental impact due to its degradation products having endocrine disrupting properties which are a danger to aquatic life and potentially humans. Due to this, the EU opted to phase out the use of Triton™ X-100 in large scale, non-exempt, pharmaceutical production.

Due to these concerns, we screened a catalogue of potential alternative surfactants for efficacy along three main performance indicators: cell lysis, viral harvest and foaming. For cell lysis we targeted ~100% cell integrity disruption at 30-60 minutes, for viral harvest we targeted an agent with as good or better post-lysis viral titre compared to Triton™ X-100, for foaming our target was equal—or preferably less—foaming in a bioreactor environment compared to Triton™ X-100. We have identified a candidate in the form of a bio-based surfactant which can be produced by fermentation processes, is biodegradable, and demonstrates low aqua toxicity. We have found that this surfactant is capable of lysing mammalian cells, disrupting membranes almost entirely as early as early as 20 minutes post-exposure. In viral production processes we found this biosurfactant is capable of harvesting an AAV titre equal or exceeding Triton™ X-100 and demonstrates a lower amount of foaming in a bioreactor setup. Evaluation of compatibility with AAV downstream processes (e.g. affinity chromatography) are ongoing.



Graphical Abstract. Lysis agents are added during viral harvest, which is a key step for all intracellular viruses. The current gold standard is aqua toxic, but we demonstrate environmentally friendly alternatives can be equally functional.

1477 An Alternative to Detergent Lysis: Promoting rAAV Release to Media by Optimizing pH, osmolality and harvest timing

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rAAV technology for gene therapy is rapidly improving, increasing the demand for high yield manufacturing techniques. The most widely used process for rAAV production - HEK293 triple transfection - utilizes a detergent lysis step at culture harvest to release rAAV particles from inside the cells. Detergent lysis improves rAAV yield,

but also generates cell debris and releases impurities such as host cell proteins, which, in combination with the detergent itself, can also impact performance of downstream purification steps such as clarification and affinity chromatography. While a majority of the rAAV resides within the cell, some percentage of rAAV is also naturally released into the cell culture media post transfection and prior to harvest. In order to eliminate the need for cell lysis, manipulations to the cell culture, such as increasing the osmolality or pH post transfection, can be used to increase rAAV released to the media. In this study we examined different high osmolality conditions and pH strategies to maximize the release of rAAV into the media. The most favorable method was able to release 95%- 100 % of the rAAV to media with yields similar to conventional detergent lysis. This material could be directly utilized for column purification without the need for a buffer exchange step. Final column purified material from both processes were compared and found to be comparable for important product quality attributes such as empty/ full capsids, residual host cell protein and DNA. This “lysis free” harvest method can be a viable alternative to traditional detergent lysis, and help decrease time and costs of rAAV manufacturing.

1478 Characterisation with Relative Quantification of AAV Capsid Particle Population Heterogeneity Using Charge Detection Mass Spectrometry in Comparison with Orthogonal Technologies

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Recombinant adeno-associated viruses (rAAVs) are key vehicles for gene therapy, delivering therapeutic genes to target cells. During manufacturing, viral particles may lack the full intended insert, instead containing partial sequences or multiple small transgenes, known as partially full and overfull capsids. Assessing analytical tools to enhance characterisation of AAVs is crucial, as impurities can impact clinical efficacy, safety, and immunogenicity. We used charge detection mass spectrometry (CDMS), mass photometry, and analytical ultracentrifugation (AUC) to identify and quantify different AAV capsid populations, addressing the need for improved characterization methods to support development.

Mass analysis of AAV2 and AAV5 with different transgenes (CMV-GFP and CMV-LacZ), including their mixtures was performed on a Waters prototype CDMS instrument, with data processed and visualized using Waters’ in-house software. Ions trapped for 100 ms were recorded, and frequency information was converted to the mass domain. Only ions trapped for the full 100 ms were compiled and binned to generate histograms. The m/z, mass, and charge were binned to obtain the average, sigma, and area of each peak. Mass photometry samples were diluted appropriately before data acquisition on a Samux^{MP} system and analysed using Discover^{MP} software. Capsid populations were identified based on theoretical masses. AUC was performed using a Beckman Optima AUC instrument, and data were analysed with Sedfit software to assign capsid population identity based on measured S-values.

An assessment of the method resolution for both CDMS and mass photometry using commercially sourced model AAV material with known masses were carried out. For both serotypes with the different transgenes, identification and resolution of the varying capsid payload status, (empty, partially full, full, and overfull) was achieved using CDMS, with average mass distributions for each capsid type in good agreement with the expected capsid masses. Empty, full and overfull capsids were detected for the AAV-CMV-GFP samples whereas empty, partially filled, and full capsids were observed for the AAV samples with the LacZ insert. The relative capsid ratios were determined based on the individual capsid ion counts to the total capsid count in the AAV mass range and compared to the results from mass photometry and AUC analysis. Additionally, CDMS revealed distinct charge distributions between the two different AAV serotypes. Specifically, charge populations around 163 and 181, were detected for AAV2 and AAV5, respectively. This charge difference is likely due to a structural change, attributed to variations in the amino acid sequences of the capsid proteins. These variations can affect the surface charge, hydrophobicity, and the presence of specific motifs, ultimately influencing the virus's ability to bind to cellular receptors. Overall, the data herein demonstrates that CDMS can support extended characterisation of capsid population profiles including relative quantification of individual capsid types.

1479 Comparison of Mass Photometry (MP) and Charge Detection Mass Spectrometry (CD-MS) in AAV Characterization

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Adeno-associated virus (AAV), a key vector in gene therapy, requires precise characterization to ensure production quality control and successful clinical applications. Quantification of empty, partial, full capsids is an important critical quality attribute. This work leverages Mass photometry (MP) and charge detection mass spectrometry (CD-MS), two advanced techniques, to characterize 16 different serotypes manufactured by the same process containing the same AAV-CAG-EGFP genome. Overall, AAV with high productivity, such as AAV9 and AAV8, have high full particle ratios and less impurities. MP, an optical scattering-based technique, stands out for its label-free, rapid, low-sample-consumption, and highly efficient analysis. It can quickly differentiate between empty capsids, partially filled capsids, and full capsids based on mass differences. As an optical technique the mass is inferred from the scattered light of capsids with different masses scattering more light. MP has been positioned as the standard for rapid in-process monitoring of AAV capsids, but is limited in resolution, detection of large aggregates and correlating size dispersity to mass. CD-MS is a mass spectrometry technique that determines the mass of particles by directly measuring the *m/z* and charge of ions. CD-MS offers high resolution, high sensitivity, and single-particle analysis capabilities, enabling simultaneous measurement of mass and charge of AAV particles but commercial instruments are not yet widely available. Moreover, CD-MS is able to assess the heterogeneity of the packaging based on the measured peak widths as well as extracting the genomes and measuring them directly, which has not been shown by MP. The serotypes each have different viral protein masses which can be measured by CD-MS and this work shows a valuable comparison to the response of small mass changes to mass photometry. There is tremendous value in comparing CD-MS and MP, not only for profiling E/P/F capsids but also for correlating low molecular weight and aggregate measurements from both techniques. This comparison can help inform best practices for addressing the differences between the methods. Throughout the development and formulation of AAV, both techniques can be used in combination. By leveraging the availability, low sample consumption and in-process monitoring of MP alongside the high-resolution data attained from CD-MS, the combed strengths of these methods offer a more efficient and accurate approach to comprehensive AAV vector characterization.

1480 Rapid and Accurate AAV Capsid Quantification Using Multi-Angle Dynamic Light Scattering (MADLS) for In-Process Gene Therapy Manufacturing

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Adeno-associated viruses (AAVs) are widely utilized as vectors for gene delivery in therapeutic applications. Precise quantification of AAV capsid titers is crucial for ensuring consistent product quality and safety during manufacturing. Current methods such as ELISA, while effective, are time-consuming and involve multiple steps, including incubation and washing, which may limit their suitability for quick paced in-process sample testing.

We conducted a study to evaluate Multi-Angle Dynamic Light Scattering (MADLS) as a rapid and reliable alternative method for measuring AAV capsid titer, particle size, and aggregation. Unlike ELISA, the MADLS assay is a one-step process that requires no sample manipulation and yields results in under 10 minutes. This feature makes MADLS particularly suitable for real-time, in-process testing, enabling faster decision-making during AAV manufacturing.

Our findings show that MADLS provides results comparable to ELISA in terms of accuracy, while significantly reducing the time required for analysis. The method's capability to perform repeated measurements without time constraints further enhances its utility in ensuring consistent product quality across multiple stages of the production workflow. Additionally, MADLS eliminates the need for complex sample preparation, thereby reducing the potential for operator-induced variability.

The adoption of MADLS in AAV manufacturing reflects the growing need for fast, precise, and simple nanoparticle characterization in gene therapy, pharmaceuticals, and medical applications. By streamlining key analytical processes, MADLS enhances manufacturing efficiency, accelerates production timelines, improves process control, and ensures consistent product quality, ultimately enabling the faster delivery of high-quality gene therapy products to meet patient needs.

1481 Highly Accurate Direct Mass Measurement of Extracted AAV Genomes Coupled with Intact Capsid Analysis to Assess Sequence Integrity and Viral Protein Ratio by Charge Detection Mass Spectrometry

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Recombinant adeno-associated virus (rAAV) is a non-pathogenic virus that has shown continued success as a gene therapy vector. Previous studies have shown charge detection mass spectrometry (CD-MS) can resolve and quantify empty, partial, and full capsids. More recently, CD-MS characterized an AAV8 construct with multiple vector packaging and found that, due to the heterogeneity caused by the stochastic nature of viral protein incorporation, intact mass measurements cannot resolve peaks with less than a 700 nt difference between two packaged sequences. Through a combination of throughput and resolution advances we present a new assay for the accurate mass measurement of packaged DNA. Previously, mass measurements by both CD-MS and Mass Photometry have determined the mass of the packaged contents by calculating the mass difference between DNA containing peaks and the empty capsid peak. This measurement relies on the assumption that the protein mass of the DNA containing capsids and the empty capsid are equal, which recent data suggest is not the case. This new assay allows for a more complete understanding of the nature of packaging and confirmation that expected gene of interest is packaging in capsids centered around the correct viral protein ratio.

In addition to a more complete understanding of the packaging of AAV the direct mass measurement of the DNA is highly accurate and can be correlated to a sequence length with a <1% uncertainty. The high resolution measurement of the DNA has also shown peak widths far narrower than the protein heterogeneity intrinsic to intact capsids. As a consequence multiple vector packaging can now be quantified below the previous 700 nt limit.

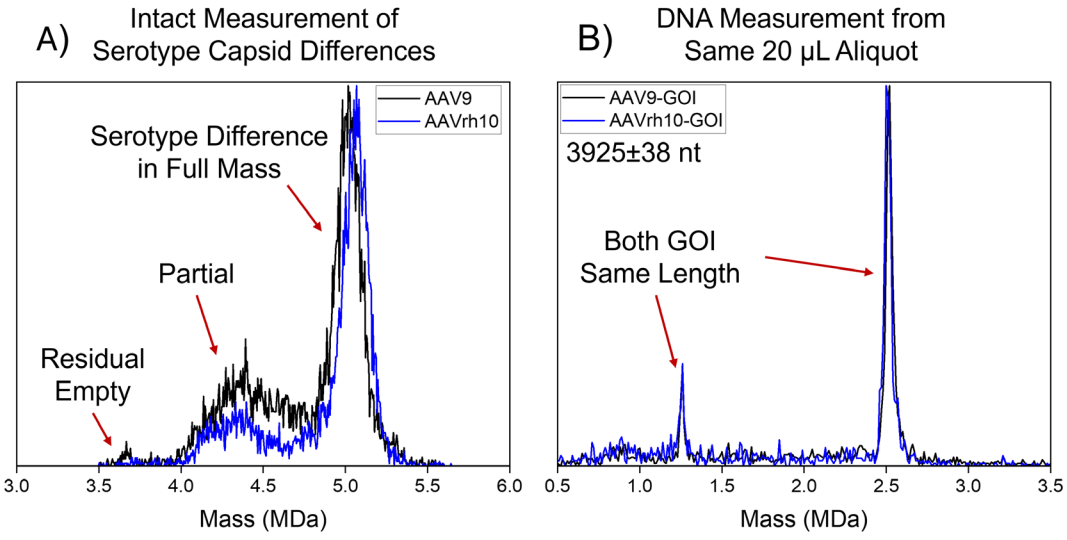

Figure 1. CD-MS Mass measurements of a) two AAV serotypes AAV9 (black) and AAVrh10 (blue) with the corresponding CD-MS mass measurements of the extracted DNA of the two serotypes intact GOI, AAV9 (black) and AAVrh10 (blue) in b).

1482 Direct Monitoring of rAAVs in Crude Cell Lysate and Conditioned Medium

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Recombinant adeno-associated virus (rAAV) has been attracted as a gene therapy vector. rAAVs are mostly produced by the transfection of three plasmids with human or insect cells. The rAAVs produced by these processes usually contain both full particles (FP) that encapsidate a designed full-length genome and empty particles (EP) that have no encapsidated genome, and sometimes contain partial particles that encapsidate an incomplete genome or over-packaged particles which encapsidate the genome more than FP. The particle besides FP are considered as product-related impurities and must be removed; however, it is difficult to completely remove these impurity particles because their outer shell, the capsid, is almost identical to FP. Thus, several researchers have studied to improve the percentage of FP to the total particles at the production stage by optimizing plasmid ratio, the transfection pH, production pH, complexation time, viable cell density at transfection, and transfection reagent to DNA. To promote these upstream optimizations, it is required for the method which can immediately analyze genomic titer and FP ratio of rAAV without performing subsequent purification steps.

Here, we established the direct analysis method for identifying FP and EP and quantifying the F/E ratio and genomic titer of unpurified rAAV in crude cell lysate and conditioned medium. Our established method can quantify the F/E ratio of rAAV in cell lysate, with a good correlation by linear fitting, with a slope of 0.892 and an R^2 value of 0.993, which is almost same accuracy for purified sample with a good correlation with linear fit (Slope = 0.914, R^2 = 0.993). The rAAV particle counts in the cell lysate which were obtained also showed an excellent linearity, with a range of 3.91×10^9 to 7.00×10^{11} vg/mL, the R^2 value of 0.998, and the %CV within 25%, indicating that our established method can estimate the genomic titer. Then, we analyzed the crude cell lysate which is a culture medium containing post-transfected cells producing rAAV to which lysis buffer containing a surfactant is added. For crude cell lysates that produced rAAV2, rAAV8, and rAAV9, the F/E ratios of rAAV2, rAAV8, and rAAV9 were determined to be 9.8%, 20.3%, and 24.8%, respectively, and genomic titers were 2.10×10^{11}vg/mL, 3.21×10^{11}vg/mL, and 2.10×10^{11}vg/mL, respectively. Finally, the analysis of conditioned media producing rAAV8 at 24, 48, and 72 hours post-transfection (hpt) showed an increase in the FP count was observed in a time-dependent manner, whereas the F/E ratio was higher at 72 hpt compared to that at 48 hpt. For the conditioned medium of rAAV8 at 72 hpt, genomic titer estimation resulted in 2.30×10^{11} vg/mL. Compared with the cell lysate collected at 72 hpt, the genomic titer in the conditioned medium was 71.7% of that in the cell lysate. In previous studies, the proportion of rAAV8 present in the conditioned medium relative to the cell lysate was reported to be 70-85% of rAAV8, which is consistent with our obtained values. These results demonstrates that our established method can quantify unpurified rAAV and have potential to facilitate the optimization of rAAV production process, including screening of production conditions.

1483 Real-Time Monitoring of AAV Empty:Full Capsid Separations using Near Infrared High-Precision Tunable Laser Spectroscopy

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Adeno-associated virus (AAV) vectors are a cornerstone of gene therapy, but their efficacy and safety depend heavily on the ratio of empty-to-full capsids. Traditional analytical methods for monitoring this critical quality attribute (CQA) during purification processes, such as ultracentrifugation and mass photometry, can be time-intensive and laborious, while rapid methods such as AEX-HPLC and SEC-MALS can typically not resolve partial from full capsids. Here, we present a novel approach utilizing Near Infrared (NIR) High-Precision Tunable Laser Spectroscopy (HPTLS) for real-time monitoring of AAV empty-to-full capsid ratios using chromatography. Nirrin Technologies' proprietary HPTLS technology enables high-resolution spectral separation of capsid species, providing quantification of full and empty capsids in real-time. This method eliminates the need for extensive

sample preparation while maintaining accuracy comparable to traditional methods. The integration of HPTLS into downstream bioprocessing workflows enhances process control, enabling rapid decision-making and reducing production timelines. This work demonstrates the potential of HPTLS to transform AAV purification through non-invasive, real-time analytics.

1484 Leveraging TruTiter™ Technology to Overcome Thermal Stability Challenges in Engineered AAV Quantitation

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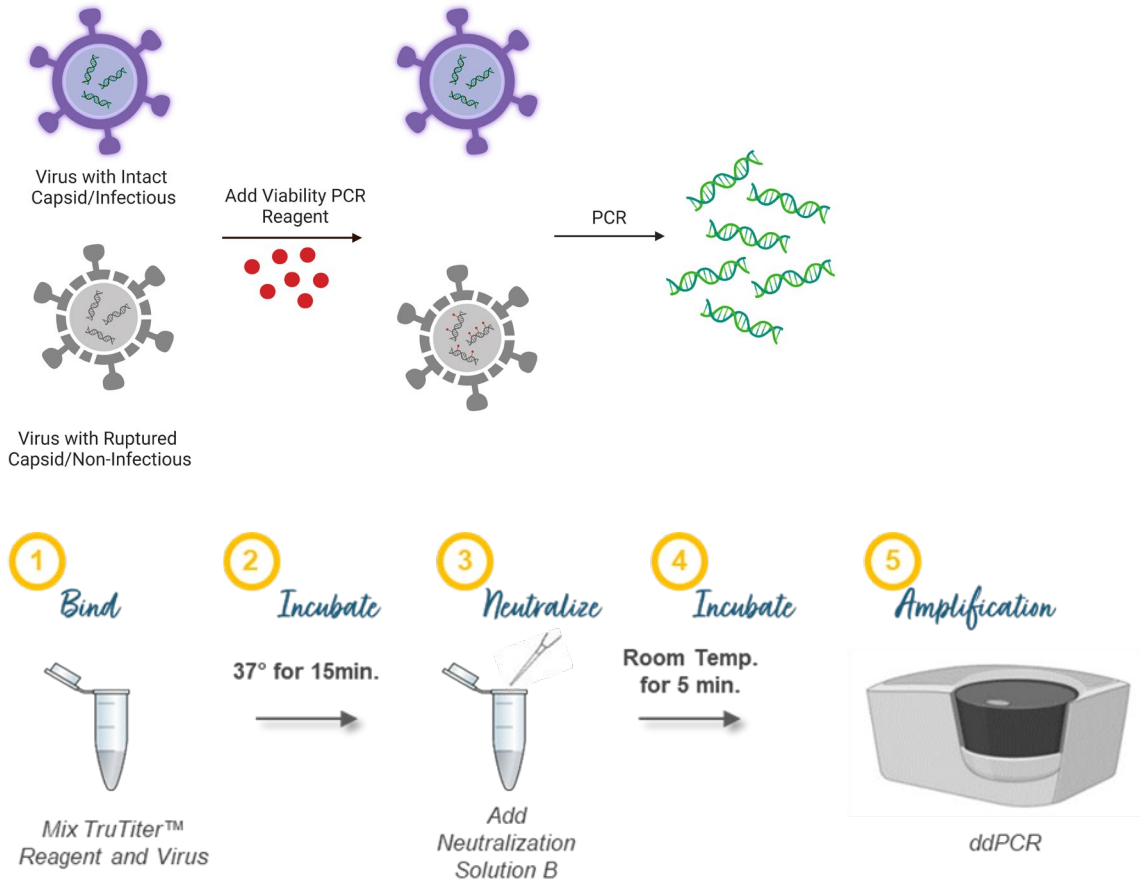
Adeno-associated virus (AAV) is one of the most commonly used vectors for gene therapy delivery. One of the main challenges for AAV gene therapy is the development of effective quality control and quality assurance protocols, as accurate quantification and characterization of AAV production batches is essential for proper clinical dosing. Current protocols are often inadequate, leading to a high degree of lot-to-lot variability, often resulting in non-optimal trial outcomes due to inaccurate determination of functional gene dosage.

Present methods of AAV characterization rely on a combination of mammalian infectivity assays and digital PCR coupled with DNase pre-treatment. These approaches are often cumbersome and lack the precision needed for accurate determination of viral titer. The workflow described below uses a novel small molecule (Viability PCR Reagent) pre-treatment approach to estimate the functional AAV titer in a viral preparation. The Viability PCR Reagent, owing to its small size, has enhanced accessibility to exposed nucleic acids from damaged viral or non-viral sources. Furthermore, use of this reagent suppresses the PCR signal more consistently from such exposed nucleic acids, allowing for a more precise determination of functional AAV titer. This new platform, called TruTiter™, enables users to quantify AAV preparations in a more robust and accurate manner.

Engineered AAV capsid variants are often used in gene therapy approaches to escape immune system detection and degradation, an effect commonly seen with WT AAV variants. In this study, we used TruTiter™ technology to quantitatively titer four AAV2 engineered capsid variants. For all four variants, the TruTiter™ workflow resulted in higher titers compared to a traditional DNase-pretreatment workflow. Using digital PCR, TruTiter™ treated samples exhibited two to four-fold higher gene titers compared to the same variant treated with DNase, resulting in a quantitative measure that was closer to the expected value. We hypothesized that the decreased titer observed with DNase-treated samples was due to the potential reduced thermal stability of the engineered AAV2 capsid variants. To test this, SYPRO Orange dye was used to perform differential scanning fluorimetry (DSF), which can differentiate general capsid stability between AAV variants. Using DSF, clear differences were observed between WT and engineered AAV2 capsids.

Overall, this data suggests that the TruTiter™ workflow can better address the challenges associated with the accurate titering of AAV capsid variants compared to traditional workflows.



1485 Quantification of Full, Empty, and Partial Particles of AAV8 Reference Standards From Multi-Lab Study

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Background: Gene therapy is rapidly emerging as a leading field in the biopharmaceutical industry, with Adeno-associated virus (AAV) at the forefront due largely to its safety and efficacy. These factors have spurred an increase in new technologies and new applications for existing technologies used for biophysical analysis of AAV, specifically for analysis of empty, full, and partial capsids. However, the lack of well-characterized standards has hindered the adaptation of many of these new technologies. Because limitations exist for every well-established AAV empty/full characterization methods, all the commercially available AAV materials, which are typically only analyzed using one method, lack characterization data representative of these innovative methods. By not having this benchmark material, development and adoption of new methods has been hampered.

Results: In this study, AAV8 (Empty Capsids) and AAV8 (Full Capsids) standards, which will be made available to the public, were analyzed using six different methods, Analytical Ultra Centrifugation (AUC), Size-Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS), Charged Detection Mass Spectroscopy (CD-MS), Cryogenic Electron Microscopy (cryo-EM), Mass Photometry, and UV-vis. The samples were analyzed in a collaborative study with five participating laboratories. Herein, we present and compare data from these studies. Data from each method show that the AA8 (Empty Capsids) and AAV8 (Full Capsids) do indeed contain

predominantly empty and full capsids, respectively. In addition to empty/full capsid analysis, AUC, CD-MS, and Mass Photometry were able to successfully quantify partially filled capsids. The AAV samples were also analyzed by other biophysical methods, including Peptide Mapping, Next Generation Sequencing (NGS), Digital PCR (dPCR), and Capillary Gel Electrophoresis (CGE), to further characterize these particles. Results from these analyses provided quantitative data on the following attributes: capsid titer, genomic titer, capsid purity, aggregation, capsid identity, genome integrity, and genome identity. The totality of data collected from these various tests, which measure various critical quality attributes, will be presented.

Conclusion: Thorough characterization of AAV reference standards was completed in this study. The data gathered in this study, in conjunction with the public availability of this material, has the potential to greatly help advance the adaption of new AAV technology, by providing material that can be used for accurate assessment of new or existing methods. These data can be useful to early-stage researchers and commercial manufacturers alike, as these standards can be used from early development to validation and through commercialization. We hope that these studies can be used to leverage the advancement of additional new technologies to further support AAV development. Furthermore, many of the methods assessed in this study are commonly used for characterizing other gene therapy products, such as Lentivirus, which further expands the application of this study.

1486 Comprehensive Analytic Tools Comparison for Empty, Full, and Partial Genome Characterization of Adeno-associated Viruses Show Need for Parallel Assay Verification

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Recombinant adeno-associated virus (rAAV) gene therapies are most commonly produced in mammalian HEK cell lines using transient triple transfection methods leading to nonhomogeneity in the final drug product. Empty and partially-filled viral capsids have been identified as major contaminants in the production method and could have adverse effects upon administration. It is important to characterize these contaminants in a fast and robust manner to accelerate the manufacturing process. We characterized AAV produced by three common methods: adherent HEK cells with iodixanol ultracentrifugation, adherent HEK cells with affinity chromatography, and suspension HEK cells with affinity chromatography. We subsequently tested AAV enrichment through cesium chloride (CsCl) ultracentrifugation and anion exchange chromatography (AEX). Herein we compare different analytical and long-read sequencing assays to identify full-empty capsid ratios and confirm genomic integrity of our AAV gene therapy products. We highlight differences between the methods and the need for parallel verification to characterize AAVs.

Several analytical methods were compared to assay empty-to-full capsid ratios as compared to the current gold standard method of analytical ultracentrifugation (AUC): mass photometry (MP), vector genome and capsid titers, and transmission electron microscopy (TEM). We show that MP had the highest qualitative correlation, genome/capsid titers gave high qualitative correlation but suffered from poor quantification, and TEM had low concordance as compared with AUC. Notably, we show that the transfection system and purification methods contribute to varying levels of empty capsids in the rAAV gene therapy. The quantitative disagreement between the methods show the need to pre-qualify the assay with suitable AAV controls when determining empty-full ratios, and supports the use of MP as a faster and cheaper method of characterization.

We also showed the utility of long-read sequencing to assay empty-full capsid enrichment of vectors. Empty AAV capsids were removed from our gene therapy product using either CsCl ultracentrifugation and fractionation or AEX. Individual fractions as well as the final pooled sample were analyzed using PacBio Single Molecule Real Time (SMRT) sequencing. Long-read sequencing methodologies have a tendency to overrepresent shorter genomic fragments, and we needed an orthogonal assay of alkaline agarose gels to confirm genomic integrity of the our AAV samples. Additionally, these data offered the ability to improve the empty removal process and highlighted the difficulty of enrichment of full length AAV genomes from partial AAV genome containing capsids.

Characterization remains challenging for rAAV for gene therapy due to the high nonhomogeneity of the final drug product. We comprehensively compared different AAV upstream, downstream, and analytical methods. Our data supports the need to add suitable controls, prequalify assays, and run orthogonal or parallel methods to characterize the rAAV drug product.

1487 Development of a High Throughput Flow Cytometry *in vitro* Relative Potency Assay as an Analytical Tool to Support rAAV Process Characterization

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In an ever-changing landscape of manufacturing optimization for recombinant adeno-associated virus (rAAV) vectors, a high throughput, quantitative process characterization method is required for efficient confirmation of control over the manufacturing process. Specifically, confirmation of similar potency is necessary to support comparability of processes for drug product use in the clinic. Relative *in vitro* potency assays can directly compare vectors prepared from two processes. However, *in vitro* potency assays are often product specific and necessarily slow to develop and implement. Here, we present the development of a high throughput flow cytometry based *in vitro* potency assay to detect vector derived eGFP expression, and successful implementation of the assay to quantify relative potency between two different manufacturing processes.

Forge Biologics has previously discovered that decreasing the size of the pEMBR™ Ad Helper plasmid increased rAAV yield. In order to assess the biological activity of vectors produced from pEMBR™ 1.2 and pEMBR™ 2.0 Ad helper plasmids we successfully developed an AAV9.eGFP *in vitro* relative potency assay. The assay determines the potency of AAV9.eGFP through quantification of eGFP expressed by cells after viral transduction. Briefly, Lec2 cells were transduced with AAV9.eGFP vector in serum-free media then harvested 48 hours post-transduction for a flow cytometry fluorescein isothiocyanate (FITC) channel fluorescent readout denoting eGFP positive or negative cells and median fluorescence intensity. Relative potency determination was calculated from four-parameter logistic (4PL) log(dose)-eGFP percent positive response curves using PLA 3.0 software.

Assay development evaluated multiple parameters including (i) dose levels, (ii) harvest conditions, (iii) plate coating, (iv) transduction conditions, and (v) cell line screening. System and sample suitability acceptance criteria were confirmed for (i) linearity across nine dose levels, resulting in an $R^2 \geq 0.90$, (ii) accuracy, confirmed by quality control fluorescent microspheres, (iii) precision, demonstrated by percent eGFP positive coefficient of variation across three biological replicates, (iv) specificity, demonstrated by the non-transduced percent eGFP positive $\leq$ 0.05%. The samples evaluated had a similar biological response, thus demonstrating equivalent potency estimation using PLA 3.0 software. The confirmation of similar potency supports the transition from pEMBR™ 1.2 to pEMBR™ 2.0 Ad helper plasmid to increase rAAV yield.

In conclusion, the herein presented potency assay can support future process characterization with AAV9.eGFP and would only require minor modification to translate to other AAV serotypes. This robust platform assay data can be leveraged as a surrogate process characterization for clients' who switch to the FUEL™ platform, Forge's new AAV manufacturing platform that incorporates pEMBR 2.0, our modified rep/caps, and foundation Ignition cells™ for more efficient manufacturing at various stages. In a fast-paced field with constant research breakthroughs, an actionable, rapid system in place to confirm similarity will save time and money when there are process changes, design changes, and molecular breakthroughs in the future.

1488 Smartphone-based Titration of AAV Vector Preparations

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Adeno-associated virus (AAV)-based vectors have gained widespread recognition for their potential in gene therapy, thanks to their exceptional safety profile and ability to sustain long-term gene expression. With over 130 clinical trials and eight approved gene therapy products, AAV has emerged as one of the most reliable and versatile tools for delivering therapeutic DNA directly into living organisms. The AAV expression system offers several advantages, including efficient transduction, broad tropism, stable high yields, and compatibility with gene-editing components. Recombinant AAV is typically produced through the co-transfection of 293 cells with vectors containing an ITR-flanked expression cassette, adenoviral helper sequences, and AAV Rep/Cap genes. Successful application of a recombinant AAV vector relies on knowing the titer of the AAV preparation, as it permits calculation of the multiplicity of infection, which can influence the final expression level of the recombinant protein in transduced cells. Quantitative real-time polymerase chain reaction (ddPCR or qPCR), ELISA, cell-based infectious unit assays, and transmission electron microscopy (TEM) have all been used to determine AAV titers. However, these methods are time-consuming and labor-intensive, with time-to-results ranging anywhere from two hours to multiple days. In this work, we present an iOS and Android-compatible smartphone application that analyzes a serotype-specific lateral flow assay and can deliver particle number values in 10 minutes. The two-step assay consists of adding a small amount (20 µl) of AAV-containing supernatant to the lateral flow device, followed by imaging of the device and analysis of the resulting signal intensity using a smartphone. Densitometric analysis of the observed bands is performed by the intuitive GoStix Plus software, which compares the results to an automatically downloaded, lot-specific standard curve (R2 greater than 0.990). The result is a GoStix Value that, like an IFU or qPCR assay, can be used to normalize virus stocks before being used for transduction. To demonstrate the utility of GoStix Plus assays as compared to ELISA, qPCR, and infectious unit assays, we used a ZsGreen1-expressing AAV2 vector. The tests demonstrated that GoStix titer values fall within three-fold of current titration methods with coefficients of variation of less than 15%, indicating comparable precision to ELISA-based approaches. In addition, we were able to quantify AAV in both crude and purified preparations, with detection ranging from 5 × 10^8 to 1 × 10^10 particles/ml. The simplicity of the assay facilitates easy monitoring and optimization of AAV production processes to ensure consistency and confidence in downstream applications. In summary, this highly convenient, lateral flow-based titration technology can quantify AAV vector preparations in approximately 10 minutes, saving time, reducing expenses related to labor and materials, and accelerate AAV vector production and application.

1489 Quality analysis and biological evaluation of AAV particle derived from AAV stable Producer cell line

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**Corresponding Author*

Introduction: Plasmid-polymer complex transfection (Triple Transfection) method is widely used for the production of recombinant adeno-associated virus (AAV). On the other hand, we are developing a cell line in which the genes necessary for AAV production is integrated into the chromosome of human cell line, AAV stable Producer Cell Line (PCL), which does not require huge number of plasmids and transfection reagents for the AAV production. Because AAV production using the developed PCL is novel method, in this study, we attempted to confirm the equivalence of the quality of the particles and their biological titer based on the comparison of AAV produced by the Triple Transfection (TT) method and PCL.

Methods: PCL or TT derived full particle of AAV5 (GOI is GFP) purified by ultracentrifugation were used. The size distribution of the AAV particles were determined by analytical ultracentrifugation (AUC). The viral protein (VP) stoichiometry was analyzed using capillary gel electrophoresis. The biological titer was evaluated by exposing the same number of particles to HeLa cells and measuring the positive rate of GFP.

Results and Discussion: The sedimentation coefficients of AAV produced by TT and PCL obtained from AUC were the same, that means the encapsuled DNA length and shape of AAV were equivalent to each other. The value was consistent with the theoretical one based on the designed GOI length, suggesting that PCL could package GOI

into AAV correctly. The results from the capillary gel electrophoresis of AAV produced by TT and PCL showed that VP stoichiometry were similar to each other, indicating PCL produced the three VPs, assembled them in the same ratio as in TT method. In addition, GFP positive rate showed transgene expression was equivalent.

Conclusion: This study suggests that AAV particles produced by PCL have the same biological function and quality as AAV produced using TT method. PCL based AAV production is expected to be an alternative to TT method.

1490 Tailored AAV Solutions: AAV ELISA Kits and Customized Variant Kit Development

Hannah Flaherty, KACTUS, Waltham, MA

Adeno-associated viruses (AAVs) are indispensable tools for gene delivery, with diverse serotypes and engineered variants targeting various therapeutic applications. Accurate AAV capsid titer determination is critical for clinical use, yet the weak immunogenicity of AAVs poses challenges in generating high-quality monoclonal antibodies (MAbs). To address this, KACTUS Bio has developed a proprietary immunization scheme, significantly enhancing anti-AAV serum titers across multiple serotypes, including the notoriously low-immunogenicity AAV2. This breakthrough enables the production of premium monoclonal antibodies, forming the foundation of KACTUS's robust AAV ELISA kits.

These kits exhibit exceptional precision, broad quantification linear ranges, and superior signal-to-noise ratios compared to leading competitors. Stability testing confirms their reliability under varied conditions, and their intra- and inter-assay precision consistently meet stringent CV thresholds below 10%. Additionally, the kits maintain high recovery rates, ensuring minimal interference from assay matrices. KACTUS's expertise extends to customized AAV ELISA kits tailored to specific variants, providing gene therapy companies with reliable solutions for precise AAV quantification.

1491 Development of *in vitro* methods for the analysis of TLR9 stimulation by AAV vector genomes.

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Pro-inflammatory innate immune responses to the AAV genome are significant obstacles to successful, long-term transgene expression and re-dosing efforts. Engagement of particular AAV genomic components and Toll-like receptor-9 (TLR9) leads to the production of Type-I interferons and inflammatory cytokines that enhance dendritic cell-mediated T cell activation. Activated cytotoxic T cells ultimately promote loss of transgene-expressing cells. Given the imperativeness of assessing AAV genomic immunogenicity prior to patient administration, rapid and reliable *in vitro* methods to achieve this goal are needed. In this study, we utilized a modified AAV receptor-expressing, murine macrophage cell line (RAW264.7-AAVR) and primary human peripheral blood mononuclear cells (hPBMCs) to measure TLR9 activation via flow cytometry following stimulation by AAVs harboring different amounts of known TLR9 agonists in their genome using three separate approaches.

In the first approach, we sought to determine whether reduction of Ik-B-alpha protein, a negative regulator of NF-kB that is rapidly degraded following TLR9 stimulation, in RAW264.7-AAVR cells could be used as an indicator of TLR9 stimulation. Following kinetic addition with AAV9 genomes at MOI of $1x10^7$, IkB-alpha protein was reduced

by 60% as early as 1 hour following vector genome stimulation, and the extent of reduction was dependent on the amount of TLR9 agonists present within the genomes.

We also discovered that the upregulation of inducible nitric oxide synthase (iNOS) could be used as a readout of AAV genome-mediated TLR9 stimulation. Vector DNA with higher agonist concentrations significantly increased both the percentage and expression levels of iNOS in the RAW264.7-AAVR cell line compared to vectors with lower agonist amounts. iNOS expression was upregulated downstream of TLR9 engagement since pre-incubation with the TLR9 antagonist ODN2088 prevented iNOS induction. Extending experimental assessment to human cells, we found that AAV addition to hPBMCs led to production of IFN-alpha and IL-6 in CD11c+ DCs. Similar to the murine macrophage cell line, cytokine expression was directly proportional to the amount of TLR9 agonist in the AAV genome.

Our studies demonstrate that multiple *in vitro* assays can be developed to detect and measure TLR9 stimulation and predict the innate immunogenicity of AAV vector genomes.

1492 Elucidating the Mechanisms of Hepatic induced Tolerance Following Liver-Directed AAV Gene Therapy

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The use of hepatic AAV gene transfer has been shown to result in tolerance induction to the transgene. This tolerance is attributed to the formation of Ag-specific $CD4^+Foxp3^+$ Tregs, and, anergy/deletion of transgene-specific effector $CD4^+$ T cells. We have seen this in our own work where we showed that liver directed AAV gene therapy results in a significant increase in Ag-specific $CD4^+Foxp3^+$ Tregs capable of reversing EAE (*Mol Ther*. Sep 19 2017;doi:10.1016) However, it is currently unknown if $CD4^+Foxp3^+$ Tregs are solely responsible for the induction and maintenance of immune tolerance following liver-directed AAV gene therapy. Interestingly, in other work we showed that liver-directed AAV gene therapy also resulted in a significant up-regulation of CD8 regulatory cells suggesting other regulatory cells may play a role in the induction and maintenance of tolernce.

In this work we investigated whether $CD4^+Foxp3^+$ Tregs are needed to induce and maintain tolerance. We utilized the experimental autoimmune encephalomyelitis model of multiple sclerosis due to previous findings showing $CD4^+Foxp3^+$ Tregs played a role in tolerance induction (*Mol Ther*. Sep 19 2017;doi:10.1016). We also utilized the $Foxp3^{DTR}$ mouse strain to allow $Foxp3^+$ cells to be rapidly depleted upon administration of Diphtheria toxin. Using this model, we were able to determine that $CD4^+Foxp3^+$ Tregs are required to induce/establish immune tolerance via liver-directed AAV gene therapy (Fig 1). However, within 3 days these cells are no longer needed to maintain tolerance and their ablation does not result disease re-establishment (Fig 1). Interestingly, ablation of Foxp3 cells results in a significant increase in CD8 regulatory cells (Fig 1). Elucidating the mechanisms of tolerance induction following hepatic AAV gene transfer will result in the development of improved therapies.

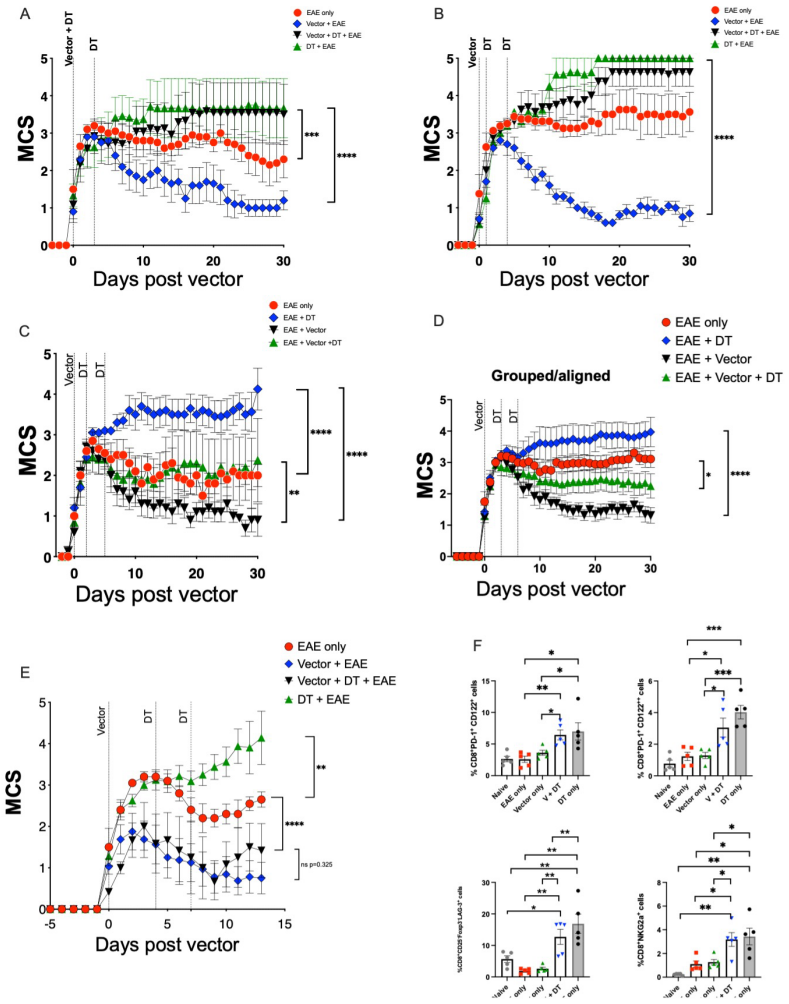

Fig 1: Defining the role of regulatory cells in tolerance induced following hepatic AAV gene transfer. A-E) EAE was induced and Foxp3 Tregs were ablated at various timepoints in relation to vector administration. Tregs are necessary for tolerance onset but not for maintenance. **F)** Treg ablation results in significant up-regulation of multiple CD8 regulatory cell subsets.

1493 Evaluating Immune Responses to AAV Transgenes in THP-1 Cells

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The host immune response poses a significant challenge to safe and efficient systemically delivered adeno-associated virus (AAV)-mediated gene therapies. The immune system recognizes all components of the AAV vector including capsid, transgene, and the protein product of the transgene through many different cellular and molecular sensors. Plasmacytoid dendritic cells (pDCs) recognize unmethylated CpG motifs in the AAV genome via Toll-like receptor 9 (TLR9), exacerbating innate immune responses and priming adaptive immunity for a stronger and more deleterious response. A common approach to mitigate this response is to reduce CpGs in the AAV genome. However, CpG depletion may not guarantee reduced downstream immunity, as nucleotides adjacent to these motifs may influence their binding and activation of TLR9. Wright et al. (2020) proposed formulae to calculate the risk factor (RF) of a DNA sequence that considers the %CpG, the tetranucleotide motifs 5'-NCGN-3', and CpG

methylation, potentially providing a more physiologically relevant measure of the immunogenicity of AAV transgenes. However, this formula is theoretical and has not been validated *in vitro* or *in vivo*. To test the validity of RF scores and provide a biological readout of AAV transgene immunogenicity, we developed an assay using THP-1 cells to compare a number of AAV vectors with varying RF scores.

THP-1 cells express TLR9 and certain pDC markers in their undifferentiated state, supporting their use as an *in vitro* model of pDCs. THP-1 cells exhibit a minimal inflammatory response to AAV alone. However, when AAV is opsonized with IgG to form immune complexes, these enter THP-1 cells at higher rates in an FcγR-dependent manner, subsequently increasing pro-inflammatory cytokine expression. Notably, *CXCL10* expression is triggered by AAV packaged with a transgene as well as by CpG DNA but fails to increase with AAV capsid alone. *CXCL10* expression is abrogated by treatment with E6446, a TLR9 small molecule inhibitor, suggesting this cytokine is transcribed downstream of TLR9 activation. By testing THP-1 cells with AAV vectors containing promoter sequences with a range of %CpG content and RF scores, our data show that *CXCL10* expression is a scalable biological readout of TLR9 activation and most strongly correlates with predicted transgene immunogenicity as calculated by the RF_3 score as compared to %CpG (RF_1) and RF_2.

Future work will use this THP-1 cell-based assay to test the immunogenicity of AAV vectors for limb girdle muscular dystrophy type 2A (LGMD2A) containing codon optimized transgene sequences. The process of codon optimization tends to increase %CpG content and RF scores, therefore codon optimized sequences were also modified to decrease the RF_3 score. Both the codon-optimized vectors and their RF_3-lowered counterparts will be tested with this THP-1 cell-based assay to determine if this strategy can diminish the inflammatory response to the AAV genome. To further validate of this assay, the top candidates will be tested in mouse models of LGMD2A to measure the extent of the immune response, levels of transgene expression, and the therapeutic effect on the phenotype of these mice.

1494 Immunosuppression with αCD20 and Rapamycin Limits Adaptive Immune Responses to AAV6.2 and Permits Unimpeded Redelivery to the Mouse Respiratory Tract

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RATIONALE: Host immune responses to adeno-associated viruses (AAV) have emerged as a primary impediment to effective gene therapy. These immune responses include anti-AAV neutralizing antibodies and CD8+ T-cell responses which have been observed to block repeated vector transduction and reduce the duration of transgene expression, respectively. Accordingly, there has been extensive work published on the nature of immune responses to systemically delivered gene therapy vectors and the development of strategies to mitigate the risk of immunological rejection. However, our understanding of immune responses to inhaled AAV gene therapy is less well developed. The lung is a unique mucosal niche and site of frequent pathogen encounter that has evolved mechanisms such as resident memory B (B_{RM})- and T (T_{RM})- cells which may present unique challenges for gene therapy vector delivery as compared to systemic administration. We therefore sought to describe the lung-resident immune response to respiratory administration of AAV in mice and to evaluate immunomodulatory for their ability to prevent priming of anti-AAV immune responses in the lung and permit redosing with sustained transgene expression.

METHODS: Using BALB/c mice, we first evaluated the immune response to intubation-mediated delivery of $1x10^{11}$ vg of lung-tropic Adeno-Associated Virus serotype 6.2 (AAV6.2-GFP). Using intravascular staining of lymphocytes and flow cytometry with MHC-I tetramer staining, we evaluated the formation of antigen-specific (capsid and transgene) lung T-resident CD8+ memory T cells (T_{RM}), total CD4 T_{RM}, and total B-resident memory cells (B_{RM}). We also evaluated the formation of anti-AAV antibodies (IgM, IgG, IgA) in serum and bronchoalveolar lavage fluid (BALF) by ELISA. Using the same assays, we next evaluated several promising immunomodulatory strategies to suppress primary immune responses to AAV6.2 and permit re-dosing, including αCD20, αCD20/tacrolimus, αCD20/rapamycin, αCD40L, CTLA4-Ig, αIL1α/αIL1β, αIFNαR, and IRAK4 inhibition. To validate the efficacy of immunomodulation, untreated or αCD20/rapamycin treated mice were dosed with AAV6.2-GFP, then one month later dosed with AAV6.2-luciferase (repeat dose groups). An additional control group received only AAV6.2-

luciferase (single dose group). Secondary gene transfer efficiency and duration of expression was measured using *in vivo* bioluminescent imaging.

RESULTS: AAV6.2 delivery elicited vector- and transgene- specific lung T-resident CD8+ T_{RM}. Airway delivery also induced anti-AAV humoral responses in serum (IgG, IgM) and BALF (IgG, IgA, IgM). Among the immunomodulatory strategies tested, αCD20/rapamycin performed best in suppressing primary immune responses (Figure 1A). Repeat dosing without immunomodulation resulted in a near-complete block to second-round gene transfer. In contrast, treatment with αCD20/rapamycin completely rescued second-round gene transfer, yielding luciferase expression equivalent to that of the single dose group (Figure 1B). Luciferase expression was equivalent and stable for at least 8 weeks in single dose and αCD20/rapamycin repeat dose groups (Figure 1B).

CONCLUSIONS: Combination immunotherapy with αCD20/rapamycin effectively prevents priming of anti-AAV immune responses after delivery to the airway, rescuing secondary gene transfer and facilitating prolonged stable transgene expression.

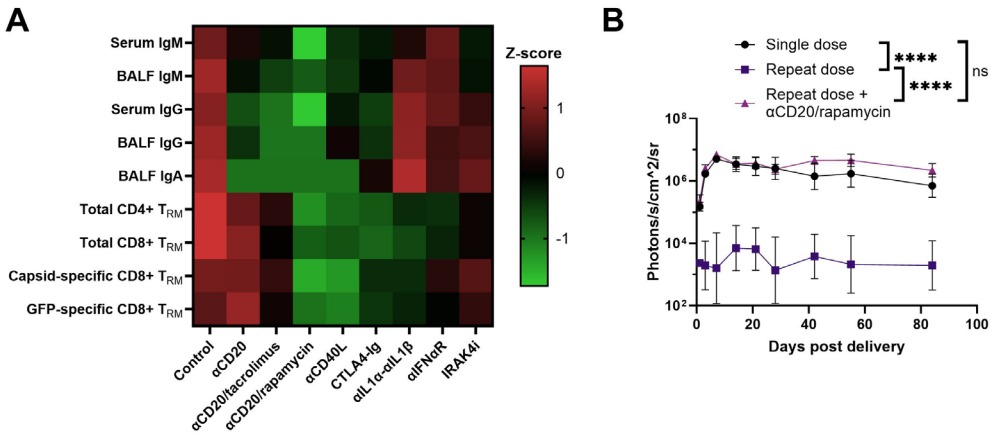

Figure 1. Immunomodulation permits effective redelivery of AAV6.2 to the respiratory tract. **(A)** Heatmap of immunological parameters measured 2 weeks after delivery of $1x10^{11}$ vector genomes (vg) of AAV6.2 via intubation to mice treated with immunomodulatory agents listed on the x-axis. A lower z-score (green) corresponds with a lower immunological response, n=3-5 mice per treatment group. **(B)** Luciferase signal over time after delivery of $1x10^{11}$ vg of AAV6.2-Luciferase to untreated or αCD20/rapamycin treated mice dosed with AAV6.2-GFP one month prior, as compared to single dose controls. ****p<0.0001 by one-way ANOVA after area-under-the curve analysis, n=5 mice per group.

1495 Extracellular Vesicles Reduce Type I Interferon Responses to AAV Gene Therapy in a Human Model of Innate Immunity

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Background/ Rationale: Innate immune responses to adeno-associated virus (AAV) gene therapy vectors have been implicated as potentiators of serious adverse events and are a significant safety concern. The advancement of novel immunomodulatory strategies to avoid immunotoxicity after systemic dosing is critical to expand the therapeutic index of AAV-based treatments. The cytokine interferon-α (IFNα) is a hallmark inducer of innate Type I IFN responses and subsequent adaptive immune responses. We recently reported that extracellular vesicle associated AAV (EV-AAV), a subpopulation generated during conventional vector production, stimulates less IFNα release than standard AAV counterparts. Interestingly, EV-AAV and standard AAV were equivalently taken up by immune cells, suggesting an inhibitory rather than an avoidant mechanism of immune suppression. Phosphatidyl serine

phospholipids can be employed by enveloped viruses to inhibit Type I IFN responses and are displayed on the exterior of extracellular vesicles (EVs), which are commonly produced by diverse cell-types. We therefore hypothesized that highly purified EVs co-formulated with standard AAV vectors would dampen Type I IFN responses in our human peripheral blood mononuclear cell (PBMC) model of innate immunity.

Methods: Using our previously described novel assay to measure IFNα and other cytokines released from human PBMCs in response to antibody-complexed AAV, we compared antibody-complexed AAV2-CAG>GFP (AAV+Ab) to AAV+Ab co-formulated with serial dilutions of EVs. We purified 293T cell-derived EVs by size exclusion chromatography and verified endotoxin concentrations of <5 EU/mL. Next, we isolated human PBMCs from six random healthy donors and exposed them to AAV+Ab preincubated with 2-fold dilutions of purified EVs ranging from 1.2e7 to 6.4e9 EVs/well, or controls.

Results: Concentrations of IFNα released from PBMCs exposed to AAV without antibody-complexation were equivalent to those from untreated PBMCs at baseline, and nearly undetectable. However, AAV+Ab vectors induced significant donor dependent increases in IFNα release above baseline to 4, 10, 70, 275, 374, and 415 pg/mL ($p<0.05$ – $p<0.0001$, N=6). For all donors, IFNα release dramatically decreased in a dose dependent manner with the addition of EVs and reached up to 25-fold decreases at 1.6e9 EVs/well, approaching baseline. We next subjected the three most responsive donor's cell supernatants to Luminex assay of 80 human cytokines. AAV+Ab stimulation increased release of commonly queried IL-6, TNFα, MCP-1, IP-10, and MIP-1α, but EVs did not modulate this effect. AAV+Ab elicited no change from baseline for 18 cytokines, including commonly queried RANTES, IL-8, IL-18, MIP-1β, and MIP-5. Importantly, for 29 cytokines, robust AAV+Ab elicited effects were restored toward baseline by EVs in a dose dependent manner. The largest 11 effect sizes were observed in IFNα1 and IFNα2, which validated our ELISA data, followed by MCP-2, Eotaxin-2, IL-10, IL-3, TNFSF10, IL1-ra, IL-1b, IL-7, and IL-12p40.

Conclusions: This preliminary study begins to reveal an elusive human cytokine signature elicited by AAV and suggests uncommonly queried cytokines for analyte panel refinement. Further, these data suggest a potentially substantial immunomodulatory role for EVs co-formulated and administered with AAV. Ongoing investigations are interrogating the putative role of phosphatidyl serine and other EV components in the observed immunomodulations.

1500 An RNA switch-regulated AAV gene therapy for obesity, insulin resistance, and MASH

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Obesity, insulin resistance, and particularly metabolic dysfunction-associated steatohepatitis (MASH) are major global health challenges with limited effective treatment options. Fibroblast growth factor 21 (FGF21)-based biologics are emerging as promising therapeutics for treating these metabolic disorders, particularly MASH. However, native FGF21 has a short half-life (30 minutes to 2 hours); therefore, its clinical application would require frequent injections (e.g., daily), which may lead to poor patient adherence. Meanwhile, FGF21 analogues with improved PK profiles have been found to elicit the development of anti-drug antibodies (ADAs) at an incidence of 70–90%.

In this study, we developed a regulatable FGF21 gene therapy in which AAV-mediated native FGF21 expression is tightly regulated by an RNA ON-switch system, termed Z^{on}. Z^{on} is an ultra-efficient, protein-independent RNA switch that we developed recently. It has minimal leakiness and enables switch-inducer-dose-dependent tight regulation of transgene expression by over five orders of magnitude. Furthermore, monthly repeated administration of the inducer can consistently induce AAV transgene expression with near-flat kinetics for over one year in mice. Here we applied Z^{on} to control AAV-delivered FGF21 gene therapy in a genetic obesity mouse model (ob/ob). Compared to native FGF21-based biologic which may require daily administration, AAV-mediated FGF21 expression induced every three weeks led to significant improvements in multiple metabolic markers. Z^{on}-regulated

expression of FGF21 at ~100 ng/mL (35-212 ng/mL) for eleven weeks resulted in up to 25.6% reduction in body weight. It resulted in a 39.1% to 64.8% decrease in blood glucose levels as early as one week after induction. Serum insulin was also restored to 2.6 ng/mL, a level comparable to that of wild-type C57BL/6J mice (2.3 ng/mL), three weeks after induced expression of FGF21. Furthermore, multiple liver-related parameters were also significantly improved: (i) the liver-to-body weight ratio was reduced by 52.8%, from 78.9 mg/g to 39.8 mg/g, comparable to that of wild-type C57BL/6J mice (43.4 mg/g); (ii) serum levels of hepatic injury markers ALT and AST decreased by 80.9% and 60.8%, respectively. Serum ALT levels, a liver-specific injury marker, were reduced from 35.0 U/L to 6.7 U/L, approaching the levels in wild-type C57BL/6J mice (4.1 U/L).

These findings demonstrate the potential of this RNA switch-regulated FGF21 gene therapy for the treatment of obesity, insulin resistance, and MASH. In addition, they highlight the potential of the Z^{on} RNA switch as an effective tool for enabling the development of controllable gene therapy for various common metabolic disorders and beyond, expanding the scope of gene therapy applications.

1501 Gene therapy rescues adrenocortical cells from lipid cytotoxicity in mice and humans with adrenoleukodystrophy

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X-linked adrenoleukodystrophy (X-ALD) results from pathogenic variants in the ATP binding cassette subfamily D member 1 (*ABCD1*) gene and impairs transport of very long chain fatty acids (VLCFAs) into the peroxisome for β-oxidation. Affected patients have abnormally high VLCFA buildup in the nervous system, adrenal glands, and testes. An estimated 80% of X-ALD patients develop primary adrenal insufficiency (PAI), but the related pathophysiology remains poorly understood. In this study, we showed that the development of PAI in X-ALD patients directly correlates with significantly higher levels of C26:0-lysophosphatidylcholine (C26:0-LPC), a complex lipid containing C26:0 VLCFA. We demonstrated that exposure of HAC15 human adrenocortical cells to C24:0-LPC and C26:0-LPC significantly reduced HAC15 cell viability. Transient knock-down of *ABCD1* expression in HAC15 cells resulted in a biochemically faithful cellular model of X-ALD-related PAI characterized by VLCFA accumulation and diminished cortisol production. A compensatory increase in expression of cytochrome P450 side-chain cleavage enzyme, which catalyzes the rate-limiting step in the cortisol synthesis pathway, could not overcome the cortisol deficiency potentially caused by reduced expression of CYP17A1 and HSD3B2, key enzymes in cortisol synthesis. Finally, we showed that adeno-associated virus (AAV)9 vector-mediated gene replacement therapy reduces VLCFA accumulation in *ABCD1*-knockout HAC15 cells and therapeutically corrects histopathologic abnormalities in the adrenal glands of *ABCD1*-deficient mice delivered intrathecally. These results significantly advance our understanding of the mechanisms and pathways affected in X-ALD-related PAI and equally importantly, demonstrate that the deficits can be corrected, at least in the murine X-ALD model, by an AAV9-mediated gene replacement therapy that is already in human clinical trial (NCT05394064).

1502 Partial Deficiency Mouse Model of Cobalamin B Class MMA: Systemic AAV9 Gene Therapy Confers Resistance to Metabolic Crisis

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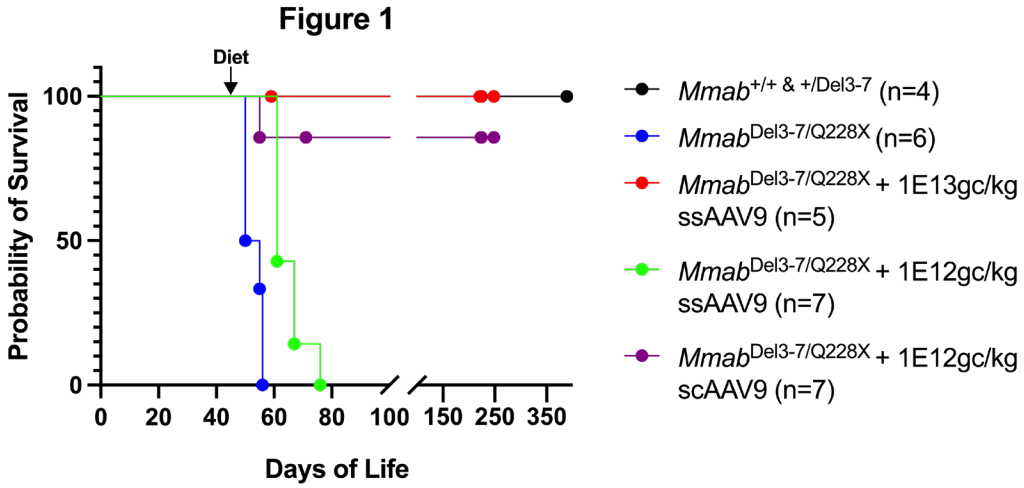
Background: Cobalamin B-type (cblB) methylmalonic acidemia (MMA) is a severe ultra-rare autosomal recessive inborn error of metabolism caused by pathogenic variants in the MMAB gene that encodes the ATP:cob(I)alamin adenosyltransferase (ATR) enzyme. ATR generates 5'-deoxyadenosyl-cobalamin (AdoCbl), the obligate cofactor for the methylmalonyl-CoA mutase (MMUT) reaction. Current treatment relies on supportive care, including dietary management, carnitine supplementation, and intermittent antibiotics. Elective liver (LT) or combined liver-kidney (LKT) transplantation has been offered to severely affected patients as an experimental surgical therapy for the treatment of MMAB.

Methods: Compound heterozygous mice (Mmab$^{Del3\text{-}7/Q228X}$) carrying one nonsense mutation, frequently found in patients (Q234X, Q228X in mouse) and one null allele, deletion of exon 3-7, were characterized and challenged with a diet consisting of a 10-fold increase in isoleucine and valine (Ile/Val) amino acids. These mice were then used in proof-of-concept AAV gene therapy studies. These mice were treated with either a single-stranded (SS) or self-complimentary (SC) AAV9-MMAB vector (NCATS BL2680) systemically at DOL 30 and then exposed to Ile/Val enriched diet 2 weeks post-treatment to assess the efficacy of gene therapy.

Results: Mmab$^{Del3\text{-}7/Q228X}$ displayed normal survival, weight gain, and 1-C^{13} propionate oxidation compared to wild type or heterozygous littermate. Mmab$^{Del3\text{-}7/Q228X}$ mice displayed mild methylmalonic acidemia [MMA](80 +/- 77 uM, ~100x WT), much like patients with attenuated forms of MMAB. When fed the Ile/Val diet, Mmab$^{Del3\text{-}7/Q228X}$ mice demonstrated significant weight loss (-6.2±.8g n=6 over 4 days) and 100% lethality within 5 days, while controls fed the same diet thrived and gained weight. Plasma MMA (pMMA) levels were drastically increased with the Ile/Val diet (1223 +/- 738 uM, ~1500x WT), reflective of severe clinical disease. In a proof-of-concept study, Mmab$^{Del3\text{-}7/Q228X}$ mice were treated with AAV9-MMAB gene therapy at a dose of 1E13gc/kg systemically on DOL 30. 1E13gc/kg SS was sufficient to rescue survival, normal weight gain, and reduced pMMA level on the challenge diet. In a follow-up trial, mice were treated with either an SS or SC vector at a dose of 1E12gc/kg. SC treated animals showed fully rescued lethality with normal weight gain, but SS could not protect lethality (Fig.1). Neither dose conferred any change in oxidation relative to WT controls. Analysis by ddPCR of the 1E12gc/kg treated mice showed no significant difference in vector genome copies between SS and SC AAV treatments (P>.05) in tested tissues except in kidney, where a slight but statistically significant difference (.011±.005 [SS] vs .056±.007 [SC], P=.0029) was noted. Protein expression after 1E12gc/kg showed that both SS and SC AAVs produce MMAB in MMAB$^{Del3\text{-}7/Q228X}$ hypomorphic mice, exceeding WT levels in the liver, heart, kidney, and muscle. Mean MMAB tissue expression values were higher in SC treated animals than in SS treated animals but were only statistically significant in heart (P=.0019) and muscle (P=.0405).

Conclusions: The Mmab$^{Del3\text{-}7/Q228X}$ mouse model can be used in conjunction with an Ile/Val diet to induce a lethal metabolic crisis and is fully rescued from lethality by systemic AAV9 gene therapy. We have also shown that an SC vector can protect against the lethality and growth inhibition of an Ile/Val diet at a 10-fold lower dose than using an SS vector.



1503 STX-Arg1: exosome-based enzyme replacement therapy restores Arg1 activity in vivo and improves lifespan of Arg1-/- mouse model.

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Arginase 1 (Arg1) deficiency (ARG1-D) is a rare genetic disorder caused by the loss of Arg1, the final enzyme in the urea cycle. Current treatment approaches primarily aim to control plasma arginine and ammonia levels. Among novel therapies under development, long-term resolution was only observed in AAV-based gene therapy in preclinical settings. Currently, no approved treatment specific for ARG1-D is available in the United States.

Lately, extracellular vesicles (EVs) have been gaining recognition as a potent therapeutic tool. By using Capricor's StealthX™ platform, EVs were engineered to express human Arg1. In vitro, nanograms of Arg1 carried by EVs were biologically active and able to convert arginine into urea as potent as micrograms of human recombinant Arg1 (rHuArg1). STX-Arg1-in EVs further demonstrated advantage in intracellular delivery of the active enzyme which was not achievable by the rHuArg1. In vivo, STX-Arg1-in EVs were non-toxic and able to restore the arginase activities in the liver of the Arg1-/- mice, which leads to a lowered plasma arginine concentration similar to wildtype mice. Most importantly, STX-Arg1-in EVs expanded the life span of the lethal neonatal Arg1 deficiency mouse model. Taken together, our data suggested StealthX™-engineered STX-Arg1-in EVs have a better safety profile due to extreme low dosage and had great potential to serve as a novel enzyme replacement strategy for patients suffering from ARG1-D.

1504 A Novel *Mmachc* c.80A>G Mouse Model: Insights of Mitochondrial Dysfunction in Skeletal Muscle and Potential AAV-mediated gene therapy for cblC

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**Corresponding Author*

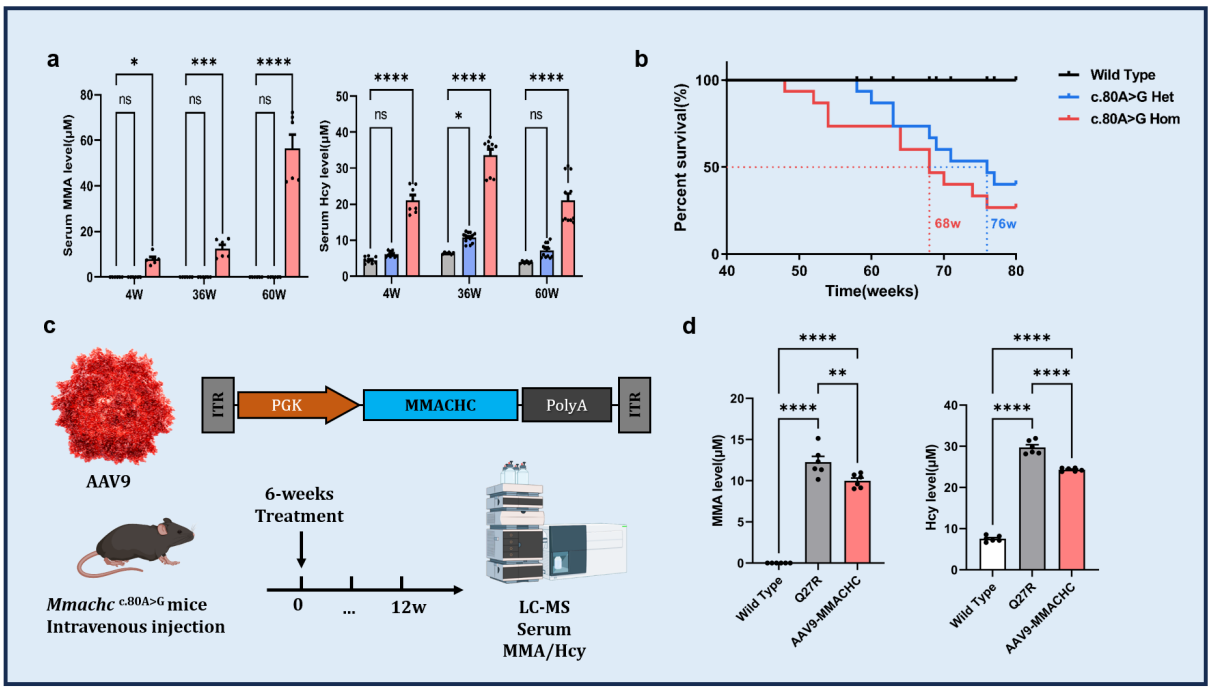
Combined methylmalonic acidemia and homocystinemia (cblC) is an autosomal recessive disorder characterized by aberrant organic acid metabolism. The c.80A>G mutation in the MMACHC gene has been widely documented and linked to cblC phenotypes, but its pathogenic mechanisms remain elusive, as it has not yet been fully validated through functional studies. To explore the underlying mechanisms of this disorder, a murine model carrying the Mmachc c.80A>G mutation was developed. These model animals exhibited markedly compromised survival rates (Figure b), altered blood biomarkers (Figure a), and imaging abnormalities that mirror the clinical manifestations of cblC.

Our current investigation delves deeper into the cblC mouse model, revealing persistently elevated levels of methylmalonic acid (MMA) and homocysteine (Hcy) in blood, urine, and tissues. This phenomenon is attributed to diminished expression of Mmachc. Surprisingly, the c.80A>G mutation leads to the p.Q27R change, yet research indicates that this mutation actually enhances B12 processing capacity. Therefore, the overall deficiency in functional B12 processing emerges as a key factor in the pathogenesis of cblC.

Further analysis revealed that the accumulation of MMA and Hcy in tissues not only results from impaired B12-processing but also contributes to mitochondrial dysfunction, which is particularly pronounced in skeletal muscle. The c.80A>G mouse model exhibited disruption of mitochondrial autophagy, leading to mitochondrial proliferation and impaired ATP metabolism. Notably, MMA was found to be a critical factor in cellular energy stress, causing damage to the mitochondrial respiratory chain and ATPase activity.

To investigate whether MMACHC expression could correct the severe cblC phenotypes in these model animals, we achieved MMACHC expression through intravenous (IV) administration of an adeno-associated virus (AAV) carrying the MMACHC expression cassette. This resulted in a reduction of MMA and Hcy levels (Figure c and d).

Collectively, our findings indicate that the c.80A>G mutation plays a significant role in the mitochondrial pathogenesis of cblC by inducing intron retention, providing novel insights into the underlying disease mechanisms. Additionally, AAV-mediated gene replacement emerges as a potential therapeutic strategy for cblC.



1505 Long Term PK/PD and Preliminary Safety Study of FGF21 Following Intramuscular Administration of KRIYA-497 (AAV1.hFGF21) in Healthy NHPs

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Fibroblast growth factor (FGF) 21 is a 181-amino acid hormone that plays a key role in lipid metabolism, glucose homeostasis, energy expenditure, and thermogenesis. Its effects are mediated by binding to fibroblast growth factor receptors (FGFRs) and the coreceptor β-klotho, activating multiple intracellular signaling pathways.

Several FGF21 analogues are currently in late-stage clinical development and have been designed to improve the pharmacokinetic profile of native FGF21 given its short half-life. These analogues have been studied in numerous metabolic diseases including metabolic dysfunction-associated steatohepatitis (MASH) (Harrison et al. 2024). MASH is an advanced form of liver disease associated with liver steatosis, inflammation, and hepatocyte injury. Progression of MASH can lead to fibrosis and potentially to hepatocellular carcinoma and death. MASH afflicts approximately 40 million people in the United States and European Union, (O'Hara et al., 2020).

KRIYA-497 is comprised of an AAV1 capsid and an expression cassette encoding native human FGF21. It is designed to be administered via intramuscular (IM) injection to provide consistent circulating levels of native FGF21 in MASH patients.

Previous studies in mouse models of MASH indicated that AAV1.mFGF21 treatment resulted in sustained increased circulating levels of FGF21, reversing hepatic fibrosis, halting liver tumor development and counteracting obesity and insulin resistance (Jimenez.et al, 2024).

Healthy cynomolgus macaques received AAV1.hFGF21 at three dose levels with or without immunosuppression (IS). The vector was administered as a single dose on Day 1, with IS on Day -1. Dose groups received varying numbers of injections in the quadriceps, hamstrings, biceps, maintaining consistent vector concentrations within dosing cohorts.

Results:

- AAV1.hFGF21 was generally well tolerated with no adverse changes in body weight, food consumption, clinical observations, hematology, or clinical chemistry
- Robust increases in human-FGF21 protein serum levels were observed in all dose groups and maintained for the duration of the 6-month study.
- Greater than 100% increases in adiponectin were observed across all dose groups, consistent with the role of FGF21 in stimulating adiponectin secretion. These changes align with findings from nonclinical and clinical studies of FGF21 analogues.
- Biodistribution analysis revealed a widespread presence of vector genomes and RNA expression in the treated muscles with low off target expression in the liver.
- Correlation between vector genome levels, vector mRNA expression and circulating protein levels was observed.
- All animals developed expected humoral immunity against AAV1 capsid and transgene with no overt impact on FGF21 expression or detection.
- Low T cell responses restricted to AAV1 capsid, were detected and did not appear related to dose.

The demonstrated pharmacodynamic activity, sustained FGF21 expression, and observed favorable safety profile in NHPs support further development of KRIYA-497 for the treatment of MASH.

1506 A GLP-compliant Toxicology Study of Intravenously Administrated EXG110 for Treating Fabry Disease

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Fabry disease is a X-linked metabolic disorder resulting from deleterious mutations of *GLA* gene which encoding a-galactosidase A (α-Gal A). Due to deficient or reduced a-Gal A activity, the accumulation of its substrates within lysosomes results in cellular dysfunction and systematic organ damages. We developed an AAV-based gene therapy product EXG110 for treating Fabry disease. It comprises an engineered liver-muscle targeting AAV9 variant capsid packaged with an optimized human *GLA* transgene expression cassette. The efficacy of EXG110 has been demonstrated in Fabry mice (*Gla* KO) after a single intravenous administration. Here we presented the GLP-complaint toxicology study of EXG110 in non-human primates (NHP). Adult cynomolgus monkeys with low or no pre-existing neutralizing antibodies against the AAV9 variant capsid were enrolled in the study before the administration of EXG110. One group of vehicle control and 3 groups of animals treated with low, medium and high dose of EXG110 with equal numbers of gender were used. The routine clinical observations, food consumption, body weight, body temperature, ophthalmological examination, hematology profiles, clinical chemistry, coagulation, urinalyses, ECG, blood pressure, respiration, functional observational battery (FOB), immunotoxicity (T cell subpopulation, cytokines, complements, capsid or transgene product specific T and B cell responses), vector clearance in the blood, vector shedding were monitored before and after administration. The plasma was also collected at different time points to determine EXG110-mediated α-Gal A activities in the circulation. Four weeks and 13 weeks after administration, animals were necropsied for gross observation, organ weight, tissue collections and histopathological examinations. The biodistribution of EXG110 in the animals treated

with high dose was studied. The transgene expression at mRNA level in the key tissues were analyzed for all the 3 dose groups. The results revealed general safe profiles with no adverse clinical signs and no histopathological findings in all animals through the 13-week duration. Compared to vehicle control, only transient ALT elevations were observed in the high dose group at Day 8. All EXG110-treated animals generated anti-capsid binding and neutralizing antibodies. No animal had capsid or transgene product specific IFN-γ producing T cells from PBMCs. EXG110 mediated robust and sustained α-Gal A enzyme activities in the plasma of NHP from all 3 dose groups with dose-dependent effect. The biodistribution results showed that liver and heart are the major target organs. The transgene mRNA levels correlated with vector genome DNA in the liver and heart. These safety profiles and superior transgene expression data from NHPs support the clinical development of EXG110 for treating Fabry disease.

1507 Long-Term Efficacy of Genome Editing in Infant Mice With Glycogen Storage Disease Type Ia

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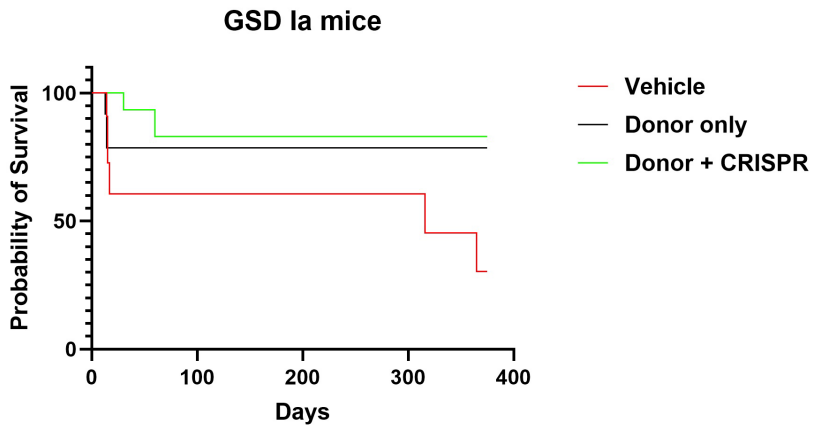
Background: Glycogen storage disease type Ia (GSD Ia) is caused by the deficiency of glucose-6-phosphatase (G6Pase), which causes life-threatening hypoglycemia, hyperlipidemia, and a long-term risk for hepatocellular adenoma and carcinoma formation. Although gene replacement therapy with adeno-associated virus (AAV) vectors has been established, its benefits gradually wain. We developed a genome editing approach to integrate a therapeutic transgene expressing G6Pase, which is flanked by two homology regions of the *G6pc* locus such that it is integrated at a double-stranded break created by CRISPR/Cas9. One AAV vector delivers the transgene expressing G6Pase and a single guide RNA (Donor vector), and the second the *S. pyogenes* Cas9 (CRISPR vector).

Methods: Vectors were administered to 12 day old liver-specific *G6pc* -/- mice with GSD Ia: either Donor + CRISPR vector to achieve genome editing, or Donor-only treatment that was equivalent to gene replacement therapy. The vectors were administered at a relative low dose (Donor, 2E+13 vector genomes/kg; CRISPR 4E+12 vector genomes/kg). Mice were fed a high fat/high sucrose diet, and the efficacy of these treatments was evaluated at Week 52.

Results: Treatment with genome editing (Donor + CRISPR vectors) achieved the highest rate of survival (81%), followed by gene replacement therapy (Donor only; 78%), while vehicle administration achieved the lowest survival (38%; Figure). An interim analysis of 3 mice per group revealed the benefits of genome editing at 52 weeks. Blood chemistry testing revealed the following improvements for treatment with genome editing, in comparison with gene replacement therapy: alanine aminotransferase and low-density lipoprotein cholesterol were significantly decreased, and triglycerides trended lower in the genome editing group. Blood glucose trended higher in the genome editing group. Blood chemistry was not available for two of 3 vehicle-treated mice due to poor health. However, one healthy vehicle-treated mouse had hypoglycemia during a 13 hour fasting test, whereas vector-treated mice maintained normal blood glucose. Analysis of the liver revealed lower liver weight in genome editing mice (4.0 +/- 0.9 g), in comparison with vehicle treated mice (9.8 +/- 1.4 g), demonstrating improvement in hepatomegaly associated with GSD Ia. Donor-only, gene replacement therapy failed to decrease hepatomegaly (liver weight 7.7 +/- 4.2 g). The appearance of vehicle-treated livers was grossly abnormal with multiple very large tumors, whereas the genome editing- and gene therapy-treated livers appeared much more normal (histology is pending). Furthermore, liver glycogen content was decreased by >50% for the genome editing and gene therapy groups, in comparison with the vehicle-treated control group.

Conclusions: This interim analysis demonstrated improved efficacy from genome editing with CRISPR/Cas9 that integrates a therapeutic transgene at a double-strand break in the mutant *G6pc* gene, in comparison with gene replacement therapy with the same transgene, following a 52 week study. Infant GSD Ia mice treated with genome editing had improved blood chemistries, in comparison with gene replacement therapy, and uniquely decreased hepatomegaly in comparison with vehicle-treated control mice. These data support the further development of genome editing to stably treat GSD Ia early in life.



Vehicle: n = 7
Donor only: n = 5
Donor + CRISPR: n = 6

1508 Lipid Nanoparticle Delivered Circular RNA Encoding Ornithine Transcarbamylase (OTC) for the Treatment of OTC Deficiency: A Novel and Sustainable Therapeutic Approach

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Ornithine Transcarbamylase Deficiency (OTCD) is a rare genetic disorder caused by a complete or partial lack of the OTC enzyme, leading to hyperammonemia, severe neurological issues, and potentially lethal outcomes. Liver transplantation remains the only viable therapeutic option, yet it involves considerable surgical risks and potential post-operative complications.

Circular RNA (circRNA), known for its high intracellular stability and low immunogenicity, offers a novel modality for protein replacement therapy. We have developed circOTC-encapsulating lipid nanoparticles (circOTC-LNPs) to drive OTC expression in the liver, as a potential therapeutic approach for OTCD.

We demonstrate that LNPs delivering circOTC with optimized Internal Ribosome Entry Site (IRES) and potent liver-targeting capabilities exhibit robust and sustained expression both in vitro and in vivo. In a conditional OTC knockout mouse model, administration of circOTC-LNPs effectively prevented weight loss and maintained normal blood ammonia levels for over two weeks, whether used preventively or therapeutically. Compared to linear mRNA, circOTC showed significantly extended pharmacological efficacy and superior hepatic safety.

Our findings highlight the potential of LNP-delivered circOTC as a promising therapeutic strategy for treating OTCD, offering a safer and more sustainable alternative to current treatments.

1509 ATP7B mRNA therapy for the treatment of Wilson's disease (WD)

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Wilson's disease (WD) is a rare inherited disorder caused by genetic mutations in the ATP7B gene, which encodes a P-type copper transporter primarily expressed in hepatocytes. ATP7B is responsible for transporting copper from intracellular chaperone proteins into the secretory pathway, facilitating both excretion into bile and incorporation into apo-ceruloplasmin for the synthesis of functional ceruloplasmin. A deficiency in ATP7B leads to copper overload in the liver and other critical organs such as the brain and eyes, resulting in hepatic, neurological, and ophthalmologic symptoms.

Current treatment options for WD, including copper-chelating agents like D-penicillamine and Trientine, have limited efficacy and are associated with numerous adverse effects. Traditional protein replacement therapy is not viable for WD patients due to drug delivery challenges, and viral-based gene therapies have yet to succeed due to the limited packaging capacity of adeno-associated viruses (AAV), neutralizing antibodies, and potential genotoxicity.

We aimed at developing a new treatment for WD using mRNA/lipid nanoparticle (LNP) technology. By screening the proprietary Diversity-oriented Lipid Library (DOLL), we identified liver-targeted LNP formulations that exhibit high efficiency and favorable safety profiles. The top LNP was subsequently used to encapsulate nucleoside-modified and codon-optimized mRNA encoding human ATP7B (hATP7B-mRNA-LNP). Our study demonstrates the therapeutic efficacy of hATP7B-mRNA-LNP in cultured human cells and in a mouse model of WD (Atp7b-/-) that resembles human conditions. Robust protein expression of hATP7B was observed in transfected cells, along with a significant increase in copper-transporting activity. Intravenous administration of hATP7B-mRNA-LNP resulted in robust and sustained expression of the hATP7B protein in livers of mice.

Furthermore, in Atp7b-/- mice, treatment with hATP7B-mRNA-LNP restored the deficient copper transporting function, reducing copper accumulation in the liver by enhancing copper loading onto ceruloplasmin (as evidenced by increased serum ceruloplasmin activity) and promoting copper excretion via feces. Collectively, our findings provide strong preclinical proof-of-concept for systemic mRNA-LNP as a potential disease-modifying therapy for patients with WD.

1510 Rescue of glutaric aciduria type I mice by liver directed gene editing

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Glutaric Aciduria Type I (GA-1) is an inborn error of metabolism with a severe neurological phenotype caused by the deficiency of Glutaryl-CoA dehydrogenase (*GCDH*), the last enzyme of lysine catabolism. Currently, the only treatment option available for patients with GA-1 is lysine restriction, carnitine supplementation and emergency care during acute crisis. Nevertheless, complex movement disorders are still encountered in one-third of patients.

We previously showed that restoring a normal lysine catabolism in the liver of adult *Gcdh-/-* mice via Adeno-Associated Viral (AAV) gene therapy is sufficient to rescue the lethality and the neurological phenotype (Barzi et al. *Sci Transl Med* 2023). However, the therapeutic success was markedly reduced in neonatal mice, most likely due to the dilution of the AAV vector.

Here, we explored a more permanent gene editing solution, integrating a wildtype copy of *Gcdh* into the albumin locus. We injected neonatal *Gcdh-/-* mice with two AAV vectors, one expressing the *S. thermophilus* Cas9 and sgRNA targeting the albumin locus under a liver specific promoter, the other one a homology directed repair template carrying a wildtype copy of the *Gcdh* gene. Applying this treatment, we observed a rescue of the lethality phenotype when putting the *Gcdh-/-* mice on high protein diet (52 weeks, n=9), while all mice in both control groups (individual AAV vectors) succumbed only after a few days (n=2x9). More importantly, the treated mice had

a normal neurological phenotype and were not distinguishable from wildtype mice even after one year on high protein diet. Our results demonstrate that the neurological and metabolic phenotype of *Gcdh-/-* mice can be rescued by gene editing and restoring normal lysine catabolism in the liver.

1511 Alpha-1 antitrypsin correction by in vivo gene editing

June Baik[1], Cathleen Cornell[2], Tadeh Derstepanian[3], Saikat Manna[3], John Bladon[4], Hong Wang[4], Patricia Berthelette[5], Mahmud Hossain[4], Laura Blaha[3], Behnaz Lahooti[3], Rong Luo[3], Cao James[6], Renee Bodinizzo[7], Nathan Westcott[4], Robert Jackson[6], Martin Goulet[4], Ashish Sarode[3], Bailin Zhang[7], Christopher Borges[4], Christian Mueller[8], Tongyao Liu[9], Christophe LECHAUVE[4]
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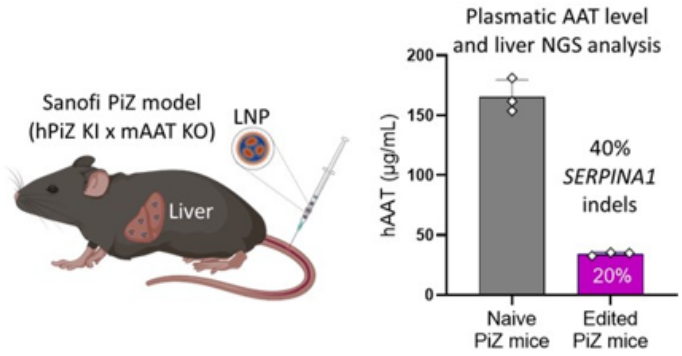
Alpha-1 antitrypsin deficiency (AATD) is an inherited disorder caused by mutations in *SERPINA1*, with >95% of severe clinical cases homozygous for the PiZ mutation (G>A point mutation). The PiZ mutation leads to the accumulation of misfolded AAT within hepatocytes (gain of toxicity) associated with low blood circulating levels of AAT as well as the destruction of alveoli in the lungs over time (loss of function). Considering this genetic condition, AATD is an ideal candidate for in vivo gene correction therapy to restore the normal expression and function of AAT.

To develop gene editing of the PiZ allele, we generated mouse models by introducing one copy of a human *SERPINA1* transgene with the Z allele, comprising coding exons and some non-coding sequences associated with various regulatory elements. PiZ mouse models were further backcrossed with AAT knockout mice resulting in the generation of a model with the exclusive expression of the human mutant AAT protein. We next evaluated lung function of PiZ mice using Flexivent plethysmography upon lipopolysaccharide induced inflammation and observed a distinct pathological phenotype based on lung compliance and inspiratory capacity compared to control mice.

A liver tropic LNP was engineered (LNP1), that resulted in high transfection of liver cells and low off-tissue transfection in vivo. As a proof of concept, we further evaluated in vivo gene editing by treating PiZ mice with LNP1 encapsulating Cas9 mRNA along with a guide targeting human *SERPINA1*. We measured ~40% indels as well as ~80% loss of protein expression by NGS and ELISA respectively (Figure 1). These results demonstrate that the Sanofi PiZ mice can be used to assess *SERPINA1* editing using in vivo non-viral gene therapy.

Our findings identify a platform to treat AATD by gene correction and we are currently evaluating technologies to correct the PiZ point mutation. These approaches may lead to an effective gene therapy to tackle the lung and liver phenotypes of AATD patients.

Figure 1:



Legend: **In vivo *SERPINA1* editing using liver tropic LNP**. Sanofi PiZ models: single copy *SERPINA1* transgene with the Z allele crossed with AAT knockout strain (Jackson Laboratory; 035015). Sanofi PiZ mice were i.v. infused with liver tropic LNP1 encapsulated Cas9 mRNA and gRNA targeting *SERPINA1*. Naïve and treated animals were euthanized 10d post-treatment. Plasma and liver tissues were analyzed by ELISA and NGS respectively to measure indels percentage and protein levels.

1512 A Potential Gene Editing Approach for AATD-Associated Liver and Lung Diseases Intended to Correct the PiZ Allele

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Mutations in the *SERPINA1* gene (Serine Protease Inhibitor, group A, member 1) cause Alpha-1 antitrypsin deficiency (AATD), an inherited disorder that is associated with an increased risk of liver disease in adults and children, and with lung disease in adults. There are approximately 250,000 people in developed countries living with AATD including 1 in every 1,500 to 3,500 people of European ancestry. One of the clinically most relevant variants is the PiZ mutation, which results in lysine substitution for glutamic acid at amino acid position 366 (E366K). Individuals who are homozygous for the PiZ allele (PiZZ genotype) develop liver phenotype caused by retained toxic aggregates of misfolded protein within the endoplasmic reticulum of hepatocytes, whereas reduced circulating AAT levels promote development and progression of lung phenotype due to impaired neutrophil elastase inhibition.

We developed an RNA Gene Writer designed to correct the E366K mutation (PiZ allele) to wild type in the *SERPINA1* gene. LNP-RNA delivery of our RNA Gene Writer corrected this mutation in a commonly used transgenic mouse model with high copy numbers of the mutation (NSG-PiZ), where we precisely edited more than 90% of the hepatocytes at very low doses, based on the assumption that 60% of liver cells are comprised of hepatocytes, and observed a corresponding >90% of corrected mRNA transcripts after a single intravenous administration. We also observed significant increases in serum AAT levels with no detectable circulating Z-AAT protein, restored neutrophil elastase inhibition, and reductions in protein aggregates in the liver. Durable mutation correction and phenotypic rescue was demonstrated for up to 10 weeks post-treatment. Separately, we generated a humanized mouse model with only two PiZ alleles to better approximate the efficacy of our product in patients with the homozygous PiZZ genotype. LNP-RNA delivery of our RNA Gene Writer achieved an estimated 89% correction of hepatocytes after a single low dose (0.25 mpk) treatment in this mouse model. A study in non-human primates (NHP) demonstrated RNA Gene Writer achieved an estimated 79% editing efficiency of the *SERPINA1* gene in hepatocytes, which corresponded to 84% of mRNA transcripts containing the edited sequence, with editing levels remaining stable for up to 6 months.

In summary, we demonstrated that our LNP formulated RNA Gene Writer efficiently edited the *SERPINA1* gene responsible for AATD in the liver of mice and NHP with a single administration at therapeutically relevant doses.

1513 Anti-SGSH antibodies following Hematopoietic Stem Cell (HSC) Gene Therapy in Patients with MPSIIIA Neither Impact Engraftment of Genetically Modified HSC nor Interfere with Multi-compartment Substrate Reduction

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Mucopolysaccharidosis IIIA (MPSIIIA, Sanfilippo A syndrome) is a neurodegenerative lysosomal storage disease caused by a deficiency in the glycosaminoglycan (GAG) degrading enzyme N-sulphoglucosamine sulphohydrolase (SGSH). SGSH deficiency results in the accumulation of heparan sulphate in cells, leading to cellular dysfunction which is particularly apparent in the brain. Symptom onset begins within the first 2 to 4 years of life and is characterised by a plateau in development, speech delay, and worsening behavioural problems, with progression to death in the mid-teenage years.

Anti-enzyme antibodies have previously been reported following ERT in other metabolic conditions. In Hurler syndrome, anti-IDUA antibodies are present in all whom receive ERT prior to hematopoietic stem cell therapy (HSCT) and resolve with the immune suppression of HSCT. However, during periods of anti-IDUA antibody production, patients with uptake inhibition of >30% and IgG titres of >1/4000 demonstrate increases in GAG levels commensurate with reduced disease correction. In primate studies of genetically modified-autologous HSCT, immune responses against genetically modified cells and the protein product, if that product is both “foreign” and secreted, have significantly attenuated therapeutic efficacy with loss of genetically modified cells or neutralisation of secreted protein, unless there is significant pre-transplant immune suppression of the recipient.

Our group has developed a gene-modified autologous haematopoietic stem cell gene therapy (HSCGT) that is currently being evaluated in 5 patients with severe MPSIIIA in a phase I/II clinical trial (NCT04201405). Here we sought to understand if anti-SGSH antibodies were produced following HSCGT, and if they had any negative impact on engraftment of genetically-modified HSC, or multi-compartment substrate reduction by the transgene enzyme product, SGSH.

An anti-SGSH ELISA was developed to detect antibodies in the serum of MPS-IIIA patients following HSCGT. We assessed anti-SGSH antibody levels at set time points before and after treatment of these patients. An uptake inhibition assay was developed to assess if anti-SGSH antibodies of even low titres inhibit SGSH uptake into MPSIIIA fibroblasts and therefore potentially impact clinical outcomes.

Anti-SGSH IgG antibodies were detected in the serum of all 5 patients, peaking at 1-3-months post-treatment at titres of 1/512-1/65,536, followed by a large reduction from 9-months post-treatment, and continued reduction over time. None of the patients had uptake inhibition >30%. All patients showed sustained HSCGT engraftment and stable vector copy number (VCN). Leukocyte, plasma and bone marrow SGSH levels were well above normal from 1-3-months post-treatment and were unchanged with either the onset or resolution of anti-SGSH antibodies. CSF SGSH levels were within or above the normal reference range by 3-6 months and remained unchanged by presence of anti-SGSH antibodies. CSF, plasma and urinary GAG levels showed continued and sustained reduction of >80% post-treatment, regardless of the anti-SGSH IgG titre, and were consistent with full biochemical correction of disease.

Transient elevations of anti-SGSH IgG antibodies do not appear to be associated with any impact on the therapeutic efficacy of the HSCGT treatment, affecting neither cell engraftment nor transgene enzyme levels, or the biological action of that enzyme in catabolising accumulated substrate

1514 Correction of Glycogen Accumulation in Muscle, Heart and CNS in a Pre-Clinical Model of Hematopoietic Stem Cell Gene Therapy for Pompe Disease

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Pompe disease (PD) is a severe and life-threatening lysosomal storage disorder caused by mutations in the acid alpha-glucosidase (*GAA*) gene. Deficiency of the lysosomal enzyme encoded by *GAA* results in toxic glycogen accumulation, primarily in skeletal and cardiac muscles, as well as the central nervous system (CNS), leading to profound neuromuscular dysfunction and progressive degeneration. While enzyme replacement therapy (ERT) is the current standard of care, its efficacy is hindered by several limitations, including the development of neutralising antibodies in cross-reactive immunological material (CRIM)-negative patients, partial correction of skeletal muscle pathology, and inadequate penetration of the blood-brain barrier.

Autologous transplantation of haematopoietic stem cells (HSCs) genetically modified *ex vivo* using lentiviral (LV) vectors presents a promising therapeutic approach for systemic enzyme delivery, with demonstrated success in achieving phenotypic correction in neurometabolic disorders such as metachromatic leukodystrophy and mucopolysaccharidosis type I. In this study, we explored the potential of HSC gene therapy to correct GAA deficiency and restore functionality in affected tissues, including the CNS, using a murine model of Pompe disease.

To enhance GAA uptake and cross-correction within target tissues, we developed a modified GAA enzyme tagged with a variant Insulin-like Growth Factor-2 binding domain (vIGF2-GAA) under the control of a mammalian PGK promoter. This modification permits GAA uptake through glycan-independent interactions with the Cation-Independent Mannose-6-Phosphate Receptor (CIMPR). The vIGF2 tag was engineered to minimise binding to insulin or IGF1 receptors while maximising cellular uptake of GAA mediated through the CIMPR receptor. Transplantation of *Gaa-/-* haematopoietic stem/progenitor cells transduced with an LV vector encoding vIGF2-GAA into conditioned *Gaa-/-* mice resulted in robust and stable engraftment and sustained systemic GAA levels. Alongside the vIGF2-GAA transgene, a wild-type (WT) GAA transgene was included as a baseline comparator.

Quantitative assessment of glycogen storage in the gastrocnemius muscles, tibialis anterior, diaphragm, heart, and brain revealed that vIGF2-GAA exhibited superior efficacy in reducing glycogen accumulation to normal levels compared to the wild-type GAA enzyme. This glycogen reduction was accompanied by histopathological correction in affected tissues and significant phenotypic improvement, as evidenced by enhanced rotarod performance three months after transplantation. Both the vIGF2-GAA and WT-GAA groups demonstrated comparable GAA enzymatic activity levels in plasma. These findings indicate that the improved correction observed in the vIGF2-GAA group is exclusively due to the enhanced functionality conferred by the vIGF2 tag only. This tag facilitates increased interactions with the IGF2 binding domain of the CIMPR, a mechanism that is not achievable with the unmodified WT-GAA enzyme. Additionally, treated mice showed complete normalisation of the surrogate Pompe clinical biomarker, urine glucose tetrasaccharide.

These findings underscore the therapeutic potential of delivering the vIGF2-GAA enzyme via genetically corrected HSCs for effective correction of skeletal muscle, cardiac tissue, and CNS pathologies. The results strongly support the clinical translation of HSC gene therapy as a transformative treatment for Pompe disease.

1515 Improving blood-brain barrier penetration in Hurler syndrome using an IDUA-ApoE fusion enzyme delivered via the PS Gene-editing System

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Mucopolysaccharidosis type I (MPS I) is a lysosomal disorder caused by mutations in the IDUA gene, which encodes α-L-iduronidase (IDUA). This deficiency results in the accumulation of glycosaminoglycans (GAGs), primarily heparan and dermatan sulfate, leading to multi-organ dysfunction and neurodegeneration. Current treatments, including hematopoietic stem cell transplantation and enzyme replacement therapy, have shown limited efficacy in addressing central nervous system (CNS) symptoms due to poor blood-brain barrier (BBB) penetration. This highlights the need for therapies capable of crossing the BBB to address CNS pathology in MPS I.

To enhance CNS enzyme delivery, we developed a novel IDUA-ApoE fusion enzyme designed to bind to low-density lipoprotein receptors (LDLR) on the BBB, facilitating CNS uptake. Using the PS Gene-editing (PSG) system, IDUA-ApoE cDNA was integrated into intron 1 of the albumin gene in neonatal MPS I mice. MPS I mice (n>10 per sex, per group) received low, middle, or high doses of the PSG-IDUA-ApoE therapeutic via intravenous administration at P0-P2. Plasma IDUA activity was monitored for 6 months and urine was collected to assess secreted GAG, which serves as an in-life biomarker of therapeutic efficacy in MPS I. Beginning at 8 months of age, cognitive and motor function was assessed using the Barnes maze, delayed fear conditioning and the pole test. At 8-10 months of age, endpoint analysis was conducted, measuring the levels of IDUA enzyme activity in the tissues and GAG accumulation. In the plasma, there was dose-dependent response in plasma IDUA enzyme activity, with all reaching supraphysiological levels that were sustained for at least 6 months. For the duration of the study, urinary GAGs were normalized. Cognitive assessment at 8 months of age showed that mice receiving PSG-IDUA-ApoE performed the Barnes maze and delayed fear conditioning test similar to untreated, normal mice. Similarly, the pole test revealed that mice receiving the mid and high dose of PSG-IDUA-ApoE maintained their motor function compared to untreated MPS I mice. In all tissues assayed, including the liver, spleen, kidney, heart, bone marrow, spinal cord, and brain, enzyme activity was significantly elevated above normal, heterozygous levels. Additionally, this increase in IDUA enzyme activity resulted in reduction and normalization of the GAGs within these tissues.

In summary, the IDUA-ApoE fusion enzyme, delivered via the PSG system, achieved supraphysiologic IDUA levels in plasma and tissues throughout the body, including the CNS, resulting in cognitive preservation and brain GAG normalization. These results support the PSG system as a promising platform for CNS-targeted gene therapy in lysosomal disorders.

1516 Evaluation of Clinical Safety and Efficacy of LY-M001: A Phase I/II Trial of AAV8-Mediated Gene Therapy for Gaucher Disease Type I

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Background: Gaucher disease (GD), a prevalent lysosomal storage disorder, arises from mutations in the GBA1 gene. LY-M001, a novel rAAV serotype 8 vector-based therapy, aims to rectify this deficiency by delivering a codon-optimized *GBA1* gene, driven by a hybrid hepatic-specific promoter, and expressing a modified glucocerebrosidase ($GCase^{LY}$). Several clinical trials is being conducted across multiple centers in China including a pilot study (NCT06162338), a Phase I/II dose-escalation trial (CTR20241092) and the first administration for an adolescent patient (NCT06528080). In this Phase I/II dose-escalation trial (CTR20241092), both naive and pre-treated patients with GD1 are eligible for inclusion. Pre-treated patients are required to discontinue standard of care (SoC) therapy at least two weeks prior to receiving the LY-M001 infusion. The primary objectives of the study are

to assess the safety and efficacy of escalating doses of LY-M001. Participants will be monitored for 38 to 52 weeks post-treatment, after which they will be enrolled in a long-term follow-up.

Results: Since August 2024, four participants have been enrolled in a phase I/II clinical trial (CTR20241092) and administered LY-M001 injection. Overall, the treatment was well tolerated across two dosage groups. A mild elevation in liver enzymes was observed in some of participants, which was promptly managed and resolved with immune modulation therapy. No serious adverse events (SAEs) related to the subjects occurred. Rapid normalization of GCase activity in peripheral blood within the 1st week after LY-M001 infusion was noted, and the GCase activity continued to increase to peak in the following 8 – 16 weeks before reaching a plateau, accompanied by reduction in Lyso-GL1 levels. Notably, despite the exceptionally elevated GCase secretion in circulation, no anti-drug antibodies (ADA) targeting GCase were detected in any patient, indicating no immunogenicity of the modified GCase^{LY} *in vivo*. Up to date, the clearance of harmful substrate led to clinical improvements in Gaucher disease symptoms in all participants, including increases in hemoglobin (by 9 to 41 g/L) and platelet levels (by 10 to 41 × 10^9/L), as well as reductions in spleen volume (by 18 to 19.5%) and liver volumes (by 5.6 to 18.8%), compared to the corresponding screening period levels. Rapid clinical responses were observed following LY-M001 administration. For instance, a participant in the high-dose cohort, who initially exhibited hemoglobin levels near the lower limit of the normal range and platelet levels below the normal range, demonstrated significant improvements of hemoglobin and normalization of platelet levels accompanied by a marked elevation in circulating GCase activity. Additionally, significant improvements in quality of life were reported by most subjects. Notable improvements in abdominal distension and fatigue were reported by all participants, with most resuming work or social activities following the LY-M001 infusion.

Conclusion: LY-M001 is a novel AAV8-based therapeutic approach for Gaucher disease currently under investigation. Preliminary clinical data from various dosage groups demonstrate a favorable safety profile and promising efficacy outcomes. Enhanced GCase expression facilitates substrate clearance, leading to improvements in both Gaucher disease symptoms and patients' quality of life.

1517 Pharmacokinetic, Pharmacodynamic, and Toxicokinetic Analysis of an rAAV8 Gene Therapy for Friedreich Ataxia-Associated Cardiomyopathy

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Introduction: Friedreich ataxia (FA) is a monogenic disease caused by frataxin protein deficiency leading to progressive neurodegeneration and, in most patients, severe cardiomyopathy.

Methods: ASP2016 is a rAAV8-based vector containing the human *FXN* gene under the control of a human PGK promoter (rAAV8-hPGK-hFXN) designed to increase the expression of frataxin. After a single intravenous dose of ASP2016, the preclinical pharmacokinetics, pharmacodynamics, toxicokinetics, or safety of ASP2016 were evaluated in a mouse model of FA (FA-MCK) and healthy non-human primates (NHPs). The overall objectives of these studies were to understand the cross-species translation and estimate the therapeutic window for treating FA-associated cardiomyopathy.

Results: Pharmacokinetic analysis of FA-MCK mice tissues demonstrated linear dose-proportional increases in vector copy number (VCN), mRNA, and frataxin protein. The highest correlation between VCN and frataxin expression was observed in the heart, followed by the liver and quadriceps. In NHPs, there were non-linear dose-dependent increases of VCN, mRNA, and frataxin expression in the heart, with similar transduction levels across both species. A significant dose-dependent improvement in survival was observed starting at the lowest dose of

1E12 vector genomes per kilogram (vg/kg) relative to vehicle-treated FA-MCK mice. Pharmacodynamic assessment in FA-MCK mice demonstrated dose-dependent rescue of ejection fraction (EF) starting at 1E12 vg/kg and plateauing at 1E13 vg/kg, with a predicted normal 50% EF at a dose of 5E12 vg/kg. Interestingly, this 5E12 vg/kg dose was predicted to yield 30% of normal wild-type frataxin expression, corresponding to the lowest level of expression in healthy human heterozygous carriers. These results demonstrate the efficacy of ASP2016 and supports the hypothesis that the increase in frataxin expression leads to improved cardiac function and survival in this mouse model. Toxicokinetic analysis identified a dose-dependent reduction in nerve conduction velocity in certain NHPs administered ASP2016, which progressed over time. Related dorsal root ganglion histological findings in NHPs were observed at doses ≥ 2E13 vg/kg and were more pronounced at the highest dose, but there were no adverse clinical or neurological examination correlates.

Conclusions: A 20-fold therapeutic window for ASP2016 was established based on the observed efficacy at the lowest dose of 1E12 vg/kg in FA-MCK mice, and the lowest observed adverse effect level without unacceptable toxicities at 2E13 vg/kg in NHPs. These results identify ASP2016 as a promising clinical candidate for treating cardiac manifestations in Friedreich ataxia patients.

1518 Slow Retrograde Renal Pelvis Injection of Centrifugally Ultrafiltered AAV Miniprep Vectors Enables Rapid Capsid Screening For Kidney Gene Delivery

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Developing novel AAV capsid variants with desired phenotypes is a key research focus in gene therapy. However, generating variants and testing their transduction profiles is often expensive and time-consuming. Generally, AAV vectors are purified using cesium chloride (CsCl), iodoxinol gradients, or chromatography and then tested in vitro or in vivo. This conventional process is lengthy, requires expensive reagents, sophisticated equipment, and skilled technicians, ultimately extending the timeline for evaluation. The use of centrifugally ultrafiltered (CU) AAV miniprep vectors is a cost and time-effective approach that eliminates these expensive, time-consuming, and labor-intensive requirements. Prior studies have shown that CU AAV miniprep vectors have been shown to be as effective as AAV vectors obtained through conventional purification (e.g., CsCl AAV vectors) in various experimental settings, but not in the field of renal gene delivery following local administration. Here, we demonstrate that CU AAV miniprep vectors can be effectively utilized for a rapid transduction profiling of AAV capsids in gene delivery to the kidney via renal pelvis (RP) injection. In this study, we evaluated the CU AAV minipreps using two capsids, AAV9 and KP3, packaged with the CAG-Tdtomato (tdT) transgene. To prepare CU AAV vectors, HEK293 cells were transfected in a single flask with the necessary plasmids and harvested together with the media five days post-transfection. The collected cells and media were freeze-thawed once, and the lysate was treated with Benzonase to eliminate free-floating DNA. The lysate was filtered with a 0.45 μm PVDF syringe filter to remove particulate matter before being concentrated by centrifugation in a molecular weight cutoff column centrifuge until the desired volume (approximately 500 μL) was achieved. The vectors were quantified using a dot blot assay. C57BL/6J male mice were then injected with CU KP3 or CU AAV9 vector at 3.0 x 10^{11} vg/mouse using the slow retrograde RP injection technique and vector transduction in the livers and injected kidneys were assessed two weeks post-injection. The harvested kidneys underwent molecular and immunofluorescence analysis. Fluorescence analysis indicated that the CU KP3 vector transduced the proximal tubules in the kidney, while the CU AAV9 vector primarily transduced mesangial cells in the glomeruli, with minimal renal tubular transduction observed. The Tdt expression in proximal tubules was 12.9% for CU KP3 and 0.3% for CU AAV9, with no significant difference between these two AAV vector preparations (CU AAV and CsCl AAV). DNA isolated from the AAV-injected kidneys was analyzed using qPCR to quantify vector genome copy numbers. CU KP3 showed 73 times more vector genomes than AAV9-injected kidneys. The off-target effects in these injected mice were examined by imaging the liver, revealing that KP3 exhibited significantly lower liver transduction compared to the AAV9 vector. All these findings are consistent with those observed in the control animals injected with CsCl AAV vectors. Therefore, RP injection of CU AAV miniprep vectors not only demonstrate safety and efficacy but replicate renal transduction profiles that would be obtained with fully purified AAV vectors. This technique offers a rapid profiling of AAV

vector transduction in the kidney, eliminating the time-consuming, complicated purification process while obtaining insightful results for further investigation.

1519 Adeno-Associated Viral Mediated Genome Editing as a Curative Therapy for Polycystic Kidney Disease

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Introduction: Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease, with a prevalence of approximately 1 in 1000 live births. It is characterized by the progressive formation of cysts in kidney and extrarenal tissues, leading to kidney enlargement and ultimately, end-stage renal disease. ADPKD accounts for ~10% of chronic kidney failure cases and is a significant public health problem. ADPKD is primarily caused by mutations in the *PKD1* or *PKD2* genes. Previously, we conducted a computational analysis of the ADPKD mutation landscape and showed that ~40% of *PKD1* and *PKD2* mutations can be corrected with CRISPR-Cas base editing. Base editing to correcting these mutations could restore the normal function of *PKD1* and *PKD2* and offer a potentially curative therapeutic approach. Furthermore, studies suggest that ADPKD kidneys exhibit plasticity, which suggests that re-expression of *PKD1* or *PKD2* in cystic kidneys could rapidly reverse the disease.

Methods: A missense mutation in *PKD1*, p.R3277C, is present in the homologous Pkd1 RC/RC mouse model, which carries the homozygous p.R3269C mutation. To investigate the potential of base editing to correct this mutation, we isolated and cultured kidney epithelial cells from these mice. Pkd1 RC/RC cells were used in vitro to test the efficacy of various CRISPR-Cas guide RNAs and identify the guide RNA with the highest editing efficiency. After selecting the most efficient guide RNA via lipid-based transfection in HEK293T cells, the optimal guide RNA and the ABE8e base editor were packaged into a novel adeno-associated virus capsid (AAV-S1) using a split-intein dual-AAV system. Edited cells were analyzed to assess Pkd1 expression via Western Blot and qPCR as well as various downstream markers associated with ADPKD progression.

Results: Our initial guide RNA screening studies showed that the most efficient guide RNA (RC_gRNA1) achieved 85% editing efficiency at the target nucleotide, without bystander editing when delivered via AAV-S1. RC_gRNA1 edited cells showed a two-fold increase in the RNA and protein expression of *PKD1* and improved mitochondrial function via an established MitoTracker assay. We also demonstrated efficient *in vivo* delivery with a modified AAV-S1 capsid using an mCherry reporter, showing transduction in ~40% of kidney cells via flow cytometry and 50% of renal tubules via automated image analysis.

Conclusions: This is the first study to show, at the cellular level, that CRISPR-Cas base editing can correct a pathogenic variant in *PKD1* to increase *PKD1* protein levels and improve downstream functional readouts. We demonstrate that a single dose of AAV-S1 achieves high editing efficiency (85%) and, crucially, without bystander editing. We also offer a comprehensive quantitative analysis of in vivo AAV-S1 delivery efficiency that is among the greatest efficiency reported to date for in vivo renal gene delivery. Taken together, these data provide strong evidence to support CRISPR-Cas base editing as a promising strategy to correct pathogenic variants of ADPKD as a potentially curative therapy.

1520 Enhancing Delivery Methods for Kidney-targeted Gene Therapy

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Many gene therapies are delivered by the intravenous (IV) route. While this route can be useful for various diseases, it is generally ineffective for kidney diseases since the kidney's own filter excludes molecules above 50 kDa and vectors are megaDaltons in mass. To circumvent the kidney filter and deliver molecular therapies into the kidney, we and others have tested direct kidney injection methods in mouse models using subcapsular (SC- through the capsule of the kidney), retro-ureter (RU), and injections into the renal pelvis (RP). In most cases, these methods require surgery to expose the kidney and its structures for injection, so are time-consuming and invasive. Vectors can also be injected percutaneously through the skin if the kidney is visualized by ultrasound or is isolated manually. While these direct injection methods can mediate gene delivery inside the kidney, cells are generally not saturated with transgene products and most vectors leak into the blood to transduce the kidney and other tissues. To explore how different vectors perform under these conditions, we compared lipid nanoparticles (LNPs), different adenovirus serotypes (Ad), and different adeno-associated virus (AAV) serotype vectors, and peptide-modified Ad and AAVs by non-invasive percutaneous injection or by microsurgery with or without renal vasculature clamping into reporter mice of different ages. Detection of the vectors in the kidney, as well as in other organs such as the liver, was achieved using *in vivo* and *ex vivo* imaging, along with tissue sectioning and confocal microscopy. Our data demonstrate that direct kidney injections can bypass the kidney's size-exclusion barrier, leading to greater vector deposition in the kidney. We are currently comparing different serotypes and mouse ages to determine the most efficient serotype for kidney targeting, as well as identifying off-target effects associated with different delivery methods. This work lays the foundation for the development of vector platforms for gene therapy targeting diverse kidney diseases.

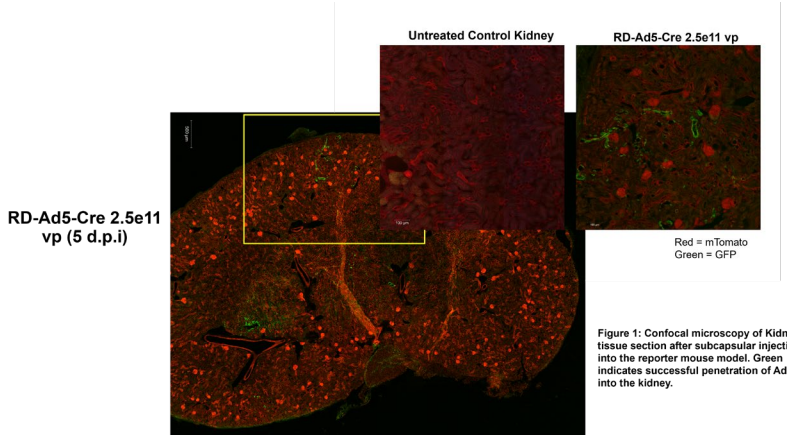

Figure 1: Confocal microscopy of Kidney tissue section after subcapsular injection into the reporter mouse model. Green indicates successful penetration of Ad5 into the kidney.

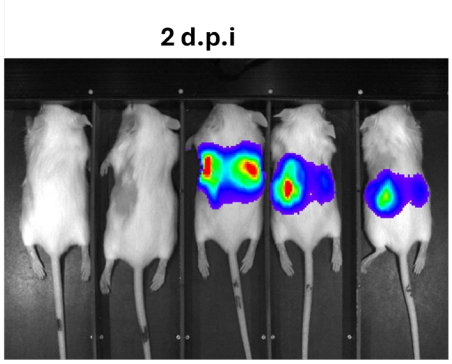

Figure 2: In-vivo imaging of the reporter mouse model showing a signal that potentially comes from the kidney as well as the liver. Adenovirus was injected using the direct non-invasive subcapsular method into the left kidney of the mice.

1521 Lentiviral complementation restores phenotypic defects of ABCA3 pathogenic variants

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ABCA3 deficiency is caused by pathogenic variations in the ATP-binding cassette A3 (*ABCA3*) gene and results in reduced or loss of surfactant production from pulmonary alveolar type 2 cells (AT2s). Lack of surfactant leads to neonatal respiratory distress and can be lethal at birth or cause later onset childhood interstitial lung disease (ChILD). Supportive care or lung transplantation are the only current treatments for severe neonatal onset forms of ABCA3 deficiency. A genetic therapy that stably complements defective ABCA3 production offers promise. Previous studies show that repairing or complementing ABCA3 in induced pluripotent stem cell (iPSC)-derived AT2s restores defining phenotypes (e.g., lamellar body morphology and surfactant production). All ABCA3 pathogenic variants are rare or private; however, they can be categorized into class 1 (mistrafficking) or class 2 (impaired phospholipid transport activity) based on the impact of the genetic mutation on protein processing and activity. Little is known about the cellular responses to complementing class 1 or 2 variants. A limitation for advancing a gene therapy for ABCA3 deficiency is lack of a high throughput cell model that recapitulates phenotypes of iPSC-derived AT2 cells. Here we show that ABCA3 complementation using a lentiviral vector in A549 cells stably expressing *ABCA3* missense variant cDNAs restores multiple phenotypic features of 3 different variants: L101P (type 1), E292V (type 2), and E690K (type 2). Parental and ABCA3 knockout cells serve as controls. We observed restoration of lamellar body-like structure morphology and cell proliferation in ABCA3 complemented cells. Interestingly, results from bulk RNA sequencing indicate that lentiviral complementation of ABCA3 ameliorates aberrant TNFa signaling via NF-KB in E292V and E690K cells, but not in knockout or L101P cells. These studies help address important questions for understanding complementation of ABCA3 on various mutation backgrounds and provide relevant endpoints for efficacy to advance a gene therapy for ABCA3 deficiency.

1522 Leveraging ML to Improve Potency and Safety of Lung-Targeted Lipid Nanoparticles

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Lipid nanoparticles (LNPs) have revolutionized RNA-based therapeutics. However, ensuring safe and effective delivery of mRNA to extra-hepatic tissues, such as the lungs, remains a significant hurdle largely due to challenges with cell-specific targeting and systemic toxicity. To address these challenges, Mana.bio has developed a machine learning (ML) platform capable of predicting critical LNP features required for specific and tolerable systemic mRNA delivery. The power and utility of this platform are demonstrated through Mana's lung delivery program, which leverages ML to model physicochemical properties, in-vivo targeting, and systemic safety toward unlocking the potential mRNA therapeutics for lung diseases.
Mana.bio's platform integrates over 80,000 proprietary data points and 45,000 publicly available data points, including results from in-vitro, ex-vivo, and in-vivo models. Leveraging advanced ML capabilities, we can accurately predict physicochemical properties—such as size, charge, and encapsulation efficiency, in-vivo lung targeting, and key toxicity parameters—such as cytokine secretion, liver enzyme elevations, and coagulation risks—while screening, in-silico, tens of millions of potential LNP formulations. The model ranks LNP candidates based on physicochemical properties, activity probabiility, and safety profile, enabling rapid identification of optimized formulations for specific therapeutic applications. The platform initially identified MB-LNP-01, an intravenously administered LNP candidate with highly specific lung delivery. Subsequent optimization efforts, incorporating only in-silico and in-vitro data, led to MB-LNP-02, a second-generation candidate with 100-fold increased potency. These findings highlight the platform's ability to rapidly enhance therapeutic performance while reducing development timelines. Towards improving the potential therapeutic index of MB-LNP-02, Mana.bio's safety prediction model identified subsequent novel LNP candidates by optimizing for critical toxicity assessments such as direct toxicity, immunotoxicity, hepatotoxicity, and coagulation. Through iterative refinement, MB-LNP-05 was engineered and validated in C57bl/6 mice, demonstrating above 100-fold reduction in cytokine elevation and a 100% improvement in tolerable dose with no off-target effects in key organs. Importantly, MB-LNP-05 exhibited enhanced lung-specific mRNA expression compared to MB-LNP-02, measured 6 hours after an IV dose of 0.65 mg/kg and 1.2 mg/kg fluc mRNA. Non-Human Primate (NHP) studies further confirmed these improvements, showing a 30-fold reduction in inflammatory cytokines, including IL-6, TNF-α, and IP-10, even at double the dose of MB-LNP-02. ALT and AST levels remained normal, with no acute liver damage observed at the maximum tested dose. This work underscores the transformative potential of integrating ML-driven modeling with experimental validation to accelerate drug development and unlock the potential of RNA medicines.

1523 Peptide insertions enhance AAV capsid tropism for airway epithelia

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Recent progress in therapies for genetic diseases, including both gene addition and gene editing, has achieved impressive results in immunodeficiencies, hemoglobinopathies, and neuromuscular disorders. A key limitation to applying these and other emerging technologies to cystic fibrosis (CF) is efficient delivery to target airway cell types. Adeno-associated virus (AAV) is a broadly useful vector for delivery to many cell types and is safe unless delivered at very high doses. Because of its persistence as an episome, expression lasts for months or years in slowly dividing cells. Here a peptide modified AAV capsid library approach was leveraged to identify highly performant capsid variants targeting human airway epithelia. Each parental AAV serotype was modified by insertion of a library of 7-mer amino acid sequence into a variable region loop. Beginning with a pool of ~6.8 million peptide sequences, we performed 3 passage rounds of capsid libraries from multiple serotypes in primary cultures of human airway epithelia (HAE) isolated from CF and non-CF individuals. Following each passage, genomic DNA and RNA were isolated and amplicon sequences enriched in HAE were selected for the following round. We selected 20 new AAV capsids with remarkable expression in HAE. These AAV capsid variants transduced target human cell types, including basal, secretory, and ciliated cells, at doses 1 to 2 logs lower than currently available capsids. We also observed enhanced transduction in primary human airway basal cells, pig airway epithelia, and mouse airways (**Figure 1**). Benchmarked to an airway tropic AAV (AAV2.5T), we identified several capsids with increased GFP reporter gene transfer. Importantly, we identified capsids that transduce HAE without the proteasome modulator,

doxorubicin (Dox). These newly identified capsids could be broadly utilized for the delivery of diverse genetic therapy reagent cargoes to the respiratory tract.

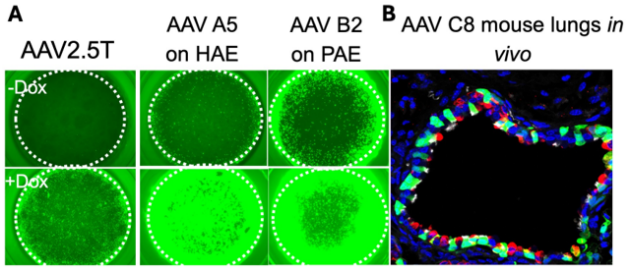

Figure legend: A) AAV2.5T, A5 and B2 capsids were applied to the apical surface (MOI=50,000) of human airway epithelia (HAE) or pig airway epithelia (PAE) overnight. Doxorubicin (1 μM) was applied overnight 48 hours later. Airway cultures were imaged by fluorescence microscopy 2 weeks later. Dotted white line indicates edge of Transwell filter. B) Mice received $1x10^{11}$ vg of AAV C8 expressing GFP intratracheally. Two weeks later, lungs were collected, embedded and sectioned for visualization. Immunofluorescence staining: red= CCSP (club cells), white= tubulin (ciliated cells), blue= DAPI.

1524 Optimising Magnetic-Guidance of Lentiviral Vectors for Improved Airway Gene Therapy Efficacy

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Introduction: For gene therapy to be effective for treating cystic fibrosis airway disease, higher efficacy is needed. Proposed enhancements include better targeting to disease-contributing conducting airways and increasing vector residence time. These could be achieved by magnetic guidance, where conjugation of iron-oxide magnetic nanoparticles (MNP) to gene vectors allows particles to be guided to and held within specific regions of the airway using external magnetic fields. This study aimed to optimise parameters for translation to an in-vivo study assessing the efficacy of magnet-guided gene therapy.

Methods: Wildtype rat basal epithelial cells were cultured in-vitro, and cells were transduced with lentiviral (LV)-GFP conjugated with MNPs with and without magnet exposure. Parameters such as nanoparticle choice (CombiMag vs. ViroMag), ratio of magnetic particle to functional LV vector (MNP:LV) (1:1, 1:10, 1:50, 10:1, 50:1, 200:1, 500:1 and 1000:1) and time spent co-incubating (5, 10, 15 and 20 min) were tested. GFP expression was measured with flow cytometry. In-vivo synchrotron phase contrast X-ray imaging was employed to understand how MNP behaviour can be manipulated on the rat tracheal airway surface under exposure to external dynamic magnetic fields.

Results: Without a magnetic field, increased volumes of MNPs improved efficacy. Magnetic field exposure led to significantly higher GFP expression regardless of factors such as co-incubation time and MNP:LV ratio (except for the lower MNP ratios, 1:1 and 1:50). Co-incubation time had no significant effect on GFP expression. Without magnet exposure, increasing the ratio of MNP:LV significantly increased GFP expression by 2.3, 4.4 and 3.6 fold respectively. With magnet exposure, each higher ratio significantly increased GFP expression until it plateaued at 200:1. In-vivo synchrotron studies identified a rotating wheel of magnets alternating between north and south poles (arranged into a Halbach array to enhance magnetic field strength) as the optimal setup for testing with gene therapy.

Conclusions: Magnetic field exposure consistently improved GFP expression. Higher ratios of MNP:LV significantly improved GFP expression under magnetic field exposure up to 200:1 after which increasing the ratio provides no further benefit. Co-incubation times tested did not influence efficacy. Optimised parameters will be adapted to an in-vivo gene therapy study in wildtype rats.

1525 Development of Lung-tropic Lipid Nanoparticles for Endothelial Cell Nucleic Acid Delivery

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Introduction: Endothelial cell injury and dysfunction are largely implicated in the progression and development of various pediatric and adult pulmonary diseases, yet they remain under-addressed therapeutic targets [1]. While inhaling nanoparticles has proven to be highly effective in targeting epithelial cell populations [2], this method encounters physiological barriers before nanoparticles can reach the pulmonary vascular endothelium. Moreover, in disease contexts where ventilatory inefficiency can complicate nanoparticle dispersion and deposition, an intravenous and physiochemical-selective approach can help minimize these barriers to transfection while still allowing for organ-specific activity and a reduction of systemic adverse effects. We hypothesize that lipid nanoparticles (LNPs) trafficking to the pulmonary endothelium can be effectively directed without needing additional targeting ligands or lipid components. Since endothelial cell dysfunction linked with pulmonary diseases can lead to the dynamic acquisition and loss of cell surface markers, the significance of a compositional-based LNP screening approach for this target is highlighted.

Methods: We pursued a multi-step screening approach to identify optimized formulations for delivering mRNA and pDNA to endothelial cells. Utilizing a library of 649 formulations featuring SM-102 as the ionizable lipid, with helper lipids comprising cationic (DDAB, DOTAP), zwitterionic (DSPC, DOPE), and anionic (18BMP, 18PG) charge, along with cholesterol and the pegylated lipid DMG-PEG-2000, we assessed the transfection efficiency of Luciferase (Luc) mRNA-LNPs in Human Umbilical Vein Endothelial Cells (HUVEC) [3,4]. To evaluate these formulations *in vivo*, we stratified formulations by percentage of helper lipid content and examined the top four formulations for both DDAB and DOTAP using a cluster-mode screen in C57BL/6J mice. Transfection efficiency was quantified through whole-body and ex vivo imaging of major organs via IVIS at 24 h following intravenous injection of Luc-mRNA-LNPs. We also investigated the effectiveness of these formulations in delivering pDNA by evaluating Luc-pDNA transfection using the DDAB cluster.

Results: Benchmarking our library against the formulation used in the SARS-CoV-2 mRNA LNP vaccine, we observe that over 44% of formulations across all helper lipids surpassed the Moderna formulation in HUVEC transfection efficiency alone (Figure 1a). All DDAB and DOTAP formulations evaluated *in vivo* demonstrated extrahepatic tropism and nearly exclusive lung transfection (Figure 1b, c). Lung transfection in the DDAB mRNA cluster showed over four-fold higher transfection compared to the DOTAP cluster (n=3, p=0.0171). pDNA-DDAB-LNPs exhibit the same tropism pattern, though with lower luminescence overall. Individual formulations within these clusters will be further evaluated.

Conclusions: Lung-tropic nanoparticle formulations derived from this screening approach can direct specific therapeutic cargo of various sizes to the pulmonary endothelium.

References: [1] R.F. Hough, et al., Am J Respir Cell Mol Biol. 71: 388 (2024) [2] S.G. Huayamares, et al., Curr Res Biotech. 7: 100200 (2024) [3] L. Cheng, et al., ACS Nano. 18: 28735 (2024) [4] Y. Zhu, et al., Nat Commun. 13: 4282 (2022)

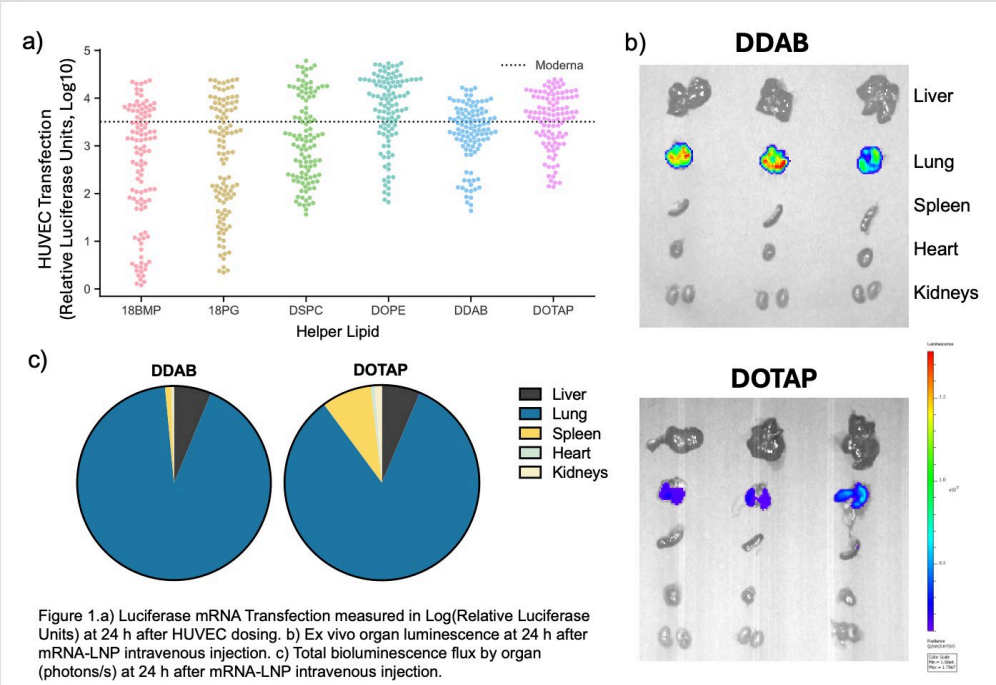

Figure 1.a) Luciferase mRNA Transfection measured in Log(Relative Luciferase Units) at 24 h after HUVEC dosing. b) Ex vivo organ luminescence at 24 h after mRNA-LNP intravenous injection. c) Total bioluminescence flux by organ (photons/s) at 24 h after mRNA-LNP intravenous injection.

1526 Pulmonary delivery of circular RNA therapeutics for cystic fibrosis

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Cystic fibrosis (CF) is a life-threatening autosomal recessive disease with > 30,000 cases of all races and ethnicities in the US. The disease arises from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes the cystic fibrosis transmembrane conductance regulator protein, leading to dysfunctional chloride ion transport and multi-organ damage, particularly in the lungs. The development of gene therapies for CF is hampered by the limitations of current CFTR modulator therapies, which fail to benefit approximately 10% of CF patients due to specific genetic mutations. Here, we disclose pulmonary delivery of circular RNA (circRNA) encoding wildtype functional CFTR protein (circRNA-CFTR), for the CFTR-mutant-agnostic treatment of CF patients. circRNA-CFTR is featured with high biostability, long-lasting expression of CFTR protein in lung epithelial cells. For the pulmonary delivery of circRNA-CFTR, we used a Design of Experiment (DOE) approach and identified lead lipid nanoparticles (LNPs) that enabled efficient protein expression in mouse lung, upon intratracheal administration of LNP-circRNA-CFTR. Further, upon intratracheal administration in mice, these LNPs enabled circRNA delivery to mouse upper tracheal epithelial subtypes, such as ciliated cells and ionocytes, of which the normalization of CFTR physiological function is pivotal for CF-associated lung diseases. The resulting LNP-circRNA-CFTR showed efficient CFTR protein production in CF patient-derived bronchial epithelial cells without invoking strong immune responses, resulting in the restoration of the physiological function of CFTR to pump Cl^- across the cell membrane in these cells. Furthermore, in a genetically engineered CF mouse model and CF rabbit model with CFTR loss-of-function mutation, LNP-circRNA-CFTR resulted in efficient CFTR expression in mouse and rabbit tracheal epithelia, as shown by ex vivo tissue flow cytometry, immunofluorescence imaging and western blot, and hence restored the CFTR function of a chloride channel, as shown by a Ussing chamber experiment in tracheal epithelia.

1527 A hybrid gene correction strategy for Cystic Fibrosis

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While treatments based on the use of CFTR modulators have significantly improved the quality of life of people with Cystic Fibrosis (CF), there is still the need for the development of durable therapies that could correct the disease at the genetic level. Using highly efficient genome editing systems such as CRISPR/Cas9, mutations in the *CFTR* gene can be corrected to produce a functional protein in lung cells. Our group has developed Cas9 ribonucleoprotein (RNP) and AAV reagents for highly efficient *ex vivo* editing of airway basal cells in two different approaches: a mutation specific approach and a universal strategy. However, the methods employed were designed to edit cells *ex vivo* that would later need to be transplanted. Transplantation of airway cells into the lower airways is still a work in progress in the field though interesting proof of concept work has been done in the trachea. Therefore, we developed a novel strategy that combines non-viral (lipid nanoparticles, LNPs) and viral vectors (adeno associated viral vectors, AAV) for the delivery of the editing reagents that could be used *in vivo*. Selective organ targeting (SORT) LNPs delivered TdTomato mRNA to nearly all primary Human Bronchial Epithelial Cells (HBECs) *in vitro* (>99%). Moreover, SORT LNPs carrying 5moU or N1MePsU Cas9 mRNA and a previously validated sgRNA targeting exon 11 of the *CFTR* gene resulted in 86% editing in wild type HBECs and 78% editing in HBECS homozygous for the most common CF mutation, dF508. We then combined the editing SORT LNPs with an AAV donor template for homologous recombination (HR) packaged in the natural capsid AAV6 or AAV2.5T, a synthetic capsid designed for airway tropism. We found that using this hybrid approach we were able to correct up to 73% of alleles in CF HBECs. The correction efficiency could be further enhanced by using AZD7648, an inhibitor of DNA-PK, achieving up to 81% corrected alleles. We are now validating this therapeutic approach in more advanced models such as precision-cut human lung slices.

1528 Allogeneic Engineered T Regulatory Cells Improve Disease Outcome in Preclinical Models of Acute Lung Injury

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Acute Respiratory Distress Syndrome (ARDS) is a life-threatening condition characterized by run-away inflammation and maladaptive tissue-repair processes. Specifically, symptoms include diffuse alveolar tissue damage, pulmonary edema, severe hypoxemia, and increased local inflammatory cytokine response and immune cell infiltration. Causes of ARDS are numerous, ranging from direct tissue insult or drug-induced etiologies to pulmonary infections of bacterial or viral origin. Despite recent advancements in standard of care, ARDS remains largely associated with poor prognosis.

Foxp3+ T regulatory cells can play a key role in lung homeostasis and tolerance, controlling inflammation and promoting tissue repair processes. Our platform creates stable, tissue targeted, and selectively activated Tregs from abundantly available T cell populations. Specifically, we utilize CRISPR-Cas9 mediated gene editing of bulk CD4+ T cells to engineer Tregs (EngTregs) for stable expression of Foxp3, and a rapamycin-activated chemically inducible signaling complex that provides tunable, targeted, IL-2 signaling as well as the expression of the tissue damage alarmin receptor ST2. These “Tissue EngTregs”, recapitulate the gene signature observed in naturally occurring Tissue Tregs based on bulk RNAseq analysis compared to PBMC isolated natural Tregs and conventional Engineered Tregs. Flow cytometric comparison of Tissue EngTregs to EngTregs without ST2 revealed a cell product with higher expression of activation markers (e.g. Ki67 and HLA-DR), lung specific repair cytokines (e.g. IL-13), and tolerogenic markers (e.g. CD39, TIGIT, CD25). When cultured with inflammatory cytokines and alarmins, Tissue EngTregs were better able to sequester pro-inflammatory cytokines including IL-2, IL-33, TNF-a and IL-4, further suggesting an enhanced ability to respond to acute injury. Importantly, Tissue EngTregs expressed

high levels of lung inflammation homing chemokine receptor CCR4 as well as enhanced expression of tissue anchoring receptor CCR8.

A murine engineered Treg (mEngTreg) surrogate was used for preclinical *in vivo* evaluation of this approach in mouse models of pulmonary inflammation and acute tissue injury. We utilized the lipopolysaccharide (LPS) induced acute lung injury (ALI) model for studying severe lung injury and an extended bleomycin induced lung injury model to examine the later fibrotic phase of disease. Cell generation from CB6F1 (C57BL/6 x BALB/c F1 cross) donor mice allowed for allogeneic cell assessment.

Both allogeneic CB6F1 and autologous C57BL/6 derived mEngTregs improved disease outcome in the LPS and bleomycin induced ALI models. ALI mice in both models treated with mEngTregs showed restored blood oxygen saturation, reduced immune infiltrates as assessed by flow cytometry, lower levels of inflammatory cytokines in the bronchoalveolar lavage fluid, and reduced tissue damage by H&E. mEngTreg treated ALI animals also showed attenuated interstitial lung fibrosis as measured by Masson's Trichrome compared to ALI controls. Critically, mEngTregs preferentially engrafted at the site of injury, supporting an inflammation tuned homing mechanism. Overall, the ability of mEngTregs to not only ameliorate disease as measured by key, translatable biomarkers but also improve downstream tissue repair mechanisms supports the potential for developing a potent off-the-shelf allogeneic engineered Treg cellular therapy for human ARDS.

1529 Ultrasound-Guided Nano-Enhanced Optical Delivery of Optogenes in the Central Nervous System for Modulating Pain

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Purpose: Pain represents a significant health concern, leading to substantial psychological and physiological impairments in patients globally. Previous studies have shown that pain can be inhibited through optogenetic modulation of specific neurons within the Anterior Cingulate Cortex (ACC) and spinal cord via AAV-based gene delivery. Achieving localized gene expression in targeted cells is critical for efficacy, while minimizing off-target effects and adverse outcomes. The research outlined here compares viral and far-red laser-based spatially targeted nano-enhanced delivery (NOD) of genes encoding for multi-characteristic opsin (MCO) in the CNS.

Method: MCO is a sensitive optogenetic actuator activated by red light, which can penetrate deeper tissue with reduced absorption and scattering loss. NOD is a non-viral delivery technique that uses nano-enhancers to amplify light intensity, creating hot spots near targeted cells to perforate membranes and facilitate gene uptake. Gold Nanorods (GNRs) were used as nano-enhancers along with a 650 nm laser to deliver GAD-MCO genes into the dorsal regions of the ACC and lumbar spine. The mixture of GNRs and GAD-MCO plasmids was injected into the target tissue (intracranial for ACC and intrathecal for the spinal cord at the L5-L6 junction) before laser irradiation. Optogenetic stimulation of MCO-sensitized ACC and spinal cord was performed using a wirelessly controlled low-power red LED. Both acute inflammatory (via formalin injection) and chronic neuropathic hyperalgesia (by sciatic nerve constriction) pain models were used to assess the therapeutic efficacy of laser-delivered and optically-stimulated MCO in ACC and spinal cord.

Results: The qPCR-based analysis of MCO-transgene biodistribution indicated localized presence in the targeted tissues. Immunohistochemistry of the targeted tissue demonstrated expression of mCherry (reporter for MCO) in the inhibitory GABAergic neurons of the ACC and spinal cord regions treated with laser irradiation. Red light stimulation (via LED) of MCO-expressing GABAergic neurons in localized areas of the ACC and spinal cord resulted in inhibition of inflammatory pain (measured by formalin test of licking/lifting) and reduction of chronic

pain (assessed via paw withdrawal threshold using Von Frey assay). Efficacy results were comparable between the two gene delivery methods.

Conclusion: Non-viral laser delivery of MCO to neurons in the ascending/descending pain pathway may enhance the effectiveness of optogenetic modulation by targeting gene expression to the specific site(s) of interest. The NOD method is expected to allow control over the duration of gene expression and potential for redosing while minimizing immunogenicity associated with virally delivered gene therapies.

1530 Evaluating Behavioral Outcomes of Whole-Brain AAV9 Gene Therapy for Fragile X Syndrome via Focused Ultrasound and BBB Opening

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Fragile X syndrome (FXS) is the leading inherited cause of intellectual disability and currently lacks effective treatments. This monogenic disorder results from a CGG triplet expansion that silences the *Fmr1* gene. Gene therapy holds promise for restoring *Fmr1* expression but requires efficient delivery across the blood-brain barrier (BBB). In this study, we employed focused ultrasound combined with microbubbles (FUS-BBBo) to achieve whole-brain BBB opening and facilitate AAV-mediated gene delivery in *Fmr1*-KO mice.

Whole brain BBB opening was conducted using 1.5 MHz ultrasound at 450 kPa peak negative pressure in raster scan mode during 92s at a speed of 10mm/s (Image-guided Therapy, Pessac, France) combined with the intravenous bolus injection of 10^8 microbubbles (Harmonix, France). Two AAV9 viral vectors encoding Luciferase and Fmr1 were produced and injected at a dose of 10^{11} viral genomes following FUS-BBBo on 8-12 weeks old Fmr*1*-KO mice. We then conducted imaging, qPCR, immunochemistry and behavioural analyses following the treatment (fig 1).

First, The T1 MRI contrast ratio following Gd/DOTA injection demonstrated an average of >60% successful BBB opening of the total brain volume was achieved with system. The use of AAV9-Luciferase vector confirmed by bioluminescence imaging the widespread and stable transgene expression in treated brains. Subsequent delivery of an AAV9-*Fmr1* vector resulted in variable but efficient rescue of *Fmr1* expression (15.2 +/- 7.6% of expression wild type control mice), assessed via RT-qPCR 8 weeks after transduction (fig 2). We observed by immunohistochemistry the protein FMRP in neurons in several brain regions, including the cortex and hypothalamus in all examined mice, and in the hippocampus less frequently. The combination of FUS-BBBo and AAV9-*Fmr1* gene therapy further enhanced behavioural outcomes on *Fmr1*-KO mice, particularly in reducing hyperactivity and anxiety-like behaviours with a significant diminution of the distance travelled in the open field in treated mice compared to sham control and a significant lower number of marbles buried over time in treated mice over sham control. It has to be noted that interestingly, whole-brain BBB opening without AAV led also to some improvements in hyperactivity phenotype.

These findings highlight the potential of FUS-BBBo as a non-invasive strategy for whole-brain gene delivery and its therapeutic benefits beyond transgene expression. This approach could pave the way for novel treatments for FXS and other neurodevelopmental disorders.

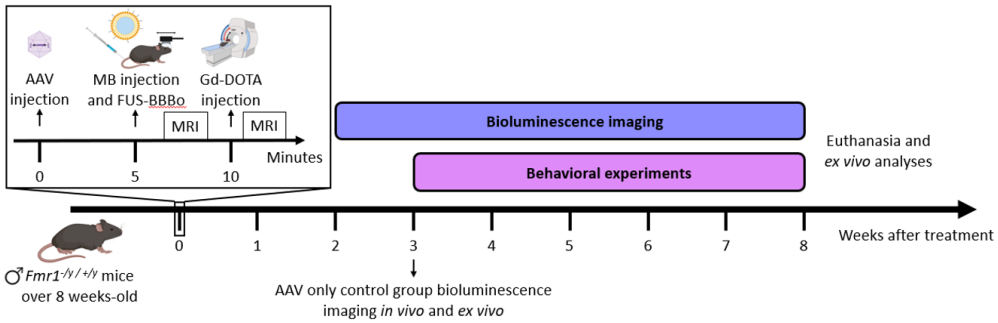

Figure 1: Timeline of FUS-BBBo procedure and following experiments.

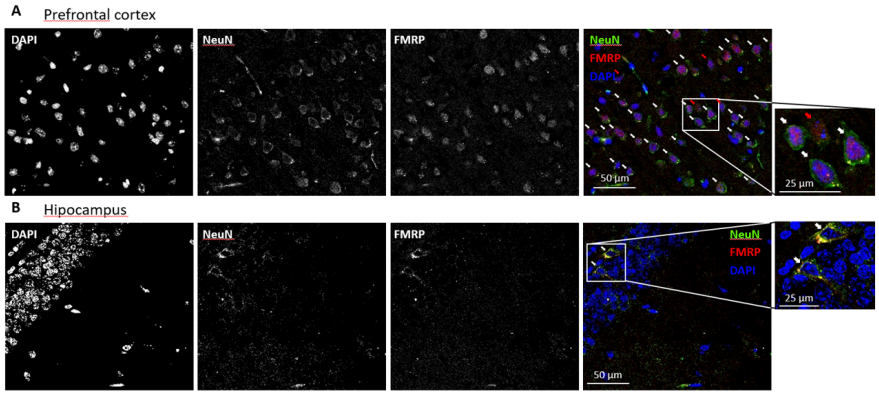

Figure 2: *Fmr1* transgene expression analyses. (**A**) Immunohistochemistry of FMRP protein on a sagittal cut of treated *Fmr1*$^{-/y}$ mice. (**green**: NeuN; **red**: FMRP; **blue**: DAPI; white arrow: FMRP+ and NeuN+ cell; red arrow: FRMP+ and NeuN- cell) (**B**) Immunohistochemistry of FMRP protein on a sagittal cut of treated *Fmr1*$^{-/y}$ mice 8 weeks after FUS-BBBo and AAV injection. (**green**: NeuN; **red**: FMRP; **blue**: DAPI) (**C**) *Fmr1* mRNA expression normalised on control untreated *Fmr1*$^{+/y}$ mice of mice treated with FUS-BBBo and AAV-*Fmr1* injection. Data expressed as mean +/- SEM. * = p-value < 0.05; Mann-Whitney bilateral test.

1531 Intravenous Delivery of a CNS-Penetrant AAV Gene Therapy Provides Survival Benefit in a TSC1 Disease Model

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Introduction: Tuberous sclerosis complex (TSC) is a neurodevelopmental disorder caused by loss of function mutations in the genes *TSC1* or *TSC2*, which regulate mTOR activity. Mutations in TSC1 or TSC2 lead to hyperactivation of the mTOR pathway, resulting in abnormal cell growth, gliosis, and epileptogenesis. While options exist for the treatment of TSC-associated epilepsy, ~60% of TSC patients develop recalcitrant epilepsy resistant to multiple anti-seizure medications, underscoring the need for improved treatments for TSC. Given the neurological impact of TSC, an effective therapeutic would require widespread distribution throughout the central nervous system (CNS).

Approach: Apertura has developed AGTX-1111, an AAV-based therapeutic designed to deliver a functional copy of the human *TSC1* (*hTSC1*) gene to the CNS after intravenous delivery using TfR1 CapX, a capsid engineered to cross the blood-brain barrier (BBB) via the human transferrin receptor (hTfR1). Preclinical studies for AGTX-1111 make use of a surrogate approach wherein a mouse-specific BBB-crossing capsid, PHP.B, is used in place of TfR1 CapX for mouse disease models lacking expression of hTfR1. We applied this approach to a previously described

loss of function mouse model in which *Tsc1* is conditionally disrupted in GFAP-expressing cells (*Tsc1 fl/fl GFAP-Cre*; "CKO"), leading to loss of function in astrocytes and some neurons. These mice show a severe disease phenotype, wherein loss of Tsc1 function results in reduced lifespan, seizures, and aberrant brain morphology (Uhlmann, et al. 2002).

Results: In pilot studies, we confirmed the ability of PHP.B to deliver a previously described hTSC1 transgene (Prabhakar, et al. 2015) to the CNS after intravenous dosing and provide a survival benefit in CKO mice relative to untreated controls. Development of an hTSC1 transgene made use of hTSC1 knockout cell lines wherein loss of TSC1 function leads to elevated mTOR activity, evidenced by increased phosphorylation of the mTOR target p70S6 Kinase (p70S6K). We developed an ELISA-based potency assay to measure the ability of hTSC1 transgene expression to rescue TSC1 function, restoring normal levels of phosphorylated p70S6K in hTSC1 knockout cells. This potency assay was used to nominate AGTX-1111, which uses an EFS promoter to drive expression of an hTSC1 transgene. While we observe lower overall levels of hTSC1 expression in vitro using the AGTX-1111 transgene cassette compared to the published transgene, we observe similar reduction of phosphorylated p70S6K to wild-type levels.

Conclusions: These preliminary efficacy data of the nominated development candidate AGTX-1111 motivate its advancement to proof-of-concept in vivo efficacy evaluations for the developed TSC1 gene therapy. These data will fit within a broader regulatory strategy to identify TfR1 CapX dose levels that achieve similar levels of CNS distribution as an efficacious dose of the surrogate capsid PHP.B. We anticipate that this strategy will help enable identification of AGTX-1111 dose levels appropriate for treatment of TSC patients.

1532 Identification of AAV engineered capsids that allows efficient CNS transduction through comparative biodistribution studies.

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Background: The efficacy of a gene therapy mediated by an adenoviral-associated virus (AAV) vector is partly determined by the tropism of the viral capsid used. For genetic conditions affecting the CNS, gene therapies would optimally be delivered using an intracerebroventricular (ICV) route of administration. There is currently lack of understanding around the ideal AAV capsid to be used for such treatments .

Aims: To enable the translational development of AAV mediated gene therapies for CNS related disorders, we strove to examine the efficacy of delivery to the CNS of various AAV capsids by assessing biodistribution through use of a packaged bioreporter expression cassette.

Methods/Materials: AAV vectors carrying a SSFV-Luc-2TA-eGFP expression cassette were packaged in AAV-9, AAV-F and AAV-B1 capsids. These were administered through an ICV route into adult mice at 3.7e9 vp. At four weeks post-administration, various body wide tissues were harvested and divided up for RNA harvest for eGFP expression and for protein for luciferase assay assessment. Tissue was also embedded for subsequent cryo-sectioning for fluorescent histochemical analysis.

Results: On the basis of eGFP expression at the mRNA level (in liver kidney, brain, blood, lung, heart, spleen, muscle), intensity of fluorescence on sectioned tissues (brain cortex, optic nerve, and sciatic nerve) and luciferase enzymatic activity (in liver kidney, brain, blood, lung, heart, spleen, muscle), we establish that AAV-B1 and AAV-F are far superior to AAV-9 for the transduction of and the delivery of cargoes to the CNS by ICV routes of administration. For example, at the brain transcript level AAV-F is 715 fold and AAV-B1 81 fold greater than

AAV-9; and at the protein level AAV-F is 104 fold and AAV-B1 is 69 fold greater than AAV-9. Consideration of the readouts as a whole suggest that AAV-F is the most efficient of the three capsids for delivery/transduction efficacy to target tissues.

Conclusions: With the identification of AAV serotypes that allow efficient transduction and delivery of transgenes to the CNS, we provide validated opportunity for gene therapies for CNS-related genetic disorders to their reach maximal potential.

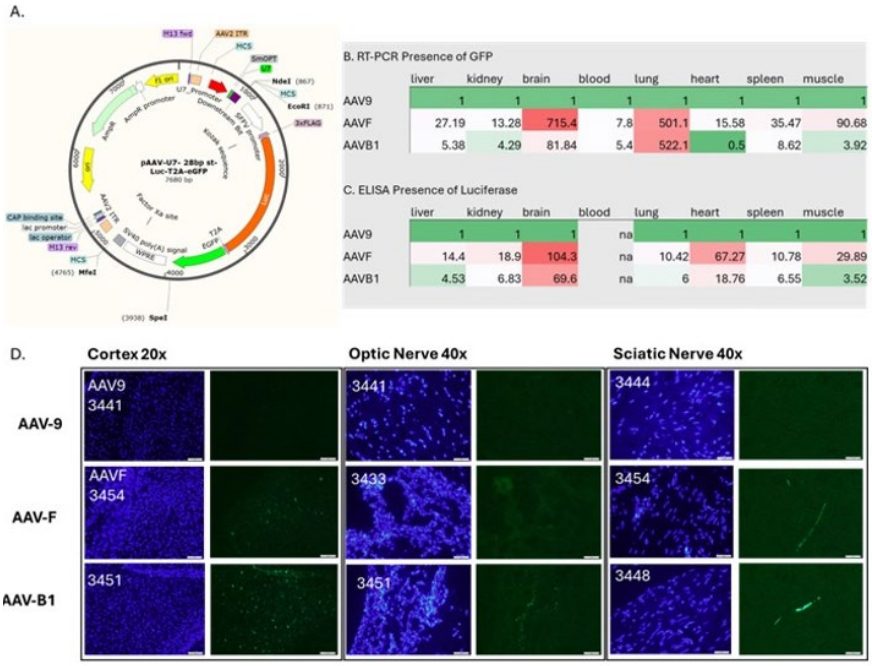

Fig: ASO Delivery with different AAV capsids. A.) Map of pAAV Biosensor with U7-SnRNA Luciferase-T2A-eGFP allowing easy biodistribution analysis. B. q-RT-PCR showing biodistribution of eGFP expression. Both AAVF and AAVB1 are normalized to AAV9 eGFP expression levels. C. ELISA assay showing luciferase expression in protein lysates. Both AAVF and AAVB1 are normalized to AAV9 Luciferase expression levels. D. Fluorescent imaging examples of NF-relevant tissues showing nuclei (DAPI-blue) and GFP expression in cortex, optic nerve, and sciatic nerve for each capsid.

1533 Multivariate Analysis of Routes of Administration and AAV Capsid Selection for Efficient Brain Transduction in the FVB Strain of Mice

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Effective delivery of biologics to the brain remains as one of the leading challenges in the field of neurogenetic therapeutic intervention. Adeno-associated viruses (AAVs) have long been the delivery vehicle of choice for CNS gene therapies due to their safety, CNS tropism, and long-term durability of associated therapeutic effect. However, there are several limitations that limit the broad use across many indications including: transduction efficiency, including restriction of transfer through the blood brain barrier (BBB), immune response and generation of neutralizing antibodies due to the delivery of high vector genome, peripheral effects involved with liver accumulation and toxicity, and limited genome size. To address these shortcomings, many in the field have developed new and evolved capsids that have enhanced BBB crossing ability along with de-enrichment of liver transduction. However, there are limited reports directly comparing this new class of evolved capsids with the naturally occurring AAV9, that is preferentially used for delivery to the brain, across multiple routes of administration. In this study, an optimized, hypercompact CRISPR protein construct was fused nanoluc and GFP to

allow for non-invasive imaging and co-localization studies and was packed using three publicly available evolved capsids that have been developed to specifically target neurons, capB10, PhP.eB, and DJ8 and compared against AAV9. All vectors were packaged following our lab's established protocols and where injected via three translationally relevant routes of administration: intrastriatal, intracerebral ventricular, and intravenous (tail vein) in mice (FVB/NJ). Evaluation and analysis of efficiency of distribution was performed using longitudinal bioluminescence imaging (IVIS) for up to 6 months post-delivery. Immunohistochemistry was also performed to quantify transduction efficiency, neuronal co-localization, and pilot studies to assess neuroinflammation. Based on our analysis we found that the AAV9 capsid delivered through CSF routes resulted in the highest expression, distribution, and transduction efficiency.

1534 A Minimally Invasive Enzyme Replacement Therapy for Angelman Syndrome

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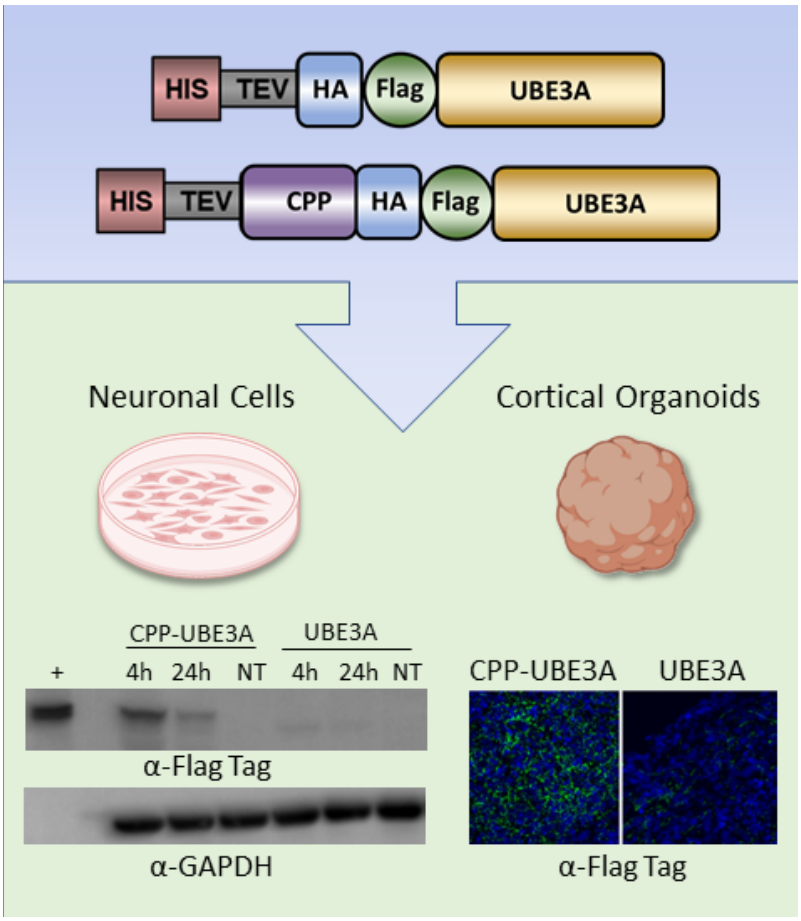
Angelman syndrome is a rare neurodevelopmental disorder caused by a loss of function of ubiquitin-protein ligase E3A (UBE3A) protein in the brain. Individuals with Angelman syndrome are generally non-verbal and experience debilitating seizures, gait abnormalities, sleep disturbances, and intellectual developmental delays. Despite decades of research characterizing Angelman syndrome and developing relevant model systems, there remains a continuing unmet need for treating this disease. Several potential and promising treatments are currently in clinical trials, including two ASOs. However, delivery of ASOs remain a challenge as they are delivered by regular injections into the cerebral spinal fluid. The injections are invasive and must be done at a hospital by specially trained physicians. Our work aims to create a minimally invasive enzyme replacement therapy, by delivering UBE3A to the brain through a simple intravenous injection that could potentially be done at home or at an outpatient clinic. The delivery of the UBE3A to the brain is made possible by the use of novel cell-penetrating peptides (CPPs), that can cross the blood-brain barrier. One of these cell penetrating peptides called "ZIP" is derived from the Zika virus. Our work has demonstrated that "ZIP-UBE3A" can enter human and mouse neuronal cells lines and human cortical organoids at a significantly higher rate than UBE3A without ZIP. We have also shown that ZIP-UBE3A retains enzymatic activity, adding ubiquitin to one of its targets TP53. We are currently evaluating the efficacy of ZIP-UBE3A in crossing the murine blood brain barrier from an intravenous injection. This presentation will highlight our progress towards developing "ZIP-UBE3A" as a minimally invasive enzyme replacement therapy for Angelman syndrome.



1535 A MyoAAV Capsid Variant Revealed Schwann Cell Specific Colocalization in AAV Serotype Screening Studies for Peripheral Nervous System Gene Therapies

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Charcot-Marie-Tooth disease (CMT) is a heterogeneous group of disorders caused by mutations in over 100 genes. Clinically, patients can be divided into two major groups, demyelinating and axonal neuropathies. Demyelinating CMTs including CMT1, CMT4, and CMTX are caused by gene mutations affecting Schwann cells and disrupting normal myelination in peripheral nerves. While various adeno-associated virus (AAV)-based gene therapies are under investigation for these genetic disorders and have shown great promises preclinically, targeting Schwann cells has remained a significant challenge. Recently, capsid engineering has laid the ground for introducing novel AAV serotypes and capsid variants with improved specificity toward cells and tissues of interest. In this study, we aimed to compare widely used AAV serotypes to screen their Schwann cell-specific targeting properties.

To elucidate the Schwann cell targeting ability of different AAV serotypes including AAV1, AAV4, AAV5, AAV6, AAV9, AAVrh10, AAVv66, AAVPHP.EB, MyoAAV3A, AAVF, and AAVrh74, we studied the AAV transduction efficiency along with Schwann cell-specific markers' colocalizations at in vitro, ex vivo, and in vivo levels. Immortalized human and isolated primary mouse Schwann cells were transduced with AAV vectors at a multiplicity of infection (MOI) of 5E+3 and the transduction efficiency was evaluated seven days post-treatment. Furthermore, an ex vivo model of dorsal root ganglion (DRG) was applied to study the lead serotype (5E+5 vector genomes (vg)

per DRG), and immunostained with a Schwann cell-specific biomarker, Sox10, for colocalization observations. Finally, wild type FVB mice were injected intrathecally at six weeks old with 5E+11 VG of different serotypes to express enhanced green fluorescent protein (EGFP) under a cytomegalovirus (CMV) enhancer/chicken beta-actin (CAG) promoter and sacrificed eight weeks post-injection. Sciatic nerves were collected and sectioned, and the sections were processed for immunofluorescence staining.

AAV1, AAV4, and AAV6 successfully transduced immortalized human and primary mouse Schwann cells in vitro, with AAV6 showing greater efficiency compared to AAV1. Interestingly, we observed highly efficient Schwann cell transduction with MyoAAV3A, which was greater than other serotypes. Immunofluorescence staining of MyoAAV3A-treated mouse DRGs revealed about 70% colocalization of EGFP with Sox10 at four days post-treatment, demonstrating its remarkable efficiency in targeting Schwann cells ex vivo. The Schwann cell-specific transduction of MyoAAV3A was investigated in vivo along with AAV6, AAV9, AAVF, and AAVrh10 vectors. While AAV9 and AAVrh10 exhibited Schwann cell transduction, AAVrh10 showed higher GFP expression in Schwann cells compared to AAV9. Surprisingly, in sciatic nerve sections, we observed colocalization of EGFP expression with a Schwann cell-specific marker when using MyoAAV3A vectors. While we used the ubiquitous CAG promoter, GFP expression was exclusively observed in Schwann cells and was absent in axons, a distinction not seen with other studied serotypes. Further in vivo studies on MyoAAV3A alongside quantification of Schwann cell transduction in different parts of peripheral nerves and liver by qRT-PCR are underway.

The novel muscle-tropic AAV serotype, MyoAAV3A, demonstrated highly Schwann cell-specific tropism in vitro, ex vivo, and in vivo, highlighting its potential for Schwann cell targeting in different disease models.

1536 Development of an antibody-conjugated AAV platform for non-invasive and targeted delivery of therapeutics to the peripheral nervous system.

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Chronic pain is a prevalent condition that affects more than 20% of the adult population and for which the current standard of care still focuses mostly on opioid use, often associated with heavy side effects or subsequent life-threatening disorders. Therefore, there is a real unmet need for the development of safer and efficient pain treatments with reduced side effects. In that context, gene therapy emerges as a promising modality to deliver new pain therapeutics to neurons of the peripheral nervous system (PNS). One major obstacle remains in the generation of appropriate vectors for delivery to the PNS. Adeno-Associated Viruses (AAV) have taken a leading place as viral vectors for the treatment of various acute and chronic neurological disorders in the central nervous system (CNS). However, in comparison to the CNS, controlled delivery of AAV to the PNS for therapeutic purposes has proven to be more challenging and less successful. So far, few examples of engineered capsids have led to specific delivery to PNS neurons with translatability across species.

To address this gap, we applied our modular and antibody-driven AAV retargeting platform to generate novel AAV that can be efficiently redirected to the PNS in rodents and NHPs. We employed rational capsid engineering and covalent conjugation of antibodies to variable loops of the viral capsid through a protein tagging system, an approach that has been already successfully validated for redirecting AAV to brain and muscle tissues with cross-species translatability. Through cross functional in-house efforts combining *in vivo* antibody internalization and biodistribution studies, we previously identified the neurotrophin receptor p75 (p75NTR) as an ideal target to redirect AAV to the PNS. In particular, we validated the capacity of anti-p75NTR-conjugated AAV to efficiently transduce dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons in wild type mice after systemic administration, while being de-targeted from the CNS and peripheral organs.

With the aim to optimize this p75-retargeted AAV platform for delivery of pain therapeutics in the clinic, we screened a larger pool of twenty anti-human p75 monoclonal antibodies (mAb) and Fab conjugated to barcoded AAVs for their ability to mediate transduction of DRG and TG neurons after systemic delivery in mice expressing

human p75NTR. To demonstrate the translatability of our approach in a large animal model, we are now evaluating the anti-human p75 AAVs in cynomolgus macaques, particularly focusing on evaluating the tropism and potency of those antibodies across species. Once validated, we aim to apply this technology for the development of novel treatments for chronic pain and PNS disorders.

1537 Gene drive viral vector suppress recurrences of herpes simplex virus 1 and 2 in mice

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Herpes simplex viruses (HSV) 1 and 2 cause lifelong recurrent infections that can have severe medical and psychosocial consequences. HSV travels through nerves and remains latent for life in the peripheral nervous system, with periodic reactivations causing oral or genital ulcerations. HSV infection lacks vaccines or cures. Here, we report a new type of viral vector as a preventive or therapeutic treatment for HSV infection. We recently developed a viral gene drive in herpesviruses that relies on the co-infection of neurons by wild-type and engineered gene drive viruses. After CRISPR-mediated recombination between viral genomes, wild-type viruses are converted into new gene drive viruses. Here, we describe a gene drive against HSV-1 and 2 that inactivates the transport of virions from the nervous system to the mucosal periphery. We hypothesize that gene drive vectors could reach the site of HSV latency in ganglionic neurons and prevent wild-type HSV from traveling to the periphery and causing disease. We show that gene drive vectors act as live-attenuated HSV vaccines that induce a protective immune response in addition to CRISPR-based protection. We report that prophylactic treatment with gene drive vectors prevents latency establishment and viral shedding after vaginal infection of mice with HSV-1 and HSV-2. Furthermore, therapeutic treatment reduces vaginal shedding in mice latently infected with HSV-2. Overall, our results suggest that gene drive vectors could combine immune and CRIPSR-based protection to durably prevent or suppress HSV disease.

1538 Gene Therapy for Tuberous Sclerosis Complex 1: AAV-Mediated Hamartin Delivery Enhanced via Extracellular Vesicles in a Mouse Model of TSC1.

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Tuberous sclerosis complex (TSC) is a dominantly inherited disease affecting multiple organs with an incidence of about 1 in 5500. TSC is caused by mutations in either the *TSC1* gene encoding hamartin or the *TSC2* gene encoding tuberin, which normally form a complex that inhibits mammalian target of rapamycin (mTOR). The loss of either of these proteins due to genetic mutations results in growth and proliferation in susceptible cells causing abnormal development and tumors. Neurodevelopmental manifestations, which are responsible for the greatest morbidity, include refractory epilepsy, hydrocephalus, and cognitive impairment. In our stochastic, cerebral mouse model of Tsc1, mice (Tsc1 flox/flox) are injected into the cerebral ventricles with a Cre recombinase encoding an adeno-associated virus (AAV) vector on the day of birth (P0; **Prabhakar et al., 2013**). The resulting loss of function leads to hypertrophy and proliferation of cells lining the ventricles, enlarged neurons and elevated phosphorylation of ribosomal protein S6 in hamartin-null cells, as well as dysmyelination, and early death with a mean of 50 days. AAV vectors have been used widely in clinical trials for many hereditary diseases with little-to-no toxicity, long-term action in nondividing cells, and benefits seen after a single injection. We used AAV9, a serotype that can efficiently cross the blood-brain barrier to deliver hamartin to cells in Tsc1 lesions, which would make them shrink and stop dividing. It could then be delivered to other cells in the brain via extracellular vesicles (EVs), increasing the range of delivery over time. We have been able to normalize the neuropathological disturbances in our mouse model of TSC1 via the intravascular administration of an AAV9 vector encoding hamartin at postnatal day 21, extending the average lifespan from 50 to over 120 days in 85% of animals (**Prabhakar et al., 2019; Abou Haidar et al.,**

under review). Our current work aims to improve gene therapy by enhancing hamartin delivery using AAV vectors combined with EVs. We have generated different modified forms of hamartin plasmid expression constructs to identify modifications that can increase hamartin uptake into EVs. We have transfected these constructs into HEKT cells and showed via western blot that we can increase incorporation of modified hamartin cargo into EVs. A version of hamartin tagged with a palmitoylation signal at the N-terminal was most effective at EV incorporation. The palmitoylation signal anchors the protein to the plasma membrane, facilitating its inclusion in EVs. Furthermore, we have used a 1.0- µm-pore Transwell system seeding stably transfected HEKT cells in the upper chambers and TSC1-null HEK cells in the bottom chambers. We confirmed successful hamartin production, packaging, and transfer to recipient cells through EVs by immunohistochemistry, as we detected a robust signal of the in-frame V5 epitope tag used in our constructs. We are currently evaluating the effects of the hamartin-loaded EVs on inhibiting mTOR activity using immunohistochemistry and flowcytometry to measure its impact on the levels of phosphor-S6 protein expression in cultured TSC1 null HEK cells and neuroprecursor cells. We have packaged these constructs into AAV vectors for *in vivo* studies on our stochastic cerebral mouse knock-out model of TSC1. We will evaluate the enhanced delivery of hamartin by combining AAV gene therapy and EV spread of the protein/mRNA in the brain.

1539 Human Secreted Klotho Expression Improves Cognition and CNS Status in Aged Non-Human Primates

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Aging is the primary risk factor for numerous diseases that significantly impact people's health. New therapeutic strategies for achieving healthy aging must be developed to mitigate the rising social and economic costs of senescence in modern societies. In this context, the protein Klotho exhibits pleiotropic beneficial functions, slowing down the progression of aging. While its therapeutic potential has been well established in murine models, limited data exist regarding its translatability to animal models that are closely evolutionarily related to humans.

Here, aged individuals of the non-human primate species *Microcebus murinus* were treated with AAV9 vectors expressing the alternatively spliced secreted isoform of the human *Klotho* gene (s-KL). Nine months post-administration, we confirmed s-KL expression in both the hippocampus and liver. Notably, s-KL-treated animals showed improved performance in the novel object recognition test over time, demonstrating enhanced cognitive preservation compared to Null-treated animals. The observed cognitive improvements in s-KL-treated aged individuals correlated with changes at the cellular level in the central nervous system (CNS). Specifically, we detected an increase in the thickness of the CA1 neuronal layer in the hippocampus. Additionally, the treatment modulated glial cell populations, leading to a significant reduction in astrocytes and an increase in microglia, as measured by GFAP and ferritin markers, respectively. Furthermore, the effect of s-KL on CNS white matter was also assessed, measuring lipid oxidation levels using synchrotron-Fourier transform infrared microspectroscopy.

In this study, we demonstrate that the secreted isoform of *Klotho* enhances cognitive performance in aged non-human primates and, for the first time, link this effect to cellular and molecular changes in the CNS of treated animals.

1540 A new, patient-derived *SYNGAP1* mouse model demonstrates multipledisease-relevant phenotypes.

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Introduction: SynGAP is a Ras-GTPase activating protein, enriched at excitatory synapses in the brain. It is critical for neuronal development and synaptic plasticity, as well as a negative regulator of excitatory neurotransmission. At sub-homeostatic levels, SynGAP deficiency results in excessive AMPA receptors on the cell membrane, which impacts neuronal development, integrity, signaling and other aspects critical for cognition and excitability. Not surprisingly, SYGNAP1 is one of the most frequently mutated genes in Neurodevelopmental Disorders (NDD). SYNGAP1-Related Disorders (SRD) describe a spectrum of pathologies that arise from clinical alleles of the *SYNGAP1* gene, impacting 1 in 16,000 live births. Patients typically harbor a loss-of-function *de novo* nonsense or missense mutations resulting in an autosomal dominant haploinsufficiency with symptoms that can include: epilepsy, intellectual disability, developmental delays, behavioral problems, high pain threshold, hypotonia, eating problems, sleep disorders, autism spectrum disorder and ataxia/gait abnormalities. In fact, large exome sequencing in the UK found *SYNGAP1* to be the 5th most prevalent NDD-related gene in and accounts for ~1% of the NDD population. Currently, treatments are limited to symptom management, as no treatments can address the underlying genetic condition or SynGAP protein deficiency.

Methods: Previously, heterozygous Syngap1 knockout mice have demonstrated several disease-relevant phenotypes, including learning and memory deficits, abnormal social behavior, seizures and hyperactivity. However, most patients do not possess deletion alleles, as most clinical alleles are nonsense mutations. Here, we describe a new mouse model with a patient derived mutation, Q503X. By employing data from previous models, we assessed behavioral, physiological, and molecular metrics in this newly developed mouse model.

Results: Q503X mice demonstrated multiple phenotypes consistent with other *SYNGAP1* -deficient models, including reduced working memory and sleep disturbance. Open field metrics identified a robust hyperactivity and vertical rearing, in addition to perturbed center-margin dwell time. We noted several previously undescribed sex-specific differences, such as acoustic startle, body weight, and 3-Chamber social discrimination index. Notably, these phenotypes track with symptoms reported in SRD patients

Conclusions and Future Directions: Upcoming assessments into pain perception and EEG telemetry. SRD is often inclusive of seizures and many caregivers report unusually high pain tolerances in patients. While we anticipate EEG to reveal spike train events and other previously identified phenotypes in other *SYNGAP1* models, Von Frey and Hot Plate assessments have been widely unexplored in mouse models for SRD. Currently, the assessed data validate this new, patient derived model platform for further study and therapeutic development addressing SRD, with a direct connection a human pathogenic variant. Combined with the upcoming assessments, the Q503X model is poised to become a robust and effective tool in the efforts to understand and treat SRD.

Acknowledgements: SynGAP Research Fund, Kathryn Helde, Mike Graglia, The Jackson Laboratory Center for Biometric Analysis

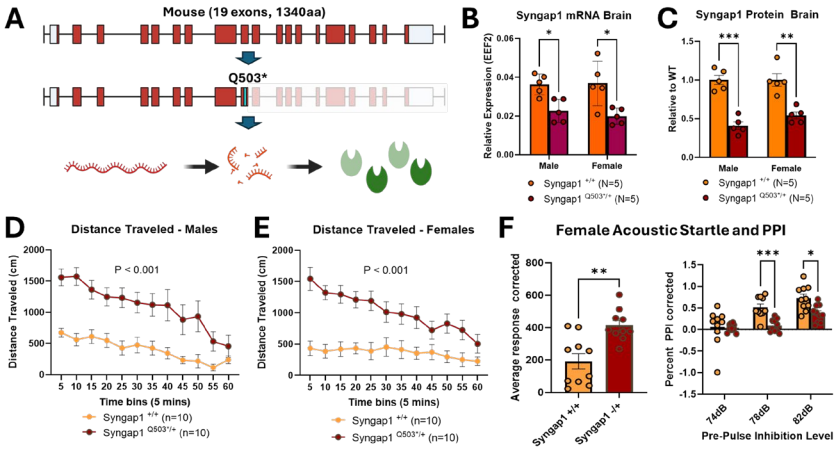

Figure 1: Key Phenotypes in the Patient-Derived Syngap1Q503 mouse*

*(**A**) The Q503* mutation is predicted to result in nonsense mediated decay, effectively reducing the amount of SYNGAP protein. Syngap1 mRNA **(B)** and SYNGAP protein **(C)** is reduced in brain tissues of both males and female Syngap1 Q503* heterozygous mice. **(D,E)** Open field analysis demonstrates hyperactivity of Syngap1 Q503* heterozygotes compared to WT littermates. **(F)** Female Syngap1 Q503* heterozygotes show enhanced freezing in acoustic startle tests, as well as reduced pre-pulse inhibition. Males were unaffected. Data presented as mean ± SD. Asterisks indicate P≤0.05(*), 0.01(**), 0.001(***), two-tailed unpaired t-tests with Welch correction.*

1541 Enhancing Chemotherapeutic Efficacy by Combining Efflux Transporter Inhibition with CED Using a Novel sEEG-based Drug Delivery System

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Chemotherapeutic efficacy in brain tumors is primarily challenged by inadequate drug penetration into the tumor due to the blood-brain barrier (BBB) and short drug-tumor residence time due to drug efflux. To address these limitations, we explored the use of convection-enhanced delivery (CED) to bypass the BBB, combined with pharmacological inhibition of efflux transporters to enhance drug retention following CED infusion, using small and large animal models.

Topotecan has shown promise for treating diffuse midline gliomas (DMG). In mouse models of DMG, topotecan concentrations in the midbrain were significantly higher after CED infusion (20 µL of 100 µM topotecan) when mice were pre-treated with either everolimus (5 mg/kg/day) or elacridar (100 mg/kg/day) for 5 days prior to topotecan infusion. A single CED infusion of a large drug volume may not provide adequate tumor coverage and can lead to local toxicity. To improve coverage and reduce local drug concentration, multiple infusion trajectories can be used. In clinical settings, this approach is often limited by the size of the available CED devices, which are bulky (e.g. take up a large area on the scalp) and restrict the number of trajectories. To address this limitation, we employ a new, slenderer device. This device is an adaptation of a stereoelectroencephalography (sEEG) electrode, which has been modified to enable drug delivery (DD-sEEG). The DD-sEEG device, with a diameter of 0.8 mm and an anchor bolt of less than 2.5 mm in diameter, allows the implantation of multiple probes in close proximity. Additionally, this device can record real-time neural activity, which can be used as a surrogate measure to evaluate drug toxicity. The DD-sEEG device was evaluated both in bench studies and in vivo in a porcine brain model. Bench tests, using a 0.6% agarose gel and 1% Methylene Blue dye, demonstrated effective CED for infusion rates ranging from 0.5 to 5 µL/min. In vivo testing in porcine brain confirmed that multiple DD-sEEG electrodes per animal could be stereotactically implanted using a clinical workflow, including structural MRI for trajectory planning, followed by device implantation and drug delivery. CED of gadolinium was successfully visualized via

post-infusion MRI, and localized infusions were observed in the putamen and hippocampus. Furthermore, real-time neural recordings were obtained before, during, and after drug delivery using penicillin (60 µL/site), a drug known to modulate neural activity.

These results demonstrate the feasibility of enhancing chemotherapeutic efficacy in the brain by combining efflux transporter inhibition with CED using a slender infusion device. Moreover, the ability to record real-time neural activity provides an innovative method for assessing local toxicity and long-term effects of chemotherapeutic drugs on neurons. Since in diffuse glioma the tissue consists of both tumor cells and neurons, the ability to monitor neural activity could greatly impact therapy development and evaluation. Future studies will focus on testing CED with topotecan and oral everolimus in a large animal model.

1542 Comparative Gene Therapy Efficacy for KO and KI Models of SLC13A5 Citrate Transporter Disorder

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SLC13A5 Citrate Transporter Disorder is an ultra-rare pediatric neurodevelopmental disorder characterized by intractable neonatal seizures, cognitive delay, and motor impairment. This disorder is caused by pathogenic loss of function variants in the SLC13A5 gene, which encodes for the plasma membrane sodium citrate co-transporter (NaCT). Pathogenic mutations cause a deficiency of citrate transport, which results in both increased extracellular citrate and decreased citrate import. Increased circulating citrate is a consistent feature of the disorder in both patients and rodent models. NaCT functions as a homodimer that is folded and assembled in the endoplasmic reticulum before being translocated to the plasma membrane. There are at least forty known mutations that fall within two major classes, affecting either transporting capabilities at the plasma membrane or protein folding/complexing. The most common patient mutation, G219R, is predicted to affect protein folding, causing protein retention within the endoplasmic reticulum.

Currently, while most patients are on several anti-seizure medications with varying amounts of control, there are no therapeutic options that target the underlying cause of the disorder. Because the disorder is monogenic, gene therapy offers a promising therapeutic option for patients. Our group has developed an adeno-associated virus (AAV) vector encoding a codon optimized human SLC13A5 gene (scAAV9/SLC13A5). In previous studies, we showed scAAV9/SLC13A5 is efficacious in Slc13a5 KO mice, decreasing citrate concentration in the plasma and cerebral spinal fluid, and ameliorating epileptic phenotypes as assessed through EEG recordings and seizure induction paradigms. Currently, it is unknown whether this gene therapy approach would be efficacious within a model that produces misfolded protein, as there could be issues with complexing between endogenous mutant copies and gene therapy introduced copies of Slc13a5.

To assess this, we used a recently created knock-in mouse (KI) that contains the human-equivalent G219R mutation in mouse Slc13a5. This model, like the KO model, displays increased citrate in the CSF as a result of nonfunctional NaCT in the brain. Slc13a5 KI and KO mouse models were treated with scAAV9/SLC13A5 at post-natal day 10 via intrathecal lumbar puncture injection along with vehicle treated WT, KI, and KO littermate control mice. Weight and survival were monitored throughout the study to monitor treatment tolerability. At approximately 4 months of age, seizure susceptibility was assessed via a kindling pentylenetetrazol (PTZ) seizure induction paradigm. Plasma and cerebral spinal fluid were collected and are being quantified for citrate concentration using GC-MS analysis to assess NaCT function. Up to 4 months following virus treatment, scAAV9/SLC13A5 appears to be well tolerated in life, as evidenced by normal weight gain and survival in both mouse models of SLC13A5 Citrate Transporter Disorder. In a small pilot study that used a low dose of PTZ, seizure severity was not worsened in virus treated KI mice. Follow-up studies are ongoing using a higher dosage of PTZ and will be reported. Results from these studies will inform on the efficacy of AAV-mediated SLC13A5 gene restoration in a patient relevant context and could be applicable to other disorders where protein misassembling is a component of disfunction.

1543 Gene therapy for mouse models of 6-pyruvoyl-tetrahydropterin synthase deficiency

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6-pyruvoyl-tetrahydropterin synthase (PTPS) is an enzyme responsible for the synthesis of tetrahydrobiopterin (BH4), and BH4 in turn is a cofactor for the synthesis of monoamine neurotransmitters and nitric oxide (NO). PTPS deficiency causes severe motor dysfunction and neurodegeneration. Although this disease can be treated by oral levodopa (L-DOPA), the treatment is life-long, and symptoms appear shortly after dose missing. In view of the heavy burden of the disease and the improvement in the safety of gene therapy, we plan to develop a gene therapy for PTPS deficiency. The gene therapy expression cassette includes a CMV promoter and three cDNAs (PTS + GCH1 + SPR) separated by IRES and 2A peptide. In 293T cells transfected with this tricistronic plasmid, pcDNA3.1-BH4, western blot analysis detected the expression of the three proteins. AAV9-CMV- BH4 was successfully packaged. Injection of AAV9-CMV- BH4 to the putamen of adult wild-type mice elected the levels of BH4 and dopamine in the brain. Currently, a toxicity study of intracisternal injection of AAV9-CMV- BH4 in adult wild-type mice is ongoing. Several approaches are employed to obtain a suitable mouse model of PTPS deficiency. A knock in (KI) mouse model with a Chinese founder PTS mutation c.259C>T (P87S) have been created. However, neither BH4 nor dopamine levels in the brain of P86S KI mice decreased. We are currently working on a conditional (inducible) knockout (cKO) mice created by flanking a PTS exon with loxP, and after mating the cKO mice with mice expressing tamoxifen inducible Cre (Cre recombinase), symptoms can be induced by tamoxifen in adult mice. A rescued PTS KO mouse model previously created by Dr. Ichinose can be another choice. We hope a safe and effective gene therapy will be available for PTPS deficiency in the future.

1544 Utilizing Cytosine Base Editors to Generate *MECP2*-R270X Knock-in Models of Rett Syndrome

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Rodent models have played a crucial role in advancing our understanding of many biological processes and diseases. However, to fully capture the complexity of human neurological disorders, additional model organisms are needed. Non-human primate (NHP) disease models offer a valuable complement, given their similarities to humans. Recent advancements in genome-editing technologies, such as cytosine base editors (CBEs), have made it possible to generate primate models. CBEs, with their ability to target C-to-T conversions at the single base-pair level without inducing double-strand breaks, offer a powerful and efficient tool to edit the genome at the embryonic stage. Here we used YE1 BE4 Max, an evolved CBE, to introduce targeted mutations in the MeCP2 genes in marmoset embryos. In this study, we conducted preimplantation genetic testing (PGT) to genetically analyze each individual embryo and selectively transfer those carrying the mutation. In combination with this approach, we have successfully generated MeCP2 R270X founders for Rett Syndrome studies, and trio deep sequencing analysis shows minimal off-target effects in these founders.

1545 AAV-Mediated Gene Supplementation in Combination with Microglial Replacement for Vanishing White Matter Disease Using Circulation-Derived Myeloid Cells (CDMCs)

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Leukoencephalopathy with Vanishing White Matter (VWM) is a rare, untreatable neurodegenerative leukodystrophy that presents with ataxia, seizures, and progression to death. Our ultimate goal is the development of a therapy. VWM is caused by autosomal recessive mutations in the five subunit genes of the Eukaryotic Initiation Factor 2B (EIF2B) complex, which is necessary for the regulation of the integrated stress response (ISR). VWM has abnormal activation of the ISR. Current dogma in the field has suggested that astrocytes are responsible for VWM pathophysiology. We developed adeno-associated virus (AAV)-mediated *EIF2B5* gene (the most commonly mutated subunit) replacement therapy for VWM with targeted astrocyte expression. Using iterations of the GFAP promoter we found astrocyte targeting throughout the neuroaxis. In the classic R191H mouse model and a more severe I98M mouse model, we showed that the astrocyte-targeted gene therapy delays disease progression, partially rescues body weight and motor function, and attenuates myelin loss (Herstine et al., 2024). However, our current data demonstrates waning efficacy in treated mice who developed phenotypes similar to untreated mutants including recrudescent ISR activation. To explore this, we tested the role of microglia on astrocytes- activated microglia secrete cytokines which induce reactive astrocytes. In R191H VWM mutants, as well as once AAV-treatment had waned, we found extensive infiltration of Iba1+/CD68+ microglia into the CNS, and increased formation of stress granules that mirrored the presence of activated microglia. Our hypothesis is that activated microglia contribute to VWM pathophysiology, and that transplant of wild-type microglia is necessary for full gene therapy rescue of VWM by interrupting the cycle of microglia-driven reactive astrocyte activation. Currently, AAV transduction in microglia is poor, necessitating transplant. To test our hypothesis, we are characterizing the timing and extent of activated microglia involvement in R191H mice at multiple ages; in comparison to wt and AAV9-treated VWM affected mice. Histological, molecular, and biochemical targets are for activated and stable microglia; and CNS cell type markers; and quantification of ISR activation. We found extensive infiltration of activated microglia in the CNS of mutants, especially in the cervical spinal cord. The activated microglia also expressed high levels of stress granules. Since dysregulated microglia may interfere with gene therapy rescue, we are testing microglia replacement using circulation-derived myeloid cells (CDMCs). Through an established protocol, including busulfan ablation of endogenous hematopoietic cells, followed by transplant with donor CDMCs and microglia depletion through PLX5622 treatment, we have shown GFP-labeled donor CDMCs cross the blood-brain barrier and replace endogenous microglia in the CNS of wt mice. We are comparing efficacy between three groups: untreated VWM mice; VWM mice receiving microglia replacement and AAV9-EIF2B5 gene therapy; and VWM mice with microglia replacement only. In the VWM mutants, busulfan and PLX5622 treatment with CDMC transplant does not worsen disease progression. We have however observed low rates of donor cell engraftment in mutants and are currently optimizing our protocols in order to assess the response on VWM disease rescue. Gene therapy in combination with microglia transplant may provide a route towards rescue of VWM.

1546 Selective reactivation of *Scn1a* gene in GABAergic interneurons to explore symptomatic reversibility in Dravet Syndrome

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Dravet Syndrome (DS) is a developmental and epileptic encephalopathy characterized by drug resistant tonic-clonic seizures, psychomotor delay, cognitive impairment, and a high risk of mortality by sudden unexpected death (SUDEP). It results from haploinsufficiency of the *SCN1A* gene, encoding the alpha subunit of the voltage-gated sodium channel Nav1.1, predominantly expressed in GABAergic interneurons (IN). DS is triggered by IN hypoexcitability, but secondary circuit modifications contribute to disease progression. Significant efforts in gene therapy aim to provide a cure, with most of the strategies directed restoring *SCN1A* gene expression specifically in INs. We provided evidence that ubiquitous restoration of physiological levels of *Scn1a* after symptom onset is sufficient to revert seizures and behavioral alterations in a mouse model of the disease. However, information on the optimal neuronal populations to target at different disease stages is lacking. Therefore, this study investigates whether IN correction alone is sufficient to treat the disease at various stages.

We utilized a DS mouse model carrying a conditionally removable STOP cassette in the *Scn1a* locus (Scn1a$^{Stop/+)}$ crossed with the constitutive (Gad2-Cre) or inducible (Gad2-CreERT2) GABAergic Cre drivers to restore *Scn1a* gene expression selectively in INs.

Phenotypic evaluations of *Scn1a*$^{Stop/+}$;Gad2Cre mice were conducted through survival monitoring until post-natal day P60, video-EEG recordings to detect spontaneous seizures, thermal induction to assess seizure susceptibility and whole-cell patch-clamp recordings in hippocampal circuit. to evaluate neuronal excitability and synaptic transmission *Scn1a*$^{Stop/+}$;Gad2Cre mice were fully protected from SUDEP and thermal induced seizures, and exhibited a substantial reduction in spontaneous seizure frequency and severity compared to *Scn1a*$^{Stop/+}$mice. Intriguingly, fast-spiking IN firing properties and inhibitory postsynaptic currents in Scn1a$^{Stop/+}$;Gad2Cre mice were not completely normalized, suggesting the existence of non-cell autonomous defects that cannot be corrected by a specific therapeutic intervention in INs. To mimic the effect of an IN specific gene therapy administered in the chronic phase of the disease, we restored *Scn1a*gene expression in INs of *Scn1a*$^{Stop/+}$; Gad2CreERT2 adult mice. After seizure baseline video-EEG recordings from P45 to P60, mice were randomized and injected with vehicle or tamoxifen, that activates Cre recombinase and restores physiological Nav1.1 expression. Mice are being recorded for 30 days after Nav1.1 re-expression and the effect on seizure frequency and severity will be evaluated.

This study will enhance our understanding of the impact of GABAergic IN dysfunction at different stages of DS and will inform ongoing gene therapy efforts regarding the sufficiency of correcting only GABAergic INs at various phases of the disease.

1547 The Importance of High-Density Microelectrode Arrays for Recording Multi-Scale Extracellular Potential and Label-Free Characterization of Network Dynamics in iPSC-Derived Neurons

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Advances in microelectrode array (MEA) technology for in-vitro electrophysiological recordings have made it possible to study neuronal networks across multiple scales, from subcellular properties to network-level dynamics. These devices are essential for exploring the phenotypes of neurological disorders and accelerating drug discovery, offering unique insights into the behaviour of neuronal networks. Key factors such as electrode density, spacing, and size significantly impact signal quality, noise, and sensitivity. To exhaustively characterize neuronal networks, MEAs must combine single-cell and subcellular resolution with high-throughput capabilities, maintaining sensitivity to small extracellular action potentials to capture the full range of network activity. In this study, the MaxOne and MaxTwo high-density (HD) MEA systems (MaxWell Biosystems, Switzerland) were utilized to record activity from induced pluripotent stem cell-derived neurons. These systems, with 26,400 electrodes per well, demonstrated the benefits of increased statistical power in longitudinal data collection. HD-MEA recordings were compared to simulated low-density recordings, where adjacent electrodes on HD-MEAs were clustered to mimic larger, lower-density electrodes. Additionally, the AxonTracking Assay, an automated tool for analysing individual axonal arbours from multiple neurons simultaneously, was used to evaluate axonal structures and network functionality in the recorded cultures. Results showed that higher electrode density and smaller electrode size enhanced sensitivity, allowing for the detection of smaller spikes and capturing the complete spectrum of network dynamics. The high-resolution analysis of network activity, combined with subcellular insights from the AxonTracking Assay, offers a robust platform for drug screening and disease modelling.

1548 Novel Stem Cell Model for CASK-Related Disorders and Evaluation of a CRISPR/dCas9 Based Rescue

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CASK-related disorders are an ultra-rare neurodevelopmental disorder with under 300 patients described. The severe form, CASK-related microcephaly with pontine and cerebellar hypoplasia (MICPCH), results from loss of function mutations within the X-linked *CASK* gene, which then contributes to aberrant neuronal activity and development. Additionally, there is no current treatment to address the underlying issue of a lack of functional CASK protein. In this study, we created a human female neuronal model system to understand the cellular deficits caused by a lack of CASK. Similarly, this model provides an epigenetically relevant system to test reactivation approaches using CRISPR/dCas9 epigenetic editors. Utilizing CRISPR-based NHEJ, we generated two distinct cell lines, one with a heterozygous 4 base pair deletion within the CASK PDZ domain and the other, homozygous for the same mutation. Pluripotency was maintained and checked through immunocytochemistry at both the iPSC and NSC stage. Edits were confirmed via amplicon sequencing of gDNA and RNA for *CASK* expression at each stage. Interestingly, the heterozygous iPSC showed low levels of escape from X chromosome inactivation (XCI), around 18% of WT *CASK* expression, while other XCI subject genes tested did not. Further, upon differentiation to neural stem cells (NSCs), *CASK* expression was seen only from a single allele, where it expressed mutant CASK on the active allele and WT was silenced via X chromosome inactivation. These results suggest low levels of *CASK* escape at an iPSC stage that can be epigenetically stabilized upon lineage commitment to an NSC. To test if WT *CASK* expression can be rescued from the inactive allele, we utilized CRISPR/dCas9 paired with an epigenetic modifier and a transcriptional activator. Guide RNA screens in this system, transfected into the heterozygous NSCs, identified lead combinations that reactivated WT expression to approximately 10% as compared to the active allele. Western blot of CASK was performed to test if this increase in mRNA is sufficient to rescue CASK protein. In summary, this project generated novel female human stem cell lines to model CASK-related disorders and demonstrated the reversal of a low level escape gene following defined lineage commitment. Further, we show increased expression of *CASK* with CRISPR/dCas9 paired with an epigenetic modifier and transcriptional activator. This project demonstrates the first therapeutic avenue for CASK-related MICPCH, as well as highlighting the potential to utilize targeted X-reactivation as a platform approach for X-linked disorders.

1549 Disruption of UBE3A-ATS by Gene Editing with a Type V CRISPR-Cas Enzyme as a treatment for Angelman Syndrome

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Angelman Syndrome is caused by the loss of maternal UBE3A. Reactivation of the paternal UBE3A allele, normally silenced by the expression of the UBE3A antisense transcript (UBE3A-ATS) in mature neurons, has been extensively pursued as a therapeutic treatment for Angelman syndrome. Here, we describe a gene editing strategy using a Type V CRISPR-Cas enzyme to potently disrupt UBE3A-ATS and restore UBE3A mRNA and protein levels in in vitro Angelman patient neurons.

We first screened multiple type V CRISPR nucleases and guides, spanning a 60,000 base pair region from the SNORD115 clusters to the 3' end of the UBE3A coding region, in HEK293 cells. Approximately 100 nuclease/guide pairs with efficient indel formations from the initial HEK293 screen were then tested in wild-type human iPSC-derived cortical neurons using a lentivirus-based delivery for the nuclease and lipofectamine-based delivery for the associated guides to identify pairs that were capable of robustly disrupting UBE3A-ATS and recovering UBE3A mRNA and protein levels. Several regions were identified within the UBE3A-ATS locus where editing led to more efficient protein recovery. Finally, 8 guides paired with two nucleases (ABR-001 and ABR-004) demonstrated UBE3A recovery equal to or greater than that of a benchmark ASO (GTX-102) in Angelman patient neurons, including 5 guides that reached UBE3A levels equivalent to neurotypical controls.

To demonstrate the functional impact of the UBE3A-ATS locus disruption, we developed an assay for measuring neuronal network activity of human iPSC-derived cortical neurons on a multi-electrode array (MEA). We identified a network hyperexcitability phenotype in Angelman patient-derived neurons. Our nuclease/guide pairs that robustly restored UBE3A expression were able to reverse this hyperexcitability phenotype and this degree of rescue was correlated with guide potency. Taken together, these results demonstrate the feasibility of using a Type V CRISPR-Cas enzyme to durably treat Angelman Syndrome.

1550 AI-Guided Gene Therapy Strategy for Treating Patients with Tuberous Sclerosis Complex Type 2

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutations in either the *TSC1* or *TSC2* genes, which affects nearly 1 in every 5,500 newborns, ~80,000 people in the U.S., and 2 million people worldwide. There is currently no cure. *TSC1* and *TSC2* encode for Hamartin and Tuberin, respectively, and together with TBC1D7, form a protein complex which negatively regulates mammalian target of rapamycin complex 1 (mTORC1) signaling. While TSC affects multiple organ systems, the most frequent causes of mortality and morbidity are due to neurologic complications, including refractory epilepsy, autism, neuropsychiatric issues, and uncontrolled ependymal growth lesions resulting in life-threatening hydrocephalus. As epilepsy occurs in approximately 85 percent of patients, the inability to control seizures is one of the most debilitating aspects of the disease as most existing medications show loss of efficacy within 1-2 years of treatment. Everolimus is a potent mTOR inhibitor that has been shown to reduce tumor lesions and treat intractable epilepsy in TSC. However, only 50% of patients show a clinically relevant reduction in seizure frequency and no evidence supports a positive impact for reducing cognitive and neuropsychiatric deficits in patients. Based on all these challenges and limitations, there is a desperate and unmet clinical need to develop long-lasting and highly efficacious treatment options for TSC.

We have developed a novel gene replacement strategy using an adeno-associated viral (AAV) vector to treat TSC type 2. Since the coding region of *TSC2* (~5.4 kb) far exceeds the packaging capacity of current AAV vectors, we leveraged principles based on rational protein design and artificial-intelligence (AI)-based protein structure prediction to develop micro-Tuberin (MT) candidates with the same key structural motifs and functional components as encoded in full-length Tuberin (Figure 1A). Our strategy to miniaturize full-length Tuberin was based on excising unstructured regions and replacing them with short flexible linkers (consisting of glycine and serine residues). Based on these principles, we generated seven novel MT candidates, which were validated by homology modeling using Swiss model and the AI-guided folding prediction tool, AlphaFold 2 (Figure 1B-I). Recently, a condensed form of Tuberin (cTuberin) was designed and evaluated as a potential gene therapy candidate for TSC type 2. However, our analysis has shown it lacks a dimerization interfacial region and overall structural homology to that of full-length Tuberin (Figure 1J-K). We rigorously characterized our MT candidates using multiple independent functional in vitro assays based on their ability to inhibit mTOR signaling in astrocytes and neural progenitor cells (Figure 2). Strikingly, our preliminary data show that several of our MT candidates show superior functional activity in comparison to cTuberin and at similar levels to that of full-length Tuberin, thereby providing a strong rationale for further preclinical development (Figure 2). Current studies include down-selecting top-performing candidates using patient-specific 3D cerebral organoid models, followed by subsequent *in vivo* efficacy testing using a *Tsc2* conditional knockout mouse model. Successful completion of these goals will be critical elements in the path towards clinical translatability for micro-Tuberin gene therapy for patients with TSC type 2.

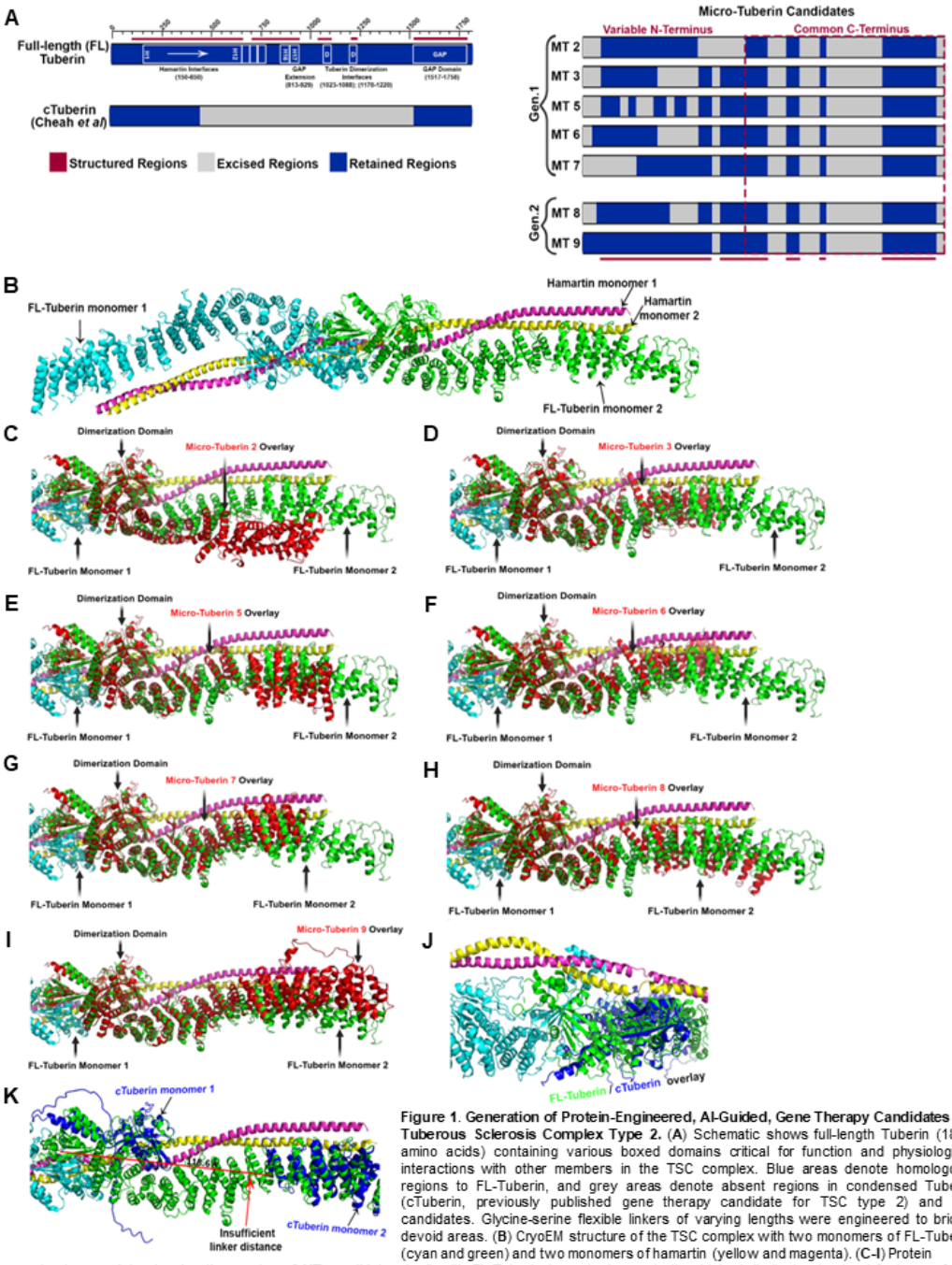

Figure 1. Generation of Protein-Engineered, AI-Guided, Gene Therapy Candidates for Tuberous Sclerosis Complex Type 2. (**A**) Schematic shows full-length Tuberin (1807 amino acids) containing various boxed domains critical for function and physiological interactions with other members in the TSC complex. Blue areas denote homologous regions to FL-Tuberin, and grey areas denote absent regions in condensed Tuberin (cTuberin, previously published gene therapy candidate for TSC type 2) and MT candidates. Glycine-serine flexible linkers of varying lengths were engineered to bridge devoid areas. (**B**) CryoEM structure of the TSC complex with two monomers of FL-Tuberin (cyan and green) and two monomers of hamartin (yellow and magenta). (**C-I**) Protein structure models showing the overlay of MT candidates (red) with FL-Tuberin (green), demonstrating high similarity in structural features of FL-Tuberin. (**J**) Overlay of one monomer of FL-Tuberin (green) with the AlphaFold2 predicted structure of cTuberin (blue), demonstrating that cTuberin lacks the Tuberin dimerization interfacial region. (**K**) Overlay of the amino terminal region of one molecule of cTuberin (blue, far right) and the carboxy terminal region of another molecule of cTuberin (left) with the corresponding regions in FL-Tuberin (green), demonstrating that the flexible linker of cTuberin is insufficient to traverse the distance between the conserved regions (118.6 Å, red line).

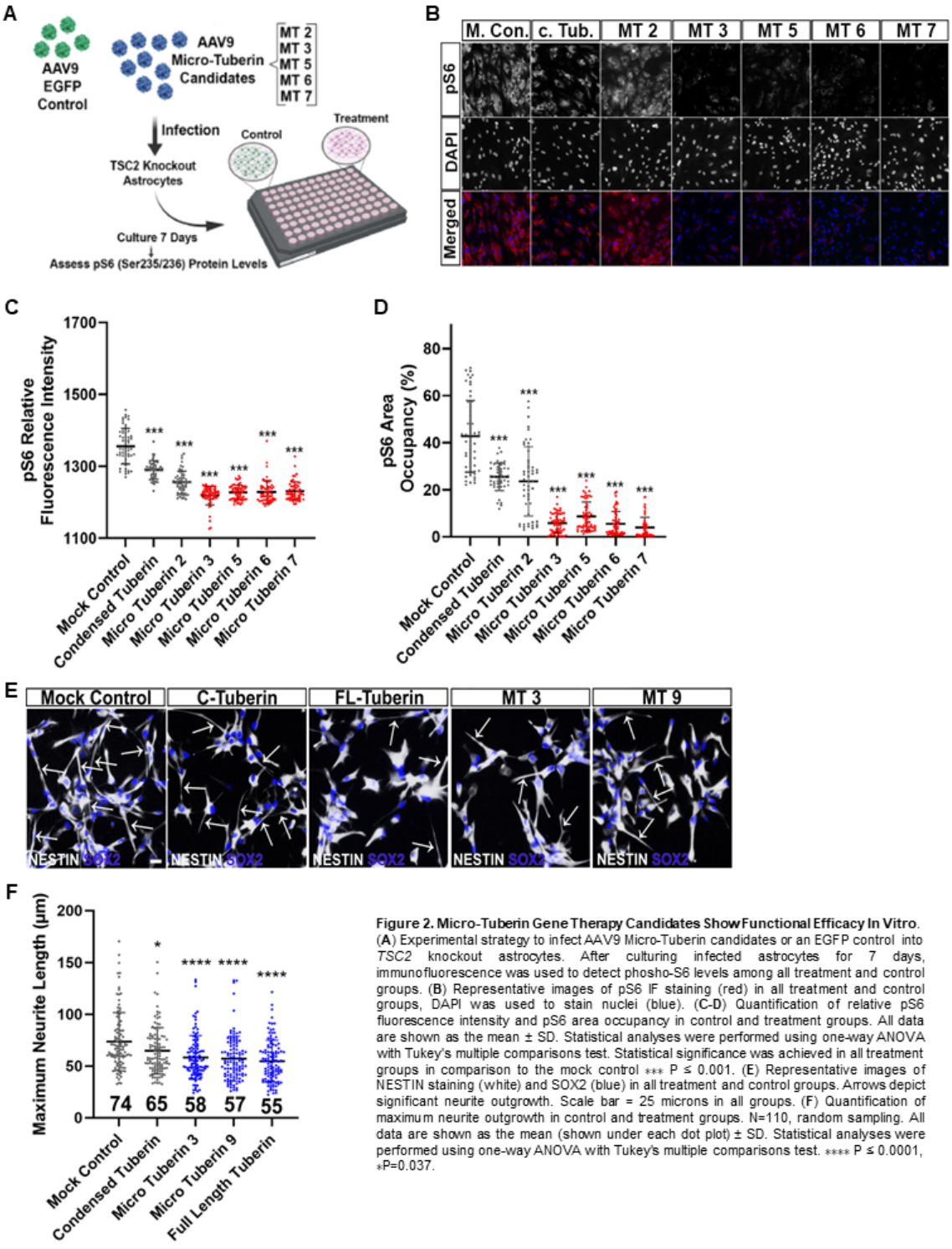

Figure 2. Micro-Tuberin Gene Therapy Candidates Show Functional Efficacy In Vitro. (**A**) Experimental strategy to infect AAV9 Micro-Tuberin candidates or an EGFP control into *TSC2* knockout astrocytes. After culturing infected astrocytes for 7 days, immunofluorescence was used to detect phosho-S6 levels among all treatment and control groups. (**B**) Representative images of pS6 IF staining (red) in all treatment and control groups, DAPI was used to stain nuclei (blue). (**C-D**) Quantification of relative pS6 fluorescence intensity and pS6 area occupancy in control and treatment groups. All data are shown as the mean ± SD. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test. Statistical significance was achieved in all treatment groups in comparison to the mock control *** $P \leq 0.001$. (**E**) Representative images of NESTIN staining (white) and SOX2 (blue) in all treatment and control groups. Arrows depict significant neurite outgrowth. Scale bar = 25 microns in all groups. (**F**) Quantification of maximum neurite outgrowth in control and treatment groups. N=110, random sampling. All data are shown as the mean (shown under each dot plot) ± SD. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test. **** $P \leq 0.0001$, *P=0.037.

1551 Impact of *FXN* gene editing in Friedreich's Ataxia patient-derived lymphoblasts and iPSC-derived microglia

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Friedreich ataxia (FA) is a neurogenerative disorder caused by homozygous GAA expansion mutation in the first intron of frataxin (*FXN*) gene. FXN is a mitochondrial protein, and its deficiency leads to mitochondrial iron overload, defective energy production, and oxidative stress. We previously showed complete rescue of neurologic complications of FRDA in YG8R mice with a single, systemic infusion of wild-type hematopoietic stem and progenitor cells (HSPC), and this rescue was mediated by FXN transfer from engrafted HSPC-derived microglia to diseased neurons. We next developed an autologous stem cell transplantation approach using CRISPR/Cas9-mediated excision of GAA repeats in FRDA patients' $CD34^+$ HSPCs that increased FXN expression and improved mitochondrial functions. The current study aims to understand tissue degeneration in FRDA, focusing on complex interactions between HSPC-derived cells and FRDA-affected tissues. To achieve this, we utilized: a) two HSPC-lineage cell models - patient iPSC-derived microglia (iMGs) and lymphoblast cells, both uncorrected or CRISPR/Cas9 gene corrected , and b) two FRDA affected cell models - iPSC-derived neurons and fibroblasts. We previously validated the FRDA phenotype in these iPSC-derived neurons which exhibited characteristic disease phenotypes including non-homogenous microtubule staining in neurites, increased caspase-3 expression, elevated mitochondrial superoxide levels, mitochondrial fragmentation, partial cristae degradation and loss in endoplasmic reticulum-mitochondria contacts compared to healthy controls. Similarly, FRDA-iMGs displayed an activated, amoeboid morphology with compromised phagocytosis and mitochondrial dysfunction. Notably, our CRISPR/Cas9 approach achieved complete phenotype rescue in both cell types, reinforcing its potential as a good therapeutic strategy for FRDA. In this study, we performed *in vitro* co-culture experiments to study both direct physical interactions between neurons and microglia, as well as transwell-diffusion interaction between lymphoblasts and fibroblasts. Immunostaining for Caspase-3 and beta-III tubulin showed that healthy control neurons cocultured with FA-iMGs exhibited increased non-homogenous microtubule staining of neurites and apoptosis compared to those cultured with gene-corrected iMGs. Similarly, FRDA fibroblasts exposed to FRDA lymphoblasts in transwell cultures showed a significant increase in Annexin-V positive, apoptotic fibroblasts compared to those cultured with gene-edited lymphoblasts. These findings highlight the cytotoxic effects of FRDA hematopoietic lineage cells on both healthy and FRDA-affected cells and the therapeutic benefits of excision of GAA repeats in these cells.

1552 AAV9-based gene therapy for PDHA1 deficiency.

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Pyruvate Dehydrogenase Complex Deficiency (PDCD) is a genetic mitochondrial disorder primarily featured by progressive neurological and neuromuscular deterioration, typically leading to childhood mortality. Approximately 80% of PDCD cases are caused by mutations in the X-linked pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1) gene. PDHA1 deficiency lacks adequate disease-modifying treatments. Adeno-associated virus (AAV)-based gene therapy provides a highly targeted and minimally invasive method for delivering therapeutic genes, offering promise for treating a broad spectrum of genetic disorders.

Our research aims to develop PDHA1 deficiency disease models and utilize AAV-based gene therapy to specifically target and deliver the PDHA1 gene, assessing the therapeutic efficacy. Initial *in vitro* studies utilized patient-derived fibroblasts treated with the AAV2/PDHA1 vector, chosen because AAV2 exhibits better tropism for fibroblasts. the treatment resulted in a significant increase in PDHA1 protein expression, proper localization of the protein to mitochondria, restoration of PDC enzymatic activity, and dose-dependent normalization of mitochondrial

respiration. *In vivo* efficacy experiments employed a tamoxifen-induced Cre-Lox knockout (KO) strategy to create conditional knockouts, since constitutive KO of PDHA1 results in prenatal lethality. After administering tamoxifen, we established a hemizygous male model, representing a severe disease phenotype, and a heterozygous female model, representing a milder disease phenotype. Following AAV9/PDHA1 intrathecal injection, in these models at postnatal day 7, we observed a small but significant extension in survival and improved PDC enzyme activity in major organs of male mice. Long-term survival studies in the female mice are ongoing, and preliminary results indicate a small improvement in body weight and a partially normalized PDC enzyme activity in major organs. The in-life part of an *in vivo* experiment to evaluate the safety of the treatment was recently completed in WT C57BL/6J mice. No obvious side effects or animal death were observed over the 12-month, long-term study, indicating no toxicity concern from the treatment.

In summary, we have developed both cell and mouse models for PDHA1 deficiency disease and tested AAV-based gene therapy on these models to assess treatment efficacy and safety. Our results demonstrate that AAV gene therapy significantly improves cellular function and animal survival with no obvious safety concern, supporting further development of this AAV9/PDHA1 approach to treat PDHA1 deficiency.

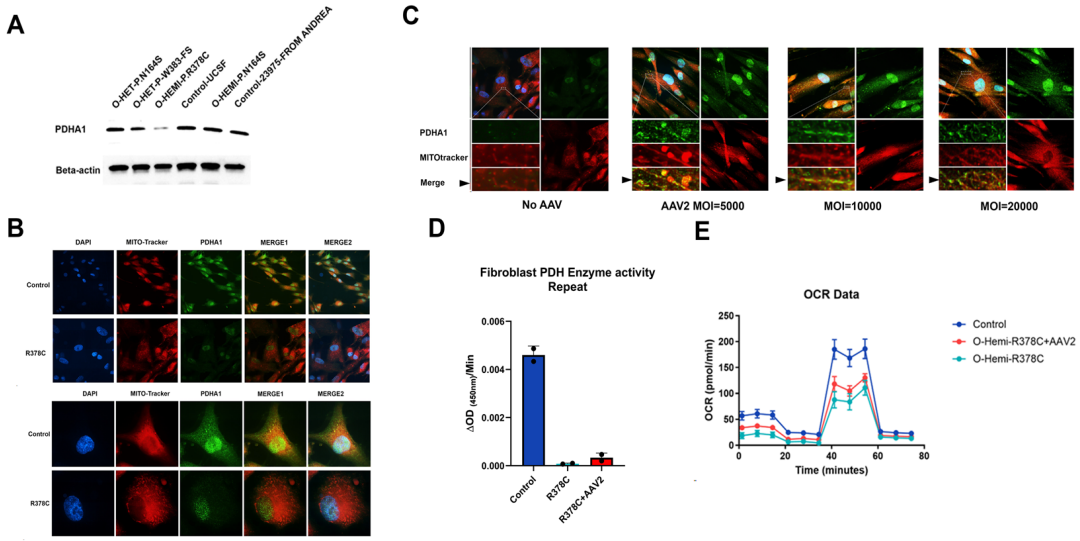

Figure.1 preliminary result of AAV gene therapy efficiency on Human patient fibroblast cells.
(A) Western blot assays were conducted on four distinct PDHA1 deficiency patient-derived fibroblast cell lines and two normal fibroblast cell lines, cultured to the same confluence in 100 mm flasks. Beta-actin was used as the loading control.**(B)** Confocal immunofluorescent staining visualized PDHA1 protein expression in both normal and patient-derived fibroblasts, cultured in 4-well slide chambers. Green fluorescence indicates PDHA1 protein tagged with Alexa488, while red fluorescence shows mitochondria stained with Deep Red MitoTracker. Images were captured at magnifications of 60x and 100x. **(C)** AAV2 was transfected into patient-derived fibroblasts at multiplicities of infection (MOI) of 5000, 10000, and 20000. These cells were also cultured in 4-well slide chambers. Three days post-infection (DPI), cells were fixed and stained with a PDHA1-specific antibody and MitoTracker. The lower left panel highlights an enlarged view of mitochondria with PDHA1 proteins labeled, where green fluorescence indicates PDHA1 protein tagged with Alexa488, and red shows mitochondria stained with Deep Red MitoTracker. **(D)** PDHA1 enzyme activity assays were performed on patient-derived fibroblasts both with and without AAV treatment, measured at three DPI. A protein concentration of 22.5 mg/ml was collected from each group, and 200 µl of this protein was used in the assays. Asterisks in the figures indicate different p-values. **(E)** A Seahorse assay measured the oxygen consumption rate (OCR) in fibroblasts, seeded into a Seahorse 96-well plate. Oligomycin, FCCP, and Antimycin-A were added to stimulate OCR.

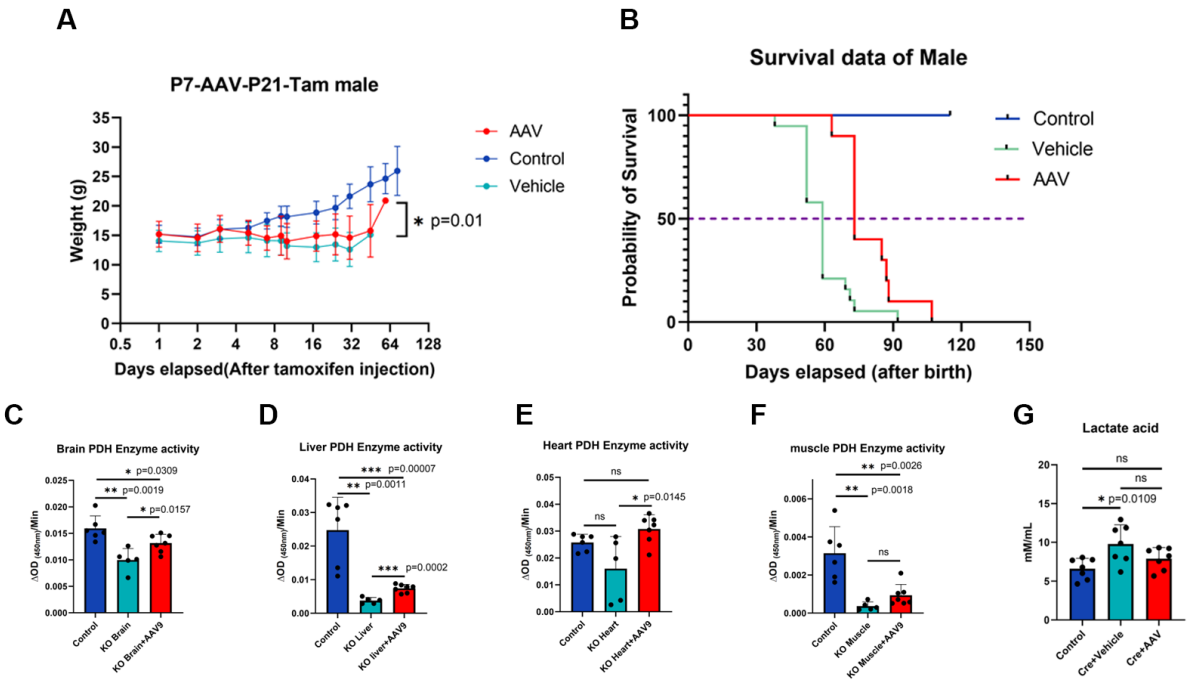

Figure 2: Effects of AAV9 Treatment on Male Mouse Disease Model
(A) Weight of male mice in the disease model after treatment with either AAV9 or vehicle.
(B) Survival curve of male mice following treatment with AAV9 or vehicle. The median survival time for vehicle treatment is 59 days, compared to 73 days for AAV9 treatment. (C-F) PDHA1 enzyme activity in major organs of male mice in the disease model after treatment with AAV9 or vehicle.
(G) Serum lactate acid concentration in the male mouse model following treatment with AAV9 or vehicle.

1553 Investigating allotopic expression and mitochondria-targeted base editors as therapeutic strategies to alleviate *MTATP6* associated Leigh Syndrome

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Introduction: Leigh Syndrome (LS) is the most common mitochondrial disease with about 1 in every 40,000 people affected with no approved treatment yet. Though it can be caused by mutations in over 100 genes, deleterious mutations in *MTATP6* (mitochondrially encoded ATP synthase membrane subunit 6) result in 10% of LS cases with most being caused by the mutation, m.8993T>G. *MTATP6* is a mitochondrial gene that translates into a mitochondrial transmembrane protein associated with ATP synthase. Here, we explored the feasibility of developing a gene therapy for patients with the m.8993T>G mutation in *MTATP6*. Since *MTATP6* is encoded in the mitochondrial DNA (mtDNA), there are several existing challenges: 1) The mtDNA resides in the mitochondria rather than the nucleus meaning there are different membrane transportation processes for nucleic acids and proteins; 2) Mitochondria have different DNA repair mechanisms than those within the nuclei; 3) There are between 100 to 100,000 copies of mtDNA within a cell vs one copy of nuclear DNA (nDNA). To achieve our goal, we explored two approaches. First, we tested allotopic expression of human ATP6. Allotopic expression is the expression of genes derived from the mtDNA within the nucleus. Through allotopic expression, the MTATP6 RNA exits the nucleus and is translated into a protein in the cytosol and transported into the mitochondria via a mitochondrial targeting sequence (MTS). This method would work for any loss of function MTATP6 mutations that can be alleviated by increased WT expression. We also tested DddA-derived cytosine base editors (DdCBEs) as a potential therapeutic strategy to alter *MTATP6* directly. DdCBEs are composed of a pair of mitochondrial base editors (mitoBEs) which each contain half of a deaminase. When these pairs align along a sequence, the deaminase is then able to edit cytosines.

Results: Our first step for preclinical evaluation was the in vitro transfection of HeLa cells via a plasmid expressing WT *MTATP6* and an MTS. To evaluate efficacy, we determined that ATP6 was being significantly translated via Western Blot. We then conducted fluorescent imaging and determined the ATP6 protein had successfully translocated to the mitochondria. Seeing that ATP6 was being translocated to the mitochondria, we wanted to determine if the ATP6 was able to translocate to the correct region, the inner mitochondrial membrane (IMM). Unfortunately, we discovered that the MTATP6 protein was embedded in the outer membrane and thus did not restore mitochondrial function. With the advent of new base editing technology and the inefficiency of allotopic expression to restore mitochondrial function, we decided to test DdCBEs to alter the mtDNA. We created 18 pairs of DdCBEs and transfected A375 cells homoplasmic for the m.8993T>G mutation to find the pairs with the best editing potential. Our results indicated that 2 of the 18 DdCBEs had ~5% desired editing.

Conclusions: In our study, we were unsuccessful with restoring MTAP6 function via allotopic expression. However, we found promising DdCBEs that were able to edit the mtDNA.

Future directions: We hypothesize that the highly hydrophobic nature of human MTATP6 prevented the successful translocation of the protein into the IMM. Future studies will include testing the less hydrophobic algal form of ATP6 and determining if the mitoBEs are able to alleviate the cellular defects caused by the m.8993T>G mutation.

1554 Two Years of Efficacy: Astrocyte-Targeted Gene Replacement for Vanishing White Matter Disease Exposes Uncorrected Disease Mechanisms

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As a rare, progressive, childhood leukodystrophy, Vanishing White Matter Disease (VWM) presents with white matter loss, ataxia, spasticity, seizures, and neurological decline which lead to premature death. VWM is caused by loss of function mutations in the subunits of Eukaryotic Initiation Factor 2B (eIF2B) with the most common pathologic variants found in subunit 5 (*EIF2B5)*. Previous in vitro and in vivo studies have indicated *astrocytic dysfunction* leads to VWM pathophysiology and are therefore a critical therapeutic target. Currently, there are no FDA-approved treatments. Thus, we aimed to develop adeno-associated virus (AAV)-mediated gene replacement therapy for VWM. Baseline measurements of disease pathology in the VWM *Eif2b5* I98M murine model indicates significant mobility loss, ataxic gait, and demyelination with a shortened lifespan of 5 months. Important for assessing therapeutic impact, this is the only VWM model that experiences epileptic seizures. To ensure astrocytic targeting of our AAV9 vectors, we employed two shortened iterations of the endogenous glial fibrillary acidic protein (GFAP) promoter, including gfaABC(1)D, and one ***novel*** intermediate—gfaABCD1405 (gfa1405). Expression profiling of the reporter AAV9-gfa1405-*eGFP* indicates we were able to achieve significantly increased expression throughout the central nervous system while maintaining comparable astrocyte specificity to gfaABC(1)D. Currently, we are monitoring disease progression after neonatal intracerebroventricular administration with one of our two astrocyte-specific, or one ubiquitous AAV-*EIF2B5* vectors in two murine VWM models: the patient orthologous *Eif2b5*^{R191H} model, and the severe *Eif2b5*^{I98M} model. In both models, gene therapy delays disease progression, partially rescues body weight and motor function, and attenuates myelin loss. The life spans of the *Eif2b5*^{I98M} animals treated with AAV9-CAG-*EIF2B5* ($p<0.0001$; 207 days), AAV9-gfa1405-*EIF2B5* ($p<0.0001$; 222 days), and AAV9-gfaABC(1)D-*EIF2B5* ($p<0.0001$; 298 days) were significantly increased. In fact, our longest extension in life expectancy **exceeds 2 years of age** in both astrocyte-specific, gfaABC(1)D and gfa1405-treated cohorts. Dose escalation studies with our most efficacious vectors—gfaABC(1)D-*EIF2B5* and gfa1405-*EIF2B5*—support that our high dose has more robust therapeutic rescue than a 5-fold lower dose. However, even our high dose astrocyte-targeted gene therapy does not fully rescue all disease, as the I98M mice are still experiencing severe and life-limiting seizures. To achieve total rescue, we are investigating uncorrected VWM pathophysiology after astrocyte-targeted gene replacement through single-cell RNA sequencing and spatial proteomics. We anticipate the

resolution of respective disease contributions and genetic pathways of other cell types (microglia, oligodendrocytes, neurons, etc.) that can be addressed for a more comprehensive and durable therapy. Through our multimodal molecular, cellular, and clinically relevant measures in two VWM models of varying disease manifestations, we anticipate the development of a lead gene therapy candidate allowing for expeditious translation to the clinic.

1555 Exploring Enzymatic Restoration and Sphingolipid Modulation through AAV-Mediated Gene Addition of a Subunit of Serine Palmitoyltransferase

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The enzyme serine palmitoyltransferase (SPT) catalyzes the initial and rate-limiting reaction in de novo sphingolipid synthesis which involves the condensation of an acyl-CoA with L-serine to form a sphingoid or long-chain base. SPT is comprised of multiple subunits: a heterodimer comprised of SPTLC1 (SPT long chain base subunit-1) and either SPTLC2 or SPTLC3 which forms the active site, and a smaller third subunit, either SPTSSA or SPTSSB, which increases the enzyme's catalytic activity by more than 25-fold. ORMDL proteins regulate sphingolipid synthesis by interacting with SPT and providing feedback inhibition. We care for a 7 year old girl with a de novo pathogenic variant (c.152 C>T, p.Thr51Ile) in *SPTSSA* which causes loss of ORMDL-mediated feedback inhibition and therefore excessive de novo synthesis of sphingolipids which are neurotoxic and cause a Complex Hereditary Spastic Paraplegia (HSP). Based on SPT inhibition with myriocin in a *Sptssa*$^{T51I/+}$ mouse model we treated our patient with the known SPT inhibitor D-Cycloserine under an IRB approved investigational protocol. We have observed an improvement in the patient's appendicular spasticity which, after 20 weeks of treatment, has led to a 71% reduction in the patient's daily dose of Baclofen, as well as a modest decrease in serum sphingolipid levels. This provided the framework to study *SPTSSA* gene addition using AAV9 and AAVF (a new variant of AAV9) in the *Sptssa* $^{T51I/+}$ mouse which we present here.

AAVF and AAV9 carrying CMV enhancer/chicken beta-actin hybrid promoter and *SPTSSA* cDNA carrying HA tag were cloned and produced in 293 cells. In vitro and in vivo studies were carried out. Retro-orbital injections of rAAV vectors were performed in a *Sptssa*$^{T51I/+}$ knock-in mouse model. Mouse tissue was processed for immunofluorescence and immunohistochemistry using HA tag, NeuN, GFAP, Olig2 and IBA1 antibodies. Tissue lysates were prepared for western blot with GADPH as loading control. SL level in mouse plasma and tissues were analyzed using LCMS. Statistical analysis was performed using $p<0.05$ to determine significance.

Mixed glial cells treated with AAV9-SPTSSA demonstrated successful transduction as well as a dose dependent SL reduction. CNS transduction (brain and spinal cord) was 6-fold higher with AAVF compared to AAV9 by retroorbital injection, whereas AAV9 was overall more efficient in peripheral tissues. Delivery of AAVF-SPTSSA mainly transduced astrocytes and neurons in CNS. AAV9-SPTSSA treatment at 3e11 gc/mouse significantly reduced SL accumulation in lung tissue but not plasma or CNS.

Overall our studies demonstrate the feasibility of adding a single unit of a multi-subunit enzyme complex to confer functional benefit in *Sptssa* $^{T51I/+}$ mice. The dose dependent reduction in SL levels seen in mixed glial cells treated with AAV9-SPTSSA demonstrates functional correction of SPT activity. A significant reduction in SL levels in lung tissue but not plasma or CNS was observed after treatment with AAV9-SPTSSA. Importantly, AAVF showed better CNS transduction compared to AAV9, mainly by targeting astrocytes and neurons. Further studies are needed to optimize the dose of AAVF-SPTSSA to demonstrate SL level reduction in the CNS and meaningful improvement in neurological phenotypes in *Sptssa* $^{T51I/+}$ mice.

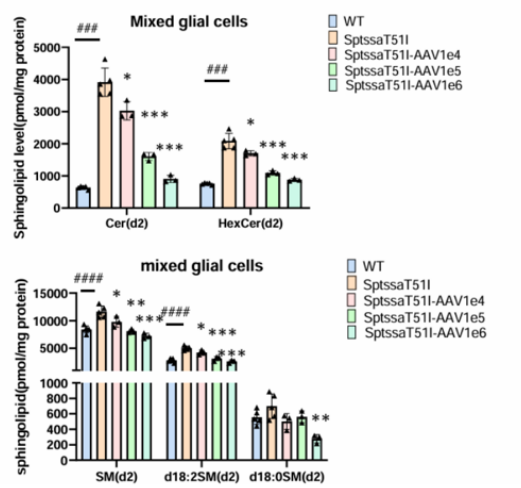

Figure 1: AAV9-SPTSSA treatment in mixed glial cells from *Sptssa*$^{T51I/+}$mice leads to a dose dependent SL reduction. Statistically significant differences in SL levels are labeled with "#" (WT vs. *Sptssa*$^{T51I/+}$) and "*" (*Sptssa*$^{T51I/+}$ vs. AAV treated groups).

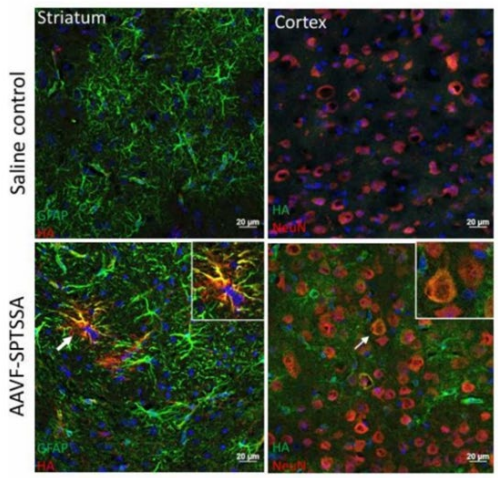

Figure 2: AAVF-SPTSSA IV delivery at 7e11gc/mouse transduce astrocytes and neurons in CNS. (GFAP=Astrocytes, NeuN=Neurons)

1556 Safety and efficacy of GJB2-GT, an adeno associated vector-based gene therapy treatment candidate for the autosomal recessive non-syndromic deafness 1A (DFNB1A)

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In the world, the estimated prevalence of severe or profound deafness in human is 1 out of 1000 neonates, and genetic factors account for half of the cases. Pathogenic variants of GJB2, the gene encoding for Connexin 26 (Cx26), are involved in 50% of congenital deafness and are mostly associated with an autosomal recessive non-syndromic DFNB1A. In the cochlea, *GJB2* is largely expressed in the supporting cells (SCs) of the sensory neuroepithelium, fibrocytes, basal and intermediate cells of stria vascularis but not in sensory hair cells. It is hypothesized that Cx26 is essential for the recycling of potassium, which is essential for the proper functioning of sensory hair cells, but *in vivo* studies also suggest that Cx26 deficiency leads to cochlear developmental disorders. Gene therapy is a promising therapeutic strategy for autosomal recessive forms of deafness, and Adeno-Associated Vectors (AAVs) are being developed to this aim.

Here, we have developed GJB2-GT, an Adeno-Associated Virus (AAV) vector for DNFB1A with proprietary cis-regulatory elements for a safe and targeted expression of the transgene *in vivo*. GJB2-GT efficiently transduces human cell lines to produce Cx26 protein that is adequately addressed to the plasma membrane. Using an adapted dye transfer assay, we show that the transgenic Cx26 protein is functional and permits the passage of a gap -junction permeable fluorescent tracer to neighboring cells.

Early tolerability and biodistribution of GJB2-GT, or GJB2-FLAG-GT studies were conducted in mice and Non-Human Primates (NHP) species after injections through the round window (RW). Intracochlear injection in both species results in transgene expression in most cells that naturally express *Gjb2* along the tonotopic axis of the cochlea, with good local and systemic tolerability. In all analyzed cochleae, no sensory hair cell express the transgene, confirming the specificity of the expression cassette. Importantly, GJB2-GT administration in NHP were performed using the surgical procedure and injection device intended for clinical use. Long-term safety studies conducted in wild-type mice demonstrate persistence of transgene expression with no impact on auditory function and maintenance of the cochlear cytoarchitecture. Additionally, high dose intravenous injection of GJB2-GT in mice does not induce any adverse events or changes in behavior, vital functions, increase in hepatic damage or inflammatory biomarkers.

To circumvent embryonic mouse lethality caused by complete loss of *Gjb2* expression, a conditional model was generated, resulting in a biallelic *Gjb2* inactivation mimicking the most common forms of DFNB1A and severe/profound hearing loss. Intracochlear injections of GJB2-GT lead to improvement of hearing thresholds as early as 3 weeks post-injection in a dose dependent manner which was statistically significant across multiple frequencies. Microscopic examination of injected cochlea confirmed that GJB2-GT efficiently restores Cx26 expression in the sensory epithelium. Current dose-response experiments confirm that intracochlear GJB2-GT injection is safe over a wide range of doses administered and provide a significant therapeutic benefit in our DFNB1A mouse model.

Altogether, these data support GJB2-GT development to restore physiological hearing in DFNB1A patients.

1557 Novel Gene Therapy Shows Cell Specific Transduction in Non-Human Primate Cochlea and Provides A Robust and Durable Rescue of Hearing in Mouse Models of GJB2 Related Deafness

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Introduction. The most common human hereditary deafness, DFNB1, is caused by mutations in the *GJB2* gene. Currently, there is no cure for *GJB2*-related hearing loss. *GJB2* encodes the gap junction protein connexin 26, which mediates transport of potassium and metabolites between cells. The *GJB2* coding sequence easily fits in an AAV viral vector, making gene addition an attractive therapeutic approach. Here, we optimize the vector design to achieve robust expression of human GJB2 protein in the appropriate cells of mouse and nonhuman primate cochlea, and greatly improving hearing in GJB2 deficient mouse models.

Methods & Results. Human *GJB2* coding sequence was packaged in the AAV-S capsid, which shows high transduction of *GJB2*-expressing cells in neonatal mouse and monkey cochleae. This vector showed strong transduction and gap junction activity *in vitro*. Vectors were injected into mouse inner ears via the round window membrane (RWM) at age P1. To understand the transduction efficiency and expression specificity of the vector, wild-type mice were injected with an HA-tagged vector. After 30 days, cochleae were removed and processed for histological analysis. Cochlear cells natively expressing *Gjb2* produced the HA-tagged GJB2 protein, but the sensory hair cells did not, demonstrating targeted, cell-specific expression by the vector. To test the efficacy of the vector, a *Sox10-Cre*, $Gjb2^{flox}$ conditional knock-out (cKO) mouse was injected at different doses. Notably, the *Gjb2* cKO model has been notoriously difficult to obtain hearing rescue with prior AAV gene therapy strategies. Animals were tested by auditory brainstem response (ABR) 30 days after injection. Cochleae were subsequently removed and stained with markers to assess hair cell survival. Functional rescue was dose-dependent, with an increasing response rate and magnitude as the doses increased. Similarly, dose dependence was observed in hair cell survival, with 100% survival at the highest dose. We also assessed the efficacy of AAVS-GJB2 in the *Gjb6* KO model, which has hearing loss caused by a 90% GJB2 reduction. We and others observed that this model has substantial hearing loss but lacks the severe histological phenotype of the *Gjb2* cKO model. The *Gjb6* KO showed even greater incidence, magnitude, and potency at efficacy than the *Gjb2* cKO model, with wild-type thresholds of hearing at the highest dose, and nearly complete hair cell preservation. This response was shown to be durable over 3-months. Finally, to assess potential for efficacy in human, the vector was injected through the RWM of cochleae of cynomolgus monkeys (*Macaca fascicularis*), using allometrically scaled mouse doses. As in mouse, we demonstrated targeted transduction and cell-specific expression.

Conclusions. Our vector demonstrated cell-specific expression of human *GJB2* in mice and nonhuman primates. Auditory sensitivity testing demonstrated significant rescue of hearing thus far reported in both a mouse *Gjb2* deletion model of DFNB1, and a milder *Gjb2* downregulation model, improving auditory sensitivity to wild-type levels.

1558 Cell-Specific Delivery of GJB2 Restores Auditory Function in Mouse Models of DFNB1 Deafness and Mediates Appropriate Expression in NHP Cochlea

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Mutations in the *GJB2* gene cause the most common form of human hereditary hearing loss, known as DFNB1. *GJB2* is expressed in two cell types of the cochlea—epithelial cells of the organ of Corti and fibrocytes of the inner sulcus and lateral wall—but not by sensory hair cells or neurons. In constitutive knockout mice, absence of *Gjb2* expression is lethal. In conditional mouse models of DFNB1, absence of *Gjb2* results in progressive degeneration of the sensory epithelium of the cochlea, with hearing sensitivity being almost completely abolished as measured by auditory brainstem recording (ABR). Attempts to treat mouse models of DFNB1 with AAV vectors mediating nonspecific *Gjb2* expression have not substantially restored function, perhaps because inappropriate expression in hair cells and neurons could compromise their electrical activity.

To target AAV-encoded *Gjb2* expression to the appropriate cells in the mouse cochlea, we used ATAC-seq on mouse and NHP cochleas, and identified gene regulatory elements (GREs) that could mediate specific expression in epithelial cells and fibrocytes. Using an AAV9-PHP.B vector, HA-tagged GJB2 was delivered under GRE control to the cochleas of wild-type mice at P1. Assessed at P30 by anti-HA labeling, GJB2.HA was localized to the appropriate cell types. When delivered to a conditional knockout model ($Gjb2^{fl/fl}$, *Sox10-Cre*), GJB2.HA largely prevented the cochlear degeneration observed in untreated knockout mice, but only slightly rescued hearing sensitivity.

We then turned to a milder model of DFNB1 deafness. As previously reported, deletion of the nearby *Gjb6* gene is thought to delete a *Gjb2* regulatory element, as *Gjb2* expression is reduced by up to 90%. In this $Gjb6^{-/-}$ model, mice are severely hearing-impaired but not completely deaf. Again, we used an AAV9-PHP.B vector to deliver GJB2.HA with GREs to P1 cochleas, but hearing was not rescued.

Concerned that the HA tag might interfere with GJB2 function, we injected the same vector encoding GJB2 without the HA tag. Assessed at P30, the vector prevented degeneration, and it preserved hearing nearly at wild-type levels. Thus GJB2-related GREs are able to direct expression of GJB2 to the correct cell types, and GJB2 lacking a HA tag can preserve normal morphology and hearing.

Finally, we tested control of expression by GREs in nonhuman primate cochleas. In three NHP cochleas , AAV9-PHP.B-delivered human GJB2.HA was located in all the appropriate cell types. Assessed with ABR recording, the vector caused little or no reduction in hearing sensitivity. Together, these results suggest that GRE-mediated expression of *GJB2* could prevent hearing loss in DFNB1 patients.

1559 *GJB2* gene therapy-response of two pre-clinical mouse models of the most frequent form of human deafness, DFNB1A.

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More than 1.5 billion people worldwide live with hearing loss (HL). Sensorineural HL affects one new-born in 700-1000 and approximately one child or young adult in 500 before the age of 20. Approximately 70% of congenital severe to profound deafness cases are hereditary due to monogenic defects, thus potentially treatable by gene therapy (GT).

GJB2-related autosomal recessive non syndromic HL (also referred as DFNB1A) is the most common genetic cause of congenital sensorineural HL in many world populations, frequently accounting up to half cases. In addition, it is also responsible for deafness occurring later, even after 40 years of age. *GJB2* encodes connexin-26 (Cx26) gap-junction channel protein that plays a key role in metabolites and ions homeostasis necessary to cochlear development and sensory hair cell function and survival. Hundreds of pathogenic *GJB2* variants including large deletions, loss-of-function and missense variants have been reported which outcome spans from mild to profound deafness.

To assess GT approaches for HL caused by *GJB2* defects, we developed several experimental mouse models: the *Gjb2* cKO/cKO model resulting in a biallelic *Gjb2* inactivation mimics the most common form of congenital DFNB1, and the *Gjb2* cKO2/Hmut, a compound heterozygote, expressing a human missense pathogenic variant (Hmut), corresponds to a frequent human *GJB2* genotype. To circumvent embryonic mouse lethality caused by *Gjb2* inactivation, conditional knockouts were generated using the same Cre-recombinant mice which displays a large cochlear spatial expression, i.e. in a vast majority of *Gjb2*-positive cells.

Gjb2 cKO/cKO and *Gjb2* cKO/Hmut mice showed cochlear structure disruption (neurosensory epithelium loss and collapsed organ of Corti, respectively) and congenital HL (profound and severe deafness, respectively, measured at P16). In both models, intracochlear injections of GJB2-GT lead to hearing improvement 4 weeks post-injection. Best responding mice show up to 40-50dB ABR threshold shifts and wave 1 amplification comparable to CTRL littermates for mid-range frequencies. Histological analysis showed cochlear structure preservation in almost all GJB2-GT injected mice. Especially, in treated *Gjb2* cKO2/cKO2 mice, GJB2-GT fully prevent hair cells and supporting cells (SCs) loss. In those mice, Cx26 expression was broadly detected along the tonotopic axis in SCs. No transduction was observed in inner nor outer hair cells.

These data support that GJB2-GT improves audition in two preclinical models of human pathogenic *GJB2* variants.

1560 Gene Replacement Therapy Ameliorates Hearing Loss in a Mouse Model with an Mpzl2 East Asian Founder Variant.

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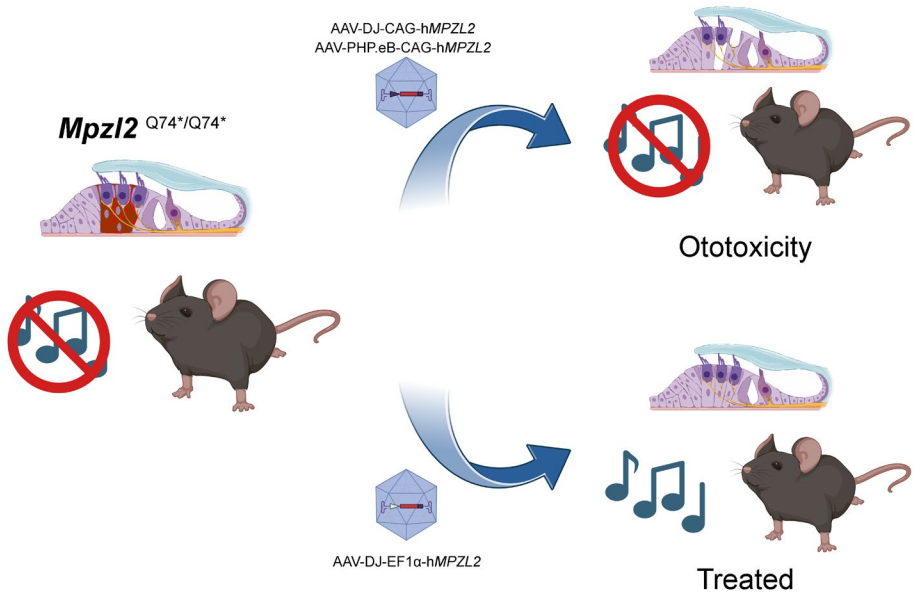
Hearing loss is the most common sensory organ disorder, with genetic factors substantially contributing to the disease. Among the 87 genes responsible for autosomal recessive nonsyndromic hearing loss, mutations in MPZL2 have been frequently linked to mild-to-moderate autosomal recessive hearing loss (DFNB111). Here, we present multiple families whose hearing loss arose from biallelic mutations in the MPZL2 gene and found that the MPZL2 p.Q74* mutation may be a founder allele among East Asians. Furthermore, we generated an Mpzl2 p.Q74* knock-in mouse model that exhibited autosomal recessive, progressive, ski-sloping hearing loss with disorganized Deiter's cells. Gene replacement using AAV-DJ or AAV-PHP.eB to deliver human MPZL2 (hMPZL2) under control of the CAG promoter induced ototoxicity, whereas employing an alternative EF1α *promoter with AAV-DJ (DJ-EF1α-hMPZL2) circumvented this issue, restoring long-term auditory function and Deiter's cell organization in Mpzl2$^{Q74*/Q74*}$ mice for up to 24 weeks. Overall, this study provides the foundational steps for developing a safe and effective biological treatment for DFNB111 and underscores the importance of precise regulation of target cells and transgene expression in AAV-based treatments.*



1561 Large animal model for human inner ear gene therapy: Transgene expression of viral vectors in pigs

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**Corresponding Author*

Objective: The recently published data on the clinical application of viral vectors to prevent the natural course of hearing loss in human patients with OTOF variants show the potential of inner ear gene therapy. For the first time, children affected by autosomal recessive otoferlin mutations were treated by delivering adeno-associated viruses (AAVs) into the inner ear. An in-depth investigation of the respective efficacy of the applied viruses has so far only been carried out in a limited number of large animal models. In this study, AAVs were injected into the cochleae of pigs to investigate transduction patterns of the inner ear tissue.

Methods: In three pigs, AAV2-eGFP with a CAG or CMV promoter was injected via the round window membrane using an established surgical procedure under general anesthesia. Pre- and postoperative brainstem audiometry was performed. After a follow-up of one week, inner ears and other organs relevant for systemic biodistribution were extracted and analyzed by means of immunofluorescence and confocal microscopy.

Results: Successful transduction was identified in the cochlear hair cells leading to a decreasing gradient from base to apex in the outer hair cells, but increasing transduction rate from base to apex in the inner hair cells. A unilateral injection showed no transduction in the contralateral ear. In addition, no threshold changes were observed in the injected ears when measuring auditory brainstem click responses. In our preliminary results, no peripheral organs were transduced with AAV2.

Conclusions: Due to the large dimensions of the inner ear (most comparable volume to the human cochlea), the pig represents a suitable alternative large animal model for hearing research and in particular inner ear gene therapy. The above-mentioned findings confirm that AAV2 is a reliable vector for targeting sensory hair cells and that future gene therapies can be investigated preclinically in pigs to optimize vector delivery, distribution, and corresponding transduction patterns, all of which could result in translational benefits by developing more effective human therapies.

1562 Supporting the Informed Consent Process in Clinical Trials of Induced Pluripotent Stem Cell–Derived Corneal Epithelium Transplantation

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Background: In clinical trials involving induced pluripotent stem cells (iPSCs), one of the most significant areas of progress is in ophthalmology, especially in relation to diseases that affect the retina and cornea. However, the details of such advanced trials are often complex and difficult for participants to understand; thus, we need to consider how to ensure genuine informed consent from patients with eye diseases. In particular, for patients with visual impairments, does providing the details of cutting-edge clinical trials solely through conventional, written information sheets truly respect their autonomy in decision-making? To address this question, we collaborated with individuals with visual impairments and professionals supporting the visually impaired to create audio support materials. These were specifically designed to improve accessibility and comprehension for visually impaired participants in clinical trials on iPSC-derived corneal epithelium transplantation. This presentation outlines our attempt to assess the initiative through interviews with trial participants. These interviews, conducted after the completion of the clinical study, focused on the participants' perspectives on their trial participation.

Methods: Semi-structured interviews were conducted with four patients who participated in a clinical trial on iPSC-derived corneal epithelium transplantation. All had given their informed consent before participating. The interviews were audio-recorded, transcribed, and coded using qualitative data analysis software. We extracted and analyzed the portions of the interview dataset where the participants mentioned the audio support materials.

Results: From the feedback participants provided on their comprehension of the research and the impact of audio assistance, we identified four distinct experiences: (1) understanding without difficulty, (2) achieving comprehension after repeated listening, (3) experiencing challenges in understanding the information, and (4) comprehending the information with the help of family members. The audio material was also considered helpful for

sharing information and facilitating discussions with family members regarding trial participation.

Discussion and Conclusion: Three key insights emerged from the dataset. Using audio support materials tailored to patients with low vision had the potential to enhance accessibility and contribute to respecting patient autonomy. Next, while it has been noted that evaluating the usefulness of patient and public involvement and engagement is challenging, discussions with research participants after their participation may be useful for assessing such initiatives. Finally, this approach could help advance the discussion on reasonable accommodations in clinical research for patients with visual impairments. In our view, the findings from the participants' experiences provide a valuable reference for further consideration and improvement.

1563 IMPROVING ACCESSIBILITY TO CELL AND GENE THERAPY FOR RARE DISEASES

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Objective: Despite growing awareness of rare diseases, knowledge of approved cell & gene treatment (CGT) treatments and their accessibility remains limited. The administrative and logistical challenges involved in managing patient eligibility and treatment initiation also hinder CGT's broader application. Complexity of CGT, and its case-by-case individual character is another limiting factor for its wider use. We evaluated the role of processes in accelerating the access to CGT in practice, including autologous CGT, which are among the most complex treatments.

Methods: The administrative processes for 6 different cell&gene therapies in 20 diagnostic and treatment centers in Europe, from diagnosis to treatment, were mapped and evaluated with 2 years follow up. A total of 52 different processes were required from diagnosis to local treatment or cross-border treatment.

Results: 133 patients were evaluated, of whom 29 were eligible for and received approved CGT, which is subject to strict eligibility criteria. Between 6 and 18 months elapsed between diagnosis and initiation of therapy, due to limited experience with CGT and de novo, intense interactions among all stakeholders. Of the 52 processes identified, 29 were specific to CGT, with 21 processes (40%) requiring more than 100 additional hours of effort to establish treatment centers. Additionally, 31 processes (60%) related to the cross-border treatment (diagnosis assessment, treatment proposal, review, and implementation). The allocated CGT team helped clarify responsibilities and coordinated all interactions to speed up processes.

Conclusions: The administrative and logistical burden associated with CGT significantly impacts the scalability of treatment. The complexity of the process, requiring coordination across multiple stakeholders (including diagnostic centers, treatment centers, and payers), increases the time and resources needed to initiate therapy. Therefore, standardization of documentation and the establishment of CGT team can improve access to CGT, since effective management of these processes is essential. Where treatment centers lack dedicated CGT teams, external coordination, such as those provided by Medasol, can help streamline these tasks. Ensuring proper process management from diagnosis to treatment initiation will facilitate broader access to CGT and support timely, effective treatment for rare disease patients.

1564 Generation of induced Pluripotent Stem Cell (iPSC)-derived Retinal Pigment Epithelium (iRPE) from a patient with Retinitis Pigmentosa associated with a mutation in *PROM1*

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Introduction: *PROM1* encodes the protein Prominin-1, which plays a critical role in regulating key processes in the Retinal Pigment Epithelium (RPE), including waste removal through autophagy and the stabilization of lysosomal activity. In the retina, Prominin-1 is also present in photoreceptors, where it contributes to the structure and morphology of their membranes. Mutations in *PROM1* have been associated with all major phenotypes of Inherited Retinal Diseases (IRD). Specifically, the c.1354dupT (p.Tyr452Leufs*13) mutation located in exon 13 of *PROM1* has been linked to various IRD phenotypes, including Retinitis Pigmentosa (RP). The aim of this study was to generate induced Pluripotent Stem Cell (iPSC)-derived RPE (iRPE) from a patient with RP and the c.1354dupT mutation in *PROM1*, for future molecular and functional characterization of the effects of this mutation.

Methods: A dermal biopsy was obtained from a patient previously diagnosed with RP and carrying the homozygous c.1354dupT mutation in *PROM1* to establish a primary culture of fibroblasts. The patient's fibroblasts were reprogrammed via non-integrative nucleofection using the Epi5 Episomal iPSC Reprogramming Kit (OCT4, SOX2, KLF4, L-MYC, and LIN28) and the Neon Transfection System under the following pulse conditions: 1,400 V; 20 ms; two pulses. After 21 days of culture, iPSC colonies were manually selected. Subsequently, iPSC characterization was conducted through immunocytochemistry using pluripotency markers (SOX2, TRA-1-60, and SSEA-4) and *in vitro*differentiation of embryoid bodies (EB) into the three germinal layers, assessed by the expression of β-III Tubulin, α-SMA, and α-1 Fetoprotein markers. To generate iRPE, EBs were cultured in Neural Induction Medium (DMEM/F12 supplemented with 1% N-2, 0.1% Heparin, and 1% MEM NEAA) until day 16. The culture was then continued in Retinal Differentiation Medium (DMEM/F-12 supplemented with 2% B27 and 1% MEM NEAA). Between days 28 and 35, iRPE was manually harvested from the optic vesicles. The generation of iRPE was subsequently confirmed through immunocytochemistry utilizing antibodies against key retinal pigment epithelium (RPE) proteins, including retinal pigment epithelium-specific 65 kDa (RPE65), cellular retinaldehyde-binding protein (CRALBP), and bestrophin 1 (BEST1).

Results: The generated iPSC line (designated [RP]-FiPSC1-Ep5F-10) tested positive for membrane (TRA-1-60 and SSEA-4) and nuclear (SOX2) pluripotency markers, demonstrating the ability to differentiate *in vitro* into the three germinal layers. The resulting iRPE demonstrated a homogeneous and polygonal morphology, along with distinct pigmentation. Furthermore, iRPE cells expressed the markers RPE65, CRALBP and BEST1.

Conclusions: Reprogrammed iPSCs derived from a patient with RP and the c.1354dupT mutation in *PROM1* can be successfully differentiated into iRPE cells. While further functional characterization of iRPE cells and elucidation of the role of the *PROM1* mutation are necessary, patient-derived iPSCs provide a promising avenue for the design and development of future precision medicine and disease modeling strategies.

1565 A Novel AAV2 Capsid Variant-based Dual-Acting Ocular Gene Therapy Targeting VEGF and Inflammatory factors for Neovascular Age-related Macular Degeneration

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Background: Anti-vascular endothelial growth factor (VEGF) therapies have revolutionized treatment and visual outcomes for patients with neovascular age-related macular degeneration (nAMD), yet up to 30% of patients fail to achieve significant visual improvements and remain at risk of severe, irreversible vision loss. Furthermore, anti-VEGF agents have been associated with adverse ocular events, including occlusive retinal vasculitis, intraocular inflammation, and geographic atrophy development, highlighting the need for improved therapeutic strategies. Chronic inflammation, including aberrant complement activation, has emerged as a critical contributor to AMD pathogenesis, presenting an opportunity for a dual-targeting approach to enhance the efficacy of anti-VEGF monotherapy and mitigate adverse ocular outcomes.

Methods: We developed a novel dual-acting gene therapy leveraging an engineered AAV2 capsid variant (AAV2.NG) identified through rational design and directed evolution in non-human primates (NHPs). This platform enables intravitreal delivery of both anti-VEGF agents (*e.g.,* anti-VEGF single-chain variable fragments [scFv], aflibercept) and anti-inflammatory factors (*e.g.,* anti-TNFα_scFv, complement component 3 inhibitory protein/peptide [C3IP]). Several transgene constructs were vectorized and assessed for their expression and functionality.

Results: AAV2.NG demonstrated an enhanced transduction efficiency for central retina in NHP eye compared to AAV2. Transgene expression in HEK293 or ARPE19 cells confirmed robust secretion of bioactive anti-VEGF_scFv, anti-VEGF_aflibercept, anti-TNFα_scFv and C3IP proteins, as determined by Western blot and ELISA. Functional assays demonstrated potent VEGF inhibition in HUVEC proliferation models, TNFα inhibition in HEK-Blue TNF-alpha cells and effective complement blockade in Raji cell-based assays. These results validate the feasibility and activity of this dual-acting gene therapeutic approach.

Conclusions: This innovative AAV-mediated gene therapy represents a promising strategy to address the unmet clinical needs in nAMD. By targeting both VEGF and inflammation signaling pathways, it offers the potential for improved efficacy and safety profiles compared to current treatments. Ongoing in vivo studies will evaluate its therapeutic potential in preclinical models, paving the way for future clinical translation.

1566 NT-501 Capsule Stability and Encapsulated Cell Technology Platform Capabilities

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Purpose: The NT-501 implant uses Neurotech's novel encapsulated cell technology (ECT) to provide long-term intraocular drug delivery of ciliary neurotrophic factor (CNTF) to the retina for treatment of Macular Telangiectasia type II. The ECT is a combination product that contains cells encapsulated within a capsule. The capsule's main components are the semi-permeable hollow fiber membrane, the polyethylene terephthalate scaffold, and the UV-cured adhesive. Due to the expected long-term duration in which the NT-501 implant will be exposed to an aqueous environment in vivo, the capsule materials, as well as the sealed capsules' stability, needed to be evaluated over a two-year duration. Additionally, the ECT platform's configuration capabilities to deliver different levels of therapeutics was evaluated.

Methods: To evaluate material stability, real time and accelerated aging in a simulated in vivo condition (0.9% Saline Solution) was performed on the capsule, the UV-cured adhesive, and the PES/PVP HFM. The integrity of the capsules was evaluated quantitatively using both pressure-decay leak testing and an HPLC-MS method for detecting the adhesive degradant molecule 4-4'-methylenebis(cyclohexylamine) (HMDA), and morphologically by H&E staining. The materials were evaluated for either chemical, functional, or physical properties. To evaluate the ECT

platform capabilities, modifications were made to the capsule to increase the delivery of therapeutic protein compared to the NT-501 implant. NT-501 (control) and modified ECT capsules were tested in vitro and conditioned media samples from both groups were tested using ELISA. Samples were tested and evaluated by ELISA to determine the level of protein delivered.

Results: The NT-501 capsule remained integral throughout the entire two-year simulated in vivo study and no substantial decrease in material properties were detected. By modifying the ECT capsule, increased levels of secreted therapeutic protein were detected compared to the control group in vitro.

Conclusions: The simulated in vivo stability study supports that the NT-501 capsule remains integral and the capsule materials are stable for at least two years. Additionally, modifications can be made to the ECT capsule to alter the delivery levels or targets of therapeutics making it a versatile long term delivery platform for intraocular diseases.

1567 Analysis of Spatial Transcriptomic Data in a Non-Human Primate Ophthalmology Model

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In this study, we demonstrate what is, to our knowledge, the first the successful application a high-resolution spatial transcriptomics platform for gene expression analysis in a non-human primate retina using a human probe set. Through cross-species in silico analysis, we identified high probe compatibility between human and cynomolgus transcripts, with 61% of genes showing perfect probe matches and an additional 36% exhibiting minor sequence variations predicted to maintain hybridization efficiency. We validated these computational predictions using retinal samples from cynomolgus macaques alongside human control tissue.

Our results show robust cross-species performance, with over 15,000 genes detected across all samples, enabling the identification of major retinal cell types and retinal layers based on distinct transcriptional signatures. These include retinal pigment epithelial cells, photoreceptors (rods and cones), and interneurons located in the inner nuclear layer, as well as the distinct retinal layers such as the photoreceptor layer, outer nuclear layer, and ganglion cell layer. The platform's high spatial resolution further revealed transcriptional domains corresponding to known anatomical structures and functional regions of the retina.

These findings lay the foundation for studying AAV transgene delivery in primate retinal tissue, offering critical insights into cellular and regional effects of gene therapies. By enabling high-resolution, cross-species spatial transcriptomics, this study highlights the potential of advanced transcriptomics platforms to advance preclinical research and inform the development of targeted therapies for retinal diseases, bridging preclinical findings with clinical applications.

1568 Encapsulated Cell Technology Enables Steady State Delivery of a Wide Range of Therapeutic Targets

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Purpose: Encapsulated Cell Technology (ECT) is a novel cell delivery platform that provides continuous, steady-state release of therapeutics. Neurotech's ECT is a validated therapeutic platform that overcomes many of the barriers of intraocular drug delivery and eliminates the need for repeated intraocular treatments. Implanted in the vitreous humor, human retinal pigment epithelial cells are encapsulated in a semi-permeable membrane. The membrane allows for the inward diffusion of nutrients to support cell survival while cell-secreted therapeutics

diffuse out of the implant simultaneously. Here, we demonstrate that the human cell line is fully customizable and can be genetically engineered to produce a wide class of therapeutics that can be delivered by ECT.

Methods: The parental cell line was derived from human retinal pigment epithelium (RPE) cells. These parental cells have been genetically engineered to express select therapeutic proteins at stable delivery rates. Proteins were designed to be secreted from the cell by adding a signal peptide vector element. Expression of therapeutic proteins was detected by immunoassays of both implant and cell-conditioned media samples. Cell line characteristics were monitored by evaluating phenotypic morphology and immunofluorescence using RPE-specific markers.

Results: The implant is tunable to allow for the optimal release of secreted therapeutics. Various proteins have been successfully expressed, including trophic factors, fusion proteins, Fab fragments, small peptides, and large inhibitory monoclonal antibodies ranging from molecular weights of 20kDA to 185kDA. Clones that produce stable levels of functional protein over time were identified and evaluated. Additionally, genetic manipulation was shown to not change the phenotype of the parental cell line. Cells secreting ciliary neurotrophic factor (CNTF) have been encapsulated in the validated implant and successfully undergone phase 3 clinical trials.

Conclusions: Encapsulated cell technology (ECT) is a versatile platform capable of secreting a wide variety of intraocular therapeutic proteins. ECT has been recently evaluated in two Phase 3 clinical trials and the long-term, safe, and effective delivery of CNTF has been established.

1569 Intra-Articular Re-administration of High-Capacity Adenovirus (HCAd) for Gene Therapy in Post-traumatic Osteoarthritis

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High-Capacity Adenovirus (HCAd) vector-based intra-articular (IA) gene therapy expressing an NFkB-responsive interleukin-1 receptor antagonist (IL-1Ra) has completed phase I clinical trial for osteoarthritis (NCT04119687) without significant toxicity and significant improvement of pain and functional endpoints after two years. Preliminary results demonstrated that baseline neutralizing antibodies (NAbs) did not correlate with the efficacy of WOMAC-A pain score reduction, suggesting the feasibility of repeated vector administrations. Therefore, we investigated the impact of pre-existing anti-vector immunity on HCAd vector transduction and transgene expression in the joint, as well as explored the potential for enhancing therapeutic efficacy of IL-1Ra gene therapy in osteoarthritis through repeated HCAd vector injections.

To model maximal pre-existing NAbs, FVB/NJ mice were first immunized intramuscularly with serotype 5 first-generation adenovirus (Ad5) and subsequently treated with IA administration of HCAd5 expressing β-galactosidase. Vector genome copy number and X-gal staining showed that pre-existing anti-Ad5 immunity reduced HCAd5 transduction and transgene expression in joint tissues. Furthermore, single IA immunization of HCAd vectors induced production of NAbs against the viral capsid five weeks post-injection. Repeated IA injections of the same serotype HCAd resulted in a reduction of transduction and transgene expression in joint tissues, supporting that in the murine model, intramuscular immunization can induce sufficient NAbs to reduce subsequent transduction.

To increase transduction with pre-existing NAbs, we evaluated serotype switching strategies. Alternative HCAd serotypes (HCAd1, HCAd2, and HCAd6) displayed joint tropism and long-term expression of luciferase comparable to HCAd5 in healthy joints and osteoarthritic joints induced via anterior cruciate ligament transection (ACLT). Importantly, prior HCAd5 exposure did not generate cross-NAbs against these serotypes. Serotype switching markedly improved transduction efficiency and transgene expression in mice with pre-existing anti-Ad5 immunity or prior IA HCAd exposure, as demonstrated by vector genome quantification, β-galactosidase mRNA levels, and X-gal staining.

Therapeutic studies in an ACLT-induced OA mouse model revealed that a single IA injection of HCAd5-NFkB-IL1Ra significantly preserved cartilage volume at 5 weeks post-injection, as assessed by phase-contrast μCT. However, continued OA progression was evidenced by reduced cartilage thickness at 9 weeks post-injection. Interestingly, a second injection with both HCAd2-IL1Ra or HCAd5-IL1Ra at 5 weeks post-initial injection effectively preserved cartilage and delayed OA progression at this second later timepoint compared with singly-injected mice, irrespective of serotype. These data suggest that while serotype switching can indeed overcome potential pre-existing NAbs, at least for the IL-1Ra vector, sufficient transduction even with the same serotype reinjection may be sufficient to mediate improved efficacy with readministration.

These findings provide critical insights into the potential impact of adenoviral immunity and highlight the feasibility of repeated HCAd vector-based IA gene therapy to achieve sustained therapeutic benefits for knee joint diseases such as post-traumatic osteoarthritis.

1570 Treatment of Rheumatoid Arthritis in a Collagen-Induced Arthritis Mouse Model Using Intra-Articular Injection of AAV6-Delivered sIL17RA

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Rheumatoid Arthritis (RA) is a chronic autoimmune disorder characterized by joint inflammation. Modern therapies, such as biologic DMARDs, disrupt dysfunctional immune signaling in RA by targeting pro-inflammatory cytokines. However, less than 50% of patients treated with biologics achieve sustained remission, largely due to rapid drug clearance in the joints. Recently, Interleukin-17A (IL17A) has emerged as a key target due to its direct involvement in RA pathophysiology, such as bone destruction and synovitis, with several studies indicating the efficacy of the extracellular domain of Interleukin-17 Receptor A (sIL17RA) as a soluble inhibitor. Adeno-associated virus (AAV) is an ideal vector due to its ability to maintain robust, long-term expression of packaged transgenes, with our prior data identifying AAV6 as the optimal serotype for joint transduction. Here, we investigated the therapeutic potential of local IL17A inhibition via AAV6-delivered sIL17RA in a collagen-induced arthritis (CIA) mouse model. Our ITR2-flanked expression cassette encoded the extracellular domain of human IL17RA (Met1-Trp320), with a 3' His-tag at the C-terminus and a constitutive CBh promoter to drive expression. Secretion ability of the construct was confirmed *in vitro* by transfecting HEK293 cells and verifying the presence of sIL17RA in the supernatants through western blot. Next, the ability of sIL17RA produced from our construct to inhibit human IL17A activity was confirmed by measuring its ability to lower IL17A-induced CXCL1 production in HT-29 cells. Although the positive control IL17RA ECD (-0.239±0.007) had greater purity due to commercial production, compared to sIL17RA produced from our construct (purified in-house), we found that sIL17RA still significantly dampened IL17A activity (0.049±0.086) relative to cells incubated with IL17A only (0.393±0.060) ($p<0.05$). Finally, to assess the therapeutic efficacy of sIL17RA in vivo, 10-week-old DBA/1J mice were immunized on day 0 with an emulsion of Complete Freund's adjuvant and Bovine Type II Collagen (CII), and on day 21 with a booster of Incomplete Freund's adjuvant and CII to induce CIA. On day 42, treatment group mice received intraarticular injections of 1E10 vg AAV6-sIL17RA in the left knee joint and PBS in the right (n=8), while vehicle controls received AAV6-Luc in the left and PBS in the right (n=4). Two naive mice were kept for comparison. On day 70, arthritis severity was evaluated post-sacrifice with a 12-point H&E scoring system. Several mice experienced distinct improvement in the AAV6-sIL17RA-treated left joint, with an average score of 5.047±2.923, relative to the PBS right joint (7.104±2.174). While not statistically significant, this result showed a promising trend towards improvement. Lastly, we found that locally expressed sIL17RA showed minimal systemic leakage, as His-tagged sIL17RA was undetected in sera. ELISA also showed no significant difference in anti-CII antibody titers between vehicle and treatment groups, suggesting that local injections did not affect the systemic immune response (1.58E5±3.80E4 vs 5.88E4±1.23E5, $p>0.05$). To conclude, we developed an sIL17RA expression construct with the ability to inhibit IL17A activity *in vitro* and demonstrated the promise of AAV6-sIL17RA as a localized RA treatment *in vivo*. Future studies will optimize constructs for higher transduction, modify injection timing for prophylactic use, and increase replicates to mitigate variability.

1571 High Capacity Adenoviral Intra-articular NFkB-responsive IL1RA gene therapy on a genetic osteochondrodysplasia model

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Osteogenesis Imperfecta (OI), or brittle bone disease, is a rare genetic disorder caused by either mutations in type 1 collagen encoding genes *COL1A1* or *COL1A2*, qualitative post-translational over-modification defects of type 1 collagen, or dysregulated signaling factors in bone cells and matrix. Patients with OI primarily manifest musculoskeletal phenotypes including bone fragility, recurrent fractures, and skeletal deformities and pain. Moreover, more than 30% of children and adolescents with OI experience chronic pain which disrupts functionality and quality of life. There are no FDA approved intervention for the pain management. Moreover, natural history studies have also demonstrated a broader growth and joint defect which is especially prominent in forms of OI that impact both cartilage and bone collagens. Thus, there is significant unmet need for treating chronic pain in OI patients. One contributor joint-related osteoarthritis (OA) pain, which was reported by a nationwide register-based cohort study from Danish group. In our previous published studies, we showed that *Crtap* $^{-/-}$ mice, a preclinical mouse model of moderate to severe OI type, have severe musculoskeletal phenotypes including joint disease including tendinopathy and osteoarthritis. Here, we focused on accessing the pain behavior of *Crtap* $^{-/-}$. We found that *Crtap* $^{-/-}$ mice showed a motor deficit associated with a significant reduction of vertical activity (VACTV) and rearing time (RTIME) based on the Open Field Assay (OFA). In addition, several parameters of CatWalk assay (Print-Area, Contact Intensity, Swing speed, Stride Length and Duty cycle) indicated that *Crtap* $^{-/-}$ mice manifest OA associated pain behavior. Consistent with OA like behaviors, we also confirmed histological changes in the knee joint of *Crtap* $^{-/-}$ early as 3 months of age which showed decreased proteoglycan in articular cartilage, ectopic chondrocytes in menisci, and hyperplasia in synovium. More interestingly, consistent with an elevated inflammation shown in tendons of *Crtap* $^{-/-}$ in the previous study, we identified infiltration of macrophages in the joint space of *Crtap* $^{-/-}$. To target OA pain in *Crtap* $^{-/-}$ mice, we performed the intra-articular High-Capacity Adenovirus expressing NFkB responsive IL1RA (HCAd-IL1RA) into the knee. This vector has been shown encouraging results in treatment of OA in a phase I clinical trial two years after a single injection (NCT04119687). We found an improvement of overall activity based on OPF and CatWalk assays after post 8wks IL1RA injection compared to Ad0 control. Overall, our study shows that *Crtap* $^{-/-}$ mice manifest osteochondrodysplasia related joint disease phenotype characterized by pain-associated behavioral changes and OA. Treating the inflammatory component of this disease with a current vector in clinical trial appears to impact pain measures in this rare disease model of OI.

1572 Antibody-Based AAV Retargeting to CACNG1 Mediates Enhanced Skeletal Muscle Transduction and Reduced Hepatotoxicity in Non-Human Primates

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The use of adeno-associated virus (AAV) for gene therapy delivery shows significant potential for treating various muscle diseases. However, the high doses of AAV required to effectively transduce extrahepatic tissues, such as muscle, have led to severe adverse events in patients, including liver toxicity and death. To address this issue, we have employed a rational engineering approach to design AAVs specifically targeted to skeletal muscle, via conjugation of antibodies against a skeletal muscle-specific protein, CACNG1. These antibody conjugated AAVs are also detargeted from the liver using an AAV9 capsid with mutated galactose binding domains.

We have generated a panel of monoclonal anti-CACNG1 antibodies and previously demonstrated that AAV particles can be retargeted to skeletal muscle in an antibody-dependent manner in both human myotubes and humanized mice genetically modified to express the human CACNG1 protein. Here, we evaluated the safety and efficacy of CACNG1-retargeted AAVs in cynomolgus macaques to demonstrate the translation of CACNG1-mediated skeletal muscle transduction in a large animal model.

Following intravenous delivery, CACNG1-retargeted AAVs, packaged with a muscle-specific promoter driving expression of a therapeutic transgene, enhanced therapeutic transgene expression more than 10-100 fold in multiple skeletal muscle tissues, compared to wild-type (WT) AAV9. The efficiency of CACNG1 retargeted AAV was similar between rodents and cynomolgous monkeys, demonstrating the cross-species translatability of our delivery platform. Additionally, CACNG1-retargeted AAVs were markedly detargeted from the liver (>200 fold) and heart (>20 fold) at DNA level. At a dose of 2E14vg/kg, NHPs treated with WT AAV9 exhibited transient ALT elevation, platelet loss, and complement activation post-dosing. In contrast, NHPs injected with CACNG1 retargeted AAV had no significant differences to control animals in these parameters, indicating a superior safety profile compared to WT AAV9.

In summary, these data demonstrated that CACNG1-retargeted AAV vectors significantly outperform WT AAV9 in both the efficiency and specificity of muscle transduction. These muscle -targeted vectors could enhance the therapeutic efficacy and reduce off-target toxicity of gene therapies for multiple muscle diseases.

1573 AAV Gene Therapy for Digit Regeneration Informed by Spatial Transcriptomics

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Introduction: Limb loss presents a significant clinical challenge that stands to benefit from fields such as gene therapy. Mouse digit tip (MDT) regeneration serves as a mammalian model of complex tissue regeneration that can provide insight into strategies for enhancing tissue repair and bone lengthening after limb amputation. However, little is known about optimal gene therapy methods for manipulating this process. This study employed spatial transcriptomics to identify gene promoters active in key anatomic regions of the regenerating MDT. Subsequently, promoter sequence fragments were tested in adeno-associated viral vector (AAV) assays for their ability to enhance bone regeneration by driving overexpression of a potent osteogenic effector gene (BMP2).

Methods: Hindpaw digits of 8-week-old BALB/cJ mice were amputated at the level of the middle (P2) or distal (P3) phalanx. Fourteen days post-amputation (dpa), samples were processed through the STOmics Stereo-seq platform to delineate gene expression in anatomic sub-regions of the regenerating digit. Proximal promoter fragments (2 kb) were cloned from the *Krt16*, *Postn*, or *Col1A2* genes and placed upstream of the BMP2-3xFLAG coding sequence, followed by plasmid synthesis and packaging into AAV8 vectors. AAV8 CMV:GFP vectors were used as controls. At 10 dpa, vectors were systemically delivered by retro-orbital injection. At 14 dpa, digits were harvested for histological analysis or Stereo-seq. P3- and P2- amputated digits were collected at 21 dpa and 28 dpa timepoints, respectively, for micro-CT analysis. Scans were reconstructed and scaled in ImageJ. Whole and segmental measurements of length, thickness, and volume were obtained for regenerating bone, then normalized by comparison with corresponding original amputated digit tips.

Results: Stereo-seq analysis revealed a rich architecture of genetically distinct regions of the digit tip. Differential gene expression in keratinocyte populations allowed for segregation of epidermal cells into computationally defined clusters, localized to the regeneration epidermis (RE). Osteogenic genes, such as *Ibsp*, *Sparc*, *Spp1*, *Postn*, *Bglap*, and *Alpl,* were induced in both P3 and P2 amputations compared to uninjured P3 digits. Spatially localized GFP expression in areas of the MDT suggested successful transduction of injected AAV constructs, whereas anti-FLAG immunofluorescence revealed promoter-specific patterning of viral construct expression. Mice injected with AAV8 POSTN:BMP2-3xFLAG demonstrated increased P3 regenerated volume when normalized and compared to the GFP control mice (mean 550% ± 250% vs 190% ± 60%, p=0.0036), and increased P2 regenerated volume relative to GFP control mice (mean 180% ± 86% vs 98% ± 47%, p=0.0085). AAV8 KRT16:BMP2-3xFLAG showed increased thickness (mean 72% ± 10% vs 49% ± 3.7%, p=0.0086) and volume (190% ± 81% vs 98% ± 47%, p=0.0467) in P2 regenerated digits. Trabecular structural properties appeared similar between mice injected with AAV8 COL1a2:BMP2-3xFLAG and those injected with control virus.

Conclusion: This study presents a novel methodology for identifying AAV control sequences in the MDT and suggests that bony digit regeneration can be enhanced by spatially directed overexpression of BMP2. Ongoing work seeks to probe cell type-specific dynamics and associated signaling pathways modulated by these vectors. This work may lead to new approaches for treating complications of limb loss through AAV gene therapy.

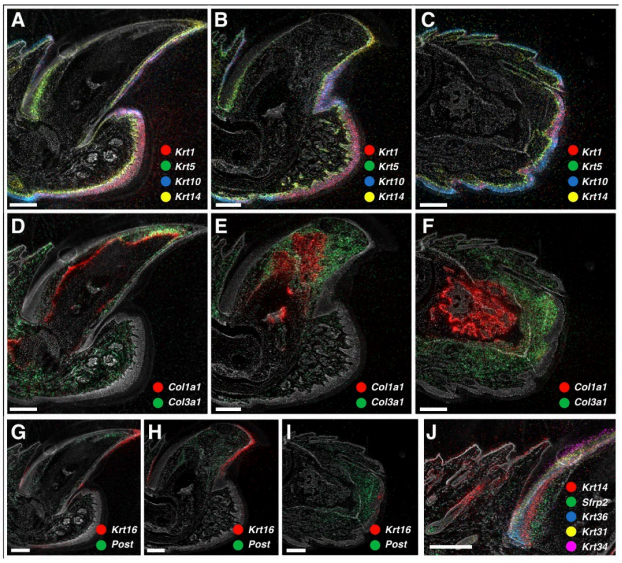

Figure 1. STOmics Stereo-seq gene expression profiles of an uninjured P3 mouse digit tip (A,D,G,J), a mouse digit tip 14 days post-amputation at P3 (B,E,H), and a mouse digit 14 days post-amputation at P2 (C,F,I).

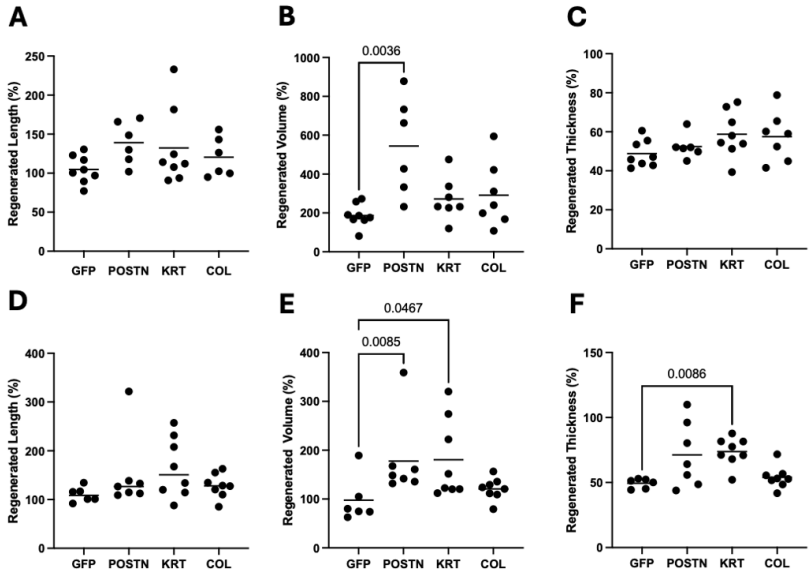

Figure 2. Measurements from 3D-rendered mouse digit tips generated from micro-computed tomography imaging. (A-C) Length, volume, and thickness of mouse digit tips amputated along P3 and harvested at 21 days post amputation, normalized to the corresponding value for original amputated digit tip counterparts. (D-F) Length, volume, and thickness of mouse digit tips amputated along P2 and harvested at 28 days post amputation, normalized to the corresponding value for original amputated digit tip counterparts.

1574 Secreted microRNAs are implicated in the reversal of myogenesis defects in ALS-derived myocytes.

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Amyotrophic lateral sclerosis (ALS) is the most prevalent adult-onset paralytic disorder, characterized primarily by a progressive loss of motor neurons (MNs). Skeletal muscle, a plastic tissue that responds to insults through proliferation and differentiation of satellite cells, is affected by MN degeneration. The process of skeletal muscle degeneration and regeneration is meticulously regulated by signals that govern satellite cell proliferation and differentiation. While it is known that satellite cell differentiation is impaired in ALS, the role of microRNAs (miRNAs) and their involvement in intercellular communication within skeletal muscle in ALS remain poorly understood.

In this study, we demonstrated impaired differentiation of satellite cells derived from ALS mice, which is associated with the impairment of myogenic p38MAPK and PKA/pCREB signaling pathways. These signaling pathways can be regulated by miR-882 and -134-5p. These miRNAs participate in autocrine signaling in conjunction with miR-26a-5p, which is secreted from wild-type (WT) and captured by ALS myoblasts. This secreted miR-26a-5p enhances ALS-related myoblast differentiation by repressing Smad4-related signals.

These findings underscore the paramount importance of elucidating the role of intercellular communication and the molecular mechanisms underlying ALS pathogenesis and progression. They also suggest that miRNAs could be targeted or utilized as potential therapeutic agents to promote myofiber regeneration.

1575 A Highly Potent MyoAAV4A Vector Achieved Long-Term Correction of Muscle Disease in Young Adult GSD IIIa Mice and Reversed Muscle Dysfunction in Aged Mice

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Background: Glycogen storage disease type III (GSD III) is caused by glycogen debranching enzyme deficiency, which leads to pathological accumulation of cytoplasmic glycogen in muscle and liver. We recently reported an innovative gene therapy approach using an AAV9 vector expressing a bacterial debranching enzyme (pullulanase) from an immunotolerizing dual promoter, which consists of a liver-specific promoter (LSP) and a ubiquitous CMV enhancer/chicken β-actin (CB) promoter. We showed that the LSP-CB dual promoter effectively suppressed pullulanase-specific cytotoxic T lymphocyte responses in GSD IIIa mice while allowing sustained pullulanase expression and glycogen reduction in liver, heart, and skeletal muscles. We have further demonstrated that two high potency MyoAAV capsids (MyoAAV4A and MyoAAV4E) remarkably enhanced transduction efficiencies in the cardiac and skeletal muscles of GSD IIIa mice, in comparison with AAV9 at 6 weeks following AAV injection. In this study, we evaluated the long-term efficiency and therapeutic efficacy of the lead MyoAAV4A capsid in young adult and aged GSD IIIa mice. **Methods:** An AAV vector carrying the LSP-CB dual promoter and a codon optimized CpG-free pullulanase cDNA was packaged in MyoAAV4A capsid and the resulting viral vector (MyoAAV4A-Dual-Pull) was intravenously injected into 3-month-old (young adult mice) or 13-month-old (aged mice) male and female GSD IIIa mice at a dose of $5x10^{12}$ vg/kg. Untreated (UT) age- and gender-matched GSD IIIa mice were used as controls. After 9 (young adult mice experiment) or 3 (aged mice experiment) months, the improvement of muscle function was evaluated by the wire-hang and treadmill tests. Tissues were harvested to assess the pullulanase expression and glycogen content. Plasma samples were collected to measure the levels of plasma creatine kinase (CK). **Results:** In young adult mice, AAV treatment led to slightly increased pullulanase activities in the heart (10.38 ± 4.08 vs UT: 4.22 ± 0.28 mU/mg of protein) and skeletal muscle (1.29 ± 0.55 vs UT:

0.58 ± 0.25), but not in the liver. AAV treatment significantly decreased glycogen contents in the heart (-53%) and skeletal muscle (-53%), accompanied by improved muscle function (as determined by the wire-hang and treadmill performances) and reduced plasma CK level (**Table 1**). In aged mice, AAV treatment resulted in significantly increased pullulanase activities in the liver (4.53 ± 2.93 vs UT: 2.19 ± 0.22), heart (143.44 ± 19.62 vs UT: 6.00 ± 0.58), and skeletal muscle (46.18 ± 26.82 vs UT: 0.27 ± 0.24), and significantly decreased glycogen contents in these tissues (-47% in liver, -88% in heart, and -87% in skeletal muscle). AAV treatment also significantly improved muscle function and reduced the plasma CK level, in comparison with UT (**Table 1**). Furthermore, the MyoAAV4A-Dual-Pull vector showed similar therapeutic outcomes in male and female mice in both experiments (**Table 1**). **Conclusions:** Our data demonstrated the long-term efficacy of MyoAAV4A-Dual-Pull-mediated gene therapy in a mouse model of GSD IIIa and its ability to reverse muscle dysfunction in aged GSD IIIa mice. Follow-up experiments to test the therapeutic efficacy in GSD IIIa dogs are being conducted. This novel muscle-tropic capsid can also be broadly used for treating other inherited disorders that affect cardiac and skeletal muscles as well as diseases with late-onset muscle manifestations.

Table 1. Correction of muscle defects in young adult and aged GSD IIIa mice

Young adult GSD IIIa mice		Male		Female	
		UT	MyoAAV4A	UT	MyoAAV4A
Glycogen (μmol gluc/g of tissue)	Liver	317.13 ± 42.48	278.67 ± 27.73	254.47 ± 28.79	225.42 ± 29.98
	Heart	69.93 ± 5.68	32.86 ± 6.04	63.07 ± 9.77	37.06 ± 8.97
	Quadriceps	138.00 ± 21.00	64.74 ± 15.54	126.20 ± 24.34	42.00 ± 11.12
Functional tests	Wire-hang (sec)	1.40 ± 0.55	3.43 ± 1.51	3.00 ± 1.87	6.57 ± 3.99
	Treadmill (m)	8.30 ± 3.47	30.71 ± 23.51	13.80 ± 2.20	61.63 ± 38.00
Plasma CK (U/L)		216.06 ± 26.59	126.60 ± 44.82	270.92 ± 108.72	103.39 ± 15.27
Aged GSD IIIa mice		Male		Female	
		UT	MyoAAV4A	UT	MyoAAV4A
Glycogen (μmol gluc/g of tissue)	Liver	351.33 ± 37.31	187.13 ± 71.67	241.60 ± 23.36	102.54 ± 24.20
	Heart	70.67 ± 15.13	8.58 ± 1.44	56.53 ± 3.96	7.96 ± 1.79
	Quadriceps	179.93 ± 25.19	23.50 ± 5.82	168.20 ± 46.89	19.50 ± 3.66
Functional tests	Wire-hang (sec)	5.22 ± 1.79	14.90 ± 4.06	5.40 ± 1.35	18.13 ± 10.33
	Treadmill (m)	16.25 ± 10.63	69.11 ± 21.07	13.35 ± 5.41	76.94 ± 25.64
Plasma CK (U/L)		406.81 ± 243.90	129.24 ± 21.15	277.68 ± 90.48	81.76 ± 22.66

Data shown are mean ± standard deviation

1576 In vitro models and viral vectors and for targeted gene replacement therapy in Calpainopathy (LGMD2A/R1)

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Introduction: Calpainopathy, (Limb Girdle Muscular dystrophies 2ALGMD2A/R1) is caused by mutations in the calpain 3 gene (CAPN3), is characterized by progressive, symmetrical weakness of the proximal limb and girdle muscles. Although physical therapy and orthopedic surgery can address symptoms, no disease modifying or curing treatment available. To support the development of targeted gene addition approaches we aimed to develop differentiated human CAPN3 deficient cell culture disease models and explore different muscle specific Adeno associated virus (AAV) derived vectors for CAPN3 gene delivery.

Methods: We established two genetically different patient derived cell culture disease models with defined CAPN3 mutations. We used immortalized myocytes as well as induced pluripotent stem cells (iPSCs) derived myocytes and applied advanced protocols using electrical pulse stimulation (EPS) to evoke differentiation into Myotubes and sarcomere formation in 2D cell culture. Both models were characterized with regard to differences compared healthy controls. To analyze viral vector mediated CAPN3 gene replacement in the differentiated myotubes, we designed vectors based on muscle specific capsids of AAV9 and AAVmyo encoding the full length CAPN3 cDNA fused to a GFP gene via a 2A peptide signal under control of the muscle specific MHCK7 promotor. Vector genomes were assembled and vectors were generated and produced to high titers. Subsequently both vectors were utilized to transduce differentiated CAPN3 deficient myotubes and healthy control myotubes. CAPN3 gene transfer and expression was analyzed by RT-qPCR, Western blot and fluorescent microscopy. Systemic *in vivo* delivery of both vectors was performed for biodistribution- and gene expression analysis in mice.

Results: Both CAPN3 deficient cell culture models were suitable for in vitro differentiation into mature contractile Myotubes and displayed morphological as well as physiological differences compared to healthy controls. AAV mediated gene transfer of the human CAPN3 cDNA resulted in enhanced CAPN3 gene expression *in vitro* and had compensatory effects on mRNA and protein levels in the two different Capn3-disease models. Preliminary *in vivo* experiments showed that systemic delivery resulted in robust CAPN3 gene expression in muscular tissue for both vectors, with AAVmyo showing reduced gene expression in non-target organs.

Discussion: This study provides novel preclinical *in vitro* disease models that help studying potential treatment options for Calpainopathy and provides insights helping to choose ideal gene transfer vehicle and or transgene expression setup for future translation into *in vivo* studies regarding tissue specificity and expression strength.

1577 Non-Viral Genetic Medicine for Targeted Delivery of Full-Length Dystrophin to Skeletal, Cardiac, and Diaphragm Muscles in DMD Mouse Models and Non-Human Primates.

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Duchenne muscular dystrophy (DMD) is a life-threatening genetic condition caused by mutations in the DMD gene, which result in the complete loss of functional dystrophin protein. To effectively treat DMD, therapies must aim to restore dystrophin production in the skeletal, diaphragm, and heart muscles, as these are the primary tissues affected by the disorder. Current gene therapy strategies utilizing truncated micro-dystrophin transgenes have faced challenges in achieving effective outcomes. These challenges stem in part from the limitations of viral delivery systems, including immune responses, inability to re-dose, restricted payload capacity, and limited organ biodistribution. Epidemiological studies of DMD patients indicate that even minimal expression levels of the full-length dystrophin gene (>0.5% of wild-type levels) may significantly alleviate the disease burden. This underscores the necessity to develop advanced non-viral genetic medicines capable of delivering full-length dystrophin (DMD) transgenes to achieve robust therapeutic efficacy. Transcutaneous ultrasound-mediated gene delivery (UMGD) represents a noninvasive and precision-targeted approach to address current challenges in genetic medicine delivery. SonoThera is advancing a proprietary UMGD-based non-viral genetic medicine platform designed to enable highly selective and efficient targeting of muscle tissues, offering a safe, extremely durable, and redosable therapeutic solution.

To evaluate the potential of UMGD for safe and effective gene delivery to muscle tissues, a nonviral DNA construct incorporating full-length human DMD transgene driven by a muscle-specific promoter was developed. Under optimized UMGD conditions, efficient delivery of the full-length DMD transgene was achieved in the skeletal, cardiac, and diaphragm muscles of the DMD disease (mdx) mouse model. Further analysis confirmed robust transgene expression across the targeted muscle tissues in the mdx mice.

To assess the feasibility of UMGD for delivering therapeutic payloads in large preclinical models, the full-length DMD nonviral DNA construct was delivered to muscle tissues in non-human primates (NHPs). Molecular analyses confirmed efficient transgene delivery to the NHP target tissues, resulting in high levels of transgenic DMD protein expression in the treated tissues.

These findings position UMGD as an efficient and scalable platform for delivering genetic medicines to key muscle groups impacted in DMD patients, facilitating robust expression of functional full-length dystrophin protein. This innovative approach lays a foundation for the development of next-generation therapeutic strategies with broad potential applications across various muscular dystrophies.

1578 Development and Characterization of a Mouse Model, Cell Model, and AAV-Based Gene Therapies for TNNT1 Nemaline Rod Myopathy

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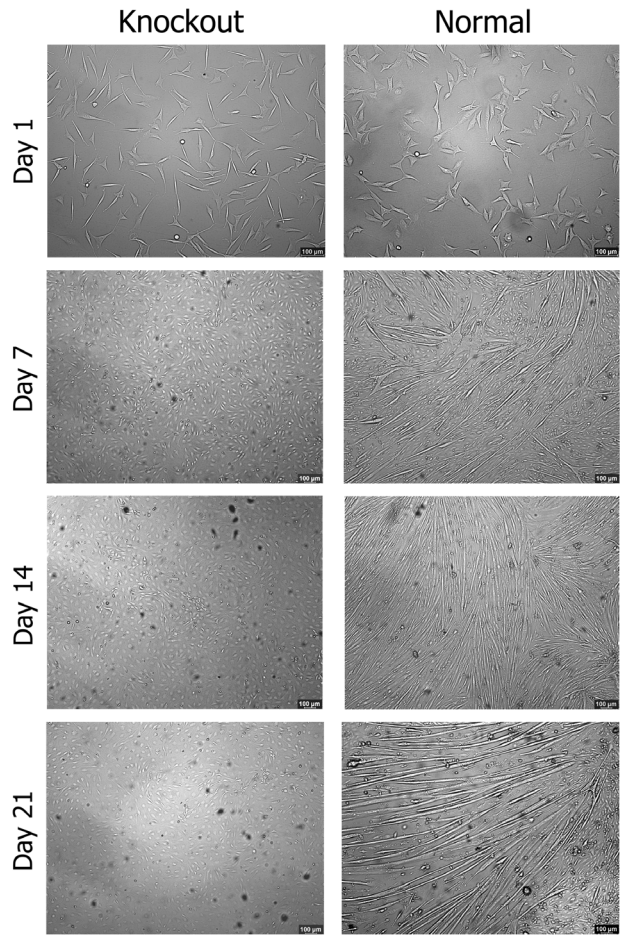
[1]University of Massachusetts Chan Medical School, Worcester, MA, [2]UMass Chan Medical School, Worcester, MA, [3]Worcester, MA, [4]UMass Chan Medical School, Worcester, Massachusetts, [5]University of Massachusetts Chan Medical School, Worcester,, MA, [6]Auburn University, Auburn, AL

TNNT1 nemaline rod myopathy is a genetic disorder caused by a c.505G>T nonsense mutation in the TNNT1 gene, which encodes for the T subunit of the troponin complex in slow twitch skeletal muscles. Initial clinical signs develop around 3-4 months of age and include axial hypotonia, limb stiffness, and tremors. As the disease progresses, diffuse muscle weakness, atrophy, and contracture occurs. The median survival time is 18 months with the cause of death being due to thoracic contracture resulting in restrictive lung disease. There is currently no treatment, and so only palliative care can be offered to patients. We created and characterized a TNNT1 gene knockout mouse and cell model to utilize in the development of AAV-based gene therapy. There was no difference between knockout and normal mice in rotarod, inverted screen, treadmill, and grip strength. Plethysmography was performed in 5 knockout and 5 normal mice at 2 ages, 4-5 and 15-16 months. There were no differences observed at the younger timepoint. At the older timepoint, the knockout mice had reduced tidal volume, reduced peak inspiratory flow, increased enhanced pause, and reduced mid-tidal expiratory flow, changes consistent with restrictive lung disease. In electromyography, 22 of the 37 knockout mice had fibrillation potentials. On histology of the knockout mice, the appearance of the biceps, triceps, quadriceps, semimembranosus/tendinosus, and cranialis tibialis was unremarkable, but there was reduced quantity and size of type 1 muscle fibers in the soleus and diaphragm. The lifespan of the knockout mice was normal. To create a cell model, we used CRISPR-Cas9 with 2 guide RNA's targeting coding sequences of the TNNT1 gene, one in exon 8, the other in exon 10, in C2C12 cells. On sequencing there were multiple edits at both guide locations, which were expected to result in either nonsense-mediated decay or a truncated, non-functional protein. In the myoblast stage, there was no phenotypic difference between the knockout and normal cells. When stimulated to differentiate, the knockout cells initially developed a more spindled appearance. Within 7 days, a majority of the normal cells formed myotubes. No myotubes were formed by the knockout cells within the 28-day evaluation period, but we did observe loss of defined cell margins, suggestive of cell fusion without organization, indicating a role of TNNT1 in myotube formation. In developing an AAV gene therapy, we designed and tested 8 vectors in the mouse model with combinations of AAV9 and AAV8 capsids, muscle specific promoters, human and murine TNNT1, and intravenous and intramuscular delivery. In skeletal muscles, mRNA was measurable, but no troponin T was detected. We hypothesize that this may be due to the need for all 3 troponin subunits to be co-translated given their complex interactions. As such, we built and packaged 2 vectors which contained all 3 genes. One vector has a single cassette comprised of the MHCK promoter, TNNC1, P2A, TNNI1, P2A, TNNT1, and an SV40 polyA. The other vector has two cassettes with opposing read

directions, the first cassette consists of the MHCK promoter, TNNC1, P2A, TNNI1, and an RBG polyA, and the second cassette consists of the Desmin promoter, TNNT1, and an SV40 polyA. These vectors are in the process of being tested in the mouse and cell models. If proven to be efficient, these vectors will not only benefit TNNT1 myopathy but could be used in treatment of TNNC1 and TNNI1 deficiencies.



Comparison of knockout and normal C2C12 cells at various timepoints after the initiation of differentiation. The knockout cells do form a more spindle-shaped appearance and appear to fuse but do not form myotubes.

1579 Therapeutic Screening of Dual AAV-Split Intein MIDI Dystrophins by Analysis of Mechanical Properties in Vivo and in Human Muscle Organoids

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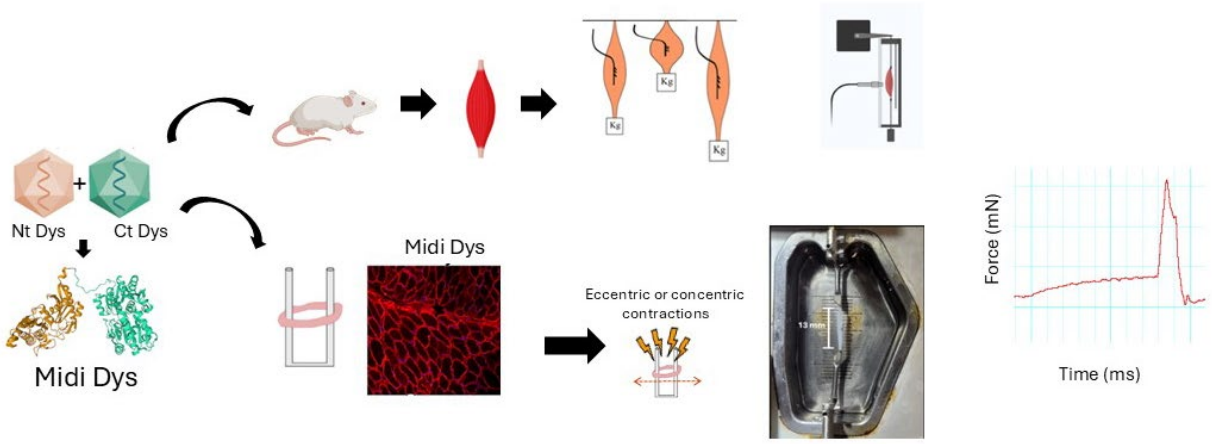
Duchenne Muscular Dystrophy (DMD) is a yet incurable muscle disorder affecting children and causing premature death. DMD is caused by genetic mutations leading to the absence of Dystrophin, a protein essential to preserve muscle integrity during contraction. In the last few years, AAVs have proved to be a promising tool for DMD gene therapy. However, due to the limited packaging capacity of AAVs, only shorter versions of dystrophin (microdystrophin) can be accommodated. Several microdystrophin products currently under investigation in clinical trials have shown promise but demonstrated limited and below-expected efficacy compared to results observed in animal models. This evidence raises questions about the reliability of current disease models or therapeutic readouts

and how microdystrophins can truly replicate the full-length dystrophin function in human patients, given that the shortened version lacks critical structural and signalling domains essential for optimal dystrophin performance.

We have recently generated two larger versions of dystrophin (MIDI dystrophin) (MIDI-DYS1 and MIDI-DYS2-EP23306256.1) using a Dual AAV system with trans-splicing intein approach, showing high protein reconstitution efficacy and therapeutic benefits in severe the DMD mouse model (D2-mdx). Because our constructs differ in key structural domain combinations, favoring dystrophin anchoring either to the sarcolemma or the cytoskeleton, we employed a more physiologically relevant muscle force assay to evaluate muscle performance based on distinct mechanical properties of the muscle when subjected to concentric (shortening) and eccentric (lengthening) contractions. Preliminary data from in situ muscle force assays indicate that MIDI-DYS1 outperforms both MIDI-DYS2 and microdystrophin, under these conditions.

To address the translational gap, we developed a human disease model that recapitulates critical hallmarks of DMD severity, such as fibrosis and muscle dysfunction, providing a more predictive framework for therapeutic investigation. By employing a muscle engineering approach, we generated MYOrganoids from iPSC-derived muscle cells co-cultured with fibroblasts, which enables functional maturation for muscle force analysis during contractions. Testing our candidates in MYOrganoids demonstrated the superiority of MIDI-DYS1, using eccentric contractions and fatigue assays—outcomes that were not revealed by isometric contractions alone, similar to in vivo data.

Overall, these findings underscore the importance of further exploring the changes in muscle mechanical properties following gene replacement, which could lead to improvements in current strategies. They also highlight the utility and translatability of dynamic muscle function assay, similar to real-world muscle movements, for therapeutic screening in human DMD muscles.



1580 3D Bioprinted Platform as a High-Throughput Model for Drug Screening and Discovery for Myopathies and Neuromuscular Diseases

Cristina Antich Acedo, NIH, Thurmont, MD

Myopathies and neuromuscular illnesses encompass several conditions that lead to a progressive decline in motor function due to faulty motoneurons (MN), muscle tissue, or a mix of both. Although there are several treatments to relieve symptoms, a cure has not yet been found. Our lab has developed an innovative platform that enables rapid and high-throughput biofabrication of human muscle tissue equivalents for therapeutics screening and discovery. The 3D muscle tissue assay system consists of a bioprinted muscle structure composed of human iPSCs-derived myoblasts and fibroblasts embedded in a hydrogel wrapped around a pillar-based multi-well culture plate to enable

the functional measurement of muscle contractility. Our work is focused on recreating various disorders included in Congenital Myasthenic Syndromes (CMS) to evaluate the efficacy of novel therapeutic gene therapies being developed by NCATS and NINDS, as part of PaVe-GT program. We used genetically modified iPSCs-derived myoblasts with deletion in the genes DOK7 or COLQ, using CRISPR technology and a doxycycline-inducible transcription factor system for iPSC differentiation. Mutations in those two genes account for about 20% of CMS cases, leading to alterations in the development and maturation of the postsynaptic domain. Our results showed that we were able to replicate the clinical features of both deficiencies in the biofabricated muscle tissues. This was evidenced by impaired contractility, with a significant drop in contractile force and noticeable fatigue after chemical (acetylcholine) and electrical stimulation, respectively. Currently, we are testing the safety and effectiveness of gene therapy using adeno-associated virus (AAV) delivery. This new 3D functional muscle tissue platform has the potential to improve understanding of muscle and neuromuscular pathologies while also providing a predictive tool for new therapeutic approaches.

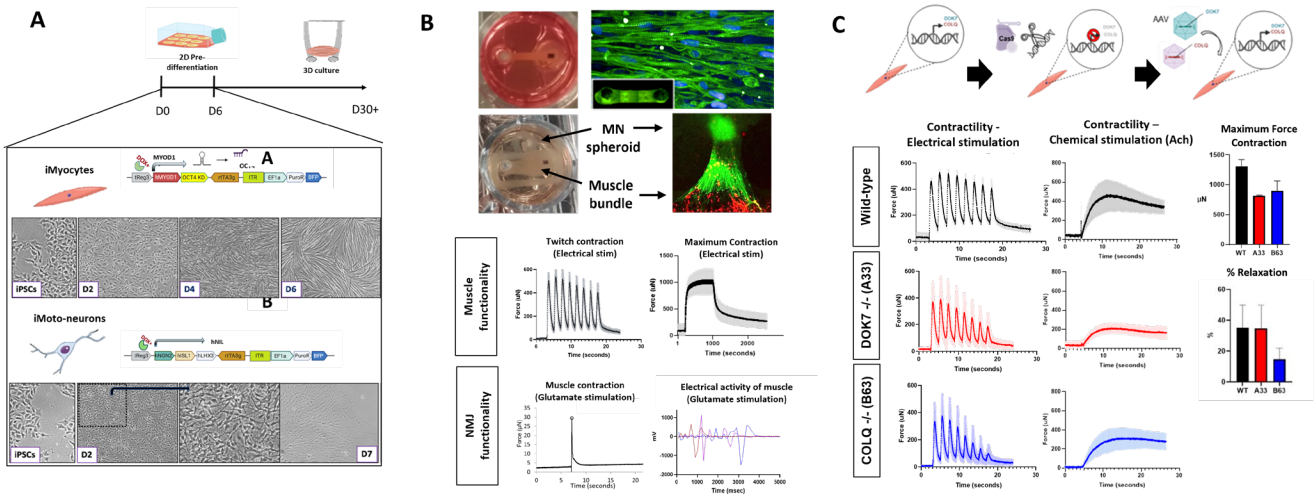


Figure 1. A. To produce the 3D muscle and NMJ models, we used hiPSCs-derived myocytes and MNs embedded in an ECM hydrogel, and then promote the muscle and neuro-muscular formation in a 3D culture system. B. Anatomical and functional studies of 3D skeletal muscle and NMJ model. Microscopic images from muscle and NMJ tissue after formation and maduration in vitro. Functional studies of contractibility and electrophysiological activity of muscle and NMJ after stimulating electrically, or chemically (glutamate), respectively. C. CMS modeling and evaluation of impaired functionality. Contractibility assays of muscle by electrical and chemical (ACh) stimulation proved the ability to recapitulate the clinical features of both CMS deficiencies.

1581 Optimizing CRISPR/Cas9 and AAV6-Based Gene Therapy for Sickle Cell Disease: The Dual Impact of Polyvinyl Alcohol on HDR Efficiency and HSC Viability.

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Sickle cell disease (SCD) is a severe monogenic disorder caused by a single-point mutation in the *HBB* gene, leading to abnormal hemoglobin (HbS) production and associated pathophysiology. Gene therapy strategies targeting the HBB locus offer the potential for permanent therapeutic correction by replacing or repairing the defective gene in patient-derived hematopoietic stem and progenitor cells (HSPCs). CRISPR/Cas9-mediated editing has emerged as a promising approach, with homology-directed repair (HDR) being a critical mechanism for precise gene correction. However, efficient delivery of CRISPR-Cas9 machinery and HDR templates via adeno-associated virus (AAV) vectors remains a challenge due to limited transduction efficiency in primary HSPCs and potential toxicity associated with high viral doses. Polyvinyl alcohol (PVA) has been reported to enhance AAV6 transduction efficiency, potentially by improving viral stability and/or facilitating its binding to cell surface receptors. We hypothesized that incorporating PVA into the transduction protocol could enhance HDR rates while reducing the viral dose required, thereby mitigating AAV6-associated toxicity and improving gene therapy efficacy and safety for

SCD. To test this, we evaluated the impact of PVA on AAV6-mediated gene correction in HSPCs at different multiplicities of infection (MOI: 312, 625, and 1250). PVA addition significantly increased targeted gene editing across all MOIs tested (MOI 312: 25% ± 9.7 vs. 13.4% ± 11.2, $p = 0.001$; MOI 625: 29.3% ± 10 vs. 12.4% ± 8, $p = 0.004$; MOI 1250: 42.5% ± 5.6 vs. 34.1% ± 8.0, $p = 0.02$). In primary transplants, all groups showed significant bone marrow engraftment 16 weeks post-infusion. HDR analysis demonstrated increased *HBB* targeting with PVA, particularly at higher MOIs (1250 + PVA: 23.88% ± 2.39; 625 + PVA: 12.83% ± 8.53; 312 + PVA: 10.78% ± 6.99). Notably, reducing AAV6 MOI was associated with improved human cell engraftment in the bone marrow of transplanted mice. As an example, frequency of $hCD45^{+}HLA\text{-}ABC^{+}$ cells was 1.8-fold higher for MOIs 312 and 625 compared to 1250. Our findings indicate that lowering AAV6 MOI while incorporating PVA enhances the clonogenic potential and engraftment of gene-edited HSPCs. The 312 + PVA group demonstrated particularly favorable outcomes in terms of myeloid lineage commitment and peripheral hematopoietic reconstitution. However, variability in HDR efficiency across treatment groups, particularly in secondary transplants, underscores the challenge of achieving consistent and robust gene correction in HSPCs. While PVA-mediated enhancement of transduction efficiency improves HDR rates at lower MOIs, its associated cytotoxicity at higher MOIs highlights the delicate balance between maximizing gene editing efficiency and preserving HSPC viability. This study underscores the need for fine-tuning HDR optimization while maintaining HSC fitness and provides a framework for refining ex vivo gene editing strategies for SCD and other hematologic disorders.

1582 Introduction of the Human Sickle Cell Mutation in Sheep Beta-globin by CRISPR/Cas9 Models Human SCD in Cloned Sheep

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Sickle Cell Disease (SCD) is the most common inherited blood disorder in the US, affecting ~100,000 people in the US and millions more worldwide, and the World Health Organization has forecast that SCD incidence will rise to >400,000 births/year by 2050. While early diagnosis and current therapies have improved, to some extent, the survival and quality of life of people with SCD, disease burden continues to be high, as treatment options are limited, they do not always permit the disease to be well-controlled, and many of the current therapies have significant side effects contributing to long-term morbidity and lack of patient compliance. Since SCD hemolytic anemia and associated chronic organ damage, as well as other clinical complications such as stroke, splenic crisis, pain episodes, life-threatening infections, and episodes of acute chest syndrome, can begin as early as 6 months of age, it is imperative to find novel therapies for infants and children affected by this disease and/or ensure the safety of the current ones for this age group. There is thus an urgent need for highly translational model systems to provide better insights into the pathobiology of the disease and to test novel therapeutic approaches. The sheep β-globin gene shares ~87.5% identity with the human gene. In addition, sheep, like human, undergo a g- to b-globin switch near birth, thereafter silencing HbF. The presence of this switch has led to the extensive use of sheep to understand this critical developmental process and makes sheep an ideal model in which to study hemoglobinopathies. After performing molecular dynamics simulations to ascertain that insertion of the human SCD-causing mutation into the sheep β-globin gene *in silico* induced conformational changes consistent with Hb polymerization driving RBC sickling, CRISPR/Cas9 editing was used to introduce the single A-to-T substitution present in human SCD into the β-globin locus of sheep fibroblasts, and somatic cell nuclear transfer generated animals harboring the human SCD mutation. SCD lambs, and the offspring of SCD animals bred with normal ewes (sickle cell trait - SCT - animals), exhibit multiple clinical and laboratory parameters that recapitulate human SCD or SCT, respectively, including sickled erythrocytes and polychromasia, positive SICKLEDEX®, and the presence of Hb with the same migration pattern as human HbS. Moreover, ektacytometry demonstrated a leftward shift of the elongation index–osmolality curves mirroring that seen in people with SCD. Importantly, in contrast to the transgenic SCD pig, sheep express HbF at birth, which gradually decreased and was almost completely replaced by HbS by the first month of age (~1 yr in humans). A deep convolutional neural network was developed and trained that enabled highly accurate identification and quantitation of various abnormal RBC shapes in blood smears of the SCD and SCT sheep. The

percentage of sickled RBCs increased with age in these sheep paralleling what is seen in people with SCD. Circular shape factor and elliptical shape factor scattergraphs of RBCs demonstrated that both SCD and SCT sheep exhibited elongated, deformed RBCs, at levels mirroring the human phenotypes. Collectively, data to-date support the high translational relevance of the SCD sheep model and suggest that this new large animal model will be a valuable and highly clinically relevant resource for the SCD community for its future use in the development of novel SCD therapies.

1583 Robust Gene Editing of CD34+ Hematopoietic Stem Cells Using a Microfluidics-Based Gene Delivery Technology

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Introduction: Hematopoietic stem cell (HSC)-based gene therapy represents a promising approach for treating inherited hematopoietic disorders and facilitating allogeneic cell therapies. However, HSCs present unique challenges due to their limited manipulation window and manufacturing constraints, which affect long-term cell therapy efficacy. Traditional gene delivery methods, such as viral vectors and electroporation, often induce cytotoxicity, inhibit cell proliferation, and limit therapeutic yield. Here, we present an optimized workflow utilizing microfluidics-based gene delivery technology as a gentler yet highly effective alternative for gene editing in HSCs. This approach ensures high efficiency, minimal toxicity, and robust therapeutic yield, while offering flexibility in processing time. It represents a promising solution for improving manufacturing processes and enhancing clinical outcomes.

Methods: We optimized a workflow using a microfluidic mechanoporation technology for efficient gene delivery into HSCs. EGFP-mRNA was initially used for optimization. We then evaluated the performance of this workflow in delivering CRISPR/Cas9 ribonucleoprotein complexes targeting the Beta-2-microglobulin (B2M) gene into human CD34+ hematopoietic stem and progenitor cells (HSPCs) across various ex-vivo culture time points. Key metrics, including cell viability and recovery, gene-editing efficiency and yield, and differentiation potential, were assessed and compared to electroporation, a conventional transfection method.

Results: The optimized workflow using a microfluidic-based mechanoporation technology efficiently delivered mRNA into HSPCs, achieving over 90% cell viability and 70% recovery the following day, along with greater than 95% transfection efficiency. Gene editing of HSPCs using this microfluidic system was successful, preserving cell viability, proliferation, and differentiation potential across transfection timepoints. The workflow achieved dose-dependent B2M gene knockouts, with a maximum editing efficiency of over 80%. Notably, this microfluidic technology required significantly lower RNP concentrations to achieve peak editing efficiency—up to four times less than electroporation. Additionally, the system supported same-day transfection post-thaw, with gene-edited HSPCs retaining their stem cell phenotype with high recovery and editing efficiency.

Conclusion: The microfluidic-based gene editing technology provides a highly efficient, low-toxicity platform for gene editing in HSCs. It is compatible with shorter editing workflows and offers a robust approach that preserves the stem cell phenotype while reducing payload requirements. This workflow has the potential to significantly enhance the clinical application of HSC-based gene therapies, improving both manufacturing processes and therapeutic outcomes.

1584 Impact of novel mobilizers and disease modifying agents in the sickle mouse

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Background: Ex vivo gene therapy (GT) can transform the lives of people with sickle cell disease (SCD). However, collecting sufficient hematopoietic stem cells (HSCs) is challenging; granulocyte colony-stimulating factor (GCF)

cannot be safely used, and inflammation and a disordered bone marrow (BM) stroma and vasculature impair mobilization. In a sickle mouse model, we showed that BM abnormalities can be reversed with osivelotor, a drug that covalently binds to the alpha globin chain, keeping the hemoglobin (Hb) in a high oxygen affinity conformation, reducing polymerization and sickling, increasing Hb to normal levels. We now ask if osivelotor can improve plerixafor mobilization in the sickle mouse, and test the ability of a novel CXCR4 inhibitor, EMU116, to mobilize HSCs.

Methods: 8-week-old Townes sickle mice (SS) were fed 8 weeks of control chow (6M, 6F) or osivelotor chow (6M, 6F). Half were mobilized with plerixafor at 5 mg/kg given subcutaneous (SC), half received PBS. Two hours later, peripheral blood (PB) was collected for a complete blood count (CBC), flow cytometry (flow), and colony forming unit (CFU) assessments. 12-week-old animals were treated with EMU116 via gavage at 30 mg/kg (3M, 3F), or SC at 4 mg/kg (3M, 3F). PB was collected and analyzed 6 hours after EMU administration. HbAA mice were also treated with plerixafor (3M, 3F), EMU116 SC (3M, 3F) and EMU116 (3M, 3F) orally. Median values were compared across groups using non-parametric tests.

Results: WBC was higher in HbSS+PBS animals compared to HbAA+PBS; plerixafor and EMU116 increased WBC. The %lineage negative Sca-1cKit+ as a percentage of all Lin- (%LSKLin-) cells in the PB were significantly higher with EMU116 than PBS control in HbAA and HbSS mice (Fig 1). Compared to plerixafor, EMU116 SC mobilized a higher %LSK in HbAA (0.004 vs 0.03%, p=0.02) and HbSS mice (0.005 vs 0.08%, p=0.01). Further, EMU116 SC mobilized more Flt3-CD34- cells in HbSS compared to plerixafor (0.3 vs 0.05%, p=0.006) but comparable to HbSS+plerixafor when pretreated with osivelotor. There was a trend toward higher CFUs in all mobilized HbSS groups (Fig 2). WBC and flow were comparable for EMU116 oral and SC.

Conclusions: The novel mobilizing agent EMU116 increases stem cells with long term potential (LSKLin-) and primitive HSCs able to rapidly reconstitute (%Flt3-CD34-) in the PB of HbSS and HbAA mice significantly more than plerixafor. CFU are also higher with mobilization indicating stem cells able to proliferate and differentiate, although not statistically significant likely due to sample size. SCD modification with anti-sickling drug osivelotor increased plerixafor mobilization of %Flt3-CD34- HSCs. Future studies include treating HbSS mice with osivelotor prior to EMU116 mobilization. We conclude that EMU116 is a promising mobilizer for individuals with SCD and may replace plerixafor in combination with GCSF for healthy individuals. Treatment with osivelotor may be an effective pretreatment strategy in individuals with SCD seeking GT to improve mobilization efficiency and quality of HSCs.

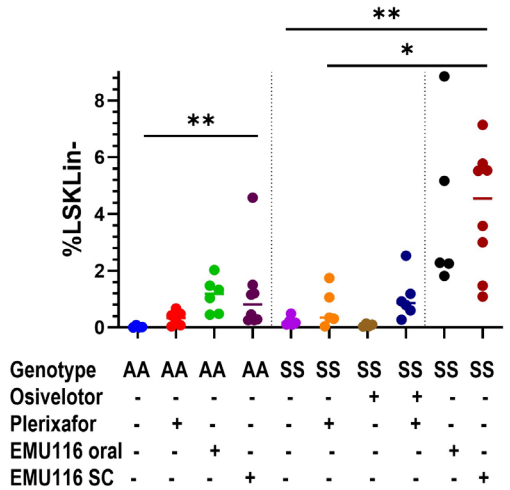

Figure 1: Mobilizer and disease modifier effects on %LSKLin- cells in the peripheral blood of wild-type (HbAA) and sickle mice (HbSS). HbSS were mobilized with plerixafor with and without a SCD disease modifier (osivelotor), or with EMU116. *p<0.01; **p<0.001.

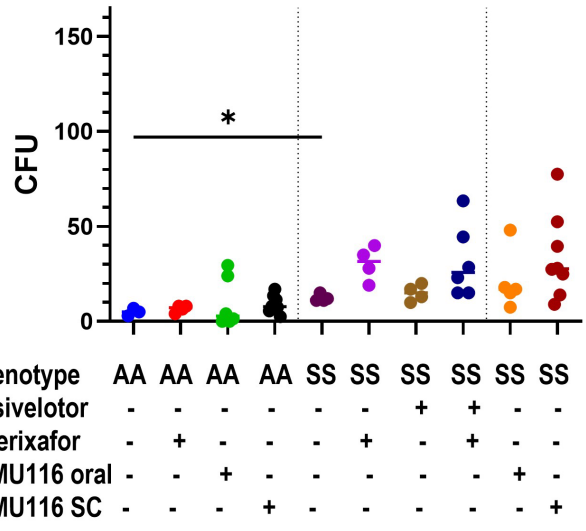

Figure 2: Mobilizer and disease modifier effects on CFU. *p<0.01

1585 Heterozygous SCD Sheep Produced by Breeding Exhibit Sickle Cell Traits

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Sickle cell disease (SCD) is the most common inherited hemoglobinopathy, affecting more than 100,000 people in the United States and millions more worldwide. SCD is caused by a single 'A to T' nucleotide replacement at the sixth codon of the β-globin gene (*HBB*) that results in the substitution of a glutamate for valine triggering abnormal polymerization of hemoglobin (HbS) and erythrocyte sickling. These changes decrease the ability of erythrocytes to carry and deliver oxygen to the tissues, leading to SCD pathology.

Sheep and humans exhibit a high degree of homology at the β-globin (*HBB*) gene and have similar anatomy, organ physiology, immune system development, and transitions from fetal to adult hemoglobin isoforms. Previously, we generated an SCD sheep model via CRISPR/Cas9 and Somatic Cell Nuclear Transfer (SCNT) and characterized their phenotype. We produced two rams that closely resembled the SCD phenotype in human patients. These animals exhibited sickled erythrocytes and abnormal hemoglobin with the same migration pattern as human hemoglobin S (HbS). They also displayed positive SICKLEDEX® test and RBCs with decreased membrane deformability. We hypothesized that breeding of these SCD rams would pass the *HBB* gene sickle mutation to the offspring and lead to Sickle Cell Traits (SCT) in heterozygous sheep. Here we describe the production of heterozygous SCT sheep by the breeding of SCD ram to wild-type (WT) ewes, and their phenotypic characterization.

The homozygous SCD2239 ram was placed with 20 WT Romney ewes for breeding. Twelve ewes became pregnant (12/20 (60%)). Two of the 12 pregnancies were lost spontaneously, and one ewe gave birth to a stillborn fetus. The remaining nine ewes delivered 12 healthy lambs (9 females and 3 males, three sets of twins and six singletons). DNA was extracted from blood samples of the lambs for genotyping by PCR and Sanger sequencing. We confirmed that all lambs carried heterozygous SCT and SM (silent mutation) in the *HBB* and *HBG* genes, respectively. Four animals were identified with the haplotype A allele in the WT sequence of *HBB* with a SNP upstream of the SCD mutation site and the rest contain the haplotype B allele. The overall pregnancy rate was lower than anticipated for the WT Romney breed. Sickle cell tests confirmed that these heterozygous animals have hematological parameters consistent with human SCT. The SCT sheep (currently around 2-years of age) have improved survival to adulthood compared to the original SCD rams that died prior to 12-months of age. Additionally, we observed impaired spermatogenesis with abnormal sperm morphology in both SCD and SCT rams. This parallels observations in men with SCD who are at risk for impaired fertility (e.g., abnormal sperm concertation and quality at baseline). These findings support the translational relevance of the SCD and SCT sheep and highlight their potential use in the development of novel SCD treatments for this devastating disease.

1586 Human Engineered Heart Tissues to Model Genetic Dilated, Hypertrophic, and Arrhythmogenic Cardiomyopathies.

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CRISPR modification of human induced pluripotent stem cells (hiPSCs) enables the study of genetic cardiomyopathies, which affect at least 1 in 500 American adults[1]. However, the contractile changes expected in cardiomyocytes derived from hiPSC disease lines are often impossible to detect in traditional 2D cell culture formats, making it difficult to mimic the clinical phenotypes of genetic cardiomyopathies in vitro. The development of 3D engineered heart tissue (EHT) models that recapitulate key aspects of contractile dysfunction of cardiomyopathies are critical for studying disease progression and evaluating the efficacy of therapeutics. We

propose that in vitro models of arrhythmogenic (ACM), dilated (DCM), and hypertrophic (HCM) cardiomyopathy can be developed by generating EHTs from hiPSC-cardiomyocytes and measuring isometric force.

HiPSC lines heterozygous for clinically identified mutations were either generated with CRISPR engineering (DCM: TTN P22582fs, ACM: DSP R451G, HCM: MYH7 R403Q) or were derived from a patient sample (HCM: MYBPC3 1800delA) and isogenic control hiPSC lines were generated in parallel. MyoPod® EHTs were generated from each pair of isogenic control/disease lines and cultured for 3 weeks to maturity. The contractile phenotype of EHTs was assessed by measuring tissue isometric twitch contractions in the MyoLab® tissue analyzer to obtain baseline contractility, the force-frequency response, and the length-dependent activation response in each genotype compared to isogenic control.

HCM EHTs exhibited hypercontractility, prolonged relaxation, and a blunted response to increasing frequency as well as both shortening and stretch whereas ACM and DCM EHTs exhibited hypocontractile behavior and a blunted response to stretch. Contractile assessments of EHTs formed using cardiomyocytes differentiated from engineered hiPSC disease and isogenic control lines revealed distinguishable properties characteristic of each type of cardiomyopathy. In the future, these models can be used to assess the functional efficacy of novel therapies across a range of cardiomyopathies.

1. Maron BJ, McKenna WJ, Danielson GK, Kappenberger LJ, Kuhn HJ, Seidman CE, Shah PM, Spencer WH, Spirito P, Ten Cate FJ, Wigle ED. ACC/ESC clinical expert consensus document on hypertrophic cardiomyopathy: a report of the American College of Cardiology Task Force on Clinical Expert Consensus Documents and the European Society of Cardiology Committee for Practice Guidelines (Committee to Develop an Expert Consensus Document on Hypertrophic Cardiomyopathy). J Am Coll Cardiol 2003;42:1687–713.

1587 Exploring the Genetic Landscape of Neurodevelopmental Disorders: Novel Insights and Therapeutic Avenues through JNK Pathway Analysis

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Neurodevelopmental disorders (NDD) pose significant diagnostic and therapeutic challenges despite advances in genomics. Critical pathways in neurodevelopment have been linked to syndromic NDD with phenotypes including autism and intellectual disability (Patterson, V. et al., 2023). Recently, haploinsufficiency of several key components of the JNK pathway, including MAP4K4 and MAP2K4, were linked to syndromic NDD with phenotypes including autism and intellectual disability. Our study aims to identify additional novel JNK-associated genes linked to NDD using a genotype-first approach. In our study, we plan to conduct phenome-wide association studies (PheWAS) using biobanks to identify other potentially NDD-associated genes in the JNK pathway and to clarify the molecular mechanisms behind JNK-associated NDD using induced pluripotent stem cell (iPSC) models. Using Sanger sequencing, Next-generation sequencing, and other quantitative methods, we have selected biallelic and heterozygous mutated clones that cause frameshift mutation effects (See Figure 1). Through the differentiation of these iPSCs into neurons expressing Neurogenin 2 (NGN2) and regular NPC differentiation, we can evaluate the different mechanisms these mutations use to affect neuronal differentiation (See Figure 2). By using an integrative approach, it is possible to identify potential therapeutic targets and gain new insights into the genetic landscape of NDD.

Figure 1. Methods for clonal selection

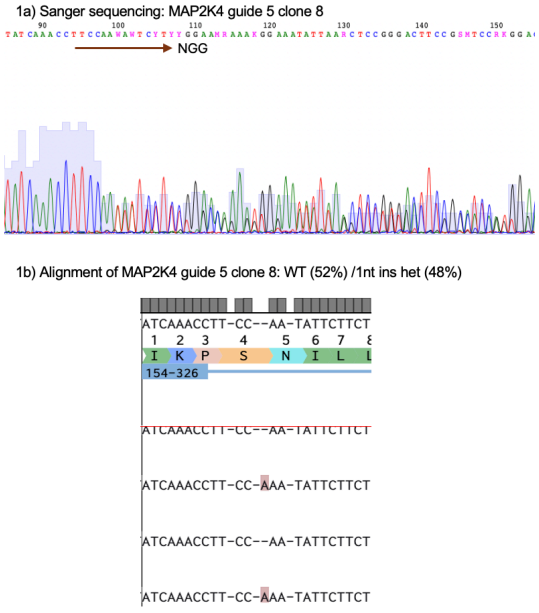


Figure 2. Edit confirmation

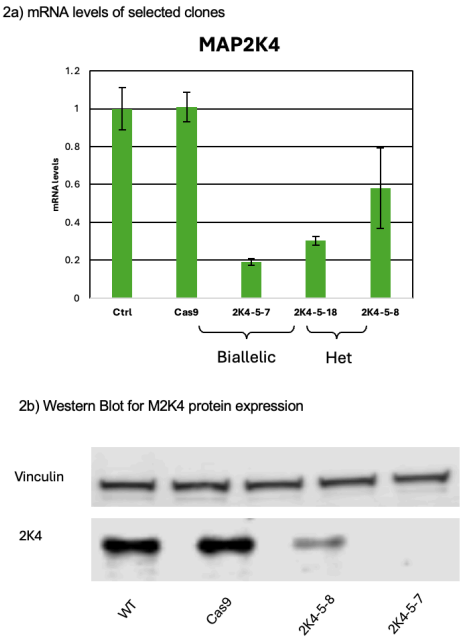
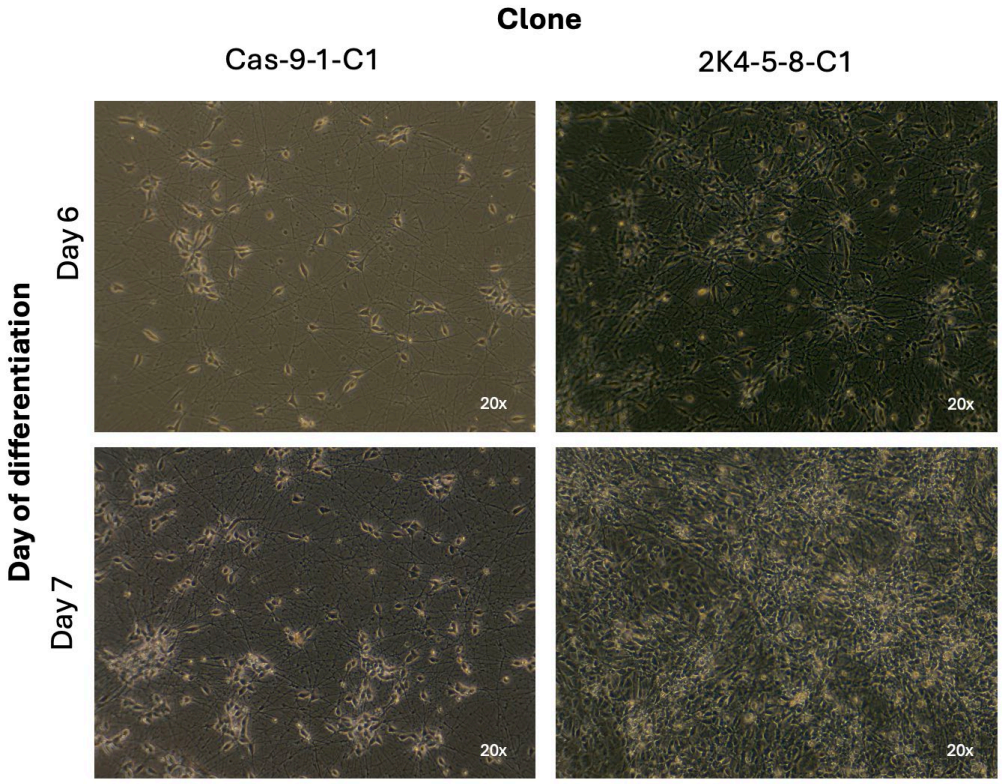


Figure 3. NGN2 differentiation of specific clones



1588 Leveraging automation and AI to streamline and scale iPSC-based drug screening

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The lack of screenable phenotypes in scalable cell models and platforms has hindered progress in drug discovery and early diagnostics for complex diseases. As the value of iPSCs for drug discovery has become ever more recognized, numerous technical factors, including intrinsic line-to-line variability and batch variation, make it challenging to define phenotypes in large-scale studies. Likewise, as biobanks have grown, technical opportunities have arisen that have allowed the development of sophisticated systems that can conduct cell biology at scale. We have leveraged advancements in robotic technology to develop the NYSCF Global Stem Cell Array®, our fully automated cell culture platform for parallelized iPSC reprogramming, gene editing, and differentiation into a range of downstream cell types, including astrocytes, glutamatergic/GABAergic neurons, hepatocytes, and cardiomyocytes, along with 3D neural and pancreatic organoids. Through this platform, we have created large biobanks of iPSCs, available to the community, spanning diseases including Alzheimer's, Age-Related Macular Degeneration, MS, and Parkinson's disease, as well as several rare diseases.

Building on this platform, we have recently developed a fully automated pipeline that integrates high-content imaging and machine learning to identify novel morphological disease phenotypes and enable drug discovery systematically. Importantly, this pipeline controls for inter- and intra-batch variation while accommodating large numbers of donor samples that allow us to capture subtle yet complex disease phenotypes across genetically diverse patients and clinically relevant subsets. In combining experiments containing upward of 96 donors, with multiple experiments running in parallel, we have used this platform to predict Parkinson's disease in patient-derived fibroblasts accurately. We are now performing phenotypic screens using multiple iPSC-derived cell types across various diseases.

We have identified novel phenotypes in a rare neurological disorder, Infantile Neuroaxonal Dystrophy, and used high-throughput compound screening to uncover novel drug candidates that display dose-responsive effects in shifting the cells to a healthier state. By combining biology with AI and software development, we have created tools such as ScaleFEx℠, enabling meaningful insights into these morphological disease phenotypes and drug-induced changes. Our phenotyping platform can be readily applied to various complex and rare diseases to advance precision drug discovery.

1589 Ethical and Social Considerations of Heritable Genome Editing in Human Embryos: A Focus on UNESCO Initiatives

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Recently, genome technologies have emerged as one of the fastest-growing fields, attracting significant attention from scientists and raising important ethical and social implications. Among these technologies, one of the most critical issues to address is whether the human genome should be modified through genome editing approaches. Many researchers have already discussed the nature of heritable gene editing, as reflected in the reports and guidelines issued by WHO and the US National Academy of Sciences. In the European context, UNESCO has actively considered the nature of the genome. The first UNESCO Universal Declaration on the Human Genome and Human Rights (1997) recognizes the human genome as *the heritage of humanity*, a symbol that should be protected, while also prohibiting human cloning. The latest UNESCO International Bioethics Committee report (2015) reflects the relationship between human rights and the human genome, building on the earlier declaration that heritable genetic changes should be prohibited (Universal Declaration on the Human Genome and Human Rights, 1997,

Article 24). This study examines the relationship between genome editing technologies, their clinical application in a heritable manner, and those ethical and social initiatives by UNESCO. Given that clinical trials involving CRISPR/Cas9 are increasingly underway, the ethics, values, and prospects of genome editing should be revisited.

1590 Deep CRISPR Nuclease Portfolio and Multiple Editing Modalities Accelerates Identification of Viable Clinical Candidates

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CRISPR associated RNA guided nucleases are widely applied in genome editing applications such as cell therapies, treatments for genetic diseases, and crop engineering. These nucleases enable programmable targeting of specific genomic DNA sequences encoded in their cognate guide RNAs (gRNA) and can be coupled to deaminases, reverse transcriptases (RT) or other genome modifiers for precise correction of disease mutations. However, challenges remain in the field such as developing disease-specific editors with maximal on-target potency and minimal off-target and bystander editing.

In order to address this gap, we have developed one of the largest collections of CRISPR Type II and Type V genome editors through categorizing 10B+ natural proteins and are leveraging AI-driven search, engineering and design methods to further expand this library. Leveraging such a large *in silico* catalogue, systems can be prioritized for characterization targeting desirable features for *in vivo* therapeutics, such as protein size, guide size, and PAM diversity. We characterized Type II and Type V nuclease systems with diverse PAM recognition sites enabling vast coverage of the genome, which expands the range of genomic targets for editing. We have implemented automated high throughput screening to characterize the activity of our novel CRISPRs in mammalian cells and found that 34% of Type II and 15% of Type V novel proteins with biochemically identifiable PAMs retained therapeutically relevant nuclease activity, which we have demonstrated can be significantly improved by enzyme engineering and guide modifications, in mammalian cells. We believe these novel proteins represent a significant expansion in the known library of CRISPR genome editors. Altogether, our deep portfolio of nucleases, diversity of natural and engineered PAM recognition, base and RT editing modalities, allow rapid identification of multiple editors with high potency and specificity for a large spectrum of disease-relevant human genomic targets, thus increasing success rates for developing these initial hits into viable clinical candidates.

1591 A single-step process for non-viral engineering of hypoimmunogenic pluripotent stem cells with the Pin-point™ base editing platform

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[1]Horizon Discovery Ltd, Cambridgeshire, United Kingdom, [2]Revvity, UNITED KINGDOM, [3]Revvity, Cambridge, United Kingdom, [4]Revvity, Cambridge, Cambridgeshire, United Kingdom, [5]Revvity, Superior, CO

Pluripotent stem cells (PSCs) hold great promise for manufacturing advanced cell therapies. Off-the-shelf allogeneic products derived from PSCs, engineered to be compatible with large patient cohorts, can broaden access to these therapies. However, their sensitivity to DNA damage presents challenges for complex genome editing. Base editors offer a solution due to their reduced genotoxicity compared to nuclease-based technologies. We developed the Pin-point platform, which enables the modular assembly of base editors composed of DNA binding Cas and DNA modifying deaminase components associated via an aptamer encoded in the sequence-targeting guide RNA (gRNA). Owing to the aptamer-dependent recruitment of the deaminase component to target DNA sequences, the Pin-point platform uniquely allows multi-purposing of a single Cas nickase component for simultaneous multiplexed base editing and targeted transgene knock-in. To demonstrate the platform's utility for engineering allogeneic PSCs, transient delivery of mRNAs encoding a Pin-point base editor (Rat APOBEC1 and SpCas9 nickase) with synthetic

aptamer-encoding and aptamer-less gRNAs was used to generate clonal hypoimmunogenic iPSC lines with various genotypes, including targeted large cargo transgene integration, using an automated clone tracking and picking workflow. Durable target protein knockout, substantially improved cell viability, editing efficiency, and genome integrity following multiplexed base editing compared to CRISPR-Cas9 with no adverse impacts on pluripotency were achieved. Hypoimmunogenic iPSC lines, generated via both multiplexed base editing and simultaneous base editing with targeted transgene integration, retained pluripotency, exhibited the expected reduced immune responses associated with their modified human leukocyte antigen (HLA) phenotypes and evaded both innate and adaptive immune responses when differentiated into therapeutic cell products. The Pin-point platform therefore represents a safe and efficient solution for multiple simultaneous genome engineering operations via a novel single step process compatible with downstream automation, dramatically streamlining the development of allogeneic iPSC-derived cell therapies.

The Pin-point™ base editing platform technology is available for clinical or diagnostic study and commercialization under a commercial license from Revvity.

1593 Expanding Base Editor Scope with Embedding Strategy

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Base editors can achieve base replacement without DNA double-strand breaks and have shown substantial potential in scientific research and gene therapy. Nevertheless, a variety of applications necessitate a range of unique features for base editors. For site-specific mutations, the base editor with narrow editing window is favored due to possible bystander mutations. In comparison, modifying the activity of regulatory elements on genome (e.g transcription enhancer), which is difficult to determine the exact regulatory sequences, requires a large editing window. To date, the editing windows of the majority base editors are +4 to +13 positions (+1 position at the far PAM end), which makes it difficult for effectively mutate these sites. Therefore, expanding the editing window will be another evolutionary direction. In this study, we evolve the Targeted AID-mediated Mutagenesis (TAM) cytosine base editor to expand its editing window using transposon technology. TAM CBE is a pioneer developed cytosine base editor that uniquely deaminate the ssDNA substrate, with an editing window of +1 to +12. First, we randomly inserted an activation-induced cytidine deaminase (AID) into the nSaCas9-KKH by MuA transposase to obtain a chimeric cytosine base editor (EB-TAM). We then introduced the EB-TAM library into E. coli along with a screening vector with inactivated kanamycin resistance. After IPTG induction, clones grown on kanamycin plates indicated that functionally EB-TAM restored the antibiotic resistance. The insertion sites of AID were mapped by Sanger sequencing. We successfully obtained 11 EB-TAMs in the prokaryotic screening. Then, the editing efficiency and their editing windows of 11 EB-TAMs were identified by five C-rich endogenous sites in HEK293T cells. Among the 6 functional EB-TAMs identified in HEK293T, EB-TAM-007 showed modest to higher editing efficiency from +1 to +17 compared to the other 5 EB-TAMs. Since nSaCas9 (D10A)-KKH cleave the middle of the +17 and +18 nucleotides of the sgRNA targeting strand, it is theoretically difficult to develop a base editor to edit the +18, +19 and +20 nucleotide of the non-targeting strand. Therefore, we assume that EB-TAM-007 harbor the largest window among the reported cytosine base editors based on the nSaCas9 (D10A) KKH. To further improve the editing efficiency, the linkers between AID and SaCas9 in EB-TAM-007 were optimized with three different linkers (L1, L2, L3). The EB-TAM-007-L1 exhibited an enhanced editing efficiency, showing a 1.51-fold to 1.82-fold increase compared to EB-TAM-007-L3 and 1.75-fold to 3.75-fold increase compared to prototype TAM CBE. Finally, we employed EB-TAM-007-L1 to edit the DMD E53 splicing site. As expected, 20% editing efficiency and 50% E53 exon skipping were obtained in DMD iPSC-derived myotubes. In summary, we developed a novel CBE with high editing efficiency and large editing windows enable for effective editing, especially for regulatory elements.

1594 Tricking Protein Translation: A Novel Base Editing Strategy to treat Dravet Syndrome

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Dravet syndrome (DS) is a severe developmental and epileptic encephalopathy caused by haploinsufficiency (HA) of *SCN1A* gene, encoding NaV1.1 sodium channel. Several novel therapeutic strategies are being developed at the preclinical stage, with some already entering clinical trials, but they necessitate lifelong administration of therapeutic agents. Here we describe a one-time permanent base editing of 5' untranslated (UTR) regulatory sequences to boost NaV1.1 protein translation.

To assess 5'UTR regulatory sequences effect on NaV1.1 protein translation and verify the possibility to interfere with this regulation to enhance protein expression, we set a reporter system screening platform to quantify fluorescence increase. Starting from the wild-type (wt) sequence, we cloned in a GFP/mCherry reporter vector 23 variants of *Scn1a* 5'UTR sequence harboring distinct combinations of nucleotide transitions reproducible with base editing. Flow cytometry analysis highlighted some variants able to significantly increase GFP fluorescence intensity over wt sequence. In particular, enhanced GFP levels were associated with specific A>G mutations in two ATG triplets upstream (uATG) to the main ATG (mATG), converting them into GTGs. By integrating available data of Ribo-sequencing performed in brain tissues, we learned that those two ATGs are candidate starting codons of upstream open reading frames (uORF) partially overlapping to the *Scn1a* mORF. uORFs have been described for 50% of human genes and are known to inhibit translation by sequestering ribosomes from the CDS; hence uATGs disruption is expected to enhance *Scn1a* translation, mirroring the results obtained in the GFP reporter. To verify this, we installed uATG>GTG mutations in the endogenous *Scn1a* locus using SpRY- and Sp-Cas9 adenine base editors. After setting up the base editing strategy in HEK-293T cells, we delivered the editor and the sgRNA in either lentiviral vectors or dual-adeno-associate viral vectors in cortical mouse primary neurons. Deep sequencing of the amplicon spanning the targeted region revealed that 60% of *Scn1a* reads carried at least one edited uATG and 13% exhibiting concomitant edits of both uATGs. Remarkably, this editing rate was sufficient to increase by 40% NaV1.1 levels without affecting mRNA levels, reinforcing the idea that these uORFs primarily regulate translation. Of note, in DS mouse model-derived neurons, uORF editing restored NaV1.1 levels back to wild-type. In addition, analysis of predicted off-target sites revealed neglectable editing activity, and the impact of bystander edits, evaluated in the reporter screening, showed minimal or no effect on the previously observed GFP fluorescence increase. This further confirms the specificity and safety of the approach. Next, to bring our therapeutic approach closer to clinical translation, we tested our system in human neurons, differentiated from pluripotent stem cells, and confirmed similar editing efficiency as in murine neurons. Finally, to further assess the therapeutic potential of our strategy, we are delivering the system *in vivo* in DS mice. Overall, we identified *SCN1A* uORFs as an inhibitory control of protein translation and showed that disrupting this via uATG editing increases NaV1.1 protein, providing a novel avenue for treating DS. Lastly, we found this mechanism to be shared across other *SCN1A* paralogs, which may further broaden the applicability of the approach to other haploinsufficiency diseases.

1595 The Second-Generation High-Fidelity Cytosine Base Editor AccuBase™ Can Proficiently Modify Multiple Genes Within A Human Primary T cell While Minimizing Off-target Effects

Sarah Rains, KACTUS, Silver Spring, MD

Introduction: In the realm of gene editing tools, one of the most prominent challenges is mitigating off-target mutations, chromosomal rearrangements, and insertion-deletions (indels). We utilized synthetic biology strategies to create a

novel cytosine base editor (CBE), AccuBaseTM , that has the highest editing efficiency in the industry, with zero off-target events. This was achieved by strategically inserting deaminases into Cas9 at specific, tolerance-verified sites, a process guided by an extensive transposon-based genetic screening, protein engineering, and direct evolution approach. AccuBaseTM forms a complex with the sgRNA, but at this step, the inserted deaminase is not yet engaged with the target DNA and is not in contact with any non-targeting dsDNA because it is hidden inside the protein. This mechanism avoids guide RNA-independent indiscriminate off-target mutations that were a common problem of first-generation base editing tools. When guided by sgRNA, AccuBaseTM associates with the target double-stranded DNA, triggering structural alterations within the AccuBaseTM. This results in the outward exposure of the deaminase domain, facilitating the editing of the target base. AccuBaseTM substantially reduces off-target effects while maintaining on-target editing when compared to BE4max. This improvement has been confirmed in multiple human cell lines, mouse cell lines, human primary T and NK cells, human embryonic stem cells, and in animal embryos and liver. In this study, we apply AccuBaseTM to multiplex gene modifications in primary human T cells, demonstrating its capacity for highly efficient multiplex gene disruption without inducing off-target effects. Importantly, the multiplex base-edited T cells exhibit enhanced ex-vivo cell expansion and do not exhibit the double-strand break-induced translocations observed in T cells edited with Cas9 nuclease. Therefore, we strongly believe that AccuBaseTM represents a solution to unmet demands within the gene editing field. It offers the highest on-target editing efficiency while minimizing off-target effects, setting a new standard for precision and effectiveness.

AccuBaseTM was made by inserting deaminases into Cas9 at specific, tolerance-verified sites, a process guided by an extensive transposon-based genetic engineering. AccuBaseTM forms a complex with the sgRNA (depicted as a purple double helix). Within this synthetic CBE, the deaminase domain (colored in red) is intricately integrated and is not in contact with any non-targeting dsDNA.

Summary: In this study, we harnessed AccuBaseTM to perform multiplex gene modifications in primary human T cells. Specifically, we targeted PDCD1, B2M, and TRAC using AccuBaseTM through the Lonza 4D nucleofector system. The knockout efficiencies for individual genes were as follows: 89% for PDCD1, 81% for B2M, and an impressive 96% for TRAC. When applied to create a triple knockout, the editing efficiency reached 60%, and this was confirmed through flow cytometry analysis. Remarkably, the edited T cells exhibited normal expansion characteristics and did not display the double-strand break-induced translocations typically observed in T cells edited with Cas9 nuclease. Equally significant, our analysis revealed the absence of any off-target effects in the AccuBaseTM -edited T cells.

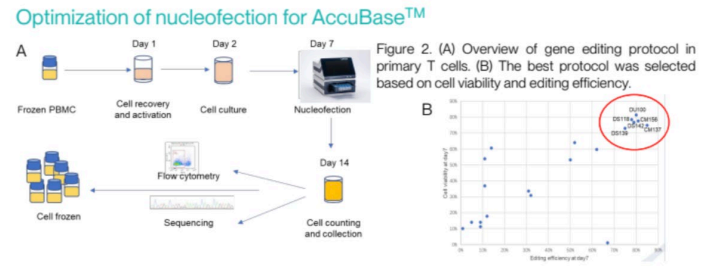

Figure 2. (A) Overview of gene editing protocol in primary T cells. (B) The best protocol was selected based on cell viability and editing efficiency.

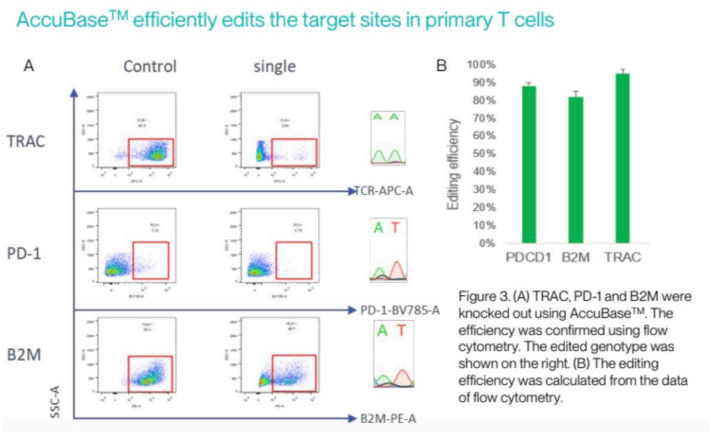

Figure 3. (A) TRAC, PD-1 and B2M were knocked out using AccuBase™. The efficiency was confirmed using flow cytometry. The edited genotype was shown on the right. (B) The editing efficiency was calculated from the data of flow cytometry.

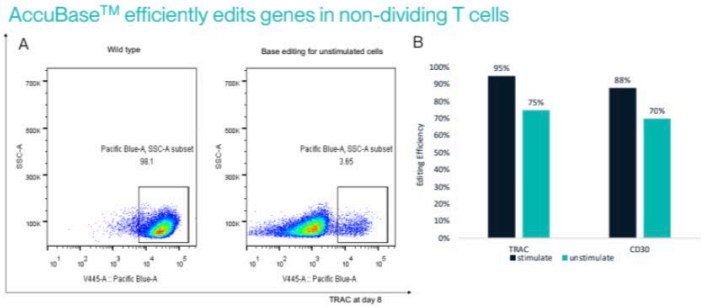

Figure 4. (A) The unstimulated T cells were edited, and the flow cytometry was used to confirm the knockout at day 8 after nucleofection. (B) The editing efficiency was calculated based on Sanger sequencing on day 4 after nucleofection.

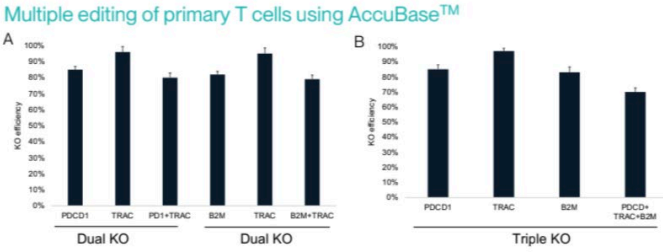

Figure 5. (A) Two genes were simultaneously edited using AccuBase™. The editing efficiency was calculated based on the flow cytometry. (B) Three genes were simultaneously edited using AccuBase™. The editing efficiency was calculated based on the flow cytometry.



1596 Minimal activation of the p53 DNA damage response by a modular cytosine base editor enables effective multiplexed gene knockout in induced pluripotent stem cells.

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Precise genome editing of induced pluripotent stem cells (iPSC) holds great promise for engineering advanced cell therapies. CRISPR-Cas systems have been widely adopted in genome engineering applications, however their dependence on genotoxic DNA double strand breaks (DSBs) presents challenges in hypersensitive iPSCs, including the selection for defective DNA DSB responses. Base editors are capable of both modifying and ablating gene function without generating DSBs making them an attractive solution in iPSC engineering applications. Here we report efficient and durable target knockout and substantially improved cell viability and expansion with a cytosine base editor assembled using the Pin-point™ platform compared to SpCas9. The cytosine base editor minimally activated p53-mediated DNA damage signalling independently of the number of simultaneous edits installed. By contrast, multiplexed editing with SpCas9 lead to high levels of p53 signalling and an associated reduction in editing efficiency. While transient inhibition of the p53 DNA DSB response enhanced SpCas9 multiplexed gene editing efficiency this was not required to achieve maximal base editing efficiency. Multiplexed editing of iPSCs with Pin-point base editors therefore both enhances the efficiency of genome engineering processes and substantially reduces the risk of selection for defective DNA damage responses inherent to DSB-dependent CRISPR-Cas systems.

1597 One-step engineering of allogeneic CAR-T cells by simultaneous multiplex knockout and site-specific transgene integration with the Pin-point™ base editing platform configured with OpenCRISPR-1, an AI-engineered CRISPR-Cas enzyme

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As our understanding of the effects of the tumor microenvironment on immune cells function improves, increasingly more complex cell engineering is needed to improve the efficacy and durability of cellular immunotherapies. First-generation genome engineering approaches utilizing CRISPR/Cas9 rely on double-strand DNA breaks (DSBs), which bring an increased risk of genomic rearrangements and cytotoxicity, particularly when multiple edits are required.

We have developed the RNA aptamer-mediated Pin-point base editing platform to facilitate complex cell engineering in a safer manner than conventional DSB-based technologies. The platform consists of either a nickase or a catalytically inactive RNA-guided nuclease, an extended guide RNA with an aptamer, and an aptamer binding protein fused to an effector (e.g a deaminase). By using a combination of aptamer-containing and aptamer-less guide RNAs, along with mRNAs encoding Rat APOBEC1 and SpCas9 nickase, the platform enables efficient multiple knockouts by base editing and simultaneous targeted insertion of a CD19-CAR at the TRAC locus in primary human T cells. Multigene edited, allogeneic CAR-T cells are therefore generated in a single intervention with a single nickase while overcoming the deleterious effects of DSBs and reducing significantly manufacturing timelines.

To broaden the applicability of the Pin-point platform, we demonstrate robust complex T cell editing using the nickase OpenCRISPR-1, an AI-generated enzyme from Profluent. A screen to identify optimal gRNA designs for base editing knockout revealed distinct editing features of the Pin-point platform employing OpenCRISPR-1 compared to SpCas9. Additionally, this novel nuclease supports both viral and non-viral targeted transgene knockin. The successful integration of the open-access OpenCRISPR-1 with the Pin-point base editing platform demonstrates versatility of our platform and provides further choices for advanced gene editing applications.

The Pin-point™ base editing platform technology is available for clinical or diagnostic study and commercialization under a commercial license from Revvity

1598 Customizing the versatile Pin-point™ platform: Modular components for tailored base editing in genomic applications

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CRISPR based gene editing technologies are extensively used for both functional genomic screening and adoptive cellular therapeutics. Among these, base editors represent a promising class of next-generation genome editing tools with the potential to precisely correct disease-causing genetic variants and safely knock out multiple gene targets simultaneously. The Pin-point base editing platform is a modular assembly of DNA binding Cas and DNA modifying deaminase components associated via an aptamer encoded in the sequence-targeting guide RNA (gRNA). This modular design allows for the creation of diverse Pin-point architectures, each with distinct base editing characteristics. This flexibility enables a “plug-and-play” approach, facilitating the use of optimal DNA-binding and modifying enzymes for specific applications.

We have developed an arrayed screening platform for high-throughput characterization of these configurations, demonstrating specific editing characteristics achieved with the Pin-point platform when employing different

cytosine deaminases. Furthermore, we show that the Pin-point platform can be configured with adenine deaminases to mediate adenine-to-guanine transitions in induced pluripotent stem cells (iPSCs) and T cells. We also use distinct aptamers fused to sgRNAs to achieve recruitment of both adenine and cytosine deaminases, enabling simultaneous editing of multiple genetic targets. By employing specific aptamer-deaminase combinations, we were able to target multiple genes independently, showcasing the potential to design sophisticated gene editing strategies for complex therapeutic interventions or functional genomic studies.

Additionally, we performed a massive parallel cell-based screen analyzing the editing activity of two different Pin-point base editor configurations with gRNAs targeting thousands of sequences. The resulting data enabled us to construct predictive models of editing outcomes for each configuration. Using these models, we ranked gRNAs designed to achieve functional knockouts of clinically relevant gene targets, including CIITA and PCSK9. Validation of the models revealed a strong correlation between in silico predictions and observed cell-based editing performance, paving the way to leverage the modularity of the Pin-point platform to design tailored configurations for specific edits. These results highlight the flexibility, precision, and scalability of the Pin-point platform, establishing it as a next-generation solution for advancing genome editing technologies.

Disclaimer: The Pin-point base editing platform technology is available for clinical or diagnostic study and commercialization under a commercial license from Revvity.

1599 Engineering TadA-Derived Base Editors with Enhanced Potency and Safety for non-viral In Vivo Gene Editing

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Base editors (BEs) have emerged as powerful tools for precise, single-nucleotide modifications without inducing double-stranded DNA breaks. Despite their potential, the therapeutic application of next-generation adenine (ABE) and cytosine (CBE) base editors in non-viral, in vivo settings has been constrained by suboptimal potency and safety concerns.

In this study, we present the discovery and engineering of novel TadA-derived BEs, identified through a metagenomic pipeline screening for new deaminases and Cas enzymes, followed by iterative rounds of rational protein engineering and directed evolution. This systematic approach resulted in a suite of ABE/CBE variants with significantly improved on-target editing efficiency, reduced sequence-context biases, and enhanced purity profile.

To further enhance potency, our lead ABE underwent linker engineering, Cas protein refinement, and mRNA sequence optimization, collectively leading to improved stability, catalytic activity, and expression. Unlike conventional studies that rely on plasmid delivery and a limited number of gRNAs to assess editor potency—an approach prone to bias— we evaluated the activity of our enhanced editors in mRNA form using a gRNA library comprising over 1,000 endogenous sites. This strategy provides a reliable and unbiased assessment of the general potency of editors under transient expression conditions.

To evaluate in vivo efficacy, the optimized ABE mRNA was encapsulated in lipid nanoparticles (LNPs) and co-delivered with a clinically validated gRNA targeting human PCSK9 in hPCSK9 mice. Our engineered ABE achieved >90% knockdown of PCSK9 at a dose of 0.6 mpk, outperforming the clinical benchmark ABE8.8, which required 1 mpk to achieve similar efficiency, highlighting the improved potency of our variant. Comprehensive whole-genome sequencing (WGS) and transcriptomic analyses further confirmed its excellent safety profile, supporting readiness for clinical translation.

In a preclinical program focused on a particularly challenging liver target, our lead ABE exhibited significantly enhanced editing efficiency, achieving over 75% ex vivo and 48% in vivo in clinically relevant models. In comparison, ABE8.8 reached only 65% editing ex vivo and 38% in vivo at the same target, underscoring the

enhanced performance and therapeutic potential of our engineered variant. Furthermore, to expand the potential therapeutic window, we developed a next-generation LNP formulation incorporating a novel cationic lipid with enhanced safety features. This novel formulation demonstrated a clear toxicity advantage over the industry-standard benchmark ALC-0315 in preclinical models, significantly enhancing the clinical viability and therapeutic potential of our platform for addressing complex liver targets.

In summary, these engineered base editors—offering enhanced potency, improved safety, and robust in vivo efficacy—represent a versatile and scalable platform for therapeutic gene editing. Our innovations pave the way for transformative drug development in liver diseases and beyond.

1600 High fidelity C-to-T editing with TALE base editors

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TALE base editors (TALEB) are fusions of a transcription activator-like effector domain (TALE), split-DddA deaminase halves, and a uracil glycosylase inhibitor (UGI). The C-to-T class of TALEB edits double strand DNA by converting a cytosine (C) to a thymine (T) via the formation of a uracil intermediate.

We recently developed and applied a strategy that allowed the comprehensive characterization of C-to-T conversion efficiencies within the target editing window. This method also takes advantage of a highly precise and efficient TALEN®-mediated knock-in of ssODN in primary T cells to assess how the composition and spacer variations of target sequences affect TALEB activity/efficiency.

Additionally, we highlighted that the composition of bases surrounding the target bases (TC) may strongly influence the editing efficiencies. We also demonstrated that different TALEB scaffolds could be used to relax target sequence limitations (to increase the targetable sequence) or be used to decrease or eliminate bystander editing within the editing window (to increase specificity). Overall, these findings allow for the fine-tuning of TALEB for a desired gene editing outcome.

We then applied a range of different techniques to assess characteristics of nuclear genome editing. First, we focused on on-target editing and then explored the possibility and risk associated with genome-wide TALE-dependent/independent binding and editing. By using an experimental model relevant for therapeutic application with TALEB mRNA vectorization in primary cells (e.g. PBMCs and HSCs), we demonstrated that targeted binding of a single TALEB arm does not lead to detectable editing (detection limit: 0.1-0.2%).

Finally, we further applied hybrid capture assays to test for off-target editing, in particular, within regions of the genome that were previously highlighted in cell lines. We demonstrated, in our relevant experimental setup with primary T cells, that editing at these sites was not detectable (detection limit: 0.2%).

Altogether, the results of this study have enhanced our control and use of TALEB and allow for the design of extremely efficient (high editing frequencies and edit purity) and specific (absence of TALE-independent off sites and very limited, if not absent, DSB generation) TALEB, compatible with the development of future therapeutic applications.

1601 Optimization of the modular Pin-pointTM base editing platform for an engineered Type V CRISPR-Cas effector

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The recent appreciation of the variable function of the natural diversity of CRISPR-associated nucleases has led to an interest in expanding the gene editing toolbox. The type V CRISPR-Cas system is particularly attractive for clinical development due to its smaller size than SpCas9 and its unique PAM specificities, permitting a greater variety of delivery vehicles and allowing for enhanced accessibility of editing sites not always attainable with type II CRISPR-Cas enzymes, such as Cas9.
Revvity's modular Pin-point™ base editing platform employs the delivery of either a nickase or deactivated Cas enzyme, a deaminase fused to an aptamer binding protein, and an aptameric guide RNA (gRNA) that assemble in vivo and act in conjunction to facilitate precise single nucleotide conversions. While originally developed using Cas9 nickase, the modularity inherent to the Pin-point platform permits swapping of components to achieve optimal editing results. Here, we demonstrate robust site-specific editing at different target loci using a deactivated CasONYX (dCasONYX) version of the Pin-point base editing platform. dCasONYX is an engineered version of dCasMINI developed by Epic Bio. It has an improved off-target profile compared to SpCas9, exhibits very low immunogenicity compared to Cas9 and is less than 1.5 Kb in size. Further utilizing the modularity of the Pin-point base editing platform, we show that additional components of the system such as different deaminases and aptameric gRNA scaffold configurations can be leveraged in combination with alternative Cas enzymes to adjust the base editing window; this is of particular advantage for applications including SNP correction and gene knockout at sites that are not usually accessible with the nCas9 configuration. Finally, we have significantly improved the design and optimized the delivery parameters of the dCasONYX version of the Pin-point platform through generation of IVT mRNA and achieving robust chemical synthesis of gRNA over 150nt in length, making this configuration suitable for application to therapeutically relevant cell types such as T cells and induced pluripotent stem cells (iPSCs).

1602 EPIREG®: A Novel Epigenetic Platform for Safe and Efficient Multi-Gene Silencing in Universal CAR-T Cell Therapies

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Introduction: Universal allogeneic CAR-T cells have broad applications, particularly in autoimmune diseases, providing a scalable, off-the-shelf solution for immunotherapy. Efficient silencing of immune targets, such as TCR and HLA class I/II, is essential to prevent immune rejection (GVHD and HVG) and enhance therapeutic efficacy. TCR editing is critical for eliminating T cell receptor recognition of host tissues, while HLA class I/II silencing prevents immune rejection of the infused CAR-T cells. Traditional gene-editing tools face safety risks, particularly with multiplexed editing of multiple genes, including off-target effects and chromosomal abnormalities associated with CRISPR/Cas9 and ABE. EPIREG® provides a safer, more efficient alternative, enabling precise, durable, and nuclease-free silencing of multiple genes in T cells.

Methods: EPIREG® utilizes epigenetic modifications via DNA-binding domains and DNA methylation/histone-modifying effectors. sgRNAs targeting genes involved in immune recognition, such as TCR, HLA class I, HLA class II , and immune checkpoint genes, were optimized using a deep-learning model. Multi-omics analyses, including RNA-seq, WGBS, PEM-seq, and CUT&TAG, were performed to evaluate on-target editing efficiency and potential off-target effects. A lipid nanoparticle (LNP)-based delivery system was developed for ex vivo editing of human primary T cells, achieving >90% editing efficiency while maintaining cell viability and proliferation.

Results: EPIREG® demonstrated excellent safety with no detectable off-target effects or chromosomal abnormalities, even when multiplexing up to seven target genes. TCR silencing was highly efficient, achieving >99.8% suppression of TCR expression through *CD3D* and *CD3E* editing, which remained fully suppressed after 60 days of continuous antigen stimulation. HLA class I silencing was achieved with >75% suppression through targeting B2M and TAP1, providing broad suppression of all HLA class I types. *HLA-A* and *HLA-B* were specifically targeted for combined editing to preserve the positive role of *HLA-C* in T cell functionality. HLA class II silencing exceeded 95% through *CIITA* editing, which regulates transcription of class II genes, providing efficient and durable suppression. Immune checkpoint genes (*PDCD1, TGFBR2, LAG3*) were effectively silenced, with editing efficiencies exceeding 95%, enhancing the immune modulation properties of the edited T cells. The LNP delivery system enabled efficient editing of primary human T cells, achieving >90% editing efficiency without compromising cell viability or proliferation. This streamlined the CAR-T cell manufacturing process, significantly

enhancing efficiency. Functional assessments of EPIREG-edited CD19-CAR-T cells demonstrated improved stemness, cytokine secretion, and cytotoxicity compared to conventional universal CAR-T cells.

Conclusions: EPIREG® provides a transformative platform for multiplexed gene silencing in universal CAR-T cells, overcoming safety challenges associated with traditional gene-editing tools. Its highly efficient LNP delivery system enables scalable and safe engineering of CAR-T cells, offering a robust solution for universal CAR-T therapies. This platform has significant potential for clinical applications in autoimmune diseases, providing a promising strategy to enhance the safety, efficacy, and manufacturability of universal CAR-T cell.

1603 Epigenetic Suppression of HBF Modifiers, Efficiently Reactivates Gamma Globin Expression in Primary Cells

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INTRODUCTION: Advances in genome editing have enabled new gene therapy strategies, such as in situ gene correction or introduction of disease-modulatory mutations. In β-hemoglobinopathies, targeted genome editing to modify fetal hemoglobin (HbF) transcription modulators has enabled the therapeutic reactivation of gamma globin, offering the ability to compensate for the dysfunctional or deficient adult hemoglobin. However, the DNA double strand breaks induced by the genome editing machinery remain a limitation. Recently, the development of custom-designed epigenome editors has allowed the introduction of specific epigenetic alterations to tightly regulate gene expression, without affecting genome integrity. The aim of the study is to assess the safety and efficacy of HbF reactivation in human primary cells, mediated by novel epigenome editing tools.

METHODS: Initially, we investigated dCas9-KRAB mediated epigenetic suppression of three HbF major suppressors, namely LRF promoter, BCL11A enhancer and promoter, and ZNF410 promoter in combination with various gRNAs, in HUDEP-2 cells. Proliferation and HbF levels of the HUDEP-2-dCas9-KRAB cells were assessed at different time points. Epigenomic suppression of the transcription factors was assessed using quantitative Q-PCR. To confirm the safety and efficacy of the approach in human primary cells, composite erythroid-specific lentiviral vectors were generated that incorporated both the dCas9-KRAB domain and the most effective gRNAs. The erythroid expression of the dCas9-KRAB was achieved using a compact erythroid-specific enhancer in combination with a minimal beta-globin promoter. Normal and thalassemic donor CD34+ cells were transduced with the composite vectors and the clonogenic capacity and multilineage differentiation were assessed ex vivo. The oxidative stress and HbF levels were evaluated during erythroid differentiation.

RESULTS: The epigenomic suppression of ZNF410, LRF and BCL11A was translated into significantly increased gamma globin levels in transduced HUDEP-2 cells ($p=0.009$, $p=0.05$, $p=0.003$, respectively). Among the three suppressors, the epigenomic silencing of ZNF410 promoter or BCL11A enhancer induced the highest increase in HbF+ cell ($p=0.009$, $p=0.0002$, respectively) with no observed adverse effects in proliferation and cell maturation. In contrast, targeting the LRF or BCL11A promoters resulted in evident cell toxicity. In subsequent experiments in primary cells, we employed the composite erythroid specific vectors, carrying both the dCas9-KRAB effector and the most effective gRNA targeting the ZNF410 promoter or the erythroid enhancer of BCL11A. Epigenomic

suppression of ZNF410 and BCL11A in both normal and thalassemic CD34+ cells had no effect on cell proliferation, clonogenic capacity or the differentiation/maturation process. Importantly, the ZNF410 and BCL11A epigenetic silencing significantly increased the percentage of HbF+ cells at the end of erythroid differentiation in the edited over unedited cells, in both normal (p=0.02) and thalassemic (p=0.004) cells. The HbF reactivation in the thalassemic cell group resulted in increased expansion rates and reduced oxidative stress over the control group.

CONCLUSIONS: Overall, our findings establish the feasibility and efficacy of epigenetically mediated suppression of gamma globin regulators leading to efficient HbF induction both in normal donor- and beta-thalassemia patient-derived cells, without apparent cell toxic

1604 Development of a Novel Chimeric Suppressor tRNA Successfully Rescues Functional Protein in Nonsense Harboring *TP53* Mutants

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Over 10% of genetic diseases are caused by mutations that introduce stop codons, known as premature termination codons (PTCs), into protein-coding genes. mRNA transcripts harboring PTCs result in premature polypeptide cleavage, leading to loss of protein function. One promising approach promote PTC-readthrough utilizes engineered suppressor transfer RNAs (sup-tRNAs), which are modified to recognize one of the 3 stop codons. Sup-tRNAs can outcompete release factors that terminate translation and incorporate amino acids (AAs) at PTCs-encoded positions, rescuing full-length protein synthesis. Previous success with sup-tRNA-mediated protein rescue has been demonstrated in cystic fibrosis (CF) and Duchenne muscular dystrophy (DMD) models; however, the application of sup-tRNAs as a cancer therapeutic has not been explored. Tumor suppressor protein p53 (encoded by *TP53*) is one of the most frequently mutated proteins in cancer—11% of cancer-associated mutations in *TP53* are PTCs. We hypothesize that sup-tRNAs can induce translational readthrough of PTCs in the *TP53* gene to restore p53 protein levels and rescue p53 activity. Further, we hypothesize that PTC-readthrough facilitated by sup-tRNAs will induce p53-mediated apoptosis and cell senescence. As a result, we hypothesize that this sup-tRNA delivery will inhibit tumorigenesis and cancer cell proliferation. In addition to assessing sup-tRNAs for tumor suppressor rescue, there is also a need to optimize these sup-tRNAs to increase their readthrough efficiency. The archaeal species *Methanomethylophilus alvus* expresses an endogenous sup-tRNA that installs pyrrolysine in conserved PTC-containing transcripts. With this naturally occurring nonsense suppression capability, we developed a novel chimeric sup-tRNA (PASS-tRNA) using the archaeal tRNA as a scaffold. This chimeric PASS-tRNA can be recognized by the human alanine aminoacyl-tRNA synthetase. PTC-readthrough analysis shows significant efficiency improvement when comparing the PASS-tRNA against two sup-tRNA variants. Using a human epithelial lung cancer cell line, NCI-H1299, we assessed the recovery of full length p53 protein using plasmid transfection encoding for the various *TP53* mutants harboring PTCs. Cells transfected with our PASS-tRNA rescued synthesis full-length p53 protein; additionally, the PASS-tRNA was able to rescue p53 activity in a luciferase-based transactivation assay. qRT-PCR results further demonstrate an upregulation of p53 downstream target genes such as *CDKN1A*, and *MDM2*. In a similar fashion, we transfected PASS-tRNAs in human cancer cell lines SK-MES-1, C2bbe1, and Calu6. These cell lines contain homozygous PTC mutations in the genomic *TP53* gene. The PASS-tRNA successfully recovered full-length p53 and its transactivation activity from these endogenous mutations; additional qRT-PCR results also solidify an upregulation in p53 downstream gene targets. These results indicate that sup-tRNAs can induce PTC-readthrough in *TP53* to rescue functional p53 protein. These sup-tRNAs are applicable to a variety of PTC-mediated disorders; our findings shed light on a useful approach for cancer therapeutics.

1605 CRISPR-hfCas13Y RNA-targeting Therapy Restores Cognitive Function by Reducing *BACE1* or *MAPT* in Alzheimer's Disease Model

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Background: Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the accumulation of amyloid-beta (Aβ) plaques and tau neurofibrillary tangles, leading to progressive neuronal loss and memory deficits. Current therapies focus on symptom management and often have limited efficacy and side effects. Targeting key contributors to AD pathology, such as BACE1, which drives Aβ production, and MAPT, which encodes tau protein, offers a promising therapeutic approach. CRISPR-Cas13Y RNA-targeting therapy provides a precise and reversible method for reducing harmful protein levels by degrading *BACE1* or *MAPT* mRNA without permanently altering DNA.

Methods: The CRISPR-high-fidelity Cas13Y(hfCas13Y) RNA editing system was used to screen gRNAs targeting the *BACE1* and *MAPT* mRNAs, promoters and functional elements, in HEK293T cells and N2a cells using fluorescence-activated cell sorting (FACS). To evaluate the therapeutic potential of BACE1 knockdown, we administrated AAV-hfCas13Y-g*BACE1* via retro-orbital injection into 3-month-old 5xFAD mice, a classic AD mouse model. Behavior tests, including fear conditioning and the Morris water maze, were conducted 3 months post-treatment to assess memory function. For MAPT knockdown, we injected AAV-hfCas13Y-g*MAPT* into the hippocampus of 3-month-old PS19 mice, another widely used AD mouse model, via intracranial injection. *MAPT* mRNA and protein expression levels were analyzed 4 weeks post-injection using qPCR, western blot, and immunostaining. To investigate whether AAV-hfCas13Y-g*MAPT* treatment could improve cognitive deficits, hippocampal injections were performed in 7-month-old PS19 mice, followed by cued and contextual fear conditioning tests 2 months post-treatment to evaluate learning and memory.

Results: This comprehensive approach evaluated CRISPR-hfCas13Y-mediated RNA editing in two key AD mouse models, targeting distinct pathological mechanisms of Aβ and tau protein accumulation. The CRISPR-hfCas13Y-g*BACE1* system efficiently targeted *BACE1* mRNA in HEK293T cells, achieving a 99% reduction ($P<0.001$, Fig. 1A). Administration of AAV-CRISPR-hfCas13Y-g*BACE1* into the hippocampus of 5xFAD mice significantly reduced in BACE1 protein levels (Fig. 1B). Moreover, fear conditioning and Morris water maze results revealed that retro-orbital injection of AAV- hfCas13Y-g*BACE1* significantly alleviated cognitive deficits in 5xFAD mice (Fig. 1C–G). For the CRISPR-hfCas13Y-g*MAPT* system, we screened 33 gRNAs and identified two (g31 and g7) that significantly reduced *MAPT* mRNA levels in HEK293T cells by 90.8% and 77.2%, respectively ($P<0.001$, Fig. 2A). Delivery of AAV-hfCas13Y-g*MAPT* via hippocampal injection in PS19 mice led to a significant reduction in *MAPT* mRNA and protein levels of MAPT in the hippocampus (Fig. 2B and C). Furthermore, fear conditioning tests demonstrated improved learning and memory in PS19 mice 2 months post-treatment with AAV-hfCas13Y-g*MAPT* (Fig. 2D). Development of a dual-target AAV-hfCas13Y system that simultaneously targets both *BACE1* and *MAPT* is underway, with the potential to address multiple pathological mechanisms of AD.

Conclusions: This study demonstrates that CRISPR-hfCas13Y RNA editing can precisely and efficiently reduce *BACE1* or *MAPT* mRNA levels, significantly improving cognitive function in AD mouse models. These findings support the potential of RNA-targeting therapies for AD and pave the way for a dual-target treatment strategy.

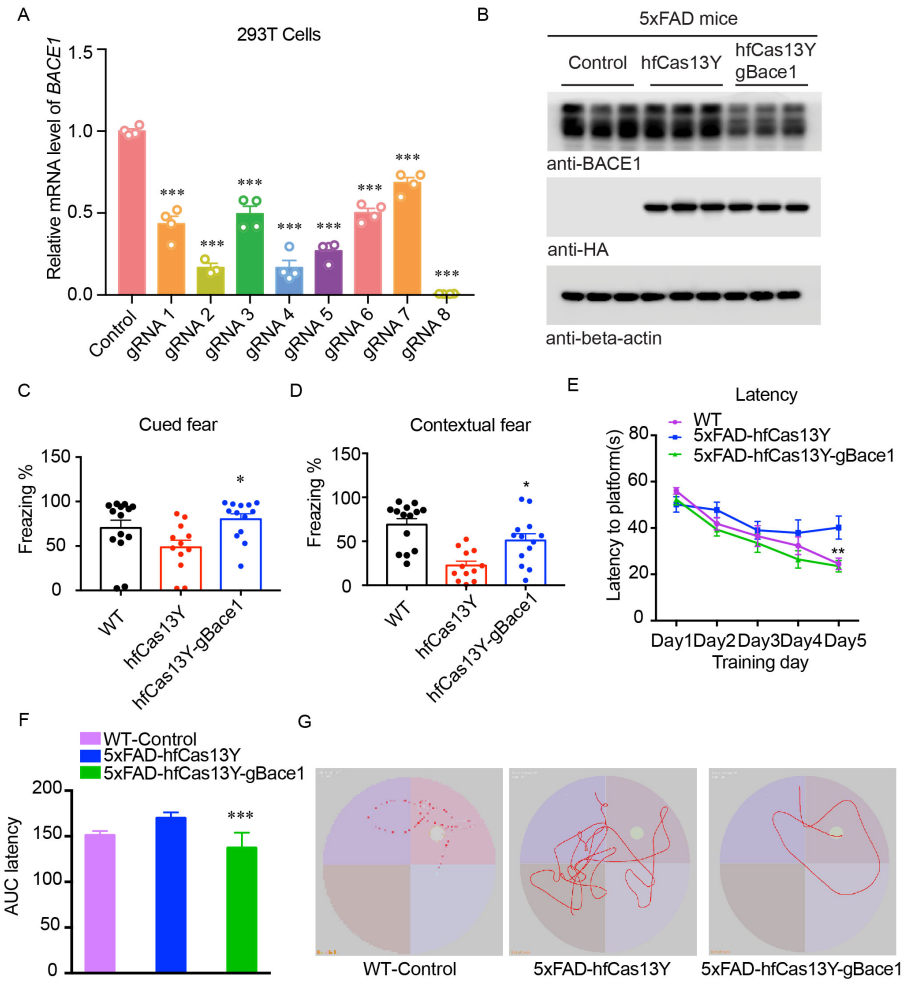

Figure 1. hfCas13Y-g*Bace1* improved the cognitive function of 5xFAD mice 3 months after retro-orbital injection. (A) gRNA2 and gRNA8 showed> 90% knockdown efficiency of BACE1 in HEK293T cells. **(B)** Injection of AAV-hfCas13Y-*BACE1*-gRNA2 into the hippocampus of 5xFAD mice resulted in the reduction of BACE1 levels. **(C-D)** Fear conditioning results show that hfCas13Y-*BACE1*-gRNA2 treated 5xFAD mice via retro-oral injection enhanced the learning and memory capabilities 3 months after treatment compared to hfCas13Y-treated 5xFAD mice, *P<0.05. **(E-G)** Water maze analysis revealed that hfCas13Y-*BACE1*-gRNA2 treatment rescued learning and memory deficits of 5xFAD mice. WT-Control (n=14), 5xFAD-hfCas13Y (n=12), 5xFAD-hfCas13Y-g*Bace1* (n=13), ***P<0.001.

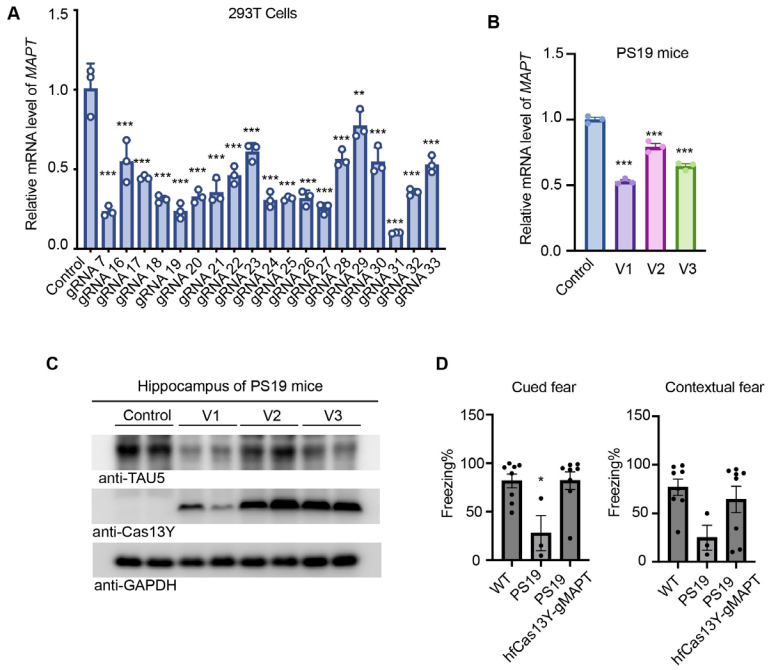

Figure 2. Administration of AAV-hfCas13Y-g*MAPT* significantly decreased MAPT and improved the cognitive functions of PS19 mice. (A) qPCR analysis of MAPT mRNA level in 293T cells transfected with different versions of hfCas13Y-g*MAPT*, **P<0.01, ***P<0.001. **(B-C)** qPCR and western analysis of MAPT in the hippocampus of PS19 mice 1 month after administering AAV-hfCas13Y-g*MAPT* via hippocampal injection. Three versions of AAV-hfCas13Y-g*MAPT* were desinged by combining different gRNAs. **(D)** Cued and contextual fear conditioning of 9-month-old PS19 mice 2 months after treatment with AAV-hfCas13Y-g*MAPT* via hippocampal injection. WT (n=8), PS19 (n=3), hfCas13Y-gMAPT(n=8), * P<0.05 .

1606 Engineering CRISPR/dCas9-based modalities in primary neurons for gene modulation of *ΔFosb* in the context of cocaine substance use disorders (SUD)

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Cocaine substance use disorder (SUD), one of an array of mental health disorders in the United States, is devastating for individuals and society in terms of health and economic consequences. While there are some common approaches for treating cocaine SUD, such as counseling, support groups, and off-label use of medication for withdrawal symptoms, there is no guarantee that any of these strategies may work or even be readily available to patients, and unfortunately, there are no curative FDA-approved treatments for this class of mental health disorders. Therefore, therapies that enable a shift from treating the symptoms of SUDs to instead targeting the pathological drivers of SUD, could both enhance the understanding of SUDs and revolutionize the ability to cure this devastating class of disorders.

Drugs of abuse lead to increased levels of the ΔFosB transcription factor, a splice variant produced from the *FOSB* gene, within the nucleus accumbens (NAc) brain region known for regulating the reward system. Specifically in cocaine SUD, ΔFosB is induced in dopamine receptor 1 expressing medium spiny neurons (D1-MSNs) within the NAc. Additionally, ΔFosB persists in the NAc substantially longer than full length FosB and plays a substantial role in the maladaptations that occur at the cellular and tissue level, which consequently creates and sustains cocaine SUD. Despite the therapeutic importance of ΔFosB in cocaine SUD and many other SUDs, the factors that control ΔFosB expression and splicing remain incompletely understood.

DNA methylation is a stable, yet reversible epigenetic mark, that typically leads to gene repression when present at promoter regions. We have analyzed publicly available sequencing data sets that suggest a strong possible link between repeated cocaine exposure and increased levels of DNA methylation at the *Fosb* splice site in mice and post-mortem human brains. This data *drives our hypothesis* that engineering DNA methylation states can be used to control and mechanistically dissect endogenous Fosb/ ΔFosB expression. To test this hypothesis, we have leveraged CRISPR/dCas9-based epigenome editing technologies to functionally interrogate the epigenetic control of *Fosb* isoforms levels (including ΔFosB), as well as uncover new therapeutic opportunities to effect sustained improvement of the neuropathology involved in cocaine SUD.

Recently, two epigenome editing platforms known as CRISPRoff and CRISPRcharm have been developed for programmable DNA methylation and potent gene repression. We have tested these systems in a murine primary neuron culture system that we have established using MSNs sourced from within the NAc of newborn mice. This system serves as a powerful *in vitro* proxy to study cocaine SUD. Here we quantified gene expression levels of both *Fosb* and *ΔFosb* and coincident changes in DNA methylation levels at these loci and tested how the targeting of either CRISPRoff or CRISPRcharm modulate these chromatin and gene-regulatory effects.

Our results demonstrate a proof-of-concept for the use of programmable DNA methylation to understand gene regulation in primary neurons *in vitro* and opens new routes for potential therapeutic approaches aimed at controlling *Fosb* isoforms and reducing cocaine driving reward behaviors in the mammalian brain.

1607 Efficacy and Safety of a CasRm-Based Therapy for Parkinson's disease in Non-Human Primates

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Background: Parkinson's disease (PD) is one of the most prevalent neurodegenerative disorders in the world, characterized by the progressive loss of dopaminergic neurons in the substantia nigra and a resultant decrease in striatal dopamine levels, leading to a spectrum of motor abnormalities. To date, there remains a significant unfulfilled demand for effective and safe treatment that can provide long-term benefits for patients with Parkinson's disease. Recent years, the in situ conversion of glial cells to neurons either by overexpressing transcription factors, such as NeuroD1, or by inhibiting repressors of neuronal differentiation, such as Polypyrimidine tract-binding protein 1 (PTBP1) and RE1-silencing transcription factor (REST), has emerged as a potent therapeutic strategy. Here we present the initial preclinical results of RM-201, an innovative adeno-associated virus (AAV) gene therapy that harnesses a state-of-the-art CasRm system to specifically knock down PTBP1 and high-effiently induce glial-to-neuron transdifferentiation.

Methods: The therapeutic potential of RM-201 in PD was evaluated through injection in the midbrain of non-human primate (NHP) model induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Control groups received an AAV expressing CasRm along with a non-targeting guide RNA. The levels of plasma membrane dopamine transporter (DAT) within the striatal dopaminergic terminals were monitored using positron emission tomography-computed tomography (PET-CT) with 11C-2β-Carbomethoxy-3β-(4-fluorophenyl) tropane (11C-CFT) monthly, consistent with the schedule for behavioral evaluations following the administration of RM-201. Immunogenicity and safety profiles were evaluated by the level of anti-CasRm antibodies in peripheral blood and the systemic distribution of AAV DNA.

Results: Compared to the control, the NHPs treated with RM-201 exhibited a restoration of the striatal dopamine transporter by over 10% as early as three months following the administration of RM-201, and persisting for at least ten months. These imaging outcomes were supported by durable and significant enhancements in behavioral assessments, with a substantial reduction in dyskinesia scores observed in the RM-201-treated NHPs, particularly in the improvement of tremor and defensive motor responses. These data imply that RM-201 could promote the

regeneration of functional dopaminergic neurons and enhance motor function. Moreover, immunogenicity analyses revealed that there is no adverse immune reactions to the CasRm system in the RM-201-treated primates. What's more, the viral genomes of RM-201 were predominantly confined to the nigral tissue, with minimal diffusion to other brain regions and negligible systemic circulation.

Conclusions: Our study affirms RM-201 as an efficacious agent for promoting neuronal regeneration and restoring motor abilities in PD NHPs, while maintaining an acceptable safety profile at therapeutic doses. This research provides robust evidence in support of RM-201 as a promising gene editing therapeutic candidate for the treatment of PD patients.

1608 A Skeletal Muscle-Specific dCas9-KRAB Mouse Model for Precision Gene Silencing

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Genetic screens are pivotal in uncovering gene function, shedding light on their roles in both healthy and diseased states across specific organs or throughout the entire body. Current mouse models to perform genetic screens involve gene editing mouse embryos with the desired gene mutation and breeding the model. However, one model is limited to a single gene, is expensive, and labor- and time-intensive. When studying a new gene a new model is required. Therefore, a new model is essential that enables the screening of any gene of choice. The groundbreaking gene editing technology, CRISPR-Cas9, alongside a gene-specific sgRNA, has the ability to target any gene in the genome. Recently, a lab incorporated CRISPR-Cas9 into mouse skeletal muscle to allow organ-specific gene knockout. However, gene-based therapies for diseases typically achieve partial knockdown instead of full knockout. Therefore, examining genetic knockdown would be more beneficial in the context of studying treatment effects on an organ in a healthy or diseased state. The catalytically deactivated CRISPR-Cas9 (dCas9) connected to transcription factor, Krüppel Associated Box (KRAB), is able to transcriptionally downregulate gene expression. *Therefore, we established a mouse model that expresses dCas9-KRAB exclusively in skeletal muscle.*

Currently, we crossbred mice containing tamoxifen inducible Cre recombinase under the control of the human skeletal muscle α-actin promoter (HSA-Cre) and mice containing dCas9-KRAB controlled by a loxP-flanked STOP cassette (LSL-KRAB). Upon breeding, Cre recombinase will remove the STOP cassette allowing expression of dCas9-KRAB under the control of the HSA promoter (HSA-KRAB), creating organ-specificity in skeletal muscle. dCas9-KRAB expression was verified to be >100x in somatic skeletal muscle (tibialis anterior, plantaris, soleus) when compared to non-tamoxifen induced HSA-KRAB mice using RT-qPCR. Negligible amounts of dCas9-KRAB were found in the liver or heart using Western Blot. Current models are able to achieve muscle specificity, but are challenging and time-intensive, unlike ours which only involves breeding and tamoxifen injections.

To determine the functionality of our model, we targeted the myostatin pathway, an inhibitor of muscle hypertrophy, that would allow the physical readout of muscle weight alongside gene and expression analysis. We selected 5 sgRNAs to target each of the following: myostatin (Mstn), activin A receptor 2A (Acvr2A), and activin A receptor 2B (Acvr2B). Each array of 5 sgRNAs was cloned into an AAV transfer plasmid produced at a high hiter in the highly myotropic myoAAV1C capsid. Each set of sgRNAs is paired with the reporter tdTomato. Currently, myoAAV was injected intramuscularly into the tibialis anterior muscle at a dose of 5×10^{11} vg. Muscle will be isolated and analyzed via RT-qPCR, western blot, histology, and RNA-seq to verify the functionality of our model. Future experiments include investigating systemic delivery and simultaneous silencing of multiple genes.

1609 A Multi-Omic Deep Learning Approach to Predict CRISPR Activation and Interference Efficacy

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CRISPRi (interference) and CRISPRa (activation) systems are powerful molecular tools for modulating gene expression. CRISPRa/i relies on a guide RNA (gRNA) to target the gene of interest and recruit epigenetic modifiers. CRISPRa/i efficacy varies widely due to complex factors influencing gene expression and efficient CRISPR intervention. Such factors include promoter epigenetics, local genomic DNA, and gRNA features. We propose a convolutional-based machine learning approach to integrate these factors and predict the efficacy of CRISPRa/i interventions based on gRNA design. We hypothesize that full integration of all modalities in our model will enhance predictions and reveal determinants of CRISPR-based transcriptional perturbations.

Using two genome-wide CRISPRi screens (a single gRNA and a dual gRNA screen) and one genome-wide CRISPRa screen, we identified several principles for effective CRISPRa/i activity. Using only the guide and transcript location, the model exhibited moderate predictive power for the single CRISPRa/i datasets (~0.3-0.4 Spearman correlation) and weak predictive power for the dual CRISPRi dataset (~0.2 Spearman). This is unsurprising, as the dual library was designed with a small search space per promoter. Results from the single-guide datasets indicate a strong influence of guide location on outcomes. Surprisingly, the model using only gRNA features (sequence, structure, energetics) also had moderate predictive power (Spearman correlation ~0.2-0.4). This suggests that guide efficacy may have some independence from target context. Further, integrating the RNA and transcript modalities boosted model performance across all datasets, indicating that both modalities influence CRISPRa/i interventions.

Interrogation of model predictions revealed critical rulesets governing epigenetic perturbations. The model trained on the single-guide CRISPRi data, using only transcript/guide location, significantly preferred placement immediately downstream of the TSS. Interestingly, the model trained on the paired CRISPRi data exhibited a similar preference for placing both guides within the gene body. The model trained on the CRISPRa screen revealed more relaxed rulesets, with ideal locations distributed between the (slightly preferred) promoter and gene body. In addition, the model utilizing gRNA features indicated the importance of scaffold secondary structure for gRNA efficacy. Input ablation studies showed that removing the scaffold features worsened model performance. This is particularly novel, as the chosen scaffold is a guide design parameter that has not been extensively explored previously.

We have also performed initial experiments to integrate epigenetic inputs into our model. A screen of epigenetic features relevant to gene expression (e.g., chromatin accessibility or transcription factor binding) revealed some markers that significantly improve the prediction of CRISPRa efficacy. Future directions involve refining the integration of epigenetic features to interrogate the CRISPRi and CRISPRa datasets. Overall, the findings from our model demonstrate the utility of machine learning approaches in predicting CRISPR epigenetic perturbations.

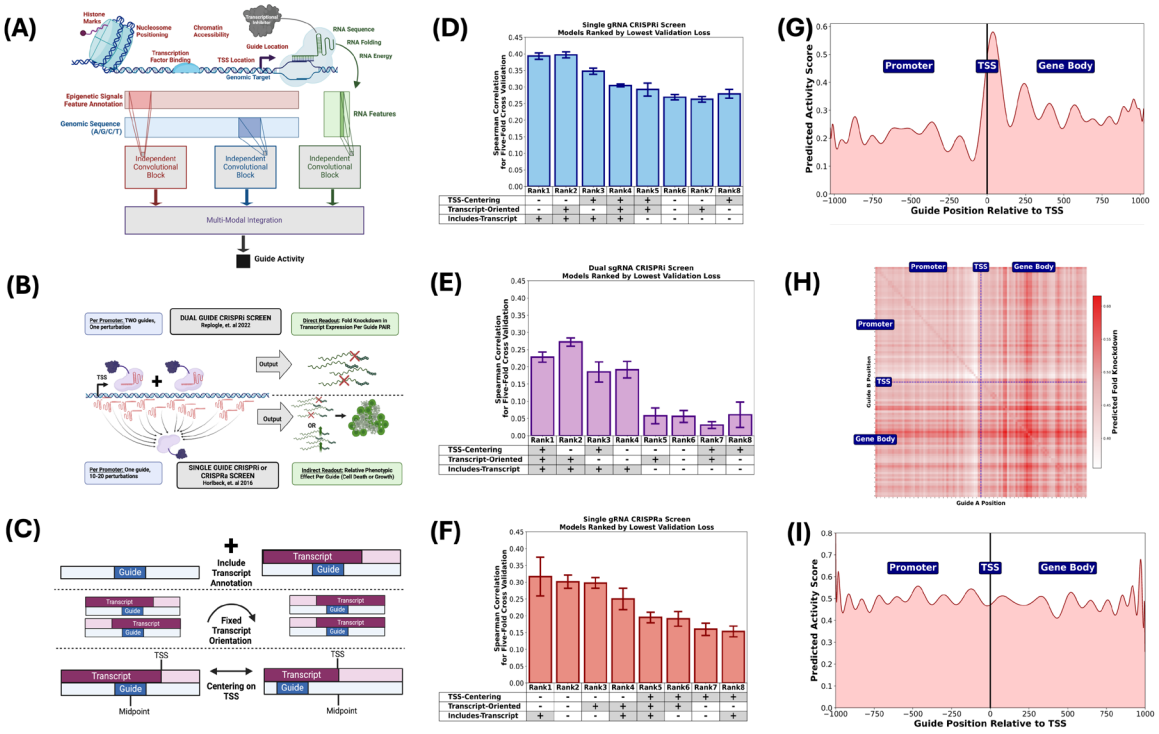

Figure 1: Appropriate representation of gRNA with respect to transcript location improves model predictions for CRISPRi and CRISPRa screens. (A) Schematic for the proposed convolutional-based model with multi-modal fusion**. (B)** Depiction of experiment design for the CRISPRi/CRISPRa screens chosen to assess the model. **(C)** Diagram of all tested representations for transcript and gRNA location. Explicit representation of transcript improved model predictions for the single CRISPRi screen **(D)**, dual CRISPRi screen **(E)**, and single CRISPRa screen **(F)** according to Spearman correlation between true and predicted guide activity. Interrogation of model predictions revealed strong model preference for placement of sgRNAs within gene bodies for the single CRISPRi **(F)** and dual CRISPRi **(G)** screens, whereas only moderate preference for upstream of the TSS in the single CRISPRa screen **(H).**

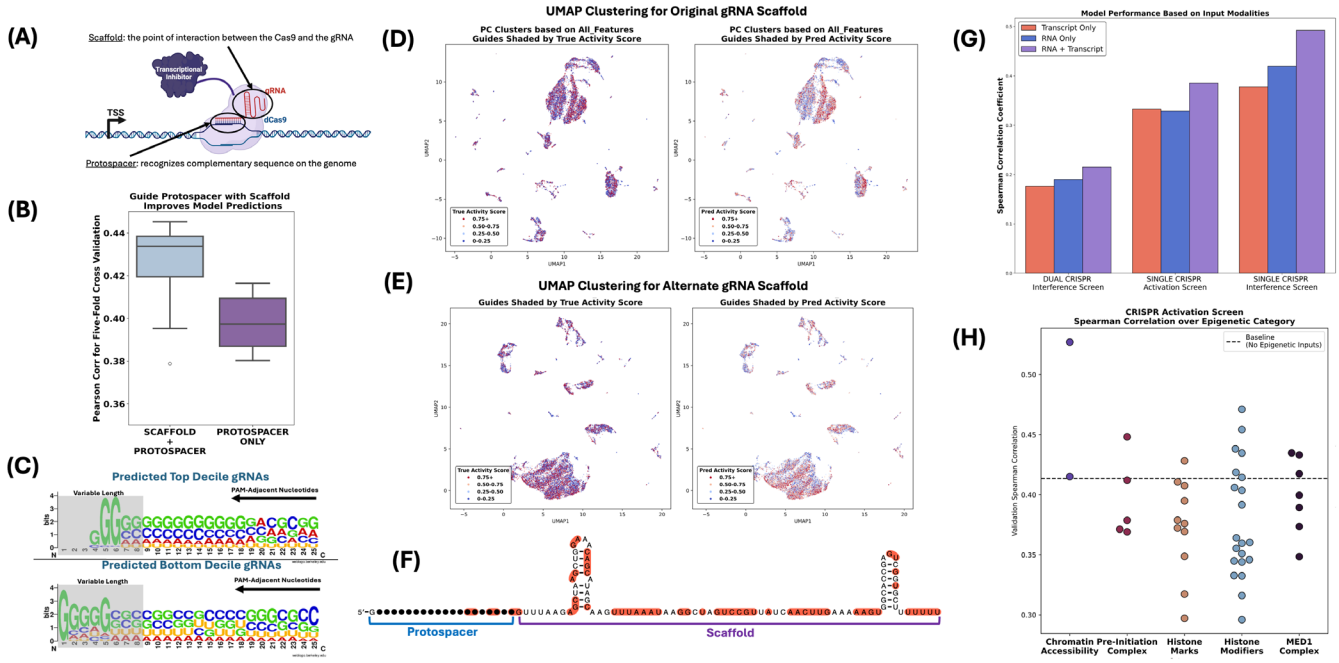

Figure 1: Model integration of RNA and epigenetic modalities reveals principles of CRISPR epigenetic interventions. (A) Depiction of gRNA scaffold and protospacer. **(B)** Input ablation indicates signifigance of gRNA scaffold features for predictions for single CRISPRi screen. **(C)** Motif enrichment of the top and bottom predicted gRNAs for the single CRISPRi screen indicate GC-richness in high performing gRNAs. **(D)** Cluster analysis of single CRISPRi screen guides based on RNA structure shows segregation of gRNAs based on true and predicted activity. **(E)** Clustering of gRNA protospacers with alternate (i.e.not utilized in the original screen) scaffold indicates loss of this segregation.**(F)** Nucleotides with the greatest model gradients were shaded along the gRNA structure, indicating model attention to nucleotides relevant to scaffold structure. **(G)** Integration of RNA with transcript annotation improves model performance for all screens **(H)** Multiple epigenetic features relevant to gene expression regulation are predictive of CRISPRa interventions.

1610 AI-Driven Engineering of RNA Control Systems for Precise Regulation of Gene Therapy Activity

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The inability to regulate gene therapy activity after administration limits the ability to manage adverse effects, restricting both safety and therapeutic potential. One method for preventing these side effects is the use of gene control systems – sequences that are placed within the gene therapy cassette and allow transgene expression to be controlled in a defined manner. Such systems hold huge promise, with the potential to allow gene therapies to respond to disease state, tissue location, or clinically administered drugs. Achieving such precise control over gene therapies will be essential in overcoming their current limitations and enabling their broader application across diverse therapeutic areas.

RNA-based control systems in particular have significant advantages. They are compatible with a broad range of therapeutics from gene therapies through to mRNA therapeutics and gene editing, where conventional DNA-based control systems (e.g. synthetic promoters) cannot be used; their small size (< 150 bp) makes them highly compatible with space-constrained vectors such as AAVs; and they can be engineered to respond to a wide variety of molecules, making them applicable to a range of disease areas.

However, these types of control system are heavily underutilised in the gene therapy sector. The key reason for this is their difficulty to engineer, with high time and resource costs and low success rates, which has greatly limited their utility in the gene therapy sector to date. To address this issue, we have developed a high-throughput and AI-guided methodology that allows 10,000s of control system sequence variants to be rapidly screened in cell lines. Our AI algorithm uses this data to predict improved variants for further rounds of testing, and by iterating through this process highly effective control systems can be developed. Using this method, we have shown significant

improvements over existing control systems and demonstrated the functionality of new-to-human control systems in cell lines.

Our approach represents a transformative step forward in the development of RNA-based gene control systems, offering a scalable and efficient strategy to overcome key engineering challenges. By expanding the toolbox available for precise and fine-tuned gene regulation, gene control systems have the potential to enable the next generation of safer and more effective gene therapies.

1611 Engineering Synthetic Epigenetic Regulation to Enable State-Switching Genetic Programs in Mammalian Cells

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Engineered cell therapies are a promising frontier, with early successes in cancer treatment demonstrating the transformative potential of this approach. Most cell therapies today are programmed to express outputs at a steady-state; however, cells programmed with dynamic control of gene expression, such as state-switching, could enable implementation of novel therapeutic functionalities, such as autonomous stem cell differentiation or pulsating therapeutic delivery. In nature, eukaryotic systems have evolved epigenetic control as a solution for inducing, maintaining, and switching states, but these behaviors are challenging to implement in a synthetic, orthogonal context when building novel genetic programs. A reason this challenge arises is the lack of understanding as to how specific biological mechanisms interact; it remains unknown how the use of synthetic epigenetic- or chromatin-modifying domains can be composed into genetic programs to modulate the behavior of a cell. While previous work has generated quantitative insights for mapping biological understanding into these mechanisms, many fundamental design principles relating to implementation of epigenetic regulation remain unexplored. This leaves biologists with little intuition on how to construct genetic programs to encode cells with dynamic behaviors.

To address this need, we characterized and employed synthetic transcription factors (synTFs) that incorporate a library of repressing chromatin regulatory domains (CRD) fused to a panel of DNA binding domains that can be implemented with precise local composition using cognate promoters. These constructs were tested in HEK293FTs and primary T-cells. We explored key design criteria that will guide usage of these domains in genetic programs, including: (1) effect on gene expression and memory when recruiting both activating and repressing synTFs to a promoter; (2) synTF dominance due to temporal and spatial organizations; (3) variation in promoter binding site composition to modulate cooperative interactions. Though previous literature suggests synTFs with CRDs will silence most activated genes, we find varied levels of repression across different promoter types. Moreover, when recruited with an activating synTF, the spatial orientation of the synTFs and stoichiometry of each part greatly impacts the level of conferred silencing, revealing previously unknown rules of dominance of competing biological mechanisms. For example, KRAB, a well-known, potent repressor, was found to increase gene expression at certain stoichiometries when repressing VP64, an activating domain. Lastly, we hypothesized and assessed how multiplexing CRDs will improve or dampen gene expression. Using our unique testbed that allows for intricate promoter architecture, we discovered unique combinations of CRDs that can be leveraged to make versatile gene expression profiles with distinct memory and potency. This type of deep characterization will enable us to build more complex programs that require multiple handles to tune dynamic gene expression. Overall, these exhaustive design principles will guide the next phase of the project, in which we construct a small panel of state-switching programs with therapeutic utility. Achieving these important and sophisticated functionalities will be a crucial step towards building safer and more effective cell-based therapies.

1612 The Long and Short of It: Comparing Next Generation Sequencing Methods for Quality Control in AAV Development

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Interest in gene therapy-based disease prevention and treatment has grown rapidly over the last decade, with adeno-associated viral vectors (AAV) emerging at the forefront with widespread application due to the non-integrating ability. With safety as the top priority in all therapies, extensive quality control (QC) throughout the entire development and manufacturing process is essential. A robust QC process expedites safe and effective commercialization of the final product. While Sanger sequencing can be ideal to verify and validate plasmid sequences pre-packaging, next generation sequencing (NGS) combined with post-viral production methodologies such as CE analysis and transmission electron microscopy offer an effective high-throughput approach for monitoring AAV quality, from initial construct assembly to analysis of the encapsulated product. Illumina® short-read, Oxford Nanopore® long-read and PacBio® long-read sequencing technologies each offer distinct advantages including sequencing of the entire viral genome, with detection of potential mutations, truncations, and contaminants. Here we compare each of these applications as a means to assess the purity, clonality and fidelity of the packaged AAV product. When evaluating the same sample with Illumina, PacBio and ONT, we show how these methods compare with detection of contaminating alternatively packaged products, detection of point mutations and indels in the ITRs and region of interest, and detection of full-length vs partially packaged genomes. Based off these results, we also evaluate how sample quality and library preparation can influence the conclusions generated and describe when each method can be ideally used within the therapeutic development workflow, starting from early research to late-stage pre-clinical work. Additionally, we consider regulatory assays, with Good Laboratory Practices (GLP) methods being ideal for late-stage development products. Ultimately, a combined NGS approach post-packaging enables a comprehensive solution and enhances the overall QC process, ideal for sequence confirmation of both transfer plasmid and final packaged product for improved viral vector gene therapy manufacturing in advance of FDA or EMA filings.

1613 B-GEn is an Innovative Bayer Proprietary Genome Editor Suitable for CRISPR Applications and Therapeutic Developments

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**Corresponding Author*

RNA-programmable nucleases from bacterial CRISPR systems, have helped revolutionize life sciences and offer enormous potential for therapeutic genome editing. Bayer has built a proprietary collection of >170,000 microbial assemblies and metagenomes, many of which are not represented in public databases.

We bioinformatically mined this collection for CRISPR loci and identified approximately 8000 systems, containing both previously known and new CRISPR effectors. We experimentally characterized ~100 nuclease candidates, ranging in size from 450 to 1100 amino acids (aa). A proprietary Bayer Gene Editing nuclease (B-GEn), a medium sized nuclease of 1091 aa with a 5'-ATTN-3' PAM, featured robust activity in mammalian cell lines, primary human cells, and mice. Engineered B-GEn, showed several folds higher indel activity on several disease targets without compromising its specificity.

We used B-GEn to edit a safe harbor locus in mice liver and find almost complete editing, at doses for which benchmark SpCas9 reagents yielded significantly lower editing. At the same time, we did not identify any detectable off-targets.

Next, we performed AAV-mediated insertion of a nano-luciferase reporter into this safe harbor in primary human cells and humanized transgenic mice. We found levels of transgene expressed that are consistent with therapeutic levels, on par with benchmark SpCas9 reagents. Furthermore, we packaged therapeutic payload in a well-established liver-tropic capsid to show robust insertional editing *in vivo* establishing a path forward for liver-targeted stable expression of therapeutic transgenes from this safe harbor.

In summary, B-GEn constitutes a valuable addition to the portfolio of CRISPR nucleases that may achieve therapeutic levels of insertional gene editing, potentially lending itself to development of therapeutic approaches.

1614 Engineering a Compact Gene Switch for Temporal Control of AAV-CRISPR Gene Editing

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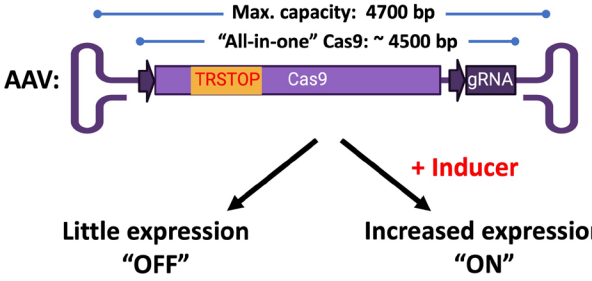
Gene therapy holds significant promise for treating human genetic disorders. To enhance the safety and effectiveness of gene therapy, there is an increasing need for gene switches that can provide “on-demand” control over gene therapy products. In this study, we aimed to engineer a gene switch by exploring the mechanism of stop codon readthrough. We began by investigating the efficiency of the three stop codons (UAA, UAG, UGA) in terminating protein translation using an in vitro cell culture assay. Consistent with previous research, we observed that all three stop codons exhibited low-level “leakiness,” allowing for termination readthrough under certain conditions. Notably, the stop codon UGA (TGA in DNA), when flanked by six virus-derived downstream nucleotides, demonstrated increased susceptibility to chemical modulation of its readthrough propensity. Building on these findings, we designed a novel two-component gene switch, termed "TREAD". TREAD consists of a nine-nucleotide DNA effector sequence containing the TGA stop codon, designed to be inserted into the gene of interest, and a chemical inducer (such as gentamicin or PTC124) that enhances TGA readthrough, effectively "switching on" gene expression. We tested the functionality of this gene switch by incorporating it into a CRISPR-Cas9 gene editing system. In experiments using Ai14 reporter mice, we demonstrated that the administration of AAV vectors expressing the switchable Cas9 and dual guide RNAs resulted in minimal background gene editing. However, when the chemical inducer PTC124 was administered over two weeks, we observed significant gene editing activity in tissues including the liver, heart, and skeletal muscles with edited cells persisting for at least five months. TREAD gene switch has several advantages, including its small DNA footprint, low immunogenicity risk, and the availability of safe chemical inducers. These attributes make it a promising tool for developing inducible gene therapies. Our ongoing research is focused on creating an inducible AAV-CRISPR gene editing therapy for cardiomyopathy associated with Friedreich ataxia, leveraging TREAD in conjunction with our newly engineered cardiotropic vector AAV.CPP.16.

Components of "TREAD", an AAV-CRISPR-compatible gene switch:
1) A DNA effector TRSTOP (9 base-pair long)
2) A clinically approved chemical "inducer" (e.g., PTC124)



TREAD-applicable AAV-CRISPR editing:
- Indel (e.g., saCas9, Nme2Cas9)
- Base editing (e.g., Nme2ABE8e, SaKKHABE8e, CjABE8e, SauriABE8e)

1615 hfCas12Max: Engineered CRISPR Nuclease for Safer and Effective CGT

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Background: In recent years, the continued exploration of new Class 2 CRISPR-Cas systems has resulted in the identification of Cas12i. Though Cas12i holds promise as for genome editing, enhancements in its efficiency and specificity are necessary for effective utilization.

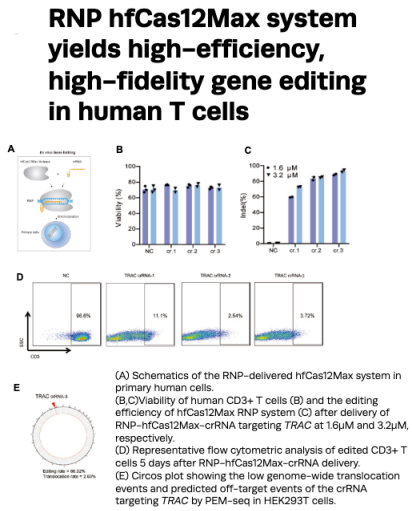
Methods: Leveraging HuidaGene's AI-driven, CRISPR-based – Platform for Rational Engineering of CRISPR-Cas Identification by Synergic Expertise (HG-PRECISE), a potent directed evolution system for both protein and guide RNAs (gRNAs), we developed hfCas12Max CRISPR nuclease, an engineered Cas12i, which recognizes 5'-TN-3' or 5'-TTN-3 PAMs with high editing activity and specificity in mammalian cells. Compared to Cas9 and Cas12a, hfCas12Max CRISPR nuclease is more compact, making it an ideal candidate for single adeno-associated viral (AAV)-based delivery. Here, we assessed the applicability of hfCas12Max CRISPR nuclease in creating gene editing therapies and cell therapies, incorporating multiple delivery methods such as AAV, lipid nanoparticle (LNP), and ribonucleoprotein (RNP). AAV is a commonly used gene-therapy delivery vehicle due to its proven efficacy, safety, and broad tissue targeting, including brain, skeletal, and cardiac muscle.

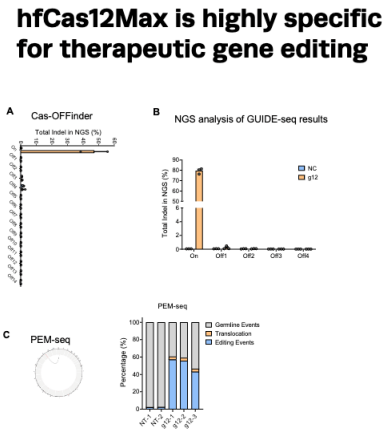
Results: After systemic delivery of a single all-in-one AAV vector that contained hfCas12Max CRISPR nuclease along with a gRNA targeting the splice donor (SD) site of the human *DMD* exon 51 in the Duchenne Muscular Dystrophy (DMD) mouse model, we observed efficient restoration of dystrophin expression. Histopathology and grip strength also showed significant improvement. Therapeutically acceptable editing efficiency in exon 51 SD was induced in a healthy wild-type non-human primate with a low dosage of AAV-hfCas12Max-gRNA without any observed hfCas12Max-related toxicity, indicating the relative safety of the AAV-hfCas12Max therapy.

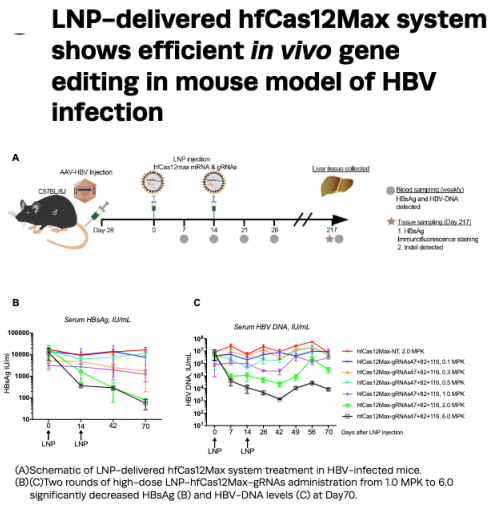
Safety and efficacy using hfCas12Max was also demonstrated in amyotrophic lateral sclerosis (ALS) studies. AAV containing hfCas12Max CRISPR nuclease and gRNA targeting the *ATXN2* gene considerably reduced toxic pTDP-43 protein aggregation to near-normal levels in the humanized *TDP-43*$^{Tg/Tg}$ mice, a widely used ALS mouse model. hfCas12Max CRISPR nuclease notably alleviated astroglial and microglial inflammation in the motor cortex, restored motor function, and significantly extended the lifespan of the humanized *TDP-43*$^{Tg/Tg}$ mice.

hfCas12Max safety and efficacy was also demonstrated targeting HBV using LNP-mediated delivery of hfCas12Max mRNA and gRNAs. Treatment led to a significant reduction in cDNA, HBV-DNA, and hepatitis B surface antigen (HBsAg) levels in HBV-infected primary human hepatocytes (PHH). Two rounds of LNP-hfCas12Max system administration resulted in a sustained reduction of serum HBsAg and HBV DNA levels in the AAV-HBV mouse model. In addition, by administering hfCas12Max RNP targeting the *TRAC* gene in primary human T cells, we detected approximately 90% editing efficiency in CD3+ T cells, with cell viability remaining around 80%. *In silico* and genome-wide off-target analyses, we have indicated the high specificity of hfCas12Max.

Conclusions: Our findings suggest that hfCas12Max CRISPR nuclease, with its robust editing activity and high specificity, is a promising tool for safer and more effective gene and cell therapy treatments. Synthetic guide RNAs and purified nuclease for hfCas12Max CRISPR nuclease are now readily available for CRISPR medicine developers through Synthego as







1616 Automated CRISPR/Cas9-based genome editing of human pluripotent stem cells using the StemCellFactory

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Introduction: The CRISPR/Cas9 technology has become a leading method for efficient gene editing, allowing the creation of isogenic pairs of mutant and control human induced pluripotent stem cells (hiPSCs). While hiPSCs offer valuable opportunities for disease modeling and personalized medicine, challenges remain in distinguishing the effects of specific mutations from genetic backgrounds. Manual genome editing processes often face scalability, efficiency, and standardization limitations. StemCellFactory, a modular robotic system, has been developed to automate the reprogramming, expansion, and differentiation of hiPSCs, enhancing the application of CRISPR/Cas9 technology in research.

Methods: HiPSCs were cultured in StemMACS iPS-Brew XF medium on Geltrex or Matrigel-coated plates, with daily medium changes and mycoplasma testing. For CRISPR/Cas9 genome editing, target gene-specific CRISPR-RNA and tracrRNA were prepared to form ribonucleoprotein (RNP) complexes using an optimized nucleofection protocol. HiPSCs were dissociated with Accutase, counted, and subjected to manual and automated nucleofection. In manual editing, hiPSCs were nucleofected with the RNP complex using a Lonza 4D-Nucleofector, followed by cell seeding into tissue culture plates for culturing monoclonal single cell colonies. The automated process on the StemCellFactory involved three subprocesses: cell preparation, nucleofection, and cell seeding, utilizing a robotic arm for material transport. The control software COPE managed the integrated hardware, enabling the execution of commands and data visualization and facilitating the automated genome editing process. Post-editing and genotyping of polyclonal hiPSCs were performed via Sanger sequencing to assess editing efficiency. Automated isolation of monoclonal colonies was conducted using the StemCellFactory-integrated CellCelector, which identified and isolated colonies meeting the specified criteria.

Results: The StemCellFactory successfully integrated a 4D-Nucleofector with a 96-well shuttle device to enable automated CRISPR/Cas9 genome editing. Adjustments were made to replace manual consumables with scalable formats, enhancing efficiency. Optimal conditions for automated editing were determined, showing that the CM150 electroporation program yielded high cell viability (83.5%) and indel rates (81.7%). The best culture conditions combined Laminin 521 with CloneR-supplemented iPS-Brew medium, supporting single hiPSC survival and colony formation. The automated workflow system allowed for the parallel processing of up to 24 independent conditions. Validation of the automated process using various hiPSC lines and target genes demonstrated high editing efficiencies, with indel rates exceeding 80% across all tested cell lines, comparable to manual methods. Monoclonal colonies were monitored using the CellCelector, allowing for the isolation of colonies derived from single cells.

Conclusions: These findings indicate the successful establishment of an automated CRISPR/Cas9 genome editing process on the StemCellFactory platform. The editing efficiency assessed across different hiPSC lines and numerous target genes was consistent with manual methods. Developing such a standardized and scalable automated system represents an exciting new tool in genome editing, enhancing the ability to address a wide range of scientific questions in disease modeling, drug development, and personalized medicine.

1617 PsCas9 (eSpOT-ON): An Engineered High-Fidelity CRISPR Nuclease for Therapeutic Applications.

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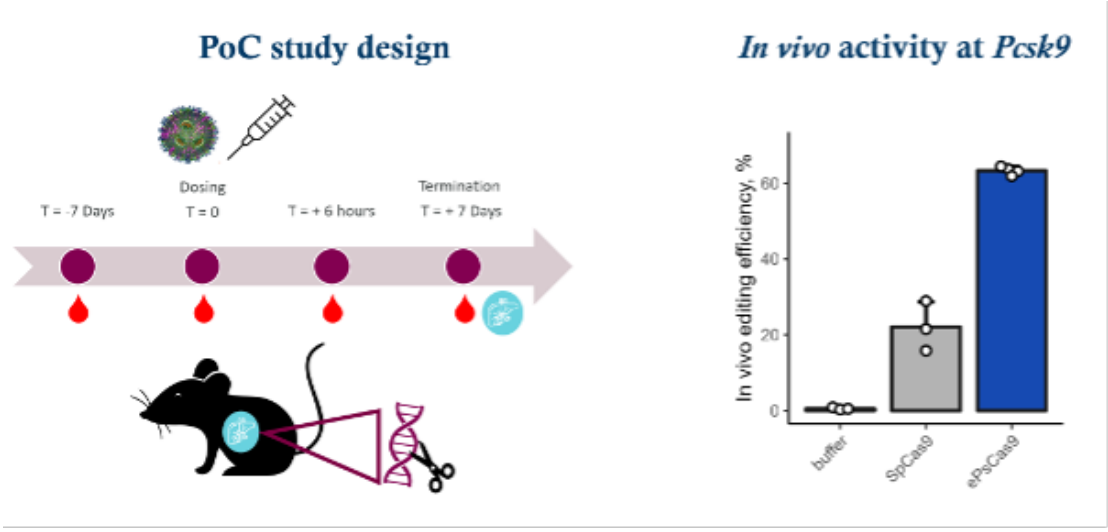
CRISPR-Cas technologies offer great potential for therapeutic genome editing. However, most Cas enzymes have not achieved sufficient in vivo activity for clinical applications. Our previous work introduced PsCas9 (SpOT-ON), a Type II-B Cas9 from *Parasutterella secunda*, as a high-fidelity enzyme. PsCas9 delivered via adenoviral vectors achieved editing levels comparable to SpCas9, with minimal off-target events and reduced chromosomal translocations.

In this study, we sought to understand why PsCas9 delivery methods beyond adenoviral vectors showed reduced efficacy. Cellular studies revealed that PsCas9 editing activity was limited under conditions of low intracellular concentration. Biochemical assays showed that PsCas9 has a lower DNA-binding affinity compared to SpCas9. We hypothesized that improving DNA interaction could enhance PsCas9 activity. To address this, we leveraged structural information to fuel the rational engineering of the enzyme. Optimizing the sgRNA scaffold modestly increased PsCas9 activity. In contrast, targeted mutagenesis resulted in up to a 20-fold enhancement across multiple genomic targets. These improvements correlated with stronger DNA binding in vitro. Importantly, the engineered PsCas9 retained its high fidelity, including low off-target effects and minimal chromosomal translocations. Finally, we tested the optimized PsCas9 (also referred to as ePsCas9 or eSpOT-ON) for therapeutic genome editing in vivo. Using lipid nanoparticles (LNPs), we delivered ePsCas9 to mouse liver to target the Pcsk9 gene, a model for hypercholesterolemia treatment. ePsCas9 achieved efficient liver editing and significantly reduced Pcsk9 protein levels in plasma, outperforming SpCas9 in this setting.

This work establishes ePsCas9 as a precise and efficient genome editor. Its enhanced activity and intrinsic fidelity make it highly suitable for therapeutic applications. Synthetic guide RNAs and purified nuclease for eSpOT-ON / ePsCas9 are now readily available for CRISPR medicine developers through Synthego as part of a strategic partnership with Astrazeneca to enhance the accessibility of these CRISPR tools.



1618 CRISPR-GPT: LLM Agents for Automated Design of Gene-Editing Experiments

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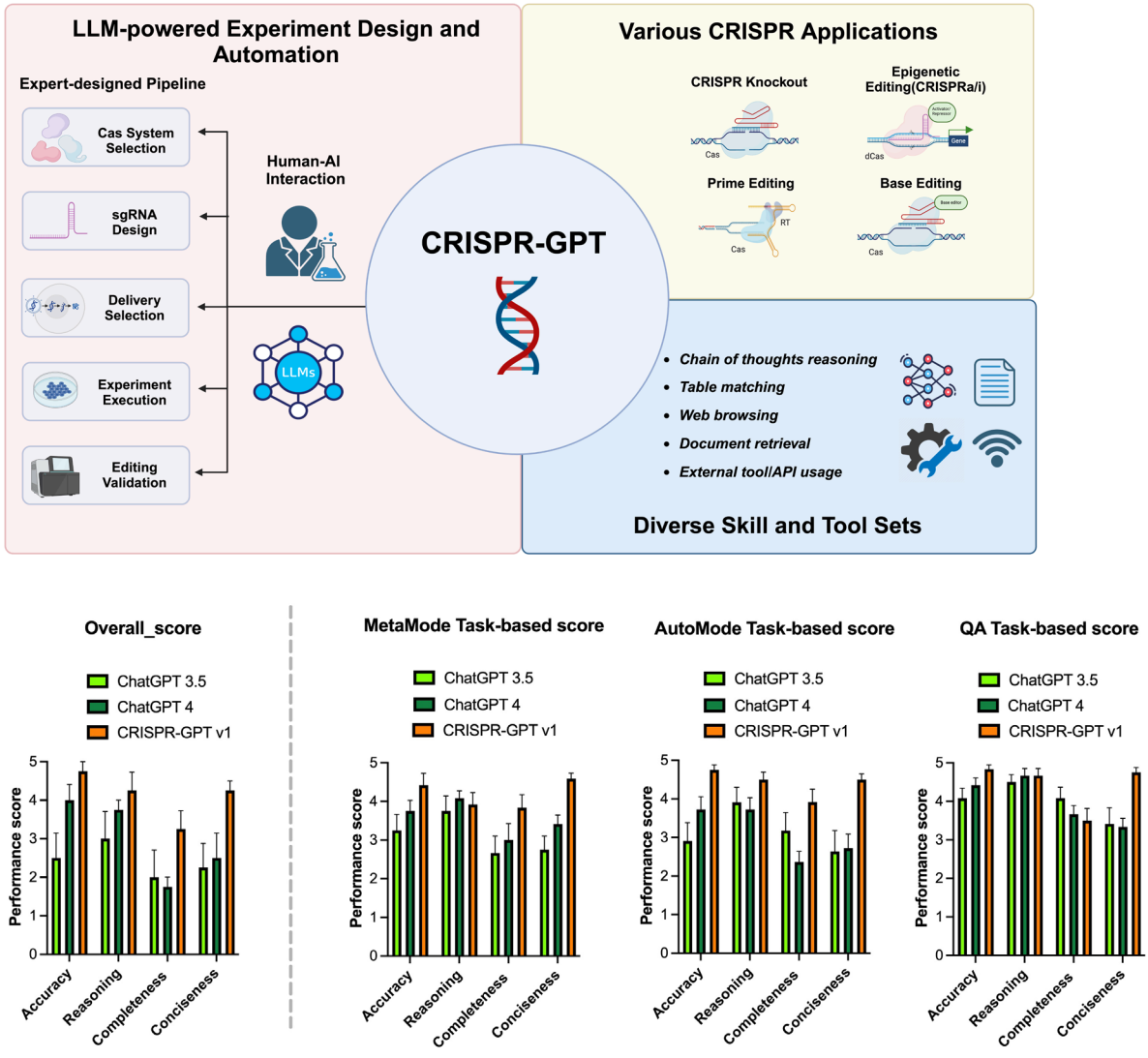
Genome engineering has revolutionized biomedical research by enabling precise genetic modifications, yet designing effective CRISPR experiments remains complex, requiring deep expertise in both gene-editing technology and biological systems. While Large Language Models (LLMs) offer broad capabilities, they often lack the domain-specific knowledge needed for complex biological design tasks. To address this gap, we introduce CRISPR-GPT, an AI-driven system that automates and enhances CRISPR-based gene-editing design and data analysis (Figure 1). CRISPR-GPT leverages the reasoning power of LLMs for task decomposition, decision-making, and interactive human-AI collaboration. It employs a multi-agent framework incorporating domain expertise, retrieval techniques,

external tools, and a specialized LLM fine-tuned with a decade of gene-editing discussions. This system assists in CRISPR selection, experiment planning, gRNA design, delivery optimization, protocol drafting, assay development, and data analysis. Compared to general LLMs like GPT-4o, CRISPR-GPT's agentic framework significantly improves accuracy, completeness, reasoning, and conciseness, as evaluated by gene-editing experts (Figure 2). To validate its effectiveness, beginner researchers successfully knocked out four genes in A549 cells using CRISPR-Cas12a, achieving 80–100% efficiency, and epigenetically activated two genes in A375 cells with CRISPR-dCas9, reaching 56.5% and 90.2% efficiency—on their first attempt. These results demonstrate the feasibility of AI-guided genome engineering, positioning CRISPR-GPT as a powerful AI co-pilot for CRISPR research, even for users with minimal prior experience.



1619 Standardized methods in iPSC for CRISPR-based editing and Homology-Directed Repair (HDR)

Karthik Murugan[1], Bernice Thommandru[1], Steve Glenn, Jessica Woodley[1], Garrett Rettig
[1]*Integrated DNA Technologies, Coralville, IA*

CRISPR-based gene editing and homology-directed repair (HDR) are used in several drug-discovery, pre-clinical, and clinical investigations to modify the genomes of stem-cell-based models. However, there is a critical need for standardized CRISPR reagents and protocols for making precise changes with high efficiencies in therapeutically relevant cell types. Here, we report the use of Alt-R™ CRISPR reagents such as Cas nucleases, synthetic guide RNAs, chemically modified HDR templates and HDR enhancers with optimized methods in various cell types including iPSC. We also showcase the application of rhAmpSeq™ CRISPR analysis system that provides a streamlined workflow for high throughput, targeted NGS-based screening of editing outcomes and HDR rates in any cell type of interest. Our optimized methods for iPSCs were also used for functional testing of Cas9 guide RNAs synthesized at IDT's therapeutic oligonucleotide manufacturing facility.

1620 *Tales from the R-loops*: how RNA/DNA hybrids may influence CRISPR/Cas9-mediated homologous recombination gene editing outcomes

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R-loops have been implicated in influencing genomic integration of recombinant adeno-associated viral (rAAV) vectors (*Puzzo et al., Mol. Ther., 2024*). Using DNA-RNA immunoprecipitation sequencing (DRIP-seq), we have mapped the R-loop formation in murine hepatoma cells (HEPA1-6) and mouse liver tissue *in vivo*. Our findings revealed that elevated R-loop levels enhance AAV-mediated nuclease-free homologous recombination (AAV-HR), particularly in R-loop-enriched regions such as the 3' end of the *Albumin* gene. Additionally, we demonstrated that HEPA1-6 cells exhibited significantly higher R-loop levels compared to murine liver.

To extend this work, we investigated the impact of R-loop formation on CRISPR/Cas9-mediated gene editing. Using HEPA1-6 cells and transgenic mice constitutively expressing *Streptococcus pyogenes* Cas9 (SpCas9), we designed guide RNAs (gRNAs) targeting two distinct regions of the murine *Albumin* gene: the mid-region (Mid Alb, exons 8–9) and the 3' region (3' Alb, exons 13–14), characterized by low and high R-loop levels, respectively (*Puzzo et al., Mol. Ther., 2024*).

Self-complementary AAV vectors expressing the gRNAs were utilized to assess the influence of R-loops on SpCas9-induced double-strand breaks (DSBs). Both *in vitro* and *in vivo* experiments revealed no significant differences in indel formation between the Mid Alb and 3' Alb regions.

To further evaluate the effect of R-loops on homology-directed repair (HDR)-mediated gene editing, donor DNA cassettes were designed for each target region. *In vivo* AAV-mediated delivery of gRNAs and donor DNA did not show significant differences in gene targeting efficiency between the two regions. However, in HEPA1-6 cells, integration rates were significantly higher in the Mid Alb compared to the 3' Alb region. Notably, overexpression of RNaseH1, which degrades genome-wide R-loop structures, enhanced gene editing efficiency at the 3' Alb without affecting the Mid Alb region. These results were in stark contrast to non-nuclease mediated AAV-HR, which was substantially higher in 3' Alb region (*Puzzo et al., Mol. Ther., 2024).*

Our current hypothesis is that high levels of R-loops at the 3'Alb in the HEPA1-6 could potentially inhibit the HDR mediated by SpCas9 and have studies in progress to address this question. Given the overall higher R-loop levels and active replication in HEPA1-6 cells compared to murine liver, we investigated the relationship between R-loop levels and cell cycle progression. Rapamycin treatment showed cell cycle arrest primarily at the G1/S phase and significantly reduced R-loop levels in HEPA1-6 cells. Our ongoing experiments are designed to determine whether the combination of cell cycle arrest and reduction in R-loop levels might influence the gene editing efficiencies at the Mid Alb and 3' Alb regions. Our new preliminary results highlight the potential interplay between R-loop formation, cell cycle status, and the efficacy of gene editing outcomes. Our findings provide novel mechanistic insights that could help design more effective gene editing strategies.

1621 Chemically Modified and Ligated Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Guide RNAs (LgRNA) and Their Anti-HBV Activities

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Cure of chronic hepatitis B requires elimination or inactivation of both HBV cccDNA and integrated HBV DNA. Several CRISPR/Cas DNA editing-based therapeutics are currently under preclinical development in hope to achieve this therapeutic goal. To improve the gene editing efficiency and accuracy, we optimize guide RNAs through chemical ligation of short RNA segments via non-phosphoramidite chemistry, i.e., ligated guide RNA (LgRNA), and chemical modifications. Compared to the classical single guide RNA (sgRNA), our proprietary LgRNA technology provides high-quality products (>99% purity by HPLC) containing a single spacer sequence and enables cost-effective scaleup productions (potentially multiple kilograms), diverse chemical modifications, targeted delivery by conjugations and various formulation technologies. We have reported the exceptional anti-HBV activity of LgRNA-33 by multiple mechanisms of action (MOA). LgRNA-33 used in our previous report was terminally modified only. In order to further improve LgRNA's drug properties, we have modified LgRNAs at sugar moieties, nucleotide bases, and by conjugations with peptides such as nuclear localization sequences (NLS).

We have firstly designed and evaluated full modifications at the sugar moieties of 11 nucleotides of the PAM-distal region. We have observed LgRNA with most of modified sugar moieties (Fig. 1) maintained the activity despite heavy modifications, which can be beneficial for improving guide's in vivo stability and decreasing the host immune responses.

Based on the crystal structures of substrate-Cas9-sgRNA tertiary complexes showing the major groove of RNA/DNA duplex is accessible for additional molecular interactions (Fig. 2), we incorporated both G-clamps (cytosine analogues of 9-(2-aminoethoxy) phenoxazine and 9-(2-guanidinoethoxy) phenoxazine) and locked 5-methyl-cytidine (LA-5mC) modifications at various positions. G-clamp modifications showed significant increase in the guide's targeting specificity, while LA-5mC modifications introduced additional off-targets in at least one example. LgRNAs with G-clamp modifications in general showed better activities than with LA-5mC modifications. No cytotoxicity was observed.

Syntheses and evaluations of LgRNA-peptide (NLS) conjugates in an in vitro cleavage assay and their anti-HBV activities in hepatocytes will also be reported.

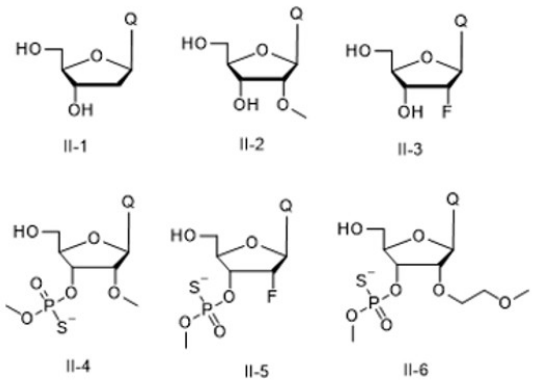

Fig. 1 Modified nucleotides. II-1 is a DNA nucleotide. II-2 has 2'-methoxy modification. II-3 has 2'-F substitution. II-4 has 2'-methoxy phosphorothioate modification (MS). II-5 has 2'-F phosphorothioate modification (FS). II-6 has 2'-ethoxymethoxy phosphorothioate modification (MoeS).

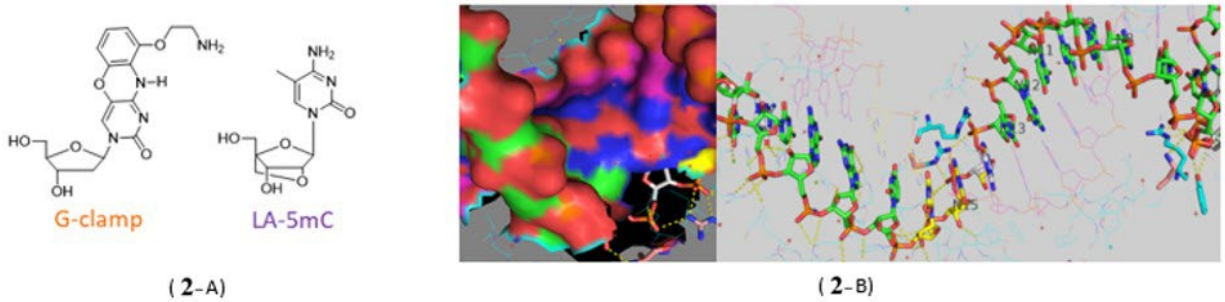

Fig. 2 Base modifications of spacer region. Structure of G-clamp and LA-5mC(2-A) and binding of RNA/DNA duplex to Cas9 protein (2-B). The major groove shows accessible space for base modifications to enhance base recognitions (20-B).

1622 Constructs of Chemically Ligated Guide RNAs (LgRNA) for Precise Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Gene Editing

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The clustered, regularly interspaced short palindromic repeats (CRISPR) gene editing has the potential to cure many diseases of unmet needs, as indicated by the first approval of CRISPR-edited medicine (Casgevy). CRISPR-Cas9 is directed by a guide RNA (sgRNA) to its targeting site, and the sgRNA is a vital drug substance of in vivo CRISPR therapeutics. However, sgRNA (~100 nt) presents unprecedented chemistry, manufacturing and control (CMC) challenges. The available commercial scales are at grams with overall isolated yields of single digits (< 6%), and the best purity available in the industry is below 50% by LC-MS in spite of reported above 80% purity by High-Performance Liquid Chromatography (HPLC) methods, which do not resolve impurities even with lengths of 5 nt shorter (i.e., 95mer). Importantly, many truncated products and products of invalid sequences (97-101mer) are biologically fully active leading to guide-dependent catastrophic off-targets. Lately developed CRISPR editors such

as Prime editor requires guide RNAs significantly longer (>160 nt for epegRNAs), while there are rarely vendors capable of providing chemically modified RNAs longer than 160 nt. Chemically ligated guide RNA (LgRNA) is our distinct proprietary intellectual property to enable state-of-the-art precise CRISPR gene editing. LgRNA is a single molecule guide RNA comprising short oligonucleotide segments joined by one or more non-nucleotide Linkers (nNt-Linker). One example is an LgRNA incorporated with a single nNt-Linker between a crRNA and a tracrRNA (both shortened) or at another site by a chemical ligation. Others have observed the structures (e.g., tetrazine-derived linkers) and locations of an nNt-Linker (e.g., between the spacer and the guide scaffold, G21U22) can decrease the activities of resulting guides, though the decrease in activity was reported to be recoverable by further modifications.

Herein we report our efforts in optimizations of nNt-Linkers (Fig. 1). We have observed an nNt-Linker can enhance guide's activity at different locations including the spacer region, and multiple nNt-Linkers can substitute nucleic acid components of guide RNAs with no loss of guide's activity. The crRNA segment of an often-used LgRNA is 32 nt long, while tracrRNA (68 nt) is still long. We showed tracrRNA can be further divided into shorter segments (e.g., 36 nt and 22 nt), and such short RNA segments of high quality with refined chemical modifications are routinely manufactured in kilograms in industry. This segmented nucleic acid strategy of multiple nNt-Linkers has been applied to productions of high-purity long guide RNAs in prime editing (epegRNA of >160 nt, Fig. 2) and of chimeric guides (RNA-DNA conjugates) in STAR editing (STAR editing guide nucleic acids (segNA) of >180 nt) by this team. The chemically ligated epegRNA (lg-epegRNA) showed similar activity to an all-RNA guide, while STAR editor guided by a segNA showed ~20% precise editing in hepatocytes with further optimizations in progress.

In summary, we have demonstrated LgRNA having nNt-Linkers at various positions is superior to sgRNA and LgRNA technology provides scalable and efficient access to long guide RNAs (epegRNA and segNA) with minimal off-target-causing impurities, which are critical for their clinical applications. We expected LgRNA to be a critical component of CRISPR therapeutics, including prime editors and STAR editors.

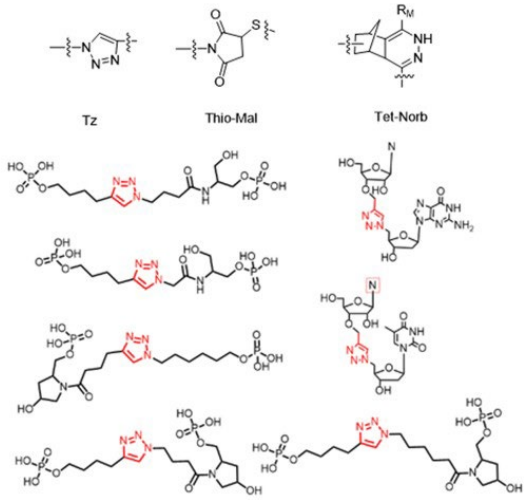

Fig. 1 Non-nucleotide linkers tested in this work.

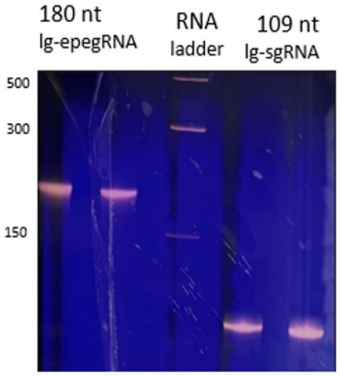

Fig. 2 Analysis of chemically-ligated long guide RNAs by denaturing PAGE

1623 Engineering the sgRNA Scaffold to Enhance Editing Efficiency in CRISPR/Cas9-Based Gene Therapy

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Gene therapy has emerged as a promising approach for the treatment of genetic disorders, offering potential cures for conditions previously thought untreatable. Recent advances in gene editing technologies, particularly CRISPR-based systems, have made significant strides in improving the precision and efficacy of therapeutic interventions. Despite these advances, however, challenges remain in achieving consistently high editing efficiencies, particularly for certain diseases that require precise and large-scale genetic modifications. Low editing efficiencies, especially within specific targets, can limit the clinical applicability of gene therapies for a wide range of conditions, preventing the development of this potentially curative approach.

To address this challenge, our work focuses on engineering the single guide RNA (sgRNA) portion of the CRISPR/Cas system to increase editing efficiency within the *Staphylococcus aureus* derived CRIPSR/Cas9, SaCas9. Specifically, we explore the potential of synthesizing a library of sgRNA mutants that have an altered scaffold region to enhance the system's performance. The scaffold region of the gRNA, though relatively small in terms of base pairs, plays a critical role in the stability, specificity, and efficiency of the CRISPR/Cas9 via its distinct tertiary structure. Because of its compact size, the scaffold region presents a unique opportunity for targeted mutagenesis; subtle changes within this small region can significantly impact the Cas9-sgRNA interaction, leading to improved performance without affecting the overall structure of the sgRNA or its ability to guide Cas9 to the target sequence.

By generating a library of scaffold mutants, we aim to systematically explore a wide array of sgRNA variants to identify those that enhance the overall editing efficiency of the CRISPR/SaCas9 system. The mutational library approach leverages the small, 76 base pair size of the scaffold region to rapidly identify sequences that may outperform the wildtype scaffold. This approach allows us to focus on a region that directly influences Cas9 binding without needing to redesign the entire sgRNA, alter the target sequence, or engineer the Cas9 enzyme, offering a more efficient and targeted strategy for improving gene editing.

We hypothesize that modifying the sequence, and thus the structure of the sgRNA scaffold will enhance the specificity and overall editing efficiency of SaCas9, providing a valuable tool for fine-tuning CRISPR systems for different therapeutic contexts. Preliminary results from two mutational screens have identified ~300 mutants that display enriched editing scores above wildtype based on next generation sequencing data. Moreover, we have performed quantitative and functional assays to confirm the viability of mutants identified from our screen.

1624 Engineering SpCas9 to be Compatible with sgRNAs with Shorter Constant Regions

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Despite the constantly growing CRISPR-Cas toolset, Cas9 from *Streptococcus pyogenes* remains one of the most commonly used enzymes for genome editing. SpCas9 is guided to a 23-nt DNA target sequence by a two-part guide RNA (gRNA) complex consisting of a 42-nt CRISPR RNA (crRNA) containing a target site-specific 20-nt complementary RNA region (spacer region) and a universal ~89-nt trans-activating CRISPR RNA (tracrRNA). It was found that the 20 nt spacer plus 12 nt of the crRNA constant region could be joined to a 60 nt segment of the tracrRNA using a 4 nt linker to form a functional single-guide RNA (sgRNA). Expression of sgRNAs in mammalian cells is typically done using a U6 promoter and a poly-T terminator resulting in the addition of on average 4 U's to the end of the guide resulting in a ~100 nucleotide sgRNA. Chemical synthesis of sgRNAs provides the advantage of being able to incorporate chemical modifications to improve guide stability and enhance editing rates however it also comes with a tradeoff between guide length and yield of full-length product. This becomes particularly problematic for production of the 125+ nucleotide guides regularly used for prime editing or of gram scale amounts of high purity guides for applications like *in vivo* editing. Reducing the length of the guide constant region needed to

facilitate Cas9 activity would help ameliorate this issue. To generate a Cas9 variant with strong nuclease activity when paired with guides with a reduced length constant region we generated a Cas9 mutant library and used a bacterial screen where Cas9 nuclease activity with short constant region guides was linked to bacterial survival. We identified multiple beneficial mutations that we subsequently validated as improving the activity of Cas9 in human cells using guides with constant regions shorter than 55 nucleotides. Combining these mutations resulted in the identification of a variant that retains high levels of editing activity when paired with short constant regions guides and delivered as a ribonucleoprotein complex into human cells.

1625 Infusion of Sso7d DNA-Binding Protein Boosts Gene Editing Efficiency of the Engineered Hypercompact OsCas12f (enOsCas12f) in Mammalian Cells

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Compact CRISPR/Cas editors offer significant advantages in viral packaging and targeting efficiency. The hypercompact Type V-F CRISPR-Cas12f is emerging as a promising genome editor for gene therapy, especially with small-capacity viral delivery vectors like adeno-associated virus (AAV). Initially, Un1Cas12f (529 aa) from an *uncultured archaeon* cleaved ssDNA exclusively, with later studies showing nuclease activity (dsDNA) in prokaryotic but not eukaryotic cells. Protein engineering and sgRNA scaffold modifications have since enhanced Un1Cas12f's editing efficiency in mammalian cells. Similar improvements were seen in AsCas12f from *Acidibacillus sulfuroxidans* (422 aa) and OsCas12f from *Oscillibacter sp.* (433 aa). Our previous study using the LoxP-STOP-LoxP-tdTomato (LSL-tdT) reporter system validated high editing efficiency (>85%) of enhanced OsCas12f (enOsCas12f) in mammalian cells with transient transfection of the LSL-tdT reporter. A non-specific dsDNA binding protein, Sso7d, when fused with Cas nucleases, has been shown to improve gene editing efficiency of spCas9, cjCas9 and nme2Cas9(Yin et al. 2023) or the transcriptional activation of dUn1Cas12f(Hu et al. 2024) and dspCas9(Yin et al. 2023). To improve enOsCas12f's editing efficiency on endogenous integrated target genes (10-15%), we fused to the C-terminus of enOsCas12f, creating SosCas12f. This modification significantly increased genome editing efficacy to 50-60%, as determined by quantitative confocal imaging and PCR fragmental genotyping in mouse microglia cell line SIM-9A stably expressing the LSL-tdT reporter and primary neural stem cells from LSL-tdT Ai14 mice. Additionally, we constructed an all-in-one AAV vector encoding SosCas12f and quadruple sgRNAs targeting LTRa, LTRc, Gag, and Pol of the SIV genome. Using the SIVmac-239-eLuc reporter assay, we found that SosCas12f robustly increased SIV genome eradication efficiency compared to enOsCas12f alone. These findings suggest that the enOsCas12f-Sso7d fusion exhibits superior genome editing activity and therapeutic potential. This innovative SosCas12f construct can be efficiently packaged into AAV, advancing CRISPR/Cas gene therapy applications. **Acknowledgements:** This study is supported by NIH R01MH130193 and R01DA056876 funding.

1626 Optimizing knock-in efficiency of therapeutic transgenes using novel Cas9 fusion proteins

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Homology-independent targeted integration (HITI) is a powerful genome-editing system enabling precise DNA integration without relying on homology-directed repair. We have demonstrated the potential of adeno-associated viral vector-mediated HITI (AAV-HITI) to target effectively gain-of-function mutations in a mouse model of autosomal dominant retinitis pigmentosa. Additionally, we have shown that AAV-HITI results in sustained lysosomal enzyme expression under conditions of hepatocyte proliferation in a mouse model of lysosomal storage

disease. Despite its potential, HITI efficiency may be insufficient for applications that require high transgene expression.

To overcome this challenge, we have engineered the *Sp*.Cas9 nuclease by fusing it to critical components of the non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ) pathways. These innovative fusion proteins are engineered to promote the targeted recruitment of a specific repair pathway at Cas9-induced double-strand breaks (DSBs), with the intent to enhance knock-in of a desired donor DNA.

In vitro data suggest a mild improvement in donor DNA integration when the *Sp*Cas9 fusion leverages the MMEJ pathway. Building on this, we will design and evaluate a panel of fusion proteins incorporating additional key repair factors from the same pathway whichwill be then tested in relevant mouse models of inherited retinal and liver diseases.

This study seeks to expand the scope of current knock-in approaches by developing more efficient, mutation-independent strategies for the treatment of genetic disorders.

1627 Engineering an Improved Inhibitor of 53BP1 to Enhance HDR Efficiency

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One of the most common methods for achieving precise genome editing is by creating a double-strand break using a targeted nuclease that is then repaired by endogenous cellular double-strand break repair machinery using a supplied template with the desired edits. The cell has two primary methods of repair of double-strand breaks, non-homologous end joining (NHEJ) in which broken DNA ends are directly joined together and homology directed repair (HDR), which encompasses a number a of different pathways that use a DNA template to facilitate repair. The rate of HDR varies by cell type and target site within the genome and limits the efficiency of some genome editing strategies. The protein p53-binding protein 1 (53BP1) plays a critical role in determining if a break is repaired by HDR or NHEJ. 53BP1 is recruited to sites of double-strand breaks and inhibits end resection, a key initial step in HDR. A modified variant of ubiquitin (i53) was shown to block 53BP1 recruitment to the broken DNA ends and to enhance the rate of HDR when delivered as mRNA or plasmid. We tested i53 and found that it did not function as well when delivered as purified protein. To improve the efficiency of 53BP1 inhibition, we performed a two-hybrid screen to identify ubiquitin variants with improved affinity for binding to 53BP1. Based on this screen, we developed ubiquitin variants with improved efficacy for enhancing HDR when delivered as protein during nucleofection. We found that our improved ubiquitin variant (Ubv-A) was able to improve rates of HDR in multiple cell types and using multiple types of donor template. We also did not observe the cytotoxicity or off target effects we observed with a small molecule inhibitor of NHEJ. We envision that our ubiquitin variants targeting 53BP1 can be a valuable tool for *ex vivo* genome editing, where both high efficiency and cell viability are needed and the effects of blocking NHEJ are a potential concern.

1628 Optimizing phenotype and function of non-viral CRISPR-Cas9 *TRAC*-replaced CAR T cells by pharmacological tyrosine kinase inhibition during ex vivo expansion

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CAR-T cell therapy has revolutionized the treatment of B cell malignancies, however it has been less effective in solid tumors. Tonic signaling describes antigen-independent signaling of CAR molecules, which promotes premature differentiation ex vivo, that has been associated with reduced in vivo efficacy of certain CAR constructs. Previous studies demonstrated that tyrosine kinase inhibitors, such as Dasatinib, can prevent CAR signaling and temporary "resting" of CAR T cells enhances their in vivo efficacy. Thus, we hypothesized that suppressing tonic signaling during ex vivo culture should help maintain an optimal CAR T phenotype and retain their potential for improved in vivo function and long-term efficacy.

Here, we investigated the effects of Dasatinib on non-viral CRISPR-Cas9 edited CAR-T cells expressing CAR targeting CD19, L1CAM or GD2 from the endogenous *TRAC* locus. Culturing *TRAC*-replaced CAR-T cells with Dasatinib for 9 days after CAR knock-in significantly reduced the differentiation, without affecting their expansion rates. Retaining higher frequencies of less-differentiated CCR7+ central memory and naïve-like T cells was more prominent in CAR constructs that tend to induce tonic signaling. Culturing with Dasatinib (100 nM) increased the percentage of CCR7+ cells by 1.7-fold for L1CAM-CAR (39.95% to 66.81%) and 1.2-fold for GD2-CAR (57.85% to 70.53%). We also detected decreased expression of inhibitory receptors associated with exhaustion (TIM-3, LAG-3, PD-1) in Dasatinib-treated cells, but similar cytotoxic capacity 16 hours upon drug removal. In co-cultures with antigen-positive tumor cells, Dasatinib-treated CAR-T cells exhibited enhanced proliferation capacity, with 12.1-fold and 2.7-fold increases in proliferation for GD2- and L1CAM-specific *TRAC*-CAR T cells, respectively, over their untreated counterparts. Intriguingly, antigen-dependent proliferation of CD19-CAR T cells was also improved (Dasatinib-treated: 9.38-fold vs untreated: 5.49-fold), even though this construct was less prone to tonic signaling.

Our results suggest that Dasatinib can modulate the phenotype and expansion capacity of non-viral generated *TRAC*-replaced CAR-T cells by mitigating tonic signaling during ex vivo culture. These insights may contribute to the development of more potent and durable CAR-T cell therapies.

1629 Therapeutic transgenes need optimization – lessons from Cystic Fibrosis

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Introduction: More than 2,000 mutations in the *CFTR* gene cause Cystic Fibrosis (CF); therefore, genetic editing approaches that can provide benefit for many or all mutations are promising strategies. Targeted insertion of a partial cDNA (aka "super exon" (SE)) coding for wild type *CFTR* exons (E) 2-27 into *CFTR* intron 1 can restore all mutations downstream of the insertion site and provide therapeutic benefit for almost all people with CF (>99%). In contrast to safe harbor approaches, insertion into the native genomic locus likely maintains native gene regulation, which is important since *CFTR* is not expressed in all cell and tissue types. Our previous work demonstrated a strong effect of sequence components (such as splice acceptor, 3' UTR/polyA, insertion site, and intron) on SE performance and the need to optimize the SE transgene sequence. Our first-generation E2-27 SE provided full CFTR functional rescue in clonally isolated CF cells. However, mRNA stability was notably reduced compared to wild type *CFTR*, so we then sought to optimize the SE transgene.

Methods: To enable more extensive and facile optimization, we developed a robust, medium-throughput NanoLuc SE reporter system to evaluate the sequence components. The NanoLuc SE reporter was constructed with a splice acceptor, non-cleavable peptide linker, promoter-less NanoLuc cDNA (named NanoLuc SE), 3' UTR/polyA, and homology arms for targeting *CFTR* intron 1. Genomic integration of this reporter results in a *CFTR* exon 1-NanoLuc SE fusion protein. We then applied the best combinations to the context of the *CFTR* SE. *CFTR* SE insertion editing was achieved in 16HBEge *CFTR* G542X (CFTR null) cells by pre-treating with an Adenovirus containing a *CFTR* E2-27 SE construct for 24 hours, followed by nucleofection with RNP targeting *CFTR* intron 1. CFTR rescue was evaluated in a bulk population of edited cells by Western blot. Subsequently, cells were sub-cloned and clonal cell lines with the SE inserted were identified and fully characterized for *CFTR* mRNA abundance, protein expression, electrophysiology function, and NGS analysis of *CFTR* isoforms.

Results: We tested over 110 NanoLuc SEs with combinations of insertion sites, mini-introns, splice acceptors, and 3' UTR/polyA sequences identified from human genes with a 3' UTR length of <400 bp and highly expressed in airway cells. NanoLuc SEs with different components resulted in a wide range of NanoLuc expression, further demonstrating that sequence components dramatically influence transgene expression. The best combinations were then applied to *CFTR* SE constructs with therapeutic potential. Full CFTR rescue was observed with a viral polyA element, and 35.1% CFTR rescue was achieved with a human gene 3' UTR. Additional NanoLuc-SE screening of 3' UTR elements was recently completed, which identified human 3' UTRs from *NDUFB86* and *SOD1* as promising candidates, which are currently being applied to the next generation of *CFTR* SEs.

Conclusion: We demonstrated robust rescue of CFTR using the SE insertion approach and show that optimizing SE sequence elements is critical for efficient expression, splicing, stability, and function of the SE. Our on-going work is focused on understanding SE expression and optimizing constructs for *CFTR.* This approach could provide benefit for almost all people with CF and provides a paradigm for many genetic diseases.

1630 DNA repair precision after gene editing, unveiled

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As the therapeutic applications for CRISPR/Cas or TALEN are expanding at rapid pace, we embarked in the mission of describing their effect on the treated cells genomes. Numerous techniques have emerged to detect or anticipate genotoxic events, reflecting the need for thorough characterization of these editing tools' activities. Nevertheless, these approaches face limitations such as high costs, time-consuming procedures, extensive bioinformatics requirements, and biased evaluations of genetic aberrations. Additionally, the temporal dynamics of DNA editing and subsequent repairs are poorly understood, leading to potential misinterpretations and observational biases.

To address these challenges, we introduce "Multipurpose Editing and Genotoxicity Assessment" (MEGA), a novel diagnostic toolkit that employs multiplexed digital PCR (dPCR) assays for comprehensive characterization of mutations at both on- and off-target sites induced by gene editing tools. This technique allowed us to reveal previously unreported significant biological information with unprecedented precision, including:

1. Precise (error-free) DNA repair mediated by NHEJ
2. Recurrent designer nuclease cleavage events
3. Kinetics of DNA repair mechanisms in primary cells
4. Timing of DSB resolution
5. Designer Nuclease activity

Furthermore, we demonstrated how to rapidly track and quantify various mutations in a temporal fashion.

MEGA addresses critical gaps in the genetic engineering analysis toolkit by providing a rapid, accessible, and specific overview of genome integrity post-gene editing, applicable to clinically relevant samples. Furthermore, by leveraging this novel technique, we elucidated fundamental biological concepts underlying DNA repair, paving the way for extensive mechanistic studies aimed at improving designer editor activity and gene therapy safety.

1631 The missing link: Kromatid's Next-Gen Cytogenetic Platform, KROMASURE™, Provides Critical Insight for Evaluating Cell and Gene Therapy Products for Chromosome Structural Variation, including Edit Integrity and Genomic Stability.

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When cells are edited, differentiated, modified, or engineered for therapeutic applications such as CAR-T, accurately measuring chromosomal stability and mutation is critical. Comprehensive, accurate analysis of the genome for potentially deleterious and cancer-inducing changes requires a panel of assays. The most robust characterization of genomic and target integrity is provided by combining cytogenetic cell-based analysis (to profile the starting material and monitor the product for chromosomal stability and integrity), which detects chromosome structural variation and aneuploidy, with targeted sequencing approaches such as NGS and dPCR. Cytogenetic assays provide critical data for structural variant discovery/validation and algorithm tuning, enabling robust breakpoint identification at the base pair level. These direct visualization assays supply characterization of low frequency, heterogenous, and complex events. Because they do not utilize pooled DNA from a heterogeneous cell population or a bioinformatic reconstruction of the genome, cytogenetic assays can characterize events with low prevalence or complex presentations, or events that involve breakpoints nested in repetitive sequence. Conventional cytogenetic methods can be burdensome and require extensive manual analysis for a relatively small number of cells (normally 20). These legacy methods (G-banding, FISH, SKY and M-FISH) are utilized on a limited basis, and often only when chromosomal aberration measurements are expected to be present in most/all cells in a sample, or they are required for assessing the risk of a gene editing or cellular engineering process.

Kromatid's KROMASURE platform of advanced cytogenetic tools enriches the dataset available beyond classic cytogenetic techniques and offers a custom suite of solutions for in-depth single cell analysis of genomic targets of interest. Targets as small as 2kb (inSite and PPF), up to the entire genome, can be characterized with KROMASURE for chromosome structural variation (K-Band and Screen). The platform leverages proprietary probe design and hybridization methods, as well as automation and advanced imaging solutions to increase the number of cells analyzed. All platform methods can be paired with custom analytical packages to support a wide variety of study designs, including therapeutic product release testing and comparative analysis. Individual structural variants, as well as structural variation phenotypes (clones with a signature structural variant profile) can be tracked, even at low prevalence, via the analysis of hundreds of individual cells per sample.

Improving and automating these direct visualization methods, along with streamlining the integration of this data with sequencing and/or array-based approaches for measuring variation is an active area of development, showcased here using the GIAB HG008-T Tumor cell line. The KROMASURE platform provides the next generation of cytogenetic assays for the characterization of cell and gene therapy products, from research and development phase all the way through drug product validation.

1632 Diji: The First Integrated Method to Analyze Single and Double-strand Breaks in GUIDE-seq and Digenome-seq Data for Unbiased Assessment of Editing Outcomes

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Genome editing technologies hold great promise for developing genomic medicines to correct monogenic disorders and restore functional cellular activity. Our RNA Gene Writers leverage the mechanism of target-primed reverse transcription and have shown in vivo efficacy in mouse and non-human primate models for diseases such as phenylketonuria (PKU), alpha-1 antitrypsin deficiency (AATD), and sickle cell disease (SCD).

A critical step before advancing these technologies to the clinic is assessing their genotoxic potential through rigorous evaluation of on-target and putative off-target effects, including any unintended mutations at the on-target site. Existing in vitro and in cellula methods, such as Digenome-seq and GUIDE-seq, can quantify editing outcomes. However, these methods lack an integrated, robust, scalable, and standardized framework to analyze both assays simultaneously. Furthermore, current tools cannot infer single-strand breaks (SSBs) alongside double-strand breaks (DSBs), a key limitation for emerging technologies that aim to minimize reliance on DSB repair mechanisms.

To address this gap, we present Diji, a novel computational method designed to analyze both GUIDE-seq and Digenome-seq datasets, accommodating experimental designs that include single or double-strand genomic breaks. Diji features read-level analysis combined with spacer-matching logic to identify putative off-target mutations. Additionally, it introduces a normalized cleavage score (NCS), which quantifies editing outcomes based on sequencing depth relative to a control, enabling unbiased comparisons across samples and conditions.

We evaluated Diji using simulated datasets encompassing diverse editing outcomes, sequencing quality (generated via ART and wgsim simulators), and sequencing depths (ranging from 1× to 100×). Diji demonstrated superior performance, achieving average precision and recall scores of 0.92 and 0.97, respectively, compared to digenome-toolkit2 (the state-of-the-art Digenome-seq analysis tool), which achieved 0.69 precision and 0.59 recall at 20× coverage and Illumina HiSeq error rates. Furthermore, benchmarking the NCS revealed its high accuracy ($r^2 = 0.97$) compared to the standard cleavage score ($r^2 = 0.48$) when correlated with the rank order of synthetic sites. By analyzing simulated data containing known off-target sites, we determined the optimal sequencing depth and number of replicates needed to maximize the discovery of off-target sites using Diji in real-world conditions.

In summary, we believe Diji provides a significant advancement in the standardization and scalability of genome editing analysis. It offered superior precision and recall, a more accurate quantification score, and supported experimental design optimization. Diji is a potentially valuable tool for assessing the precision and safety of genome editing technologies, paving the way for accelerated preclinical and clinical applications.

1633 Fast Quantification of Genome-Editing Outcomes using CRISPR-based detection

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Introduction: Genome editing is rapidly gaining traction in many clinical applications. However, the evaluation of editing outcomes remains time consuming and impedes R&D timelines, especially when multiple iterations are needed to optimize workflows. Amplicon sequencing (amp-seq) is considered the gold standard for quantification of editing efficiency. However, amp-seq is time consuming (~1 week turnaround time), and can be costly. Faster methods have been developed, but these often pose a trade-off of accuracy. The introduction of new workflows into research pipelines also requires additional equipment and/or extensive assay optimization. Hence, there is a critical need for fast, accurate, and cost-effective quantification of gene editing efficiency. To address this need, we have developed an assay which can be easily run on standard lab equipment for quantification of indels, knock-ins, and off-target activity. The assay is called QUiCKR (Quantification Using initial CRISPR Kinetic Rates) and offers results in 20 min with minimal steps. The assay protocol consists of generating a DNA sample dilution series, which is added to a pre-plated reagent mix containing Cas12, crRNA and fluorescent reporters, followed by fluorescence read-out (Fig 1a). The editing efficiency is then quantified using a machine-learning trained software. Current CRISPR based detection assays typically have a binary output, and are ill-suited for precise quantification. In contrast, the QUiCKR assay quantifies the kinetic rates of Cas12 trans-cleavage to quantify gene editing efficiency, with amp-seq level accuracy. Pre-plated reagents remain stable during long-term storage, which provides users with ready-to-use reagents, a quick workflow, and accurate results.

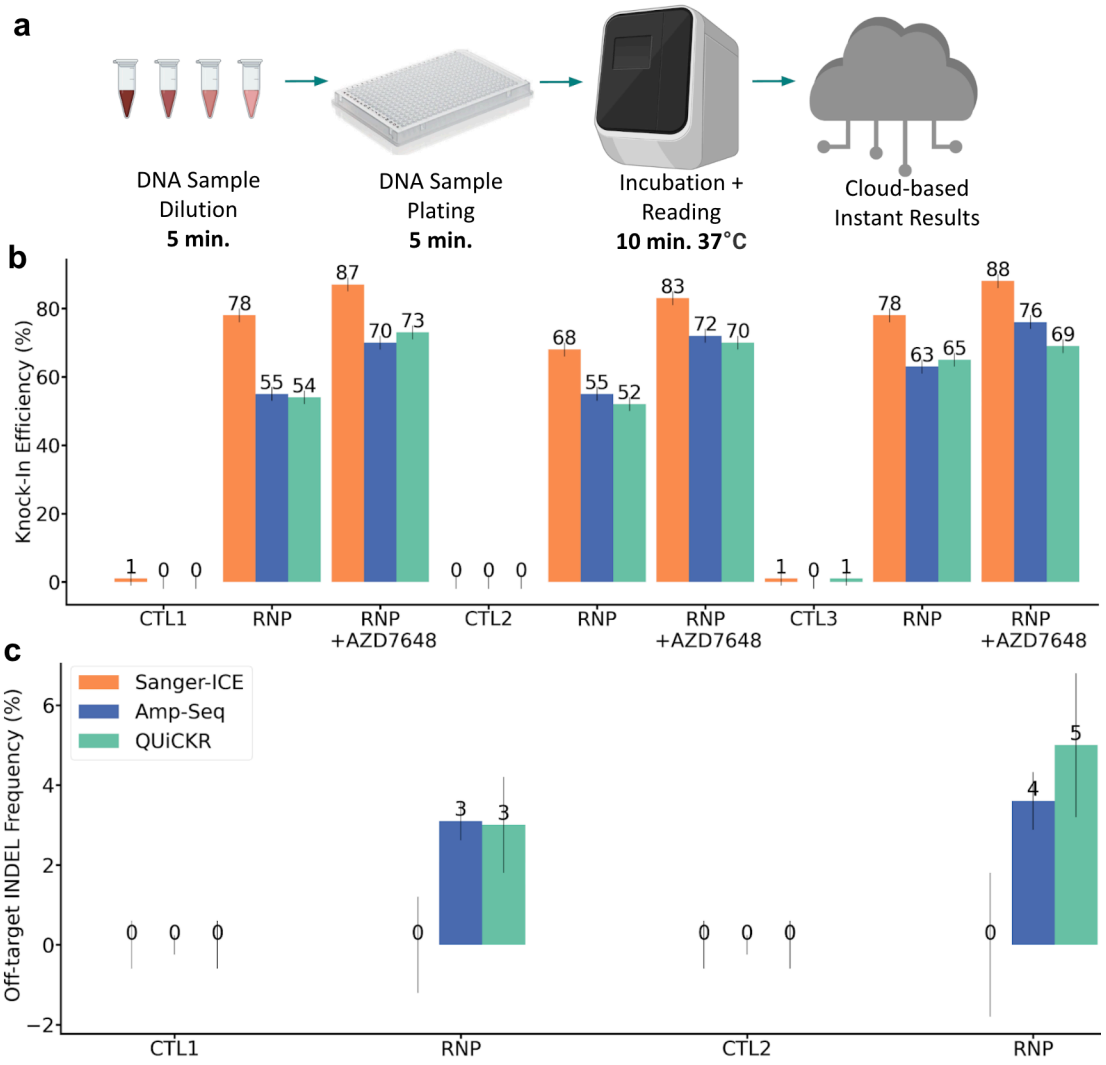
Experiments and Results: Edits quantification with QUiCKR, amp-seq and Sanger-ICE HSPCs were separately edited at HBB, and STING1 loci with Cas9 ribonucleoprotein (RNP) with or without a knock-in AAV6 donor template, followed by amplification of the on-target sites and an off-target site. On- and off-target INDEL frequency, knock-in efficiency, and wild-type ratios were quantified by Sanger sequencing with ICE analysis, amp-

seq, and the QUiCKR assay (Fig 1b). QUiCKR demonstrated 99.1% R-square fit with amp-seq data over 35 data points. In contrast, ICE showed 91.9% R-square fit with amp-seq data and did not capture low levels of off-targets (~3%, Fig 1c).

LOD study on CCR5 single-base deletion iPSCs were edited at CCR5 locus with RNP, quantified at 98% with Sanger-ICE. Edited CCR5 amplicons were diluted with unedited CCR5 amplicons to form 9 mixes (editing frequencies from 90% to 0%). Editing frequencies were quantified with QUiCKR, which yielded a 98.2% R-square fit with Sanger-ICE data.

Dynamic-Range study on BCL11A 15-base substitution Mixes of wild type and mutated DNA amplicons were prepared at ratios from 0%-90%, and quantified by QUiCKR. The R-square fit with mutant ratios was 98.4% and the standard error of the assay was 0.7%.

Conclusion: The QUiCKR assay accurately quantified INDEL edits, 15bp knock-ins with 99% R-square fit with amp-seq (versus 91% for Sanger-ICE). The QUiCKR assay also demonstrated a sensitivity below 3% for a single nucleotide edit and INDEL edits at off-site, modifications that Sanger-ICE was not able to capture. To conclude, QUiCKR offers a 20-min and accurate alternative to amp-seq, which has the potential to significantly increase the speed of gene editing research.



1634 Comprehensive On- and Off-target Validation Using Integrated rhAmpSeq and Targeted DNA Resequencing Single-Cell Technology for Gene Editing Applications

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Advances in genome editing technologies have facilitated precise correction of adverse mutations and the engineering of cells with novel therapeutic functions. Among the critical determinants of robust genome editing outcomes is assay specificity, particularly in CRISPR-based systems where guide RNAs (gRNAs) enable precise on-target edits with minimal off-target activity. To address off-target effects, both in-silico and empirical methods have been widely utilized to predict and characterize potential off-target sites. Amplicon-based targeted resequencing has emerged as the leading methodology for validating on- and off-target edits due to its multiplexing flexibility, high sensitivity, and cost efficiency.

Indeed, recent innovations, such as rhAmpSeq, which leverage novel reaction primer design and chemistry to improve performance in bulk validation by reducing background noise in multiplexed PCR, have gained prominence. Concurrently, innovations in droplet-based single-cell DNA resequencing technologies provide critical insights into single-cell context, including editing zygosity, co-occurrence of edits, and clonality across thousands of individual cells. These attributes are particularly valuable for gene and cell therapy applications.

In this study, we integrated rhAmpSeq chemistry with droplet-based single-cell assays to develop a comprehensive and flexible workflow for on- and off-target validation with single-cell resolution. To validate this approach, we used two well-characterized cell lines, GM24385 (son, HG002) and GM24149 (father, HG003), targeting loci that are homozygous in HG003 and heterozygous in HG002, with indel sizes ranging from 1 bp to >2,000 bp. These cell lines and targets were chosen because of the abundance of orthogonally validated bulk sequencing data which would be useful for benchmarking the performance of the overall workflow. Targeted amplicons were designed using rhAmpSeq design pipeline incorporating unique parameters that are compatible with droplet-based single-cell DNA resequencing platform. The resulting multiplex panel was compatible with both bulk rhAmpSeq and single-cell workflows.

With this targeted panel, we assessed the droplet-based single-cell rhAmpSeq chemistry, evaluating assay performance at critical enzymatic steps. For sequencing data analysis, we employed an automated single-cell genome editing analytical pipeline to quantitatively evaluate on- and off-target editing efficiency, co-occurrence, and zygosity of the experiments. We also compared the single-cell assay performance with available orthogonal bulk assay, rhAmpSeq assay in detecting indels. The integration of the panel design pipeline, chemistry and analytical software from both rhAmpSeq and the single-cell workflow enables a highly customizable, comprehensive, yet cost efficient approach to evaluate on- and off-target with single-cell context which is highly valuable to genome editing applications in the CGT field.

1635 RGENtool - a CRISPR/Cas double-strand cleavage evaluator

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CRISPR/Cas is a powerful gene-editing tool that allows precise genome modifications. However, unintended off-target effects can lead to unwanted genetic changes at unintended sites. Evaluating off-target effects is crucial in research, therapeutic development, and regulatory assessments to ensure the safe application of CRISPR/Cas technologies in clinical settings. Next-generation sequencing (NGS) offers a high-throughput method for DNA sequencing, enabling comprehensive genome analysis to detect both intended and unintended edits. Various assays, such as RGEN-seq, INDUCE-seq, AID-seq, and SITE-seq are designed to identify double-strand cleavage by CRISPR/Cas and may show similarities in analyses, allowing for a universal pipeline to assess off-target effects.

From the large volumes of NGS data generated in CRISPR/Cas experiments, we can extract two key aspects: read coverage around cut sites and sequential homology around those sites. These indicators are cruicial for identifying authentic CRISPR/Cas cleavage. We use Convolutional Neural Networks (CNNs) for pattern recognition of both indicators. Given the interconnection between coverage signatures and homology, our RGENTool's initial coverage-CNN automatically generates training data for the downstream homology-CNN. Additionally, we have developed a customized pipeline for efficient and specialized CRISPR/Cas alignment. This pipeline includes trimming to narrow the alignable region and automatic extension at the flanks of the Smith-Waterman alignment, along with a text-based visualization system for human analysis. Ultimately, we predict CRISPR/Cas cut sites with varying confidence levels based on the combined results of coverage and homology. We have also designed a robust reporting folder system to summarize and visualize these results.

1636 Enhancing Precision and Safety in CRISPR/Cas Gene Editing: A Combined Off-Target Detection Strategy

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The advent of CRISPR/Cas systems has revolutionized gene editing, providing unprecedented precision for modifying genomes to treat genetic disorders and advance biological research. However, the potential for unintended genetic alterations, or off-target effects, poses significant safety concerns. Current detection methods lack systematic approaches, underscoring the need for an integrated strategy that enhances safety protocols in gene editing applications. Here, we introduce a combined off-target detection approach to enhance the reliability of CRISPR applications, making it an essential tool for advancing precision medicine.
We utilized a diverse range of computational tools to predict potential off-target sites based on the sgRNA sequences. These tools incorporate various algorithms and machine learning techniques to assess the likelihood of unintended genetic alterations.
Based on the predictions, we identified a range of potential off-target sites and designed probes for hybrid capture to target these regions. The captured DNA fragments from biological samples were then sequenced and analyzed to assess off-target risks. This method efficiently enriched and detected off-target events, confirming and quantifying the predicted sites with high precision, thus enhancing the accuracy and safety of our CRISPR/Cas system.
These computational predictions provided a foundational list of potential off-target sites, which were then combined with experimental prediction in later stages of the study. By combining multiple predictive models, we ensured a comprehensive assessment of off-target risks.

1637 Type V CRISPR nuclease edit patterns enable highly sensitive off-target detection

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The CRISPR Cas12i nuclease family represents a promising therapeutic tool, but the off-target effects remained to be understood. This study characterizes the off-target activity of Cas12i2, an engineered type V nuclease, to ensure the safety of therapeutic applications. We have previously demonstrated that Cas12i2 generates predominantly 5-20

nucleotide deletions at the target site with a reproducible and target-dependent pattern. To characterize off-target editing outcomes, we designed a library of guide RNAs and corresponding potential off-target sites, a total of 60,000 on- and off-target sequences. For those active off-target sites verified in the library, deletions are observed in much greater frequency than insertions, similar to the on-target pattern. Enabled by the large deletion pattern, we further demonstrated that we could detect editing activity in off-targets at approximately 10-fold higher sensitivity than reported by using other CRISPR nucleases in the field. This high sensitivity allows for the rigorous identification and selection of safe and effective Cas12i2 guide RNAs. This work significantly enables our assessment of Cas12i2's safety profile and advances its potential for therapeutic applications.

1638 Sensitive Rearrangement Detection at CRISPR On- and Off-target Editing Loci Using SAFER Detection

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Gene editing strategies that target multiple loci using engineered CRISPR Cas systems or other sequence-specific gene editing nucleases run a high risk of generating chromosomal rearrangements. Here, we applied Selective Amplification for Efficient Rearrangement Detection (SAFER detection) to detect intrachromosomal deletions and inversions as well as inter-chromosomal translocations involving loci for several therapeutic guide RNAs targeting *CCR5, FXN, TRAC, TRBC1, TRBC2, B2M, and PDCD1*. The assay uses transposome-based tagmentation of genomic DNA, followed by selective amplification of rearranged molecules using a target-specific primer and locked nucleic acid blocker. The target specific primer is typically anchored near the on-target locus where double strand breaks are intended to occur, but it can also be designed to bind near any known off-target locus. The assay can be performed in less than 1 week; it requires less genomic DNA than competitive methods, and sensitively detects rearrangements involving the target locus, in a genome-wide, unbiased way. Our analysis pipeline quantifies independent rearrangement events involving split reads with UMIs across multiple sample replicates, providing tabular and graphical summaries. Rearrangements with breakpoints in close proximity to regions of homologous sequence or off-target editing sites are classified as homology-mediated, or off-target mediated, respectively.

For the *CCR5* guide, we detected off-target and homology-mediated events at the *CCR2* locus and off-target mediated translocations involving loci in chr13, 19, and 20 that had been characterized previously by others using the same guide. A novel chromosomal translocation, mediated by off-target editing near oncogene *PTP4A2* on chr1, was detected at an off-target site with 3 mismatches and 1 indel when aligned to the target. These sites were validated by PCR and dPCR. Editing with guides in the homologous regions of TRBC1 and TRBC2 exon 1 results in frequent deletions and inversions of the intervening region on chromosome 7. We also investigated a model system for treatment for Friedreich's ataxia in which two guides at adjacent loci on chromosome 9 were used to edit a lymphoblastoid cell line from a patient with Friedreich's ataxia carrying an expanded GAA repeat cluster at the Frataxin locus (Rocca *et al*, 2020). SAFER detection identified frequent deletions, as described in the initial study, but it also detected inversions of the GAA repeats between the two target loci.

The SAFER detection assay is complementary to nomination methods such as GUIDE-seq and ONE-seq, as it provides a cost-effective approach to assess the risk of chromosomal rearrangements in therapeutic genome editing, and is especially valuable where multiplex editing strategies are employed. The method can also be adapted to identify viral or transposon integration sites.

1639 Preventing large deletions and chromosome loss in engineered human primary T cells by CasPlus with optimized guide RNAs

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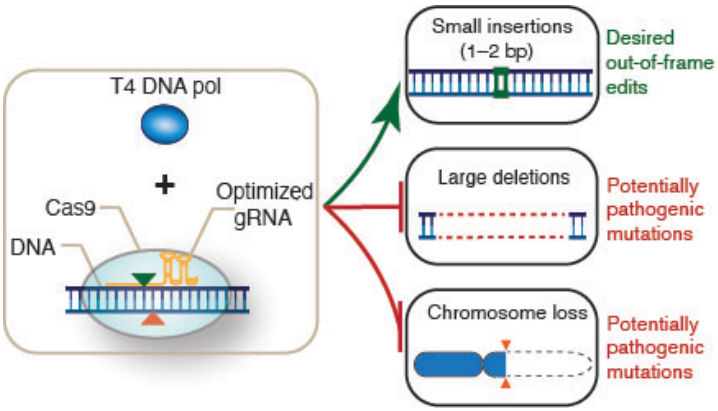
Genetically engineered T-cell therapies rely heavily on genome editing tools, such as the CRISPR/Cas9 system. However, unintended on-target chromosomal alterations, including large deletions and chromosome loss can occur

and pose significant risks including tumorigenesis. Here we combined CasPlus and optimized guide RNAs to reduce these issues in CRISPR/Cas9 engineering human primary T cells. CasPlus, which integrates an engineered T4 DNA polymerase with Cas9 nuclease and guide RNA, promotes favorable small insertions (1-2 bp) while reducing large deletions and chromosome loss in T cells. Our optimized guide RNAs favoring small insertions reduced large deletions and chromosome loss by two- to five-fold versus those favoring small deletions. Moreover, combining optimized guide RNA with T4 DNA polymerase further synergistically reduced large deletions and chromosome loss by additional two-fold. Notably, replacing currently used guide RNA pairs in clinically applications with optimized pairs biased towards small insertions, along with CasPlus instead of Cas9, for editing greatly reduced large deletions and chromosome loss in gene-edited human primary T cells. These findings demonstrated that pre-selecting target sites favoring small insertions via guide RNA optimization coupled with CasPlus editing is a safer and more effective strategy to improve genome stability in T-cell engineering and other gene-editing applications.



1640 Potent and specific activation of fetal hemoglobin expression by cis-regulatory interference

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Control of gene expression is mediated by sequence-specific binding of transcription factors (TFs) in regulatory DNA, and genetic variants that modulate TF binding underlie many disease phenotypes. Several naturally-occurring genetic variants within the γ-globin promoter have been identified that attenuate the binding of transcriptional repressor proteins, resulting in hereditary persistence of fetal hemoglobin (HPFH) and clinical silencing of co-inherited sickle cell disease or β-thalassemia. Currently it is not possible to phenocopy the effects of such genetic variants without introducing alterations to DNA, which may carry unknown downstream risks. To phenocopy the effects of genetic regulatory variants using a non-invasive approach, we developed highly specific, single chain cis-regulatory interfering proteins (cRIPs) capable of precisely displacing a transcriptional repressor or activator from a single site in the human genome. We show that delivery of a cRIP targeting the -200 region of the γ-globin promoter via either mRNA or lentivirus potently and specifically reactivates fetal hemoglobin expression and reverses hemoglobin switching in both human primary erythroid cells and humanized mice. We show further that cRIPs can be deployed combinatorially to enable complex control of gene expression including quantitative gene silencing or activation via the displacement of activating or repressing transcriptional factors in either promoters or enhancers, and are capable of phenocopying the effects of diverse genetic regulatory variants. Cis-regulatory interfering proteins present a new modality for precisely and non-destructively controlling endogenous gene expression with direct therapeutic application to hemoglobinopathies.

1641 Robust Genome Editing with Reduced Cytotoxicity and Enhanced Engraftment Using Protein-Assisted Delivery of Cas12a-RNP into Human HSPCs

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Genome editing technologies, such as CRISPR-Cas, offer transformative potential for treating sickle cell disease (SCD). SCD is caused by a heritable mutation in the β-globin gene, impairing oxygen transport and resulting in anemia, acute pain crises, ischemic tissue damage, and early mortality. Emerging therapies for SCD focus on autologous hematopoietic stem and progenitor cell (HSPC) transplantation after *ex vivo* genome editing. Given that patients with SCD often have reduced stem cell numbers and compromised cell quality, maintaining the viability and functionality of edited HSPCs is essential for successful therapeutic outcomes. Electroporation (EP) is a widely used method for delivering ribonucleoprotein (RNP) editors into HSPCs, but it presents significant challenges, including cytotoxicity and disruptions to global RNA expression, which negatively impact stem cell function. Protein-assisted genome editing, utilizing cationic endosomal-escape peptides (APs), offers a promising alternative by enhancing RNP delivery and minimizing cellular stress, potentially improving editing efficiency and HSPC recovery.

In this study, we utilized an AP for the transfer of Cas12a-containing RNPs to induce BCL11A editing in healthy human CD34+ HSPCs to boost fetal hemoglobin expression. Cell recovery in AP-treated groups (50-200 μM AP) was significantly higher (70-86%) compared to the EP group (53.6%). Higher concentrations of AP (150-200 μM) achieved editing levels comparable to EP (83.5-87.7% vs. 86.7%, respectively). Furthermore, significant γ-globin expression was achieved in both the EP and high-AP groups (125-200 μM) compared to non-edited controls (21.5-23.3% vs. 6.2%). Similar improvements in cell recovery and editing efficiency were observed in SCD CD34+ HSPCs, with 52.3% cell recovery and 80.5% editing in the EP group and 72.4-91.3% cell recovery and 77-78% editing in the AP groups, further demonstrating the potential of AP-based delivery for improving HSPC editing in both healthy and SCD samples.

Next, we transplanted non-edited, AP-edited, and EP-edited SCD CD34+ HSPCs into NBSGW mice, along with two 1:1 mixed groups of EP- and AP-edited cells (marked with GFP or YFP) in a competitive transplantation assay. After 16 weeks, human cell engraftment in peripheral blood (~35%) and bone marrow (~96%) was comparable across all groups. F-cell and γ-globin levels were significantly elevated in both the AP- and EP-edited groups compared to the non-edited control group (46.8-48.1% vs. 15.4% for F-cells and 23.2-29.1% vs. 6.0% for γ-globin), with no significant difference between the two edited groups. Importantly, the competitive assays revealed that AP-edited cells exhibited significantly higher engraftment frequencies than EP-edited cells, demonstrating a survival advantage to AP-edited cells despite similar initial editing efficiency. Secondary transplants have now been performed and will provide further insights into long-term outcomes, with updates to follow.

In conclusion, AP-based RNP delivery offers a gentler alternative to traditional electroporation for editing HSPCs. Despite comparable editing efficiencies, AP-based delivery resulted in improved cell recovery, better engraftment, and enhanced survival in a humanized NBSGW mouse model. These findings highlight the potential of AP-assisted genome editing to optimize *ex vivo* gene therapies for SCD, offering a more efficient and safer approach to clinical translation.

1642 Engineered Extrachromosomal Technologies for Tunable and Persistent Control of Therapeutic Gene Expression in Human Cells

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Though retroviral vectors have seen clinical success with respect to delivering transgenes to primary human cells, they are prone to random genomic integration of payloads, which poses clinical drawbacks, such as the disruption of endogenous genes, epigenetic silencing of integrated transgenes over time, and oncogene activation. These drawbacks converge to make the use of programmable non-integrating methods of transgene expression attractive. Current non-integrating viral systems used in therapeutic contexts, such as adeno-associated virus (AAV), are limited by small payload capacities or extensive modifications designed to reduce virulence and immunogenicity. These modifications often lead to transient transgene expression. Additionally, both integrating and non-integrating approaches lack the ability to fine-tune transgene expression levels.

To overcome these limitations, we developed a novel technology that recapitulates the long-term gene expression strategies of latent non-integrating viruses using a synthetic biology toolset. This approach enables the creation of persistent and programmable extrachromosomal expression systems that provide user-defined tunability of transgene expression and maintenance of genetic components. Specifically, we designed and optimized a class of modular, non-integrating DNA systems that are stably maintained through mitotic stability and host-mediated replication. We hypothesized that this system would allow episomal DNA to persist in dividing human cells without requiring genomic integration.

We first constructed and tested an engineered tethering system to site-specifically control the targeting and engagement frequency between extrachromosomal DNA and the human genome in live cells. Using ChIP-qPCR, we confirmed that the tethering modules effectively bind both the human genome and synthetic extrachromosomal DNA. Fluorescence in situ hybridization (FISH) and high-resolution microscopy further verified co-localization. We then transfected human cells with episomes containing EGFP, our tethering system, and an engineered replication module. By optimizing the number of tether binding sites in the host genome, we demonstrated long-term persistence of EGFP expression for over 3 months post-transfection. qPCR analysis of isolated genomic DNA confirmed that persistence was not due to off-target genomic integration of the payload.

To enhance versatility for both viral and non-viral delivery methods, we developed truncated versions of the tethering and replication modules. These compact designs enable us to successfully deliver our system to primary human T cells. Our goal is to deliver therapeutic payloads, such as anti-CD19 CAR, for targeted killing of B cell lymphomas, without any genomic integration. Importantly, this platform allows for modulation of CAR expression levels, and this tunability, combined with the absence of genomic integration, offers an alternative approach for CAR T cell therapy. In summary, we created a novel approach for maintaining extrachromosomal DNA payloads in human cells, demonstrating the ability to sustain therapeutic gene expression in dividing primary cells without genomic integration. This extrachromosomal genetic technology offers new possibilities for transgene-based treatments, mitigating the risks associated with altering the endogenous human genome.

1643 Self-Targeting CRISPR-CasX-Editor AAV Vectors Enable Potent and Controllable On-target Editing in the Murine CNS

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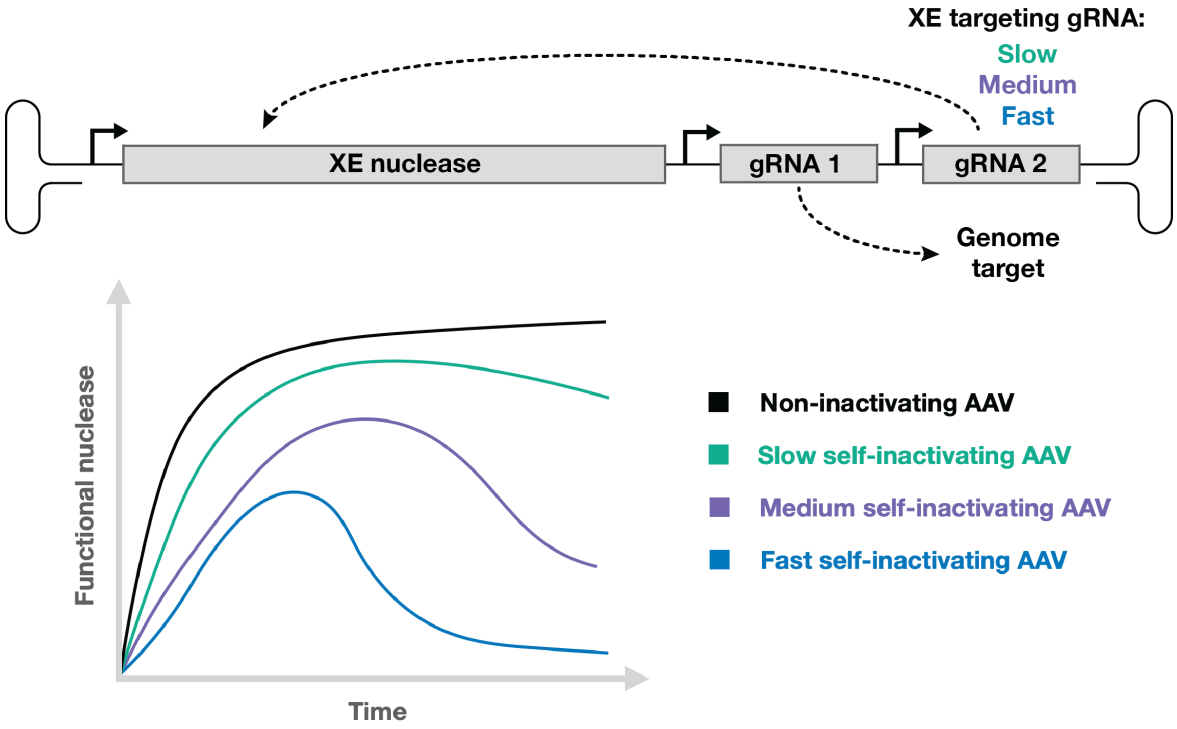
Adeno-associated viral (AAV) vectors are among the most promising vehicles for delivering genetic cargo, including CRISPR-based editing technologies, to the CNS. However, long-term expression of CRISPR from AAV

raises concerns about undesired off-target effects. To mitigate this, we have engineered self-inactivating AAV (siXAAV) vectors that self-target and cleave their CRISPR components, thereby limiting constitutive nuclease expression once the desired edit is achieved.

Our siXAAV system features a potent, highly engineered, compact CasX-Editor (XE) in a single AAV vector with multiple guide RNAs (gRNAs), including one on-target gRNA and a second, lower-activity gRNA that targets the XE coding sequence for self-cleavage and inactivation.

To develop siXAAV, we systematically screened dozens of XE-targeting gRNAs—varying guide-target mismatch designs—to identify distinct self-cleavage potencies and kinetics capable of maximizing on-target genomic editing before self-inactivation. We employed a tagged XE plasmid system to quantify XE expression, self-targeting, and on-target editing, ultimately selecting three prototype gRNAs for *in vivo* validation. We then packaged the siXAAV: XE, a safe harbor-targeting gRNA, and a self-targeting gRNA into a single AAV vector. Assessment of packaged AAV genome integrity revealed high quality genomes with no measurable indels at the self-targeting sites. We administered siXAAV and non-inactivating control AAV vectors via intracerebroventricular injection into adult C57BL/6 mice. Tissues were analyzed at 1 and 2 months post-injection for vector biodistribution, on-target genome editing, and self-targeting events.

Both siXAAV and control AAVs achieved robust on-target editing. Crucially, siXAAV-treated mice showed editing within the target sites in the XE coding sequence, confirming self-cleavage. Despite high self-editing rates, vector distribution remained stable, indicating potential episome repair rather than degradation. Notably, while controls exhibited small increases in on-target editing over time, siXAAV-treated animals did not, indicating effective nuclease self-inactivation. This work demonstrates the feasibility of developing self-inactivating AAVs for transient but potent *in vivo* genome editing, offering a promising avenue for safer therapeutic applications by avoiding long-term nuclease expression.



1644 CRISPR/Cas9 enables efficient knockout of CD52 without CD3/CD28 in expanded CMV-specific T cells

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Adoptive immunotherapy using virus-specific T-cells (VSTs) has been successfully used in clinical trials to prevent and treat viral infections in immunocompromised patients post-hematopoietic stem cell transplantation (HSCT). Alemtuzumab, a lymphodepleting monoclonal antibody that targets the surface antigen CD52, is commonly used as a conditioning regimen to prevent the development of graft versus host disease (GVHD). However, it increases time for lymphocyte recovery post-HSCT and the potential risk for viral infections and reactivations. CD52 has no known critical impact on the immune system, and there have been no reported toxicity by knocking out CD52 per prior phase I chimeric antigen receptor (CAR) T-cell cancer immunotherapy trials. Therefore, we predicted that using CRISPR-Cas9 mediated knockdown (KO) of CD52 in VSTs provides a potential novel preventative therapy early post-HSCT. We compared two methods of generating KO VSTs to determine the most effective way to remove CD52 while maintaining antiviral activity.

CRISPR single-guide RNA (sgRNA) were designed using CRISPick and CRISPR-DO. Resulting guides were chosen based upon predicted high efficiency and off-target sites. CD52 KO was performed in a Lonza Nucleofector System. Cas9 protein and sgRNA were used to electroporate PBMCs using the program for stimulated Human T cells. For method one, cryopreserved VSTs were thawed and activated for 72 hours using anti-CD3/anti-CD28 in RPMI media supplemented with GlutaMAX™, fetal bovine serum, and IL-2 (CTL media) and cultured for 5 days prior to RNP-CRISPR electroporation. In method two, we optimized our existing rapid ex vivo expansion of cytomegalovirus (CMV)-specific VSTs protocol to include gene editing on day 7 of VST expansion, followed culture in CTL media supplemented with IL-4 and IL-7 for an additional 5-7 days.

To confirm and assess the percentage of CD52KO on a protein level, VSTs were surface stained with CD52 antibodies and acquired on a CytoFLEX Cytometer. For indel evaluation, PCR products of isolated DNA were Sanger sequenced and KO rate was determined using ICE CRISPR Analysis Tool.

Six CRISPR sgRNA that targeted the CD52 gene were tested using method one with KO frequencies ranging from 18% to 85% seen on flow cytometry. The two sgRNAs with higher efficiency (sgRNA1 - 83%, and sgRNA2 - 85%) were further investigated. In silico analysis identified 88 and 168 off-target sites requiring additional analysis for sgRNA1 and sgRNA2, respectively, but none involved known oncogenes. Rhampseq performed on CMV-VSTs for sgRNA1 showed no significant off-target editing at any site of interest in the edited population compared to non-edited control. Furthermore, sgRNA1 (UUACCUGUACCAUAACCAGG) had a 70% ICE-d score via ICE analysis.

After comparing both stimulation methods, we were able to successfully optimize and generate CD52 KO CMV-VSTs without the use of anti-CD3/anti-CD28 (Figure 1) which is thought to maximize CRISPR-Cas9 editing efficiency. We instead did electroporation during ex vivo expansion of VSTs in the presence of growth-enhancing cytokines and viral peptides and achieved comparable KO of CD52,73.2% (method two) compared to 82.5% (method one), N = 3 (Figure 2).

This demonstrates the feasibility of CD52 KO with peptide stimulated T cells in absence of CD3/CD28 and could facilitate production of “next generation” gene-edited VST product to restore antiviral immunity early post HSCT alongside prevention of GVHD.

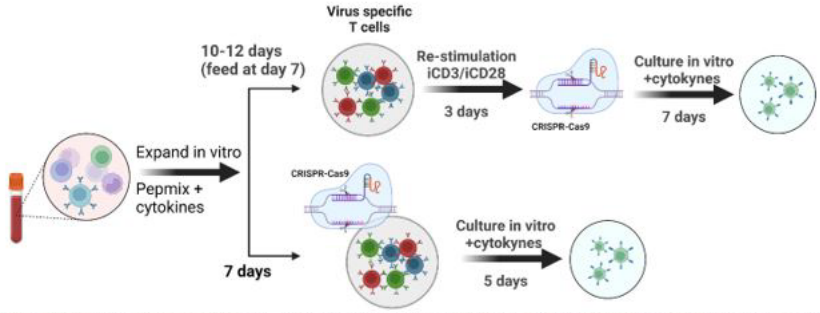

Figure 1CRISPR/Cas9 mediated knockout (KO) of CD52 in CMV-specific VSTs. (A) Schematic summary of the protocol for CRISPR-Cas9-mediated KO of CD52 in primary human VSTs. PBMCs were stimulated with a CMV pepmix in the presence of IL-4 and IL-7. On day 7 of in vitro expansion, primary human VSTs were nucleofected with control Cas9 alone or Cas9 preloaded with gRNAs targeting the CD52 gene. KO efficiency and functional assays were performed at day +12 post-initial PBMC isolation.

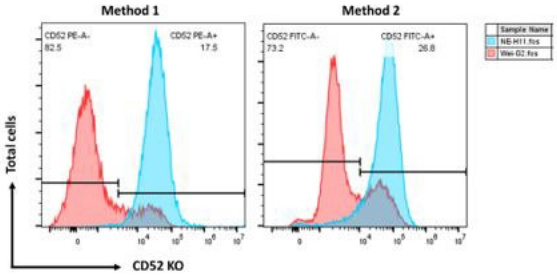

Figure 2 KO efficiency for two different stimulation methods to generate CD52 KO CMV-VSTs.

1645 ADAR-Mediated RNA Editing of Premature Termination Codon Results in Functional Correction in *MECP2* for Rett Syndrome

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Rett Syndrome is a severe neurodevelopmental disorder affecting approximately 350,000 people worldwide. It mainly occurs in females and is caused by mutations on the methyl CpG binding protein 2 (*MECP2*) gene on the X-chromosome. This gene codes for the MECP2 protein which is a key regulator of gene expression and is vital for maintaining normal brain function and development. The severity of the disease is determined by the location, mutation type and level of X-chromosome inactivation. The premature termination codon gives rise to p.R270X which is one of the most commonly occurring pathogenic variants of MECP2. Axiomer, an RNA editing platform, uses chemically modified editing oligonucleotides (EONs) to recruit and guide endogenous Adenosine Deaminase Acting on RNA (ADAR) to perform adenosine to inosine (A-to-I) editing on double stranded RNA, which can restore the precise regulatory function of the MECP2 protein.

ADAR-mediated RNA editing edits the stop codon (X) to a Tryptophan (W) coding codon, leading to a functional MECP2 protein. EONs were designed and screened in vitro in patient primary fibroblasts to assess editing efficiency, impact on RNA and protein levels. Three EON optimisation rounds resulted in highly efficient A-to-I editors. R270X MECP2 patient fibroblasts were transfected with EON, and editing efficiencies were measured. Top performing EONs demonstrated mean editing efficiencies between 50-60%. Additionally, a time course experiment was performed where editing was measured for 5 days after a single dose treatment. Sustained editing was measured from days 1-5.

Premature termination codons often lead to nonsense-mediated decay (NMD) which reduces RNA levels. Total RNA copies of MECP2 were measured in patient fibroblasts treated with EONs vs. non treated, to assess differences in copy number. An approximately 4-fold increase in total RNA copies was measured for top performing EONs when compared to the non-treated sample, indicating inhibition of NMD.

Furthermore, upon EON transfection of HeLa MECP2 KO cells, R270W MECP2 protein levels were approximately 8-fold higher than R270X MECP2 protein levels in the untreated sample. This shows transcriptional read through and translation into a functional MECP2 protein.

ADAR-mediated RNA editing of the mutated p.R270X MECP2 shows great potential as a disease modifying therapy for Rett syndrome. The dual action - recoding the premature termination codon and stabilizing the mRNA - obtained with EONs, could ensure that the corrected RNA is not only functional but also restored to physiological levels, maximizing therapeutic impact. Lead EONs are currently being optimized for further development.

1646 Novel U7 Small RNA mediated modulation of TCF4 Promoter activity as a therapeutic strategy for Pitt-Hopkins syndrome

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Introduction: Pitt-Hopkins syndrome (PTHS) is an autosomal dominant disorder with an estimated prevalence of 1 in 41,000 individuals. It results from mutations in the TCF4 gene and is marked by intellectual disability, significant delays in motor development, absence of functional speech, and breathing difficulties. TCF4 is a basic helix-loop-helix (bHLH) protein that is broadly expressed, especially in the nervous system, and regulates gene expression by interacting with other bHLH proteins to either promote or inhibit transcription. Due to its large size and multiple known protein isoforms, TCF4 poses a challenge for traditional gene replacement approaches. However, since patients still have one functional copy of the gene, enhancing TCF4 expression presents a promising alternative. In this study, for the first time, we modified U7 small nuclear RNAs (U7-snRNAs) to modulate TCF4 promoter activity and enhance its expression, proposing a novel gene therapy approach for PTHS.

Methods: We designed 3 U7 small RNA cassettes targeting different regions of the TCF4 promoter that we predicted could modulate the promoter activity. We assessed the efficiency of U7 cassettes through transient transfection in HEK293 cells to evaluate their ability to enhance TCF4 expression. Next, we generated AAV9 viral particles and validated their effectiveness in an in vitro model with human PTHS patient cell lines, employing RT-PCR and immunofluorescent imaging to measure TCF4 expression. Functional assays were used to determine the effect of the treatment on cellular health as well. In addition, we used mouse embryonic fibroblasts (MEFs) from a mouse model that expresses the green fluorescent protein under the human TCF4 promoter as an additional model system to further validate our approach. We also evaluated the efficiency of AAV9.U7-TCF4 in enhancing TCF4 expression in a transgenic mouse model that carries the human TCF4 promoter with GFP downstream of TCF4 gene.

Results: In vitro studies in HEK293 cells and a PTHS patient induced astrocytes demonstrated elevated expression of TCF4 following transient transfection of U7 cassette, as confirmed by qRT-PCR and immunofluorescence staining after 72hrs of transfection. The enhanced expression of TCF4 improved the survival of co-cultured mouse motor neurons interacting with patient-derived induced astrocytes, providing functional evidence of the treatment's efficacy. Moreover, using mouse embryonic fibroblasts expressing GFP under the control of the human TCF4 promoter, we showed upregulation of GFP expression 72 hrs after treatment with AAV9.U7-TCF4. Interestingly, stereomicroscopic imaging of brain tissues showed increased GFP fluorescence in the injected animals compared to the uninjected controls after 6 months post-injection, which confirms the TCF4 protein expression. The qRT-PCR

analysis of Intracerebroventricular injection of 4.4´1010 dose of AAV9.U7-TCF4 into a transgenic mouse model showed a 1.4-fold increase in TCF4 transcript levels at 1- and 6-months post-injection.

Conclusion: We developed a novel U7-snRNA-based technology that enables modulation of TCF4 promoter activity. Altogether, our in vitro results in HEK293 cells, PTHS patient-induced astrocyte cell lines, and mouse embryonic fibroblasts expressing GFP under the human TCF4 promoter, along with in vivo studies in a transgenic mouse model, demonstrate the effectiveness of this approach. Our novel therapeutic strategy holds great p

1647 Combined Targeting of *HTT*, *HTT1a* and Somatic Instability in Huntington's Disease using LinQURE®: a Feasibility Study in Multiple HD Mouse Models

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Huntington's disease (HD) is an autosomal dominant disorder caused by an expanded CAG repeat in the huntingtin (*HTT*) gene. The unstable CAG repeat expansion leads to toxic gain-of-function of *HTT* mRNA and protein species, causing cellular dysfunction and neurodegeneration. There are currently no disease modifying therapies for HD. Many therapies that are under development, including uniQure's clinical-stage candidate AMT-130, are designed to lower HTT levels, with the goal of slowing neurodegeneration.

Recent studies indicate that somatic expansion of the CAG repeat in mutant *HTT* through aberrant mismatch repair is a pivotal disease contributor, with MutS homolog 3 (*MSH3*) as a promising therapeutic target. Furthermore, there is increasing evidence for a repeat-length dependent increase in expression of a truncated isoform called HTT1a, which is considered to be the most toxic HTT protein species. Here, we have explored the feasibility of simultaneous dual or triple targeting of *HTT*, *HTT1a* and *MSH3*, using uniQure's LinQURE® AAV platform, as a potential next-generation therapeutic approach for HD.

Therapeutic miRNAs targeting *HTT1a* or *MSH3* were screened *in vitro*, and several lead candidates were selected for *in vivo* evaluation in relevant HD mouse models. We tested the individual *HTT1a*-targeting candidates in YAC128 mice, showing preferential lowering of the HTT1a protein and more modest reductions of full-length HTT. Individual *Msh3*-targeting candidates were tested in $Hdh^{Q111/+}$ mice, showing potent reduction of *Msh3* mRNA and a concomitant reduction in somatic repeat instability. We then proceeded to test multi-target approaches using LinQURE® in two different HD mouse models: R6/1 and BAC-CAG. Dual *Msh3* and *HTT* targeting led to a potent, dose-dependent reduction of *Msh3* mRNA and was associated with reduced somatic instability in R6/1 mice. Finally, dual and triple *Msh3*-, *HTT*- and *HTT1a*-targeting LinQURE® candidates all showed potent target engagement and rescue of the molecular phenotype in BAC-CAG mice. Importantly, we could recapitulate both the allele-preferential effect of *HTT1a*-targeting, as well as the inhibition of somatic instability by reducing *Msh3* expression, in multiple HD mouse models.

These data support the feasibility of concurrently targeting *HTT* and *MSH3* using LinQURE®, as well as the option to preferentially lower mutant *HTT1a* using a miQURE® or LinQURE® approach. Such approaches hold major promise as potential next-generation gene therapies for HD.

1648 PYC-002, a Novel Antisense Oligonucleotide Targeting SHANK3 Haploinsufficiency in Phelan-McDermid Syndrome

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Phelan-McDermid Syndrome (PMS) is a rare neurodevelopmental disorder generally diagnosed between 3 and 6 years of age. It is caused by deletions or mutations resulting in haploinsufficiency of the SH3 and multiple ankyrin repeat domain 3 (SHANK3) gene, which is critical for synaptic structure and function. These genetic alterations result in various behavioural and physical symptoms, that can include speech impairment, intellectual disability, seizures, and autism, which permanently affect the quality of life for patients. Currently, there is no effective treatment available for PMS patients, which highlights the significant unmet medical need for this disorder.

This study aims to develop a therapeutic approach for PMS by upregulating SHANK3 expression levels using antisense oligonucleotides (ASOs). ASOs are short synthetic nucleic acids that modulate gene expression through ASO-mRNA interactions and provide a precise and reversible method to address this deficiency.

ASOs were designed to target regulatory regions of the *SHANK3* transcript, to enhance the expression of functional SHANK3 protein isoforms. Initial ASO screening in SH-SY5Y neuroblastoma cells identified a hotspot region within the *SHANK3* gene that could be targeted to increase SHANK3 protein levels by 1.3-1.5-fold ($p<0.01$). Subsequent screening was conducted in healthy induced pluripotent stem cell (iPSC)-derived glutamatergic neurons and ASO candidates that induced a 1.4-2.3-fold uplift in SHANK3 protein expression were selected for further assessment. The pharmacodynamic profile of two ASOs was then assessed in iPSC-derived glutamatergic neurons differentiated from four PMS patients, with both candidates demonstrating robust patient-agnostic efficacy. The lead ASO, 'PYC-002', was evaluated *in-vivo* to determine safety and efficacy in wild-type Sprague-Dawley rats following intrathecal injection. PYC-002 was well-tolerated and a 1.2-1.5-fold upregulation of SHANK3 protein was observed in brain regions that are affected by disease in PMS patients.

Our data highlights that PYC-002 is a promising clinical candidate to treat PMS by restoring SHANK3 expression. This novel approach addresses an urgent unmet need and extends ASO application to a broader spectrum of neurodevelopmental disorders. Future research will investigate the effect of optimized PYC-002 delivery on neuronal function, with an Investigational New Drug submission projected for 2026.

1649 First in Class ASO Targeting *A53T* Allele: Preclinical Efficacy

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Introduction: First delineated by Dr. Polymeropoulos et al. in 1997, the Ala53Thr (A53T) missense mutation in *SNCA* has been identified as one of the most significant risk factors in early-onset Parkinson's disease (PD) (Polymeropoulos et al., 1997). We propose an antisense oligonucleotide (ASO)-based allele-specific strategy to target the mutant A53T *SNCA* allele and reduce resulting aberrant α-syn production, without downregulating the wild-type (WT) allele. This strategy is unique compared to existing ASOs under development for the treatment of PD, as these target the general knockdown of SNCA via recruitment of RNase H activity despite mutation status. α-syn is critical for neuronal function and survival; depletion in the functional pool of α-syn by RNAi-mediated silencing induces neurodegeneration (Gorbatyuk et al., 2010). An allele-specific therapeutic that targets just the mutant A53T allele ensures that healthy levels of α-syn are maintained by the WT allele. Given the high prevalence of this disease, a treatment that works by directly targeting an underlying molecular cause of PD holds significant potential to significantly improve PD patients' disease manifestation and quality of life.

Methods: We designed an 18-mer gapmer phosphorothioate (PS) methoxyethyl (MOE) ASO that selectively targets the *A53T* mutant allele. This ASO was designed to have a higher affinity for the mutant allele, as predicted *in silico,* as opposed to the same sequence from WT of the same length. HEL cells were incubated for 72-hours with the A53T-specific ASO (at 1μM) using gymnotic uptake. The HEL cell line was chosen due to its abundant SNCA expression.

Results: ASO treatment resulted in a 40% reduction of SNCA expression. This knockdown with qPCR is consistent with RNAseq data. Positive control gapmers as well as siRNA were used as controls. Limited off-target effects of this ASO were demonstrated *in silico*. Furthermore, fibroblasts from a patient with confirmed A53T mutation status were obtained and iPSCs were generated. Currently, further molecular characterization in a human iPSC-derived PD model is planned to assess the effects of A53T-specific ASO silencing in rescuing PD phenotype. This model contains dopaminergic, GABAergic, and glutamergic neurons, which display disease-relevant phenotypes at basal conditions which correlate with the brains of A53T PD patients.

Conclusions: It has been previously demonstrated that using a CRISPR-Cas9 system to delete the A53T *SNCA* mutation improved PD-related conditions, including α-syn overproduction, reactive microgliosis, dopaminergic neurodegeneration, and motor symptoms (Yoon et al., 2022). Achieving a ratio in favor of mutant allele downregulation is desirable as it does not knockdown the WT allele while targeting the aberrant gain-of-function allele, a phenomenon that is not possible with other proposed lines of treatment. We present a novel ASO that achieves this goal *in vitro*. An iPSC-based system containing a mixed neuronal population is a more comprehensive model for A53T synucleinopathy and for assessing therapeutic agents. We plan to utilize this model to examine the efficacy of our ASO in reducing numerous parameters, including the presence of Lewy neurites, Lewy bodies, extracellular Lewy body-like formations, varicosities, bulbous endings, and degenerating neurons.

1650 Exon 45 Skipping, Dystrophin Production, and Functional Improvement with ENTR-601-45 in Preclinical Models of Duchenne Muscular Dystrophy

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Currently approved exon skipping phosphorodiamidate morpholino oligomer (PMO) therapies for Duchenne muscular dystrophy (DMD) produce modest amounts of dystrophin due to poor cell entry and limited escape from the endosome. In order to overcome these limitations, we developed a family of therapeutics consisting of Endosomal Escape Vehicle (EEV™) cyclic cell penetrating peptides conjugated to exon skipping PMOs.

ENTR-601-45 is an exon 45 skipping PMO conjugated to the EEV platform and is in development for the treatment of exon 45 skip-amenable DMD. Here, we examined the efficacy of ENTR-601-45 in cell and animal models. Treatment of exon 45 skip-amenable, DMD patient-derived skeletal and cardiac muscle cells with ENTR-601-45 demonstrated robust, dose-dependent exon 45 skipping and dystrophin production. To assess the therapeutic potential of ENTR-601-45 in vivo, we utilized del44hDMD.*mdx* mice containing a human dystrophin-expressing transgene (hDMD) with *DMD* exon 44 deleted on an *mdx* background. These mice are amenable to human exon 45 skipping allowing for quantification of both exon 45 skipping and dystrophin production *in vivo*. Three doses of ENTR-601-45 administered every six weeks (Q6W) to del44hDMD.*mdx* mice produced robust, dose-dependent exon 45 skipping, dystrophin expression, and proper localization to the sarcolemma in skeletal muscle 6 weeks after the third dose. In addition, three Q6W doses of ENTR-601-45 produced a dose-dependent reduction to damage after repeated eccentric contractions in the gastrocnemius skeletal muscle which was maintained in all dose groups 5 weeks after the third dose.

These findings demonstrate the efficacy of ENTR-601-45 in both cell and animal models of exon 45 skip-amenable DMD. Improved skeletal muscle function in an exon 45 skip–amenable DMD mouse model suggests that ENTR-

601-45 can produce functional dystrophin protein *in vivo*. Together, these results show the therapeutic potential of ENTR-601-45 and support further study in DMD patients amenable to exon 45 skipping.

1651 Translating *IGHMBP2* Variants with a CMT2S Patient-Specific Model: Personalized Medicine Rescue

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Introduction: Charcot-Marie-Tooth disease Type 2S (CMT2S) is a rare Charcot-Marie-Tooth disease subtype caused by pathogenic immunoglobulin mu-binding protein 2 (*IGHMBP2*) variants that result in abnormal RNA processing leading to alpha-motor neuron degeneration. A patient was reported with variants in *IGHMBP2*: a pathogenic missense variant acting in trans with a confirmed intronic cryptic splice site variant (c.1235+894 C>A) deep in intron 8. This non-coding *IGHMBP2* variant was shown to result in the creation of a new splice acceptor site, loss of reading frame and nonsense-mediated decay, ultimately reducing levels of IGHMBP2. We developed an antisense oligonucleotide (ASO) that targets this cryptic intronic variant to restore canonical splicing, rescuing levels of IGHMBP2. A microphysiological system (MPS) of the neuromuscular junction (NMJ) was utilized to characterize the CMT2S phenotype. Our objective was to rescue neuromuscular defects caused by this variant with our novel ASO.

Methods: We designed a 19-mer ASO targeting this cryptic intronic variant. To develop an *in vitro* model for this CMT2S patient, patient cells were transformed into induced pluripotent stem cells (iPSCs) and differentiated into motor neurons (MNs). The NMJ was analyzed using a patient-derived 'human-on-a-chip' NMJ system created by Hesperos Inc. CMT2S-MNs were integrated into a dual-chamber NMJ system. NMJs underwent ASO treatment and were assessed. To evaluate axonal defects, CMT2S-MNs were integrated on microelectrode arrays with wild-type (WT) Schwann cells. Conduction velocity (CV) effects were assessed before and after ASO treatment.

Results: In patient fibroblasts, ASO treatment significantly increased the ratio of restored WT transcript to cryptic exon containing transcript and resulted in over a 50% increase in IGHMBP2 protein levels. CMT2S-MNs displayed an overall functional decay at later stages of development, aligning with the motor function decay observed in CMT2S patients. CMT2S-NMJ analyses revealed a higher fatigue index (FI) than WT-NMJs and showed high levels of decay and chaotic tetanus. Following treatment with VCA-894A, we demonstrate rescue of NMJ functioning, captured by a decrease in FI and decay and chaotic responses. CMT2S-MN CV was evaluated by assessing average velocities of all conductions and the number of conductions over 28 days. CMT2S-MNs exhibited consistently fewer conductions at all testing time points and a reduced average conduction velocity, with the exception of Day 14. Following ASO treatment, CMT2S-MNs experienced a significant dose-dependent rescue of CV and number of conductions, as compared to WT control MNs and vehicle treated CMT2S-MNs.

Conclusions: We show rescue of NMJ functioning post-ASO treatment, captured by FI decrease and reduction in decay and sporadic responses. Furthermore, ASO treatment resulted in a dose-dependent increase in CV and number of conductions. This provides translational evidence that this ASO may improve fatigue and restore clinical motor control. We are further analyzing this patient-specific model to continue phenotyping CMT2S caused by *IGHMBP2* variants. N-of-1 ASO-based therapeutics may prove instrumental in the design of treatments for this diverse genetic disorder.

1652 Functionalized Nonsense Suppressor tRNA Picovectors Represent a Novel Therapeutic Cargo for the Treatment of PTC-associated Diseases

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Background: Nonsense mutations or premature termination codons (PTCs) occur when a canonical triplet nucleotide codon is converted into one of three stop codons (TGA, TAG and TAA). These mutations make up 10-15% of all genetic lesions that cause disease including Cystic Fibrosis (CF), Duchenne muscular dystrophy, spinal muscular atrophy, and choroideremia, among others. We have recently demonstrated the function of a series of nonsense suppressor anticodon edited tRNAs (ACE-tRNAs) to suppress CF-causing PTCs in the cystic fibrosis transmembrane regulator (*CFTR*) gene in gene-edited immortalized human bronchial epithelial (16HBE14ge) cells. A major advantage of ACE-tRNAs as a therapeutic cargo is the small (~125 bp) size of the ACE-tRNA gene including promoter and transcriptional terminator. Taking advantage of this property, we have previously encoded ACE-tRNAs in non-viral DNA minivectors, which impart several favorable characteristics to the vectors including increased cell transfection efficiency and higher molar equivalents of the cargo sequence per vector delivered. Through these efforts, we now understand that linear covalently closed DNA vectors with sizes as small as 200 bp can serve to produce a robust PTC suppression response. However, this approach towards a therapeutic cargo for treatment of PTCs had a poor outlook due to technical challengers related to minivector production methods, which exhibited poor scalability, adaptability, and GMP adherence.

Methods: With this in mind, we developed a novel assembly method for production of synthetic linear ACE-tRNA picovectors (sLPV; 'picovectors' owing to their small size) using substrates produced by solid-phase oligonucleotide synthesis, with DNA hairpins serving to covalently close the sLPV ends. Further, these hairpins were synthesized to contain internal DBCO functionalized nucleotides for labeling with azide functionalized moieties in copper-free click reactions. Introduction of this chemical functionality allows for dual labeling of the sLPVs with any combination of azide functionalized fluorophores, peptides, proteins, or other DNAs to improve either cellular delivery, intracellular trafficking, or intracellular imaging of the sLPVs. Using this approach we labeled ACE-tRNA picovectors with peptides comprising several different nuclear localization signals (NLS) for active transport to the nucleus. To test the NLS-functionalized sLPV in a manner consistent with delivery to post-mitotic cells, aphidicolin was utilized to arrest cell division in 16HBEge cells.

Results and Conclusions: We tested previously characterized but disparate NLS sequences, with a scrambled peptide serving as a control. All NLSs improved PTC suppression in aphidicolin treated 16HBEge cells, with the best sequence outperforming other NLSs (3.5-fold over scrambled and 17.5-fold over unlabeled plasmid DNA). While the presence of dsDNA in the cytoplasm of human cells can trigger the cGAS-STING innate immune pathway, sLPV labeled with the best performing NLS produces ~80% less cGAMP as compared to plasmid DNA. This novel modular sLPV assembly procedure allows for production of minimal ACE-tRNA therapeutic vectors with properties that can be engineered for optimal cellular delivery and intracellular trafficking.

1653 Micro RNAs in the core arsenal of Molecular Paradigms in Papillary Thyroid Cancer Modalities

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Introduction: To improve the diagnosis of thyroid cancer miRNAs are one of the novel classes of molecular markers that are being used now a day. The pattern of expression of miRNAs can be the rich source of biological information. miRNAs are endogenous, noncoding RNAs with lengths ranging from 19 to 25 nucleotides. They play major roles in the posttranscriptional regulation of gene expression. In general, miRNAs down modulate the

expression of a target gene by diminishing the stability of its transcript and/or inhibiting its translation. variations in the expression patterns can provide clues as to how different cellular processes are modulated under both physiological and pathological conditions.

Research on microRNA expression in thyroid tumors provides a new insight for the development of novel biomarkers that can be used to diagnose thyroid cancer and optimize its management. Specific patterns of miRNA expression have been identified in many studies and several miRNAs were found overexpressed or downregulated in major types of thyroid tumors.

Objectives: The study aimed to speculate the overexpression of miRNA-146b and miRNA-181b in thyroid cancer, as compared with levels in normal thyroid tissues & MNGs, Moreover, mutations in the same miRNAs may alter the pathways and correlated with the aggressiveness of cancer proving these miRNAs as tangible biomarkers for diagnosis of the disease.

Methodology: QRT-PCR is probably the most popular method currently used for detection of miRNAs expression. miRNA profiling begins with the isolation of total RNA which entails reverse transcription of miRNA to cDNA, followed by real-time monitoring of the accumulation of polymerase reaction products. An internal control was used for relative quantification of the expression. Sanger sequencing was used for the mutational analysis as compared to normal specimen and correlated the mutations with thyroid cancer specimen.

Results: 5 to 20 folds and 4-60 folds' overexpression of miRNA-146b & miRNA-181b was observed in thyroid cancer tissue specimen compared to controls and MNGs which was statistically significant expression. Similarly, rare genotypes and alleles were observed significantly in PTC tissue samples compared to controls and MNGs.

Conclusion: This forged maladaptation of the miRNA-146b and miRNA-181b in the cancer microenvironment may warrant analytically, therapeutically and genetically and this upregulation is positively correlated with tumor aggressiveness.

Figure.1: Relative Expression of miRNA-146b & -181b in PTC specimen, controls & MNG samples.

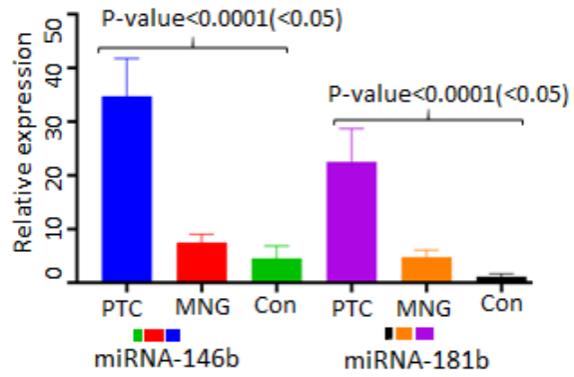
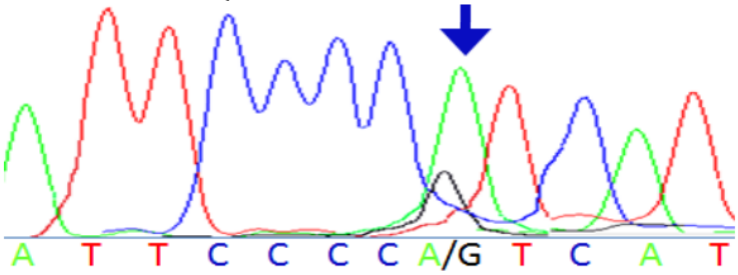


Figure.2: Heterozygous mutation of miRNA-146b (A/G) observed in PTC specimen.



1654 ADAR-Mediated RNA Editing-Based Correction of PNPLA3 I148M Functionality to Address Hepatic Steatosis

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Metabolic dysfunction–associated steatotic liver disease and steatohepatitis (MASLD/MASH) and subsequent stages of liver disease are very prevalent and still on the rise worldwide. MASH individuals have a high unmet medical need due to the progressive nature of the disease (hepatic steatosis, cirrhosis, and hepatocellular carcinoma) and the limited therapeutic options available making it the second cause of liver transplantation in the US. Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), a lipase expressed in hepatocytes and liver stellate cells, is involved in lipid homeostasis and metabolism. PNPLA3 I148M (rs738409) is a variant commonly reported in the MASH population worldwide (20-60% of the patients) and is known as an associated risk factor for hepatic steatosis and an increased risk of developing MASH. Approximately 8 million individuals in US and EU are homozygous for the 148M variant. PNPLA3 I148M variant induces triglyceride accumulation in hepatocytes, leading to increased lipid droplet size and number, which in turn promotes cellular stress, fibrosis, and disease progression to cirrhosis.

Axiomer, an RNA editing platform, uses chemically modified and complementary editing oligonucleotides (EONs) to recruit and guide endogenous Adenosine Deaminase Acting on RNA (ADAR) to perform targeted A-to-I editing on double stranded RNA. Editing oligonucleotides convert the PNPLA3 148 Methionine into a Valine, restoring wild-type like function of the PNPLA3 and alleviating the induction of hepatic steatosis. Here we present preclinical proof-of-concept data to support the approach.

In-silico 3D structural modelling analysis revealed that the PNPLA3 148M (ATG) shows a non-conservative substitution with predicted functional consequences, leading to a change in binding cavity volume limiting access of substrate to the active site, impairing its metabolic function. On the contrary, PNPLA3 148V (GTG) variant showed similar structure to wild-type (WT) PNPLA3 (148I, ATC), with Valine expected to exhibit similar properties and behave like isoleucine leading to minimal impact on the activity/functionality of the protein. To investigate experimentally if PNPLA3 148V variant can function as WT, PNPLA3 148V, 148M and WT variants were expressed in HeLa cells loaded with linoleic acid. Confocal imaging revealed that PNPLA3 148V (Bodipy, mean area per cell of 3.65 μm^2) showed similar levels of triglycerides and lipid droplet sizes inside the cell compared to PNPLA3 WT (1.33 μm^2 per cell, $P<0.0001$, one-way ANOVA) versus PNPLA3 148M (34.00μm^2 per cell).

Following this proof of concept, EON induced *PNPLA3* editing was observed up to 53% in primary human hepatocytes. Subsequently, in a homozygous 148M HepG2 cell model exposed to linoleic acid, Axiomer editing resulted in a significant decrease in lipid droplet size, ranging from -17% to -26% compared to control.

Axiomer ADAR RNA editing technology has the potential to convert PNPLA3 148M into a WT-like functional PNPLA3 148V protein. This approach could become a promising therapeutic opportunity for homozygous and heterozygous MASLD/MASH individuals, limiting changes only to affected allele(s).

1655 Developing suppressor tRNAs to target the most common type of pathogenic nonsense mutation

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Nonsense mutations are point mutations that convert a sense codon into a premature termination codon (PTC) - UAA, UAG or UGA - resulting in the premature cessation of protein synthesis. These mutations account for 11% of human pathogenic mutations. In a proof-of-concept study, we demonstrated that **AAV**-delivered **no**nsense **s**uppressor **t**RNA **o**perating on **P**TC (AAV.NoSTOP) can efficiently and safely overcome a TAG nonsense mutation in a disease mouse model, suggesting its therapeutic potential for a broad spectrum of disorders caused by a common genetic defect, namely the TAG nonsense mutation (Wang J et al. *Nature* 2022). This therapeutic approach is especially advantageous for restoring the endogenous expression of large or dose-sensitive proteins, such as dystrophin in Duchene muscular dystrophy or MECP2 in Rett syndrome, where traditional rAAV gene replacement therapies face significant challenges. Notably, nonsense mutations that convert arginine codons to UGA (ArgàUGA) are the most prevalent, representing 22% of all disease-causing nonsense mutations. In this study, we expand the AAV.NoSTOP platform by developing sup-tRNAs that target UGA and install arginine. We first screened 25 arginine tRNA-derived, UGA-targeting sup-tRNAs driven by the U6 promoter (U6.UGA-sup-tRNA^{Arg}) for their ability to induce PTC readthrough in HEK293 cells by plasmid co-transfection, with one plasmid expressing a readthrough reporter and another carrying a U6.UGA-sup-tRNA^{Arg} candidate. This assay revealed a broad range of readthrough capabilities from barely detectable to 17%. The top three candidates were further evaluated in mice by hydrodynamic co-injection of the two plasmids, a delivery method that predominantly targets the liver. We observed up to 13% of reporter PTC readthrough in mouse liver. As an alternative sup-tRNA cassette design, we placed the best-performing sup-tRNA candidate in its natural flanking sequence (NFS) context, omitting the U6 promoter to exploit the intrinsic Pol III promoter activity of tRNA genes. Compared to U6.UGA-sup-tRNA^{Arg}, NFS.UGA-sup-tRNA^{Arg} improved PTC readthrough efficiency by 4-fold in mouse liver, reaching above 50%. To assess its function in suppressing a genomic nonsense mutation in human cells, we used lentiviral vectors to deliver NFS.UGA-sup-tRNA^{Arg} to fibroblasts from a Batten disease patient harboring ArgàUGA nonsense mutations in the *TPP1* gene (*TPP1*$^{R127X/R208X}$). Lenti.NFS.UGA-sup-tRNA^{Arg} treatment restored TPP1 protein from undetectable levels to above 10% of the normal level, as determined by western blotting. To further evaluate therapeutic efficacy *in vivo*, we generated and characterized a new Hurler syndrome mouse model carrying a TGA nonsense mutation in the *Idua* gene (*Idua-TGA*). This mouse model enables us to quantify readthrough efficacy across multiple tissues using a sensitive iduronidase activity assay, and it provides logistical convenience in terms of colony maintenance. In contrast, other existing ArgàUGA mouse models are limited to testing efficacy in specific tissues (e.g., *Mecp2-R168X* mice for Rett syndrome) or present breeding challenges (e.g., *Tpp1-R207X* mice for Batten disease). Currently, we are generating AAV.NoSTOP reagents and evaluating their efficacy in the new *Idua-TGA* mouse model. (MX and HL: co-first authors)

1656 Optimizing LNP-RNA Formulations: Key Insights into Size, Zeta Potential, and Stability

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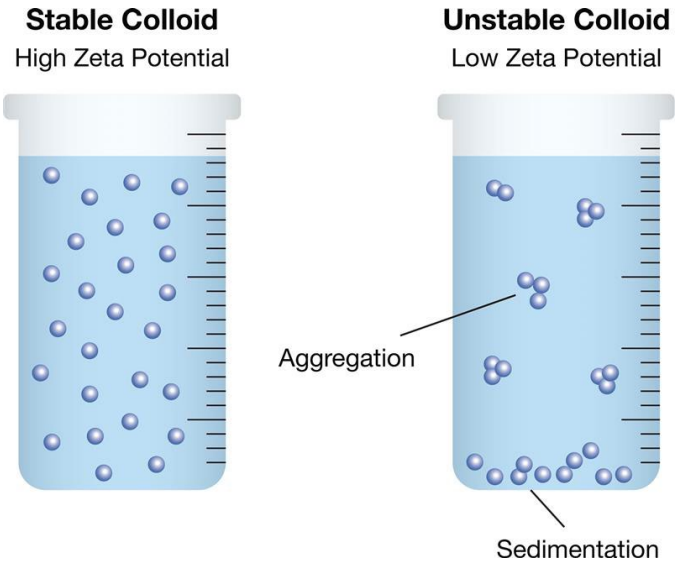
The microfluidic formulation of RNA-containing lipid nanoparticles (LNP-RNA) is a widely utilized approach for developing and producing non-viral gene delivery systems. A critical challenge in this process is optimizing lipid

selection and formulation conditions to achieve targeted particle sizes and surface potential. This study highlights the application of the DLS/SLS/ELS instrument for rapid characterization of LNP-RNA therapeutics, focusing on size, polydispersity, and zeta potential. Results demonstrate the impact of production methods on LNP formulations, colloidal stability across various buffer conditions, and structural changes following freeze-thaw cycles. These findings underscore the importance of precise characterization for optimizing LNP-RNA formulations and ensuring their stability and efficacy.



1657 Large-scale Transfection of T cells in a Xuri™ Cell Expansion System W25 Bioreactor Using LNPs

Paula Marcus[1], **Danylo Sirskyj[2]**, Vlada Sadetckaia[3], Dana Arab[2], Sadegh Davoudi[3], Mana Novin[4], Aruna Balgi[5], Natalie Orr[6], Reka Geczy[7], Darryl Knight[2], Azher Razvi[2], Samuel Clarke[5]
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Current methods for gene modification of immune cells can impact cell viability and total yield, and are time-consuming, complex processes. Lipid nanoparticles (LNPs) are an emerging technology for T cell transfection. LNPs efficiently encapsulate and transport payloads, such as mRNA, to target tissues and cells, and have the potential to improve cell transfection, reduce cell damage, and simplify cell culture workflows. The Xuri™ cell expansion system W25 bioreactor has a reputation for its ability to generate T cells required for a broad spectrum of immunotherapies. Here, we compare the difference between electroporation and a commercially available Cytiva LNP cell therapy kit for CRISPR-Cas9-mediated gene knockout of the T-cell receptor (TCR) within the Xuri™ Cell Expansion System W25 — an established system for generating T cells for a broad spectrum of immunotherapies.

Using NxGen™ mixers on the NanoAssemblr™ Ignite™ instrument, we manufactured LNPs encapsulating Cas9 mRNA and TCR-targeted guide RNA (sgRNA).. T cells were isolated from fresh Leukopaks using positive selection for CD4/8. T cells were seeded and activated directly in the Xuri™ cell expansion system W25 bioreactor for LNP transfection and after 3 d, cells were transfected using the TCR LNPs. Cells were activated in a G-Rex for EP and after 3 d, cells were resuspended in EP buffer and electroporated. Cells were then placed back into the G-Rex for 24 h, prior to seeding into the Xuri™ cell expansion system W25 bioreactor. Both sets of cells were batch-fed to 1 L and then perfusion was initiated. Cells were monitored daily for counts and viability. Transfection efficiency was determined at multiple time points throughout the culture.

In situ activation was comparable to G-Rex controls, although cell proliferation was reduced when compared to G-Rex activation. The hands-on time required for LNP transfection and scale-up was significantly reduced compared

to EP due to the one-step nature of the process. EP resulted in a significant decrease in cell viability 24 h post EP, which also resulted in a reduction in total cells at harvest, when compared to LNP transfection. The was no difference observed in transfection efficiency between the two groups.

In conclusion, we have developed a highly efficient, end-to-end, large-scale one-step T cell transfection workflow. LNPs manufactured using the NanoAssemblr™ Ignite™ nanoparticle formulation system can be scaled to the requirements for cell-based therapies manufactured in a Xuri™ cell expansion system W25 bioreactor. In addition, by activating and adding LNP directly within the Xuri™ cell expansion system W25 bioreactor, manual manipulation and process steps are reduced when compared to EP. LNP transfection also doesn't have any impact on cell health and proliferation, with similar transfection efficiency observed. This simplified and efficient process for gene modification of T cells has the potential to reduce manufacturing costs accelerating the development of next generation T cell-based immunotherapies, including gene-edited allogeneic CAR T cell therapies.

1658 Insights of CMC, Quality & regulatory for mRNA-based gene editing program – A case study and lessons learned from early phase development to final GMP manufacturing for IND application

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In the rapidly evolving field of mRNA-based gene editing, developing and manufacturing such innovative therapies demand a comprehensive understanding of CMC processes. ReciBioPharm has successfully delivered multiple GMP programs in this field, from early-phase prototype, process, and analytical development to final GMP manufacturing. Leveraging this unique experience, this abstract introduces a case study that delves into the CMC best practices and invaluable lessons learned in the journey from sequence to IND for a Gene Editing Program.

The presentation will provide an in-depth exploration of the following key aspects:

- Development and Manufacturing Considerations: We will spotlight the critical points along the road in plasmid DNA, drug substance, and drug product process and analytical development and GMP manufacturing.
- Regulatory and Quality Assurance: We will address the regulatory hurdles and how to navigate in the early phase landscape while still ensuring patient safety and successful IND submission.
- Lessons Learned and Future Perspectives: This case study reflects on the challenges faced, setbacks encountered, and strategies adapted during the entire development and manufacturing process. Insights gained from real-world experiences will offer valuable guidance to sponsor companies.

It serves as a vital resource for CMC stakeholders, offering a roadmap for success in bringing mRNA-based gene editing therapies from sequence to IND.

1659 Comprehensive evaluation of T7 polymerase for enhanced yield and quality in mRNA production

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messenger RNA (mRNA) drug modalities are a revolutionary breakthrough in medicine, offering the potential to treat various rare and infectious diseases. ReciBioPharm, contract with MIT, follows the largest award from FDA's Center for Biologics Evaluation and Research office (CBER) to MIT, enabling next generation mRNA manufacturing capacity and providing faster readiness and reaction to new pandemic threats. One major effort of the

ground-breaking initiatives is to systematically identify critical raw materials during mRNA production and scientifically understand their impact on the final quality of these drug products. In vitro transcription (IVT) is an enzymatic process used in the synthesis of mRNA products. In a typical IVT process, a RNA polymerase is responsible for the transcription of RNA from a DNA template. While T7 polymerase is the most common used in both research and commercial purposes and there are multiple offerings of both wild type and engineered T7 polymerases in the market, so far there is no systematic studies to understand the performance of these commercially available T7 polymerases. Herein, we conducted a comprehensive study to characterize total 19 of T7 polymerases including 4 wild types and 15 modified ones. Briefly, a high throughput screening (HTS) workflow was established to enable >1500 IVT runs with subsequent critical analytics including yield, purity, dsRNA and potency; these DOE based optimizations were followed by subsequently lot-to-lot variations, and scalability studies; the desired process range for each T7 was defined and some high potential process parameters was proposed to offer >15g/L mRNA yield with >95% purity and minimum dsRNA (below dsRNA ELISA LOD). The outcomes as a T7 knowledge library with deep profiling their performance & cost analysis was well established, which will accelerate the deployment not only of mRNA vaccines for rapid response against future pandemics but also of novel mRNA therapeutics.

1660 Developing a Cost-Effective and Reproducible Protocol for Lipid Nanoparticle Synthesis and Characterization to Advance Preclinical Research and Education

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**Corresponding Author*

Nanotechnology has transformed clinical applications, with its impact extending into gene therapy, targeted drug delivery, and diagnostic tools. Among its most notable advancements are lipid nanoparticles (LNPs), which have gained prominence as an efficient vehicle for nucleic acid delivery. In recent years, LNPs played a critical role in the development of mRNA vaccines, contributing to the swift global response to the COVID-19 pandemic. The success of LNP-based vaccines demonstrated the potential of these nanoparticles for broader therapeutic applications, but their adoption in research and clinical settings remains limited by challenges such as high production costs and difficulties in achieving effective delivery beyond hepatic tissues.

As more research institutions look to harness the potential of LNPs for various disease therapies, there is a growing demand for affordable, standardized manufacturing protocols that can be easily implemented across diverse labs. Traditional methods for LNP synthesis, such as pipette mixing and vortexing, often yield inconsistent results, while microfluidic mixing offers better control over LNP properties but requires expensive equipment. This financial barrier prevents many laboratories from accessing cutting-edge LNP technology, slowing down the pace of preclinical research and limiting the exploration of its full therapeutic potential.

To address this, we developed a standardized protocol for microfluidic mixing using a syringe pump and commercially available microfluidic chips. This approach provides a cost-effective, reproducible method for LNP synthesis. The protocol details the synthesis of LNPs, physical characterization via dynamic light scattering (DLS), encapsulation efficiency using the RiboGreen assay, and evaluation of in vitro transfection efficiency using OneGlo assay and flow sorter.

Various experimental parameters were explored, including different mRNA concentrations, syringe and tubing reusability, stability profiles, dialysis conditions, and total flow rates. Using the ionizable lipid DLin-MC3-DMA as a baseline, we formulated LNPs with three different ionizable lipids—LP01, DLin-MC3-DMA, and SM102—alongside Cholesterol: DSPC: DMG-PEG: DOTAP. We found that syringes and tubing remained functional for up to six continuous runs. Encapsulation efficiency remained high (96-100%) across mRNA concentrations ranging from 10 µg to 2.5 µg, though particle size increased from 82 nm to 100 nm at the lower concentration. Luciferase luminescence assays revealed a marked decrease in intensity as mRNA concentration decreased. Validation with

GFP mRNA and flow cytometry demonstrated that both 7.5 µg and 10 µg formulations produced adequate fluorescence, leading us to select 7.5 µg for subsequent studies.

Dialysis experiments showed minimal variation in encapsulation efficiency across 1-hour, 2-hour, and overnight conditions, but LNP size increased significantly after 12 hours of dialysis. Notably, longer dialysis times correlated with decreased luminescence intensity. The polydispersity index (PDI) for most samples remained below 0.2, indicating a narrow size distribution.

The next phase of this research will focus on evaluating the LNPs in in-vivo mouse models, with an emphasis on non-viral gene delivery. By applying this streamlined and cost-effective protocol, we aim to accelerate preclinical studies and broaden the therapeutic applications of LNPs.

1661 Key learnings during mRNA-LNP drug product process development

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In recent years, lipid nanoparticle-based delivery of mRNA therapeutics in infectious diseases has shown great clinical and commercial success. However, in contrast to protein therapeutics, mRNA-LNP drug products manufacturing involves complex steps that pose number of challenges in building a robust process. Hence, it is critical to develop a good understanding of mRNA-LNP drug product unit operations to ensure a successful manufacturing scale up and tech transfer. The purpose of this talk is to inform on process considerations to develop high-quality mRNA-LNP drug products.

1662 Enzymatic Linearized DNA – A Novel, Cell-Free Platform for Accelerated mRNA Therapeutic Development

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The production of high-quality DNA templates remains a critical bottleneck in mRNA therapeutic development, often constrained by the inefficiencies of traditional plasmid DNA (pDNA) workflows. We introduce a **novel, cell-free enzymatic linearization platform** that streamlines DNA template production, eliminating the need for plasmid cloning, bacterial fermentation, and enzymatic purification. This innovative approach delivers **backbone-free, high-purity linearized DNA** in just weeks, significantly reducing development timelines and enhancing mRNA production efficiency.

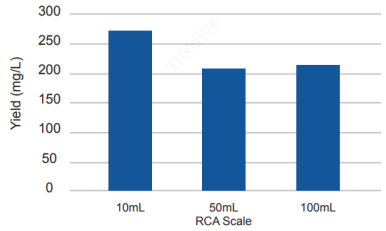
Key advantages of this platform include:

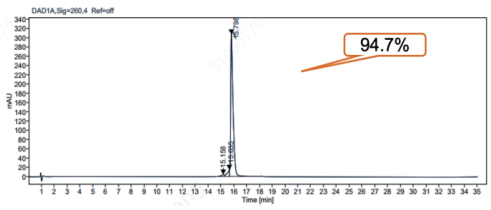
- **Rapid turnaround**: By bypassing months-long pDNA processes, the platform accelerates progression from research to clinical applications.
- **Superior purity**: The technology generates clean, consistent DNA templates, minimizing dsRNA impurities and poly-A tail instability, which are known to compromise mRNA performance.
- **Scalability**: Designed for seamless scale-up, the platform ensures a reliable supply of DNA templates for both preclinical and commercial mRNA manufacturing.

This enzymatic linearization technology addresses the limitations of traditional pDNA methods, enabling researchers to achieve **higher mRNA yields, improved stability, and reduced immunogenicity**. By advancing the efficiency and quality of DNA template production, this platform represents a transformative step forward in mRNA therapeutic development, empowering researchers to accelerate the delivery of life-changing therapies.

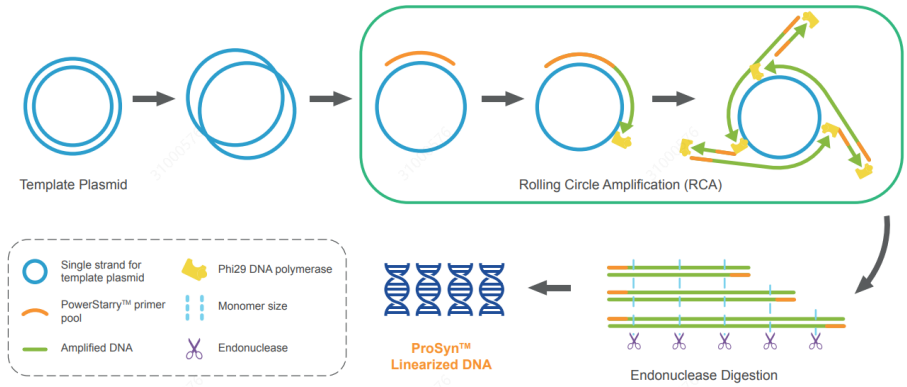
☐ **2~3 mg per 10 ml with 4000~5000x amplification rate**

☐ **Purity (IEC-HPLC): ~95%**





Cell-free technology is a breakthrough in plasmid and linearized DNA manufacturing with its short turnaround time. ProSyn™ Linearized DNA is using enzymatic DNA synthesis through rolling circle amplification (RCA). Under the action of proprietary PowerStarry™ Primer Pool, GenScript ProBio is able to accelerate different projects by reducing time and manpower.



1663 Effective Quality Control is Crucial Throughout mRNA Product Development

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At the forefront of therapeutic delivery is an array of vectors and targeting methods that hold immense promise for precision medicine in cell and gene therapies. Lipid nanoparticle (LNP) encapsulated RNA and circular RNA (circRNA) are emerging as a preferred method for new therapies, offering faster development and production with high specificity, transient expression and reduced long-term risk, as it does not integrate in the host genome. As a result, mRNA therapeutics have the potential to quickly and effectively treat a wide range of diseases.

Here we present an end-to-end RNA production workflow engineered for quality and scalability through novel methods for minimizing synthetic risks and validation by sequencing. Critical to successful production is maintaining the integrity of complex polyA regions. Using proprietary methods for synthesis and plasmid preparation, monodispersity of polyA tails are conserved within DNA templates. Examination of the final mRNA transcript is performed utilizing a new library generation method for PacBio long-read Single-Molecule, Real-Time (SMRT) technology, capable of full-length sequencing of products up to and beyond 10kb without limitations or bias due to repetitive sequence or C/G content. Crucially, this method preserves the entire length of the polyA tail enabling sensitive and specific counting of the polyA tail length -- an important parameter when confirming overall sample purity, including intermediate and alternative products present within the sample.

Both circRNA and linear mRNA products are amenable to the above workflow, providing a robust solution for any RNA research program. The workflow results in an accelerated mRNA development program with higher quality templates and rigorous qualification post transcription.

1664 mRNA Manufacturing: from Template DNA to Lipid Nanoparticles – an Integrated Platform Approach

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The manufacturing of messenger RNA (mRNA) has emerged as a pivotal technology in the development of novel therapeutics and vaccines. For mRNA production, Good Manufacturing Practice (GMP) is the essential quality standard to ensure product safety, consistency, and regulatory compliance. The complex nature of mRNA requires precise in-vitro synthesis, base modification, purification, and quality control and demands highly controlled and standardized manufacturing processes.

This abstract provides a case study for the establishment of a GMP-compliant mRNA manufacturing platform integrating the whole process chain from template DNA, mRNA production, and lipid nanoparticle (LNP) formulation until final aseptic filling of drug product. The essential elements of GMP mRNA production will be highlighted, emphasizing the key challenges to ensure the scalable, reproducible, compliant and economic manufacturing of mRNA-based therapeutics.

The investigated target products were mRNA transcripts encoding a CRISPR/Cas9 nuclease featuring a 5´-cap analog, a 3´-poly(A) tail, and N1-methyl-pseudouridine base modifications. The GMP manufacturing process was carried out at full scale in certified clean rooms. Manufacturing of the linear DNA template consisted of cloning of a circular plasmid, preparation of an *E. coli* master cell bank, fed-batch fermentation, alkaline cell lysis, chromatographic purification, and enzymatic linearization steps. The mRNA was synthesized by enzymatic in-vitro transcription (IVT) using T7 RNA polymerase and co-transcriptional addition of a 5´-cap analog. Subsequent mRNA purification was achieved by oligo dT affinity chromatography. The mRNA was encapsulated in lipid nanoparticles (LNP) by impingement jets mixing. The drug product was formulated by tangential-flow filtration (TFF) and finally aseptically filled.

A comprehensive analytical platform conforming to GMP regulations has been set up, including raw material testing, in-process controls, Quality Control release and stability testing, as well as extended characterization of the mRNA. It was confirmed that all quality parameters (content, identity, purity, safety) were within pre-defined specifications at all stages. Bulk mRNA was highly homogenous (≥ 90 % by CE). 5´-capping efficiency was ≥ 95 %, and 3´-poly(A) tail length was confirmed by LC/MS. The LNPs showed a high encapsulation efficiency (> 90 %) and a low polydispersity index (PDI). Satisfying stability was confirmed from both the bulk mRNA as well as from the formulated LNPs by performing stability studies at several conditions.

Functionality of the CRISPR/Cas9 mRNA was shown in a cellular assay by measuring Cas9-mediated recombination in HEK293 cells. As a conclusion, with the established mRNA platform, the parameters and requirements of yield, throughput time, GMP compliance and quality were successfully achieved.

1665 Versatile Lipid Nanoparticle Platform for Efficient CRISPR-Cas9 Gene Editing in Primary T Cells and CD34+ Hematopoietic Stem Cells

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Background and Aims: Lipid nanoparticles (LNPs) have emerged as a promising nonviral alternative for delivering CRISPR-Cas9 components to difficult-to-transfect cell types such as human primary T cells and CD34+ hematopoietic stem cells (HSCs).We have developed a library of novel ionizable lipids and lipid nanoparticle compositions designed for *ex vivo* applications. In this work, we show the results of screening and optimization of Cas9 mRNA and sgRNA delivery to achieve multiplex gene knockouts across both T cells and HSCs. Further, we investigate the interplay between cell culture media and vessels to develop protocols suitable for scale-up and clinical translation.

Methods: A range of novel LNPs were formulated using the NxGen™ mixing platform and evaluated by adding LNPs directly to activated human primary T cells and CD34+ HSCs obtained from mobilized peripheral blood (**Fig 1A**). LNPs were encapsulating various RNAs, such as GFP or αCD19-CAR mRNA, or Cas9 mRNA and sgRNAs targeting TCR/CD52 in T cells and CD33/CD45 in HSCs. Culture media, supplements, and transfection kinetics were systematically varied and optimized for high gene delivery efficiency (**Fig 1B**). Viability, proliferation, and colony-forming potential (in the case of HSCs) were used to measure functional outcomes for LNP post-editing.

Results: Up to 90% TCR–/CD52– double knockout was achieved in T cells, with > 90% viability maintained. Similarly, LNP addition to HSCs showed 80% and 90% knockouts (CD45 and CD33, respectively), preserved > 95% viability, and showed strong proliferative capacity relative to untreated controls. Colony-forming unit (CFU) assays indicated no significant changes in multi-lineage differentiation. Media optimization proved essential, with some commercial T cell and HSC media producing higher editing efficiencies. In addition, treatment kinetics, media supplements, and small molecules showed a significant impact on editing efficiency in long-term HSC (LT-HSCs). Lastly, LNPs and the optimized cell culture conditions were successfully scaled from well-plates to G-Rex bioreactors for both cell types.

Conclusions: This work highlights a versatile, nonviral LNP platform for efficient gene delivery and editing in difficult-to-transfect cell types. LNP scalability, combined with minimal cytotoxicity and high efficiency, supports its potential as a rapidly translatable and gentle platform for gene delivery in both T cells and HSCs.

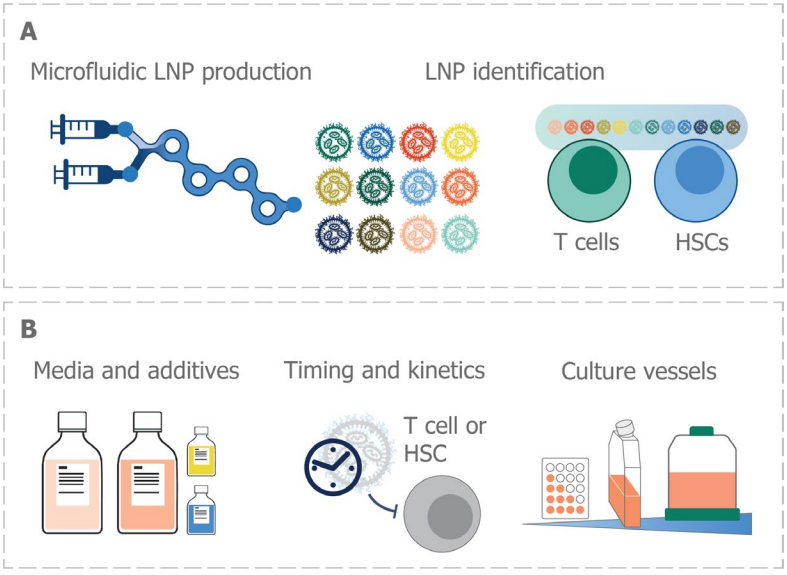

Figure 1. Optimization of lipid nanoparticles and culture conditions for LNP-mediated gene editing. (A) Various LNPs were produced and tested on both T cells and HSCs. (B) Media composition, timing and kinetics were optimized. LNPs and cell culture were scaled up from plates to small bioreactors.

1666 Novel In Silico Generation of a Synthetic Peptide Library Derived from Biological Complexes for Protein and Vector Engineering

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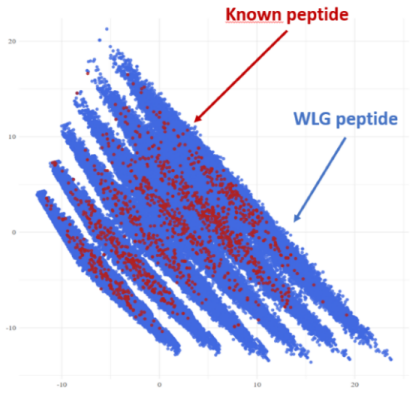
The generation of chemical libraries, specifically peptide libraries, has been an ongoing topic for the past decades. Peptide design is actively being researched due to their specific properties (stability, binding...) both as a therapeutic agent and, in the cell and gene therapy field (C&G), for their targeting properties. They can be combined with various viral or non-viral vectors (e.g., adeno-associated viruses, nanoparticles...) as they have a diverse set of association possible ranging from, but not limited to, peptide conjugation as well as insertion in both linear and cyclized conformations. A common strategy for designing peptide libraries involves using a random approach, which typically leads to a large volume of candidates, with a high proportion of structurally and biologically irrelevant sequences, driving up both cost and time in identifying relevant binders. Despite the rapidly evolving field, the concept of in silico peptide library, especially based on rational biological complexes, represents an important breakthrough, yet remains largely unexplored due to its complex nature.

WhiteLab Genomics is presenting a novel in silico approach, stemming from structurally resolved experimental data from protein-peptide complexes. The objective is to create a peptide library specifically tailored in a vector engineering context for gene therapy that can also be used in the discovery of pertinent motifs for new therapeutic peptides. It is based on biologically active motifs that, when combined with WhiteLab Genomics' rational-guided approach, can enhance both in silico discovery and evaluation of new hits against a receptor of interest reducing the risk of having biologically irrelevant peptides. It led to the generation of more than 300 thousand unique sequences, ranging from 5 to 12-mer peptides, reconstructed in 3D, in four initial conformations, namely helix, linear, cyclized and beta-sheet, forming a total library of more than 1.2 million unique structures. This allows expanding synthetically the chemical space of available peptides by exploring a highly diverse conformational space. Every newly generated structure is mapped and characterized with sequence and structural descriptors.

To significantly improve the success rate of our approach, we also developed a subset called the Diversity library composed of peptides representative of key properties. It enhances both the search for efficient therapeutic screening and improves the engineering of a vector of choice by allowing us to explore a large chemical space while extracting a minimal number of peptides sharing similar properties. Finally, the generated properties in both these libraries will also be useful in the development of tailored machine-learning or artificial intelligence models, paving the way for new and better solutions in the C&G therapy field.



Principal Component Analysis (PCA) illustrating the expansion of the chemical space, based on 1D & 2D physico-chemical descriptors, with the WhiteLab Genomics peptide library (in blue) compared to already known peptides (in red).

1667 Revolutionizing Peptide Discovery: AI-Driven Drug Design Paving the Way for the Future of Cell & Gene Therapy

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Over the past decade, rational peptide design has gained significant momentum as a promising approach in drug discovery. Peptides are emerging as highly versatile therapeutic agents, offering unique advantages in targeting complex biological pathways. However, their design and development still represent a significant challenge due to their intrinsic structural flexibility and potential for unintended biological interactions, which can complicate therapeutic applications. Beyond their therapeutic potential, peptides may enhance gene delivery through various vector types and modifications. For Adeno-Associated Virus (AAVs) vectors, peptides can be inserted into their sequences to trigger better targeting. Peptides can also be conjugated as linear or cyclic forms to improve stability and binding of AAV vector or non-viral vector (e.g. Lipid Nanoparticles, LNP). Optimization opportunities include incorporating non-canonical amino acids to improve pharmacological properties.

In this context, we apply a drug design protocol for the discovery of novel peptides towards a pivotal therapeutic target in oncology and immunotherapy. Despite extensive data availability, this receptor remains challenging for peptide-based drug design due to the atypical nature of the binding site: non-buried, without any specific pocket to be used as a basis for docking. However, the existing crystallized receptor structures provide a solid foundation for structure-based computational approaches, in addition to the availability of a reference peptide (Kd = 4.2nM), enabling competitive binding assays.

WhiteLab Genomics' AI-powered platform pre-screened tens of thousands of unique peptides extracted from our proprietary peptide library, employing a rational workflow: (i) Analysis of known binders to identify critical interaction motifs, (ii) Customization of computational tools tailored to receptor binding, (iii) Virtual screening of high-potential candidates, (iv) Structural optimization using SAR (Structure Activity Relationship) analysis, and (v) Peptides refinement through macrocyclization, sampling, and dynamic modeling.

During the virtual screening phase, over 1,000 peptides were modeled in complex to predict binding affinity and assess virtual structure-activity relationships. Approximately 200 peptides were shortlisted based on predicted binding strength and compatibility with macrocyclization strategies. Using Surface Plasmon Resonance (SPR) assays, we evaluated the binding affinity of our peptides library and achieved hit rates of 6.3%. Notably, every hit demonstrated a higher binding affinity than the reference, underscoring the efficiency of our computational and

experimental pipeline. Although no gold standard exists for both hit rate and hit definition, we considered the WhiteLab Genomics platform to have surpassed established field methodologies by defining hits as superior to the reference while still achieving an impressive hit rate. We are presenting an automated AI-driven platform for the discovery and optimization of peptide binders. This platform demonstrated exceptional performance, culminating in experimental validation of high-affinity binders (Kd) for a clinically relevant oncology target. These findings highlight the potential of AI-enhanced peptide design to drive the next generation of cell and gene therapy innovations.

1668 A Novel Technology for Sequence-Specific Determination of Lipid Nanoparticle Encapsulation Efficiency

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Lipid nanoparticles (LNPs) are an established non-viral delivery platform for nucleic acids and other biomolecules, offering significant promise for gene and cell therapy applications. As the utilization of LNPs expands to diverse cargo types and complex formulations, there is a growing need for robust tools to characterize encapsulation efficiency and ensure the integrity of therapeutic payloads.

This study introduces the TruTiter™ Reagent, a novel small molecule that facilitates the assessment of LNP encapsulation efficiency. TruTiter™ selectively reacts with exposed nucleic acids but is excluded from encapsulated or compartmentalized cargo, thus preserving nucleic acids within intact LNPs. By covalently modifying unprotected nucleic acids, TruTiter™ prevents their amplification by PCR or digital PCR (dPCR). This enables accurate quantification of encapsulated nucleic acids and supports optimization of lipid composition, cargo properties, and storage conditions.

Quantitative analysis using RT-qPCR or dPCR with TruTiter™ was employed to measure encapsulation efficiency (%EE). Encapsulation efficiency was calculated using the formula: $\%EE = (1/2^{dCq}) \times 100$, where dCq = Cq [with TruTiter™] - Cq [without TruTiter™]. Nucleic acid purification was performed prior to amplification to mitigate the inhibitory effects of certain lipid components on reverse transcriptase and DNA polymerase activities. The Maxwell RSC simplyRNA Cells Kit was validated as an effective method for isolating nucleic acids from LNPs.

To demonstrate the utility of TruTiter™, in vitro-transcribed RNA (Firefly luciferase, 1.5 kb) was encapsulated within pre-formulated LNPs. Samples were analyzed with and without TruTiter™ treatment, followed by neutralization, nucleic acid extraction, and quantification using RT-qPCR and dPCR. Additionally, GFP mRNA encapsulated in LNPs was subjected to thermal stress (22°C, 42°C, and 55°C) to assess the impact of temperature on encapsulation integrity. TruTiter™ detected significant reductions in encapsulation efficiency with increasing temperature, aligning with the loss of LNP structural integrity. In contrast, the RiboGreen assay failed to detect similar losses, underscoring its limitation in assessing structural changes and providing sequence-specific information.

These results highlight TruTiter™ as a valuable tool for characterizing LNP encapsulation efficiency, offering sequence-specific insights that complement existing techniques. This method advances the characterization of LNP formulations, contributing to the development of more effective delivery systems for gene and cell therapy applications.

1669 Comprehensive Imaging Methods to Quantify mRNA Lipid Nanoparticle Trafficking and Expression

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Introduction: Messenger RNA lipid nanoparticles (mRNA-LNPs) have excelled as vaccines for infectious diseases including COVID-19. We are interested in applying our expertise in nonhuman primate (NHP) models of gene therapy to broaden the therapeutic applications of mRNA-LNPs. For example, Human Immunodeficiency Virus (HIV) and simian/human immunodeficiency virus (SHIV) establish infection in distinct tissues, which can be identified via viral RNA (vRNA)-specific *in situ* hybridization (ISH). We have developed a highly sensitive and specific RNAscope ISH technique to identify both vRNA and LNP-delivered mRNAs. To simultaneously quantify virally infected cells and therapeutic mRNA/protein trafficking, we couple RNAscope ISH with immunohistochemistry (IHC) assays specific for mRNA-LNP-encoded proteins. Here, we applied our dual RNAscope-IHC assay to identify reporter mRNA-LNP formulations and dose routes that efficiently traffic to systemic sites of SHIV latency in NHPs.

Methods: Reporter mRNA-LNPs encoded enhanced green fluorescent protein (eGFP). Nine SHIV-infected rhesus macaques received eGFP mRNA encapsulated in one of two LNP formulations ("LNP-A" or "LNP-B"), administered via intravenous or intranodal injection. Twenty-four hours later, tissues were collected from a broad array of anatomical sites that harbor latent SHIV-infected cells. The tissues were paraformaldehyde-fixed, paraffin-embedded, sectioned and mounted on slides. After paraffin removal, hydrogen peroxide and target retrieval, the slides were incubated with an eGFP RNA probe or SHIV RNA probe followed by the use of the RNAscope kit as directed. Slides were then analyzed for the presence of positively stained cells. Established IHC methods were applied to compare eGFP mRNA to eGFP protein.

Results: Localization of LNP-delivered eGFP mRNA varied based on LNP formulation and dose route. When injected intranodally, eGFP mRNA was highly enriched in lymph nodes relative to intravenous injection. In particular, eGFP mRNA localized predominantly to draining lymph nodes near the injection site. When given intravenously, LNP- B more efficiently delivered eGFP mRNA to the marginal zones and red pulp of the spleen while LNP-A delivered mRNA in clustered regions near the marginal zones. Additionally, LNP-B enhanced delivery to the lung and gastrointestinal tissues. The localization of eGFP protein was often distinct from eGFP mRNA and associated with areas antigen presenting cells are known to reside.

Conclusions: These data demonstrate that RNAscope ISH coupled with transgene-specific IHC is a useful tool to trace each step in mRNA-LNP therapies, from LNP-dependent delivery of mRNA within tissues to the biodistribution of mRNA-encoded proteins. In our NHP model of HIV, we are particularly interested in targeting therapeutic mRNA-LNPs to sites of virus persistence, as well as to HIV-specific immune effectors. We predict that efficient, on-target mRNA-LNP delivery will be associated with more effective therapeutic endpoints in our NHP model, validating analogous clinical approaches for people living with HIV. Our future goals are to broaden our suite of disease-specific multiplex assays, namely for degenerative diseases of the central nervous system (CNS).

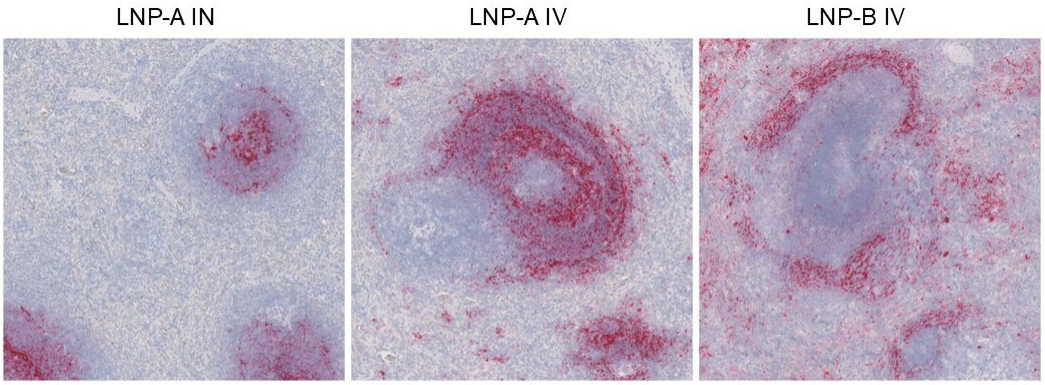

Figure 1: Lipid nanoparticles (LNP) delivery of mRNA to various locations differs by formulation and route of administration. Representative images of eGFP RNAscope (red) in the spleen of rhesus macaques administered with LNP-A or LNP-B. IN=intranodal injection, IV= intravenous injection.

1670 Total Holographic Characterization: A New Tool for Empty/Full Ratio Determination

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Total Holographic Characterization (THC) has the unique capability of distinguishing sub-visible particles by size and composition in complex heterogeneous mixtures of colloidal particles. THC shows promise as an analytical tool to distinguish filled versus empty AAV capsids or lipid nanoparticles (LNP).

THC is currently used to distinguish individual sub-visible contaminants in biologic pharmaceuticals, ranging in size from 0.5 µm – 10 µm. In biologic formulations different contaminants are distinguished by their different indexes of refraction. THC uses Lorenz-Mie Theory to analyze holograms of individual sub-visible particles. The analysis yields the size distribution of particles present and simultaneously determines the composition from the index of refraction of the particles. Particle symmetry provides additional information about the morphology of the particles. Spherical particles, such as those of emulsion droplets or air bubbles are distinguished from more irregularly shaped particles, by the symmetry of their holograms. Particles that are identical in shape, such as innocuous silicone oil emulsion droplets and dangerous degradation products of surfactant degradation, can be easily distinguished by their indexes of refraction. Protein aggregates are another application of THC in biologic pharmaceuticals. While protein aggregates are irregular in shape, their holograms can be analyzed using Lorenz-Mie theory, approximating the aggregates using an effective sphere model.

Filled or empty capsids or lipid nanoparticles have different indexes of refraction and therefore will have their own unique and distinguishable THC signatures. While individual capsids or LNPs are below the detection threshold of THC, their aggregates are easily detected. The dramatic difference in index of refraction can be used to determine the Empty/Full ratio in these aggregates.

THC measurements are performed in 10-15 min with minimal sample preparation and no labeling required. Characterization requires 25-35 mL of sample per measurement. Samples flow down a channel in a disposable microfluidic sample chip, and is crossed with a laser. The scattered light from the particles interferes with the incident laser beam thereby creating the hologram of the particle which encodes all of the structural information about the particle.

1671 High throughput process development for column-free purification of mRNA via BioRMB™ platform

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The BioRMB™ Kascade™ is a column-free and steady-state purification platform that is specifically tailored for sensitive modalities such as gene therapies, vaccines, mRNA, and complex antibodies. By moving resin as a slurry through the system, rather than utilizing packed resin beds, on-column contact time can be reduced, improving product quality. Because the resin within the system is cycled continuously, this results in significantly lower resin usage than a typical packed column, or even that of column-switching devices.

In this work we will present the protocol for a linear scale-up development onto the BioRMB™ System. An eGFP mRNA molecule was bound to off-the-shelf (OTS) Poros Oligo(dT)-25 resin. The first step of the scale-up development was to perform binding kinetics in 96-well plates to create binding kinetics curves for multiple binding conditions. From this study, it was determined that an optimal load challenge of 1.25 g of eGFP per L of resin achieved a binding efficiency of 95% after 18 minutes in two-stage binding. As a secondary condition, a load challenge of 0.75 g of eGFP per L of resin achieved a binding efficiency of 96% after 18 minutes in two-stage binding. It was also determined that a higher feed concentration of eGFP (0.4 g/L vs. 0.2 g/L) could achieve similar binding efficiencies at upwards of 2.0 g of eGFP per L of resin. This condition was not explored for scale up due to material constraints.

Wash and Elution screenings were also performed in 96-well plates. eGFP mRNA was bound to the OTS Poros Oligo(dT)-25 resin at 1.25 g of eGFP per L of resin. After removing the supernatant, the resin was resuspended in buffers of various conductivities from 0 mS/cm (elution), and 50 mS/cm (binding) to assess a suitable working range for development of a wash buffer and to determine the critical value for complete elution of the mRNA from the resin. This determined that a conductivity no lower than 20 mS/cm could be used to wash the resin without incurring more than 5% product loss.

To validate the filter plate binding kinetics model, slightly larger volumes were prepared for static mixing in syringes. At various time points, a syringe filter was used to sample the supernatant while retaining the resin. These curves demonstrated a slightly lower binding efficiency of 93% for the condition at 1.25 g of eGFP per L of resin, but similar binding efficiency of 97% for the condition at 0.75 g of eGFP per L of resin.

By applying ChromaTan's recipe maker and buffer model to design a recipe within these set parameters, a BioRMB™ run was performed. This run resulted in 92% binding efficiency for the condition at 1.25 g of eGFP per L of resin, and 97% binding efficiency for the condition at 0.75 g of eGFP per L of resin, within 2% of the modeled binding efficiency. Wash losses aligned closely with the model, losing 3.4% of product compared to the modeled 4.8%. Elution also aligned closely, recovering 97% of the bound product compared to the predicted 98%.

1672 Heterogeneous RNA Loading and Size Correlations in Lipid Nanoparticles Revealed by Single-Particle Spectroscopic Chromatography

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Introduction. Lipid nanoparticles (LNPs) have emerged as essential delivery vehicles for RNA therapeutics. Previous research and development typically assumed that LNPs are homogeneous in population, loading density, and composition. Such perspectives are challenging to evaluate due to the absence of appropriate tools to characterize these physicochemical properties. Here, we introduce an integrated spectroscopy−chromatography approach as a generalizable strategy to unravel the complexities of multicomponent LNP assembly. Our platform combines cylindrical illumination confocal spectroscopy (CICS)[1] with single-nanoparticle free solution hydrodynamic separation (SN-FSHS) to simultaneously profile population identity, hydrodynamic size, RNA loading levels, and distributions of helper lipids and PEGylated lipids of LNPs at the single-particle level in a high-throughput manner.

Methods. The laminar flow generated by SN-FSHS produced a parabolic velocity profile in the tube due to the nonslip boundary condition (Figure 1). Larger particles sample the central flow streams more due to size exclusion from the channel wall, resulting in a higher average velocity and faster elution than smaller particles. This method allows for differential separation of any LNP formulation based on its size distribution. The CICS platform was coupled immediately downstream SN-FSHS. It features one-dimensional beam shaping, which enables accurate measurement of payloads by quantifying fluorescence with single-fluorophore sensitivity, assisted by a fluorescence deconvolution technique. To label LNPs, Cy3-siRNA served as the cargo, while a specific ratio of the DSPC helper lipid and PEG lipid was substituted with TopFluor-PC and DSPE-PEG-Cy5, respectively.

Results. Utilizing a benchmark siRNA LNP formulation, we analyzed the 4-dimensional parameter output from SN-FSHS-CICS. When examining the siRNA loading level (Cy3), the PEG loading level (Cy5), and the size of each LNP in a 3D space, a power regression for all formulations was observed, particularly for the 0.5% PEG formulation, where R^2 reached 0.90 (Figure 2a). Notably, a significant degree of heterogeneity in loading density (in terms of either siRNA or PEG) was observed for each formulation. In other words, the same LNP size can accommodate different amounts of siRNA or PEG lipid within each LNP.

By combining data points from all three formulations with a size distribution ranging from 30 to 200 nm, we can better visualize the spread of the data points between the maximum and minimum loading boundaries (Figure 2b). The size-dependent convergence of these boundaries clarifies the super-volumetric scaling observed in each formulation and suggests potential changes in the packing structure or density as the particle size varies. We attribute this observation to siRNA packaging in LNPs, which shifts from surface packing to heterogeneous volumetric packing, and ultimately to uniform volumetric packing as the size of the LNP increases.

Conclusions. This SN-FSHS-CICS analysis offers insights into a significant degree of heterogeneity in the packing density of RNA in LNPs. We anticipate that this technical framework will guide development efforts to refine manufacturing processes to achieve greater compositional homogeneity.

References. 1. Li, S., Hu, Y., Li, A. et al. Payload distribution and capacity of mRNA lipid nanoparticles. *Nat Commun* 13, 5561 (2022). https://doi.org/10.1038/s41467-022-33157-4

Figure 1

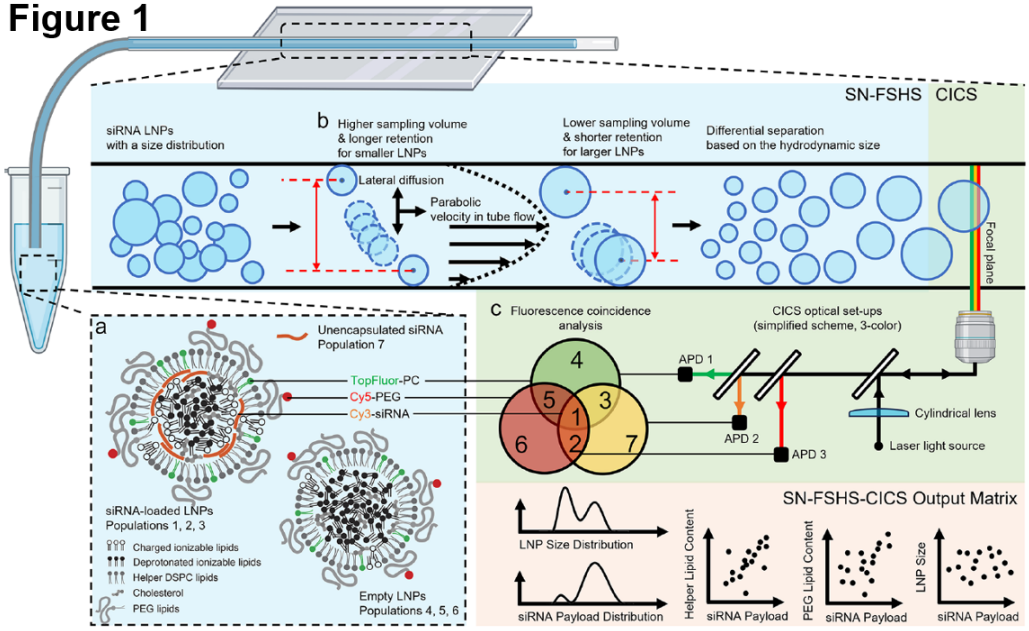
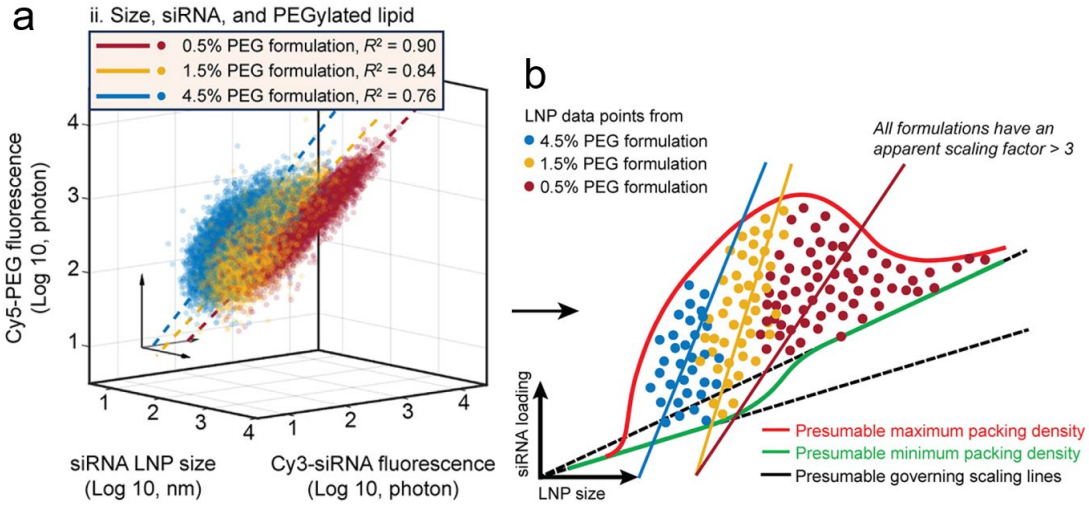


Figure 2



1673 Orthogonal chromatography analytics to allow for faster AAV or LNP process development and better in-process control

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- In-process analytics supports process engineers who want to visualize process related execution data and to have a clear representation of the process flow and/or to monitor and improve process execution.
- Biomolecules production is very complex process and analytical data may be misleading and lead to erroneous conclusions. Orthogonal analytical methods are therefore required to diminish the mistakes and enable faster and cheaper biomanufacturing processes.

- Chromatography is orthogonal method of choice to molecular biology methods. Examples of two-dimensional liquid chromatography in-process methods, for AAV and LNP production will be presented in this paper.

1674 An End-to-End High-Throughput LNP Formulation Device Platform for mRNA & LNP Screening

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Lipid Nanoparticles (LNPs) have been widely utilized in prophylactic vaccines and liver directed therapies; however, their applications in other areas remain limited due to challenges in payload design, LNP chemistry, composition, and formulation process optimization. To address these challenges and facilitate clinical translation, large-scale screenings are essential to establish robust design rules for LNPs. Promising methodologies, such as barcoded *in vivo* screening and plate-based *in vitro* assays, have been adopted but often require labor-intensive, one-by-one formulation of LNPs. Therefore, high-throughput formulation platforms are crucial to accelerating the development of genetic medicines.

At XGen Bio, we developed a high-throughput screening instrument, "INano^{TM} HT-Smart," which integrates three key modules for LNP preparation within a 96-well plate: (1) an automated lipid preparation module that mix lipid components in defined ratios, (2) a mixing module that facilitates the rapid mixing of lipids and payloads using either a T-mixer or a microfluidic mixer, and (3) an automated buffer exchange module for removing ethanol and acidic buffers. The device also features a temperature control system to maintain payload and mRNA stability throughout the process.

To validate the HT-Smart platform, several experiments were conducted:

- Mixing Regime Validation: T-mixers and microfluidic mixers were compared directly, demonstrating that both mixers could produce LNPs with consistent physical properties (size and PDI) when the optimal flow rate was identified.
- Reproducibility Testing: A single LNP formulation was independently prepared 96 times within a plate. The resulting LNPs showed high reproducibility with sizes <80 nm and PDI <0.1 across wells.
- Process Optimization: By varying mixing flow rates, LNP sizes were tuned from 70 to 100 nm.
- Large-Scale DOE Experiments: A Design of Experiment (DOE) approach was applied to formulate 96 unique LNPs encapsulating luciferase mRNA. Parameters such as lipid ratios, N/P ratios, and flow rates were systematically varied, resulting in LNPs with diverse size, PDI, encapsulation efficiency, and charge. From these, 30 formulations with >90% encapsulation efficiency and size <80 nm were selected for in vivo testing. Intramuscular injection into mice revealed a lead LNP formulation with over 5-fold higher mRNA expression compared to other candidates.

These findings demonstrate that HT-Smart is a robust platform for high-throughput screening of payloads, LNP formulations, and process parameters. This capability is expected to significantly accelerate the discovery and development of genetic medicines.

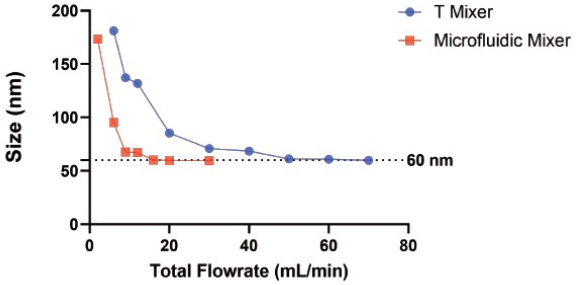
(A)

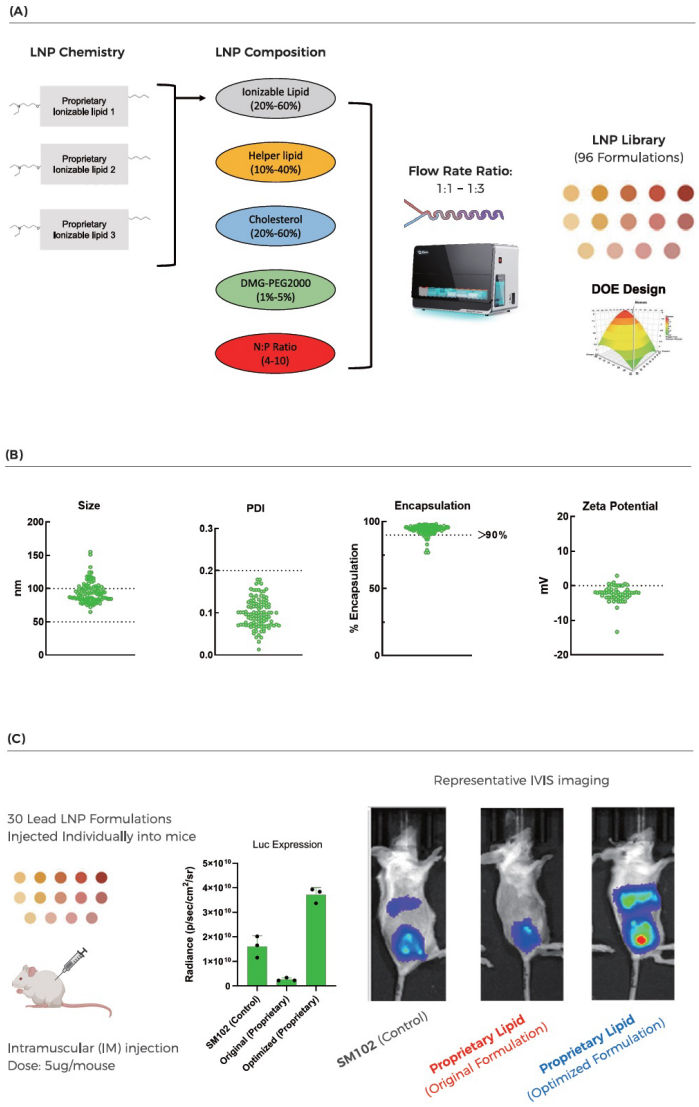

T-Mixer
Microfluidic Mixer

(B)

INano™ L⁺




T Mixer
Microfluidic Mixer
Size (nm)
200
150
100
50
0
60 nm
0
20
40
60
80
Total Flowrate (mL/min)



1675 Advanced mRNA Lipid Nanoparticle Analysis by Flow Cytometry

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Lipid nanoparticles (LNPs) have emerged as a widely used delivery system for vaccines and therapeutics since the successful deployment of mRNA LNP vaccines against COVID-19. Recognized as a platform technology, LNPs can facilitate the rapid production and widespread availability of new medicines for various complex diseases upon FDA approval. The increasing demand for new mRNA LNPs underscores the need for high throughput methods to analyze their dosage and quality accurately.

Current techniques for measuring mRNA LNPs have limitations. Dynamic light scattering estimates diameter based on time correlations of the scattered light intensity but is biased towards larger particles, making it problematic for

heterogeneous samples. Nanoparticle tracking analysis suffers from variable detection limits due to sample polydispersity. Cryo-electron microscopy, recognized as the gold standard, is impractical for routine use due to its high cost and time requirements. RNA loading and encapsulation efficiency are often measured by ribogreen assay but this bulk measurement technology also has limitations. In contrast, flow cytometry offers significant advantages, enabling high-throughput analysis at the single nanoparticle level. In this study, we utilized a flow cytometry assay with various lipophilic, lipid-impermeable, and RNA dyes to detect empty LNPs and those with poor encapsulation efficiency. Several LNP preparations and fluorophores were examined on the Amnis Imagestream Flow Cytometer and other orthogonal methods. We have identified LNP and nucleic acid dyes that can be optimized for LNP analysis in flow cytometer platforms. Different subpopulations of LNP particles, including those with LNP membrane-bound RNAs and LNPs containing multiple mRNAs, were characterized with flow cytometry to provide great insights into LNP quality. This study has significant implications for LNP manufacturing and the analytics of nanoparticles, providing a fast and accurate assessment method with a high-throughput flow cytometry platform.

1676 A Novel HPLC-ELSD Method for Lipid Identification and Quantification in mRNA-LNP Complexes

Xiulian Sun[1], Zhong Ming[1], Hu Liu[2], Xiaohong Huang[2], Xiulan Zhu[2]
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Objective: A typical Lipid Nanoparticle (LNP) delivery system for mRNA vaccines consists of four types of lipids: cationic lipids with amine functional groups that bind to mRNA via ionic interactions, lipid-polyethylene glycol conjugates, cholesterol, and zwitterionic phospholipids. However, LNPs are prone to aggregation and degradation during storage and transportation, which can compromise the efficiency and safety of mRNA vaccines. Qualitative and quantitative testing of individual lipids is essential for process and formulation development and serves as a critical QC item during the release stage

Methods: This method utilizes an Agilent 1260 Infinity II HPLC system equipped with an Agilent Poroshell 120 EC-C18 column (inner diameter: 4.6 mm, length: 150 mm, particle size: 4.0 μm) and an Agilent 1260 Infinity II Evaporative Light Scattering Detector (ELSD). The mobile phase consists of methanol and water (98:2) with 7.5 mM ammonium formate. The flow rate is set at 1.2 mL/min. The column, evaporation, and nebulizer temperatures are maintained at 65°C, 50°C, and 40°C, respectively.

Result: The chromatogram in Figure 1 below demonstrates the baseline separation of all four lipids, with each lipid component identified by its unique retention time. Furthermore, each of the four lipid components exhibits linear quantitation within the following detection dynamic ranges: PEG2000-DMG, 0.004 mg/mL to 0.12 mg/mL; Cholesterol, 0.0006 mg/mL to 0.50 mg/mL; SM102, 0.002 mg/mL to 1.25 mg/mL; and DSPC, 0.003 mg/mL to 0.30 mg/mL.

Conclusion: A method for determining lipids extracted from mRNA-LNP vaccines, featuring high detection sensitivity and a wide dynamic range, has been developed using the cost-effective Evaporative Light Scattering Detector (ELSD) compared to the more expensive Charged Aerosol Detector (CAD). A comprehensive methodology verification was performed, and the results confirmed that the method is reliable, accurate, and free from interference, making it suitable for ensuring the safety and efficacy of lipids in mRNA complexes.

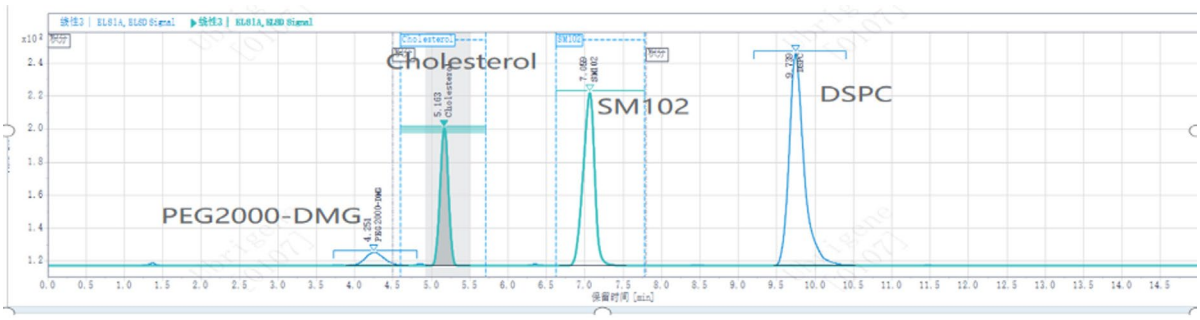

Figure 1 HPLC chromatogram of lipids from lipid nanoparticles.

1677 Optimized CRISPR-Cas9 Editing of Primary Human Immune and CD34+ Hematopoietic Stem and Progenitor Cells using a Mechanoporation Platform

Eric Ouellet[1], Ulrike Lambertz[2], Quan Nguyen[3], Tina Liao[2], Phillip Chau[2], Gil Paik[2], Elinor Binson[2], Colin Hammond[3], Marta Walasek[3], Nooshin Tabatabaei-Zavareh[1], Allen Eaves[4], Sharon Louis[3], Andy Kokaji[1]
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Genetic engineering of human immune cells and hematopoietic stem and progenitor cells (HSPCs) is critical for advancing immunology and regenerative medicine research. However, traditional intracellular delivery methods, such as viral transduction or electroporation, can pose significant challenges to cell engineering due to increased cytotoxicity, inefficient delivery to non-dividing cells, and altered cellular physiology.

Mechanoporation is a non-viral intracellular delivery approach that relies on controlled mechanical deformation of cells within microfluidic channels. This creates transient pores in the cell membrane for cytosolic cargo delivery. Unlike conventional methods, mechanoporation minimizes unwanted cellular perturbations and safeguards physiological function. These features are advantageous for applications that require manipulation of sensitive cell types that are prone to loss or need to be studied in their resting states, such as unactivated immune cells or primitive HSPC populations. The CellPore™ Transfection System is a mechanoporation platform that efficiently delivers diverse biomolecules while preserving cell quality attributes, such as viability, phenotype, and function. Here, we demonstrate the efficiency of CellPore™ in delivering CRISPR/Cas9 ribonucleoproteins (RNPs) for achieving robust gene knockout in unactivated T cells, natural killer (NK) cells, and CD34+ HSPCs.

In unactivated T cells, CRISPR-mediated knockout (KO) of surface markers MHC-I and TCRαβ achieved efficiencies of 74% (mean; n = 12) and 94% (n = 3), respectively, while maintaining high viability (> 80%; n = 15) and preventing unwanted activation (indicated by low CD69 activation marker expression and minimal pro-inflammatory cytokine secretion, comparable to untreated control). Importantly, we also demonstrated that gene-edited unactivated T cells retained their downstream activation/expansion potential. Similarly, in primary human NK cells, KO efficiencies of 85% for TIGIT and 87% for NKp46 were obtained (n = 3), and cells retained their downstream cytotoxic and expansion capabilities.

In CD34+ HSPCs, CellPore™ mechanoporation achieved KO efficiencies of 92% (n = 4) for MHC-I and 84% (n = 3) for CD45, with a shortened pre-transfection culture timeline. Reliable RNP transfection and subsequent gene knockout was achieved as early as 4 hours post-thaw, and optimally after 24 hours in culture, contrary to electroporation, which required 48 hours in culture to achieve similar gene editing performance. The shortened workflow resulted in higher retention of CD34+CD90+CD45RA- hematopoietic stem cells, with minimal impacts to differentiation, expansion, and clonogenic potential, for edited HSPCs relative to the untreated control.

This study highlights the power of mechanoporation-based intracellular delivery to meet the evolving demands of modern generic engineering. The CellPore™ Transfection System achieves high editing efficiencies with minimal cellular perturbations, and offers a streamlined and practical solution for ex vivo engineering of immune cells and

HSPCs. It enables the study of novel immune functions, disease mechanisms, and cell-based therapies that would be otherwise challenging to perform using conventional methods. This platform provides researchers with reliable tools and avenues for accelerating innovations in cell therapy and regenerative medicine research.

1678 Exploring Mechanoporation for Efficient Genetic Modification Across Diverse Cell Types Using SMAR Vectors

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**Corresponding Author*

Gene and cell therapies rely on stable and safe genetic modification of cells for various applications such as cancer immunotherapy and the generation of stable cell lines. Our research focuses on leveraging non-viral, Scaffold/Matrix Attachment Region (S/MAR)-based vectors that provide sustained gene expression without genomic integration. These vectors have shown effectiveness across multiple cell types, including T and NK cells, Jurkat cells, HEK cells, induced pluripotent stem cells (iPSCs), and hematopoietic stem cells (HSCs). However, efficient DNA delivery and cell viability remain key challenges. Mechanoporation offers a promising solution for efficient DNA delivery with the potential to improve cell recovery and viability. The CellFE technology employs a microfluidic system to achieve rapid cell deformation, generating mechanical forces to porate cells and enhance transport through convective forces. DNA delivered by CellFE mechanoporation results in a more evenly distributed DNA payload, with a lower concentration of DNA leading to effective uniform delivery. Consequently, this technique reduces toxicity and improves cell recovery following gene transfer. Data from our lab demonstrates that mechanoporation with SMAR vectors can be used for the efficient transfection of cell lines such as HEK293T and Jurkat cells. This study illustrates the use of mechanoporation for the transfection of these cell types with SMAR vectors carrying fluorescent reporter genes and chimeric antigen receptors (CARs). We measure key metrics such as transgene expression efficiency and cell viability, along with functional assays measuring tumor-killing efficiency in CAR-transfected immune cells. Additionally, we show that mechanoporation can be used to efficiently generate stable cell lines. We show that CellFE mechanoporation is applicable as an advanced transfection technique compatible with SMAR vectors. The results contribute to understanding the versatility and robustness of SMAR vectors for diverse use cases including immune cell engineering, the generation of stable cell lines, and applications in regenerative medicine.

1679 Improving Workflow Efficiency of CAR T Cell Gene Editing Using Microfluidic Delivery Technology

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[1]CellFE, Alameda, CA, [2]NVI Therapeutics, Inc, Hayward, CA

Genetic editing of CAR T cells beyond introduction of the CAR transgene holds great promise for expanding the applicability of CAR T cell therapies. Editing is essential for mitigating allogeneic interactions in donor-derived T cell therapies and can be applied to increase T cell fitness in immunosuppressive microenvironments. However, the introduction of additional editing steps increases the complexity of manufacturing processes and results in substantial toxicity to cells using currently available methodologies.

To address these challenges, we have developed a transformative system for microfluidics-based mechanoporation that can be used to introduce gene editing reagents such as CRISPR/Cas9 into T cells. We found that microfluidic mechanical compression can seamlessly integrate T cell transfection and viral transduction without increasing the total editing time compared to viral transduction alone. In the new workflow, T cells are edited by mechanical transfection of Cas9 RNP on the same day as lentiviral transduction of a CAR transgene. Using TCR knockout as a

test case, we show that the workflow routinely produced T cells edited with greater than 80% efficiency. Cells recovered viability within 2 days of transfection and proliferated at the same rate as non-transfected controls. Transfected cells exhibited superior CAR transduction efficiency compared to non-transfected controls. Gene editing preserved CAR T cell immunophenotype, particularly stem cell memory prevalence. Edited CAR T cells retained high cytotoxicity and cytokine production in response to CD19-expressing target cells.

The workflow provides multiple benefits compared to conventional gene editing protocols. First, cells recover quickly from transfection and can be immediately handled for purposes of viral transduction, simplifying the production process. Second, viral transduction immediately following transfection increases gene transfer compared to transduction alone.

Importantly, the microfluidics-based gene editing process can also be applied to non-activated T cells. Comparable rates of gene editing and CAR T cell yield were achieved by transfecting T cells maintained in a quiescent state. These cells differentiate less than activated T cells and retain their stem cell memory phenotype, offering a platform for production of CAR T cell products with exceptional potency and durability.

1680 Non-viral, high throughput genetic engineering of diverse human primary immune cells using nanostraw-mediated transfection

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Transfection of proteins, mRNA, and chimeric antigen receptor (CAR) transgenes into immune cells remains a critical bottleneck in cell manufacturing. Current methods, such as viruses and bulk electroporation, are hampered by low transfection efficiency, unintended transgene integration, and significant cell perturbation. The Nanostraw Electro-actuated Transfection (NExT) technology offers a solution by using high aspect-ratio nanostraws and localized electric fields to precisely deliver biomolecules into cells with minimal disruption. We demonstrate that NExT can deliver proteins, polysaccharides, and mRNA into primary human CD8+ and CD4+ T cells, and achieve CRISPR/Cas9 gene knockout of *CXCR4* and *TRAC* in CD8+ T cells. We showcase NExT's versatility across a range of primary human immune cells, including CD4+ T cells, γδ-T cells, dendritic cells, NK cells, T_{reg} cells, macrophages, and neutrophils which are being developed for a variety of immune cell-based therapies. Finally, we developed a scalable, high-throughput multiwell NExT system capable of transfecting over 14 million cells and delivering diverse cargoes into multiple cell types from various donors simultaneously. This technology holds promise for streamlining high-throughput screening of allogeneic donors and reducing optimization costs for large-scale CAR-immune cell transfection.

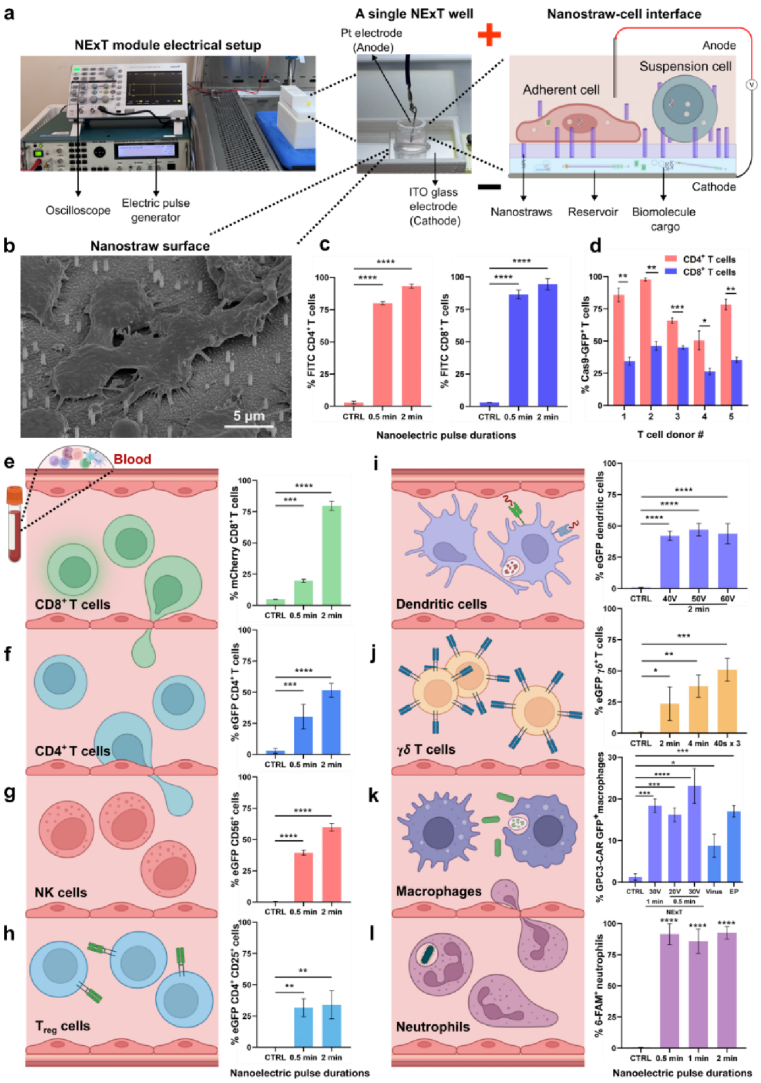

Figure 1. NExT transfection system enables intracellular delivery to diverse primary human immune cell types. (a) Schematic of NExT transfection system illustrating the different components including an electrical pulse generator and oscilloscope on the left image. The magnified image exhibits a single NExT well, comprising a plastic cylinder with nanostraw membrane adhered at the bottom, facilitating the channel between the biomolecule cargoes at the bottom and cells overlaid on the top. The circuit is closed by placing the Pt electrode anode dipped into the media and the bottom ITO glass electrode serving as the cathode. **(b)** FE-SEM image showing close adherence of CD8+ T cells around high aspect-ratio nanostraws after centrifugation and incubation for 2 hours. **(c)** Transfection efficiency of FICT-Bovine Serum Albumin (BSA) showing more than 80% delivery in CD4+ and CD8+ T cells respectively after 24 hours (n = 3 donors). **(d)** Transfection efficiencies of, GFP-Cas9 into CD4+ and CD8+ T cells demonstrate donor-to-donor differences (n = 5 donors). **(e)** Transfection efficiency of mCherry mRNA with > 80% in CD8+ T cells after 24 hours. **(f)** eGFP mRNA delivery with > 50% is observed in CD4+ T cells after 24 hours (n = 3). **(g)** Transfection efficiency of eGFP mRNA of up to 60% efficiency in NK cells (cell selection with CD56+ marker) after 24 hours (n = 4). **(h)** Delivery of eGFP mRNA in CD4+CD25+ T_{reg} cells was observed in over 30% cells with 2 min electric pulse after 24 hours. (n = 3). **(i)** Transfection of eGFP mRNA into human dendritic cells was observed up to 45% efficiency with 2 min electric pulse delivered at 50V (n = 3). **(j)** The transfection efficiency of eGFP mRNA resulted in delivery efficiency of up to 50% in γδ-T cells within 24 hours. (n = 3 donors). **(k)** Transfection efficiency of THP-1-derived M1 macrophages with GPC3 CAR constructs evaluated 24 hours after transfection by measuring the GFP expression. NExT delivered GPC3-CAR constructs with up to 23.08 ± 4.15% efficiency, surpassing viral vectors transfect (Virus: 8.75 ± 2.26%) and bulk electroporation (EP: 17 ± 1.09%) (n = 3 biological repetition) Expression efficiency was evaluated by measuring the GFP tag in CAR expressing macrophages. **(l)** NExT could achieve up to 90% delivery efficiency of 6-FAM-labelled siRNA into primary bone marrow derived mouse neutrophils (n = 3). Statistical significance was determined by one-way ANOVA test with post-tests performed with Tukey's multiple comparisons test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

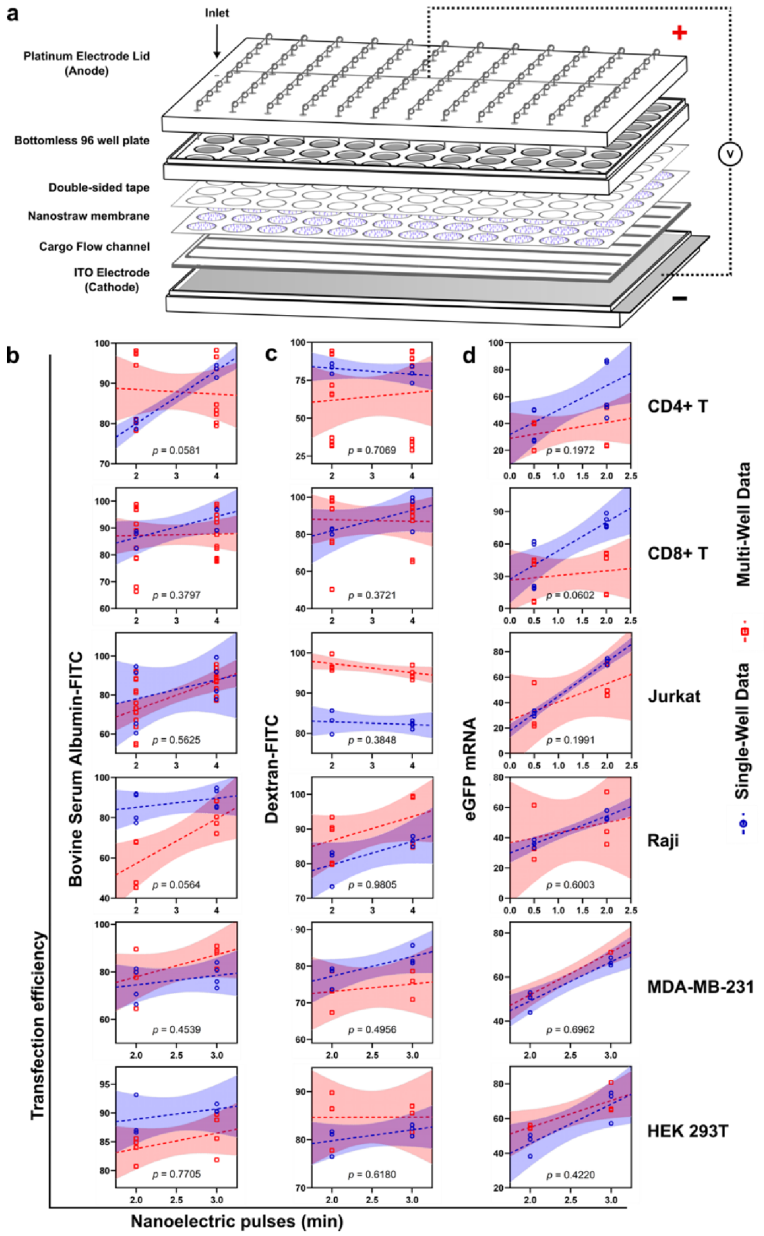

Figure 2. High throughput multiwell NExT platform enhances transfection throughput and exhibits comparable efficiencies to single well NExT system. (a) Schematic of high-throughput 96-multiwell NExT system. **(b)** Diverse cell types were seeded into the 96-well NExT system and transfected with BSA-FITC protein throughout the well plate. The transfection efficiency was evaluated 24 hours after transfection using flow cytometry. There was no significance observed between the multi-well system and single-well system with transfection of BSA-FITC and an overall increasing trend of transfection efficiency with increasing nano-electric pulse durations (min) **(c)** Diverse cell types were seeded into the 96-well NExT system and transfected with Dextran-FITC throughout the well plate. The transfection efficiency was evaluated 24 hours after transfection using flow cytometry. There was no significance observed between the multi-well and single-well system with transfection of Dextran-FITC and an overall increasing trend of transfection efficiency with increasing nano-electric pulse durations (min) except for Jurkat cells. **(d)** Diverse cell types were seeded into the 96-well NExT system and transfected with eGFP mRNA throughout the well plate. The transfection efficiency was evaluated 24 hours after transfection using flow cytometry. There was no significance observed between the multi-well and single-well system with transfection of eGFP mRNA and an overall increasing trend of transfection efficiency with nano-electric pulse durations (min) can be observed.

1681 Precise intradermal and suprachoroidal delivery of gene and cell therapeutics using a silicon microneedle

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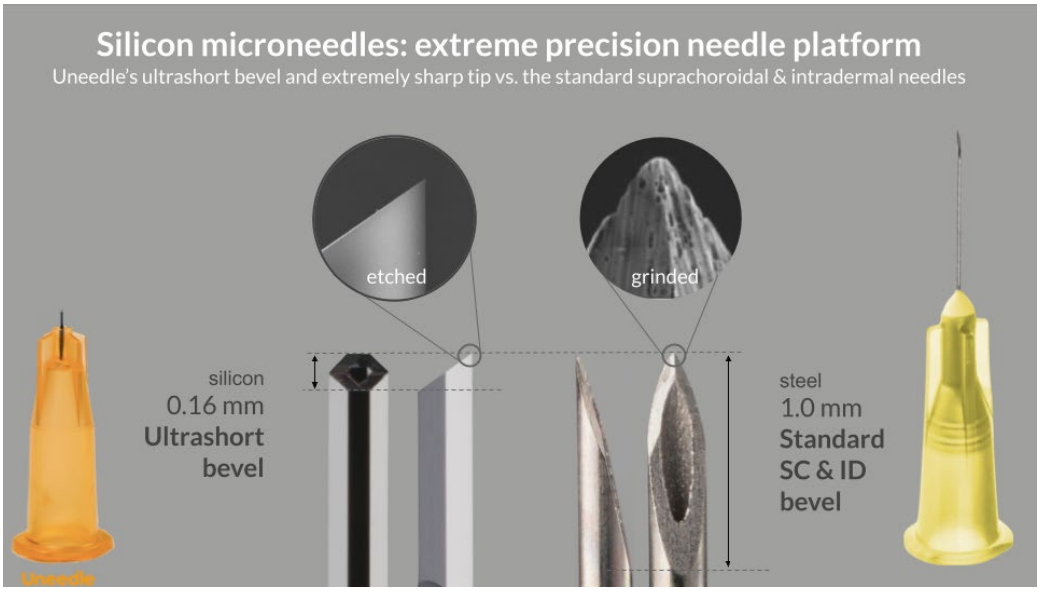
**Corresponding Author*

Introduction: The precise delivery of novel gene and cell therapeutics into superficial tissues is critical for maximizing their efficacy. Treatments such as cell-based cancer vaccines targeting the skin or adeno-associated viral (AAV) vectors administered to the suprachoroidal space rely on accurate depth-specific administration. However, traditional delivery methods, such as steel needles, lack precision and present challenges in achieving the targeted depth. Inaccurate administration of gene and cell therapies leads to unreliable dosing limiting therapeutic effectiveness and hampering translatability in larger clinical trials or real-world situations. Using silicon, a crystalline material, provides a promising solution to enable precise depth delivery in the dermis and suprachoroidal space, advancing the potential of gene and cell therapeutics.

Methods: Uneedle has developed a silicon microneedle platform that can precisely deliver therapeutics at depths anywhere between 200 and 1400 µm. This innovative delivery technology is simple to use in combination with any Luer lock syringe to administer therapeutics in superficial layers of the skin or eye. Clinical evaluations were conducted using cell-based and DNA-based cancer vaccines, as well as mRNA vaccines. The effectiveness, usability and dose sparing effects were assessed. Preclinical ophthalmological proof-of-concept evaluations were conducted using Non-human primates and New Zealand rabbits to assess the feasibility of microneedle delivery for suprachoroidal applications.

Results & conclusions: Here, we present data from (pre)clinical studies demonstrating the successful administration of gene and cell therapeutics using Uneedle's silicon microneedle technology. These studies establish proof of concept for the precise and efficient delivery of therapeutics into the dermal layers of the skin and the suprachoroidal space of the eye, highlighting the versatility and potential of this innovative platform. By enabling precise targeting and delivery, silicon microneedles offer a significant advancement in the field of gene and cell therapy, enhancing therapeutic efficacy and broadening clinical applications of current and novel therapeutics.



1682 ThecaFlex DRx™: Implantable Intrathecal Catheter and Subcutaneous Port System for Repeated Intrathecal Delivery of Nusinersen

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Many genetic therapies, including antisense oligonucleotides, RNA-based therapies, and biologics for treatment of neurological disorders require repeated intrathecal (IT) dosing via lumbar puncture (LP). Repeated LP can be challenging for younger children, individuals with complex spinal anatomy and those who require repeat anesthesia and radiation exposures for therapeutic administration. Additional obstacles may also include complications from therapy administration such as post-LP headache. Consequently, patients who fall in these categories may benefit from an alternative delivery method.

Currently, there are no US Federal Drug Administration (FDA) approved IT catheter and subcutaneous port systems for therapeutic delivery. The ThecaFlex DRx™ System, part of Alcyone's Falcon™ Intra-CSF Delivery Platform, is designed to address this significant unmet need. ThecaFlex DRx™ has undergone extensive pre-clinical testing including design verification, in vitro functional testing, biocompatibility and extractables, drug-device compatibility, ovine in vivo safety and functionality studies, and extensive human factors usability evaluations. The design allows for both aspiration of CSF and infusion of therapeutics and is intended to reduce rate of infusion failures due to kinking, breakage, occlusion, migration or catheter-port disconnection, when compared to available off-label implanted systems.

Following development, the investigational device, ThecaFlex DRx™, received CE Mark Approval in Europe and Breakthrough Device Designation from the FDA. The safety and performance of ThecaFlex DRx™ is currently under evaluation in a two-stage IDE clinical trial (PIERRE, NCT05866419) delivering nusinersen to individuals with spinal muscular atrophy (SMA). In stage 1, 10 participants were enrolled, implanted, infused with nusinersen and followed for 30 days. During stage 1, ThecaFlex DRx™ performed as intended, and no device-related infections or device-related adverse events were observed. After reviewing these data, FDA approved stage 2, which is currently enrolling the balance of 80 individuals across the U.S. and Europe. Concurrently, an additional study (PIERRE-PK, NCT06555419) comparing nusinersen pharmacokinetics between LP and ThecaFlex delivery routes is also being conducted.

Potential clinical benefits of the ThecaFlex DRx™ system may include:

- Mitigating the challenges associated with repeat LP dosing, including potential reduction in the need for anesthesia and/or radiation for therapy, infusion, and monitoring, especially for patients with complex spinal anatomy.
- Enabling routine subcutaneous administration of therapies historically delivered intrathecally to the cerebrospinal fluid with the goal of improving the patient treatment experience.
- May allow more reliable intrathecal dosing, due to the potential variability of needle placement with manual injections

Combined, these benefits may improve the patient treatment experience of repeat drug administration to the CNS and may provide an easier approach to CSF sampling and therapeutic delivery. Importantly, ThecaFlex DRx™ IT catheter and port system may be applicable to broad populations with neurological disorders.

1683 MRI-Guided Focused Ultrasound: A New Drug Delivery Modality Addressing the Blood Brain Barrier

Zeinab Noroozian[1], **Michael Templin[2]**, Catherine Spickler[3], Kathryn McCabe[4], Rafi de Picciotto[4], Itay Rachmilevitch[4], Yeruham Shapira[4], Michael Plaksin[5], Sang Won Choi[4], Arjun Desai[4], Julius Haruna[3], Madhu Ravi[3], Simon Authier[6]
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The use of gene therapy and vector delivery as potential treatment methods for neurodegenerative diseases is of peak interest to the scientific and clinical communities. Delivery mechanisms to the brain that can overcome the blood-brain barrier (BBB) are critical to ensure effective non-invasive drug delivery to the target region. MRI-Guided Focused Ultrasound (FUS) has been used as an effective tool in delivering antibodies, RNA and small molecules to specific target areas of the brain. Results from the current study provided insight on the neuroanatomical region to treat and on acceptable acoustic dose levels to be used in cynomolgus monkeys to allow delivery through the BBB. The technology used in the current study was the same as used clinically (Insightec Exablate Type 2 Low Frequency device). An AAV9 gene therapy vector with mCherry as reporter gene was used. Specifically, during these assessments (n= 12), sensitivity and tolerance to the acoustic dose (A.D) was confirmed in all animals using MR with contrast agent (i.e. gadolinium). The acoustic dose ranges for several brain targets were also assessed (A.D = 0.7 (caudate nucleus and putamen); A.D = 0.5 (thalamus and hippocampus)).The blood-brain barrier was noted to close approximately 4 hours postdose, with complete closure by 48 hours postdose. T2* imaging confirmed changes in the vascularization of tissue with fluid mobility through the BBB. High resolution diffusion tensor imaging (DTI) was used to map neuronal tracks to help predict drug diffusion patterns following FUS in cynomolgus monkeys. In conclusion, the use of FUS has provided great insight into the capability of locally surpassing the BBB and will serve as an effective method of therapeutic delivery of gene therapy to the brain.

1684 Scalable Softporation™ platform for intracellular delivery to primary natural killer (NK) cells

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Introduction: Transfection of primary immune cells, including natural killer (NK) cells, is essential for next-generation immunotherapies. However, current methods suffer from low viability and high cell loss. This study evaluates Softporation™, a novel transfection platform, for modifying primary NK cells and delivering intracellular payloads. The system uses fluid pressure generated by acoustic amplification to eject cells through a nozzle array and reversibly porate cell membranes, enabling the efficient introduction of large molecules while preserving native cell conditions. This method of payload delivery retains high cell viability and enables continuous processing of 1-300M cells/min, representing a critical step in the realization of an end-to-end NK cell manufacturing platform. Leveraging the Softporation™ platform's ability to increase throughput while preventing significant cell loss, we have established that the platform can be used for delivery of large fluorophore conjugates to primary NK cells. Effective operation with cell concentrations ranging from 1E6 cells/mL to 2E7 cells/mL were used to demonstrate the capacity of the system to support large-scale cell engineering and production pipelines.

Methods: Primary human NK cells from multiple donors were treated with the Softporation™ platform for delivery of three payloads - fluorophore-conjugated dextran molecules (70kDa, 500kDa, 2MDa) - using a range of device parameters. Following Softporation™ treatment, delivery efficiency was evaluated relative to untreated cell cultures incubated with equivalent concentrations of payload. Flow cytometry was used to quantify the delivery efficiency of the treatment. Cell viability and total cell yield were also evaluated. Cell concentrations of 1E6, 3E6, 1E7, and 2E7 cells/mL were tested. Payload concentration was kept constant at 0.5 mg/mL for fluorophore conjugates.

Results: The Softporation™ platform was successfully delivered large payloads to primary NK cells while retaining high viability (>99% of initially viable cells remained viable post-treatment). Cell viability following passage through the Softporation™ platform was not statistically different from starting viability ($p=0.86$). System throughput (flow rate over modified-cell production rate) was associated with applied acoustic field strength ($p<0.001$) under constant cell concentration. It was also observed that increasing cell concentration resulted in increased delivery ($p<0.001$).

Conclusion: The OpenCell Technologies Softporation™ platform enables efficient, high-viability delivery of large molecules (fluorophore-conjugated dextran) to primary NK. This platform addresses outstanding issues in the engineered NK cell manufacturing workflow and enables the possibility of repeated exposure to increase cumulative delivery.

1685 Swine Reporter Model for *In Vivo* Vector Characterization

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Delivery of nucleic acids to the appropriate tissues and cells at an effective dose is a significant challenge for *in vivo* gene therapies. Rodent reporter models activated by recombinases or gene editing reagents have been indispensable for characterization of new gene delivery vector modalities. However, their small size and anatomy do not always accurately translate to delivery in large mammals. To address this concern, we are developing a series of swine reporter models designed to track *in vivo* vector delivery to any tissue or cell in the body. The first Swine Reporter Model (SRM-1) harbors a modified Ai9 allele to allow for Cre mediated recombination as well as single-guide SpCas9 or AsCas12a CRISPR-based activation. This reporter is driven by a highly active and ubiquitous CAG promoter and is positioned at the *Rosa26* safe harbor locus. After removal of a strong transcriptional stop domain by CRISPR or Cre, the transfected cells will begin producing a fluorescent gene (tdTomato) that can be tracked by flow cytometry or direct imaging of the tissue. Additionally, a sodium iodide symporter (NIS) gene is expressed to also allow for non-invasive imaging of cell activation.

To assess the effectiveness of SRM-1 for tracking vector transfection *in vivo,* we intravenously injected AAV9-Cre into a male SRM-1 piglet and collected tissues for analysis of reporter activation. The injected animal was found to have widespread viral tropism with the highest reporter activation in muscle, adrenal glands, testicular tissue, and liver. These tissues were analyzed by IHC to visualize the reporter activation, which showed clear and robust reporter activation at the tissue and cellular level. We next systemically delivered LNPs carrying Cre mRNA and analyzed tissues from three piglets using fluorescent imaging of tdTomato. We observed the highest rates of reporter activation in the swine liver, as previously observed in mice, NHP, and humans. In addition, we saw substantial uptake in the spleen, testicular tissue, and adrenal gland, with lower levels of transfection in lung and gastrointestinal tissues. To broaden the utility of a swine reporter models, a second reporter pig model has also been created that can be activated by adenine base editor and by prime editing. We anticipate these pig models will be impactful for studying novel nucleic acid delivery vectors, as well as for establishing clinically relevant routes of administration in large animals for viral or nonviral gene therapies.

1686 Charge Detection Mass Spectrometry of Large Biomolecular Assemblies: A Novel Tool for the Characterization of Gene Therapy Vectors

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Introduction: *Mycobacterium tuberculosis* (*Mtb*) encodes a rare prokaryotic proteasome system essential for evading the human immune system. Substrate access to the active sites of the 20S core particle (CP) is tightly controlled via proteasome activators to avoid spurious degradation. Proteasome activators recruit substrates and induce proteasome gate opening to facilitate substrate degradation. In *Mtb*, this is partly handled by Bpa (bacterial proteasome activator - 228 kDa homododecameric ring). Functional assays have established that Bpa can bind its native substrate, HspR, and translocate it to the 20S CP for degradation. The stoichiometry, oligomeric heterogeneity, and high-resolution structures of Bpa:HspR complexes remain elusive. Here we use charge detection mass spectrometry and electron cryomicroscopy (cryo-EM) to address these questions.

Methods: *Mtb* Bpa and its well-established substrate HspR were recombinantly co-expressed in *E. coli*. Proteins were purified using immobilized metal affinity chromatography (IMAC), followed by size exclusion chromatography (SEC). We performed native MS on a prototype Waters CDMS instrument to study the previously unknown Bpa:HspR complex stoichiometry. Additional CDMS experiments where the ion sampling inlet was heated to induce complex dissociation were also performed. Single-particle cryo-EM data was collected on either a 200 kV Glacios or a 300 kV Titan Krios microscope to generate a high-resolution reconstruction of the Bpa:HspR complex. Data processing was done using CryoSPARC 4.6.2 to generate 2D and 3D classifications. The resulting density maps will then be used to structurally model the Bpa-HspR complex.

Preliminary data: SDS-PAGE and intact protein mass spectrometry showed that His-SUMO-tagged HspR could pull down untagged Bpa during IMAC and co-eluted with Bpa during SEC, indicative of tight binding between Bpa and HspR. Reconstitution of a functional Bpa-20S CP degradation system *in vitro* was tested using discontinuous SDS-PAGE based degradation assays. CDMS was performed on a Waters prototype CDMS instrument equipped with an electrostatic linear ion trap and a nanoelectrospray source. The resulting spectra established dodecameric Bpa bound to two copies of HspR, with minimal heterogeneity in the number of HspR subunits bound (Fig 1). CDMS experiments where the ion sampling inlet was heated to induce complex dissociation showed charge-reduced Bpa dodecamers, consistent with asymmetric charge partitioning of the Bpa:HspR complex due to HspR ejection (Fig 2). We used single-particle cryo-EM to generate a high-resolution reconstruction of the Bpa:HspR complex. Substrate-bound samples analyzed using a 200 kV Glacios microscope yielded 2D classes with clear density within the central pore of Bpa. The preferred orientation of Bpa dodecamers yielded limited orientations and therefore made 3D classification difficult. An experiment in which Bpa-HspR is mixed with 0.25% IGEPAL and subsequently frozen appeared to drastically broaden the distribution of orientations. These samples will be subjected to 300 kV Krios analysis, and the resulting data will be processed to generate high-resolution maps suitable for structural modeling, thereby enabling the elucidation of the interaction sites between Bpa and HspR at atomic resolution. The implications of these findings for the gene therapy manufacturing pipeline will be discussed.

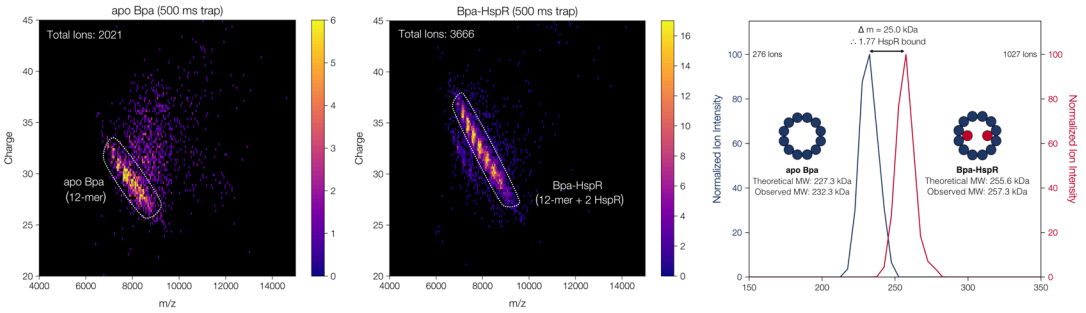

Fig 1. CDMS data represented as 2D and mass histograms of apo Bpa and Bpa-HspR complex. White hatched lines indicate points included after data filtering for m/z, z, and m analysis. (A) 2D histogram of apo Bpa created using in house Python scripts. (B) 2D histogram of Bpa-HspR created using in house Python scripts. Bin size and number for z and m/z is (0.5,100) and (50, 220), respectively (C) Mass histograms comparing filtered apo and substrate bound Bpa data. Bin size and number for m is (5000,1000), respectively. All experiments were conducted at 1 uM Bpa (Bpa monomer) in 10 mM ammonium acetate, pH 7.0. Both data sets were collected utilizing nano electrospray ionization via pulled borosilicate capillaries (5 um tip diamater) and 500 ms ion trapping time on a prototype Waters charge detection mass spectrometer.

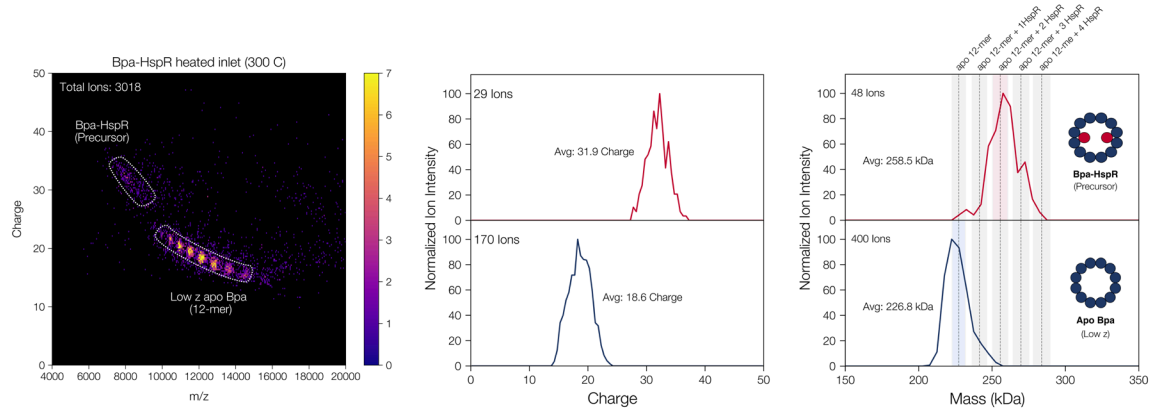

Fig 2. CDMS data represented as 2D and mass histograms of Bpa-HspR after dissociation via inlet heating (300 °C). (A) 2D histogram of Bpa-HspR created using in house Python scripts. White hatched lines indicate points included after data filtering for m/z, z, and m analysis. (B) Charge histogram of Bpa-HspR created using in house Python scripts. Bin size and number for z is (0.5,100). (C) Mass histograms comparing filtered Bpa-HspR precursor and low z apo Bpa data. Bin size and number for m is (5000,1000), respectively. Grey hatched area represents ± 2% of the theoretical weight for each respective species. All experiments were conducted at 1 uM Bpa (Bpa monomer) in 10 mM ammonium acetate, pH 7.0. Both data sets were collected utilizing nano electrospray ionization via pulled borosilicate capillaries (5 um tip diameter) and 500 ms ion trapping time on a prototype Waters charge detection mass spectrometer.

1687 Advancements in Charge Detection Mass Spectrometry for Characterizing Large, Intact mRNA Structures and Optimizing mRNA Therapeutic Analytics.

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Rapid advances in biotechnology have accelerated the use of messenger RNA (mRNA) to combat a wide range of incurable diseases. Since the release of the mRNA-based COVID-19 vaccines, the potential of mRNA technology has only begun to be fully realized. mRNA utilizes host cell machinery to produce proteins that aid the immune

system in fighting infection and disease. The process of mRNA translation is tightly regulated, with a delicate balance between the decay of its structure, especially in the 5' cap and the poly(A) tail. The 5' cap protects the mRNA from 5' to 3' exoribonucleases, while the length of the poly(A) tail determines the 3' to 5' exonucleolytic decay. Numerous studies have focused on optimizing these features to develop the most effective mRNA designs. As mRNA is modified, techniques to characterize these structures are required at every stage of their development. As the use of mRNA therapeutics expands analytical techniques to confirm identity and stability need to be explored. Mass spectrometry is a robust technique to confirm identity of small molecules, but therapeutic mRNA's fall beyond the mass limit of conventional mass spectrometers. Methods have been developed to enzymatically cleave mRNA to sizes that can be measured by conventional mass spectrometry; however, information on truncations and/or partial sequences can be lost. Charge detection mass spectrometry (CD-MS) is an emerging technique that has shown significant utility in the high-resolution mass measurement of large heterogenous biomolecules. Herein, we developed a CD-MS method for intact mRNA analysis, featuring optimized sample preparations for high resolution analysis of large mRNA sequences. Using this high-resolution technique, we measured the mRNA construct for hemagglutinin (HA) protein of the influenza virus and were able to differentiate constructs with variable poly(A) tail length.

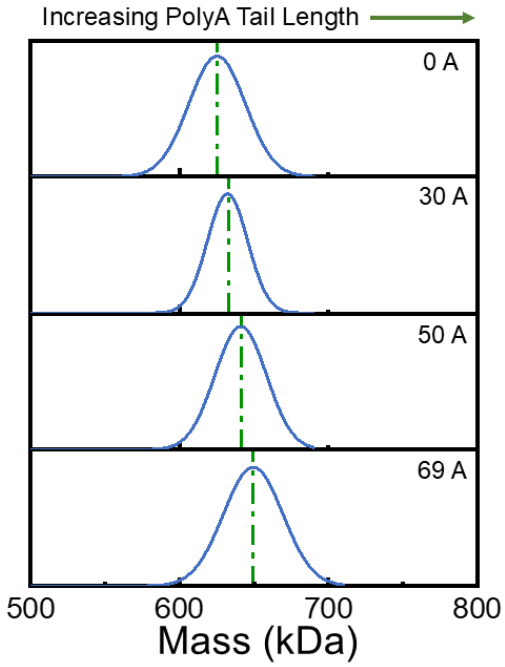

Figure 1. CD-MS Mass distributions for HA mRNA Constructs with increasing PolyA tail lengths.

1688 Closing the Gap: Boosting the *TFAMoplex* Transfection System with bZIP Domains allows Comparison with AAVs

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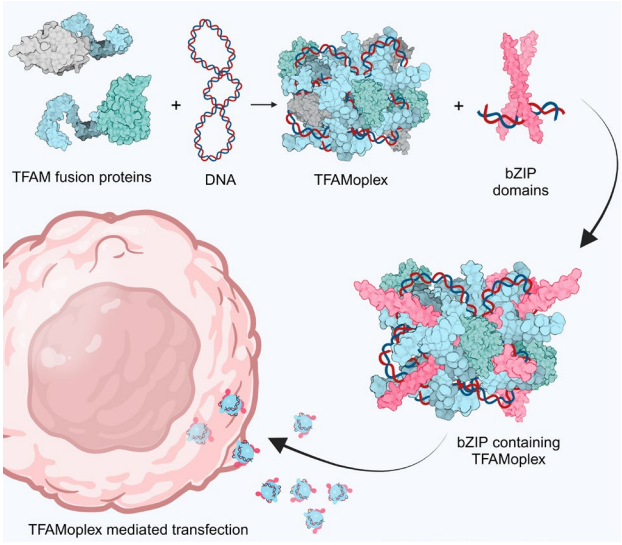
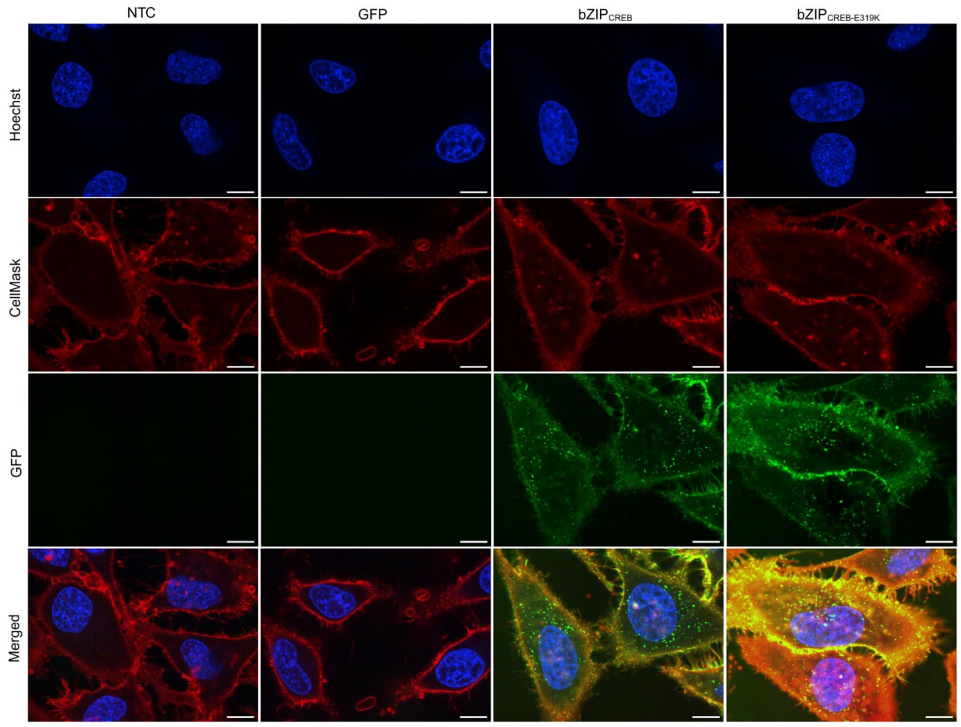
Gene therapy offers promising treatments for a wide range of diseases, but efficient delivery of DNA into target cells remains challenging due to its large size and negative charge. While viral vectors are highly effective, nonviral systems are often preferred for their safety and ease of production, though they typically suffer from limited efficiency and stability in biological fluids. Our group previously developed a nanoparticulate gene delivery system called **TFAMoplex**, which leverages the mitochondrial transcription factor A (**TFAM**) protein that binds and

condenses DNA into **nanoparticles**. This system can be customized with functional domains by protein engineering to enable efficient **plasmid DNA transfection**.

In this study, we redesigned the TFAMoplex system by integrating basic leucine zipper (**bZIP**) **domains**, particularly from the cAMP response element-binding protein (CREB), which binds DNA through dimerization. We also evaluated bZIP domains from other proteins, including BACH1, ATF-3, and BATF, under challenging *in vitro* transfection conditions like **pure serum and only 10 min incubation time**. Among these, the bZIP domain of BACH1 ($bZIP_{BACH1}$) was identified as the most effective one, significantly enhancing TFAMoplex performance by reducing the half-maximal effective concentration (EC_{50}) by more than 2-fold. Additionally, we show that bZIP domains increase the interaction of TFAMoplexes with cell membranes, thereby enhancing cellular DNA association, which could partially explains the enhanced transfection. Finally, we **compared the optimized $bZIP_{BACH1}$-TFAMoplex to adeno-associated viruses (AAVs)** in terms of transfection efficiency and transgene expression. Although AAVs demonstrated superior transfection efficiency based on the number of transfected cells, both the original and optimized TFAMoplex constructs significantly outperformed AAVs in terms of transgene expression per cell.





1689 Title ZIP Delivery: A Versatile Delivery Platform for the Central Nervous System

Barbara Bailus[1], Stephen Scheeler[2], Kizito Tshitoko-Tshilenge[2], Ningzhe Zhang[2], Swati Naphade[2], Sally Mak[2], Kenneth Wilson[2], Robert O'Brien[2], Mahru An[2], Brian Rossmiller[2], Stanislav Moroz[2], Jennifer Suoja[2], Maria Sanchez[2], Tyne McHugh[2], Anja Sandholm[2], Delihla Correa[1], Akos Gerencser[2], Lisa Ellerby[2]
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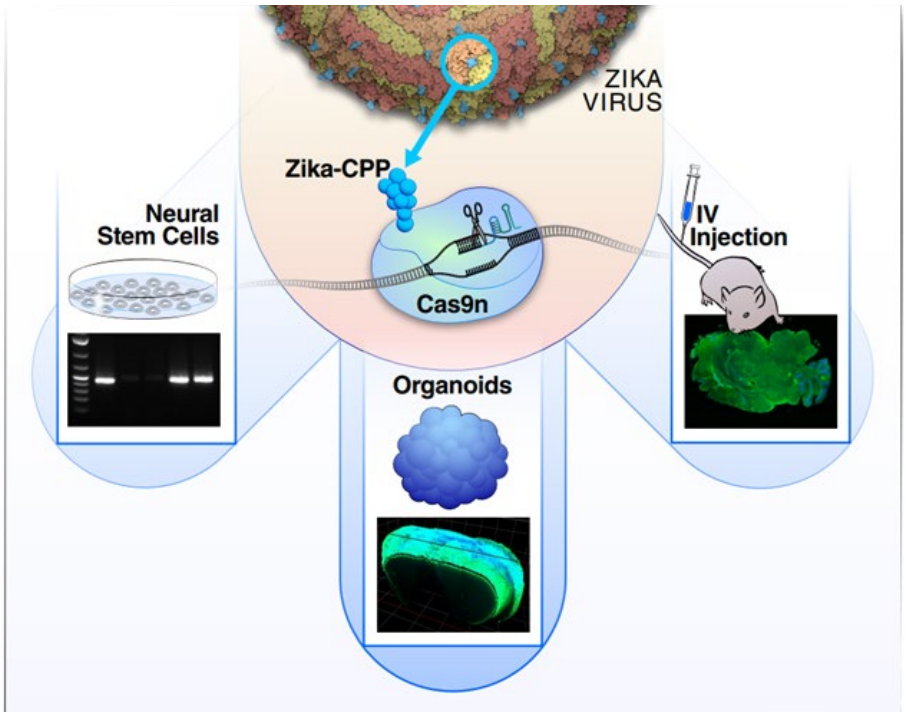
One of the major challenges in developing therapeutics for neurological disorders remains the widespread delivery of therapeutics to the brain. To address this challenge, we designed a novel cell-penetrating peptide based on the Zika virus, called "ZIP". We tested this technology in neuronal stem cells, striatal organoids, and murine models. We have fused ZIP to both Cas9 and to EGFP to evaluate its ability to transport cargo both into neuronal cells and past the blood-brain barrier. Our initial experiments focused on using Huntington's disease (HD) models to explore a potentially therapeutic mechanism for decreasing the number of CAG repeats in the exon 1 CAG expansion. HD is a rare, devastating, neurodegenerative disorder with no disease progression modifying treatments. It has been previously shown that a reduction in the expanded CAG repeat in exon 1 in HD mice leads to amelioration of symptoms. Using our ZIP-Cas9 delivery system we genetically corrected the exon 1 CAG expansion in both neuronal stem cells and striatal organoids. Impressively, we have been able to show that the "ZIP" transports Cas9 past the adult murine BBB and into neurons and astrocytes with extremely high efficiency, including into the hippocampal and striatal regions using a single intravenous injection. Further, we tested the ability of the ZIP to transport different cargos such as EGFP, again showing transport into human striatal organoids and past the adult murine brain. We have also tested the versatility of ZIP when attached to different versions of Cas9 including dCas9VP64 and dCas9KRAB. To move this technology toward potential human use, we have explored the ability to have the ZIP-Cas9 commercially synthesized, and we are testing this new construct to evaluate recombination efficiency at therapeutically relevant levels. Our presentation will highlight the advances ZIP technology continues to make as a potential therapeutic delivery platform for neurological diseases and disorders.



1690 Engineered CRISPR enzymes mediate highly efficient editing of neurons in mice and pigs

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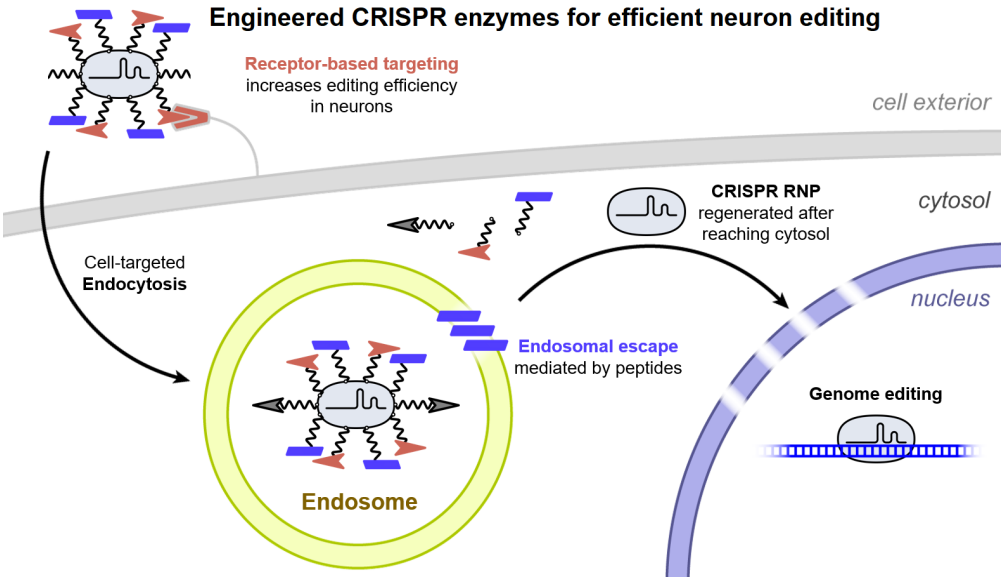
CRISPR-driven genome editing of the central nervous system (CNS) has the potential to revolutionize the treatment of neurological disorders such as Huntington's and Parkinson's diseases. However, developing CRISPR therapeutics for the CNS has been challenging, due in large part to delivery. Two key obstacles need to be overcome before gene editing in the human brain is feasible: (1) safe and effective intracellular delivery of editing machinery into neurons, and (2) establishment of strategies that enable targeted brain regions to be efficiently edited.

Our team has advanced an approach for highly efficient editing of neurons in the brain. This approach relies on the intersection of three technologies: NLS-rich CRISPR ribonucleoprotein (RNP) enzymes that possess some capacity for self-delivery into cells, amphiphilic peptides that substantially enhance efficiency of intracellular RNP delivery, and convection-enhanced delivery (CED), a means of administration that promotes optimal distribution after local injection. Furthermore, the CRISPR and peptide components are reversibly tethered using a PEG linker, enabling improved tissue distribution as well as neuron-specific targeting. This approach results in widespread, high-efficiency genome editing of neurons in the mouse brain following administration into striatum, thalamus, or hippocampus. Following extensive optimization using reporter mice, this approach is being used to edit neurons in the brain of a large mammal (pig) and has conferred phenotypic correction in a murine model of Huntington's disease. Our experiments to date demonstrate favorable host responses: negligible toxicity and only moderate immune responses to the reagents. Our approach relies on components – CRISPR protein, guide RNA, PEG-based

linker, and synthetic peptide – that are all straightforward to manufacture at scale. The transient nature of our reagents provides the benefits associated with non-viral delivery: minimal risks of off-target genome editing or immune responses to the CRISPR cargo. In contrast to CRISPR mRNA cargo delivery using lipid nanoparticles, which are typically at least 100 nm in diameter, our particles are an exceptionally small ~20 nm in diameter, faciliating diffusion through the brain parenchyma (~50 nm pore size). Together, these properties suggest our approach for CRISPR enzyme delivery has substantial potential for clinical translation in addressing neurological diseases.



1691 Peptide-Based Nanoplatform for Improved Cas9 RNP Delivery and Editing

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Introduction: Clustered regularly interspaced short palindromic repeats (CRISPR) technology has transformed the field of genetic engineering over the last decade by enabling precise and permanent changes to the genome. Despite its immense potential to correct and cure human genetic diseases, CRISPR technology has been limited in its clinical translation. The Cas9/gRNA system can be delivered to cells in multiple ways, including plasmids, messenger RNA (mRNA), or ribonucleoproteins (RNPs). Delivering Cas9 via RNPs has led to higher editing efficiencies and lower off-target effects than either plasmids or mRNA. However, due to their large size, net negative charge, and endosomal entrapment, RNPs require a delivery vehicle for efficient cellular internalization to achieve therapeutic efficacy. Currently, no therapeutic systems on the market enable the delivery of Cas9 RNP. Peptides have emerged as a promising non-viral delivery vector for transporting bioactive nucleic acids into cells due to their high tunability, stability, and biocompatibility. Our lab has previously demonstrated that a novel fusogenic peptide, DIV3W, can electrostatically complex with and protect RNA, is cytocompatible, and mediates efficient endosomal escape of bioactive RNAs. This study aims to evaluate the DIV3W peptide for delivery of RNPs to induce efficient green fluorescent protein (GFP) knockout in healthy HEK293 cells.

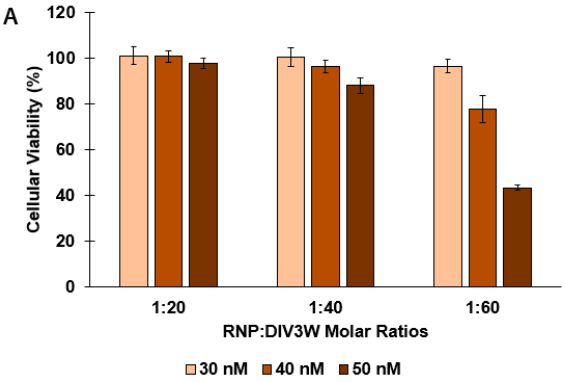
Methods: RNP:DIV3W complexes were synthesized by combining gRNA and Cas9 at equimolar ratios, followed by complexation with various molar concentrations of DIV3W peptide. The complexation of RNP with DIV3W was analyzed using agarose gel electrophoresis and was further characterized by dynamic light scattering. MTS assays determined a maximal RNP/DIV3W concentration that will not significantly kill healthy cells. gRNA was replaced with Cy5-fluorescently-tagged siRNA to visualize and quantify 4-hour cellular internalization using fluorescent imaging and flow cytometry. RNP:DIV3W and RNP:Lipofectamine complexes were also delivered to HEK293-

GFP cells to knockout GFP expression, and changes in fluorescence after 96 hours were analyzed using flow cytometry.

Results and Discussion: RNP:DIV3W molar ratios greater than 1:20 resulted in complete complexation of RNPs and, therefore, a successful delivery vehicle capable of protecting the RNP from premature degradation. MTS results confirmed that 50 nM RNP:DIV3W complexes were not cytotoxic to HEK293 cells up to 1:40 molar ratios (Figure 1A). 50 nM RNP/1:40 DIV3W molar ratio complexes were measured at 169 nm with a PDI<0.3, confirming monodisperse nanoparticle formation (Figure 1B). Preliminary uptake studies show ~50-60% cellular internalization after 4 hours. Knockout studies have demonstrated significant GFP knockout using the 50 nM/1:40 concentration compared to Lipofectamine CRISPRMax, highlighting comparable editing efficiencies as a commercially available system (Figure 2).

Conclusion: The DIV3W peptide can self-assemble with and completely complex free RNPs and is cytocompatible with HEK293 cells. The RNP:DIV3W nanocomplexes can escape the endosome prior to lysosomal degradation and traverse the nuclear membrane, as shown through successful gene editions. This peptide system has achieved significant GFP knockout comparable to that of Lipofectamine. These results demonstrate the potential of peptide-based delivery systems for Cas9 RNPs and warrant further investigation.

A



B

	Size (nm)	PDI	Zeta Potential (mV)
1:40 RNP:DIV3W	169.0 ± 0.16	0.28 ± 0.20	6.10 ± 0.31

Figure 1: (A) Cellular viability of HEK293 cells was analyzed 48 hours following RNP:DIV3W treatment at various RNP concentrations and DIV3W molar ratios. Data are mean ± SEM of three independent experiments. (B) DLS characterization of 1:40 RNP:DIV3W complexes.

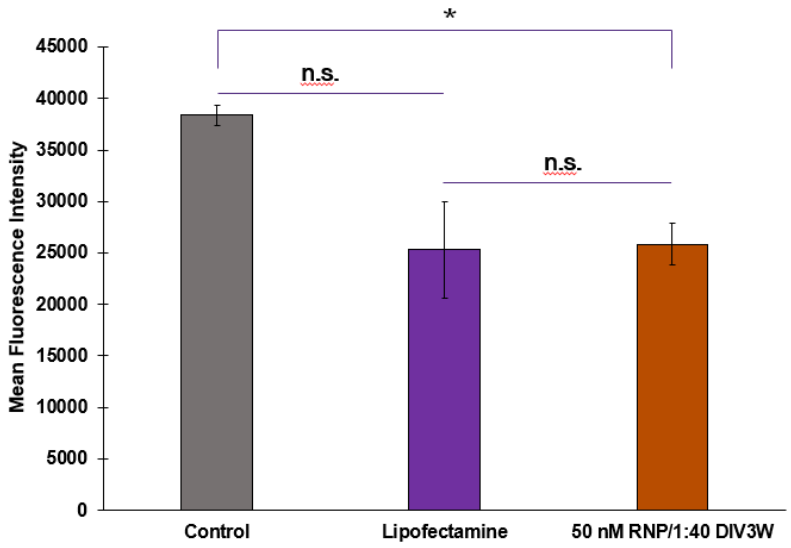


Figure 2: HEK293-GFP cells were treated with 50 nM RNPs complexed at the 1:40 molar ratio of DIV3W. Cells were washed after 24 hours, and flow cytometry was run after 96 hours. Data are mean ± SEM of three independent experiments. *p < 0.05

1692 Non-viral Co-delivery of Long DNA Template and Cas9 Ribonucleoprotein into Primary Blood Cells

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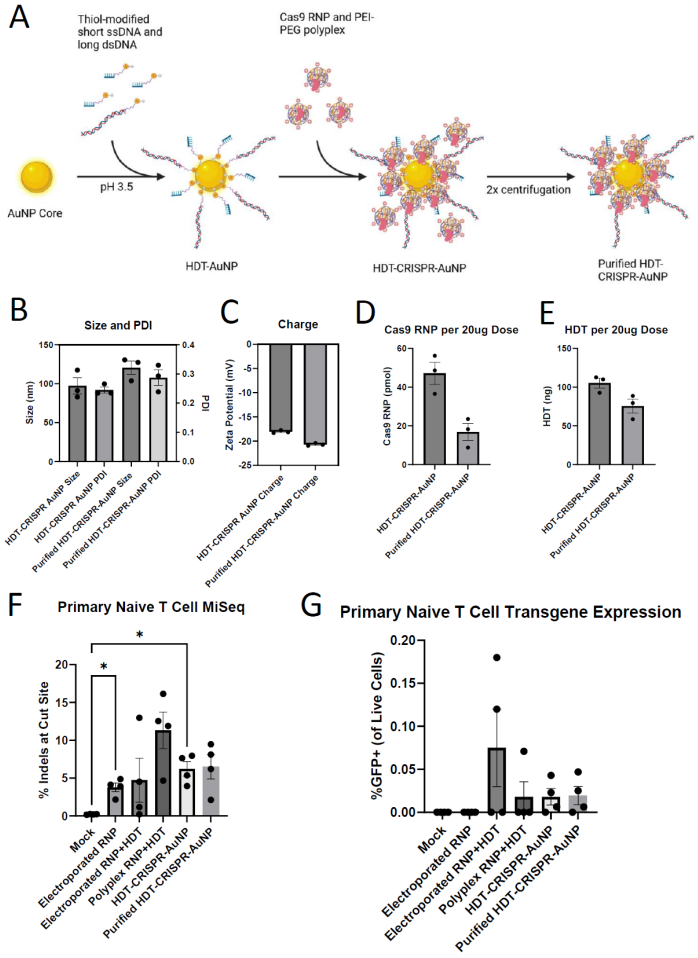
CRISPR systems can direct mutations to specific loci and have been engineered to introduce small segments of DNA into the genome through direct write-in approaches. However, the delivery of large, transgene-encoding DNA cassettes with CRISPR systems remains limited. Current state of the art relies on viral vectors such as AAV to deliver double-stranded DNA (dsDNA) templates encoding the desired transgene(s) flanked by regions of homology to the target CRISPR site in the genome. These viral vectors are costly to manufacture and immunogenic, which limits repeat dosing. We developed a nonviral, synthetic gold-based nanoparticle (AuNP) that can deliver fully assembled Cas9 ribonucleoprotein (RNP) and long dsDNA homology directed repair template (HDT). This HDT-CRISPR-AuNP can be stably formulated with dsDNA as long as 2.5kb. In brief, thiol-modified HDT is conjugated to the gold core surface, then purified by centrifugation. The HDT-modified gold cores are then concentrated and added to a polyplex solution consisting of Cas9 RNP and PEGylated-polyethylenimine to allow binding to the gold surface (**Figure 1A**). If necessary, further purification by centrifugation can be performed. Characterization by dynamic light scattering showed that HDT-CRISPR-AuNP is stable with a hydrodynamic diameter and polydispersity index (PDI) of 97.2±18nm and 0.245±0.018 for unpurified and 120.5±14.72nm and 0.287±0.046 for purified formulations respectively (**Figure 1B**), with average charge (zeta potential) of –18.1±0.31mV and –20.71±0.29mV (**Figure 1C**). Analysis by SDS-PAGE and agarose gel electrophoresis showed that HDT-CRISPR-AuNP carried an average of 47.12±9.93pmol RNP and 105.1±10.86ng HDT per 20ug of 15-20nm gold core solution (determined by transmission electron microscopy), for unpurified and 16.84±7.48pmol RNP with 75.58±15.46ng HDT for purified samples (**Figure 1D, E**). HDT-CRISPR-AuNP synthesis required a maximum of 6.5 hours, with up to 2 hours of hands-on time and is scalable to the calculated available surface area of the gold cores. Nanoparticle treatment of unstimulated primary human naïve T cells resulted in statistically significant editing at the *β2-microglobulin* locus with unpurified formulations yielding 6.23±1.9% and purified with 6.49±3.2% insertion/deletion mutations (indels) at the target site. Additionally, T cells demonstrated expression of an enhanced green fluorescent protein (GFP) reporter transgene encoded in the HDT confirming delivery of DNA cargo into these cells (**Figure 1F, G**). While overall levels of GFP-expressing cells were low, for primary T cells engineered to

express a CAR transgene, these levels are sufficient to elicit an immune response (i.e. activation and proliferation) in the presence of target antigen [PMID: 29849141]. Future studies will confirm the efficacy of HDT-CRISPR-AuNP at clinically relevant loci such as *TRAC* and *CCR5*, as well as efficacy for editing primary blood cells in murine models. Given the low cost of AuNP core synthesis ($179 for 1g), and the reduced total and hands-on time required for assembly, HDT-CRISPR-AuNP are a simple alternative to viral vectors or electroporation for evaluating CRISPR-mediated transgene cassette delivery into primary T cells and possibly other hematopoietic cells of interest.



1693 E4/K4 Coiled-coils Improve Cargo Loading in Engineered Virus-Like Particles

Dylan Dautel[1], Karol Budzik[1], Pauline Schmit[1], Liana Behmoiras, Melissa Bonner[1], Claudia Pereira[1], Mahmudul Hasan, Yi Yu[1], Tatiana Ketova
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An urgent need exists for novel delivery modalities to fully realize the therapeutic potential of gene editing technologies, including CRISPR-Cas-based gene editors. Recently, engineered virus-like particles (eVLPs) have emerged as a promising approach for *in vivo* delivery of gene editing therapies, offering transient delivery of ribonucleoproteins (RNP) and targeted payload delivery. Fluorescent imaging analysis revealed the majority of eVLPs do not contain detectable amounts of RNP. As a result, the editing activities of eVLPs containing less efficient cargoes, such as ABE7.10 and prime editors, were lower than direct plasmid transfection in HEK293T cells. David Liu *et al.* have demonstrated enhanced prime editor loading in eVLPs by utilizing P3/P4 coiled-coil (CC) peptides. In this study, we further optimized the CC loading strategy in our eVLP-derived DLVR-X system to enhance cargo loading efficiency and broaden cargo compatibility.

Heterodimeric coiled-coils consist of two short alpha-helical peptides that tightly wrap around each other to shield hydrophobic amino acids along the peptide backbones. These interactions can be strengthened by complementary electrostatic interactions and adapted to have different binding affinities, specificities, and orientations. We constructed DLVR-X particles by inserting one CC peptide between MMLV-Gag and Pol (Gag-CC-Pol) and the other to the N or C terminus of gene editing cargoes. To identify CCs with optimal properties for loading cargoes into eVLPs, we screened different configurations and types of CC domains. DLVR-X particles loaded with the E4/K4 CC pair outperformed the canonical v4-BE-eVLP design and P3/P4 CCs when loading ABE7.10, OpenCRISPR-1, prime editors, CBE, enAsCas12f, and enAsCas12a, highlighting that this E4/K4 CC cargo loading method is a modular and generalizable approach to deliver a wide variety of gene editors via DLVR-X. E4/K4 CCs resulted in an up to 6–fold increase in editing efficiency in HEK293T cells. In addition, we demonstrated potent editing in primary human cortical neurons and NHP hepatocytes with E4/K4 DLVR-X particles. Using a particle staining protocol developed on the Leprechaun particle imaging system, we showed that the increase in editing efficiency was linked to a 3-fold improvement in RNP loading efficiency compared to v4-BE-eVLP. These novel DLVR-X™ particles are anticipated to be highly versatile, addressing some of the constraints of existing delivery methods in both research and therapeutic contexts.

1694 Engineered Virus-like Particles with Minimal Viral Components for Improved Base Editing Efficiency in vivo

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Novel delivery methods are essential to fully realize the therapeutic applications of CRISPR and related gene-editing technologies. Engineered virus-like particles (eVLPs) derived from Moloney Murine Leukemia Virus (MMLV) have recently emerged as a promising approach for in vivo delivery of gene-editor cargoes, providing flexibility in targeting specific cell types and reduction of off-target effects through transient ribonucleoprotein (RNP) delivery. However, the assembly efficiency of these eVLP particles is lower than wild-type MMLV virions, representing a manufacturing challenge. In addition, cargo loading and release require substantial improvement to unlock the full potential of this delivery platform. The presence of nonessential viral-derived components in the particle, the viral reverse transcriptase (RT) and integrase (IN) within the Gag-Pol scaffold, also poses potential safety concerns.

Here, we report the successful generation of RT- and IN-free DLVR-X particles, which exhibited significantly improved production efficiency and cargo release compared to the original v4-BE-eVLP design. The standard Gag-Pol scaffold was modified by truncating Pol to only the protease function and split into two constructs: (i) Gag and (ii) Gag-Protease (Gag-Pro). The optimal Gag-Pro/Gag plasmid ratio in ABE8e-carrying DLVR-X particles was determined as 1:20, which was in line with the reported 1:20 protease/Gag ratio in wild-type MMLV virions. This Gag-Pro particle design resulted in a ~10-fold improvement in titer, measured via p30 ELISA. Interestingly, the Cas9 content in the particles and editing efficiency of HEK293T cells significantly decreased with increasing Gag-Pro/Gag ratios. This indicated that cargo may be prematurely released in producer cells due to the increased cytoplasmic concentration of the MMLV protease, hindering cargo loading into DLVR-X particles. An automated Western blot assay confirmed the association of premature cargo release with high Gag-Pro/Gag ratios. The introduction of Amprenavir, an HIV protease inhibitor, during Gag-Pro particle production resulted in up to a 5-fold increase in particle Cas9 content relative to untreated Gag-Pro controls and a dramatic increase in editing efficiency of HEK293T cells. Surprisingly, Amprenavir treatment also increased cargo cleavage within the assembled Gag-Pro DLVR-X particles and significantly improved cargo release. Next, we replaced the native MMLV protease in the Gag-Pro construct with the HIV protease and observed further improvement in cargo processing and editing efficiency of different cell types. Finally, RT- and IN-free DLVR-X particles are under evaluation in mice for in vivo potency improvements. This is the first retroviral eVLP design for protein delivery that is completely free of RT and IN, to our knowledge. The increased production efficiency and cargo loading/release resulting from the design and process changes described above, in addition to the improved safety profile, enable exploration of the full potential of DLVR-X as a therapeutic protein delivery platform.

1695 Regulation of rAAV transgene expression using nanoparticle-delivered Cre-recombinase protein

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Introduction: Recombinant adeno-associated virus (rAAV) is a common gene therapy vector engineered to deliver a transgene cassette into the nucleus of a cell that persists as an episome resulting in durable transgene expression. However, no strategy currently exists to permanently terminate rAAV transgene expression in an event where an adverse reaction occurs. Unregulated expression of rAAV-delivered transgenes, like broadly neutralizing antibodies (bNAbs), can have several potential consequences that can include immune responses to high levels of transgene expression, off-target effects that could lead to toxicity, and development of anti-drug antibodies against the bNAbs reducing their efficacy. With the potential to utilize rAAV as an HIV-1 therapy, we can mitigate these risks through deployment of a strategy to regulate gene expression. Our approach encompasses a rAAV cassette engineered with LoxP sites flanking the transgene and leverages the use of engineered virus-like particles (eVLPs) or nanoparticles to deliver Cre recombinase (Cre) proteins to irreversibly terminate expression.

Methods: We engineered six different rAAV cassette designs, each with varying LoxP site locations that flank the transgene and evaluated the impact on transgene expression after AAV transduction by a luciferase reporter assay in HEK293T cells. All nanoparticles were assessed by ELISA, flow cytometry, and western blot to determine the amount of Cre recombinase packaged into the particle. Next, we evaluated different nanoparticle delivery systems (eVLPs and gesicles) that contain the recombinant Cre protein (Cre-eVLPs or Cre-gesicles), which were assessed on their ability to decrease rAAV transgene expression in vitro. Lastly, we evaluated our rAAV transgene cassette designs expressing the HIV-1 bNAb 10-1074 in mice that were treated with nanoparticles delivering Cre.

Results: Four out of six transgene cassettes containing LoxP sites resulted in equal or better luciferase expression compared to the cassette without LoxP sites after rAAV transduction of HEK293T cells. We observed a considerable decrease in luciferase signal of up to 99% in rAAV-transduced cells treated with Cre-gesicles. In contrast, rAAV-transduced cells treated with Cre-eVLPs only resulted in a decrease of luciferase signal of up to 90%. The two LoxP cassettes tested in vivo (LoxP-5 and LoxP-6) yielded concentrations of 10-1074 higher than the control rAAV vector harboring no LoxP sites. Current experiments are ongoing to assess Cre-eVLP and Cre-gesicle knockdown of in vivo transgene expression.

Conclusion: Our study demonstrates that nanoparticles provide an efficient system to deliver functional Cre-recombinase protein into cells transduced with rAAV cassettes engineered with LoxP sites resulting in a substantial decrease of transgene expression in vitro. Additionally, we conclude that rAAV cassettes containing strategically placed LoxP sites do not interfere with transgene expression in vitro or in vivo.

1696 A versatile platform for the manufacturing of lentiviral vectors-based nanoparticles for *in situ* gene delivery - an integrated solution to accelerate advanced therapies into the clinics

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Recent market approval of CAR-T cell therapies, their development beyond oncology and the continuously growing number of *ex vivo* and *in vivo* gene therapies evaluated in the clinics has created a huge constraint on the supply of high-quality grade lentiviral vectors (LVs). This results in higher manufacturing costs and longer timelines that are

not sustainable for early-stage development programs. Bioproduction remains a bottleneck for most biotech companies and established biotech players have invested in their own bioproduction capabilities.

Alaya.bio has built up a versatile manufacturing platform for the production of high-quality grade LV material to cover internal and customers' needs for R&D *in vitro* and *in vivo* evaluation. Our 350 m2 facility and team of 17 scientists based in Paris is operating a scalable and GMP-compatible bioprocess based on mammalian cell suspensions from 30 mL (for screening and feasibility tests) to 2 L of bulks produced in synthetic medium with no antibiotic or serum needed. The facility complies with the European regulations on the handling and use of genetically modified organisms. We have shown an excellent reproducibility of the production yields in bulks from the 30-mL to the 2 L-scale in shake flasks (>1e6 TU/mL or 1e9 vp/mL). We are now operating at the 30 mL scale for feasibility studies to test the combination of new transgenes, ubiquitous or tissue-specific promoters with LV variants. From the 2 L scale, bulks are endonuclease-treated, clarified, purified and concentrated (by chromatography and TFF). LVs are sterile-filtered and stored at -80 °C. 20 to 40 mL of sterile purified drug product are obtained within 4 days (>2e6 TU/mL or 1e9 vp/mL).

Product quality, process performance and repeatability are systematically verified through the integrity and functionality of viral particles (DLS, interferometry, ELISAs, RT-qPCR, flow cytometry and qPCR). The robustness of this bioprocess has been applied to second (106 batches) and third generation LVs (50 batches), integrating or non-integrative lentiviral vectors (NILV), mainly pseudotyped with the VSV-G envelope protein.

We engineered VSV-G-deficient LVs that lack cell transduction ability until coated with pH-responsive cationic polymers. Further modification with shielding polymers enhances stability and allows bioconjugation with targeting agents. A portfolio of 40 families of cationic or shielding polymers (with diversified shape, MW and pKa) has been assembled and screened to formulate homogeneously sized LV-based nanoparticles (Dh<300 nm and PdI<0.3) that efficiently deliver *in vitro* transgenes to different types of human cells that are relevant for the development of gene and cell therapies. We are currently implementing microfluidics-based formulation and TFF-polishing to render these nanoparticles compatible with industrial manufacturing. Further developments include the engineering of T-lymphocytes targeting nanoparticles by conjugating anti-CD3 scFv to shielding polymers by amine coupling. Targeted gene delivery to other cells or tissues can easily be redirected by switching binder specificity. By combining optimized bioproduction of lentiviral vector payload and high throughput screening of formulations with superior biophysical and functional properties, Alaya.bio provides an integrated solution to support biotech companies and academic laboratories throughout the development of their disruptive gene therapy programs.

1697 Efficient, Large-Scale Virus-Like Particle Manufacturing for Gene Editing by a GMP-Compliant Flow Electroporation Platform

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Virus-like particles (VLPs) are non-infectious, virally-derived nanoparticles that lack a viral genome. Upon packaging a CRISPR ribonucleoprotein complex (RNP), VLPs can target and transiently deliver cargo for genome editing *in vivo* to specific cell and tissue types. They are safer than traditional viral vectors as they are incapable of genomic integration. Scaling up VLP production can be cumbersome and time-consuming, requiring several rounds of optimization to transition from research to clinical scale. Additionally, consistency and reproducibility between batches is a major concern when relying on chemical methods of VLP production. Here, we utilized the MaxCyte

ExPERT GTx®, a GMP-compliant electroporation instrument, to manufacture clinical-scale VLPs, in adherent and suspension HEK cells, packaged with CRISPR Cas9 or adenine base editor RNPs for genome editing in primary human cells. We found that electroporation consistently produced high yields of functional VLPs with an optimized electroporation workflow. We also demonstrated effective gene editing at several therapeutically relevant loci in primary hematopoietic cells, such as B2M and PD-1. Furthermore, the production of VLPs using electroporation exhibited favorable production kinetics compared to other transfection methods, enabling a one-day manufacturing process. Finally, we highlight the scalability of VLP production across a 400-fold volume range with minimal re-optimization, transfecting over one billion cells per production. In summary, our results show that MaxCyte's Flow Electroporation® technology is a viable means for consistent, efficient, and scalable manufacturing of VLPs for gene editing applications and has high promise to address the needs of future clinical and commercial manufacturing.

1698 Capsid and Virus-Like Particle Biophysical Characterization by High Sensitivity, High Resolution SEC-MALS: Case Studies on QBeta, Hepatitis B, and Recombinant Protein VLP Control

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Virus-like particles (VLPs) can serve as novel antigen-presenting vaccine modalities. Moreover, critical insights into their capsid morphologies can be translated into the creation and optimization of delivery vectors for genetic medicine. Their compositions must be well characterized and documented to determine if they are to be used as components in a therapeutic or vaccine. VLPs are manufactured by controlled self-assembling viral structural proteins to mimic the size and shape of viruses without infectious genetic materials. Monitoring the size, shape, structural integrity, and stability under various conditions and formulations is essential to ensure the drug substances' safety and efficacy and uncover key insights about structure-function relationships. VLPs can range from 20 nm upwards of 200 nm in diameter or larger. Integrity analysis is essential for validating product quality in advanced therapeutics and vaccines. Size exclusion chromatography (SEC) combined with multiangle light scattering (MALS) provides a platform analytical approach for these investigations. Success of SEC technology for intact analysis of Megadalton VLP complexes require appropriate pore size of stationary phase and low adsorption hardware to facilitate maximum recovery of molecules to achieve high-throughput, multi-attribute, intact biophysical measurements of VLPs. VLPs can be complex and, at times, heterogeneous in composition; developing robust SEC methods can aid in the speed of formulation measurement and monitoring at each stage of the process. There is promise in improving SEC column technology through the development and implementation of low-adsorption flow paths. In addition, there is an opportunity to enhance separation efficiency and throughput on miniaturized particles with average pore diameters approaching 1000 and 2000Å. Using low adsorption 1000Å and 2000Å 3 µm SEC columns, we investigated VLPs as large as 300 nm, smaller 28 nm QBeta VLP, and 42 nm Hepatitis B VLP as well as 100 – 300 nm VLP prepared from HEK293 for use as an immunology isotope control. As these VLPs increase in hydrodynamic size, there is a significant risk to stability and self-induced aggregation. For instance, for a small VLP like QBeta, we observed recoveries for higher order aggregates and resolution of T=1 form to T=3 forms with the GTx Resolve 1000 Å 3 µm column that was about 30 to 50% better than another commercially available SEC 1000 Å column. Furthermore, the low adsorption GTxResolve 1000 Å 3 µm column technology exhibited lower MALS noise to facilitate more sensitive and accurate biophysical measurements. These observations were also corroborated by SEC-MALS, as displayed in Figure 1. Through additional mobile phase optimization, we obtained improved recovery of greater than 85% and have measured the radii of VLPs as large as 120 – 160 nm in diameter upon applying a prototype SEC 2000 Å 3 µm column. The hydrodynamic size of this proteinaceous VLP was confirmed by DLS measurement. While DLS measures the size distribution and polydispersity, SEC-MALS quantifies the heterogeneity, size, shape, and average molar mass of the VLPs and their impurities. In summary, we have shown that with new column technologies and method considerations, SEC-MALS can become an even more powerful tool for biophysical measurements of VLPs. These measurements are essential to maintaining drug and product stability for efficacy, controlling immunogenic responses, and unlocking k

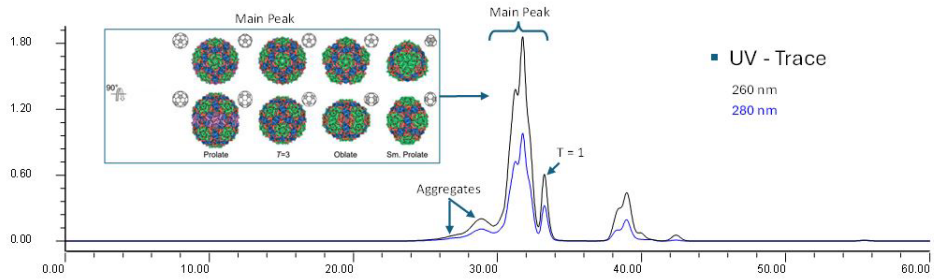

Figure 1: Chromatogram of QBeta on low absorption SEC 1000 Å 3 µm column resolving various forms present within QBeta. A Visual representation of the capsid structures as shown in an edited form as originally published by Chang JY, Gorzelnik KV, Thongchol J, Zhang J. Structural Assembly of Qβ Virion and Its Diverse Forms of Virus-like Particles. Viruses. 2022 Jan 24;14(2):225. doi: 10.3390/v14020225. PMID: 35215818; PMCID: PMC8880383. CC-BY. https://creativecommons.org/licenses/by/4.0/

1699 Virus-Like Particles: Advanced Insights through Charge Detection Mass Spectrometry (CDMS)

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Over the past few decades, virus-like particles (VLPs) have shown immense potential in vaccine-based therapeutics, addressing the limitations of traditional vaccines. A major advantage of VLPs is their high immunogenicity combined with non-infectiousness, as they lack the core genetic material of the virus. However, characterizing intact native VLPs remains a significant challenge due to their structural complexity and large size, in which the masses of VLPs often exceed 1 MDa. Charge detection mass spectrometry (CDMS), utilizing an electrostatic linear ion trap (ELIT), is an ultra-high mass technique that enables direct mass measurement of individual ions by simultaneously determining their mass-to-charge ratio (m/z) and charge (z). CDMS can overcome the limitations of conventional mass spectrometry in analysing large and heterogeneous analytes, where overlapping and unresolved charge states hinder mass determination. We demonstrate the potential of CDMS in characterizing the structural integrity and stability of various VLPs, including those of dengue, chikungunya, and different strains of human norovirus (hNoV). All samples were buffer exchanged into ammonium acetate solution using Micro Bio-Spin columns (Bio-Rad) or 100 kDa molecular weight cut-off filters (Amicon). Ions were generated in positive ion mode using nanoelectrospray ionisation (nESI) and mass analysis was performed using a prototype CDMS instrument. Signal processing and data visualization were performed using software developed in-house. Ions were trapped for 100 ms and the frequency and amplitude information were converted to m/z and z values respectively, and ultimately mass (m/z x z). These data are subsequently binned to generate the corresponding spectra (histograms). The m/z, mass, and charge data were binned to obtain the average, sigma, and area of each peak. CDMS capabilities in the structural characterisation of different MDa-sized VLP ions are highlighted. The VLPs mentioned can vary in heterogeneity and range in size from 20 – 60 nanometres. Icosahedral capsids are defined by their triangulation number (T), which is related to the number of monomers (T x 60) in the structure. Dengue virus serotype 1 VLP was studied, where a significant mass distribution was observed around 7 MDa with 155 charges, corresponding to the dimer of the VLP. Low level aggregation was also detected, with the presence of an additional peak at 14 MDa, at approximately less than 2 % of the dimer peak of the dengue VLP. The mosquito-borne Chikungunya virus-like particle (CHIKV VLP) was observed at a higher mass, with the presence of a distinct peak in the mass spectrum around 50 MDa. Aggregation of the CHIKV VLP was also observed with ion populations observed up to 200 MDa. It is important to monitor the stability of VLPs, which are often used in vaccine therapeutics, as aggregation may have potential implications for clinical safety and efficacy. Finally, hNoVLPs[TL1] [UC2] derived from three different strains were also analysed including GI.1 Norwalk and GII. 17 Kawasaki[TL3] [UC4] . T=3 particles were observed for Norwalk and Kawasaki strains around ~ 11 MDa[UC5] [TL6] . Overall, the measured masses agree well with

expected masses for the geometrically allowed capsids, while accounting for the presence of counterions observed with CDMS. The results presented here demonstrate the potential of CDMS to provide valuable mass information about the different capsid geometries of VLPs

1700 A Novel Non-Viral DNA Delivery Platform for Durable and Potent Expression of Bispecific T-cell Engagers

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Bispecific T-cell engagers (BTE) are immunotherapies engineered to drive apoptosis by linking activated T cells to target cells. These molecules are approved for the treatment of CD19-positive B-cell precursor acute lymphoblastic leukemia (BCP-ALL) and hold promise for treating autoimmune disorders by depleting autoreactive B cells. However, their clinical application is constrained by their short half-life, requiring continuous intravenous infusions over 4-week cycles. To overcome this limitation, we evaluated our novel DNA gene therapy platform, HALO™, for providing efficient and durable BTE therapy. HALO is a circular DNA vector with unique structural DNA elements designed to favor nuclear uptake, nuclear retention, and immunomodulation, which greatly increases the efficiency, durability and redosability of gene delivery *in vivo*.

Here, we describe the design and evaluation of HALO DNA encoding CD3 x CD19 BTE fused to a peptide secretion tag (HALO BTE). HALO BTE was first transfected into HEK293 and HEPG2 cells, resulting in efficient BTE secretion. The secreted BTE potently activated T cells. Then, we showed that HALO BTE, delivered as lipid nanoparticles (HALO BTE-LNP), enabled durable BTE expression for up to 14 days in iPSC-derived hepatocytes. Using a CD19-positive Raji B cellular model, HALO-derived CD3 x CD19 BTE effectively activated primary human T cells and induced Raji B cell lysis in an effector-to-target ratio dependent manner. Finally, to evaluate *in vivo* potency and durability, HALO BTE-LNP was intravenously administered as a single ascending dose to wild-type mice. Expression of BTE in plasma was detected by ELISA and shown to be functional through *in vitro* T-cell activation assays. The expression was dose dependent and activated T-cells *in vitro* is a similar dose dependent manner. BTE expression persisted for at least 3 weeks *in vivo*, with ongoing evaluation for more than one month.

Altogether, these findings demonstrate that the HALO™ DNA platform enables safe, robust, and durable BTE expression *in vivo*. This innovative non-viral approach offers an attractive alternative to current BTE-based therapies, particularly if co-expressed with key oncogenic or immunogenic regulators, and holds significant potential for advancing other antibody-based therapies as well.

1701 Development of a Stable Lyophilized Formulation of a Quadrivalent Frame-Shift Peptide Vaccine for Cancer Prevention in Lynch Syndrome

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Some cancers are deficient in their ability to repair mismatched DNA which results in the accumulation of frameshift mutations in genes that contain microsatellite sequences, repeated sequences of DNA. Translation of these genes generates proteins with frameshift peptides induced by the mutations. These neoantigen peptides can provide a means for the immune system to target these microsatellite-unstable (MSI) cancers. Such frameshift peptides have been identified in four proteins, TAFB(-1), AIM2(-1), HT001(-1) and TGFBR2(-1) are being developed as vaccines to prevent/treat colorectal cancer, endometrial cancer, gastric cancer or small bowel cancer. A stable lyophilized formulation containing quadrivalent frameshift peptides (FSP) AIM2(-1), HT001(-1),

and TAFB (-1), TGFBR2(-1), was obtained through an iterative screening process. The screening included evaluation of pH, stabilizers, bulking agents, and surfactants. The screening resulted in a scalable formulation and lyophilization process with 100 µg/ml each peptide,10 mM Histidine at pH 5.5, 260 mM trehalose, and 0.02% polysorbate 20 as a clinical formulation. Based on peptide content and peptide purity from a three-month stability study, the lyophilized formulation is estimated to be stable at ambient temperatures for 18 months. Asymptomatic carriers of DNA mis-match repair mutations (Lynch Syndrome carriers) will be the target population to be vaccinated and monitored for safety and immunogenicity in a Phase 1 clinical study.

1702 Evaluation of Immunomodulatory Effects of Oncolytic Adenovirus Therapy in Immunocompetent Hamster Models of Metastatic Breast Cancer.

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Breast cancer remains a significant clinical challenge, particularly due to recurrence and progression to metastatic disease driven by therapy-resistant breast cancer stem cells (BCSCs). Despite effective initial therapies, estrogen receptor-positive (ER+) breast cancer patients frequently experience relapses, with up to 41% developing resistance to endocrine and chemotherapy. Current immunosuppressive tumor microenvironments further complicate therapeutic intervention. This highlights the urgent need for innovative therapies capable of targeting BCSCs while addressing immune evasion.

We previously developed OAd5/3-Cox2-NIS, a tumor-selective, infectivity-enhanced oncolytic adenovirus (OAd) encoding the sodium iodide symporter (NIS), which facilitates radioiodine imaging and therapy. Our studies demonstrated the superior ability of OAd5/3-Cox2-NIS to induce oncolysis and enhance NIS expression in paclitaxel-resistant ER+ BCSCs, including chemoresistant tumorsphere models. The vector selectivity was further validated in therapy-sensitive and -resistant human breast cancer cell lines, with low viral titers effectively eliminating cancer cells while sparing normal human mammary epithelial cells. However, the lack of a relevant immunocompetent and adenovirus-permissive preclinical model has hindered the evaluation of OAd-driven immune responses in breast cancer.

To address this limitation, we established a novel “Mammary INtra Ductal” (MIND) model in immunocompetent, adenovirus-permissive hamsters using the luminal-B HMAM5 cell line. This orthotopic model closely recapitulates key features of human ER+ breast cancer, including tumor formation within mammary ducts, estrogen receptors expression, epithelial markers retention (e.g. CK-18, pancytokeratin), and consistent metastatic spread to lymphatic nodes. Importantly, OAd replication in this model was confirmed via bioluminescence imaging (using luciferase-expressing Ad) and qPCR detection of viral E4 transcripts in tumors, overcoming the limitations of non-permissive rodent models.

Therapeutic study of OAd5/3-Cox2-NIS in this model demonstrated a significant ten-fold tumor volume reduction accompanied by robust viral replication in tumors and minimal off-target presence in non-tumor tissues (e.g. liver, lung, spleen).

Building on this newly developed model, we are currently conducting comprehensive studies to assess OAd-driven immunomodulation. This includes the abscopal effect studies in a bilateral tumor model to determine whether OAd-mediated immune activation can suppress distant, untreated tumor sites. Additionally, we are performing immune profiling using flow cytometry to analyze tumor-infiltrating immune cells (T-cell subsets and NK cells), alongside assessments of cytokine secretion and immune checkpoint expression to characterize OAd-induced immune activation. Furthermore, single-cell RNA sequencing will provide insights into gene expression changes induced by OAd infection, particularly in pathways related to innate and adaptive immune responses, tumor antigen presentation, and immune evasion mechanisms.

In conclusion, our MIND model offers a robust, immunocompetent system for evaluating OAd-based viroimmunotherapy in ER+ breast cancer, overcoming limitations of other models. By elucidating immune modulation and establishing proof-of-concept for multimodal therapies, this work supports the clinical translation of OAd for breast cancer patients, including metastatic disease.

1703 Development and Validation of a Six-Plex Quantitative PCR Potency Assay for an Anti-Breast Cancer DNA Plasmid Vaccine

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[1]MRIGlobal, Kansas City, MO, [2]National Cancer Institute, Bethesda, MD, [3]SAIC-Frederick, Frederick, MD, [4]Mayo Clinic, Jacksonville, FL
**Corresponding Author*

Breast cancer represents a major health burden, with over 40,000 deaths annually in the United States alone. This disease encompasses several subtypes, each with distinct genetic profiles and treatment needs. Prevention strategies for breast cancer remain limited, with most interventions either by surgery (e.g., prophylactic mastectomy for BRCA mutation carriers) or targeting only specific subtypes (e.g., tamoxifen for hormone receptor positive breast cancer prevention). Options such as chemotherapy and radiation have serious risk of adverse side effects and are not suitable in the prevention setting. Vaccination offers a promising alternative, leveraging the immune system's ability to mount immune responses specifically to overexpressed tumor-associated antigens (TAAs) without inducing harmful immune responses against normal tissues. Recent studies have demonstrated the potential for vaccines targeting overexpressed self-antigens to safely elicit an immune response, particularly in the context of cancer prevention. The Artemis Project, a collaboration among leaders in breast cancer and immunology, aims to develop a multi-antigen prophylactic vaccine targeting six TAAs: MUC1, HER2, hTERT, Survivin, MAGEA3, and Mammaglobin A, which are overexpressed across all major subtypes of breast cancer, including triple-negative, estrogen/progesterone receptor-positive, and HER2-positive cancers.

Two DNA plasmids were designed to co-express six human antigens in separate constructs (2 and 4 antigens in each construct) using 2A peptide technology. Multiantigen gene cassettes were inserted into pUMVC4a vaccine plasmid to create, pBC.1 (pUMVC4a-Her2ECD_Muc1_Her2ICD) and pBC.2 (pUMVC4a-Mamma_Survivin_MAGEA3_hTERT). Expression was validated by transient transfection in HEK293T cells. To support the analysis of these complex plasmids, we have developed a robust pipeline of assays to evaluate potency of the DNA plasmid vaccine during ongoing formulation and stability studies.

The mRNA expression of each TAA was quantified using our custom developed six-plex qPCR assay with a standard curve generated from gBlocks of each antigen, details of which will be described. Total protein expression was quantified using a commercially available ELISA kit for each antigen. During development and optimization of the 6-plex qPCR assay we consistently observed <10% CV for each standard curve and contrived samples. Contrived samples were consistently found to have <20% relative error during development. We observed an 84 to 26,000-fold overexpression of mRNA transcripts for each antigen relative to the vector control and mock transfection controls, indicating suitable transfection efficiencies for both plasmids. Similarly, we observed up to 56-fold overexpression of each TAA protein via ELISA. These data indicate that both DNA plasmids are transfected with high efficiencies and all six antigens are overexpressed relative to the mock and vector controls. In conclusion, we developed and validated a novel potency assay workflow to evaluate potency of two DNA plasmid vaccine candidates. These potency assays are integral to evaluating the expression of multi-antigen vaccines for breast cancer prevention and contribute to the ongoing development of safe and effective prophylactic strategies. Our assays provide robust measurements of TAA expression and serve as a key tool for vaccine development.

1704 Phase 2 Study Using Talimogene Laherparepvec, Nivolumab, and Trabectedin for Advanced Leiomyosarcoma and Liposarcoma (NCT03886311)

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Background: Advances in cell therapy/immunotherapy present the opportunity to further improve established chemotherapy regimens for soft tissue sarcoma. Here, we present the results of a phase 2 study for patients with advanced leiomyosarcoma (LMS) or liposarcoma (LPS), combining intravenous anti-neoplastic agent Trabectedin (T) with PD-L1 inhibitor Nivolumab (N) and intratumoral cell-therapy product Talimogene Laherparepvec (TVEC), a genetically modified Herpes simplex Type 1 oncolytic virus expressing GM-CSF which evokes a cytotoxic immune response.

Objectives: Primary: To assess progression-free survival (PFS); Secondary: (1) To evaluate the best overall response (BOR) by RECIST v1.1, disease control rate (DCR), 6 month PFS rate, 6 month overall survival (OS) rate, and Incidence of treatment-related adverse events (TRAEs). **Key eligibility criteria:** ≥ 18 years, locally advanced unresectable or metastatic LMS/LPS, measurable disease by RECIST v1.1, at least one accessible tumor for TVEC intratumoral injection; adequate hematologic and organ function; no concurrent clinical trial; no known hypersensitivity to study drugs; no known autoimmune disorder. **Study Design:** This is an open label single center phase 2 study. Treatment schedule: N (240 mg i.v. q 2 weeks), T (1.2 mg/m2 i.v. q 3 weeks), and TVEC (1x10e8 PFU intratumorally q 2 weeks depending on tumor size) were administered. A starting dose of TVEC (1x10e 6 PFU) was initially given, followed by a dose of 1x10e8 PFU q 2 weeks (according to tumor size) three weeks after the test dose.

Efficacy: The modified-intention-to-treat population (n=24), which includes patients who completed at least one treatment cycle and had a follow-up CT/MRI was used to determine the following: Median PFS = 8.0 (95% CI: 4.1-17.8) months; BOR = 2 PR, 18 SD, 4 PD; DCR = 83.3%; 6 month PFS rate = 62.5%; 6-month OS rate = 87.5%. The intention-to-treat population (n=35), which includes patients who received at least one dose of T, N, and TVEC, was used to determine the following: Median OS = 19.2 (95% CI: 10.2- 37.9) months. **Safety:** The intention-to-treat population (n=35), which includes patients who received at least one dose of T, N, and TVEC, was used to evaluate safety. Eighteen of 35 patients (51%) experienced Grade 3 TRAEs that included anemia (n=4), fatigue (n=3), ALT increased (n=3), AST increased (n=3), thrombocytopenia (n=3), abdominal pain (n=2), myalgia (n=2), localized edema (n=2), injection site pain (n=2), alkaline phosphatase increased (n=1), LVEF decreased (n=1), pain in extremity (n=1), neutropenia (n=1), colitis (n=1), headache (n=1), flu-like symptoms (n=1). There were no ≥ Grade 4 or unexpected adverse events.

Conclusions: Taken together, the results indicate that the combination regimen of Talimogene laherparepvec, Nivolumab, and Trabectedin may be more effective than Trabectedin alone (Median PFS 4.1 months) as treatment for previously treated patients with advanced leiomyosarcoma and liposarcoma with manageable toxicity. A randomized phase 2 clinical trial is warranted to confirm these promising results.

1705 Targeting Adenosine Immunosuppression in Glioblastoma with Oncolytic Virotherapy

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Background: Glioblastoma (GBM) is highly immunosuppressive, with the adenosine (ADO) pathway playing a central role in disrupting immune responses within the tumor microenvironment (TME). To overcome this, we have developed new oncolytic herpes simplex virus (oHSV) G47Δ vectors armed with enzymes able to reduce extracellular (e)ADO and/or signaling and decrease immunosuppression and enhance immunovirotherapy. This novel combination of ADO degrading enzymes (ADEs) and the immune-stimulatory properties of oHSVs should improve clinical outcomes for GBM patients.

Methods: To construct novel oHSVs expressing ADEs, adenosine deaminase isoform 1 (ADA1) and adenosine kinase short isoform (ADK-S) were chosen to arm G47Δ. ADA1 can be intra- and extra-cellular and converts ADO to inosine. ADK-S catalyzes ATP-dependent phosphorylation of ADO to AMP and is appealing due to its higher affinity for ADO. Both enzymes are engineered to be secreted. To our knowledge, ADK has never been expressed in the TME, adding to the uniqueness of this strategy. Codon-optimized mouse mADA1 or mADKS with a secretory sequence (IL-2 signal peptide) upstream were inserted into G47Δ-using the CRISPR/Cas9 technique. G47Δ has been conditionally approved to treat recurrent GBM in Japan**.** Expression of ADO pathway proteins was determined using western blots, and immunohistochemistry and FACS of orthotopic mGSC-derived tumors.

Results: The lab has established unique panels of murine GBM stem-like cells (mGSCs) tumorigenic in C57BL/6 mice and primary and recurrent human GSCs (hGSCs). The expression of key ADO pathway proteins (CD39, CD73, and A2aR) in GSCs *in vitro* and in their derived tumors *in vivo* was characterized. *In vitro* analysis found large variability between GCSs. *In vivo* expression varied greatly in tumor cells and different immune cells in 005 mGSC-derived tumors (high CD39 and CD73 in T cells, low in macrophages, and high A2aR in microglia). This variability in the ADO pathway in both human and murine GSCs and tumors provides representative models to evaluate the effects of G47ΔmADA1 and G47ΔmADKS *in vitro* and *in vivo*. *In vitro*, mADA1 and mADKS were detected in infected 005 mGSCs, and their supernatants. mADA1 enzymatic activity, increased inosine, was only detected in the supernatants from G47ΔmADA1 infected mGSCs. mADK activity will be measured using highly sensitive liquid chromatography-mass spectrometry (LC-MS/MS) for adenosine, inosine, and AMP/ATP levels. Because eADO has its largest impact on immunotherapy, we will evaluate the activity of G47ΔmADA1 and G47ΔmADKS in immunocompetent mouse models *in vivo*. We will also examine the combination of G47ΔmADA1 and G47ΔmADKS. In a separate cohort of treated mice, immune cell subtypes will be determined by FACS and immunohistochemistry to observe a shift in the TME. Our hypothesis is that one or both viruses will improve survival vs. G47Δ and that improvement will be related to changes in tumor-infiltrating lymphocytes and polarized macrophages.

Conclusion: Infection with the novel oHSV-ADEs described here of our unique collection of mGSC-derived tumor models, with variable expression of ADO pathway components in tumor and TME cells, should lower eADO levels in the TME, reduce immunosuppression, and induce anti-tumor immunity. This innovative strategy can be translated to the clinic and potentially impact GBM patients.

1706 Evaluating IL27-expressing Oncolytic Adenovirus for Treating Advanced Prostate Cancer

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The mortality of prostate cancer mainly consists of patients experiencing recurrence after androgen deprivation therapy (ADT) and those with distant metastasis, especially in bone, for which the five-year survival rate is only ~30%. The lack of effective therapeutics drives our objective to develop systemic treatments. Oncolytic viruses, such as adenoviruses (oAd), have shown success over decades, as evidenced by their entering clinical trials in several countries. However, limitations such as restricted delivery routes (intratumoral) to reach effective doses, and rapid neutralization by the host immune system remain key challenges to their broader therapeutic application. In this study, we engineered an oAd with a chimeric fiber to express genes of interleukin 27 (IL27) and green fluorescent protein (GFP), using adipose-derived mesenchymal stromal cells (ASCs) as carriers due to their distinct tumor-homing and immune-modulatory properties. We hypothesized that ASC carriers for oAd-IL27 would be *more effective than oAd or ASCs alone.*

Characterization confirmed selective viral replication in cancer cells (through an E1A deletion design), IL27/GFP expression, and cytotoxicity in the prostate cancer cell line, PC3. Cell cycle analyses indicated a significant increase in the subG1 phage in PC3 cells following treatment with oAd-IL27-loaded ASCs, as suggested by reduced confluency after five days of incubation. Further, in a 3D spheroid model, by coculturing NucLight-transfected PC3 spheroids with control oAd- or oAd-IL27-carrying ASCs, we observed promising effects for the IL27 treatment in reducing viable cancer cell number, as indicated by Nuclight intensity. Significant viral replication was detected as an increase in GFP integrated intensity, in both oAd- and oAd-IL27-loaded ASCs treatment groups. A PC3 xenograft mouse model was used to evaluate the therapeutic efficacy *in vivo*. Athymic male mice bearing subcutaneous PC3 tumors received a single treatment of phosphate-buffered saline (PBS), ASCs, oAd-carrying ASCs, or oAd-IL27-carrying ASCs when the tumor volume reached 50 mm^3. From the preliminary results, a prolonged survival time was observed in the treatment groups versus PBS control. Additionally, decreases in tumor volumes and tumor growth rate were also detected in groups given either of the oAd-carrying ASCs, and tumor reductions tended to be greater in PC3 tumors treated with oAd-IL27-carrying ASCs.

We will focus on optimizing the administrations toward multiple treatments to maximize therapeutic efficacy. Using ASCs to disguise oAd can reduce rapid neutralization and inflammatory responses from the host, enhancing the potential for repeated treatments. Ongoing and future studies may include immunohistochemistry and bulk RNA sequencing of tumors to validate and characterize the mechanisms of treatment efficacy. Additionally, immune cell profiling will be conducted to understand better the interactions between these oncolytic viruses, cancer cells, and the host immune system.

1707 Intranasal Delivery of IR-780-Conjugated Oncolytic Adenovirus: A Non-Invasive Approach Combining Virotherapy and Photothermal Therapy for Glioblastoma Treatment

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Intranasal delivery has recently gained attention as a promising and highly safe method of drug administration. This non-invasive approach involves direct application of drugs to the nasal mucosa, allowing for direct delivery to the brain via olfactory and trigeminal neural pathways that originate in the olfactory neuroepithelium of the nasal cavity and terminate in the brain. Here, for efficient delivery of intranasal delivery of oncolytic adenovirus (oAd), we have developed a novel nanohybrid vector comprising an oAd complexed with a photosensitive carrier (INP-IR780). The intranasal administration of this oAd-INP-IR780 complex uniquely bypasses the blood-brain barrier, a major obstacle in conventional GBM therapies, enabling direct and efficient delivery to the brain. This nose-to-brain route significantly enhances the accumulation of both the oncolytic virus and the IR780 photosensitizer in glioma tissues, overcoming the limitations of systemic delivery. The pH-sensitive nature of the carrier allows for targeted release in the acidic tumor microenvironment, while IR780 enables localized photothermal therapy upon near-infrared laser irradiation. In vivo studies demonstrated synergistic antitumor effects with reduced systemic toxicity, highlighting the potential of this non-invasive, targeted approach to revolutionize GBM treatment strategies. Taken together, this multifunctional nanohybrid vector offers a promising, minimally invasive approach for targeted GBM treatment, potentially transforming therapeutic strategies for this challenging malignancy.

1708 Overcoming Recombination in Semi-Replicating Retroviral Vectors: A Novel Double Suicide Gene Therapy for Glioblastoma

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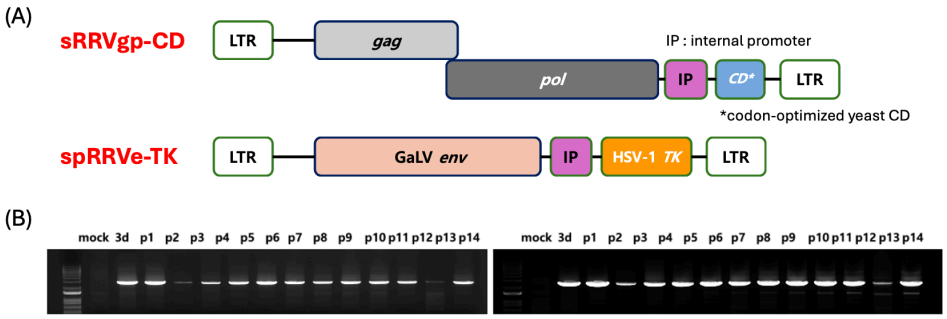
One of the major challenges in developing semi-replicating retroviral vector (sRRV) systems has been the loss of therapeutic genes due to non-homologous recombination during the reverse transcription in tumor cells. Despite prolonged efforts, previous studies have failed to prevent such recombination events. However, after years of optimization, we have successfully developed a recombination-resistant sRRV system that maintains the integrity of both therapeutic genes while efficiently replicating and spreading within tumor cells.

In this study, we utilized a gibbon ape leukemia virus (GaLV) envelope-pseudotyped semi-replicating retroviral vector system (spRRVs), consisting of two trans-complementing replication-defective retroviral vectors, MuLV-Gag-Pol and GaLV-Env, each encoding a therapeutic suicide gene. After confirming the antitumor efficacy of the spRRVs-mediated double suicide gene therapy in glioblastoma multiforme (GBM), we conducted survival and tumor burden analyses in six experimental groups (n=8 per group) stratified by viral dose and prodrug treatment in a rat glioma model.

Glioma models were established by intracranial injection of C6 cells into Wistar rats. The spRRV vectors expressing yeast cytosine deaminase (CD) and herpes simplex virus type 1 thymidine kinase (HSV1-TK) (sRRVgp-CD and spRRVe-TK, respectively) were intratumorally injected, followed by administration of the prodrugs 5-fluorocytosine (5-FC) and/or ganciclovir (GCV). On day 98 post-tumor induction, animals were sacrificed for histological analysis, revealing a dose-dependent reduction in tumor size. Notably, complete tumor eradication was observed in the cohort receiving the highest dose of therapeutic vectors in combination with both prodrugs. These histopathological findings correlated well with the survival data, demonstrating a significant therapeutic benefit.

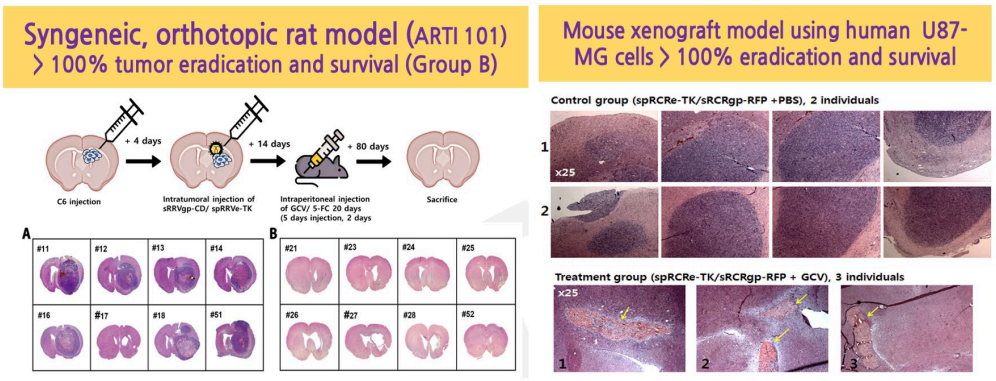
Furthermore, we performed statistical analyses to compare survival rates across control and treatment groups, establishing the efficacy of our spRRV system. Importantly, our results demonstrate that despite encoding two separate therapeutic genes, our novel spRRV design effectively prevents non-homologous recombination, ensuring stable replication and therapeutic function. This breakthrough overcomes a long-standing limitation of split-replication retroviral vectors, paving the way for more robust and clinically viable gene therapies for GBM and other malignancies.

Structure and Stability of the sRRV System Encoding Dual Suicide Genes



(A) Schematic representation of the two-component semi-replicating retroviral vector (sRRV) system used in this study. (B) PCR analysis demonstrating the genetic stability of the sRRV system in tumor cells over multiple passages. Genomic DNA was extracted from glioma cells infected with sRRVgp-CD and spRRVe-TK, and PCR was performed to detect the presence of both therapeutic transgenes across different passages (p1–p14).

Efficacy of the sRRV Vector System in Syngeneic and Xenograft Glioblastoma Models: 100% Tumor Eradication and Long-term Survival



This study confirms that the sRRV vector system effectively eliminates glioblastoma in both syngeneic and xenograft models, achieving 100% tumor eradication and long-term survival.

1709 Stem Cell Delivered Viro-immunotherapy Advancements for Ovarian Cancer Treatment

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Oncolytic viro-immunotherapy is a highly promising cancer treatment which uses replicating oncolytic viruses (OVs) to induce tumor cell lysis and stimulate an anti-tumor immune response. Currently, clinical trials administering free virus have demonstrated safety but limited efficacy due to rapid inactivation by the host immune system. To address this barrier, tumor-tropic stem cells can be used as an OV delivery vehicle, providing protection from immune neutralization, allowing for viral amplification which increases the payload, and enabling enhanced targeting and viral distribution at metastatic tumor sites. Once tumor cells are infected with the OV, viral replication ensues until lysis occurs – releasing more virus to infect neighboring tumor cells in a self-amplifying fashion until normal tissue is reached and replication ceases. Resulting changes to the tumor microenvironment and exposure of new tumor antigens attract immune cells and can ultimately stimulate an anti-tumor immune response.

We are using a stable, allogeneic, immortalized, human neural stem cell line, (HB1.F3.CD21 NSCs), that has demonstrated clinical safety and tumor localized delivery of therapeutic payloads in brain tumor trials. We have loaded these NSCs with a conditionally replication-competent adenovirus (CRAd-S-pk7) that has been genetically modified 1) with a pk7 poly-lysine fiber knob to increase tumor infectivity, and 2) the insertion of a *survivin* promoter to drive E1A transcription, making it selective to tumor cells with high *survivin* expression, including glioma and cisplatin resistant ovarian cancer. A phase I trial with a single dose intracerebral administration of NSC.CRAd-S-pk7 (CLD-101) as an adjuvant to temozolomide and radiation in newly diagnosed glioma patients yielded promising results (Lancet Oncol, 2021). A multi-dose study in recurrent glioma patients is ongoing at COH (NCT05139056).

We have now optimized the manufacturing protocols to generate a NSC.CRAd-S-pk7 product with increased efficacy for application to intraperitoneal (i.p.) ovarian metastases. Effects of the process optimization on RNAseq, protein expression, and critical quality attributes of the therapeutic product have been characterized. Comparative persistence and distribution studies were conducted in both immunodeficient and immunocompetent preclinical models of i.p. ovarian metastases, demonstrating significant benefit. Translational biodistribution, efficacy, and

safety/tox IND-enabling studies are ongoing with our goal of initiating a phase I clinical trial of NSC.CRAd-S-pk7 for patients with platinum resistant Stage III ovarian cancer in 2026.

1710 A Novel Oncolytic Adenovirus-based Therapy for Pediatric Neuroblastoma

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Neuroblastoma, the most common and lethal extracranial pediatric tumor, originates from sympathetic neuroblast precursors that fail to differentiate properly, remaining in a pro-proliferative developmental state. This tumor accounts for 12%–15% of childhood cancer-related deaths. Current treatments for high-risk neuroblastoma, which typically involve intensive multimodal approaches, including radiation and immunotherapy, often result in severe adverse effects. To mitigate these effects and enhance treatment efficacy, novel therapeutic strategies are needed. One promising approach is the use of Sodium-Iodide-Symporter (NIS) delivered by a replication-competent adenoviral vector. This strategy combines the oncolytic and immunomodulatory potentials of adenoviruses with the ability of NIS to facilitate the uptake of radioactive iodine by tumor cells, which may not only improve detection of neuroblastoma, but also facilitate radiotherapy with 131-I.

This project aims to develop a novel oncolytic adenoviral vector specifically targeting neuroblastoma. To identify the vector with the best binding to neuroblastoma cells, we tested a panel of adenoviruses with various fiber proteins targeting different cellular receptors: the coxsackie and adenovirus receptor (CAR)-targeted Ad5, CD46- and human desmoglein 2-targeted Ad5/3, and integrin-targeted AdRGD. Our results indicate that while Ad5/3-based vectors typically exhibit superior binding across most cancer cell lines, AdRGD-based vectors show the highest efficacy in the SK-N-SH neuroblastoma cells, as well as in the Ewing sarcoma EW-8 cell line. To further restrict viral replication to neuroblastoma cells, we incorporated the 796-bp Achaete-scute homolog 1 (ASCL1) promoter upstream of the adenoviral early gene E1A. ASCL1, a proneural transcriptional master regulator, plays a crucial role in the proliferation and differentiation of sympathetic neuroblast precursor cells and is essential for maintaining the oncogenic program in MYCN-amplified neuroblastomas. We are currently evaluating the new vector in a variety of neuroblastoma cell lines, including MYCN-amplified and non-amplified strains, as well as functional and non-functional p53 variants.

To incorporate the NIS transgene into the adenoviral backbone, we are replacing the Adenovirus Death Protein (ADP) gene in the E3 region as our previous studies have shown that ADP expression interferes with NIS membrane localization and inhibits radiotracer uptake. Importantly, we will keep other E3 genes, including E3-6.7K, E3-gp19KID, RID, E3-14.7K, as they are known to reduce vector clearance and promote prolonged circulation. Following these modifications, we will conduct in vivo evaluations to assess the imaging and therapeutic potential of the RGD-ASCL1-NIS vector in combination with radioactive iodine.

We believe this adenoviral platform has the potential to improve treatment outcomes for pediatric neuroblastoma, a devastating disease with limited therapeutic options.

1711 A Highly Tumor-Specific Oncolytic Virus in Combination with a Viral Protein-Targeted T Cell Engager for the Treatment of Triple-Negative Breast Cancer

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Triple negative breast cancer (TNBC), a subtype comprising 15-20% of all breast cancers, is known to be the most aggressive and carries the bleakest prognosis. The ineffectiveness of targeted therapies due to a lack of targets in TNBC underscores the urgent need for alternative therapies against a larger demographic of TNBC patients. We propose that combining oncolytic herpes simplex virus type 1 (HSV-1) with a bispecific T cell engager (BiTE) specific for glycoprotein D (gD), a viral protein expressed on the surface of infected cells, will result in better

therapeutic efficacy against TNBC. Furthermore, the HSV-1 used in this study has a *UL40* gene deletion and deletions in both copies of the *γ34.5* gene, which are intended to enhance its cancer specificity and reduce neurovirulence. We compared the replication and cytotoxic properties of the *UL40*-deleted virus (SY134ΔUL40) with its parental virus (SY134) in both normal and cancer cells. The UL40-deleted HSV-1 showed significantly reduced activity in normal cells and cancer cells. We also evaluated the impact of RRM2 (the catalytic subunit of ribonucleotide reductase) on the replication of the SY134ΔUL40 virus. Our results showed that virus growth was significantly attenuated when RRM2 was knocked down. Additionally, we assessed the tumor specificity of SY134ΔUL40 compared to SY134 in a TNBC model using athymic mice. We observed that the SY134ΔUL40 virus remained confined to the tumors, with limited presence in the lungs, while the parental virus spread to other normal organs. This finding suggests that the deletion of *UL40* reduces the virus' ability to replicate in normal tissues, thus enhancing the safety profile of the oncolytic virus. Next, we have constructed a full-length BiTE against gD and CD3 and confirmed binding of the BiTE to $CD3^+$ cells and TNBC cells expressing gD protein. In addition, we have verified the efficacy of this combined approach in killing TNBC cells *in-vitro*. Further confirmatory *in-vivo* studies are ongoing.

1712 Neural Stem Cell Delivered CRAd-S-pk7 Dose Interval Optimization for Ovarian Cancer in an Immunocompetent Mouse Model

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Background. Ovarian cancer (OC) is a high mortality gynecologic malignancy with more than 19,000 new cases and an estimated 13,000 deaths occuring annually in the U.S. This highlights an urgent need for novel therapeutic strategies to improve clinical outcomes. Oncolytic viro-immunotherapy has emerged as a promising strategy due to the ability of oncolytic viruses (OVs) to selectively target tumor cells while sparing healthy tissue. We are using tumor-tropic neural stem cells (NSCs) to deliver a conditionally replicating adenovirus, CRAd-S-pk7, genetically modified with a poly-lysine fiber knob to enhance tumor cell infectivity, and the insertion of a survivin promoter that drives viral replication. Survivin overexpressed in >85% chemoresistant OC patients. The use of NSCs to deliver this OV (NSC.CRAd-S-pk7), shields the virus from immune inactivation, allows for viral amplification, and enhances tumor-specific targeting and distribution- resulting in increased efficacy as compared to free virus. However, the optimal dosing interval for maximizing this NSC-OV therapy remains to be determined.

Objective. This study aimed to determine the optimal dosing interval for a multi-dose regimen of NSC.CRAd-S-pk7 in a syngeneic immunocompetent mouse model of intraperitoneal (i.p.) ovarian metastases to achieve maximal survival efficacy. We hypothesize that i.p. NSC.CRAd-S-pk7 will effectively infect and lyse OC cells, inducing a dynamic immune response within the tumor microenvironment (TME), characterized by early activation of innate immune cells followed by progressive engagement of adaptive immunity. We postulate that optimizing the dosing interval will enhance the anti-tumor immune response while mitigating immune exhaustion, thereby improving long-term survival outcomes.

Methods. NSC.CRAd-S-pk7 was produced by transduction of an immortalized, allogeneic NSC line (HB1.F3.CD21) with CRAd-S-pk7 and frozen down as the therapeutic product. ID8.eGFP.ffluc murine OC cells were used to induce i.p. tumors in C57BL/6 mice on Day 0. Mice were divided into five groups: A) tumor-only control, B) single dose, C) 3 weekly doses, D) 3 doses every other week, and E) 3 doses every two weeks. Each dose consisted of 2 x 10^6 NSC.CRAd-S-pk7 cells. Disease burden was monitored using BLI, ascites onset, CA-125 and HMGB1 expression in the blood, daily scoring of clinical observations and long-term survival. Endpoint gross distribution of tumor nodules was assessed and scored. Ascites fluid was analyzed for innate natural killer cells, dendritic cells macrophages, and neutrophils, and adaptive CD4 and CD8 immune cell profiles to characterize the immune response in the TME over time.

Results. NSC.CRAd-S-pk7 significantly improved survival in when comparing treated groups vs. control ($p < 0.0001$), and multiple doses vs. single dose ($p < 0.0001$) (**Fig. 1)**. Furthermore, the efficacy of every other week dosing was significantly more efficacious than other dosing schedules (Group D vs. Group C; $p=0.010$) and also delayed ascites onset. Immunophenotyping showed a peak adaptive response on Day 7. Ongoing studies are investigating the add-value of repeat interval cycles, as well as adjuvant treatments.

Conclusion: These findings suggest that administration of NSC.CRAd-S-pk7 treatment every other week optimizes therapeutic outcomes in OC models by balancing sustained immune activation and viral replication.

1713 Somatic Cell Gene Editing for Translational Preclinical Cancer Modeling in Swine

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Cancer, as a disease, encompasses a diverse taxonomy of malignancies that impart a high degree of burden on the global healthcare system. With incidence of morbidity and mortality expected to rise in the coming decades, novel approaches to treating cancer are needed. To drive innovative therapeutic development, comparative preclinical animal models that recapitulate the disease, genetically and phenotypically, are essential to the process. While *in vitro* cell work and rodent models have been the historical standard for developing and testing cancer therapies, their deficiencies in complexity, shared anatomy and physiology, have contributed to high failure rates in late preclinical and early clinical trial phase testing. This equates to significant losses of fiscal resources and valuable time. ***To provide the necessary tools to reverse this trend, we are using somatic cell gene editing to create genetically and phenotypically accurate cancer models in swine for preclinical therapy development.*** Using CRISPR/Cas9 to knock-out tumor suppressor genes and transposons to knock-in oncogenes, Recombinetics has focused on modeling two types of deadly cancer, glioblastoma (GBM) and hepatocellular carcinoma (HCC). These cancers are of high priority as the former is the most prevalent and aggressive primary brain tumor, with a 3% survival beyond 5-years, and the latter is the fourth most common and second deadliest cancer worldwide. To create the GBM model, we use stereotactic lateral-ventricular delivery of gene editing reagents in piglets and monitor tumor growth through development with a simple blood-based test. Results consistently demonstrate tumor formation that mimics the pathological characteristic of human GBM within 115 days post-induction. To create the HCC model, we exploit on the phenomena of hydrodynamic cellular delivery to distribute gene editing reagents to the liver of neonate piglets. Tumor growth is monitored in these animals by ultrasound imaging. Notably, the cancer models created replicate the anatomy, physiology, and intact-immune system of human patients. With robust numbers and molecularly accurate swine model platform, we anticipate that the resources spent on cancer therapy will yield greater progress and drive development of targeted small molecules, immunotherapies, surgical and biopsy techniques, biomarker identification, and imaging technologies.

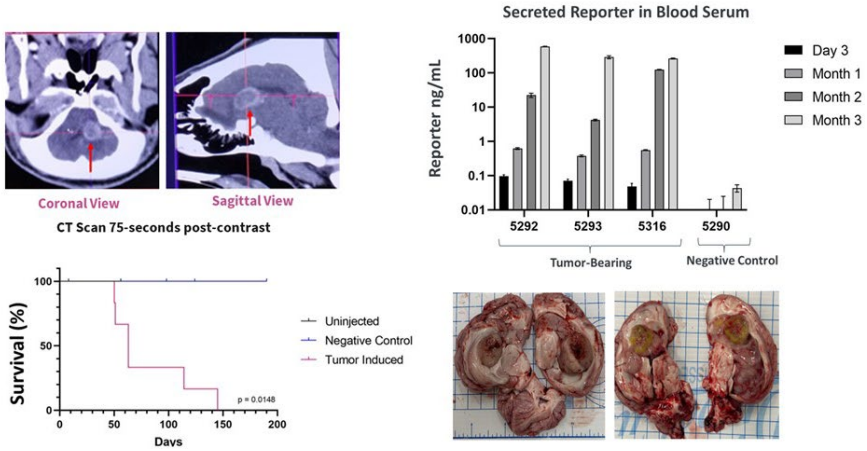

Figure: An *in vivo* model of GBM in swine. A. CT imaging of a GBM tumor in swine, a potential method for monitoring tumor status. B. Monthly secreted reporter used to monitor tumor growth in models. C. Kaplan-Meier plot showing the rate at which swine succumb to tumors. D. Swine mesenchymal GBM tumors harvested at necropsy.

1714 Cost-Effectiveness of Gene Therapy for Sickle Cell Disease in Uganda: Tailoring High-Income Evidence to Uganda's Context

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In high-income countries, cost effectiveness analysis (CEA) models are used to support policy regulating access to transformative treatments such as gene and cell therapies. However, translating cost-effectiveness models for gene and cell therapies to low resource settings is hampered by lack of local clinical trial data. Here we describe a novel CEA model to assess gene therapy for sickle cell disease (SCD) in Uganda. While two gene therapies to treat SCD are authorized in the U.S. and Europe, neither has been evaluated clinically in sub-Saharan African countries. Uganda carries a high burden of SCD, with 13.3% of its population carrying sickle cell trait and 0.7% having SCD. To estimate lifetime SoC costs in Uganda, we employed a Markov model designed to simulate the progression of patients across three health states: (1) standard of care (SoC) without hydroxyurea, (2) SoC with fixed dose hydroxyurea, and (3) death. Scaling factors were calculated using Basu et al.'s methodology and two different CEA models (UW-MEASURE (UWM) and FH-HISCORE (FHH)) to adjust U.S.-based gene therapy costs to Uganda's context. Application of lovo-cel (bluebird bio) or exa-cel (Vertex Therapeutics) gene therapy for SCD was targeted to the same patient populations for authorized use in the U.S. and Europe: eligible individuals with SCD aged 12 years and older who meet specific trial inclusion criteria. Clinical benefits of gene therapy treatment, including life-years, quality-adjusted life-years (QALYs), and health years in total (HYT), were assumed to be the same across settings and gene therapy drug products, aligning with global expectation of biological consistency for gene therapy across populations. Incremental cost-effectiveness ratios (ICERs) were calculated under healthcare and societal perspectives, with sensitivity analyses using Uganda's gross domestic product per capita-based thresholds recommended by the World Health Organization. All values were represented in US dollars (USD) and Ugandan Schillings (UGX) based on currency conversion in January 2025 ($1 = 3,680.18 UGX). Lifetime SoC costs in Uganda were calculated to be $21,877 (80,511,250.39 UGX). Scaling factors of 0.018 (UWM) and 0.033 (FHH) contextualized U.S.-based gene therapy costs for Uganda. For **lovo-cel**, healthcare ICERs were $5,219 (19,206,848.09 UGX) and $20,023 (73,688,200.69 UGX) per QALY (UWM and FHH, respectively), while societal ICERs were $3,992 (14,691,269.90 UGX) and $16,091 (59,217,741.46 UGX) per QALY (UWM and FHH). For **exa-cel**, healthcare ICERs were $3,837 (14,120,842.33 UGX) and $20,023 (73,688,200.69 UGX) per QALY (UWM and FHH), with societal ICERs at $2,610 (9,605,264.14 UGX) and $16,091 (59,217,741.46 UGX) per QALY

(UWM and FHH). Reflecting broader economic benefits like improved productivity and reduced caregiver burden, the societal ICER for **exa-cel** under the UWM model is cost-effective at a per drug product cost of $40,205 (147,961,549.66 UGX). Using this CEA framework, a gene therapy for SCD which costs $2.2M in the U.S. offers a cost-effective intervention in Uganda when societal benefits are considered. Importantly, per product local manufacturing cost of CAR T cell gene therapy drug products in India ($35,107 or 129,200,003.08 UGX) (Palani, H. *et al.* 2023) is lower than the scaled cost of a SCD gene therapy product in this model, suggesting a profit margin is possible for a locally manufactured gene therapy product for the treatment of SCD in Uganda.

Table 1. Incremental Effectiveness and Costs of exa-cel Gene Therapy Compared to Standard of Care in the U.S. and Uganda

	United States UW-Measure	Uganda UW-Measure	United States FH-HISCORE	Uganda FH-HISCORE
Effectiveness				
Undiscounted Life-Years	17.4	17.4	17.0	17.0
Discounted Life-Years	7.9	7.9	6.4	6.4
QALYs (Patients)	9.8	9.8	5.1	5.1
QALYs (Family)	2.1	2.1	0.3	0.3
QALYs (Total)	11.9	11.9	5.4	5.4
HYT (Total)	19.7	19.7	NA	NA
Costs				
Medical Costs ($)	-172065	-3144	-267872	-8771
Gene Therapy Drug Costs ($)	2200000	40205	3100000	101504
Gene Therapy Administration Costs ($)	470796	8604	470089	15392
Total Medical Costs ($)	2498731	45664	3302217	108125
Productivity Costs ($)	-1247805	-22803	-1015378	-33247
Patient Time Use Costs ($)	-28620	-523	NA	NA
Caregiver Time Use Costs ($)	-19035	-348	-25402	-832
Consumption Costs ($)	495968	9064	392324	12846
Total Societal Costs ($)	1699239	31053	2653761	86893
ICER (Health Care Sector) ($/QALY)	209977	3837	611522	20023
ICER (Societal Perspective) ($/QALY)	142793	2610	491437	16091
ICER (Health Care Sector) ($/HYT)	126839	2318	NA	NA
ICER (Societal Perspective) ($/HYT)	86256	1576	NA	NA

1715 Therapeutic Potential Of NF-κB Inhibition In Glioblastoma: Gene Therapy Approach Using rAAV-5 Mediated IκBαM Overexpression

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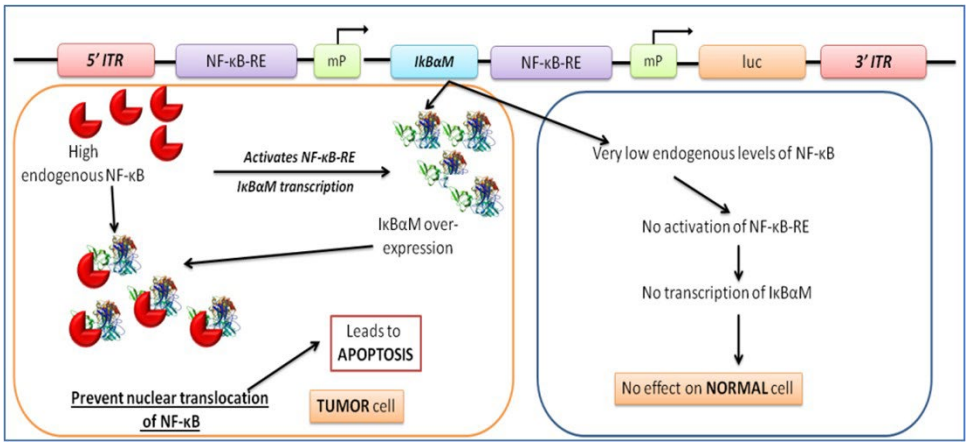
Current treatment strategies for glioblastoma, including resection followed by concurrent chemotherapy/radiotherapy are not curative. Angiogenesis and hypoxia are two major factors responsible for GBM growth and resistance to existing therapies, leading to poor clinical outcomes. The transcription factor NF-κB induces tumor progression by activating genes associated with cell proliferation and angiogenesis. It is expressed constitutively in gliomas and is known to regulate the expression of HIF-1α and VEGF in GBM. Activation of NF-κB is a significant contributor to the cancerous characteristics associated with GBM, indicating a poor prognosis for patients. Non-specific anti-inflammatory drugs have been the main means of targeting NF-κB, but their short-lived action and numerous side effects prevent them from producing persistent results. Novel techniques must be developed to attenuate NF-κB activity selectively within certain cell types and mitigate any adverse consequences in clinical settings. One such approach is overexpressing IκBαM (a dominant negative mutant of IκBα) to inhibit NF-κB expression in the glioma cells. This will inhibit the nuclear translocation of NF-κB, thereby facilitating a decrease in the expression of downstream targets. AAVs have emerged as one of the most widely used methods for delivering genes into the central nervous system because of their broad range of infectivity, ability to induce long-term transgenic expression, and lack of toxicity. In this study, we aim to investigate the potential of IκBαM, resistant to IKK phosphorylation and known to block NF-κB, as a therapeutic agent to treat GBM. U87-MG cells were transduced in 2D and 3D spheroids using recombinant adeno-associated virus-5 (rAAV-5) to induce IκBαM over-expression. Employing this rAAV 5 prevented the nuclear translocation of NF-κB in LPS-stimulated U87-MG cells. This result supports our hypothesis that over-expression of IκBαM inhibits NF-κB nuclear translocation by remaining sequestered to NF-κB and preventing it from entering the nucleus. The cues from this study could aid in developing a gene therapy approach to target crucial pathways in GBM tumor progression.



1716 AK2, COL1A1, and PLG protein signatures: targeted therapeutics for meningioma

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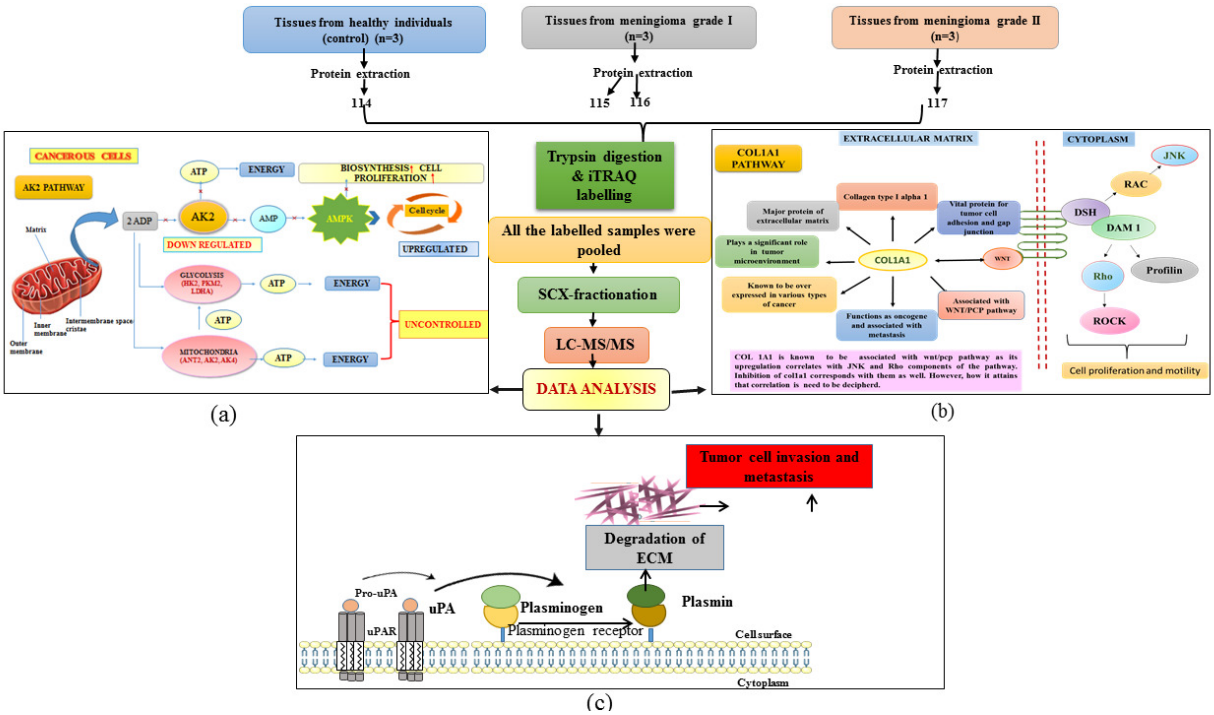
Background: Meningioma is the most prevalent primary intracranial brain tumor and accounts for one-third of all CNS tumors. Meningioma is known to be the most common yet life-threatening brain tumor with a higher recurrence rate. Globally, there is an increase in the healthcare burden due to meningioma and hence in its research. The present clinical approach includes surgical resection, chemotherapy, and radiation therapies to which the malignancy does not seem to respond efficiently. Targeted therapies and molecular markers provide elite patient treatment and care for individuals suffering from meningiomas as compared to conventional measures. Although

there is proteomic data on meningioma the knowledge of potential biomarkers differentiating the grades is scarce. To identify the best set of biomarkers, validation of reported markers in large and independent sample cohorts in the future is necessary.

Methods: A total of 12 samples, 3 each of control (which made pool 1) meningioma grade I (which made 2 sets: pool 2 and pool 3), and meningioma grade II (which made pool 4) were taken for LC-MS/MS. After this, the expression of three proteins was checked by immunocytochemistry, flow cytometry, and western blotting.

Results: Protein expression was analysed using various techniques like mass spectrometry, immunocytochemistry, flow cytometry, and western blotting. Mass spectrometry is the most commonly used standard and reliable technique for identifying and quantifying protein expression. We got three highly upregulated proteins namely AK2, COL1A1, and PLG using this technique. The biomarker potential of these proteins was further checked by ICC, western blotting, and flow cytometry. Three important proteins were found to be upregulated namely, AK2 (Adenylate kinase 2), COL1A1 (collagen 1A1), and PLG (plasminogen). The order of increased protein expression was control < MG grade I < MG grade II according to mass spectrometry and western blotting. In immunocytochemistry, we found that COL1A1 expression increases significantly with grades in comparison to control. Similarly, AK2 and PLG also showed little increase but not as much as COL1A1. In flow cytometry, PLG showed higher upregulation in grades than control. While AK2 and COL1A1 showed little increase in expression in grades than control. All techniques, especially mass spectrometry and western blotting, presented higher expression of these proteins in grades as compared to control.

Conclusion: In the quest to find a suitable therapeutic marker, this study incorporates quantitative labelling and detection followed by flow cytometry, immunocytochemistry, and western blotting for early diagnosis and treatment of meningioma. The article further explores the efficacy of some proteins namely AK2, COL1A1, and PLG to be the targeted molecules.



1717 Tumor-specific AAV Delivery of Interleukin-12 Enhances Antitumor Immunity and Safety in Ovarian Cancer

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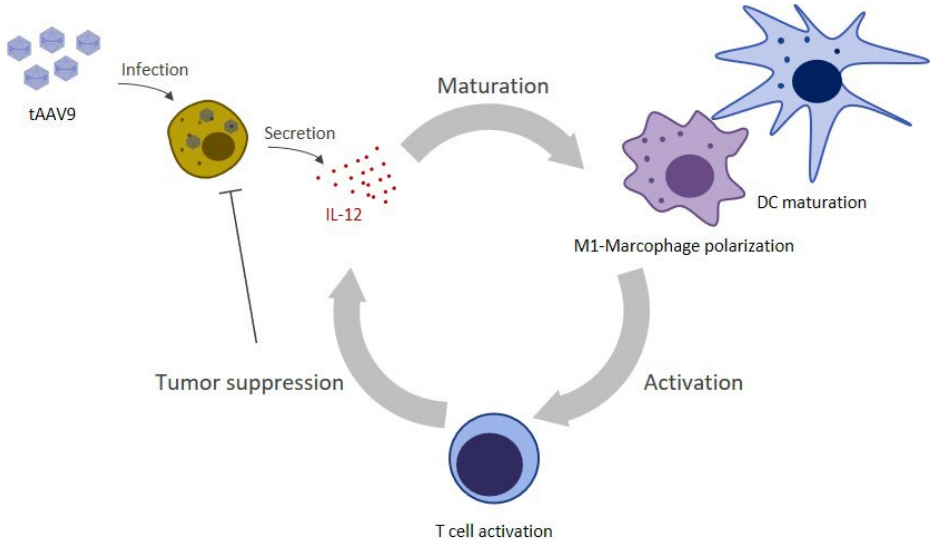
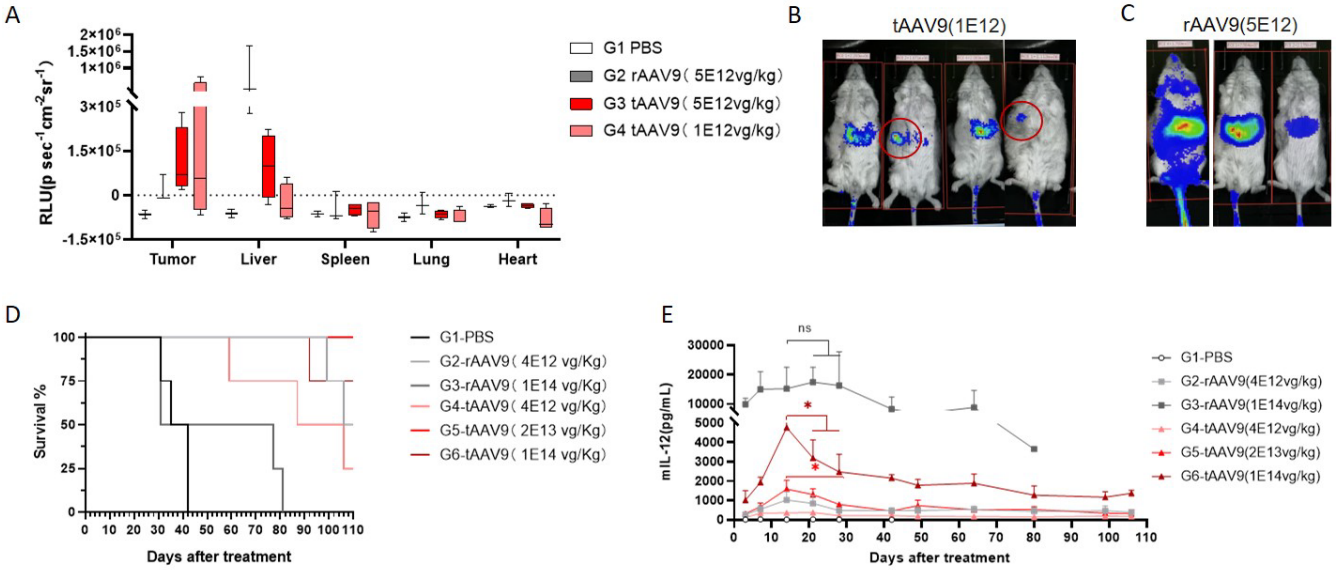
Interleukin-12 (IL-12) is a promising pro-inflammatory cytokine for cancer immunotherapy, but its toxicity and short half-life in serum limit its clinical application. Tumor targeted delivery of IL-12 either by fusion with antibody or secretion by CAR-T cells showed reduced systematic toxicity; however, the poor TME response or the lack of systematic IL-12 regulation still remains risk of low efficacy or high toxicity. Here, we developed TME specific delivery of IL-12 by a tumor targeted AAV (tAAV9). The tAAV9 was formed by an anti-FOLR1 antibody fragment conjugated with AAV9 via highly efficient Spy-ligation. With targeted infection of FOLR1+ cells *in vivo*, intravenous (IV) administration of tAAV9 specifically delivered IL-12 (tAAV9-IL-12) to TME and significantly suppressed tumor progression with favorable safety profile compared with rAAV9 (recombinant wild-type AAV9) delivery. Moreover, the IL-12 level in the serum was decreased significantly with the suppression of tAAV9-IL-12 infected tumor cell so that generates promising negative feedback to ensure the safety profile.





1718 Programmable iPSC-derived monocytes for the treatment of solid tumours

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The development of effective immunotherapies for solid tumours has been challenging due to several barriers, including antigen heterogeneity, reduced infiltration, and the presence of an immunosuppressive tumour microenvironment (TME). Faced with these challenges, CAR-T cell therapies – which have been transformative in haematological cancers - have mostly produced disappointing results in trials for solid tumours, calling for new modalities to be developed.

The majority of solid tumours recruit and subvert myeloid cells to support tumour growth, angiogenesis and to inhibit an effective immune response. Indeed, tumour associated macrophages (TAMs) orchestrate the establishment and maintenance of the immunosuppressive environment through the secretion of cytokines and immunomodulatory ligands, and are known to supress immunotherapies as well as conventional cancer treatments such as chemotherapy.

The ability of macrophages to infiltrate and establish long term residence within the tumour, makes these cells extremely valuable as therapeutic agents. If macrophages can be programmed to maintain their tumour-homing abilities, but resist polarisation into immunosuppressive TAMs and instead trigger potent inflammatory responses, they can be used as ‘trojan horses’ to effectively remodel the TME, facilitating the direct killing of tumour cells, the anti-tumour response of infiltrating lymphocytes, and enhancing the efficacy of immuno- and chemotherapies.

Here we show proof of concept data for an allogeneic cell therapy based on iPSC-derived monocytes programmed to preferentially differentiate into pro-inflammatory macrophages within the tumour, and to resist anti-inflammatory polarisation. To engineer the therapeutic cells, we deploy a novel programmable gene silencing technology based on repurposing endogenous microRNAs (miRNA) to silence desired target genes. Gene edits are introduced in myeloid-specific miRNA genes to redirect their silencing activity towards critical macrophage polarisation factors, thus programming the myeloid-specific silencing of these factors. iPSCs engineered in this manner maintained the ability to differentiate into monocytes (iMonocytes) and macrophages (iMACs), and the differentiated cells showed effective cell type-specific silencing of the target genes. Remarkably, when cultured under strong anti-inflammatory conditions, the engineered iMACs were resistant to these stimuli, and instead spontaneously activated a pro-inflammatory programme characterised by the upregulation of STAT1. *In vivo* administration of iMonocytes to animals bearing ovarian cancer xenografts showed the therapeutic cells were able to persist and differentiate within the tumour. The engineered iMACs delayed tumour growth and showed reduced expression of TAM markers compared to control wild type iMACs. Our data demonstrates the power of our gene silencing approach in an *in vivo* model, and the feasibility of applying this technology to the development of macrophage-based cell therapies for solid tumours.

1719 Identifying a target for CAR therapy in tumor-initiating cells

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Eliminating cancer stem cells (CSCs) has the potential to significantly improve patient prognosis, as studies in mice demonstrate that CSC eradication can enhance cure rates and reverse chemotherapy resistance. However, ovarian CSCs present a unique challenge, as they develop chemoresistance to standard therapeutics, such as cisplatin and paclitaxel. While nearly 70% of ovarian cancer patients initially respond to chemotherapy, recurrence occurs within weeks or months. Thus, to effectively cure ovarian cancer, there is an urgent need to specifically target ovarian CSCs to block their tumor initiation and propagation capabilities.

To identify ovarian CSCs, both we and others have utilized aldehyde dehydrogenase (ALDH) activity and CD133 expression as biomarkers. ALDH consists of a group of 20 enzymes responsible for converting toxic aldehydes into corresponding carboxylic acids. However, the commonly used ALDEFLOUR FACS-based assay does not distinguish between different ALDH enzymes. Additionally, many ovarian cancer cell lines express high levels of CD133 (up to 50%), indicating the necessity to identify more specific markers for CSCs. These CSCs are defined by their resistance to chemotherapy and their capacity to initiate tumors. Tumor initiation capacity in vitro is assessed through clonogenic assays, where serial dilutions of cell numbers are evaluated. We have previously shown that as few as 10 CSCs are capable of forming viable clones.

Our objective is to identify surface biomarkers of ovarian CSCs using proteomic analysis that correlate strongly with their tumor initiation capacity. Based on the identified tumor antigens, we aim to develop genetically engineered T cells with chimeric antigen receptors (CARs) to specifically target ovarian CSCs, thereby preventing recurrent disease.

In our approach, we utilized serial dilution clonogenicity assays to evaluate tumor initiation. Proteomic analysis of five biological replicates from two ovarian cancer cell lines revealed 11 upregulated proteins located on the cell surface that are common across both lines. Current validation studies are underway to identify the most promising candidates for CAR therapy development.

1720 Programmed evolution: Delivery of asexual gene drives to sculpt tumor populations and combat genetic diversity

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In oncology, therapeutic opportunity is fundamentally constrained by the genetic similarities between malignant and off-target, healthy cells. Even in cases where actionable drug targets have been identified (e.g. EGFR in lung cancer), resistance to targeted agents inevitably develops in patients with late-stage disease. Suicide gene therapy seeks to artificially create therapeutic opportunity by directly introducing genetic vulnerabilities into cancer cells. Suicide gene systems generally leverage bystander killing, such that both modified and nearby, unmodified tumor cells are susceptible to the therapeutic effect. However, current gene delivery technologies are insufficient to eliminate all malignant cells in a tumor, even with bystander function. As a result, efforts to translate suicide gene therapy have been unsuccessful. Here, we describe a novel gene therapy platform that bridges the gap between existing gene delivery capabilities and the requirements for effective suicide gene therapy. In reprogramming tumors with additional genetic vulnerabilities, the resulting potential for combination therapies makes durable remission possible.

Our modular platform employs a tumor-directed lentiviral vector that stably modifies cancer cells in situ (Figure 1a). The delivered cargo is a genetic circuit that couples a therapeutic payload (suicide gene) with an inducible marker that amplifies the frequency of the engineered cancer cell population. After gene delivery, engagement of the inducible marker reprograms the tumor to be majority-engineered cells (Figure 1b). Then, activation of the suicide gene targets modified and – through bystander activity – low-frequency, unmodified cells.

To develop a tumor-selective vector that can safely deliver our genetic circuits, we pseudotyped lentiviral vectors with a mutant, receptor-blinded VSVG and tumor-targeting scFv molecules. We designed and evaluated targeted vectors in vivo by administering tumor-directed lentiviral particles in NSCLC-xenografted mice via tail vein injection. Across diverse targets, our approach demonstrated remarkable specificity in transducing tumor cells, suggesting a generalizable platform for modifying cancer cells in vivo (Figure 2a). Additionally, the compatibility of this approach with systemic administration indicates the potential to target metastatic sites in disseminated disease, where unmet need is the greatest.

After delivery, the next phase of treatment involves leveraging approved targeted inhibitors to amplify the frequency of genetically modified cells and reprogram the bulk tumor. To develop a genetic circuit capable of “hijacking”

cancer evolution, we designed a drug target analog capable of transiently rescuing modified cancer cells from targeted therapy function. We coupled this selection marker with cytosine deaminase, a suicide gene that locally activates an otherwise inert prodrug. We found that in coupling an inducible fitness benefit with a shared fitness cost, we could effectively reprogram cancer cell populations, even at low levels of genetic modification. In vivo, these genetic circuits durably eliminated tumors that otherwise develop resistance under standard-of-care monotherapy (Figure 2b).

Together, our tumor-directed vectors and evolutionary-guided genetic circuits demonstrate the potential to reprogram tumors with more therapeutic opportunity to pave the way for durable remission.

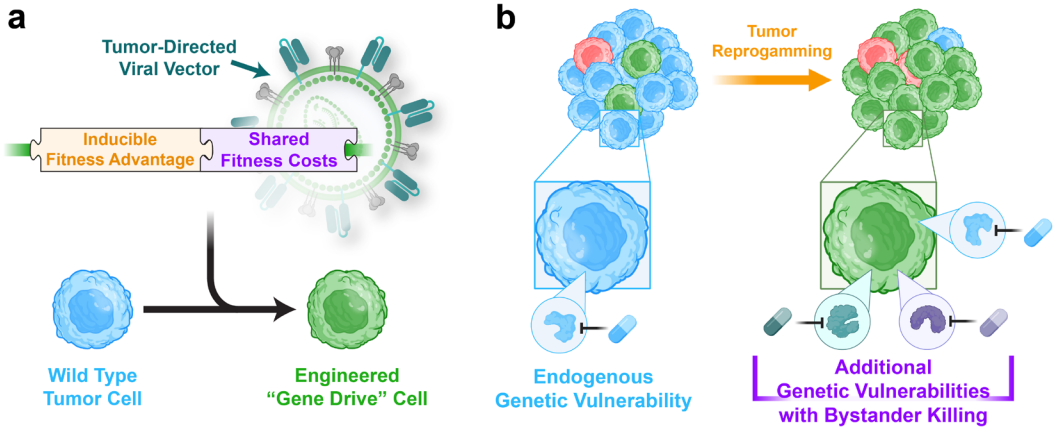

Figure 1: (a) Schematic of tumor-directed lentiviral vector and evolutionary-guided genetic circuit. (b) Schematic of tumor reprogramming to introduce genetic vulnerabilities.

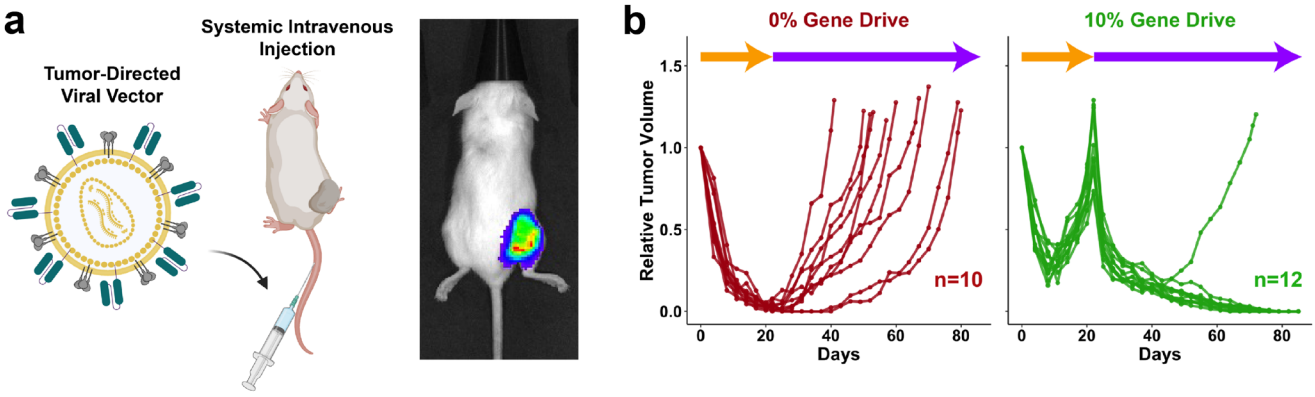

Figure 2: (a) Luciferase imaging demonstrating tumor-selective lentiviral transduction in vivo. (b) Tumor dynamics for mice without (left, red) and with (right, green) low-level engineered "gene drive" populations.

1721 DEC205-Targeting mRNA Cancer Vaccines for Enhanced Antigen Presentation in Dendritic and Tumor Cells

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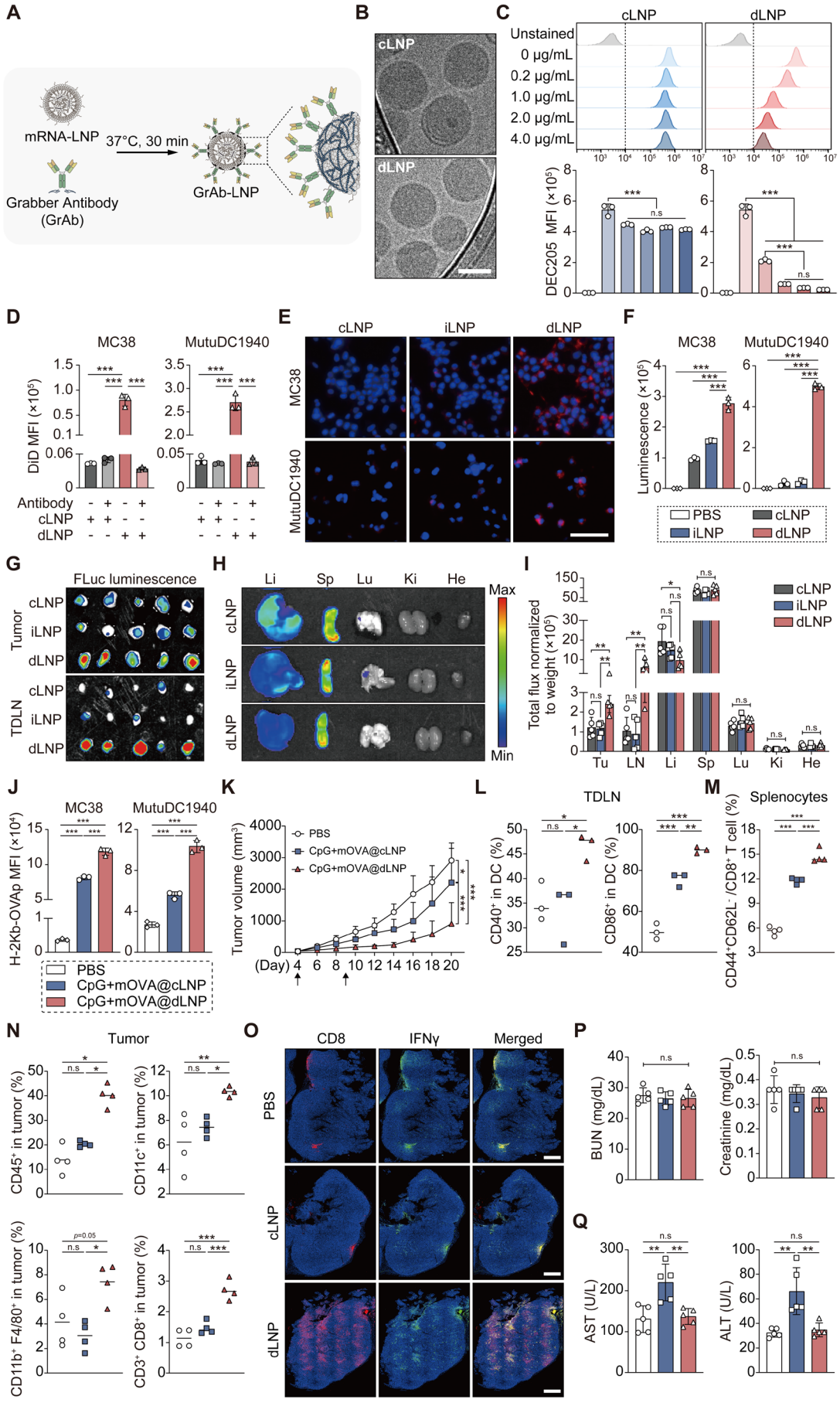
Introduction: Genetic alterations in cancer produce neoantigens that can elicit robust anticancer immune responses when effectively presented to T cells. However, the immunosuppressive tumor microenvironment often hampers antigen presentation, contributing to immune evasion. Although lipid nanoparticle (LNP)-based mRNA cancer vaccines have shown promise in delivering neoantigen-encoded mRNA, their tendency to accumulate in the liver diminishes tumor-specific antigen presentation and antitumor efficacy. To overcome this limitation, the current study employs a novel "Grab Antibody" (GrAb) approach, where apolipoprotein A1 is fused to the Fc domain of a DEC-205 antibody (NLDC145). DEC-205, known to be highly expressed on both dendritic cells (DCs) and various cancers, facilitates receptor-mediated delivery of mRNA to these crucial cell populations. By simply mixing GrAb and LNPs, the apolipoprotein moiety anchors the antibody on the LNP surface, enabling dual targeting while circumventing conventional chemical conjugation pitfalls.

Results: DEC-205-functionalized LNPs (dLNPs) retained a spherical morphology similar to control LNPs (cLNPs) (Fig. 1B). In DEC-205-expressing MutuDC1940 DCs, dLNPs prompted receptor-mediated endocytosis, evidenced by a dose-dependent reduction in surface DEC-205 (Fig. 1C). Flow cytometry and confocal microscopy confirmed that dLNPs significantly enhanced mRNA (FLuc) uptake and translation in both DCs and MC38 colorectal cancer cells, effects abrogated by DEC-205 pre-blocking (Fig. 1D–F). *In vivo*, dLNPs administered to MC38 tumor-bearing mice exhibited stronger tumor and tumor-draining lymph node localization compared to cLNPs or isotype-control LNPs (iLNPs), while reducing liver accumulation (Fig. 1G–I). Using CpG plus ovalbumin (OVA) mRNA as a model vaccine, dLNP-treated cells displayed increased MHC class I presentation of OVA peptides (Fig. 1J), leading to robust antitumor effects in MC38 tumor-bearing mice (Fig. 1K). Immune profiling revealed elevated DC maturation, effector memory $CD8^+$ T cell expansion, and higher infiltration of DCs, macrophages, and cytotoxic T cells in tumors (Fig. 1L–N). Notably, IFNγ production was also enhanced, indicating a potent T cell response (Fig. 1O). Although cLNPs induced liver toxicity (Fig. 1Q), dLNPs demonstrated no significant toxicity, as confirmed by normal blood chemistry (Fig. 1P). These findings highlight the potential of DEC-205-targeting GrAb LNPs for dual delivery of mRNA to DCs and tumor cells, resulting in improved antigen presentation, enhanced immune activation, and reduced off-target effects.


A
mRNA-LNP
37°C, 30 min
Grabber Antibody (GrAb)
GrAb-LNP
B
cLNP
dLNP
C
cLNP
dLNP
Unstained
0 μg/mL
0.2 μg/mL
1.0 μg/mL
2.0 μg/mL
4.0 μg/mL
DEC205 MFI (×10⁵)
n.s
D
MC38
MutuDC1940
DiD MFI (×10⁵)
Antibody
cLNP
dLNP
E
cLNP
iLNP
dLNP
MC38
MutuDC1940
F
MC38
MutuDC1940
Luminescence (×10⁵)
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cLNP
iLNP
dLNP
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FLuc luminescence
Tumor
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Ki
He
J
MC38
MutuDC1940
H-2Kb-OVAp MFI (×10⁴)
PBS
CpG+mOVA@cLNP
CpG+mOVA@dLNP
K
Tumor volume (mm³)
PBS
CpG+mOVA@cLNP
CpG+mOVA@dLNP
(Day) 4 6 8 10 12 14 16 18 20
L
TDLN
CD40⁺ in DC (%)
CD86⁺ in DC (%)
M
Splenocytes
CD44⁺CD62L⁻/CD8⁺ T cell (%)
N
Tumor
CD45⁺ in tumor (%)
CD11c⁺ in tumor (%)
CD11b⁺ F4/80⁺ in tumor (%)
CD3⁺ CD8⁺ in tumor (%)
p=0.05
O
CD8
IFNγ
Merged
PBS
cLNP
dLNP
P
BUN (mg/dL)
Creatinine (mg/dL)
Q
AST (U/L)
ALT (U/L)

1722 Development of a Novel Process which Combines Lentiviral Transduction and Base Editing for the Production of Highly Effective and Potent TCR T Cell therapy Products

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Introduction: T-cell receptor (TCR) therapy is a promising immunotherapy approach with demonstrated efficacy and durability in the treatment of solid tumors. Emerging base-editing technology exploits CRISPR mechanisms with adenine/cytidine deaminase enzyme to introduce single nucleotide changes at specific chromosomal sites in the genome without creating detrimental double-stranded DNA breaks. We have leveraged this novel targeted base-editing technology to perform multiplexed gene knockouts in human T cells to improve safety and effector function of a TCR product.

Methods: We have successfully developed a novel process which combines lentiviral transduction for TCR expression, and base editing technology for ablating expression of up to 3 genes, including endogenous TCRs, in primary T cells. This process can be translated into a large-scale GMP compliant process. The electroporation process was optimized to base edit up to 1E+09 cells effectively to ablate 3 different genes using gene-specific sgRNA's and a base-editor encoding-mRNA. The base editing process was optimized with two different electroporation systems (Maxcyte GTx and Prodigy ELPO) separately. Different cell densities, buffer systems and different sgRNA's and mRNA concentrations and time intervals were tested for the optimum editing efficiencies during electroporation for both Maxcyte GTx and Prodigy ELPO. The gene edited TCR-T was produced using a 13-day process. The developed small-scale model was successfully scaled up to a large-scale production process using the Miltenyi Prodigy platform for transduction, cell growth & expansion while using Maxcyte GTx. This TCR-T cell process, developed using GMP compatible reagents and equipment, is ready for manufacturing a cellular product for clinical applications.

Results: The 13-day TCR-T production process resulted in approximately ~20-30-fold expansion in small scale studies with greater than 90% viability during harvesting. Multiplexed base editing quantification assays showed >90% knock out of 3 genes using either Prodigy or Maxcyte instruments for electroporation. The TCR transduction efficiency was found to be ~32-53%. This small-scale process model was successfully scaled-up to produce a minimum of ~1E+09 transduced and edited TCR-T cells using the Prodigy cell culture expansion and Maxcyte electroporation systems.

Conclusion: A novel scalable GMP-compliant process to produce TCR T cells was developed by combining lentiviral transduction and multiplexed ablation of 3 genes by base editing technology. New strategies to optimize the base editing protocols with two different GMP-compatible electroporation instruments were developed. To our knowledge this scalable and GMP - compatible protocol represents an innovative manufacturing process that combines lentiviral transduction and base-editing for generation of clinical-grade TCR T cell products. This pioneering approach will benefit the production of both TCR-T, and CART cell products thereby providing new opportunities for the treatment of cancer.

Results of 13-day process for the production of highly effective and potent TCR T cell therapy products which combines lentiviral transduction and base editing

Donor	% of Transduction efficiency	% of Cell Viability during harvest (Day 13)	Fold expansion	Electroporator used
Donor 1	43	90.3	24	Maxcyte
Donor 2	48	94	23	Maxcyte
Comparison of Maxcyte GTx and Prodigy ELPO: Cell viability and fold expansion during harvesting (13 day process)				
Donor 3	32	94.6	23.5	Maxcyte
	32	95.3	22	Prodigy

Results from the base editing quantification of 3 different gene knock outs using Sanger sequencing and analysis by BEAT program

Donor	% Editing of Target Gene 1	% Editing of Target gene 2 / site 1	% Editing of Target gene 2 / site 2	% Editing of Target gene 3	Electroporator used
Donor 1	98	98	98	94	Maxcyte
Donor 2	99	98	98	99	Maxcyte
Comparison of Maxcyte GTx and Prodigy ELPO: % Gene editing					
Donor 3	94	99	99	94	Maxcyte
	90	99	99	98	Prodigy

1723 COMET, a novel TCR-enabled architecture, combines the sensitivity and intracellular targetability of a TCR with the ease of engineering of a CAR

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Chimeric antigen receptor (CAR) T cells have proven to be effective cancer therapies. However, it is widely accepted that T cell receptors (TCRs) are superior in reacting to antigens with lower surface density compared to CARs. To address this, we developed a novel receptor architecture called COMET (constitutively operational multiplexable endogenous T-cell receptor). COMET combines antibody-based (surface or intracellular) antigen targeting with the signaling proficiency and inherent sensitivity of native T-cell receptors. COMET consists of two polypeptides (organized N to C-term): the first is a CH3-derived heterodimerization domain fused to full-length CD3ε, and the second is an antigen targeting modality linked to the counterpart CH3-derived heterodimerization domain followed by a CD4-derived hinge and transmembrane domain. This design enables integration and efficient redirection of the TCR complex to antigen targets of interest.

Here, we demonstrate that COMET-T cells outperformed CAR-T cells in vitro and in vivo across a variety of target antigens and solid tumor models. COMET-T cells exhibited lower tonic signaling, were less differentiated, and had increased antigen-driven IL-2 production and cytotoxicity. They also displayed higher sensitivity to low-density antigens compared to CAR-T cells. Transcriptomic analysis indicated that COMET-T cells show hallmarks of TCR activation, suggesting improved fidelity to native T cell responses over that of CAR-T cells. Additionally, we show

that COMET-T cells armed with peptide-in-groove (PiG) scFvs recognizing a peptide-HLA complex were functional, enabling recognition of intracellular targets with in vitro sensitivity to low-density antigen approaching that of control TCR counterparts. Importantly, in a xenograft model, PiG COMETs achieved greater anti-tumor activity and clearance at multiple doses compared to TCR and CAR counterparts.

In conclusion, our data demonstrate that the COMET architecture enables COMET-T cells to target either surface or intracellular antigens with significantly enhanced in vitro and in vivo potency compared to 2nd Generation CAR-T cells, which may lead to more successful solid-tumor immunotherapies in the clinic.

1724 DLL3-targeting CAR co-deletion of PD1 results in enhanced T cell cytotoxicity and persistence in a pre-clinical model for small cell lung cancer.

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Purpose: Small cell lung cancer (SCLC) is an aggressive and high-grade neuroendocrine disease with few treatment options. Delta-like ligand 3 (DLL3) is highly expressed on SCLC and several other types of neuroendocrine cancers with minimal or absent expression in normal adult tissues, making it a promising CAR-T target for SCLC and other solid tumor indications.

Methods: A VHH antibody for human DLL3 was selected and generated from a humanized Camelid synthetic library. Subsequently, it was made into a second-generation CAR format and the one with additional PD1 intracellular retention (a non-gene editing technique to remove membrane proteins), both of which were compared for *in vitro* activities and an *in vivo* subcutaneous SHP77 tumor animal model for target-dependent T cell cytotoxicity and persistence.

Results: The CAR with PD1 intracellular retention demonstrated a much better long-term cytotoxicity against SHP77 tumor cells than the unmodified CAR *in vitro* by repeatedly adding two more rounds of the tumor cells in an Incucyte's real-time killing assay at an E:T ratio of 1:2. To further evaluate the activities of both DLL3 CAR T cells *in vivo*, $5x10^6$/mouse of SHP77 cells were implanted subcutaneously in nonobese diabetic SCIDg (NSG) mice and allowed to engraft for 7 days, reaching a mean size of approximately 40-60mm^3 before treatment with a single tail vein injection of $2x10^6$ CAR+ T cells/mouse. For this well-established *in vivo* solid tumor model, we found that PD1-deleted CAR-T group started to show the tumor growth slowing down D13, soon reaching the peak volume D20 at a mean size of 1500mm^3, and then steadily declining close to the baseline D30. But the unmodified CAR-T group was observed to have a much delayed and gradual decline of the tumor volume starting D20 instead at a mean size of 2500mm^3, with shrinkage down to a mean tumor volume of 600mm^3 D30 and further down to the 300mm^3 D34. In addition, both groups showed the CAR-T cell expansion during the tumor shrinkage and no signs of GVHD in the study.

Conclusions: Although both CAR-T groups exhibited good tumor suppression capabilities, PD1-deleted DLL3 CAR-T showed a quicker and stronger response in this adoptive T cell therapy against solid tumors in a pre-clinical animal model. The findings of improved preclinical efficacy and persistence warrant further evaluation of PD1-deleted DLL3 CAR-T cells as a potential clinical candidate for the treatment of SCLC.

1725 A Novel Approach To Preclinical Safety Assessment Of TCRs, Pairing Quantitative Immunopeptidomics With Cell-Based Functional Assays, Identified a Development-Limiting Risk Of Cardiotoxicity for a PRAME TCR

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Background: T cell receptor (TCR)-engineered T cells (TCR-T) are a promising modality for the treatment of solid tumors. However, cross-reactivity of TCRs to other antigens has the potential to drive off-target (OT) reactivity to normal tissues and must be evaluated preclinically, despite the absence of *in vivo* models. Given this challenge, holistic approaches integrating computational modeling with empirical *in vitro* data are needed to comprehensively assess the potential risks of OT reactivity. Here, we present data showcasing an innovative approach combining mass spectrometry (MS)-enabled quantitative immunopeptidomics, to determine copy numbers of peptides presented on the cell surface, with cell-based functional assays that identified a development-limiting risk of cardiotoxicity for a TCR targeting an HLA-A*02:01-restricted PRAME $_{(425\text{-}433)}$ epitope.

Methods: A bioinformatic analysis of the human peptidome was used in conjunction with an X-scan assay to identify potential OTs. TCR sensitivity to these OTs was determined using a T2 peptide-pulse assay. *In vitro* cell models that either endogenously expressed or were engineered to overexpress the OT peptides were leveraged to determine OT reactivity. Quantitative immunopeptidomics was performed on these cells and primary tissues samples to evaluate OT peptide copy numbers on the cell surface and compare it to the levels of the OT peptides observed in healthy and diseased tissues.

Results: 119 cross-reactive peptides were predicted as potential off-targets via the X-scan assay. 4 out of 191 predicted peptides were confirmed as OTs using the T2 peptide-pulse assay. 3 of these potential OTs were derisked since the peptides were not detected in essential normal tissues by MS. The remaining OT, the LRP1 peptide $_{(408\text{-}416)}$ (*LRP1*), was therefore prioritized for further de-risking. Initial *in vitro* functional assays using primary normal cells and tumor cell lines endogenously expressing *LRP1* (<250 copies/cell) did not induce TCR-T cell reactivity. However, overexpression of *LRP1* in HLA-A*02:01^{+} tumor cells at supraphysiological levels (>12,000 copies/cell) elicited robust TCR-T cell reactivity as measured by IFNγ release and cytotoxicity. By pairing the functional and quantitative immunopeptidomic readouts of the T2 peptide-pulse assay, a threshold of reactivity of ~1,000 copies/cell of *LRP1* was determined. Immunopeptidomic analysis of essential normal tissues predicted to express LRP1 quantified copy numbers ranging from 200-7,000 copies/cell, which was higher than observed in cell lines and normal cells initially tested (<250 copies/cell). Notably, multiple samples derived from aorta and diseased coronary arteries presented *LRP1* at copy numbers exceeding the 1,000 copies/cell threshold of reactivity, thus uncovering a risk of cardiotoxicity in patients.

Conclusions: By pairing *in vitro* co-culture data with quantitative immunopeptidomics, we elucidated the threshold of reactivity to *LRP1* peptide for a PRAME TCR. Comparing the threshold of reactivity with the observed peptide copy number in normal and diseased tissues identified a potential high risk of cardiotoxicity presented by this OT reactivity. Standard *in vitro* models did not reveal this risk because they did not present physiologically relevant *LRP1* copy numbers. These data demonstrate the importance of leveraging peptide copy number analysis in normal tissues in conjunction with *in vitro* assays when evaluating the safety of TCRs.

1726 CARD11-PIK3R3 Potency Enhanced p53R175H-HLA-A*02:01 TCR T Cell Therapy: Overcoming Barriers in Solid Tumor Treatment

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Engineered T Cell Receptor (TCR) T cell therapies hold significant promise for targeting tumor specific antigens[1], with the first TCR T cell therapy receiving FDA approval in 2024[2]. TP53 is the most frequently mutated tumor suppressor gene in cancer. The hotspot missense mutation p53R175H, found in approximately 5% of all cancers[3], stabilizes the neoepitope's presentation on HLA-A*02:01, resulting in robust and selective presentation of the mutant peptide[4]. This selective tumor expression, combined with the role of p53R175H as an oncogenic driver and key dependency in tumor survival, makes it a highly attractive target for TCR T cell therapy development[5,6]. While ongoing efforts to develop therapies targeting p53 mutant neoepitopes are encouraging[7,8], several challenges remain that limit the broader clinical potential and impact of TCR therapies. TCR therapies commonly require high T cell doses to achieve adequate engraftment and proliferation, and often experience progressive T cell dysfunction and exhaustion, limited persistence, and impaired T cell memory formation[9]. Strategies to address some of these limitations, such as the co-administration of high-dose IL-2 to enhance T cell engraftment and expansion[10], have improved clinical efficacy but add significant cost, complexity, and toxicity[11]. More recently, a CARD11-PIK3R3 fusion protein (C11P3) was discovered and shown to enhance T cell cytokine secretion and proliferation, and dramatically amplify the *in vivo* potency of engineered CAR and TCR T cells[12]. Importantly, C11P3 was demonstrated to substantially reduce the T cell dosing required for anti-tumor efficacy in various mouse tumor models, including in syngeneic systems where lymphodepletion was not required for anti-tumor activity. Expression of C11P3 alongside engineered TCRs may solve for many of the mechanistic, manufacturing, and clinical limitations currently confronting the development of TCR T cell therapies.

Here, we present the preclinical development of a novel HLA-A*02:01 restricted p53R175H TCR T cell therapy that co-expresses the C11P3 fusion protein. We describe three patient-derived p53R175H TCRs[13] that demonstrate high specificity, robust antigen-dependent activation of both CD4+ and CD8+ T cells, and superior *in vivo* anti-tumor efficacy compared to a clinically tested HLA-A*02:01 restricted p53R175H TCR[14] (Figure 1A). Co-expression of C11P3 markedly enhanced *in vitro* antigen-dependent T cell cytokine secretion and cytotoxicity. Notably, antigen-driven IL-2 secretion increased by as much as 260-fold over TCR T cells alone (Figure 1B). *In vivo* studies using a tumor model endogenously expressing p53R175H demonstrated potent efficacy of C11P3 enhanced TCRs (Figure 1C). Specifically, TCR T cells alone achieved tumor clearance in 2/6 mice, while TCR T cells co-expressing C11P3 achieved full tumor clearance in 6/6 mice without requiring IL-2 co-administration (Figure 1C). Importantly, co-expression of C11P3 did not increase the off-target risk profile of the TCR T cells, preserving specificity and safety. These findings highlight the potential of C11P3 to address key limitations in TCR T cell therapies by enhancing cytokine secretion, cytotoxicity, and *in vivo* efficacy. Furthermore, this study demonstrates the promise of a C11P3-enhanced p53R175H TCR therapy, offering a novel approach with the potential to treat a wide range of solid tumors harboring the p53R175H mutation.

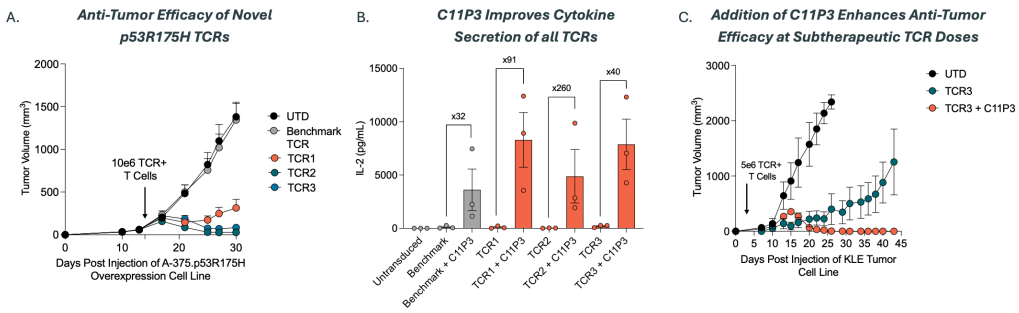

Figure 1. CARD11-PIK3R3 Enhances the Potency of p53R175H-HLA-A*02:01 TCRs

Tumor growth curves of NCG (NOD-Prkdcem26Cd52Il2rgem26Cd22/NjuCrl) mice bearing A-375 tumor xenografts that overexpress p53R175H. Animals were treated with 10e6 TCR+ T cells 14 days after tumor inoculation and tumor volumes were measured twice weekly using calipers. Untransduced (UTD) cells were used as a negative control (A). IL-2 cytokine secretion levels of TCRs with or without co-expression of CARD11-PIK3R3 (C11P3), measured 24 hours post co-culture with a p53R175H positive tumor cell line (B). Tumor growth curves of NCG mice bearing KLE tumor xenografts that endogenously express p53R175H. Animals were treated with 5e6 TCR+ T cells 14 days post tumor inoculation and tumor volumes were measured twice weekly via calipers (C). Error bars depict mean ± SEM.

[1] Malekzadeh et al., "Neoantigen Screening Identifies Broad TP53 Mutant Immunogenicity in Patients with Epithelial Cancers."
[2] Hong et al., "Autologous T Cell Therapy for MAGE-A4+ Solid Cancers in HLA-A*02+ Patients."
[3] Pearlman et al., "Targeting Public Neoantigens for Cancer Immunotherapy."
[4] Chiang et al., "The Function of the Mutant P53-R175H in Cancer."
[5] Lo et al., "Immunologic Recognition of a Shared P53 Mutated Neoantigen in a Patient with Metastatic Colorectal Cancer."
[6] Cappell et al., "Adoptive Transfer of T Cells Genetically Engineered to Express T-Cell Receptors Targeting Neoantigens Arising from P53 and Ras Hotspot Mutations in Hematologic Malignancies."
[7] Kim et al., "Adoptive Cellular Therapy with Autologous Tumor-Infiltrating Lymphocytes and T-Cell Receptor-Engineered T Cells Targeting Common P53 Neoantigens in Human Solid Tumors."
[8] "Non-Viral Engineered T Cell Therapy Specific for the Hotspot Mutation P53 R175H That Integrates Signal 1 (TCR), Signal 2 (Co-Stimulation) and Signal 3 (Cytokine) and Co-Opts Fas╬Dependent Apoptosis to Achieve a Coordinated Antitumor CD4/8 T Cell Response."
[9] Baulu et al., "TCR-Engineered T Cell Therapy in Solid Tumors."
[10] Rosenberg et al., "Adoptive Cell Transfer."
[11] Cotran et al., "Endothelial Activation during Interleukin 2 Immunotherapy. A Possible Mechanism for the Vascular Leak Syndrome."
[12] Garcia et al., "Naturally Occurring T Cell Mutations Enhance Engineered T Cell Therapies."
[13] Kim et al., T cell receptors targeting y220c or r175h mutation in p53.
[14] Kim et al., "Adoptive Cellular Therapy with Autologous Tumor-Infiltrating Lymphocytes and T-Cell Receptor-Engineered T Cells Targeting Common P53 Neoantigens in Human Solid Tumors."

1727 Novel Innate Immune Receptor-Based Chimeric Antigen Receptors For In Vivo Programming Of Myeloid Cells Show Potent Anti-Tumor Activity In Preclinical Solid Tumor Models

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Background: Chimeric antigen receptor (CAR) T cell therapy faces significant challenges in solid tumors due to the immunosuppressive tumor microenvironment (TME). Given their tumor-infiltrating capability, phagocytic activity, and ability to promote adaptive immune responses, CAR-engineered myeloid cells represent a promising therapeutic approach. Here we introduce a novel class of CARs designed for programming myeloid cells in vivo. These CARs comprise antibody binders against tumor antigens fused with truncated innate immune receptors CD89 or NKp44. Like the natural receptors, activation of CD89 and NKp44-based CARs require association with their cognate signal adapters FcRγ or DAP12, which are only expressed in myeloid/NK cells. Such segregation of antigen binding from cellular activation ensures immune cell-restricted CAR activity, minimizing off-target effects during in vivo administration.

Methods: The CAR constructs comprise a single-chain variable fragments (scFv) against HER2 or gp75, fused to truncated CD89 or NKp44. CAR mRNA was encapsulated in lipid nanoparticles (LNPs) to achieve in vivo programming. Coding sequences and 3' UTR of the mRNA were optimized for prolonged expression. In vitro, CAR expression and function were evaluated in a THP1 reporter cell line and primary monocytes. Anti-tumor efficacy of the CARs was assessed in a human HER2 (hHER2) transgenic mouse model and a syngeneic B16/F10 melanoma model, in which CAR mRNA/LNPs were administered intravenously.

Results: The CD89 or NKp44-based CARs were highly expressed in primary CD14+ monocytes for more than 7 days post electroporation. Transfection of human PBMCs with mRNA/LNP showed preferential CAR expression in myeloid cells. NKp44-based CAR specifically associated with DAP12, whereas the CD89-based CAR associated with and FcRγ. Upon antigen binding, CAR-expressing monocytes expressed genes associated with a proinflammatory phenotype, and NKp44-based CAR induced more durable pro-inflammatory response than CD89. In the hHER2 transgenic mouse model bearing hHER2-MC38 tumors, intravenous administration of mRNA/LNPs encoding HER2-targeting CARs showed potent anti-tumor activity and prolonged survival. Likewise, gp75-targeting CAR mRNA/LNP inhibited tumor growth and increased $CD8^+$ T cell infiltration into tumors demonstrating that CAR-programmed myeloid cells remodel the TME towards an immunostimulatory state.

Conclusions: In vivo programmed CAR myeloid cells elicit potent anti-tumor activity and promote an increase in T cells in tumors in syngeneic mouse models. The functional dependence of the CARs on endogenous signaling adaptors, combined with the preferential mRNA/LNP uptake by myeloid cells, ensures immune cell-specific activity and provides an enhanced safety profile for in vivo myeloid cell engineering.

1728 ACTallo: Developing an Allogeneic T Cell Therapy Platform Using Engineered γδ T Cells

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In the last decade, cell therapies have shown promising success in the treatment of hematological malignancies and tremendous progress has been made to expand their utility to more difficult to treat solid tumors. In addition to chimeric antigen receptors (CARs), T cell receptor (TCR)-based immunotherapies hold a great potential for the treatment of solid tumors by targeting extra- and intracellular tumor-associated peptide antigens on the cell surface with high sensitivity. Autologous TCR-T therapies like ACTengine IMA203 targeting PRAME have demonstrated deep and durable objective responses in heavily pretreated patients with solid tumors in clinical trials.

We have developed an allogeneic, off-the-shelf adoptive T cell therapy platform, ACTallo, with the intent to lower costs, expedite the supply of cell therapy products to patients and at the same time incorporate more extensive cell engineering to enhance functionality and durability in different indications. ACTallo is based on *ex vivo* feeder cell-free expansion and multiplexed genetic engineering of healthy donor-derived γ9δ2 T cells, which by themselves possess remarkable innate anti-tumor functionality. This T cell subset is not alloreactive, thus avoiding the risk of graft-versus-host activity. The known degree of donor-to-donor variability of γδ T cell expandability and functionality is addressed by a donor selection program, including extensive pre-screening, thereby ensuring optimal quality of starting material and process performance.
Efficient multiplex targeted gene engineering using CRISPR/Cas enables us to introduce 1) targeted cytotoxic capabilities by expressing a highly functional TCR or CAR, 2) cloaking technology to avoid fast graft rejection and 3) armoring to counteract the suppressive tumor microenvironment and supporting γδ T cell expansion and persistence.

Using the ACTallo platform, we have developed a multi-edited product candidate targeting a proprietary peptide target. The product candidate shows specific cytotoxic activity against target-positive tumor cell lines *in vitro*; incorporation of CD8 in the product design enables recognition of low target expressing lines. A single dose of cell product candidate was sufficient to induce complete tumor remission in a cell line derived xenograft model in immunodeficient mice with γδ T cells persisting for several weeks. *In vitro* coculture experiments have shown reduced rejection responses to the engineered product by cytotoxic HLA-mismatched T cells and natural killer cells, validating the functionality of the cloaking approach.

This data indicates that the ACTallo platform could be a promising pathway to enhance future TCR-T and CAR-T approaches for the benefit of cancer patients.

1729 Engineering Inducible Signal3 Chimeric Cytokine and Antigen Receptors for T Cell Therapy in Solid Tumors

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Clinical efficacy of CAR-T cells in patients with solid tumors has been lower than expected, mainly due to the lack of expansion and survival of CAR-T cells. Providing the signal 3 to CAR-T cells has been proposed as a strategy to overcome this limitation. Most of the CAR constructs studied in the clinic include only the first and second signals, while strategies to provide signal 3 are typically focused on constitutive interleukin signaling, which can generate toxicities. Here, we hypothesized that inducing signal 3 in CAR-T cells upon antigen recognition through cytokine receptor activation motifs from the IL-7Rα will increase T cell activation and persistence.

First, we designed chimeric cytokine receptors (CCR) by coupling a scFv to the IL-7Rα intracellular domain, including either the IL-7Rα or CD8α transmembrane domain. Signaling was compared to a constitutively signaling IL-7Rα chain able to phosphorylate STAT5 autonomously, due to the homodimerization via a disulfide bridge within the transmembrane domain. All IL-7-based CCRs were efficiently expressed, and further characterized in single transduction or co-transduction with a first-generation CAR. In single transduction, CCRs were unable to induce STAT5 signaling after antigen recognition. In co-transduction, the CCRs did not provide a cytotoxic nor a proliferative advantage.

Next, we explored the addition of specific IL-7Rα signaling motifs within ICOS or CD28-based second-generation CAR constructs. We generated a panel of constructs including different configurations of the box 1, interbox and box 2 motifs that lead to JAK/STAT5 signaling. Once expressed in T cells, we observed differences in terms of expression and cytotoxicity *in vitro* among the different constructs. We could not detect a clear upregulation in phosphorylation of STAT5 after antigen recognition, probably due to background STAT5 signaling from the CD3ζ domain. Using an *in vitro* model of continuous antigen exposure, some IL-7R-based CAR-T cells revealed enhanced cytotoxicity compared to the second-generation CAR-T cells. *In vivo*, IL-7-based HER2-CAR-T cells behaved similarly to the control group, showing no clear advantage in antitumor activity.

We next hypothesized that the impaired STAT5 signaling was due to insufficient dimerization with the endogenous common gamma chain, known as CD132. To test this, we evaluated CD132 bioavailability in T lymphocytes. Our observations indicate that the surface lifetime of CD132 is relatively short, as it undergoes constant endocytosis and recycling. Despite this, CD132 mRNA was highly expressed and the protein could heterodimerize with other cytokine receptors, suggesting that the bioavailability of this molecule is not the main hurdle behind the lack of CCR signaling.

As previously published, diverse IL-7R conformations may signal constitutively once expressed in T cells. However, we have demonstrated that, if these signaling domains are expressed in an inducible conformation, they lack STAT5 signaling. Further analysis of CCR 3D-structure and molecule dimerization is being performed to decipher whether they can form homo- or heterodimers to activate downstream signaling. We believe that these results could shed light on the dynamics of cytokine signaling and allow the design of an inducible CCR.

1730 A Novel T Cell Receptor Targeting Cancer Testis Antigen NY-ESO-1

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BACKGROUND: New York esophageal squamous cell carcinoma 1 (NY-ESO-1, encoded by the CTAG1A gene) is a well-characterized cancer testis antigen (CTA) with documented potential as a target for immunotherapy [1, 2]. We hereby report the isolation of a novel HLA-A*02:01-restricted T cell receptor (TCR) that recognizes NY-ESO-1, and the functional characterization of TCR-transgenic T cells for their use in adoptive immunotherapy of cancer. In addition, we provide CTAG1A RNA expression data across cancer types, using a CLIA-certified assay that may serve as a patient-selection tool.

METHODS: Tumor-infiltrating lymphocytes (TIL) were expanded as previously described [3] from a sarcoma patient. NY-ESO-1-reactive TIL clones were identified and FACS-sorted using an HLA-A*02:01 dextramer

(epitope: SLLMWITQV). TCR rearrangements (VDJ) were identified by single cell RNA sequencing and the resulting sequences were cloned in a gamma-retroviral vector (MSGV1) for expression in peripheral blood T cells. TCR expression and dextramer binding were assessed via flow cytometry. Co-cultures were performed for IFN-gamma ELISA and RTCA experiments to evaluate reactivity and cytotoxic efficacy, respectively, of TCR-transduced T cells against myeloma, melanoma, and sarcoma cell lines. Finally, we tested the *in vivo* antitumor effect of TCR-transgenic T cells using a xenograft of A375 melanoma cells in NSG mice. For the analysis of NY-ESO-1 expression in clinical specimens, we compiled RNA expression data from 809 tumor samples analyzed at Moffitt Cancer Center using the CLIA-certified RNA Salah Targeted Expression Panel (RNA STEP) [4]. Tumors displaying expression values that were 4-fold ($\log2 \geq 2$) greater than the normal tissue controls were considered positive for CTAG1A/NY-ESO-1.

RESULTS: We identified a T cell clone with specificity for a 9-mer NY-ESO-1 peptide within a TIL culture expanded from synovial sarcoma. VDJ sequencing of these TIL yielded a highly enriched TCR clonotype (>50%), and a retroviral vector was generated to express the TCR variable regions fused to murine TCR constant regions. Upon retroviral transduction, both CD4+ and CD8+ T cells expressed the TCR and bound NY-ESO-1 dextramers. Using co-culture experiments, we demonstrated specific IFN-gamma secretion by transduced T cells against HLA-A2+/NY-ESO-1+ U266 (myeloma), A375 (melanoma), and STB024 (sarcoma) cell lines ($p<0.0001$). Cytotoxicity assays showed significant dose-dependent cytolysis of A375 cells by TCR-transduced T cells after 72-hours ($p<0.0001$). Utilizing an NSG mouse model, we found that injection of TCR-transduced T cells resulted in a significant delay in A375 melanoma xenograft growth, in comparison to untransduced T cells ($p=0.0003$), and untreated mice ($p<0.0001$). Using an institutional database of RNA expression data, we found that 9.9% of samples analyzed showed positive expression of CTAG1A/NY-ESO-1. Among these, 10.7% of lung tumor, 14.8% of adrenal tumor, and 27.3% of thyroid tumor samples expressed high levels of CTAG1A/NY-ESO-1 compared to normal tissues.

CONCLUSION: We generated a retroviral vector for expression of a novel TCR that recognizes NY-ESO-1. Functional validation of TCR-T cells generated using this retroviral vector indicate that the TCR is specific, and that the resulting transgenic lymphocytes can lyse tumor cells *in vitro* and *in vivo*. Altogether, our results support the future clinical translation of a novel NY-ESO-1-targeted TCR-T cell product.

1731 Multiplexed Engineering for Generation of HLA-Class I Restricted CD4 T Cells

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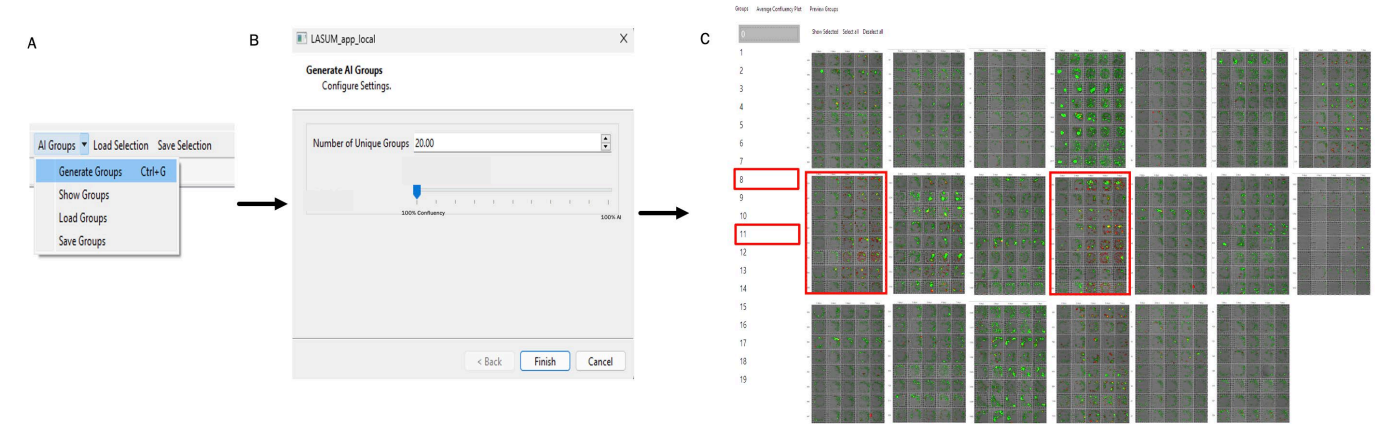
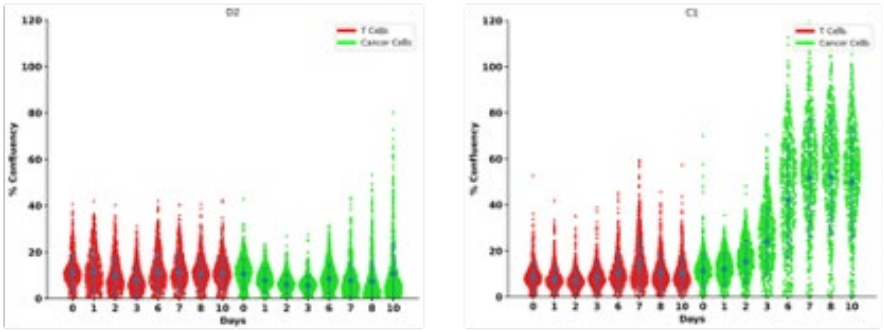
TCR-based T cell therapies have a remarkable potential for the treatment of solid tumors. The success of such therapies relies on the use of a TCR that can recognize tumor specific antigens presented on HLA-Class I. Although CD8 T cells were long thought to be the main effectors in this process through their direct killing of tumor cells, it has recently become evident that CD4 T cells play a crucial role. Thus, in order to harness the full potential of T cell therapies it is vital to generate CD4 T cells that can target tumor cells. HLA-Class I restricted TCRs can be expressed on CD4 T cells, but tumor antigens often have low affinity for TCRs and full activation of a T cell requires the CD8 co-receptor to stabilize the TCR-peptide-HLA complex. To this aim, we engineer HLA-Class I restricted CD4 T cells through CRISPR Cas9 mediated knockin of an HLA-Class I restricted TCR accompanied by a CD8 co-receptor. Through multiplex engineering approach that optimized for order of expression, promoter use and proteolytic sites, we engineer a single knockin construct with optimal expression of all four genes: TCRa, TCRb, CD8a and CD8b. To reduce cargo load and simplify our construct design, we also explore engineering a single chain CD8 co-receptor. We find that HLA-Class I restricted CD4 T cells engineered to express the CD8 co-receptor effectively recognize the respective tumor antigens with kinetics similar to CD8 T cells. The approach here shows the successful engineering of HLA-I restricted CD4 T cells using a single CRISPR Cas9 knockin construct. These findings improve T cell therapies in the quest to transform for the treatment of solid tumors.

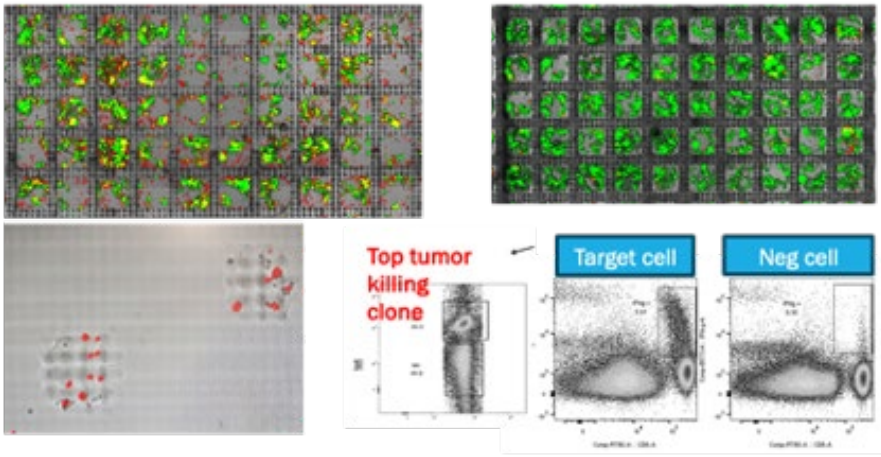
1732 AI guided tumor reactive cell isolation using immune cell tumor microcultures

Qi Zhao, Enrich Biosystems Inc., Branford, CT

Robust determination of single-cell kinetics, including killing rate, proliferation rate, and engaging time… are the basis for comparing and predicting in-vivo performances among different cell clones, especially in CAR-T development or TCR-T identification. To suppress single cell noise, different strategies to increase sample size are used on Enrich TroVo platform. We here use hydrogel-based microwell systems and deep-learning based cell behaviour movie grouping to analyze, rank and isolate optimal Tumor killing T cell clones for downstream studies i.e. Rapid expansion, single cell multiomics.







1733 Development of Novel TCR Based Cell Therapies Targeting Tumor Specific Mutation in AML.

Alun Carter[1], Egidio Cofano[1], Erik Martin[1], Jennifer Roy[1], Stephanie Stras[1], Shawn Radovic[1], Paola Sette[1], Amanda Dynoske[1], Andrew Bellesis[1], Kiera Regan[1], Autumn Toki[1], Joshua Dalton[2], Zhimei Du[1], **Robert Keefe[3]***
[1]Bluesphere Bio, Pittsburgh, PA, [2]Bluesphere Bio, Lovedale, Pennsylvania, [3]BluesphereBio, Pittsburgh, PA
**Corresponding Author*

Treatment of acute myeloid leukemia (AML) remains an unmet medical need due to the mortality arising from the complexity of the disease. Patient responses to current treatment regimens compound the challenge of finding effective AML treatments. One of the most common and clinically significant AML mutations occurs in the nucleophosmin 1 (NPM1) gene. The main objective of this program is to identify clinically viable therapeutic TCRs targeting mutated NPM1 (NPM1c+). Here we described the discovery, selection and characterization of therapeutic TCR candidates.

Anti-NPM1c+ TCRs were identified through tetramer-based screening, single cell sorting and NGS sequencing of potential candidates. In total, 15 TCRs were found to bind NPM1c+ target peptide. Jurkat reporter assays, as well as OCI-AML2 and OCI-AML3 cytotoxicity assays were used to further characterize TCR candidates. TCRs that showed activity at an effector-to-target (E:T) ratio of <1:1 in OCI-AML3 cytotoxicity assays were investigated further. Three candidates showed reproducible potency using multiple donors to generate TCR-Ts. Additional cytotoxicity assays utilizing cysteinylated target NPM1c+ peptides demonstrated the ability of TCRs to target post translationally modified peptides. We further examined TCR epitope specificity by performing alanine scanning on two NPM1c+ TCRs. Both TCRs showed an overlap in critical peptide residues and had very similar initial cytotoxicity profiles. However, each TCR recognized a distinct single amino acid target and had different tetramer binding characteristics. The initial cytotoxicity profiles for the two TCRs are similar. Nevertheless, additional studies indicated differences in binding characteristics using tetramer-based binding assays.

In summary, we demonstrate the selection and characterization of viable AML anti- NPM1c+ TCRs. While several TCRs demonstrated similar cytotoxicity profiles, there were clear differences in target peptide binding which are under investigation. The data demonstrates the development of TCR based therapies for addressing an unmet need in AML patients.

Summary of anti-NPM1c+ lead candidate TCR endogenous cytotoxicity assays.

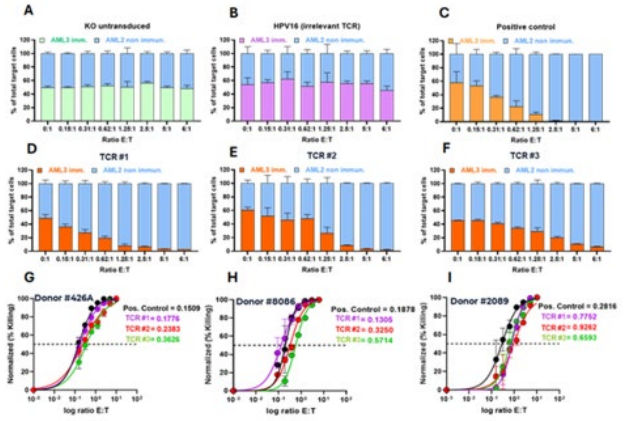


Figure summary. Bar graphs **A, B, and C** represent OCI-AML3 and OCI-AML2 cytotoxicity percentages at a 1:1 E:T ratio for the positive control, untransduced negative control, and HPV irrelevant TCR negative control, respectively. Bar graphs **D, E, and F** represent cytotoxicity against OCI-AML3 (immunogenic) and OCI-AML2 (non-immunogenic) at varying E:T ratios for anti-NPM1c+ TCR 1, 2, and 3 respectively. Summary of EC50 cytotoxicity for lead and positive control is summarized for Donor #426A **G**, #8086 **H** and # 2089 **I**, respectively. For control conditions green, pink, and yellow are used for immunogenic cells for the untransduced , irrelevant, and positive controls, respectively. The proportional killing of non-immunogenic (blue) and immunogenic (orange) cells is also shown. Graphs represent the percentage of killing (%) of target cells as determined by flow cytometry. Data points represent the mean average value for 3 technical replicates, and the error bars represent the standard deviation.

1734 Deep Learning Algorithm-Guided Optimization of a Natural Ligand-based CAR-T against CD70 in Acute Myeloid Leukemia

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Introduction: Acute myeloid leukemia (AML) has a 5-year survival rate under 30% and has a great clinical need for new therapies. CD70 is a promising AML target antigen due to its high expression on AML tumor cells and absence on healthy hematopoietic stem and progenitor cells (HSPCs). One of the most potent CD70-targeting strategies is a "natural ligand" (NL) binder derived from the extracellular domain of CD27, the receptor for CD70. Our group has shown that NL binder CAR-Ts display improved proliferation and tumor control compared to scFv-based CD70 binders (Kasap et al, *Biorxiv* 2024). However, there are currently no described strategies for the optimization of NL CAR-T binders, which may follow different "rules" compared to scFv-based CAR-Ts. Here, we utilize deep learning-based algorithms to address this gap, generating an NL variant CAR that outperforms the wildtype (WT) CD27-based CAR-T in preclinical AML models.

Methods: CD27-based variants were generated and evaluated using proteinMPNN and Rosetta based on the 7KX0 PDB structure. CAR constructs were delivered into primary T-cells using AAV and integrated at the *TRAC* locus via CRISPR knock-in. *In vitro* cytotoxicity was measured by luminescence after co-culturing CAR-Ts with luciferase-expressing target cell lines for 24 hours. Intracellular cytokine staining was performed on CAR-Ts following stimulation, brefeldin A treatment, fixation, and permeabilization. Ligand-receptor affinity was measured on an Octet RED384 biolayer interferometry instrument. *In vivo* evaluation of CAR-Ts was performed in NSG mice, with tumor burden measured using bioluminescence imaging and CAR-T expansion by flow cytometry.

Results: We first utilized deep learning and physics-based algorithms to model substitutions on the CD27 surface that would stabilize binding to CD70. We identified 8 single mutations and 4 combinations of mutations, with ΔΔG values ranging from +11.2 to -33.0 Rosetta energy units relative to the WT CD27-CD70 interaction (Fig. 1). We expressed these CD27-variant CARs in T-cells and evaluated their cytotoxicity against the AML cell lines MOLM-14 and NOMO-1. We observed that a subset of our CD27 variant CARs outperformed the WT CAR, particularly at

lower effector-to-target ratios, and that the best-performing variants expressed higher levels of TNF-α, IFN-γ, and IL-2 in a cytokine stimulation assay. We compared the best-performing CD27-variant CARs in a "stress test" *in vivo* model using the MOLM-14 cell line. We observed initial tumor control in the WT CD27-based CAR group, followed by tumor relapse and death. In comparison, several CD27-variant CARs had improved tumor control, with the best performing variant, named "N88A", showing complete tumor control in all surviving mice for the duration of the study (Fig. 2). We also observed improved peripheral blood expansion for this CAR compared to the WT CAR. Finally, we measured the binding affinity of the N88A CAR to CD70 using biolayer interferometry and measured a Kd value of 110 ± 35 nM, about a 3-fold improvement over the WT CD27 CAR, which has a Kd measured at 350 ± 110 nM.

Conclusion: We report here the development of a novel deep-learning based approach to develop improved natural ligand-based CAR binders, identifying a CAR variant with improved binding to CD70 and improved anti-tumor efficacy *in vitro* and *in vivo*. These results highlight this methodology's potential to improve natural ligand CAR-Ts in several tumor indications.

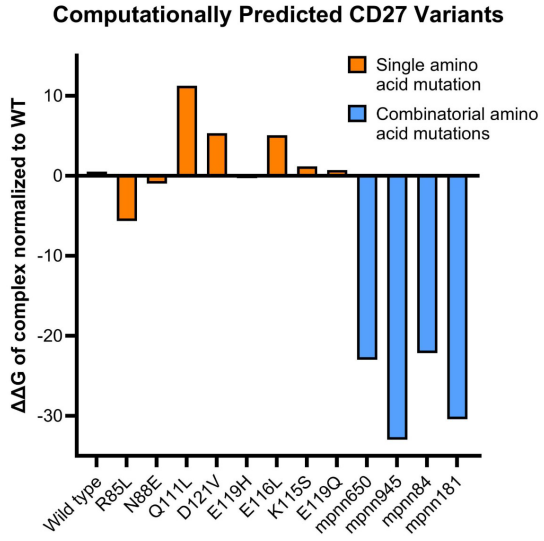

Figure 1: Predicted target binding stability for CD27-based variants, normalized to the WT CD27-CD70 interaction. Units given are arbitrary Rosetta energy units, with variants consisting of a single amino acid substitution colored in orange and variants with multiple amino acid substitutions colored in blue.

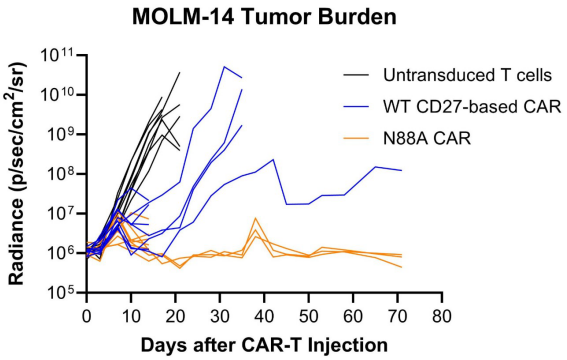

Figure 2: The N88A NL variant CAR-T outperforms the WT CD27-based CAR-T in a stress-test MOLM-14 AML murine xenograft model. Curves represent quantified bioluminescence imaging values for each mouse. Increased radiance indicates higher tumor burden.

1735 Super Immunodeficient NOG-EXL Mice Have Enhanced Patient-Derived Xenotransplantation of Acute Myeloid Leukemia: An Ideal Model to Test Novel Therapies

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Xenotransplantation of human acute myeloid leukemia (AML) cells into immunodeficient mice is an established model for preclinical development and increasing our understanding of translational AML biology. AML xenotransplantation has been well characterized in highly immunocompromised NSG mice, but with variable results, including overall poor engraftment or a prolonged time to engraft. Previously we have found that TP53 mutant AML and splicing factor mutant AML engraft poorly in NSG mice. Alternative mouse models that may improve engraftment efficacy include the NSG-SGM3 (SGM3) and NOG-EXL mice, which combine severe

immunodeficiency with transgenic expression of human myeloid stimulatory cytokines. However, we have found that the SGM3 strain is also prone to high variability in regard to time to engraft and level of engraftment, as well as spontaneous animal death.

To address these concerns, we collaborated with Taconic Biosciences to compare AML primary sample engraftment in the NSG, SGM3, and NOG-EXL mice. We first studied overall survival in SGM3 and NOG-EXL mice engrafted with four separate AMLs (n=5 per cohort). Consistent with previous results, 5/20 of SGM3 mice engrafted with AML became ill and required sacrifice approximately 5 weeks after injection. SGM3 health and survivability did not correlate with the level of AML engraftment, and over time additional SGM3 mice required sacrifice. In contrast, AML successfully engrafted in all NOG-EXL mice and only 1/20 NOG-EXL mice became ill during 12 weeks of observation.

We next studied relative engraftment in all three mouse strains of five TP53 mutant AML samples that had either previously not engrafted or engrafted poorly in NSG mice. We aspirated bone marrow on all mice five weeks after injection. Level of engraftment across strains varied from not detectable in NSG mice to over 80% in NOG-EXL and SGM3 mice. Mice were observed until symptoms of leukemia engraftment developed (42 to 77 days) at which time mice were sacrificed and analyzed for leukemic burden by measuring huCD45+huCD33+ cells in the bone marrow and spleen. Interestingly, bone marrow burden increased in NSG mice over time although total mouse leukemic burden in NSG mice was consistently lower than in the SGM3 and NOG-EXL mice. Comparing leukemic burden in NOG-EXL to SGM3 mice showed a modestly higher leukemic burden and lower variability in NOG-EXL mice at time of sacrifice. Human CD45+ and human CD33+ cell expression, as assessed by flow cytometry, were higher in SGM3 and NOG-EXL compared to NSG mice. We also found that splicing factor mutant AML engrafted poorly in NSG mice, with shorter time to engraftment and higher levels of engraftment in both NOG-EXL and SGM3 mice. Again, no early deaths were seen in the NOG-EXL strain but were observed in the SGM3 mice.

Overall, these results support the hypothesis that the NOG-EXL strain is an improved mouse model for use in AML xenotransplantation compared to NSG and NSG-SGM3, with enhanced levels of engraftment without spontaneous mortality. Currently, response to cytotoxic, venetoclax, and novel therapies in the NOG-EXL mice is ongoing.

1736 Asparaginase Coupled CAR-T Cells Overcome ASNS Mediated Resistance to CAR T Cell Therapy in Leukemia

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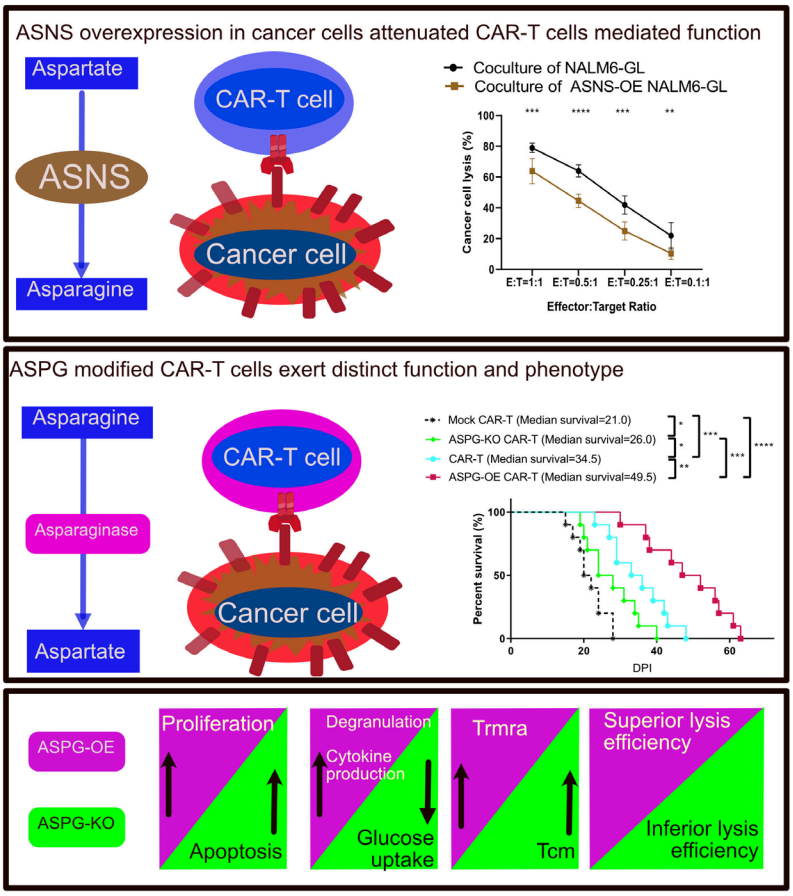
Asparagine is a conditionally essential amino acid that plays a crucial role in certain types of hematologic malignancies. In this study, we found that asparagine synthetase (ASNS) overexpression (OE) attenuated CAR-T cell-mediated cancer cell lysis efficiency in the co-culture of anti-CD19 CAR-T cells and NALM6 cancer cells. Asparaginase (ASPG) is a medication that breaks down circulating asparagine in the leukemia and lymphomas, thereby depriving cancer cells of asparagine and inhibiting cancer growth. We thus generated ASPG-modified engineered CAR-T cells for the killing against ASNS-overexpressing cancer cells. ASPG-OE CAR-T cells exhibited enhanced killing efficiency, while ASPG-KO CAR-T cells attenuated killing efficiency. Phenotypic analysis showed that ASPG-OE CAR-T cells exhibited distinct differentiation states, including central memory T cells and effector memory cells re-expressing CD45RA. This distinct phenotypic switch and functional enhancement of ASPG-OE CAR-T cells also confirmed from the in vitro co-culture experiments and in vivo xenogeneic mouse models. The ASPG-OE CAR-T cells showed an increased survival and a differentiation trend towards effector memory cells re-expressing CD45RA. Taken together, ASPG-OE CAR-T cells overcome ASNS-mediated resistance to CAR T cell therapy in leukemia. Further research is warranted to investigate the safety and efficacy of ASPG-modified CAR-T cells in clinical trials.



1737 Advancing AML CAR-T: Transposon Based CAR-T Cells

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Survival outcomes for pediatric acute myeloid leukemia (AML) lag significantly behind other pediatric leukemia and lymphomas. Given the unprecedented success of Chimeric Antigen Receptor T cells (CAR-T) for lymphoblastic leukemias, there is a dire need to develop AML-targeted CAR-T cells. However, its success has been limited due to the need for tumor-specific antigen targets in AML and the risk of off-tumor toxicities in the absence of such antigens. Strategies to overcome the AML clonal heterogeneity has lead to the design of very complex and large CAR constructs. The traditional approach to CAR- T cell engineering utilizes the lentiviral vector to deliver DNA into the T cell in a process that is both time and money consuming on the clinical scale. To address this issue, we have engineered CAR-T cells using a non-viral PiggyBac transposon-based method aiming to facilitate the manufacturing of these novel treatments for AML. We demonstrate that ERM2 CAR, CD123 CAR, and CD33 CAR engineered using the PiggyBac system was effective against multiple in AML cell lines invitro. Furthermore, we have successfully demonstrated that more complex dual gated structures, such as a synthetic notch CD33>CD123 CAR can also be generated into a single frame of the transposon cassette. This non-viral platform demonstrates that more complex structures can be constructed amount of time and materials as simpler CAR constructs and have similar efficacy against AML cell lines. The implementation of non-viral methods to create complex CAR-T modules is novel and could significantly contribute to advancing future clinical trials more rapidly and at a lower cost. This approach could translate into improved access and equity of these life-changing treatments for all patients.

1738 Development of ARI-007: CD7negative Anti-CD7 CAR-T Cells for the Treatment of T-Cell Malignancies

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CAR-T cell therapy has demonstrated remarkable efficacy in B-cell malignancies. However, treating T-cell acute lymphoblastic leukemias and lymphomas (T-ALL/LBL) remains challenging. A major obstacle to therapeutic success is the shared expression of the target antigen between CAR-T cells and malignant T cells, leading to self-killing, known as fratricide. Additionally, there is a risk of contamination of the infusion product with circulating malignant blasts, which could be transduced with the lentiviral vector. We hypothesized that selecting the CD7negative (CD7neg) population may reduce T cell fratricide while ensuring a safer infusion product in comparison to other expansion strategies.

Our center developed three scFvs targeting CD7 from in-house hybridomas (124.1D1, 142.9 and 142.24), and incorporated them into second-generation 4-1BB-based CAR constructs. To compare the three anti-CD7 CAR candidates in the absence of T-cell fratricide, we first used the CRISPR/Cas9 system to knockout CD7. CAR avidity against Jurkat T-ALL cell line was similar for all candidates, consistent with antibody affinity results. *In vitro*, CAR-mediated activation was confirmed in cytotoxicity, cytokine secretion and proliferation assays using Jurkat, CCRF-CEM and MOLT4 cell lines. Although all scFvs eradicated the tumor cells and had similar cytokine secretion, 124.1D1 and 142.24 groups showed greater *in vitro* proliferation than 142.9 group. Accordingly, in a Jurkat xenograft mouse model, 124.1D1 and 142.24 groups significantly reduced tumor burden and prolonged animal survival compared to 142.9. Based on these findings, the 142.24 CAR was selected as our final candidate construct.

To avoid gene-editing, CD7 protein expression blockers (PEBLs) were designed by fusing the scFvs to an endoplasmic reticulum intracellular retention domain. The final PEBL construct combined the 124.1D1 scFv, which showed the highest retention capacity, with the 142.24 CAR sequence in a bicistronic construct. This strategy generated CAR-T cells with robust *in vitro* performance, including cytotoxicity, cytokine production and proliferation. *In vivo*, the PEBL strategy achieved animal survival rates comparable to those of the CD7KO approach.

Additionally, other expansion strategies to prevent fratricide were explored, including pharmacological inhibition of CAR signaling using dasatinib (tyrosine kinase inhibitor) in a 10-day expansion (D10) and in a shortened 3-day protocol (D3). Both approaches successfully generated anti-CD7 CAR-T cells, but D3 cells showed superior IL-2 production and proliferation *in vitro* and significantly extended animal survival *in vivo* compared to the CD7KO, PEBL and long-term pharmacologically inhibited CAR-T cells.

Finally, to enhance safety during manufacturing, we generated CD7neg anti-CD7 CAR-T cells using a custom magnetic depletion process. The obtained CAR-T cells significantly enhanced survival rates compared to untreated mice, similarly to D3 CAR-T cells. The feasibility of producing CD7neg 142.24 CAR-T cells was further evaluated in a GMP-grade bioreactor using apheresis samples.

Overall, we developed and compared five approaches to mitigate fratricide and ensure safety in anti-CD7 CAR-T cells. Based on these results, and due to the reduced risk of contamination of the infusion product with malignant blasts, CD7neg 142.24 CAR-T cells (ARI-007) were selected as our final candidate to be evaluated in clinical trials in patients with T-ALL/LBL.

1739 In Vivo CAR-T Cell Therapy Using Modified AAV Capsids Targeting Human T Cells

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Adeno-associated virus (AAV)-mediated generation of chimeric antigen receptor T (CAR-T) cells in vivo represents a transformative approach to cancer immunotherapy and autoimmune diseases. Unlike traditional ex vivo CAR-T cell therapy, which requires complex procedures including T cell isolation, genetic engineering using lentivirus or retrovirus, expansion and reinfusion, in vivo CAR-T cell therapy enables direct genetic modification of T cells within a patient. Leveraging AAV vectors for efficient gene delivery, this method eliminates the need for labor-intensive and costly manufacturing processes, offering a scalable, fast and affordable treatment paradigm.

However, the progress of in vivo CAR-T cell therapy using AAV vectors has been significantly hindered by their low transduction efficiency of unactivated T cells. To overcome this, we genetically engineered the AAV capsid proteins VP1, VP2, and VP3 by introducing pre-designed polypeptides at various positions in the proteins. Through extensive testing using our Bac-to-AAV system in Sf9 cells, we developed the Precision AAV Capsid Engineering (PACE) platform technology. We also applied this platform technology to the mammalian system mediated by triple-plasmid transfections. This technology allows for the insertion of pre-designed DNA fragments at specific VP sites, thereby altering the capsid affinity for various cellular targets. Utilizing this approach, we engineered a modified AAV capsid, dubbed AAV^{TceT}, which demonstrated an increased affinity for human T cells. Unlike other modifications that often compromise AAV packaging efficiency, the AAV^{TceT} capsid maintained normal production yields.

AAV^{TceT} vectors carrying CMV-GFP and CD19-specific CAR were produced using the Bac-to-AAV system in Sf9 cells. These vectors were then used to transduce peripheral blood mononuclear cells (PBMCs) from various donors in vitro. The results were striking, showing a transduction efficiency increase of over 70 folds compared to their wild-type counterparts (FIG. 1). When these transduced PBMCs were co-cultured with CD19-positive Raji tumor cells, the modified T cells demonstrated efficient killing capabilities, rivalling those of lentivirus-transduced CAR-T cells.

An in vivo mouse study is currently underway, where we aim to observe the cancer-killing effects by directly administering AAV^{TceT}-CD19-specific CAR to NSG mice bearing Raji tumors. Preliminary results have already indicated that animals treated by a single injection of AAV^{TceT}-CD19-specific CAR have significantly smaller tumors than the control group (FIG. 2). This enhanced T cell targeting of the modified capsids could pave the way for an affordable, off-the-shelf in vivo CAR-T cell therapy using AAV vectors.

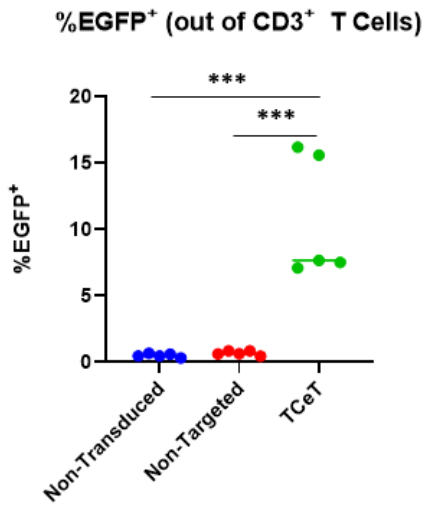

Figure 1. rAAV containing TCeT capsids transduce T cells with up to 70-fold higher efficiency than non-targeted (wt) rAAV. Each dot represent a healthy human donor. Statistical significance was evaluated using One-way ANOVA and Tukey's multiple comparisons test in Graphpad Prism.

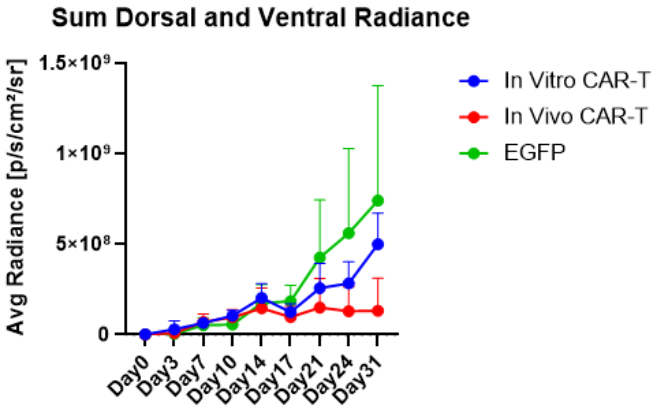

Figure 2. In vivo generated CD19-specific CAR-T cells effectively control Raji tumor cell growth.

1740 Low Dose Methotrexate as a Combinatorial Therapy with mDHFR+ Allogeneic CAR-T Cells

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**Corresponding Author*

Allogeneic (allo) CAR-T cells, derived from healthy donor material, are susceptible to rejection by the recipient's immune system as demonstrated by reduced clinical persistence compared historically to autologous CAR-T products. This has prompted exploration of various shielding strategies to reduce, delay, or prevent rejection. Poseida's allo CAR-T cells are manufactured using a DNA transposon-based delivery system and the Cas-CLOVER™ Gene Editing System (Madison, 2022). Gene editing to eliminate the endogenous T-cell receptor and partially delete beta-2-microglobulin minimizes the likelihood of graft-versus-host disease and host-versus-graft CD8+ T cell-mediated rejection. The CAR transgene encodes a mutant dihydrofolate reductase (mDHFR) gene, enabling the ex vivo selection of transgene-positive cells during manufacture using the antimetabolite methotrexate (MTX). MTX targets rapidly dividing cells and is commonly used in oncology and, at lower doses, to reduce inflammation in autoimmune diseases such as rheumatoid arthritis (RA) and psoriasis.

We hypothesized that administering low-dose MTX to patients after mDHFR+ allo CAR-T infusion could reduce host allorejection while sparing mDHFR+ CAR-T cells, thus enhancing their therapeutic window for antitumor

activity. To test this, mDHFR$^+$ allo CAR-T resistance to MTX was assayed in vitro by incubating antigen-stimulated CAR-T and either unstimulated or antigen-stimulated pan-T cells with increasing concentrations of MTX. These studies demonstrated that exposure to clinically relevant concentrations of MTX (~250-500 nM) (Bannwarth, 1996) was sufficient to kill >80% of activated pan-T cells within 48 hours, while unstimulated pan-T and antigen-activated mDHFR$^+$ allo CAR-T were largely resistant at these concentrations with 85-100% of CAR-T cells surviving on average across multiple donors. Further, abrogation of alloreactivity by 250 nM MTX was assessed in 7-day mixed lymphocyte reactions. MTX completely eliminated HLA-mismatched pan-T cell alloreactivity, as measured by a >95% reduction in proliferating T cells, while mDHFR$^+$ allo CAR-T proliferation was unchanged compared to controls not treated with MTX.

We further investigated whether mDHFR$^+$ CAR-T retain in vivo potency in the presence of low dose MTX. NSG mice engrafted with Raji tumors were administered either mDHFR$^+$ or mDHFR$^-$ anti-CD20 CAR-T, followed 24 hours later by insertion of subcutaneous pumps set to release MTX over the subsequent 5 days (Figure 1A). This method of administration resulted in stable MTX serum levels that were comparable to those observed clinically in RA patients receiving low dose MTX treatment (Figure 1B) (Bannwarth, 1996). Importantly, the in vivo expansion and potency of mDHFR$^+$ CAR-T were unaffected by MTX. Conversely, MTX delayed the expansion of mDHFR$^-$ CAR-T resulting in impaired tumor control (Figure 1C-D). As MTX is rapidly metabolized in mice, mDHFR$^-$ CAR-T were able to expand and regain tumor control after MTX release from the subcutaneous pumps ended on Day 5.

Our results show how MTX effectively reduces alloreactive T cell activity in vitro without affecting the proliferation and cytotoxicity of mDHFR$^+$ CAR-T. Furthermore, in vivo in the presence of clinically relevant serum MTX concentrations the potency of mDHFR+ CAR-T was unchanged. This strategy holds potential for improving allo CAR-T persistence and expanding their therapeutic window by inhibition of alloreactive host T cells.

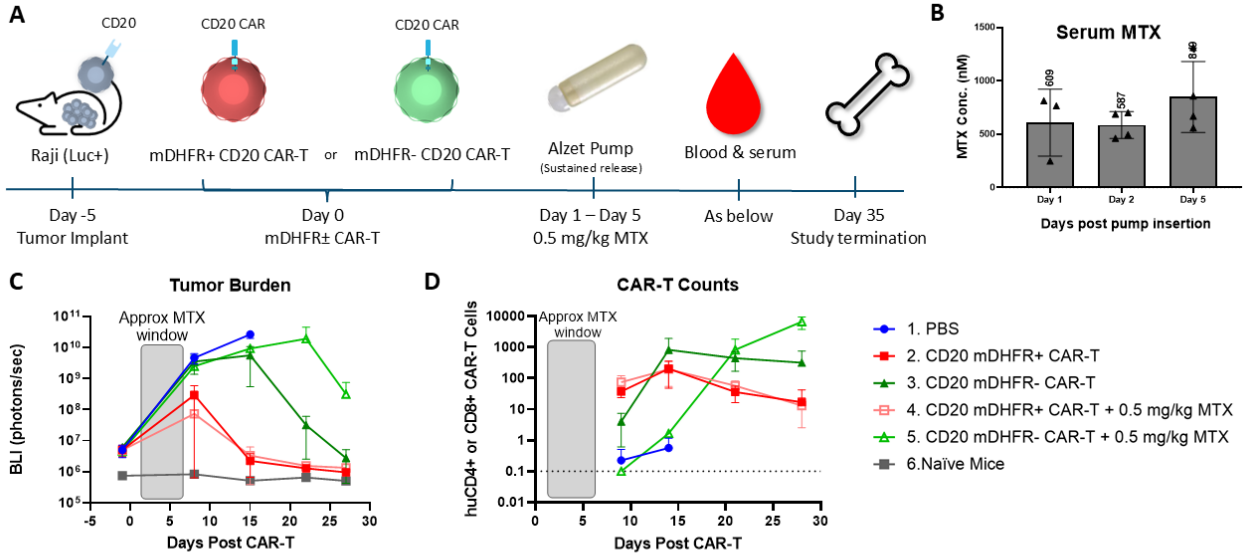

Figure 1: mDHFR+ CAR-T are resistant to clinically-relevant MTX doses in vivo: (A) Schematic for assessing the effect of MTX on mDHFR+ and mDHFR- CAR-T using Alzet pump-mediated delivery (B) Concentration of methotrexate detected in serum samples collected 1, 2 and 5 days after pump implant. (C) Raji-CBG-GFP tumor burden as detected by bioluminescent imaging (BLI) and (D) huCD4+ and CD8+ CAR-T counts throughout study duration.

1741 Novel Humanized anti-CD20 CAR-T cells Demonstrate Robust Efficacy in B-cell Lymphoma in Preclinical Studies.

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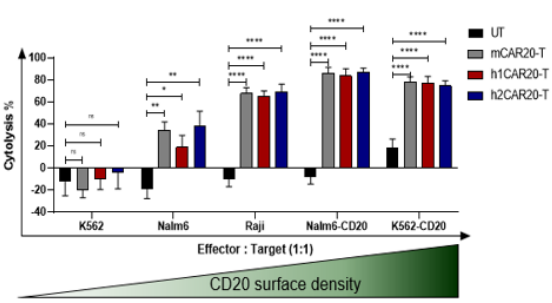
Introduction: Anti-CD19 CAR-T cell therapies are effective for treatment of relapsed /refractory B-cell lymphomas (r/r B-NHL). However, loss or down-regulation of CD19 poses a challenge, contributing to 60% relapses. CAR-T therapies targeting alternative antigens could mitigate this risk. The efficacy of anti-CD20 therapies like Glofitamab post-CAR-T failure is well-established; however, high costs and limited availability restrict access to these therapies in India. We aimed to develop a CD20-directed CAR-T therapy to complement our approved CD19-directed CAR-T therapy (Tali-cel) to improve the durability of responses in r/r B-NHL.

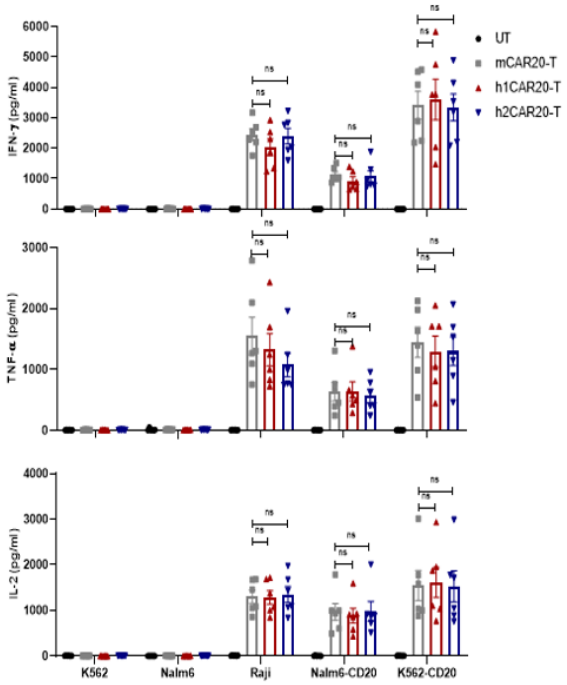
Methods: Two humanized anti-CD20 CAR constructs (h1CAR20 and h2CAR20) were designed and synthesized from parent murine anti-CD20 scFv (mCAR20). CAR-T cells were generated using lentiviral transduction of healthy donor T cells and extensively characterized for functionality in vitro (antigen dependent cytotoxicity and cytokine release, repeat antigen stimulation assay). In vivo studies were performed in NOD/SCID mice, wherein luciferase-tagged CD20+ Raji cells were injected intravenously, followed by CAR-T cells. Tumor progression in the animals was monitored by bioluminescent imaging (BLI).

Results: CAR-T cell manufacturing feasibility demonstrated comparable transduction efficiency (~50%) and ex vivo expansion (40-60-fold) in all anti-CD20 CAR constructs. In co-culture assays, CAR-T cells were tested for anti-tumor activity against tumor cells expressing CD20 at variable levels (low CD20+: Wild type Nalm-6; mid CD20+: Raji cells; high CD20+: transgenic Nalm-6 CD20+, K562 CD20+). Humanized anti-CD20 h2CAR-T cells showed robust antigen dependent efficacy comparable to murine CAR-T cells even at low CD20 expression (Figure 1A). Optimal cytokine release (IFN-y, IL-2 and TNF-α) was seen from humanized CAR-T cells on antigen stimulation, comparable to mCAR20-T cells (Figure 1B). Further, h2CAR20-T cells retained their functional capabilities after multiple antigen exposures. We further evaluated the in vivo efficacy of h2CAR20-T cells in NOD/SCID animal models implanted with Raji cells. Rapid and durable tumor suppression was observed with h2CAR20-T cells compared to mCAR20-T cells and h1CAR20-T cells ($p = 0.0399$ and 0.0352, respectively). h2CAR20-T cells were also associated with low toxicity, with no adverse events, whereas treatment with mCAR20-T cells and h1CAR20-T cells resulted in instances of lethal toxicity in mice. The mice treated with h2CAR20-T cells showed improved survival; with a median survival of 14 days for mCAR20-T cells, while the median survival for h2CAR20-T cells was not reached after 60 days, further supporting the robust anti-tumor activity.

Conclusion: The novel h2CAR20-T cells exhibit robust efficacy, and safety in preclinical models. This indigenous CD20 CAR-T product holds promise for use in combinational CAR-T therapy for r/r B-NHL.



(A)



(B)

Figure 1. humanized anti-CD20 CAR-T cells showed antigen-specific activity. A) CAR T cells were co-cultured with tumor cells for 24 hours, and antigen-specific cytotoxicity was determined by flow cytometry (n = 6) B) CAR T cells were co-cultured with tumor cells for 24 hours, and IFNγ, TNFα, and IL2 were quantified in cell-free supernatants by ELISA (n = 6). Numbers of experiments are represented as n. P < 0.05, 0.01, 0.001 and 0.0001 are represented as *, **, ***, and **** respectively. P > 0.05 is considered as non significant (ns).

1742 The human natural killer (NK) cell line YTS as a novel platform for cellular therapy

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The introduction of CAR T cells has revolutionized the treatment of lymphoma and myeloma. However, this approach has notable limitations, including severe adverse events, complex and costly production processes, dependence on the patient's own T cells, and variability in the final product. In contrast, natural killer (NK) cells are not MHC-restricted, making them suitable for allogeneic use, and they do not trigger cytokine release syndrome. Despite these advantages, NK cell manufacturing remains complex and expensive.

YTS is a human NK cell line characterized by a limited number of activating receptors and can be easily cultured and transfected. In this study, we demonstrate that YTS cells transfected with a CD19-targeted CAR can serve as a novel immunotherapy against CD19-expressing cells. We demonstrate that YTS cells transfected with CAR against CD19 efficiently kill both the B-cell line Raji and primary chronic lymphocytic leukemia (CLL) cells. We show that CAR activity relies on intracellular signalling and, in certain tumors, on co-stimulation through the 2B4-CD48 pathway.

To use YTS cells therapeutically, they must be irradiated before injection to prevent proliferation while maintaining cytotoxic activity. We evaluated irradiation effects using multiple methods. In vitro, a 1000 rad dose effectively inhibited proliferation, as shown by reduced viable cell numbers, lower metabolic activity in an MTT assay, and cell trace labeling (data not shown and Figure 1A). In vivo, NSG mice injected with YTS-CAR-CD19 cells irradiated at 2000 rad or higher showed no tumor growth (data not shown). Based on these findings, a 2500 rad dose is optimal

for safely preventing proliferation. Next, we evaluated whether the cytotoxic activity of YTS cells expressing CAR was retained after irradiation at 2500 rad. Additionally, we froze the cells post-irradiation to simulate how these engineered YTS cells could be administered to patients. A cytotoxicity assay was performed, in which irradiated YTS cells, both with and without CAR targeting CD19, were incubated with Raji cells. The assay showed that YTS-CAR CD19 cells maintained their cytotoxic activity after irradiation and freezing (Figure 1B). To assess the therapeutic function of YTS cells expressing CAR CD19 in vivo, we used a tumor model in which Raji cells were injected subcutaneously into NSG mice. Once tumors became palpable, we injected irradiated YTS CAR CD19 cells twice a week. As controls, we used non-transduced YTS cells or PBS. Tumor size was evaluated, and injection of YTS CAR CD19 cells resulted in a significant reduction in tumor size (~50%) (Figure 1C).

We also transduced the cells with the Fc receptor CD16 and demonstrated that the transduced YTS cells can induce antibody-dependent cytotoxicity (ADCC) mediated by rituximab when targeting B cells. More importantly, these cells were also capable of inducing ADCC against solid tumor cells.

We propose that genetically engineered YTS cells can serve as a novel, cost-effective, and accessible "off-the-shelf" immunotherapy, which can be tailored to target different antigens. These modified cells have potential applications in treating resistant lymphoma, autoimmune diseases, and solid tumors.

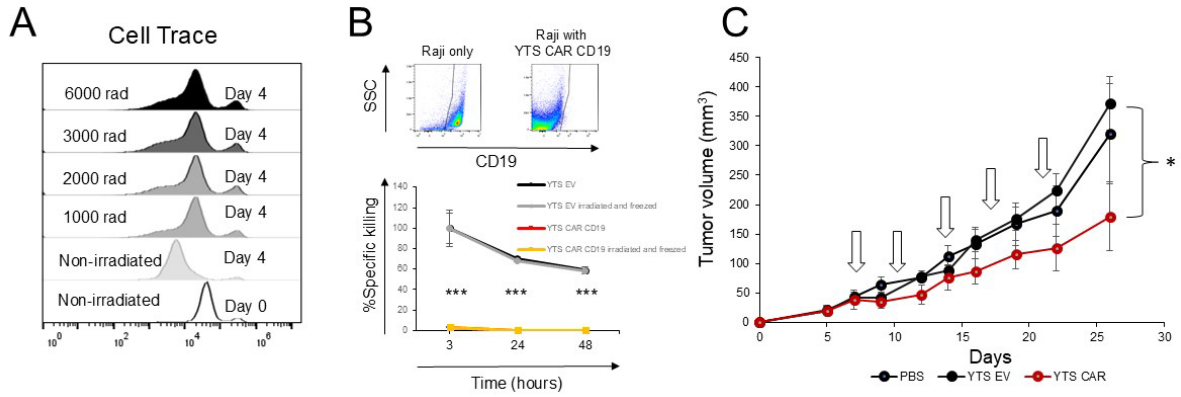

Figure 1. Safety and efficiency of irradiated YTS-CAR CD19 cells. (A) YTS cells expressing CAR against CD19 were non-irradiated or irradiated at different levels and then subjected for proliferation assay using CellTrace. (B) YTS cells expressing either a CAR against CD19 or an empty vector were irradiated with 2500 rad or left non-irradiated, then frozen and later used in a killing assay against Raji cells. The degree of killing was assessed at multiple time points over 48 hours. E:T 20:1. (C) Effect of YTS cells expressing CAR against CD19 *in vivo*. 15x10^6 Raji cells were injected subcutaneously into immunodeficient mice. Once tumors became palpable, mice were injected with 10x10^6 YTS cells expressing CAR against CD19, which were irradiated with 1000 rad (marked with arrows). *, P<0.05, ***, P<0.001, ANOVA test with multiple comparisons or Student's t-test.

1743 DesignCell® Platform for Optimal CAR Discovery

Atsushi Okuma[1]*, Yoshihito Ishida[2], Daisuke Ito[3], Taketo Kawara[3], Takuya Yamashita[3], Kei Yoshida[3], Shoji Hisada[2], Tomohiko Okuda[3], Hiroko Hanzawa[2], Shizu Takeda[3]
[1]Hitachi, Ltd., Kobe, Hyogo, Hyogo, Japan, [2]Hitachi, Ltd., Kobe, Hyogo, Japan, [3]Hitachi, Ltd., Kobe, Japan
**Corresponding Author*

Introduction: Chimeric antigen receptor (CAR) T cell therapy has demonstrated promising outcomes in B cell leukemia and multiple myeloma. However, its efficacy against other tumor types remains suboptimal. Most CAR-T studies employ a limited range of CAR designs, similar to the approved CD19 CARs, differing mainly in the binder domain. these conventional designs may not be optimal for CARs targeting other tumors or even for CD19 CAR-T therapies. To maximize CAR efficacy, we have developed the DesignCell® platform, which can rapidly design CAR

sequences, build CAR T cells, test their functionality, and learn the relationship between CAR sequence and cell function for subsequent design-build-test-learn (DBTL) cycles. Our platform integrates original AI capable of generating CAR designs with predicted functionality, a pooled screening system specialized in evaluating CAR T cells on a single-cell level, and an automated high-throughput arrayed screening system for CAR T cell phenotyping.

Results: We started a CD19 CAR screening from a theoretical diversity of 10^8 designs generated by our AI. To handle CAR libraries with such enormous diversity, we developed a pooled screening system utilizing an optofluidic system to verify the single-cell cytotoxicity of each CAR T cell. Our pooled screening system provides up to 14,000 single-cell data per run, capturing cytotoxicity and stimulation-dependent cell growth. Our in-house long-read sequencing setup links each CAR sequence to its phenotypes. Following the enrichment of active CAR designs through pooled screening, we performed arrayed screening to obtain bulk data for hundreds to thousands of cells per CAR. Our arrayed screening system, compatible with high throughput and automated via robotic handling, covers the entire process from production to evaluation of CAR T cells. This system can evaluate short-term cytotoxicity and serial killing activity of over 500 CARs in one cycle. Upon completion of the screening flow, we identified CAR designs with superior activity compared to commonly used designs from the huge CAR library.

Conclusion: Our DesignCell® platform facilitates rapid CAR T cell screening based on cytotoxic activity from a vast CAR library. Using the platform, we selected several enhanced CD19 CAR designs. This strategy holds potential for optimizing CARs targeting other antigens.

1744 SynKIR-310 Provides a Novel Split-Signal CAR T Platform (KIR-CAR) Based upon Natural Killer Cell Signaling to Prolong Anti-Tumor Treatment in B-NHL

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Single-chain CD3-based chimeric antigen receptors (CARs) utilize FMC63 single-chain variable fragments (scFv) to target CD19 and demonstrate robust clinical responses in patients with B cell non-Hodgkin lymphomas (B-NHL). Despite initial response rates of 60-80%, about 40% of responders experience relapse within 1 year[1]. Therapies that improve upon CAR T cell functional durability remain an unmet medical need. Previously, we developed a multi-chain specific killer immunoglobulin-like receptor (KIR)-derived CAR T cell therapy, SynKIR-110, targeting mesothelin on solid tumors with enhanced anti-tumor function in a murine xenograft model of a CAR-resistant patient-derived mesothelioma, compared to 2nd generation (gen) CD3-based CAR T[2].

Using our newly developed anti-human CD19-specific canine scFv, DS191, with 84% heavy chain homology to humans as opposed to murine FMC63 with only 64% homology, we designed a novel KIR-CAR (SynKIR-310) with separated antigen binding and T cell activation in attempts to confer the prolonged functional durability observed in SynKIR-110 with solid tumors, to address patients with refractory/relapsed CD19-targeted B-NHL. Expressed on the same KIR-CAR T backbone, DS191 binder showed comparable sensitivity and specificity to FMC63 *in vitro* in antigen density assays, equivalent anti-tumor activity *in vivo* as FMC63, and no off-target cross-reactivity as measured by membrane proteome array and co-culture with primary cells from different organs[3].

To evaluate whether the differing signaling components in single chain benchmark FMC63 CD19-CAR T vs multi-chain SynKIR-310 had any impact on transduced T cells' antigen sensitivity and activation, we compared the two *in vitro* both qualitatively and quantitatively. Results demonstrated comparable T cell activation of CD107a, TNF-α, IL-2 and IFN-γ, to the extent that not only the types of cytokines produced matched, even the concentration of each cytokine produced in response to targets matched between SynKIR-310 and FMC63 CD19-CAR. These data suggest that although they are comprised of different scFv binders and different signaling mechanisms, SynKIR-310 and benchmark FMC63 CD19-CAR are comparable in their specificity, sensitivity, and potency *in vitro* in the presence of various levels of CD19 antigen.

Previous work on SynKIR-110 demonstrated that although there was no apparent difference between 2nd gen CAR and KIR-CAR *in vitro*, once applied in *in vivo* tumor models, a different profile emerged, with KIR-CAR reducing or eliminating solid tumors that were resistant to single-chain CD3-based CAR T treatment[2]. This work supports further *in vivo* evaluation and translation into clinical studies, as we have shown that DS191 binder can recognize CD19 targets with equivalent sensitivity and specificity as the globally approved FMC63.

Our next steps include conducting SynKIR-310 *in vivo* tumor-killing studies compared to benchmark CD19-CAR in CD19-positive heme tumor models. These results should provide insights into the correlation between *in vitro* and *in vivo* potency of SynKIR-310, building upon the mechanism for prolonged anti-tumor response observed in SynKIR-110, and supporting clinical development of SynKIR-310 for patients with relapsed/refractory B-NHL in a Phase 1 clinical trial (NCT06544265).

1. Cappell, K.M. *Nat Rev Clin Oncol* 20, 359-371 (2023) 2. Wang, E. *Cancer Immunol Res* 3, 815-826 (2015) 3. Yucel, N. *Molecular Therapy* Vol 32 No 4S1 (April 2024) Abstract #189

1745 Immunobiology of Adeno-Associated Virus (AAV) in Murine Liver: A Cross-Sectional Comparison of AAV8, AAV6, and a Chimeric Liver-Tropic Capsid in a Preclinical Model

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[1]AskBio, Durham, NC, [2]AskBio, Morrisville, NC, [3]AskBio Inc., Morrisville, NC, [4]AskBio, Morr, NC, [5]AskBio, Philadelphia, PA, [6]Asklepios Biopharmaceutical, Morrisville, NC, [7]Askbio, Research Triangle Park, NC, [8]Asklepios Biopharmaceutical, Research Triangle Park, NC, [9]AskBio, Milton, DE, [10]Stealth, Chapel Hill, NC, [11]Ask Bio, Morrisville, NC

Adeno-associated virus (AAV) vectors are leading candidates for gene therapy product delivery. However, capsid-specific immune responses reduce therapeutic benefit by driving transgene loss and have been linked to adverse events. AAV8 is a well-described liver-tropic capsid, yet clinical trials in hemophilia have varied in measures of safety and efficacy (Nathwani 2014, Konkle, 2021). In rare cases, liver toxicity, including liver failure, has been observed after high-dose AAV8 administration in patients with X-linked myotubular myopathy (Shieh, 2023; Wilson & Flotte, 2020). Conversely, AAV6 was generally well tolerated in a hemophilia A study even at high doses (Leavitt, 2024). Additionally, a recently described chimeric liver-tropic capsid demonstrated minimal liver inflammation and sustained transgene expression in a hemophilia B clinical trial (Xue, 2022). Thus, a better understanding of AAV-induced immune pathways in the liver has the potential to inform capsid design, immunomodulatory regimens, and enhance clinical trial outcomes.

The current study directly compared the biology of AAV8, AAV6, and the chimeric liver-tropic capsid in a murine model. Adult female B6(Cg)-Tyrc-2J/J albino mice (n=4/group) received $1E^{11}$ vg (~$5E^{12}$ vg/kg) of AAV8, AAV6, or the chimeric liver-tropic capsid carrying a luciferase reporter or formulation buffer by tail vein injection at day 0. Mice were sequentially culled at 2, 5, 8, 16, 24, 72 hours (h) or 7, 30, or 90 days (d) post-dose.

Whole body and ex-vivo liver IVIS imaging showed significantly higher transgene expression from AAV8 (~1.0 log) compared to AAV6 and chimeric liver-tropic capsid at all timepoints. Interestingly, the chimeric liver-tropic capsid exhibited higher (~0.5 log) viral DNA copies in liver compared to AAV6 and AAV8. In situ hybridization of luciferase DNA/RNA in liver 2h post-dose revealed distinct vector distribution patterns. AAV8 nucleic acids were uniformly distributed, with small, punctate foci whereas AAV6 was less uniform with larger foci. The chimeric liver-tropic capsid was organized into large areas of intense staining, and AI-assisted image analysis demonstrated an increased number of membrane-associated aggregates whose staining co-localized with F4/80, a marker of murine macrophages and Kupffer cells. Gene expression analysis demonstrated that the chimeric liver-tropic capsid transiently elicited an upregulation of select innate genes including interferon alpha (~10 fold) and flow cytometry revealed increased plasmacytoid dendritic cell frequency and natural killer cell activation relative to AAV8, AAV6, and mock controls. Despite aggregation of the chimeric liver-tropic capsid and immune involvement observed at

early timepoints, AST and ALT remained at background levels across all groups/ timepoints, and apoptotic hepatocytes were not observed by TUNEL staining, suggesting normal liver function after capsid delivery. At later timepoints we observed similar levels of T cell activation in the liver and spleen and circulating AAV binding antibodies induced by all three capsids. Overall, this study suggests capsid-specific differences in the early host response may impact transgene expression and potentially therapeutic efficacy.

1746 A Multitherapeutic Approach Combining *Ex vivo* and *In vivo* Cell Engineering to Treat HIV in Nonhuman Primates

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The recent advances in *in vivo* cell engineering will contribute to a great acceleration in the application of therapies in oncology and infectious diseases. However, few studies have explored these approaches in immunocompetent large animal models such as nonhuman primates (NHP), which most closely model and predict therapeutic potential in patients. Here we focused on HIV-1, a virus responsible for acquired immunodeficiency syndrome (AIDS). Since its identification in the 80's, a very limited number of people living with HIV have been cured following transplant of hematopoietic stem cells to treat hematologic malignancies. However, these treatments remain burdensome, restricting this approach. Additionally, HIV-1 has shown unprecedented ability to adapt to the host immune response and treatment attempts, complicating its eradication and suggesting that combining therapeutics might be key. In this study we evaluated a combination of *ex vivo* and *in vivo* cell engineering with the goal to deliver several therapeutics as a treatment against SHIV (Simian-human immunodeficiency virus) in pigtail macaques.

Nine pigtail macaques were enrolled. Three control animals were SHIV-infected, suppressed with antiretroviral therapy (ART-suppressed) and left untreated. The remaining 6 animals were SHIV-infected and ART-suppressed before being transplanted with autologous *ex vivo* chimeric antigen receptors (CAR)-modified hematopoietic stem cells. Following a 6-month recovery period, the animals were divided in two groups and received *in vivo* injections of $CD4^+$ T cells- and hepatocytes-directed lipid nanoparticles (LNPs; n=3) or Adeno-associated virus vectors (AAVs; n=3) encoding broadly neutralizing antibodies (bNAbs) and Brec1-recombinase. In total the treated animals received a combination of two CARs, two bNAbs directed against the HIV-1 Envelope and a Brec-1 recombinase (Brec).

Throughout the study, viral load, $CD4^+$ T cells, bNAb concentrations and CAR expression were monitored in peripheral blood and tissues. Limited but detectable CAR expression was observed despite low to no viral antigen expression for the duration of antiretroviral therapy administration. Preliminary results indicate that LNP-receiving animals had detectable bNAbs with concentrations ranging from 1.3 to 9 µg/ml in the first two weeks following injections. bNAb expression from the AAVs-receiving animals is still being investigated. Among these animals receiving LNPs, bNAbs could be detected for several weeks and one animal presented a delayed viral rebound at 5 weeks post ART withdrawal correlating with the most sustained and highest bNAb expression. The other animals from the treatment or control groups had a shorter bNAb half-life and the virus rebounded between 1 to 3 weeks after ART withdrawal. More experiments are being performed to analyze bNAb concentrations and persistence in the LNP- and AAV-receiving animals.

While more investigations are required to unravel the efficiency and contribution of each therapeutic approach employed in this study, our data show that a combination of *ex vivo* and *in vivo* cell engineering can be delivered and potentially impact the viral rebound. Although this study focused on *ex vivo* and *in vivo* cell engineering, future

developments will be geared toward an all–*in vivo* approach to facilitate a one shot multitherapeutic approach as standard of care.

1747 Cross-reactive Immunologic Material (CRIM) Analyses for AAV Gene Therapy

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The absence of Cross-Reactive Immunologic Material (CRIM) may potentially impact the long-term efficacy of AAV gene therapy, by eliciting strong immune responses against the transgene protein. Parameters that may impact the immune response to the transgene protein can include gene therapy dose, route of administration, genotype of the individual, and immunomodulatory history.

AAV gene therapies are predominantly designed for monogenic rare diseases where a single defective gene is responsible for driving disease pathology. At the individual level, genotypes can be analyzed for the degree of homology between the pre-existing protein, the transgene protein, and other proteins in the genome. Individuals with significant lack of protein homology may be predicted to be "CRIM-high risk"; *eg* these people may be more likely to generate a stronger immune response to the transgene protein than others. The immune response to the transgene protein may be driven by CD8+ T cells or both CD4+ T cell and B cells (antibodies). Just as an antibody response may limit biodistribution of soluble protein therapies, antibodies and CD8+ T cell responses may kill transduced cells. To address the potentially strong immune response, stronger immunomodulation regimens may be introduced, or CRIM-high risk individuals may be excluded from studies.

To understand the potential impact of CRIM risk in people with OTC and GSDIa, we created an *in silico* CRIM-analysis workflow utilizing IEDB tools, AlphaFold, and custom programs. This workflow includes 1) individual genotype impact on protein product, 2) homology to other genome proteins, 3) predicted B cell epitopes, 4) antibody accessibility based on protein secondary structure, and 5) interaction score with most common HLA-A, HLA-B, and HLA-C haplotypes in North America and Europe; these HLA predictions were compared with published Duchenne clinical cases where CRIM-high risk individuals were identified.

For individuals with OTC enrolled in the DTX301 Phase 1/2 study (ClinicalTrials.gov Identifier: NCT02991144), 1 of 8 participants with available data was characterized as CRIM-medium risk due to a hemizygous splicing mutation predicted to cause absence of a portion of ornithine transcarbamylase protein. For individuals with GSDIa enrolled in the DTX401 Phase 1/2 study (ClinicalTrials.gov Identifier: NCT03517085), two of 12 participants were characterized as CRIM-high risk and three of 12 were characterized as CRIM-medium risk due to homozygous or compound heterozygous mutations predicted to result in truncation or absence of glucose-6-phosphatase protein.

The relationship between *in silico* CRIM-analysis workflow predictions and clinical outcomes will be important for refining these tools as clinical results become available. As more people consider taking AAV gene therapy, understanding the impact of CRIM more comprehensively will help guide discussions to optimize these potentially transformative treatments.

1748 Genetically Engineered Organs for Early Reporting of Transplant Rejection

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The diagnosis of organ transplant rejection remains a major clinical challenge due to the limited sensitivity and specificity of blood tests and the invasive nature of biopsies. This study presents a novel approach for rejection monitoring by genetically modifying transplant organs to serve as immunosensors. Using viral gene delivery during ex vivo machine perfusion, we successfully incorporated transgenes into vascularized composite allografts (VCAs), enabling real-time, in situ detection of rejection through the release of a sensitive, blood-based biomarker (**Fig. 1A**).

We demonstrate, for the first time, that genetically modified VCAs maintain stable expression of Gaussia Luciferase (GLuc) for over 300 days—equivalent to approximately 30 years in humans—highlighting the durability of this approach (**Fig. 1B**). Given the high immunogenicity of skin, VCA recipients, such as face or arm transplant patients, experience acute rejection episodes in 89% of cases within the first year post-transplant, despite intensive immunosuppression. Additionally, the short preservation window of six hours and the complex tissue architecture of VCAs pose significant challenges for efficient transduction.

To address this, we designed an inflammation-responsive construct using a synthetic NF-κB promoter to drive GLuc expression, enabling early, non-invasive detection of rejection. This system allowed for biomarker detection up to *six days* before any detectable histological signs of rejection, providing a crucial diagnostic advantage (**Fig. 1C, D, E**). Our findings establish that integrating gene therapy with ex vivo machine perfusion can significantly enhance the diagnostic capabilities of transplantable organs, offering a groundbreaking strategy to improve long-term graft survival and patient outcomes.

Beyond transplantation, this proof-of-concept study sets the stage for broader applications in transplantation and regenerative medicine, such as localized inflammation and infection monitoring in burn injuries and wound healing. By defining key parameters for effective tissue transduction, this approach paves the way for innovative, real-time diagnostic and therapeutic strategies in transplant and wound care medicine.

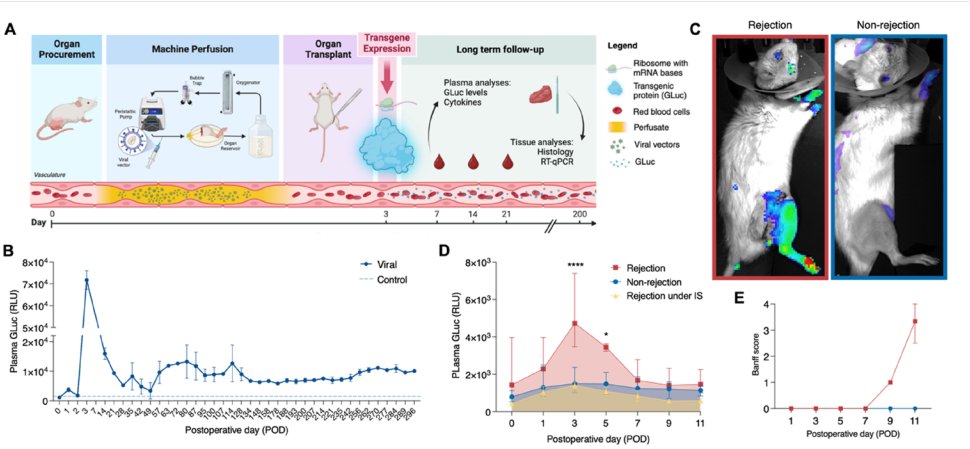

Figure 1. Genetically Engineered *'Smart Grafts'* Detect Rejection *In Vivo* Earlier Than Biopsies. (**A**) Experimental design describing the study timeline. (**B**) Plasma GLuc levels of constitutive animals stabilize after postoperative day (POD) 14 and remain consistent for near 300 days. (**C**) *In vivo* bioluminescence imaging shows significant luminescence in rejected hindlimb. (**D**) Plasma GLuc levels in rejection animals are significantly elevated at PODs 3 and 5 compared to non-rejection or rejection group that received immunosuppression (IS). (**E**) Blinded histopathological assessment shows rejection on POD 9 in rejection group. Two-way ANOVA (* $p \leq 0.0332$; **** $p \leq 0.0001$).

1749 Towards a Universal Cell Replacement Therapy: Engineering Human Pluripotent Stem Cell Lines to Evade Xenogeneic Transplantation Barriers

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Human pluripotent stem cell (hPSC)-based therapies for regenerative medicine are beginning to fulfill their promise, with several exciting treatments for degenerative and autoimmune diseases having reached clinical testing. However, immunological rejection of hPSC-based therapies remains a limitation to their widespread adoption. To circumvent rejection, these therapies can be combined with systemic immunosuppression, but this increases the risk of infections and cancer. Alternatively, induced PSCs (iPSCs) can be used autologously, but this has the practical limitation of scalability. A third option is genetic modification of the starting hPSCs to evade immunological rejection, which can enable scalable production of cell replacement therapies without the need for systemic immunosuppression. To create cells capable of evading immune rejection for preclinical testing, we used CRISPR-Cas9 to genetically ablate expression of HLA-I, HLA-II, and natural killer (NK) cell activating ligands in hPSCs (HM-KO hPSCs). Additional combinations of immune evasion factors were expressed on HM-KO hPSCs to inhibit NK cells, complement deposition, and/or phagocytes. To define the immunological barriers causing immune rejection, we performed teratoma assays in fully immunocompetent mouse models using these modified lines. Immunocompetent mouse models present the extremely high immune barrier of xenogeneic transplantation and, unlike 'humanized' mice, have a fully intact and functional immune system. Transplantation of HM-KO hPSCs expressing additional inhibitors for NK cells and complement deposition led to persistent teratomas in fully immunocompetent wild-type mice. Expression of additional inhibitory factors such as CD24, CD47, and/or PD-L1 had no discernible impact on teratoma growth or persistence. Transplantation of HM-KO hPSCs into mice genetically deficient in complement and depleted of NK cells also led to persistent teratomas. Thus, T cell, NK cell, and complement evasion are necessary to prevent immunological rejection of hPSCs and their progeny. These hPSCs and versions expressing human orthologs provide a template for regenerative therapies and could eventually serve as a universal hPSC bank for cell replacement therapies that circumvent the need for systemic immunosuppression.

1750 The Effects of Antigen-Specific CD8$^+$ T Cells on Successful Engraftment in Platelet-Targeted Gene Therapy

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Background: Immune responses, particularly CD8$^+$ T cell-mediated rejection, remain a major obstacle in gene therapy, leading to the elimination of genetically engineered cells and therapy failure. Our platelet-targeted gene therapy, driven by the αIIb promoter, has demonstrated therapeutic potential and immune tolerance in F8^{null} mice. However, pre-existing immunity can impair engraftment by targeting transgene-expressing cells. Identifying the threshold of antigen-specific CD8$^+$ T cells that can be tolerized is crucial for optimizing platelet gene therapy in primed recipients.

Objective: To determine the threshold of antigen-specific CD8$^+$ T cells that allows successful engraftment of engineered bone marrow (BM) cells and sustained transgene expression in platelet-targeted gene therapy, overcoming immune barriers to improve therapeutic outcomes.

Methods: We used ovalbumin (OVA) as a surrogate protein for platelet-specific gene expression (2bOVA) and generated a transgenic model, 2bOVA^{Tg}, with OVA expressed explicitly in platelets. CD8 T cells from OT-I mice [OVA-specific T cell receptor (TCR) transgenic mice] were used to explore the effects of antigen-specific CD8 T cells on the genetically manipulated engraftment.

Results: In a mixed BM transplantation (BMT) setting, OVA-specific CD8$^+$ cells appear to have killed the 2bOVA^{tg} BM cells in all mixed BMT groups when OT-I BM cells were 10% or greater, demonstrating too many OVA-specific CD8$^+$ cells for successful engraftment of 2bOVA^{tg} BM under 660R total body irradiation (TBI). The 1% and 0.1% OT-I groups exhibited < 10% peripheral blood donor-derived leukocyte chimerism by week 7, with nearly 100% of donor-derived CD8$^+$ being Vα2$^+$Vβ5$^+$ OT-I-specific T cells. There were undetectable 2bOVA transgene by week 8 post-BMT and undetectable platelet-OVA protein expression at all time points. Similarly to the 0% control, the 0.001% group exhibited ~ 90% peripheral blood donor-derived chimerism, with only ~ 1-2% Vα2$^+$Vβ5$^+$CD8$^+$ T

cells and stable OVA expression over the study course. In the 0.01% group, 4 of 7 mixed BMT recipients exhibited parameters similar to the 0% control and 3 of 7 to those of the 1% and 0.1% groups. In the second model we examined, we transplanted OVA-specific OT-I CD8 T cells alongside 2bOVA^{Tg} BM cells to mimic the gene therapy scenario where engineered cells encounter primed CD8 T cells. We found that OVA-specific CD8^{+} T cells could be tolerized when the proportion of these cells was 0.5% or less in the presence of engineered 2bOVA^{Tg} BM mononuclear cells, and successful engraftment of 2bOVA^{Tg} BM cells was achieved. Flow cytometry analysis revealed that granzyme B (GrzmB) expression changed over time as OVA-specific CD8 T cells were tolerized. In

Conclusion, the findings indicate that a threshold of 0.5% antigen-specific CD8+ cells can be tolerated in platelet gene therapy. This level is conducive to achieving successful engineered engraftment in the primed model under conditions of 660 TBI. This insight is crucial for optimizing therapeutic strategies and ensuring effective outcomes in platelet gene therapy applications.

1751 A localizing nanocarrier formulation enables anti-enterovirus antibody responses from multivalent replicon RNA by limiting systemic interferon responses

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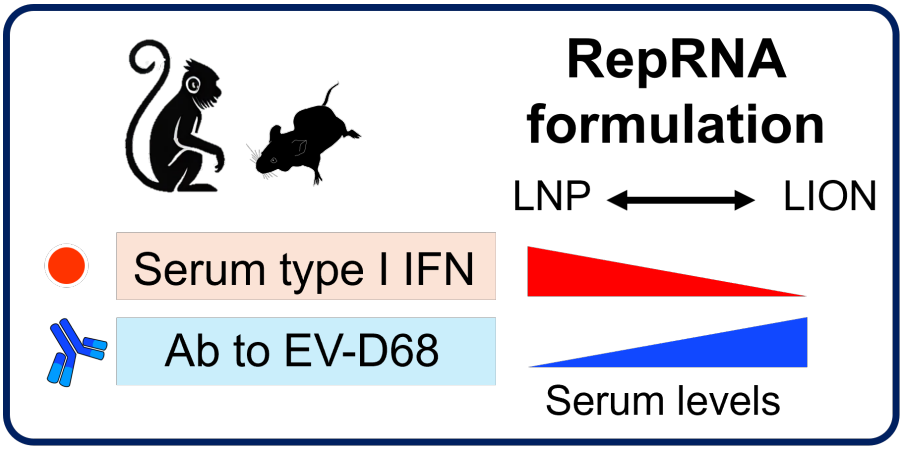
Replicon RNA (repRNA) vaccines are a promising platform for inducing robust immune responses at reduced doses. However, systemic inflammatory responses triggered by lipid nanoparticle (LNP)-formulated RNA vaccines may compromise both safety and immunogenicity. Cationic nanocarriers such as LION™ offer an alternative delivery approach by facilitating localized repRNA retention at the injection site. Here, we evaluated a multivalent repRNA vaccine encoding three distinct virus-like particles (VLPs) from different enterovirus D68 (EV-D68) subclades and two respiratory syncytial virus (RSV) antigens (F and G) in pigtail macaques. LION-formulated repRNA elicited robust antigen-specific antibody (Ab) responses while mitigating the systemic type I interferon (IFN) response observed with LNP formulations. Notably, elevated systemic type I IFN levels negatively correlate with EV-D68-specific binding and neutralizing Ab titers but not with RSV-F and G-specific binding Ab titers, suggesting antigen-specific sensitivity to IFN-mediated suppression. These findings underscore the importance of modulating innate immune activation to optimize the immunogenicity of multivalent repRNA vaccines. LION-based delivery may provide a safer and more effective platform for RNA vaccines targeting IFN-sensitive pathogens, with broader implications for refining RNA vaccine design in inflammatory settings.



1752 Alphaviral Genetic Background of Self-Amplifying RNA Influences Protein Expression and Immunogenicity Against SARS-CoV-2 Antigen

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Introduction: Self-amplifying RNA (saRNA) vectors are a next-generation RNA technology that extends the expression of heterologous genes. Structurally similar to conventional messenger RNA, saRNA are designed to contain viral replicases and conserved sequence elements derived from alphaviruses. Clinical trials have shown the dose-sparing capacity of saRNA vectors as evidenced by 6-33 lower dosages of the approved ARCT-154 vaccine compared to Spikevax and Comirnaty messenger RNA vaccines against SARS-Cov-2. Historically, saRNA vectors have been based on a limited number of alphaviruses, and only the Venezuelan equine encephalitis virus-based saRNA vaccines have been used clinically. Blanketed application of the VEEV saRNA vector as a vaccine or protein replacement therapy is potentially problematic. The efficacy of a saRNA-based therapy will be affected by the expression profile, toxicity, translational efficiency, and cellular tropism, which are determined by the genotype of the alphaviral source and its interaction with the host cell. Therefore, this work sought to determine the suitability of unique alphaviruses as saRNA vectors and their utility as a vaccine platform.

Methods: Twelve unique alphaviruses were adapted into saRNA vectors encoding a green fluorescent protein or firefly luciferase. Expression kinetics of the saRNA vectors were assessed in mammalian cells, human skin explants and female Balb/c mice. Furthermore, competent saRNA vectors were re-configured into SARS-CoV-2 vaccine platforms and evaluated for their level of immunogenicity in C57Bl/6 mice.

Results: Five of the 12 saRNA vectors were replication-competent, resulting in higher protein production than the control saRNA by a 2 to 5-fold difference in fluorescent cells. Lipid nanoparticle delivery of saRNA vectors encoding the firefly luciferase (Fluc) into mice resulted in variable pharmacokinetics, enabling modulation of the magnitude and duration of protein expression. saRNA-Fluc vectors based on the VEEV TC83 variant, Tonate (TONV), Chikungunya (CHIKV) and O'nyong-nyong (ONNV) viruses resulted in 7.8-, 6.7-, 8.4- and 1.5-fold total expression as determined by the area under the curve of the total flux compared to the control saRNA vector derived from VEEV Trinidad Donkey variant. Additionally, standard homologous prime-boost vaccination of the saRNA vectors encoding the SARS-CoV-2 antigen resulted in differential humoral immunity before and after booster immunization. Two weeks after booster vaccination, the concentration of spike-specific IgG antibodies was 5- to 14-fold higher than the control saRNA. Except for the ONNV-saRNA vector, similar levels of antigen-specific splenocytes were observed after vaccination as determined by an interferon-gamma ELISpot assay. ONNV saRNA vaccination resulted in 2.7 higher anti-spike splenocytes. Interestingly, a heterologous vaccination regimen using distinct genotypes of saRNA resulted in differential immunity depending on the sequence of administration. Heterologous vaccination using CHIKV-VEEV (prime-boost) saRNA vectors resulted in a 7 times higher anti-spike IgG boost than the 1.4-times boost in the VEEV-CHIKV regimen.

Conclusion: Unique alphaviruses can be adapted into saRNA vectors. The genotype of the saRNA vectors influenced the magnitude and duration of expression as well as cellular and humoral immunity against an infectious disease antigen.

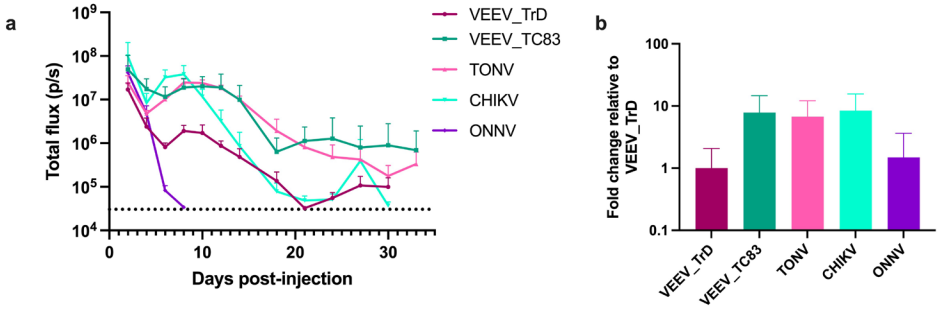

Figure 1. Bioluminescence of mice after intramuscular injection of lipid naopartciles containing saRNA encoding firefly luciferase. (A) Quantification of total flux obtained from mice after in vivo imaging. (B) Fold change of area under the curve of total flux.

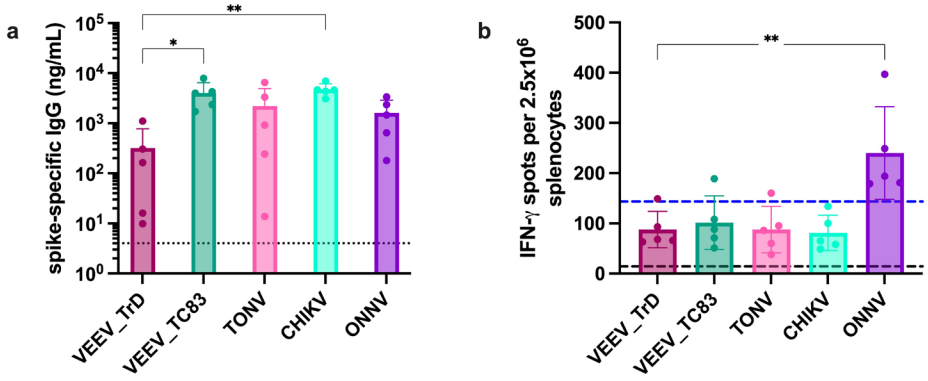

Figure 2. Immunogenicity of saRNA-spike vaccines in C57BL/6J mice. (A) Concentration of anti-spike IgG in blood sera as determined by ELISA. Dotted line indicates the PBS control levels. (B) Quantification of spike-specific splenocytes producing IFN-γ. Black and blue dotted lines indicate results from PBS-control splenocytes stimulated with spike peptides and Concanavalin A, respectively.

1753 T Cell Targeted mRNA tLNPs Drive Fast, Selective and Highly Effective in *situ* CAR T Cell Programming

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Chimeric Antigen Receptor (CAR) T Cell therapies have shown impressive clinical benefit in patients with hematologic malignancies. More recently, they have also shown encouraging signs of clinical efficacy in autoimmune diseases. However, all six of the CAR T therapies currently approved by the FDA and EMA have costly and time-consuming *ex vivo* T cell manufacturing protocols. In addition, these therapies are based on viral transduction resulting in permanent CAR expression that is associated with some safety concerns. On-target off-tumor activity of persistent CD19 CAR T cells kill healthy B cells leading to hypogammaglobulinemia with an increased risk of infections. Approved therapies now come with boxed warnings from regulatory agencies because viral transduction may be associated with secondary T cell leukemia and lymphoma. Recent developments in our mRNA LNP technology allow the design of a novel *in situ* CAR therapy benefitting from transitory CAR expression circumventing numerous potential risks of viral constructs while offering tremendous cost advantages over autologous cell-based therapies with *ex vivo* manufacturing protocols (Figure 1).

Materials & Methods: We developed T cell targeted LNP formulations (tLNP) via surface functionalization that achieve highly effective (>90%) uptake not only in activated but also in resting T cells. T cell targeting is achieved through surface functionalization of our LNPs via direct conjugation of targeting ligands onto the surface, enabling our tLNPs to bind to desired T cell-specific antigens and heighten delivery of our mRNAs into the cells *in vivo*. T cell-selective delivery is further enhanced with Kernal's peripheral LNP formulation that is optimized to redirect biodistribution away from the hepatic system and into secondary lymphoid organs, including the spleen, thymus, lymph nodes. Importantly, in addition to LNP targeting, we sequence engineer the mRNA payload for T cell selective CAR expression to limit the potential off-target CAR expression in different cell types.

Results: We developed T cell targeted tLNPs that exhibit reporter protein expression in nearly all $CD4^+$ and $CD8^+$ T cells (90%). The selectivity of mRNA-encoded receptor protein expression in T cells is >20-fold over myeloid cells and >60-fold over B cells. In addition to our *in vivo* data, we demonstrated similar selectivity in human peripheral blood mononuclear cells (PBMCs). Next, we delivered human CAR mRNAs into human primary T cells with targeted tLNPs and observed CAR-mediated T cell activation within 24 hrs. Furthermore, functional B cell depletion was achieved in mice with *in situ* CAR T cell programming. tLNPs encapsulating murine CAR mRNAs were intravenously administered to C57BL/6J mice. Within days, peripheral B cells in the blood were depleted, demonstrating the efficient CAR mRNA delivery into T cells, their quick activation and *in vivo* cytolytic capacity of *in situ* programmed CAR T cells.

Conclusion: Our goal is to advance our novel state-of-the-art in situ CAR T cell therapy, making a transformative difference in the way the therapy is administered, manufactured and tolerated. If successful, patients with B cell malignancies and with autoimmune diseases will benefit from these CAR mRNA tLNPs that induce effective B cell depletion *in vivo* with reduced side effects, owing to the highly cell-selective, and tolerable transient nature of targeted mRNA tLNP therapeutics.

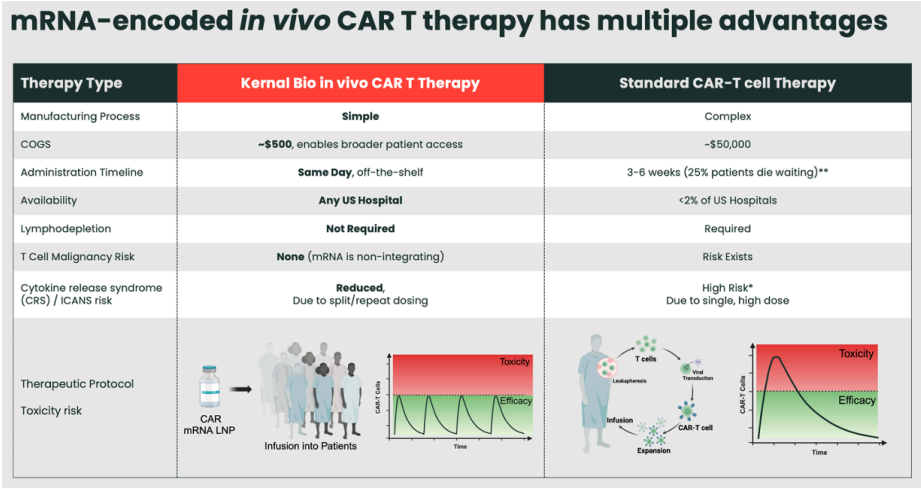

Figure 1. Comparison of standard with *in situ* mRNA tLNP CAR-T cell therapies.

1754 CD19-CAR and IL-10 Reprogramming of $CD4^+$ T Cells Generates Type 1 Regulatory CAR-T Cells for the Treatment of Autoimmune Disease

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Autologous CAR-T cells have been remarkably successful in treating B-cell lineage malignancies. Building on this success, autologous CAR-T cell targeting of B cells in various autoimmune conditions has yielded promising early clinical data. However, broad implementation and delivery of autologous cell therapy to large numbers of patients still faces significant challenges, such as inflammatory toxicities (e.g., cytokine release syndrome (CRS) and ICANS), scaling manufacturing to reach all eligible patients, and high cost. Moreover, specifically targeting B cells by conventional CAR-T cells may be sub-optimal in T-cell and B-cell driven autoimmune diseases.

Type 1 regulatory T (Tr1) cells are a subset of peripherally derived FOXP3$^-$ CD4$^+$ T cells known to limit pathogenic T-cell responses across many inflammatory and autoimmune diseases. Tr1 cells suppress inflammation largely through secretion of the anti-inflammatory cytokines, IL-10 and TGF-b, in addition to contact-mediated T-cell suppression. To target autoimmune diseases driven by both B and T cells, we are developing TRX319, a CD19-CAR targeted, type 1 regulatory T (CAR-Tr1) cell product intended for allogeneic use and off-the-shelf availability. TRX319 combines both Tr1 immunoregulatory properties and B-cell depleting functionality to broadly target inflammatory and autoimmune pathways. TRX319 is generated by transducing isolated CD4$^+$ T cells with a lentiviral vector encoding both CD19-CAR and IL-10 through a production process that yields up to 1.6 x 10^{10} cells per manufacturing run.

TRX319 cells were highly pure, reaching 95.4 ± 2.5% CAR$^+$ at the end of manufacturing. TRX319 exhibited hallmark Tr1 characteristics, including a secreted cytokine profile of high IL-10, high IL-22, and low IL-2 relative to control CAR-T cells. The immunoregulatory capacity of TRX319 was demonstrated by suppression of effector T-cell proliferation and suppression of TNF-a and IL-1b secretion by activated monocytes. TRX319 cells demonstrated robust CAR-driven cytotoxicity against the CD19$^+$ NALM6 cell line and primary B cells from autoimmune disease patients in vitro. TRX319 also exhibited significant B-cell depletion in vivo and performed comparably to conventional CAR-T cells in the disseminated NALM6 B-cell leukemia model.

IL-10 reprogramming of CD4$^+$ T cells during TRX319 production resulted in reduced immunogenicity as demonstrated by reduced T cell activation and outgrowth in mixed lymphocyte reactions with multiple donors. This IL-10-dependent reprogramming was further associated with reduced proliferation of TRX319 in response to allogeneic HLA, suggesting that TRX319 presents a reduced risk of GvHD when administered in patients. Further, in an in vitro CRS model, TRX319 exhibited reduced potential to induce IL-6 secretion by monocytes compared to conventional CAR-T cells.

Overall, TRX319 is a promising allogeneic cell therapy for a broad range of autoimmune diseases by coupling Tr1-mediated regulatory function with CAR-T cytotoxicity against B cells.

1755 Role of CD4$^+$ T cell and NK cell subpopulations in CMV IFN-γ production in CMV-specific T-cell products

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Background: Third-party virus-specific T cell (VST) products represent a safe, effective therapy for cytomegalovirus (CMV) infections in post-transplantation, immunodeficient patients. However, understanding the mechanisms that transform donor anti-CMV immunity into clinically relevant anti-CMV activity in VST remains limited, hindering the optimization and standardization of potent, off-the-shelf VSTs. Current protocols use interferon-γ (IFN-γ) production as a surrogate for VST cytotoxicity. Donor CMV humoral immunity does not reliably predict the presence of cells responsive to CMV IFN-γ upon *in vitro* CMV peptide stimulation. There is a need to quantify factors such as HLA restriction of CMV immunodominant peptides, donor-specific cytotoxic activity, and manufacturing variations to evaluate their impact on clinical outcomes. We investigated the HLA and cellular factors influencing CMV IFN-γ production in CMV-seropositive donor-derived VST products.

Methods & Results: We analyzed 533 individual tetravalent VST products (generated following exposure to peptide libraries from CMV, EBV, adenovirus and BK viruses) derived from 281 CMV-seropositive and 252 CMV-seronegative donors of predominantly white Caucasian descent. We confirmed that the percentage of CMV IFN-γ-expressing T-cells in VSTs from CMV-seropositive donors was increased over 50 times [mean: 3.43 ± 4.9% vs. 0.06 ± 0.1%, $p<0.01$] compared to seronegative donors. HLA class I genotypes HLA-A*02:01, B*07:02, and C*08:01 were more frequently observed in CMV-seropositive donors ($p<0.05$). In contrast, the HLA class II genotype DRB1*04:01 was less frequently observed in CMV-seropositive donors compared to seronegative donors [24/281(8.5%) vs 40/252(15.9%), $p<0.01$]. However, these HLA alleles did not correlate with the frequency of CMV IFN-γ-producing cells in VST products. Phenotypic analysis of donor-derived mononuclear cells (MNCs) showed increased fractions of $CD8^+$ and $CD56^+$ cells in CMV-seropositive donors compared to CMV-seronegative donors ($p < 0.01$). Phenotypic analysis of VSTs revealed higher fractions of $CD8^+$ cells, lower fractions of $CD4^+$ cells ($p < 0.01$), and a reduced $CD4^+/CD8^+$ cell ratio ($p<0.05$) in CMV-seropositive donors. However, these differences showed only weak correlation with CMV IFN-γ production (r_s=0.12-0.37, $p<0.05$). In a representative subgroup of VST products with a wide range of CMV IFN-γ production capability (0.06-14.89%) from 8 CMV-seropositive and 6 CMV-seronegative donors, we found lower frequencies of naïve NKG2C-/NKG2A- $CD4^+$ T cells in VST products from CMV-seropositive donors compared to CMV-seronegative donors (55.5 ± 23.3% vs. 85.1 ± 7.3%; $p<0.01$, 95% CI: -49.6 to -9.5). These naïve $CD4^+$ T cells expressed lower levels of PD-1, a marker of T-cell exhaustion. We also found a strong correlation between CMV IFN-γ production and the content of NKG2A+ $CD4^+$ T cells (r=0.65-0.82, $p<0.01$), and both double-positive and double-negative NKG2C/NKG2A fractions in $CD16^+/CD56^{dim}$ mature NK cells (r=0.83 and -0.71, respectively, $p<0.01$).

Conclusion: The enumeration and typing of specific $CD4^+$ T cell and NK cell subpopulations of VST products may help in the selection of products with clinically-relevant adoptive immune activity against CMV infections. Our data suggests that IFN-γ plays a complex role in coordinating immune responses involving both adaptive and innate immune responses.

1756 *Overcoming Barriers for T-cell Therapy of Chronic Hepatitis B Virus Infection*

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Hepatitis B virus (HBV) infection is a significant global health problem, with 254 million individuals with chronic infection and a death toll of 1.1 million/year. Current treatment options require lifelong therapy and only rarely achieve viral clearance. In cases of resolved infection, viral eradication is closely linked to a robust T-cell response, while chronically infected patients typically have few and partially dysfunctional T-cells. Adoptive T-cell transfer and therapeutic vaccination have emerged as promising alternative therapeutic strategies for restoring HBV-specific T-cell responses.

Michler et al. have previously shown that a therapeutic vaccination's effectiveness in mice strongly depends on the replication level of HBV, and the vaccine was less effective in high-level HBV-carrier mice. The first clinical studies demonstrated the efficacy of HBV T-cell receptor (TCR) T-cell therapy in patients with HBV-related hepatocellular carcinoma, achieving significant and sustained hepatitis B surface antigen HBsAg (HBsAg) reduction or clearance in 4 out of 6 patients. One patient with initially high HBsAg levels experienced recurring titers post-treatment. These results suggest that high viral antigen levels could prevent the induction of an effective immune response against HBV. Therefore, this study aims to clarify whether high HBV infection levels influence the efficacy of T-cell therapy. We infected immunocompetent C57BL/6 mice with an adeno-associated virus vector carrying a replication-competent hepatitis B virus genome at increasing titers to establish low- and high-titer HBV carrier mice. These mice received two doses of Cor93-TCR^+ $CD8^+$ T cells directed against the immunodominant epitope in the HBV core protein and were monitored over ten weeks. Blood and liver samples were obtained to analyze viral parameters, HBV-specific immune responses, and liver damage.

In low-titer HBV carriers, transferring $1x10^6$ TCR^+ T cells achieved viral clearance associated with transient liver damage. However, when the lower dose with $0.1x10^6$ TCR^+ T cells was administered, 4 out of 5 animals eliminated the virus. In contrast, animals with high infection levels did not achieve HBV clearance at all, regardless of the number of T cells transferred. When examining the transferred Cor93-TCR^+ $CD8^+$ T cells ex vivo by flow cytometry, we observed signs of exhaustion and dysfunction. T-cells showed an increased PD-1, Lag-3, TOX, and 41BB expression in the high-titer HBV-carrier mice, and ex vivo restimulation resulted in decreased cytokine secretion.

Our data indicates that viral titers significantly impact the effector function of TCR^+ T cells. Single-cell RNA sequencing analyses have been initiated to better understand the dysfunction of the TCR^+ T cells. This will provide deeper insights into the molecular patterns of the T cells, potentially revealing strategies to overcome the limitations of T-cell therapy for chronic HBV infection.

1757 UltraSlice Gene-Editing mRNA Enables Efficient Transgene Insertion into *TRAC* Locus in Primary Human T Cells

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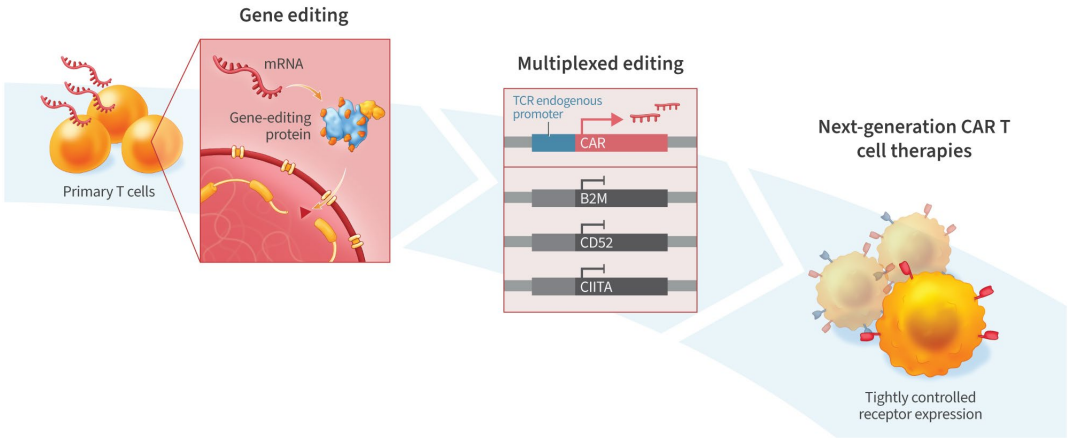
Chimeric antigen receptor (CAR) T cell therapies can successfully treat B cell malignancies and have shown promising results for autoimmune conditions such as systemic lupus erythematosus (SLE). However, manufacturing complexities of autologous CAR T cell therapies have limited their widespread use. Allogenic CAR T cell therapies are being developed to address these limitations, yet CAR insertion with lentiviral and retroviral vectors present their own manufacturing challenges at scale, and allogenic CD19 CAR T cells generated using these vectors have shown lower persistence and expansion *in vivo* than their autologous counterparts. Placing CAR expression under the control of the endogenous T cell receptor promoter by targeted insertion of the CAR transgene to the *TRAC* locus has demonstrated lower levels of T cell exhaustion and enhanced persistence in animal models. Efficient gene editing strategies to precisely engineer CAR T cell therapy products may therefore overcome the manufacturing, persistence, and expansion challenges of existing technologies.

Here, we show that novel mRNA-encoded gene-editing nucleases enable efficient targeted insertion of a CD19 CAR transgene into the *TRAC* locus in primary human T cells. Electroporation of mRNA encoding UltraSlice™ sequence-specific endonucleases results in on-target editing of *TRAC*, *B2M*, *CD52*, and *CIITA* with up to 80% knock-out efficiency as assessed via flow cytometry of TCRαβ, HLA-ABC, CD52, and HLA-DR, respectively. Co-delivery of a transgene sequence resulted in insertion into the *TRAC* locus with 42% efficiency, as assessed by flow cytometry and PCR (n=3 PBMC donors). Simultaneous delivery of mRNA encoding UltraSlice™ targeting *TRAC* and *B2M* enabled multiplexed gene knockout, with 23% of cells demonstrating lack of both TCRαβ and HLA-ABC surface receptors, as assessed by flow cytometry. Co-delivery of *TRAC*- and *B2M*-targeting UltraSlice™ mRNA and a *TRAC*-targeted CD19 CAR transgene sequence yielded CD19 CAR T cells lacking both TCRαβ and HLA-ABC. Up to 6% of T cells expressed the CD19 CAR 72 hours after electroporation, comparable to lentiviral transduction. *TRAC*-replaced CD19 CAR T cells lysed 65% of RAJI B cells, significantly more than wildtype T cells (15%, $p<1e-5$). The efficient generation of potent allogeneic CAR T cells using UltraSlice™ gene editing mRNA may prove useful for the development of more affordable and reliable cell therapies.



1758 Second Generation CD4^{+} CAR T Cells Incorporating a 2B4 Cosignaling Domain Exhibit Enhanced Proliferation and CD8^{+} CAR Help

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Background: Despite early studies on redirected cytotoxic T lymphocytes to specifically recognize HIV envelope-expressing cells, and the success of chimeric antigen receptor (CAR) T cell therapy for treatment of B cell malignancies, successful CAR T cell therapy for HIV has remained elusive. Particularly, the lack of persistence and long-term efficacy in patients is a central hurdle for current CAR T therapies. Expanding the intracellular cosignaling domain (ICD) repertoire to include receptors found on early responding/innate cells may expand the knowledge of how signaling domains from other cell types modulate T cell function. This may in turn inform immunotherapies tuned to optimize distinct aspects of T cell function.

Methods: CD4 and CD8 T cells were separated prior to manufacture and redirected with lentiviral vectors expressing CARs comprised of an HIV-envelope targeting CD4 receptor and one of ten unique costimulatory domains from receptors differentially expressed on αβ T cells, B cells, and NK cells. CAR T cells were assessed for antigen-induced proliferation, HIV suppression, and targeted lysis *in vitro* at baseline and during chronic antigen stimulation.

Results: While CD244 (2B4) is considered an activation receptor on natural killer cells, signaling through adaptor molecules SH2D1A (SAP) or SH2D1B (EAT-2), it is often considered an inhibitory receptor on T cells. Therefore, we sought to engineer and further interrogate CD4-ectodomain (CD4E) CAR T cells with an intracellular 2B4 cosignaling domain to determine the functional impact of 2B4 signaling on CD4 and CD8 T cells. When tested in a panel of 10 distinct cosignaling domains, 2B4^{CAR4} exhibited superlative antigen-driven proliferation over several weeks of stimulation, which was not the case for 2B4^{CAR8}. Further, in an *in vitro* co-culture assay, 2B4^{CAR4} improved expansion of CD28^{CAR8} as compared to CD3z^{CAR4}, CD28^{CAR4}, or 41BB^{CAR4}. In a comparison between co-culture and transwell assays, the fold expansion was significantly higher in cell-cell contact conditions as compared to transwell separation, suggesting that CD4 T cell help to CD8s is contact-dependent. To better understand the effect of 2B4 cosignaling domain on CD8 T cell expansion and survival, we cultured CD4 and CD8 T cells separately *in vitro* and redirected them with CAR vectors containing CD28 or 2B4 ICDs. Immunodeficient mice were reconstituted with human donor CD4 T cells followed by singular injection of CD28^{CAR8} cells or co-injection of CD28^{CAR8} with CD28^{CAR4} or 2B4^{CAR4}. Co-injection of CD8 CAR T cells with 2B4^{CAR4} exhibited the highest expansion levels. Finally, we show that SAP and SHP-2 transcripts are upregulated to similar levels after antigen-driven stimulation;

but, in contrast, at both baseline and post-stimulation, CAR T cells show little to no EAT-2 transcript expression. Importantly, EAT-2 is thought to play a role in the inhibitory nature of CD244 signaling.

Conclusions: 2B4 contains four cytoplasmic immunotyrosine-based switch motifs (ITSMs), which can either be costimulatory or inhibitory depending on which ITSM is phosphorylated, the overall expression level of the receptor itself, and the level of signaling adaptor molecules SAP (SH2D1A) or EAT-2 (SH2D1B). This data suggests that in the context of CD4+ CAR T cells, the 2B4 signaling domain is capable of being co-opted as a costimulatory domain, enhancing persistence of CD4+ CAR T cells and improving the initial expansion of CD8 CARs.

1759 Synergizing Broadly Neutralizing Antibody-Secreting T Cells and CAR-T Cells Against HIV-1 Infection

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Introduction: A functional cure of HIV-1 is precluded by latent HIV-1 reservoirs that cause viral rebound when antiretroviral therapy (ART) is interrupted. Broadly neutralizing antibodies (bNAbs) that target highly conserved HIV gp120 epitopes can maintain HIV-1 suppression in people living with HIV (PLWH) in the absence of ART, providing an approach to achieve ART-free HIV-1 remission. However, monthly or bi-monthly administration may be required to maintain therapeutic levels of bNAbs, which can reduce adherence. Genomically engineered T cells, such as CAR-T cells, have shown decade-long persistence with therapeutic function and can migrate to areas of infection where free bNAb may have limited access to. We hypothesize that T cells can be engineered to sustainably secret bNAbs. As a proof-of-concept, we engineered a lentiviral vector (LV) encoding 3BNC117 bNAb (3BNC) to transduce T cells. We further evaluated the synergistic effects of 3BNC-expressing T (3BNC-T) cells and anti-HIV CAR-T cells against HIV-1 infection.

Methods: CD3+ T cells were purified from both healthy donors and PLWH, transduced with 3BNC LV and evaluated *in vitro* for anti-HIV activity and 3BNC production by co-culturing with autologous HIV-1-infected PBMCs. 3BNC-T cells were intrasplenically injected into NSG mice and maintained for 2 months to monitor long-term 3BNC secretion. *In vivo* synergistic antiviral function was determined by intrasplenical co-injection of autologous HIV-1-infected PBMCs and effector cells (3BNC-T or CAR-T cells alone or both) into NSG mice. Viral suppression and 3BNC serum levels were measured after 2 weeks.

Results: The transduction efficiency of 3BNC LV was comparable between healthy donors and PLWH. 3BNC was produced at comparable levels between healthy donors and PLWH. At an E:T ratio of 5:1, 3BNC-T cells suppressed HIV-1 infection by an average of 53 ± 1 % *in vitro* as compared to untransduced T cells, with a mean 3BNC concentration of 232 ± 48 ng/ml. In addition, the combination of 3BNC-T and CAR-T cells elicited significant enhancement of HIV-1 killing as compared to CAR-T cells alone ($p < 0.01$). To determine the *in vivo* production of 3BNC, 30 x 10^6 T cells (59% were 3BNC+) were intrasplenically injected into NSG mice. Plasma 3BNC concentration measured weekly were above 1.5 µg/ml throughout the 8-week experiment. In HIV-1-infected NSG mice, plasma 3BNC levels were detected in 3BNC-T cell only (323 ± 46 ng/ml) and combination therapy (414 ± 60 ng/ml) treated animals. Plasma HIV-1 RNA (copies/ml) was most suppressed in combination therapy treated mice (6.0 ± 2.1 x 10^4), followed by CAR-T cell only treated mice (2.5 ± 1.1 x 10^5), compared to control mice (7.2 ± 1.5 x 10^5). Consistently, spleen HIV-1 DNA (copies/ul) was most suppressed in combination therapy treated mice (24.3 ± 5.0), followed by CAR-T cell only treated mice (51.0 ± 5.5), compared to control mice (114.5 ± 40.5). There was no HIV-1 suppression in 3BNC-T cell only treated mice.

Conclusions: We successfully engineered T cells that secrete bNAb, and exhibit *in vitro* anti-HIV function and sustained *in vivo* bNAb production. BNAb-secreting T cells can synergize with CAR-T cells both *in vitro* and in mice to enhance HIV-1 killing as compared to CAR-T cells alone. This novel therapy combining cellular and humoral anti-HIV approach provides a new strategy to generate more potent immunotherapeutics, which may contribute to a functional cure of HIV-1 infection.

1760 Enhancing Engineered B Cells through Expression of B Cell Activating Molecules

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We use CRISPR/Cas9-mediated gene editing to engineer B cells to express custom heavy-chain only antibodies (HCAbs) against hard-to-vaccinate targets such as HIV[1]. The edited B cells express both B cell receptor (BCR) and secreted antibody isoforms and retain important natural features of B cell biology, such as the ability to respond to antigen in tonsil organoid models of immunization. However, in addition to the signals resulting from the interaction of a BCR and its target antigen, B cells also require co-stimulatory signals. These are primarily provided by helper T cells, and allow the B cells to enter germinal centers, expand, generate memory cells, and differentiate into long-lived plasma cells. However, helper T cell functions are frequently impaired in people living with HIV, even when the disease is well-controlled by antiretroviral drugs[2]. Therefore, we are interested in developing a strategy that enhances the ability of HCAb-engineered B cells to respond to antigens *in vivo*, even after ex vivo culture and in immunocompromised hosts.

Toward that goal, we screened a panel of synthetic molecules, designed to mimic the co-stimulatory signals received by B cells and to induce a range of B cell activation states. Among these B cell activating molecules (BAMs), we identified constitutively active, drug-inducible, and chimeric antigen receptor-based BAMs that activated B cells *in vitro*. BAM-expressing B cells had higher fitness in the tonsil organoid model and expanded even in the absence of antigen. Finally, bulk RNA-sequencing revealed a candidate BAM that specifically induced pathways associated with T cell help, B cell-T cell interactions and class II antigen presentation. Finally, because HIV infection perturbs the ability of T cells to provide help to B cells, we are currently evaluating whether dual-engineered HCAb/BAM cells are better able to control HIV infection in tonsil organoids.

In sum, we propose a novel approach to enhancing the functionality of engineered B cells under conditions where T cell help may be suboptimal, such as HIV infection, oncology or autoimmune diseases.

1. Rogers, G.L., Huang, C., Mathur, A. *et al.* Reprogramming human B cells with custom heavy-chain antibodies. *Nat. Biomed. Eng* **8**, 1700–1714 (2024).
2. Cubas, R., Mudd, J., Savoye, AL. *et al.* Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med* **19**, 494–499 (2013).

1761 Enhanced CART Function by Arming CAR-T Cells with Cytokines

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Despite the remarkable success of Chimeric Antigen Receptor T-cell (CAR-T) therapy in treating hematologic malignancies, its efficacy in solid tumors and long-term response is often hindered by T-cell dysfunction and poor persistence. Cytokines are essential regulators of immune responses, and their optimization could enhance CAR-T cell function. Many pro-inflammatory cytokines such as IL15, IL12 and IL7 have been utilized in the adoptive cell transfers and their clinical applications are hindered by toxicity associated with systematic expression of cytokines.

On the contrary, some unconventional cytokine such as IL10 and IL4 are reported to enhance CART function while does not trigger systematic toxicity.

In this study, we systematically screened 20 cytokines that does not seem to be pro-inflammatory and belong to the common beta chain family, IL6 family and common gamma chain family, and IL10 families. Using tumor-bearing mice model, we found 12 novel cytokines that can potentially enhance CART functions in vivo without incurring apparent toxicity in the mice model. Interestingly, we found dosage dependent feature for different cytokines, some of them are beneficial with both MOI transduction and some of them only work for low or high concentration. We quantified the increased of efficiency by titrating the amount of CART-cytokine infusion cell number and found some of them will enhance the efficacy with < 1% of the wt CART without cytokine infusion. To further explore the therapeutic potential of this finding and minimize the systematic impact of this cytokine, we attempted to engineer the cytokine receptor pathway of the identified factors. We successfully recapitulate the results of cytokine-X effect by having the constitutive activation receptor. Such construct increases the cart efficacy in vivo and seems to not tumorigenesis based on cytokine independent growth assay and PK in the tumor bearing mice model.

Taken together, tur results highlighted specific cytokines that not only enhanced CAR-T cell expansion and persistence but also improved their anti-tumor activity. Moreover, certain cytokines demonstrated a synergistic effect, further potentiating CAR-T cell function. These findings present new avenues for cytokine-based strategies to optimize CAR-T cell therapies and provide a foundation for future clinical trials aimed at enhancing the therapeutic potential of CAR-T cells in cancer treatment.

1762 In Vivo Generation of anti-CD19 CAR T Cells Utilizing Circular RNA Encapsulated in Targeted Lipid Nanoparticles

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Background: Chimeric Antigen Receptor (CAR) T-cell therapy has revolutionized cancer treatment and shown promise in addressing autoimmune diseases. However, current *ex vivo* CAR T-cell therapies encounter challenges such as complex manufacturing processes, scalability issues, reliance on viral vectors, and the necessity for lymphodepleting chemotherapy, which limit broader clinical applications. To address these limitations, there is a critical need for an off-the-shelf *in vivo* CAR technology that can simplify delivery and expand therapeutic potential. We have developed tLNP-circCAR, a novel in vivo anti-CD19-CAR circular RNA product delivered via targeted lipid nanoparticles (tLNPs). Preclinical studies in animal models demonstrate its efficacy in B cell depletion, marking a significant advancement towards overcoming existing barriers in CAR T-cell therapy and expanding its applicability to a broader range of diseases.

Methods: tLNP-circCAR encapsulates a circular RNA encoding an anti-CD19-CAR within a tLNP containing a novel ionizable lipid and a T cell targeting domain, enabling efficient transfection of primary human T cells. The functional activity of tLNP-circCAR-mediated CAR T cells was evaluated using multiple *in vitro* and *in vivo* systems.

Result: In activated primary human T cells, circCAR mediated CAR expression lasted up to 9 days. Notably, tLNP-circCAR efficiently engineered non-activated primary human T cells into functional CAR T cells. *In vitro*, these engineered CAR T cells exhibited robust cytotoxicity against autologous B cells and the $CD19^+$ Raji cell line. *In vivo*, utilizing a humanized NOG-PBMC mouse model, a single intravenous dose of tLNP-circCAR resulted in efficient CAR engineering of T cells, leading to significant B cell depletion in the spleen. Additionally, in humanized NOG-PBMC mice bearing CD19+ Raji lymphoma xenografts, tLNP-circCAR treatment induced tumor remission.

Conclusion: tLNP-circCAR represents a novel *in vivo* CAR therapy that delivers therapeutic anti-CD19 CAR circular RNA efficiently to human T cells, generating highly functional CAR T cells both *in vitro* and *in vivo*. These

results support the further development of tLNP-circCAR for treating B cell disorders, including B cell lymphoma and autoimmune diseases. This innovative approach promises to enhance the accessibility and efficacy of CAR T-cell therapy.

1763 INB-600: A Novel T Cell Engager Platform Specific for gamma-delta (γδ) T cells

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Introduction: Although autoreactive B cells comprise only 0.1-0.5% of total B cells, current long-term depletion strategies remain inadequate, exposing patients to severe treatment-related side effects, potential mortality, and impaired quality of life. Clinical data with alpha-beta (αβ) T cell-based chimeric antigen receptor T cell (CAR-T) and T cell engagers (TCE) have demonstrated risks of cytokine release syndrome (CRS) or neurotoxicity (ICANs) and in the case of CAR-T, may require lymphodepletion protocols that can result in ovarian damage and infertility. In patients across three trials (NCT03533816, NCT04165941, and NCT05664243) in two indications and at multiple centers, neither CRS nor ICANs have been observed in patients treated with gamma-delta (γδ) T cells to date. γδ T cell engagers (γδ TCEs) represent a new frontier in immunotherapy, leveraging the unique properties of γδ T cells to provide MHC-independent target recognition, strong immune activation, and improved treatment safety. We now present a novel bi-specific γδ TCE that enables binding and robust expansion of both major γδ T cell subsets and the elimination of target cells. Although IN8bio is developing this CD19-γδ TCE (INB-619) for potential applications in both autoimmune disease and hematologic malignancies, autoimmune indications are presented here.

Methods: INB-619 is added to the culture of γδ T and target cells, and the TCE binding specificity, T cell activation, TCE-induced target cell cytotoxicity, and cytokine release were analyzed by flow cytometry. To determine B cell depletion, γδ T cell expansion, and optimal dosing, incremental concentrations of the INB-619, along with a no-treatment (NT) control and zoledronate treatment (Zol), were cultured with peripheral blood mononuclear cells (PBMC) for up to 10 days from two healthy donors.

Results: INB-619 exhibited concentration-dependent cytotoxicity at an effector-to-target (E:T) ratio of 1:1 against the CD19+ Nalm6 cell line but did not bind nor kill the CD19- myeloid cell line, MOLM-13, at any concentration. Early observations suggest partial competitive binding with Zoledronic acid-derived isoprenyl pyrophosphate, potentially indicating non-exclusive Vd2 receptor binding. Treatment with γδ TCE in a co-culture of γδ T cells and CD19+ Nalm6 target cells (E:T = 1) induced concentration-dependent γδ T cell degranulation, upregulation of activation markers, and the release of inflammatory cytokines such as TNF-α, IFN-γ, IL-4, minor concentrations of IL-17 and IL-6, and cytotoxic molecules including perforin, granzymes, and granulysin. The culture of INB-619 with PBMC revealed a concentration-dependent expansion of both Vd1+ and Vδ2+ γδ T cells up to 450-fold over 10 days, which was sustained through the complete elimination of B cells.

Conclusion: In addition to its specific target binding and robust cytotoxicity, this novel γδ TCE promotes the expansion of both Vδ1+ and Vδ2+ γδ T cells, thereby sustaining its killing effect over time. This approach harnesses the advantages of Vδ2-mediated antigen processing and the long-term durability of Vδ1+ T cells. Should this approach be confirmed in autoimmune disease models, INB-619 could represent a promising off-the-shelf therapy for autoimmune diseases, potentially with fewer side-effects, mobilizing both circulating and tissue-resident γδ T cells to eliminate autoreactive B cells and reset B cell hematop

1764 Treg Specific Synthetic Promoters (TRSP) for Antigen Specific Treg Therapy

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Antigen-specific regulatory T (Treg) cell therapy holds promise for treating autoimmune disorders, preventing organ transplant rejection, and managing inflammation-associated diseases. However, several challenges arise from the intrinsic properties of Treg cells, including the difficulty of generating pure antigen-specific Treg cells without effector T (Teff) cells contamination, maintaining Treg stability under inflammatory conditions, and ensuring in vivo persistence (1,2).

We developed **Tr**eg **s**pecific **S**ynthetic **P**romoters (TRSP) as a robust solution for addressing key challenges in Treg-based therapy through a high throughput library screening. With a remarkably small footprint, TRSP demonstrated Treg-specific expression and minimal activity in Teff cells, outperforming the published CNS123p promoter derived from endogenous human *FOXP3* regulatory elements (3). More importantly, TRSP transcriptional activity was absent in destabilized Treg cells, whether caused by internal tonic CAR signaling or by exposure to proinflammatory cytokines. Furthermore, TRSP-CAR Treg cells exhibited superior suppressive cytokine profile and functional suppressive effects compared to Treg cells expressing the same CAR under the MND promoter. To assess safety, we engineered Tregs and Teffs with a constitutive IL2 signal for persistence. MND-based constructs promoted Teff expansion, whereas TRSP constructs did not, confirming their safety in the presence of Teff contamination in the cell product. Lastly, to accommodate diverse therapeutic needs, we developed a toolbox of TRSP variants with various transcriptional strengths, all maintaining Treg specificity.

Overall, our TRSP solution enables precise, Treg-specific payload expression, which significantly enhances the safety and efficacy of antigen-specific Treg therapies and ensures the promise of Treg cell therapy.

References:

1. Raffin, Caroline, Linda T. Vo, and Jeffrey A. Bluestone. “Treg Cell-Based Therapies: Challenges and Perspectives.” Nature Reviews Immunology 20, no. 3 (March 2020): 158–72. https://doi.org/10.1038/s41577-019-0232-6.
2. Ho, Patrick, Ellen Cahir-McFarland, Jason D. Fontenot, Tracey Lodie, Adel Nada, Qizhi Tang, Laurence A. Turka, and Jeffrey A. Bluestone. “Harnessing Regulatory T Cells to Establish Immune Tolerance.” Science Translational Medicine 16, no. 738 (March 13, 2024): eadm8859. https://doi.org/10.1126/scitranslmed.adm8859.
3. Masiuk, Katelyn E., Jennifer Laborada, Maria Grazia Roncarolo, Roger P. Hollis, and Donald B. Kohn. “Lentiviral Gene Therapy in HSCs Restores Lineage-Specific Foxp3 Expression and Suppresses Autoimmunity in a Mouse Model of IPEX Syndrome.” *Cell Stem Cell* 24, no. 2 (February 2019): 309-317.e7. https://doi.org/10.1016/j.stem.2018.12.003.

1765 AlloNK: A Scalable and Consistent NK Cell Therapy in Development for Treatment of Oncology and Autoimmune Diseases

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Background: AlloNK® (AB-101) is a non-genetically modified, allogeneic, off the-shelf, cryopreserved natural killer (NK) cell therapy candidate in development for the treatment of cancer and autoimmune diseases. A highly scaled manufacturing process enables production of thousands of 1 billion cell doses from a single donor cord blood

unit (CBU). AlloNK in combination with B-cell depleting monoclonal antibodies (mAbs) has the potential to enhance the antibody-dependent cellular cytotoxicity (ADCC) response thereby leading to deeper B-cell depletion without the safety challenges associated with T-cell approaches, including high rates of cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). Initial clinical data with rituximab and AlloNK in subjects with advanced B-cell malignancies showed that this treatment approach is well tolerated, and preliminary efficacy of the combination treatment is encouraging (Khanal R 2023). AlloNK is currently in multiple clinical trials as a cellular therapeutic for cancer and autoimmune disease. Here we report on AlloNK manufacturing scale, consistency of viability and cytotoxic activity across multiple drug product (DP) lots.

Methods and Results: CBUs are pre-selected for killer-cell immunoglobulin-like receptor (KIR)-B haplotype and the natural high-affinity variant of CD16 (158V/V polymorphism), which is associated with ADCC enhancement, resulting in a highly active NK cell product without the requirement for additional engineering. Following isolation, the NK cells are expanded utilizing a proprietary engineered feeder cell (eFeeder) to derive a master cell bank (MCB). One CBU yields ≥50 cryopreserved vials of MCB. MCB production is followed by a bioreactor-based (50L) industrial scale NK cell expansion and activation process to produce the DP. At this scale, a DP manufacturing run can produce ≥110-115 billion NK cells from a single vial of MCB with over ~98% CD3-/CD56+ purity and high CD16 expression. With at least a 4-year shelf life, AlloNK demonstrates robust viability pre and post cryopreservation, with ~95% viability pre-cryopreservation and ~94% viability post-thaw. A consistent AlloNK-mediated dose-dependent potency is observed in both short-term and long-term cytotoxicity assays, with minimal variability between lots. Long-term ADCC assays also demonstrate prolonged killing activity in combination with anti-CD20 mAbs against ARH-77, a B lymphoblast cell line.

Conclusions: The current scale of AlloNK production from CBU to DP equates to ≥ 4,000 net vials of 1 billion NK cells per CBU, which is enough to potentially treat >1,000 patients (assuming administration of 3 billion cells per patient). The drug product generated is uniformly high in CD16 expression and has consistent potency across lots. The current scale can support both clinical trial demand as well as potential commercial launch. The manufacturing platform will allow for scaling the process to support full commercial production, without significant process modifications. Developing scalable manufacturing solutions with AlloNK is essential to meet the growing demand for cell therapies across various disease areas.

References:
Khanal R, Mehta A, Maly J, et al. AB-101, an Allogeneic, Non-Genetically Modified, Natural Killer (NK) Cell Therapy, Evaluated as Monotherapy or in Combination with Rituximab in R/R Non-Hodgkin Lymphoma. JCO 41, abstract 7529 (2023). DOI:10.1200/JCO.202

1766 Preclinical Development of Allogeneic CD19 CAR-γδT Cells for the Treatment of B-cell Malignancies and Autoimmune Diseases

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Autologous CAR-T cell therapy has demonstrated substantial efficacy in hematologic malignancies, and preliminary data suggest promising results in B cell-mediated autoimmune diseases such as systemic lupus erythematosus (SLE) and systemic sclerosis. However, challenges such as limited accessibility, manufacturing complexities, and the potential for immune-mediated side effects remain. Allogeneic CAR-γδT cells, derived from healthy donors, offer several advantages, including off-the-shelf availability, lower manufacturing costs, and a reduced risk of immune escape. These characteristics make allogeneic CAR-γδT cells a promising alternative for the treatment of B-cell malignancies and autoimmune diseases.
We used lentiviral vectors to deliver a CD19 CAR with a 4-1BB co-stimulatory domain to Vδ2 T cells. *In vitro* experiments demonstrated that CD19 CAR-γδT cells efficiently eliminated CD19-positive Raji and Nalm-6 tumor cell lines and released significant amounts of IFN-γ. Serial killing assays showed that CD19 CAR-γδT cells exhibited sustained anti-tumor activity. To reduce the risk of Host versus Graft (HvG) rejection, we utilized the GDance® platform to reduce the immunogenicity of the CD19 CAR-γδT cells. *In vitro* experiments revealed that

this genetic modification significantly decreased allo-reactive T cell rejection but did not induce killing by allogeneic NK cells, and it did not impair the anti-tumor activity of the CAR-γδT cells. In mouse xenograft models, CD19 CAR-γδT cells effectively inhibited the progression of B-cell leukemia and lymphoma and significantly prolonged the survival of the mice. Additionally, CD19 CAR-γδT cells demonstrated the ability to rapidly eliminate B cells *in vitro*. In humanized mouse models reconstituted with CD34+ hematopoietic stem cells, infusion of CD19 CAR-γδT cells resulted in complete clearance of peripheral blood B cells.
Our preclinical study demonstrates that allogeneic CD19 CAR-γδT cells exhibit robust anti-tumor activity against CD19-positive B cell and malignancies *in vitro* and *in vivo*. These findings support the potential of allogeneic CD19 CAR-γδT cells in treating B-cell malignancies and autoimmune diseases such as SLE and systemic sclerosis, with clinical trials already underway.

1767 Advancing Non-Opioid Pain Management for Osteoarthritis: SN101 Nociceptor Cell Therapy Approach

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Osteoarthritis (OA) is a prevalent condition affecting 250 million people worldwide, with knee OA (KOA) representing the most common form. Chronic pain from KOA, including nociceptive and neuropathic pain, severely impacts quality of life, mobility, and increases reliance on opioids for pain management. Despite promising advances in non-opioid analgesic development, challenges persist due to species-specific differences in pain signaling molecules. Recent breakthroughs in stem cell therapies, particularly human pluripotent stem cells (hPSCs) and human induced pluripotent stem cells (hiPSCs), offer innovative alternatives for chronic pain management by providing an off-the-shelf solution for targeted pain control.

In this study, we explored the potential of hiPSC-derived nociceptor cells and gene therapy approaches to address chronic pain in OA. First, we generated purifiable populations of human sensory neurons, including transient receptor potential cation channel subfamily V member 1 (TRPV1)+, sodium voltage-gated channel alpha subunit 9 (SCN9A)+, and Mas-related G protein-coupled receptor X1 (MRGPRX1)+ neurons from hPSCs. These subsets demonstrated distinct functional polymodalities, with a transcriptomic continuum revealing that mature hPSC-derived sensory neurons (hPSC-SNs) accurately recapitulate the key molecular profiles of human dorsal root ganglia (DRG) neurons.

We tested the therapeutic potential of SN101 hiPSC-derived nociceptor cells in a mouse model of KOA. These SN101 cells, characterized by the expression of pain-related markers SCN9A and TRPV1, exhibited pain-sensing properties *in vitro*, exhibited by calcium influx to noxious stimuli such as capsaicin, mustard oil, and menthol as well as synovial fluid from patients with OA. When transplanted *in vivo*, SN101 cells act as decoys to competitively bind to inflammatory ligands. This mechanism intercepts pain signals typically transmitted by endogenous nociceptors, effectively reducing pain behaviors and alleviating mechanical hypersensitivity. Histological analysis revealed reduced expression of injury markers (ATF3) and pain markers (CGRP) in the DRGs innervating knee joints.

Together, these findings demonstrate that SN101 hiPSC-derived nociceptor cells offer a promising strategy for managing chronic pain in OA. By exploiting the hypoimmunogenic properties of iPSCs and the distinct functional profiles of nociceptor subtypes, SN101 is a novel, non-opioid-based solution for managing pain while also modulating pain transmission at the peripheral level.

1768 *ex vivo* Gene therapy for Artemis-SCID and leaky-SCID patients: results of the French ARTEGENE phase I/II clinical trial

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Genetic deficiency of the endonuclease DCLRE1C/Artemis, a key factor for the Non-Homologous End-Joining (NHEJ) mechanism, causes Severe Combined Immunodeficiency (SCID) characterized by a complete lack of T and B-cells associated with hypersensitivity to ionizing radiations. Allogeneic Hematopoietic Stem and progenitor Cells (HSPCs) transplantation is the gold standard for treating Artemis-SCID. However, graft-versus-host disease and graft rejection, which can lead to post-transplant complications and mortality, are increased in case of HLA incompatibility between the donor and recipient. In this context, the first US clinical trial of lentiviral ex vivo corrected HSPCs in patients with Artemis-Deficient SCID was conducted by Cowan et al. (NEJM 2022). This study utilized pharmacologically targeted low-exposure busulfan, which resulted in the generation of corrected and functional T and B cells after injecting genetically corrected hematopoietic progenitors. While this trial offers valuable insights into the treatment, it is essential to highlight that autoimmune hemolytic anemia occurred in four out of ten patients. A more thorough analysis of post-transplant immune reconstitution is necessary in this case.

We are excited to announce the launch of a European-supported phase I/II clinical trial (NCT05071222) at Necker Hospital in Paris, France. This innovative trial features utilizes a more intense conditioning regimen and a different lentiviral vector (LV) with a more potent promoter (short intron-less EF1α promoter) to regulate the expression of the human DCLRE1C cDNA. We have enrolled two patients with SCID phenotypes (P1 and P3) and one leaky SCID form (P2), with follow-up periods ranging from 6 to 24 months. In two different experimental approaches, we demonstrated that corrected HSPCs derived from the ATMPs initiated productive T-cell rearrangements *in vitro* and recapitulated T-cell differentiation in an *in vivo* xenotransplantation model. For the three patients, the engraftment of gene-corrected HSPCs was quantified in the whole peripheral blood mononuclear cells (PBMCs) and purified CD15+ circulating cells. P1 and P3 experienced a progressive increase in vector copy number (VCN) in PBMCs. Functional T-cell and B-cell compartments were progressively restored as the patients could control viral infections caused by rotavirus and adenovirus without any other specific treatment. Moreover, the severe maternal graft-versus-host disease (GVHD) present in P1 before gene therapy was managed without any post-transplant treatment. In addition, we used a mathematical algorithm to model the CD4+ T-cell reconstitution following the engraftment of gene-corrected cells and compared it with alternative haplo-selected CD34+ cells. We also evaluated the T and B-cell repertoires by sequencing to follow the Immune reconstitution compared to alternative transplantation from our cohort. Our data unequivocally confirm that gene transfer is a valuable strategy to restore a functional T- and B-cell compartment in Artemis patients presenting an SCID phenotype. These results also underlined that the corrected HSPCs restored a functional immune system that efficiently fought against infections or maternal GVHD.

1769 A stable Engineered Regulatory T cell therapy platform with modular targeting for autoimmune and inflammatory diseases

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Regulatory T cells (Tregs) play a central role in establishing and maintaining immune tolerance, tissue homeostasis and preventing fatal autoimmunity. Current Treg therapies face challenges of unstable phenotype in an inflammatory microenvironment, a lack of IL-2 support due to IL-2 scarcity in multiple diseased states, and lack of tissue specificity. Our strategy to address these challenges harnesses a gene editing platform with dual AAV to engineer human regulatory T cells (EngTregs) from bulk CD4+ T cells resulting in the stable expression of FOXP3 and a chemically inducible signaling complex split across both vectors to enrich only the engineered population by selectively providing IL-2 signaling with rapamycin in the edited cells. This modular engineering allows for indication specific, interchangeable tissue specific targeting including TCRs, CARs, or inflammation tuned alarmin receptors. Using transcriptomic analysis, we evaluated the stability of cultured sorted Tregs (cTregs) compared to EngTregs. We found EngTregs expressed higher levels of key "Core Treg" and "FOXP3 Synergy" genes based on bulk and single cell RNA-seq analysis as confirmed by flow cytometry. Additionally, favorable expression pattern of key Treg stability markers, CD27, CD70 and IKZF4 was observed in EngTregs compared to cTregs. Finally, we observed that these correlations have a functional implication as demonstrated by higher expression of key tolerogenic markers in EngTregs compared to cTregs. Utilizing different targeting moieties, we have developed therapeutics for Type 1 Diabetes, B-cell driven autoimmune diseases, and acute inflammatory and ischemic diseases. GNTI-122 is an EngTreg with a TCR specific for IGRP, intended for the treatment of patients with recently diagnosed T1D. GNTI-122 demonstrates strong bystander suppression of islet specific T cells against multiple antigens isolated from T1D patients. In a mouse model of T1D, an islet antigen specific autologous mEngTreg surrogate trafficked to the pancreas, decreased the severity of insulitis, and prevented progression to diabetes. CD19 specific CAR (CAR19) EngTregs were developed to address both B and T cell components involved in B cell driven autoimmune diseases. CAR19 EngTreg demonstrate B cell killing and T cell suppression in vitro, while maintaining lower inflammatory cytokine production compared to CD19 CAR-T effectors. Allogeneic CAR19 EngTregs tested in humanized models of inflammatory disease also resulted in B cell depletion and immune reset with low levels of serum inflammatory cytokines compared to CAR T. Finally, we have developed a novel allogeneic EngTreg demonstrating a Tissue Treg phenotype (Tissue EngTreg) by expression of ST2, an alarmin receptor sensitive to injury induced inflammation, aimed to restore tissue homeostasis, repair and regeneration. Tissue EngTregs demonstrate high expression of tolerogenic markers (e.g. CD25, CD39), homing receptors (e.g. CCR5, CCR8), and repair cytokines (e.g. IL-13, Osteopontin) allowing them to serve as "repair ready" for acute inflammatory conditions. Tissue EngTregs have broad applicability to indications of acute inflammation as demonstrated by allogeneic efficacy achieved in various in vivo models of acute lung injury, stroke and acute kidney injury. Taken together, these data are supportive of Engineered Tregs as a potent and stable Treg cellular therapy approach that can be targeted to a broad range of indications.

1770 A Novel *Staphylococcus epidermidis* Compound for the Topical Treatment Epidermal Growth Factor Receptor (EGFR) Inhibitor-Induced Dermal Toxicity

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Systemic therapeutic agents targeting the EGFR-mediated signaling pathway are used for the treatment of advanced cancers, including of the lung, pancreatic, colorectal, and head and neck. Significant dermal toxicities occur in up to 90% of patients treated with EGFR inhibitors (EGFRIs) and other medications inhibiting downstream signaling pathways. These toxicities may be disruptive to a patient's quality of life and adherence to therapy. Inhibition of the EGFR pathway may suppress host defenses and lead to opportunistic pathogenic colonization or infection. Dermal toxicity is associated with elevated levels of *Staphylococcus aureus* and IL-36g. ATR04-484 is a novel *S. epidermidis* strain isolated from a healthy human volunteer, selected for its ability to reduce *S. aureus* colonization and inhibit IL-36g when applied topically.

ATR04-484 has a promising profile of activities for the treatment of EGFRI-induced dermal toxicity by significantly reducing *S. aureus* growth and completely ameliorating IL-36g levels. Reconstructed human epidermis (RHE) and *ex vivo* pig skin were utilized to measure the effect of ATR04-484 on *S. aureus* in therapeutic and prophylactic settings with *S. aureus* added prior to or after topical ATR04-484 application, respectively. ATR04-484 inhibited growth of both methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *S. aureus* in both therapeutic and prophylactic settings. In a therapeutic setting, ATR04-484 inhibited MRSA growth by 1 log (90%) compared to untreated MRSA in both RHE and pig skin. In a prophylactic setting, ATR04-484 inhibited MRSA growth by

approximately 5 logs on RHE and 2 logs on pig skin. IL-36g levels were measured on RHE treated with erlotinib alone or in combination with ATR04-484. Application of ATR04-484 reduced erlotinib-induced IL-36g to a level comparable to non-erlotinib treated RHE ($p<0.001$). The effect was dose-dependent; application of 10^9CFU/cm^2 of ATR04-484 trended towards more potent IL-36g reduction compared to 10^8 CFU/cm^2.

An ongoing multicenter, randomized, double-blind, vehicle-controlled Phase 1/2 clinical study is designed to evaluate the safety and tolerability of topical ATR04-484 (10^9 CFU/g) for the treatment of EGFRI-induced dermal toxicity affecting the face of adult patients. ATR04-484 or its vehicle (3:1 randomization) will be applied in a stable volume to all patients, including the face, with application to prioritize affected areas of the neck, chest, back, and paronychial areas (using remaining product on unaffected skin in the same areas). The key objectives of the study are to assess the safety and tolerability of topical ATR04-484 and to evaluate efficacy signals including severity of disease, pruritus, and pain. The bioavailability of ATR04-484 and pharmacodynamic parameters (including IL-36g) are also studied. This clinical study will establish the basis for continued clinical development of ATR04-484.

1771 Reaching Remission: Harnessing Master Regulator Transcription Factor *Zbtb20* for the Development of a Cellular Therapy against IBD

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Inflammatory bowel disease (IBD) poses significant health challenges that diminish the quality of life for patients; its increasing prevalence necessitates the generation of innovative therapeutic approaches. Published data from Dr. Derek Sant'Angelo's lab revealed that the expression of the master regulator transcription factor, Zbtb20, in endogenous T cells, effectively protects mice against this disease. These data led us to hypothesize that genetically engineered Zbtb20-expressing CD4 T-cells could serve as a novel therapy to treat and prevent IBD, thus improving outcomes of afflicted patients. To begin investigating this question, we constructed and optimized the generation of recombinant retrovirus containing Zbtb20, cloned into the retrovirus-producing vector, pMSCV-IRES-GFP. Conventional mouse CD4 T cells were transduced by either Zbtb20-containing or empty vector (EV) retrovirus. We studied the functional and genetic changes induced by ectopic Zbtb20 expression by FACS and RT-qPCR, indicating the significant upregulation of IL-10, a 3-fold increase compared to EV control, and Treg markers in the Zbtb20-expressing cells. To determine the therapeutic potential of these transduced cells, we adoptively transferred 500k T cells, either Zbtb20 or EV control transduced, into the Dextran Sulfate Sodium-induced colitis model and monitored weight over 11 days. Our studies into the induced DSS model indicated a minor rescuing effect with the Zbtb20-expressing cells and homing of the transferred cells to inflammation in the colon. These data indicate that Zbtb20-expressing CD4 T cells produced in-vitro have the potential to serve as a cellular therapy for IBD, though more optimization of cell engineering is needed. Ongoing experiments in the RAG1KO mouse model aim to determine therapeutic function in a T cell-driven IBD model. Future studies will characterize genes controlled by Zbtb20 through bulk RNA-seq of transduced cells and elucidate its role in FoxP3 expression, a key transcription factor in regulatory T-cell differentiation. Our results have the potential to allow patients who have failed other treatment options another shot at remission or even a cure for an otherwise challenging lifelong condition.

1772 STASH-Select: a platform for multi-vector engineering and single-step selection of cell therapies bearing multiple enhancements

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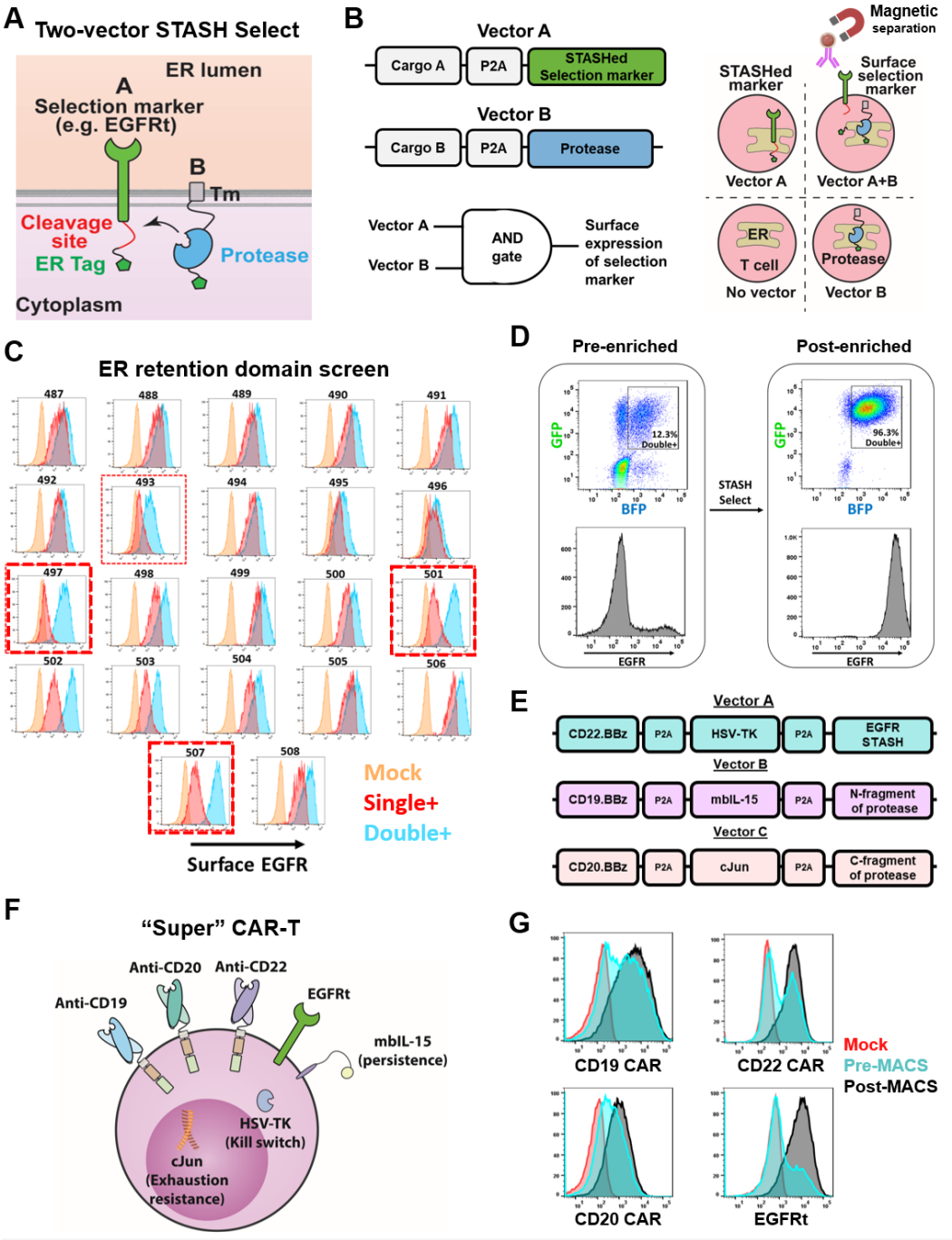
Background: Chimeric antigen receptor T-cells have demonstrated impressive clinical benefit, particularly in hematologic malignancies, but formidable obstacles such as tumor heterogeneity, T cell exhaustion, limited persistence, and a hostile tumor microenvironment limit their broad efficacy. Next-generation CAR-T cells endowed with multiple enhancements could overcome these distinct axes of resistance and expand this class of therapeutics to

solid tumors but will require the introduction of multiple genetic modules that often exceed the cargo capacity of a single vector. Multi-vector engineering results in a heterogeneous, poorly defined cell product. Here we report on STASH-Select, a simple platform technology for purifying cell products containing multiple genetic modifications using a single-step selection that is compatible with current GMP workflows.

Methods: The core principle of STASH-Select is the conditional cellular localization of a selection marker (e.g. tEGFR). We fused a protease-cleavable endoplasmic reticulum (ER) retention tag to the C-terminus of tEGFR, "STASHing" it inside the cell. Co-expression of TEV protease cleaves off the ER tag, allowing tEGFR to translocate to the cell surface, where it serves as a MACS selection handle (**Fig.1A**). Expressing the STASHed tEGFR on a first vector and the TEV protease on a second vector creates an AND gate where only cells transduced by both vectors display tEGFR at the cell surface (**Fig. 1B**). We engineered primary human T cells from healthy donors using retroviral transduction and purified them by MACS. T cells were characterized by flow cytometry and functionality was tested in tumor killing assays, proliferation experiments, and murine models.

Results: We screened 22 ER retention domains and identified several novel human ER tags that efficiently trapped tEGFR in the ER (**Fig. 1C-1D**). We built a 3-vector STASH-Select, which allowed for selection of CAR-T products exceeding 90% purity at high yield. We utilized STASH-Select to generate a uniform product of trispecific CAR-T cells expressing cJun, membrane bound IL-15, a safety switch, and tEGFR (**Fig. 1E-1G**). These "Super" CAR-T exhibited improved efficacy in murine stress test models of cancer and superior safety in toxicity models. We developed further iterations of STASH-Select that allowed for single-step purification of cells engineered with up to six vectors in primary T cells and up to nine vectors in cell lines.

Conclusion: STASH-Select is a broadly applicable platform that enables the deployment of sophisticated next-generation cell therapies bearing multiple enhancements into the clinic, towards addressing multifaceted resistance mechanisms and safety concerns that limit efficacy in solid tumors.



1773 Cell therapy biodistribution and efficacy assessment using a highly sensitive spatial multiomics assay

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Cell therapies such as CAR-T therapies and TCR T cell therapies have proven to be highly effective in treating hematologic malignancies, and major efforts are being made to achieve similar efficacy in solid tumors as well as for autoimmune disorders. To achieve this, it is essential to study the biodistribution of these therapeutics and assess their efficacy, toxicity and response. The RNAscope *in situ* hybridization (ISH) technology can detect any engineered RNA sequence and identify the CAR-T cells *in vivo*. But, to successfully detect very specific immune response associated with the therapeutics, it is essential to localize targeted immune cells with the therapeutic in a spatial context. The new RNAscope™ Multiomic LS assay can combine immune-cell specific protein markers with activation marker RNAs for cytokines and chemokines to determine therapeutic efficacy.

Here, we demonstrate the use of the multiomic assay on the BOND Rx that can detect up to 6 RNA and protein targets on the same slide. The TSA-based amplification strategy offers signal boost for both RNA and protein targets. To optimize protein detection, the workflow is completely protease-free there by preserving antigen integrity and tissue morphology. The assay can be customized to include any target RNA and proteins of interest. Here, we demonstrate the use of our pre-conjugated antibody panel which includes CD8, CD4, FoxP3 and PanCK with *IFNG* and *GZMB* RNA to visualize tumor infiltrating lymphocytes(TILs) in human cancer tissues. We also detected an mRNA-based in vivo CAR-T therapeutic and associated immune cells using a custom panel with CD3, CD45, CD8, CD4, CD20 and a target probe against the CAR sequence in NHP spleen tissue.

Infiltrating Cytotoxic T cell lymphocytes were detected using CD8, *GZMB* and *IFNG* co-expression in human cervical, breast and colon cancer tissues. In the same tissues, regulatory T cells were detected using CD4, FOXP3 co-expression. PanCK was used as a tumor marker to delineate tumor from stroma. In the NHP model, the distribution of the mRNA in tissues was able to be differentiated as on-target (in hematopoietic or T cells) or off target (tissue parenchyma).

The protease-free RNAscope Multiomic LS assay was successfully able to interrogate different subpopulation of immune cells with high fidelity RNA and protein signal while maintaining tissue morphology. The higher plex capability provides detection of more markers simultaneously, in turn enabling immune phenotype profiling while maintaining the sensitivity and specificity . Finally, the assay affords a very high throughput on the Bond Rx allowing large-scale pre-clinical and patient sample analysis.

1774 Allogeneic CAR-γδT cells armored with ‘super-mbIL15’ demonstrates enhanced efficacy towards both liquid and solid tumors

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CAR-T cell therapy has demonstrated remarkable efficacy in hematologic malignancies. However, its application in solid tumors remains limited due to challenges such as the immunosuppressive tumor microenvironment and limited T-cell persistence. γδT cells, a distinct subset of T cells, have shown potent anti-tumor properties with the ability to recognize a wide range of tumor-associated antigens, making them a promising alternative to conventional αβT cells for CAR-based therapies. Additionally, IL-15, a cytokine crucial for T-cell survival and expansion, has been shown to enhance the efficacy of immune cells, particularly CAR-T cells, by promoting their persistence and cytotoxic activity. The constitutive co-expression of IL-15, especially membrane-bound IL-15 (mbIL15), has been demonstrated to significantly enhance the anti-tumor efficacy of CAR-T, CAR-NK, and other immune cells, further potentiating their therapeutic potential.

In this study, we co-expressed mbIL-15 with either CD19 CAR or B7-H3 CAR in γδT cells and observed that it enhanced downstream STAT5 phosphorylation, leading to increased cytokine-independent proliferation and anti-tumor activity *in vitro*. However, mbIL-15 expression significantly reduced CAR transduction efficiency and expression intensity. To overcome these issues, we performed in-depth optimization of the mbIL-15 structure and identified a modified version that induced a significantly higher level of STAT5 phosphorylation compared to mbIL-15, without compromising CAR transduction or expression intensity. This optimized structure was named "super-mbIL15". *In vitro* experiments demonstrated that super-mbIL15 more effectively maintainied CAR-γδT cell

proliferation and viability under cytokine-free conditions. Furthermore, through serial-killing assay, CAR-γδT cells armored with super-mbIL15 exhibited more durable anti-tumor activity and higher level of IFN-γ release compared to mbIL-15 *in vitro*. To evaluate *in vivo* anti-tumor efficacy, we utilized multiple xenograft models, including B-cell acute lymphoblastic leukemia (B-ALL), acute myeloid leukemia (AML), and ovarian cancer. In all of these models, CAR-γδT cells armored with super-mbIL15 demonstrated significantly enhanced anti-tumor activity, peripheral blood expansion, and tissue infiltration levels, outperforming other IL-15 constructs.
Our study demonstrates that CAR-γδT cells armored with super-mbIL15 exhibit enhanced anti-tumor efficacy in both liquid and solid tumor models. The optimized version of membrane-bound IL-15 improves CAR-γδT cell expansion, persistence, and anti-tumor activity, providing a promising strategy for γδT cell-based immunotherapies. Additionally, super-mbIL15 has the potential to enhance the efficacy of other immune cells, such as CAR-T and CAR-NK cells, suggesting broad applicability for engineered immune cell therapies in cancer treatment.

1775 Development of A Feeder-Free Process for IL-2-Independent TIL Expansion

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Background: Tumor-infiltrating lymphocyte (TIL) therapy demonstrates significant promise in treating various solid tumors, recently gaining FDA approval for advanced melanoma. The conventional TIL expansion process relies heavily on high-concentration interleukin-2 (IL-2) and feeder cells (irradiated peripheral blood mononuclear cells from healthy donors), rendering TILs dependent on these factors for survival and function. Consequently, high-dose IL-2 is required post-infusion to maintain TIL potency and persistence. This high-dose IL-2, coupled with the need for high-intensity lymphodepletion to reduce pro-tumor regulatory T cells (Tregs), which are also sensitive to IL-2, is associated with significant toxicity, including high mortality rates and frequent intensive care unit (ICU) admissions. Additionally, the use of feeder cells carries inherent risks such as infection transmission, variability in cultivation and supply chain, and high associated costs.

Method: We developed a robust TIL culture process utilizing a chemically defined medium that eliminates the requirement for high-concentration IL-2 and feeder cells. This innovative approach successfully expanded TILs from multiple solid tumors, including melanoma, cervical, ovarian, endometrial, and several other cancers. Flow cytometry was employed to characterize TILs, while potency assays were assessed using a real-time cell analyzer and ELISA against syngeneic primary cells and patient-derived xenograft (PDX) models.

Results: The feeder-free TIL manufacturing process exhibited high robustness across tumors, exceeding a 95% success rate (within specifications). Average viabilities consistently surpassed 90% across tumor types, with cell numbers ranging from 5x109 to 45x109 after a 24-day expansion. Within the CD45+CD3+ T cell population, CD8+ T cells constituted more than 50% on average. Notably, central memory T cells (TCM) and effector memory T cells (TEM) comprised over 95% of the T cell population. Importantly, the proportions of Treg and PD-1+ T cells were both below 0.5%. Remarkably, the TILs exhibited dose-dependent cytotoxic effects against syngeneic primary cancer cells and demonstrated antitumor activity in PDX models without exogenous IL-2 supplementation.

Conclusion: This feeder-free process enables the expansion of abundant and potent IL-2-independent TILs. The TILs exhibit low levels of exhaustion markers like PD-1 and demonstrate robust in vitro and in vivo functions without the need for IL-2 supplementation. This innovative approach has the potential to significantly improve the safety profile and reduce the manufacturing costs of TIL therapy.

1776 Enhancing Production and Functional Potential of Tumor-Infiltrating Lymphocytes via Lipid Nanoparticle-Mediated CRISPR/Cas9 Gene Editing

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Tumor-infiltrating lymphocyte (TIL) therapy has shown promise in treating solid tumors, but its clinical efficacy remains limited to certain indications. Genetic engineering offers a powerful method to enhance the anti-tumor function and persistence of TILs, potentially broadening their application to a wider range of solid tumor types. Electroporation (EP), a traditional T cell engineering technique using electrical pulses to introduce genetic material into cells, typically achieves high knockout (KO) rates but compromises cell viability, proliferation, and large-scale expansion. In contrast, lipid nanoparticles (LNPs) have emerged as an innovative delivery modality for CRISPR/Cas9 editing tools. Recent studies have demonstrated that LNPs can efficiently edit human peripheral blood mononuclear cells (hPBMCs) efficiently with less cellular stress compared to EP. In this study, we utilized a novel ionizable lipid, which exhibited high delivery efficiency and a favorable safety profile across multiple species. Notably, the ionizable lipid was rapidly metabolized within cells via a cytochrome enzyme-independent pathway, reducing potential adverse effects during T cell transfection. We optimized the transfection process with hPBMC T cells, refining culture conditions, activation methods, and serum concentration. The protocol was subsequently adjusted to the manufacturing process of TILs, achieving KO efficiency similar to EP but with higher cell viability and recovery at 24hr or 48hr post transfection in both hPBMCs and TILs. The LNP-mediated CRISPR/Cas9 KO efficiency was dose-dependent, and LNP-edited cells showed at least a two-fold increase in cell numbers after large-scale expansion. Functional assessments revealed that LNP-edited TILs maintained comparable cytotoxicity and cytokines release in vitro to EP-edited cells in response to tumor cells. Further characterization showed improved memory phenotype, enhanced stemness, and reduced exhaustion and apoptosis. RNA-seq analysis indicated minimal transcriptomic changes with LNP, unlike the significant gene expression disruption seen with EP, especially in exhaustion and metabolism pathways. Taken together, LNP-edited TIL products achieved high KO efficiency with minimal genetic disruption, showing enhanced cell fitness and expansion potential. These findings indicated LNP as a superior CRISPR/Cas9 delivery system for ex vivo TIL editing, warranting further clinical evaluation of LNP-engineered next-generation TIL therapies.

1777 Metabolic Alterations in Highly Activated NK-like Cells (GAIA-102) Through Cryopreservation

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Background: We have developed an innovative approach to generate highly activated NK (Natural Killer)-like cells (development code: GAIA-102) with potent antitumor activity against solid tumors. While highly activated NK cells are generally known to be vulnerable to cryopreservation, leading to a decline in post-thaw viability and cytotoxic function, GAIA-102 has been optimized to overcome this challenge and is currently being utilized in clinical trials as a cryopreserved, off-the-shelf product (NCT05207371, NCT05438459, NCT05608148). Our efforts have included refining the pre-freezing culture conditions and modifying the composition of the thawing diluent, significantly improving post-thaw viability and cytotoxic activity. These advancements have led to promising clinical outcomes, supporting the potential efficacy of GAIA-102. However, further optimization of the freeze-thaw process remains necessary to fully preserve its intrinsic antitumor activity and maximize therapeutic efficacy.

Purpose: Recent studies have highlighted the critical role of cellular metabolism, particularly mitochondrial respiration and glycolysis, in regulating NK cell cytotoxicity. Cryopreservation has been shown to induce progressive mitochondrial dysfunction and promote a metabolic shift toward glycolysis in peripheral blood mononuclear cells; however, its specific impact on the metabolism of NK cells remains insufficiently explored. Given the pivotal role of mitochondrial function in NK cell activity, this study aims to elucidate the metabolic changes underlying the decline in GAIA-102 cytotoxicity post-thaw and to develop metabolism-based strategies for further optimizing its cryopreservation process.

Methods: After producing GAIA-102, the cells were cryopreserved at -80℃ for more than two days. Immediately after thawing, GAIA-102 were incubated with the injection solution, and the cytotoxicity and metabolism were assessed. Particularly, the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were measured using an extracellular flux analyzer to evaluate the metabolism (mitochondrial respiration and glycolysis).

Results: Compared to primary NK cells, GAIA-102 demonstrated significantly higher basal respiration and glycolysis, along with enhanced spare respiratory capacity and glycolytic reserve, which are critical indicators of metabolic flexibility and reserves. After thawing, the basal respiration, ATP-linked respiration and spare respiratory capacity, and glycolytic reserve of GAIA-102 decreased significantly, while glycolysis remained roughly equivalent.

Conclusion: GAIA-102 exhibits high metabolic flexibility and reserves at harvest, with respiratory capacity of mitochondria and compensatory function of glycolysis notably impaired through cryopreservation. Further investigation is warranted, specifically targeting mitochondrial respiration and glycolysis mechanisms.

1778 Nonclinical Safety Evaluation of a KRAS G12D Neoantigen-Targeting TCR

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Engineered TCR T-cell therapies are an emerging class of adoptive cell therapies delivering recombinant cancer antigen-targeting TCR-Ts to kill tumor cells. We developed an autologous CD8+ T-cell product using non-viral gene editing to knock out the endogenous TCR and knock in an engineered TCR targeting the VVVGADGVGK epitope of KRAS G12D, a prevalent tumor-specific somatic mutation important for neoplastic growth, in the context of HLA-A11:01. KRAS G12D TCR T cells were found to effectively recognize and kill tumor cells expressing the target peptide:MHC complex and demonstrated tumor regression in a mouse xenograft model. Due to inter-species variance of MHCs and proteomes, in vivo models are not relevant for toxicity evaluation, necessitating comprehensive in vitro characterization. In vitro studies confirmed a lack of reactivity to wildtype KRAS peptide, though did identify off-target (OT) reactivity to two peptides belonging to ERAS and RASL11b at potencies 1000- and 100-fold lower than cognate peptide, respectively when evaluated using HLA-A11:01 engineered cell lines. No OT reactivity was observed against endogenously expressed epitopes in primary and iPSC-derived human cells covering multiple organ systems, or to a majority of HLA-A11:01+ tumor cell lines that do not express KRAS G12D. Further no alloreactivity was detected in a cell panel covering 90% HLA diversity. Gene editing fidelity was confirmed through multiple orthogonal approaches, showing no OT editing in regions of concern, no gross chromosomal abnormalities, and a lack of cytokine-independent or unregulated cell growth. The cumulative nonclinical data support further clinical evaluation of this autologous TCR-T product in cancer patients expressing KRAS G12D in the context of HLA-A11:01.

1779 Acute safety and biodistribution profile of hematopoietic stem cell (HSC) targeting virus-like particles based on helper-dependent adenovirus serotype 5/35++ in non-human primates

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Ensoma has developed a novel in vivo gene therapy platform that uses virus-like particles (VLP) based on helper-dependent adenovirus serotype 5/35++ for in vivo hematopoietic stem cell (HSC) gene therapy. VLPs are produced in a scalable, suspension-based manufacturing process that can support large patient populations at low cost. VLPs are devoid of viral genes to minimize immunogenicity and allow up to a 35 kb payload capacity to enable large gene insertions. Following systemic delivery, VLPs preferentially transduce HSCs due to the use of CD46 receptor for cell entry. CD46 receptor is expressed at higher density in primitive long-term HSCs compared to other hematopoietic cell lineages. The platform has the potential to significantly improve accessibility for patients with a variety of diseases that affect blood and immune cells.

We evaluated the acute safety, biodistribution and transgene expression profile of VLPs (at doses up to 9.25E12 gc/kg) following intravenous administration in cynomolgus macaques. Cynomolgus macaques were mobilized with granulocyte colony-stimulating factor (G-CSF) and plerixafor and given an immune prophylaxis regimen consisting of dexamethasone, tocilizumab and anakinra. Following dosing, VLPs exhibited a favorable tolerability profile

without any clinical observations and all animals survived to scheduled terminal euthanasia on Day 8. No VLPs-related increase in alanine transaminase (ALT) and aspartate aminotransferase (AST) was observed. VLPs were associated with a transient elevation in IL-6 that peaked at 6 hours and returned to baseline at 72 hours. Gross and histopathology examination was performed on collected tissues from all animals at scheduled necropsy. There were no VLP-related microscopic or gross changes.

Biodistribution and gene expression analysis were performed on whole blood and tissues collected at necropsy. The highest vector copy number (VCN) was detected in whole blood, followed by the lung, liver, and spleen. VCN in gonads (testes and ovaries) was approximately 2 to 3-log lower than the lung, liver and spleen. Gene expression analysis showed no transgene expression in the gonads. The biodistribution and transduction profile supports a low probability of germline transmission with VLPs following systemic administration.

This work demonstrates VLPs to be well tolerated following systemic delivery in cynomolgus macaques and support the further development of Ensoma's VLPs as a platform for in vivo HSC gene therapy.

1780 Novel *In Vivo* Gene Therapy Approach to Hematopoietic Stem Cell (HSC) Engineering Creates Durable HSC-Derived Neutrophils to Treat X-Linked Chronic Granulomatous Disease

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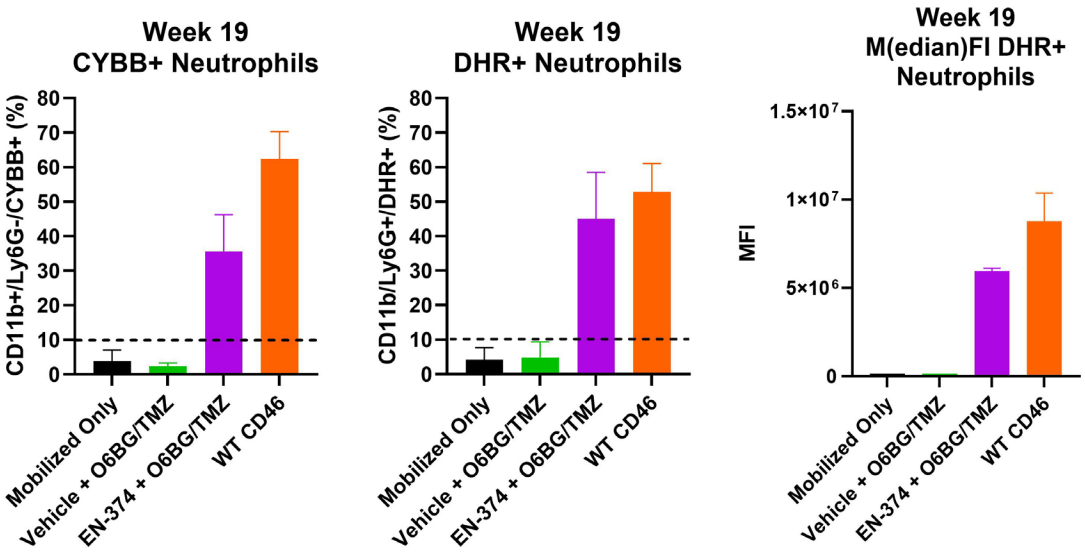
Allogeneic hematopoietic stem cell (HSC) transplant is standard treatment for many diseases involving blood cells, but it carries risks of graft rejection and graft-vs-host disease. Although *ex vivo* autologous HSC gene therapy can bypass donor related issues, it carries risks and burden of conditioning and limited access due to complex N-of-1 manufacturing. Ensoma has developed a novel *in vivo* gene therapy with virus-like particles (VLP) based on helper-dependent adenovirus 5/35++ for *in vivo* HSC genome modification. Our VLPs are devoid of viral genes, minimizing immunogenicity, and allow for up to 35 kB payload capacity for large gene insertions. We show that our VLPs preferentially transduce HSCs by binding to the CD46 receptor, which is highly expressed on primitive long-term HSCs. Ensoma has applied this novel HSC engineering technology for the treatment of X-linked chronic granulomatous disease (X-CGD).

X-CGD is caused by a defect in the NADPH oxidase complex, which inhibits phagocytic cells from eliminating bacterial and fungal pathogens. Ensoma's candidate, EN-374, consists of two independent VLPs; one encodes for Sleeping Beauty 100X transposase and Flp recombinase, and the other contains a transposon with a myeloid specific promoter expressing wild-type CYBB and a ubiquitous promoter expressing a selectable methylguanine methyltransferase (MGMT) marker containing the P140K mutation. In cells co-transduced by the two VLPs, the transposon is excised and integrated into the genome to drive stable expression of CYBB and restore NADPH oxidase activity in neutrophils. Unmodified HSCs are then depleted by O6-benzylguanine (O6BG) and single dose temozolomide (TMZ), at less than conventional regimens, while gene-modified HSCs are protected from the toxic effects of TMZ and enriched to achieve therapeutic levels of gene marking.

In a 19-week *in vivo* study, EN-374 was administered to transgenic human CD46 and CYBB knockout mice. At end of the study, in EN-374 treated mice, CYBB vector copy number was 0.51 ± 0.14 copies/bone marrow cells and gene marking, based on MGMT-P140K expression, in bone marrow HSCs and progenitor cells was 51.0 ± 17.0%. The HSCs demonstrated normal hematopoietic function by CFU assay. CYBB protein expression and NADPH activity in circulating neutrophils was evaluated by flow cytometry and dihydrorhodamine (DHR) assay, respectively. EN-374 mice demonstrated an average CYBB protein expression and DHR activity in neutrophils of 35.6 ± 10.6% and 45.0 ± 13.5% (mean ± SEM), respectively. DHR activity in restored neutrophils, measured by median fluorescence intensity, was 68% of the activity in wild-type controls. Restoring ≥10% of neutrophils with NADPH oxidase activity has been shown to confer clinically meaningful improvements in infection outcomes in X-CGD patients.

Our data demonstrate compelling proof-of-concept for efficient *in vivo* HSC engineering that leads to the expression of functional CYBB in neutrophils at therapeutic levels in X-CGD disease model mice. Additionally, with a scalable, suspension-based manufacturing process, our VLPs can support large patient populations at low cost, and this study sets the foundation for the first VLP-based, off-the-shelf *in vivo* HSC gene therapy to be studied in a X-CGD clinical trial. Our technology has the potential to be applied to a wide range of genetic disorders which thus far have only been addressed with allogeneic-HSC transplants and *ex vivo* gene therapies



1781 *In Vivo* Hematopoietic Stem Cell Gene Therapy for Sickle Cell Disease Using BaEVRLess-pseudotyped Viral Vectors

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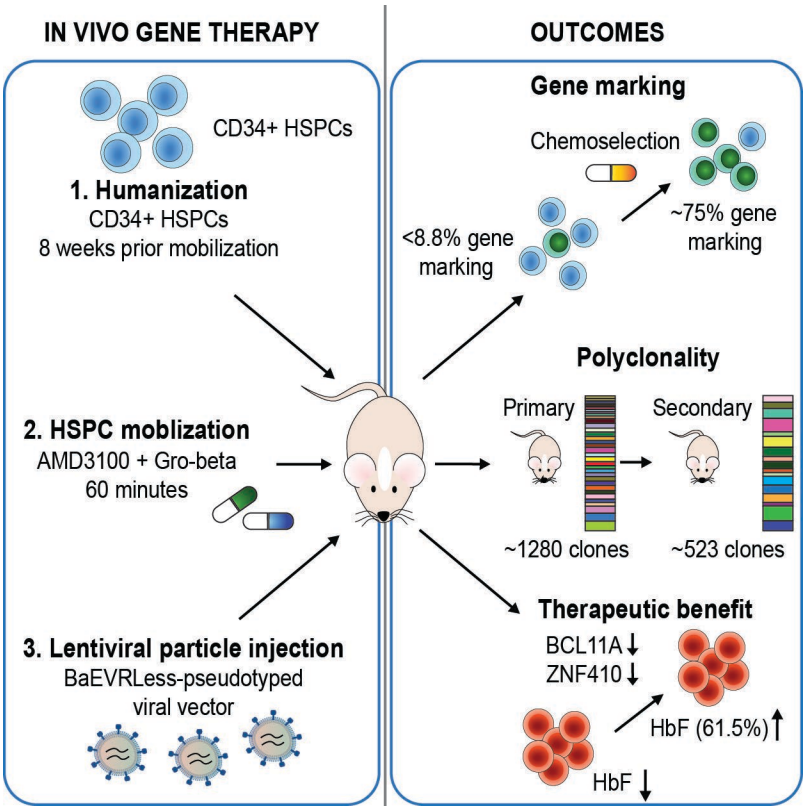
Traditional *ex vivo* hematopoietic stem cell (HSC) gene therapy has effectively cured severe inherited blood disorders, such as sickle cell disease (SCD), but more widespread implementation is limited by high costs, scalability issues, and significant risks associated with myeloablative chemotherapy. *In vivo* HSC gene therapy, which aims to directly modify the cells *in situ*, has the potential to overcome these limitations. Here, we explore the use of viral vectors pseudotyped with the baboon endogenous retrovirus (BaEV) glycoprotein, and its derivative BaEVRLess, for stable gene transfer into HSCs *in vivo*. Although BaEVRLess-pseudotyped retroviral vectors very efficiently transduce quiescent HSCs and are serum-resistant, their production in HEK293T cells is severely hampered by toxicities related to the high fusogenic activity of BaEVRLess. To overcome this problem, we generated a stable BaEVRLess-packaging cell line carrying knockout of the viral entry receptor ASCT2 and stably expressing Gag/Pol and BaEVRLess envelope. This resulted in a 10-fold increase in titers up to 10^9 viral particles/mL ($p<0.001$) and a 3-fold reduction of viral vector-induced cytotoxic apoptosis ($p<0.001$). Additional modifications to the packaging cells include the overexpression of CD47 and knockout of *B2M*, which aims to reduce phagocytosis and immune responses *in vivo*, respectively. To increase *in vivo* HSC gene transfer, we mobilized humanized NBSGW mice with AMD3100 and Gro-beta, prior to injection of ~$3x10^7$ viral particles achieving up to 8.8% gene marking in peripheral blood hCD45+ cells within one week. To enrich gene-marked cells, we overexpressed MGMT-P140K in transduced cells, which mediates resistance to the alkylating agent

BCNU. After three cycles of BCNU treatment, we observed a 33-fold enrichment of gene marked cells in peripheral blood ($p<0.001$). In bone marrow, gene marking reached on average 73.7% in hCD45+ cells 16 weeks after vector injection and averaged 78.4% in hCD34+ cells. Barcode analysis showed the presence of 200-4600 unique barcodes and a polyclonal distribution in >93% of treated mice. Transduction and selection of *bona fide* HSCs was confirmed in secondary transplantations, where gene marking and clonality remained stable at 58.5% transduced hCD45+ cells in bone marrow. As a proof of concept for a therapeutically relevant approach to treat SCD, we used a double shmiR containing vector to downregulate *BCL11A* and *ZNF410* in erythroid cells, which results in reactivation of gamma-globin and induction of fetal hemoglobin, which largely attenuates the hematologic effects of SCD. Following *in vivo* gene transfer and BCNU selection in humanized mice, we observed a significant upregulation of gamma-globin to 61.5% in transduced erythroid cells at week 16 ($p<0.001$). In summary, we demonstrate that BaEVRLess-pseudotyped viral vectors could be applied for *in vivo* gene therapy to treat SCD.



1782 *In Vivo* Truncation of the EpoR Gene Allows for Expansion of Therapeutically Edited Erythroid Cells after HSC Gene Therapy with HDAd Vectors

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Ex vivo HSC gene therapies for hemoglobinopathies are costly and complex. We have developed a simpler *in vivo* method where HSCs are mobilized and edited *in vivo* using intravenously injected HDAd vectors. A fraction of edited HSCs returns to the bone marrow. The goal of this study is to expand this fraction without using chemotherapy. We used naturally occurring erythropoietin receptor variants (tEpoR) that are associated with benign red blood cell overproduction. We aim to generate tEpoR through *in vivo* EpoR gene editing using HDAd vectors with cytosine base or prime editors. Our screening with base editors identified two EpoR variants that exhibited a

100-fold increase in proliferation in HUDEP-2 cells, even under conditions with 600-fold lower Epo levels. We incorporated optimal tEpoR editors together with an integrating therapeutic g-globin cassette into an HDAd vector. After transduction of $CD34^+$ cells from healthy donors with integrating HDAd-tEpoR-g-globin, we observed 5-10% EpoR gene editing on day 4 post transduction. Importantly, upon erythroid differentiation, the editing rate increased to 40%, indicating selective expansion of edited erythroid cells. Flow cytometric analysis of differentiated erythroid cells showed increased g-globin expression. For *in vivo* studies, integrating HDAd-tEpoR-g-globin was injected into mobilized humanized mice (engrafted with human $CD34^+$ cells from healthy donors). 11 weeks post HDAd transduction, we observed EpoR target site editing in bone marrow $CD34^+$ cells in the range of 0.4 to 4%. Ex-vivo erythroid differentiation of explanted human cells in the presence of human Epo showed an increase in editing from 4% to 16%. This indicates that humanized mice are not efficiently supporting human erythropoiesis. Therefore, we have recently started testing the *in vivo* approach in rhesus macaques. We generated an HDAd vector with rhesus specific guide RNA to edit tEpoR and injected it at the peak of G-CSF/ADM3100 mobilization. Seven days post transduction, we observed >4% of tEpoR target site editing in bone marrow $CD34^+$ cells. The first data on the expansion of edited erythroid cells and g-globin -positive erythrocyte look promising and a 4-month follow-up will be presented. Integration of HDAd-tEpoR-g-globin by a Sleeping Beauty transposase is relatively inefficient which limits the potential of our approach. To address this, we developed dual base editing vectors to truncate EpoR and disrupt sites that bind g-globin repressors (BCL11a and LRF1 sites). To implement this as hit-and-run strategy, we incorporated a pomalidomide-based degron system for precise control of the gene editing machinery. Transduction of the dual base editing vectors in HUDEP-2 and $CD34^+$ cells resulted in preferential expansion with g-globin reactivation. As an alternative approach, we utilized prime editing to truncate EpoR by introducing a 9 bp substitution originally described by Harvey Lodish. Our screening identified an optimal guide RNA that achieved 30% of complete 9bp substitution in HUDEP-2 cells, which resulted in a 5-fold increase in proliferation under low Epo conditions. We then developed a dual prime editing HDAd vector to introduce the EpoR mutation and correct the sickle cell disease mutation. Testing in HUDEP-2 cells showed efficient editing, achieving nearly 30% EpoR site editing and nearly complete correction of the sickle cell disease mutation. The new vectors will be tested in humanized mice and NHPs.

1783 In Vivo Engineering of Hematopoietic Stem Cells with Virus-Like Particles to Generate Multi-Lineage CAR Immune Cell Therapy for Cancer

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Background: Chimeric antigen receptor-engineered T (CAR-T) cell therapy has achieved remarkable clinical success, particularly in hematologic malignancies. However, its broader application remains limited due to complex manufacturing, limited persistence and functional exhaustion, and high costs associated with personalized approaches. CAR-T cell therapy has shown limited efficacy in solid tumors, owing to an immunosuppressive tumor microenvironment and inefficient trafficking of CAR-Ts to tumor. To overcome limitations of autologous CAR-T therapy, we are pursuing an off-the-shelf, multicellular approach that leverages synergy between myeloid and lymphoid immune cells to establish a new benchmark for durable therapeutic outcomes in solid tumors.

Methods: We developed a helper-dependent adenovirus platform of virus-like particles (VLPs) to enable in vivo engineering of hematopoietic stem cells (HSCs). This vector selectively targets human CD46, a receptor highly expressed on primitive HSCs. Co-transduction with a hyperactive Sleeping Beauty transposase (SB100x) facilitates efficient transgene integration, ensuring sustained development of engineered immune cells. These VLPs have a large cargo capacity of up to 35 kilobases, enabling construction of single- or multi-cellular CAR sequences under distinct lineage-specific promoters for precise immune cell engineering. CAR gene expression, specificity, and function were evaluated in vitro and in vivo.

Results: To achieve selective therapeutic payload expression, we identified and validated lineage-restricted promoters with myeloid- or T/NK-cell-specific activity in primary human and murine immune cells. CAR constructs

driven by monocyte- or T/NK-restricted promoters successfully generated functional CAR macrophages, T and NK cells, respectively, with all CAR effector cell types exhibiting potent antigen-dependent tumor cell cytotoxicity. To assess activity in vivo, hCD46 hematopoietic stem and progenitor cells (HSPCs) were transduced with VLPs encoding CAR driven by a ubiquitous promoter (CAG) or lineage restricted regulatory elements and transplanted into irradiated recipient mice to assess HSPC-derived CAR+ immune cell generation. While the ubiquitous CAG promoter drove CAR expression across all immune cell lineages, myeloid- and T/NK-restricted promoters confined CAR expression to their respective lineages. These lineage-specific CAR immune cells exhibited on-target tumor cytotoxicity comparable to CAG-driven CAR while minimizing off-target expression. Tumor-infiltrating CAR+ effector cells displayed a proinflammatory phenotype compared to their CAR- counterparts. Furthermore, concatenation of myeloid- and T/NK-restricted promoters enabled generation of multi-lineage CAR immune cells from a single VLP.

Conclusions: This study establishes a robust proof of concept for in vivo multicellular CAR immune cell therapy via HSC engineering with lineage-restricted control. Our platform provides a versatile and scalable strategy for delivering CAR and other immunomodulatory payloads to specific immune cell types while preventing expression in HSPCs and off-target lineages. By leveraging a self-renewing reservoir of engineered HSCs and the large cargo capacity of VLPs, this approach offers an off-the-shelf solution that enables a durable and synergistic anti-tumor response.

1784 Effective Gene Editing in Hematopoietic Stem and Progenitor Cells (HSPCs) through a Novel Targeted Lipid Nanoparticle

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Background: Ex vivo Hematopoietic stem cell (HSC) gene therapy has achieved substantial progress over the past decade, advancing as a feasible therapeutic strategy for various blood and immune disorders, including sickle cell anemia, beta-thalassemia, and other immunodeficiencies. However, the complexities and challenges of ex vivo HSC gene therapy such as manufacturing, scalability, cost, and accessibility, highlight the need for an in vivo solution that could simplify the process and address many of the current limitations. This study describes a novel lipid nanoparticle (LNP) system designed to selectively deliver gene editing payloads to hematopoietic stem and progenitor cells (HSPCs) in vivo. The targeted lipid nanoparticles (tLNPs) are a product of the CellSeeker™ platform technology and comprise a proprietary LNP formulation, conjugated to a recombinant antibody that binds to a specific surface marker on HSPCs, enabling efficient delivery of CRISPR-Cas components to genetically edit HSPCs in vivo.

Methods: To generate proof-of-concept, a tLNP was designed to deliver Cas9 mRNA and single guide RNA (sgRNA) targeting beta-2 microglobulin (B2M) to human CD34+ HSPCs. This tLNP was formulated with a novel, highly biodegradable ionizable lipid and an antibody targeting the stem cell factor receptor, CD117, which is expressed on the surface of HSPCs. We evaluated the efficiency of tLNP-mediated B2M knock-out (KO) both in vitro and in vivo using a CD34+ hematopoietic stem cell engrafted NCG mouse model.

Results: To evaluate the specificity and efficiency of delivery, we first examined the ability of CD117-targeting tLNPs to deliver a GFP reporter mRNA to human CD34+ HSPCs. tLNP administration resulted in robust GFP expression in plerixafor-mobilized peripheral blood CD34+ human HSPCs in vitro (up to 75% GFP+) and in CD34+CD38- HSPCs in humanized NCG mice upon intravenous dosing. To assess the ability to deliver gene editing payloads, Cas9 mRNA and a sgRNA designed to knock out B2M expression were co-encapsulated in these HSPC-targeting tLNPs. Bone marrow and mobilized peripheral blood CD34+ human HSPCs treated with these gene editing tLNPs displayed a significant loss of B2M expression (up to 80%) as measured by flow cytometry and genome editing by sequencing. The gene editing ability of these CD117-targeting tLNPs translated in vivo in humanized NCG mice, demonstrated by high efficiency B2M-KO in CD34+CD38- HSPCs.

Conclusions: The findings in this study provide compelling evidence of CellSeeker™ platform in general and CD117-targeting tLNPs in particular, as a promising strategy to genomically edit HSPC. The distinctive targeting moiety of these tLNPs facilitated efficient delivery of both reporter mRNA and gene-editing payloads to CD34+ human HSPCs. The successful in vivo gene editing in the NCG mouse model supports the potential of the tLNP system for clinal applications. Ultimately, the study highlights CD117-targeting tLNPs as a powerful strategy for advancing in vivo HSC gene therapy.

1785 Chromatographic purification of linear DNA from supercoiled plasmid DNA

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The demand for high-quality DNA vectors and scalable and cost-effective manufacturing platforms for DNA products continue to grow. Ministring DNA (msDNA™) is a linear, covalently closed double-stranded DNA vector devoid of bacterial sequences, such as antibiotic resistance genes and origins of replication. It contains only the therapeutic gene expression cassette flanked by proprietary supersequences that enhance efficacy and safety (Figure 1A). msDNA™ offers numerous advantages, including lack of immunogenicity, absence of insertional mutagenesis risks , and enhanced stability. Its in-vivo manufacturing process in E. coli ensures high fidelity, scalability, and cost efficiency. These characteristics position msDNA™ as a next-generation efficient and safe DNA-based platform for applications such as gene therapy, gene editing, DNA vaccine, and as starting material for production of viral and RNA-based biologics. To meet the stringent purity requirements of gene therapy products, msDNA™ undergoes a sequential downstream purification process, including chromatography and filtration, to yield pure, endotoxin free, high-quality DNA. We applied a DOE-driven process development approach to optimize purification of linear msDNA™ by removing contaminating episomal DNA species (like supercoiled or relaxed plasmids) and other cellular impurities (such as genomic DNA, RNA, proteins, lipids, and small molecules).

Our investigation compared two scalable chromatographic modalities: anion exchange chromatography (AEX) and hydrophobic interaction chromatography (HIC), using various supports including resins, monoliths, and membranes. Due to the large size and slow diffusion of DNA, supports based on convective mass transport, such as monoliths and membranes, demonstrated superior performance for large scale bioprocessing. Our data showed that monoliths were more effective than membranes in separating linear msDNA™ from other DNA forms (Figure 1B). Additionally, HIC chromatography with selected resines and optimized conditions provided enhanced resolution compared to AEX in resolving the two DNA species.These findings underscore the importance of DOE-driven optimization in downstream process development to achieve high-purity msDNA™ at industrial scale, paving the way for safer and more effective DNA-based therapeutics.

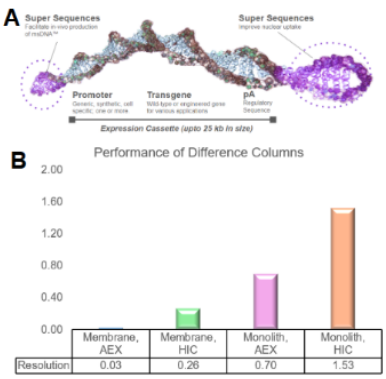

Figure 1: (A) msDNA™ is a linear covalently-closed double-stranded DNA vector free of bacterial backbone DNA carrying a minimal expression cassette composed of the eukaryotic promoter, transgene and regulatory sequences. (B) The relative performance of various columns in separating linear msDNA from its precursor parent plasmid.

1786 Enhancing Lentivirus Depth Filtration Purification: Strategies for Improved Recovery and Quality for Downstream

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In gene therapy manufacturing, the filtration of lentiviral material from transfected cell culture media during upstream processing is pivotal for ensuring downstream success. Challenges in lentiviral manufacturing include limited titer production and difficulties in achieving high recovery due to the virus's environmental sensitivity. This study focuses on optimizing the initial stages of the lentivirus downstream process through the application of diverse analytical methods.

High-quality lentivirus purification is essential to establish robust preliminary standards for downstream processing prior to chromatography. Filtration steps are critical for removing residual host cell proteins (HCPs) and DNA while preserving viral particles. In this study, a comparative analysis of three vendors' depth filters was performed, with each vendor supplying their top three high-performing filters.

Multiple analytical methods, including physical and functional titer assays, were employed to assess filtration performance. Among these methods, droplet digital PCR (ddPCR) was utilized to quantify DNA and viral particles, complemented by an in-house functional titer assay. Filtrate samples were compared against pre-filtered materials to evaluate turbidity reduction, HCP and DNA removal, viral particle recovery, and aggregation. Considerations such as filtration time and throughput were also assessed.

The optimized filtration methodology developed through this study, supported by advanced analytics, demonstrated greater than 95% viral recovery and enabled the production of lentivirus materials ready for chromatography. The total calculated throughput exceeded 230 L/m². Data on titer recovery using ddPCR, along with assays measuring residual HCPs and DNA, provide valuable insights for enhancing lentivirus purification processes. These findings contribute to improving the efficiency and quality of lentivirus production, supporting advancements in gene therapy applications.

1787 Advancing Purification Strategies for Large Viral Modalities Using Nanofiber Adsorbent Technology

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The demand for efficient purification strategies in cell and gene therapy is increasing as large viral modalities such as exosomes, Herpes Simplex Virus (HSV), Vaccinia Virus (VACV), and pseudo typed lentiviral vectors gain prominence since they play critical roles in regenerative medicine, cancer immunotherapy, and gene delivery. However, these vectors present significant downstream purification challenges, including low recovery, high host cell protein (HCP) contamination, and scalability limitations in traditional chromatography-based methods. This study evaluates the purification characteristics of an innovative nanofiber adsorbent, focusing on its capacity, impurity clearance, and process efficiency. The nanofiber-based approach demonstrated high binding capacities, improved recovery, and superior removal of host cell proteins compared to conventional chromatography. Additionally, it showed scalability potential, making it attractive for industrial applications.

Nanofiber-based purification presents a promising alternative to conventional chromatography, offering improved performance for large viral vectors. By enhancing impurity removal, binding efficiency, and scalability, this technology supports the advancement of next-generation cell and gene therapy manufacturing. Beyond purification, the therapeutic applications of these viruses highlight the urgency of improved manufacturing strategies. Oncolytic

viruses such as HSV and VACV are increasingly recognized for their potential in cancer therapy, where they selectively replicate in tumor cells, induce cell lysis, and stimulate anti-tumor immune responses. The ability to efficiently purify and scale up these viruses is critical to their clinical success. Furthermore, pseudotyped lentiviral vectors, such as GalV and VSV-G, expand the scope of gene therapy by enabling targeted transduction of specific cell types. The development of robust purification methods, such as nanofiber-based technology, is essential to support the growing demand for these advanced therapeutics, ensuring their efficacy, scalability, and translational success.

1788 Lenti Viral Vectors Displaying Ligands for Enhanced Capture, Purification, Concentration and Targeted Infection of Specifically Selected Cells

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Generation of lenti and g-retroviral vectors displaying specific markers on their outer surface will facilitate vector purification, concentration and the targeted infection of selected cell subsets both *in vitro* and *in vivo*. We have previously demonstrated that expression of specific ligands on the surface of producer cells results in the presence of these ligands on the surface of the virus (Lenti and g-Retro)[1-6]. This can be achieved with the transient or stable modification of cells producing these vectors, without the genetic modification of either the vector or indeed the helpers.[1-6] As a result, liganded vectors can be generated that are suitable for better affinity purification, concentration and targeted delivery of the vector payload, but without genetic modification of the vector.

The range of ligands successfully attached to the surface of the virus include poly-histidine for immobilised metal affinity chromatography (IMAC) columns[1], biotin or StrepTag for avidin/streptavidin mediated capture[2-4], including the use of paramagnetic particles (PMPs)[2,3,5,7]. In addition, proteins and other ligands can be attached to the surface of the vector producing cells by direct chemical modification[2,7], or the metabolic modification of proteins that are displayed on the surface of producer cells and hence the vector[3,5]. These latter modifications can facilitate other forms of affinity capture and targeting of selected receptors on specific cell subsets[6].

The outlined strategies have been used to achieve enhanced capture and recovery of vectors (substantially greater than 100% recovery of the starting infectious titre)[2,7-8]. Such liganded vectors can also promote the more efficient infection of specifically targeted cells. For instance, lenti vectors displaying SCF can be used for the enhanced infection of c-kit positive haematopoietic stem cells[6].

Improved capture, purification and concentration of lentiviral vectors, combined with enhanced target cell infection, can facilitate more efficient *in vitro* applications with the potential for *in vivo* infection of specific human cell subsets. The combination of better vector recovery and enhanced infection of specifically targeted cells, enables the reduction of costs and a potentially safer vector profile. The real challenge will be to minimise the “off-target” infection[9] by applying technologies to increase the efficiency of targeting with the aid of defective/non-infectious vectors that incorporate cell specific ligands.

References:
*[1] MC Cheeks et al., J Chromatogr A **1216** (13), 2705 (2009). [2] L Chan et al., J Virol **79** (20), 13190 (2005). [3] D Nesbeth et al., Mol Ther **13** (4), 814 (2006). [4] L Mekkaoui et al., Mol Ther Methods Clin Dev **11**, 155 (2018). [5] R Chen et al., J Chromatogr B Analyt Technol Biomed Life Sci **878** (22), 1939 (2010). [6] Chandrashekran et al., J Gene Med **11**, 6 (2004). [7] C Hughes et al., Mol Ther **3** (4), 623 (2001). [8] Dick et al., Nat Protoc **6**, 5 (2011). [9] Cordes et al., Mol Ther Methods Clin Dev 21 (2021).*

1789 ABL's HIV p24 ELISA Kit: a Solution for Lentivirus Quantification in Industrial Processes

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Introduction. ABL has been engaged in Human Immunodeficiency Virus (HIV) research since the 1980s when the virus was first described. Through this work, ABL pioneered one of the first p24 ELISA kits (**ABL® kit**, Catalog #**5421** & #**5447**) for the reliable quantification of HIV capsid protein (p24). Today, ABL provides manufacturing and analytical services for oncolytic, cell and gene therapies, and vaccines. Recently, ABL developed a proprietary platform for the industrial production and purification of Lentivirus (LVV). As LVV shares structural and biological similarities with HIV, we evaluated ABL's HIV p24 kit for the quantification of LVV in-process samples generated during industrial production and purification. One challenge in quantifying such samples is the poor correlation between genomic titer (viral particle titer derived from RNA content) and p24 results (viral particle titer derived from LVV capsid marker-p24), particularly in early-stage and low-concentration samples. However, the **ABL® kit** exhibited remarkable reliability with such samples. To further address this observation, we compared the performance of the ABL® kit to those offered by other vendors.

Note: ABL is a member company and legal affiliate of Ascend Advanced Therapies Group, Inc.

Method. LVV in-process samples were tested for p24 concentration (ng/mL) using the **ABL® kit** and kits from four other vendors (B, C, D, E). Genomic titer (vg/mL) was measured via RNA extraction and qRT-PCR.

Viral particle concentrations (VP/mL) Calculations:

- VP/mL from p24 (**VP-p24**) = p24 conc. (ng/mL) x 1.0E+07.
- VP/mL from Genomic titer (**VP-GC**) = virus genomic copy conc. (**vg/mL**) ÷ 2.

Sample Subgroups:

- All Samples
- Lower Titer: Genomic Titer < 4.0E+09 vg/mL
- Higher Titer: Genomic Titer ≥ 4.0E+09 vg/mL

1. Early Steps: Harvest, AEX Load.
2. Later Steps: AEX eluate, TFF retentate & Sterile filtered product.

Correlation Assessment

- **R^2** (Coefficient of determination): a bigger R^2 indicates stronger correlation and higher reliability
- **VP-p24/VP-GC Ratio** and **CV%** (coefficient of variation).
    - An average ratio ≈ 1.0 indicates that the p24 concentration aligns with the reported standard (1 ng p24 = 1.0E+07 VP).

- To reduce R^2 bias from high-titer samples, CV% of individual sample ratios was used to assess correlation strength within subgroups.

Results.

1. **Ease & Cost Efficiency**: the **ABL® kit** offers a cost advantage, with a simple and fast workflow.

2. **Correlation Strength:** the **ABL® kit** demonstrated higher R^2 values than Kits B, C, D and E particularly in low-titer and early-step samples (**Table 1**).
3. **Ratio & Variability**: The **ABL® kit** average VP-p24/VP-GC ratio is 1.03, consistent with the reported standard 1 ng p24 = 1.0E+07 VP, while Kit-C and Kit-E produced significantly lower ratios (**Fig. 1**). The ABL® Kit also exhibited the lowest CV% (as compared to Kit-B, C and D), particularly in lower titer and early-step samples, confirming superior reliability.

Conclusions. The **ABL® kit** is a reliable, robust and accurate tool for LVV lentivirus quantification in cell & gene therapy applications. The assay is adaptable for cGMP compliance, ensureing suitability for commercial, industrial, and research use.

Correlation of p24 with GC (R^2 value)

	ABL	Kit-B	Kit-C	Kit-D	Kit-E
All Samples	0.984	0.976	0.839	0.981	0.989
Low Titer	0.919	0.890	0.712	0.877	0.887
High Titer	0.981	0.971	0.825	0.980	0.990
Early Step	0.961	0.816	0.836	0.845	0.852
Later Step	0.992	0.981	0.990	0.988	0.998

Table 1. List of R^2 values representing the correlations of viral particle concentration derived from p24 with those derived from genomic titer for each kit across different sample subcategories. Darker green indicates stronger correlations, while darker red indicates weaker correlations.

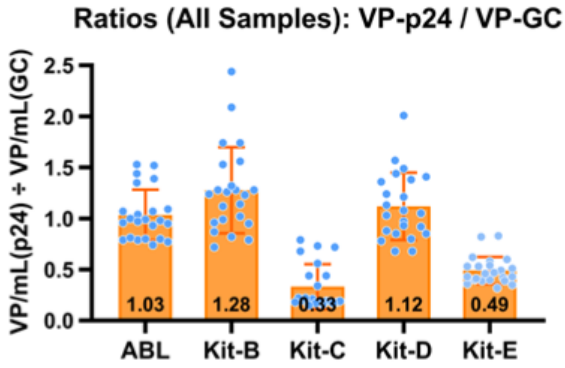

Figure 1. A bar graph displaying average ratios of VP-p24 / VP-GC and sample distributions of each kit in "All sample" subcategory.
- The average ratio of each kit is indicated at the bottom of each bar.
- Each blue dot represents a ratio of an individual sample.

1790 A Lentiviral Vector Downstream Processing Analytic Tool Powered by Knowledge Graph and LLM Technology

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The industrial bioproduction of lentiviral gene therapy vectors (LV) remains a complex and costly operation. Regardless of the production systems used, the harvested LV particles undergo Downstream Processing (DSP), a serial workflow which includes several steps to concentrate and purify the LV. The challenge of DSP is in achieving high biological potency, high quality and high yields of finished product with little batch-to-batch variability. Repeated runs are costly, but necessary: they establish the production workflow and optimize the protocol steps. There is a wealth of information in private and public databases which should be used to assess and establish quality benchmarks for these steps efficiently. However, the heterogenous, high-dimensional and manuscript nature of this available data remains a bottleneck in characterizing runs effectively for inclusion in benchmarks, inference pipelines & analysis algorithms.

Existing computational tools cannot be used effectively because of this lack of structured tools to make sense of the disparate but important corpus of data. Current approaches for streamlining production to facilitate data gathering are centered around reviews or description of protocols without referring to the intricate relationships implied or the results to be expected. Each of these otherwise rich datasets end up representing a finite set which is a strong limitation to scale these approaches.

All the reasons exposed above prompted us to develop a rationally guided approach by providing a solution for structuring data from the literature along with internal data from producers as well as providing statistical solutions upon the structured data. Using a rationally guided approach, we developed OLIVIA, a novel platform leveraging Large Language Models (LLMs) integrated with Retrieval-Augmented Generation (RAG) and a knowledge graph approach to dynamically process and organize bioproduction data. Using our in-house expertise in bioproduction to generate relevant experimental data, we generated a framework for LV bioproduction automated analysis. We have also identified key articles and patents in the field in which production protocols have been documented and which include experimental approaches, assessment of yields and description of relevant materials. OLIVIA employs a comprehensive approach integrating biological context, material, scale, steps and parameters to generate a global outlook on processes, apply analytical methods and even facilitate user interactions. A prototype has been established and will be presented.

This tool is useful not only to track production performance across batches or in comparison with similar process steps, but it also lays the foundation for a potentially more powerful machine learning approach for the enhancement of bioproduction performance and quality. Ultimately OLIVIA is expected to accelerate the development of vector production for novel genomic and cell therapies.

1791 Quality by Design in Plasmid DNA and Gene Therapy: ProBio CDMO's Digital QMS for Risk-Based Manufacturing Excellence

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In the high-stakes world of gene therapy and plasmid DNA manufacturing, quality is non-negotiable. ProBio is setting a new benchmark for quality excellence through implementation of its cutting-edge digital Quality Management System (QMS), powered by Veeva's Quality Risk Management (QRM) solutions.

By integrating a risk-based, data-driven approach, ProBio ensures end-to-end quality oversight, regulatory compliance, and operational efficiency across every stage of production. This enables real-time risk identification, proactive mitigation, and streamlined change control, minimizing deviations and accelerating the delivery of life-saving therapies.

ProBio has built a knowledge-centric ecosystem that centralizes critical data, fostering continuous improvement and enabling faster, more informed decision-making. The results are clear: reduced manufacturing cycle times, enhanced audit readiness, and unparalleled customer satisfaction.

This abstract highlights how ProBio's implementation of its first digital QMS and how it redefines ensures highest quality standards in our Hopewell manufacturing site in ensuring product safety.

1792 A Novel Pipeline for the Genomic Characterization of Cell Lines.

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Cell line characterization is critical to the control of biological product manufacturing. Confirming the identity, purity, and genomic stability of a cell line is of upmost importance for both viral vector manufacturing and cell therapy. The specific strategy for the characterization of a cell bank varies depending on several factors including the source of the cell line itself, cultivation history, genetic manipulation, clonality and growth requirements, amongst others.

We have developed a novel pipeline specifically designed for the genomic and transcriptomic characterization of cell lines used in gene and cell therapies and for the production of biologics, based on three specific technologies: 1) Optical genome mapping, 2) long-read high-fidelity DNA sequencing and 3) short-read, high-throughput RNA sequencing. The data obtained are integrated and analyzed using a custom-built bioinformatics pipeline. Macro-molecular optical genomic maps (OGM) using Bionano was used to assess clonality and genomic stability over many culture passages. OGM can reliably estimate cell ploidy and detect complex genomic rearrangements in a single, genome-wide workflow, providing a 10,000-fold higher resolution when compared to standard karyotyping. Whole genome sequencing using PacBio's high-fidelity (HiFi) long-read technology was used to simplify the complex processes of whole genome assembly, yielding highly accurate ultra-long reassembled DNA sequences (QV>60, N50=150Mb) including difficult to resolve low complexity regions. HiFi reads also provided simultaneous 5mC detection in CpG contexts without additional library preparation, allowing the analysis of the epigenetic methylation status of the genome from a cell population single sequencing analysis. Whole-transcriptome analysis based on total cDNA sequencing (RNA-Seq) using Illumina short-read sequencing can accurately measure gene and transcript abundance, giving information on the transcriptional capacity of the cell line.

This novel pipeline was applied to fully characterize a new HEK293 cell line specifically developed for the manufacturing of AAV products. The genomic analysis revealed fewer genomic rearrangements the new cell line compared to the parental heterogeneous HEK293 cell line and demonstrated its genetic stable over many cell culture generations. This new pipeline allows for the rapid and accurate characterization of cell lines used for cell and gene therapy applications and for the production of biologics.

1793 Harnessing the Power of Next Generation Sequencing and Bioinformatics to Propel Viral Safety of Cell Therapies

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Adventitious viral agents (AVAs) pose significant risks in biopharmaceutical manufacturing, particularly in vaccines, therapeutic proteins, and cell and gene therapies. Traditional animal- and cell- based detection methods often fall short in sensitivity and specificity in the identification of viruses -- especially unknown viruses, leading to potential contamination risks. For short shelf-life products or those requiring rapid turnaround time for testing, such as Cell Therapy products, time to result is critical. Next Generation Sequencing (NGS) is a powerful tool for comprehensive adventitious agent detection, especially viruses in biologics. We have developed an NGS method to detect AVAs leveraging whole transcriptomic sequencing followed by bioinformatic analysis against an internally-developed comprehensive viral database. This method rapidly detects and identifies contaminating viruses in cell therapy products.

1794 Optimization and Comparative Analysis of Host Cell DNA Quantification and Plasmid Supercoiled Percentage: Cost-Effective Agarose Gel Electrophoresis vs. High-Sensitivity qPCR and IEC-HPLC

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The detection and quantification of host cell DNA (hcDNA) and plasmid supercoiled percentage are essential for biopharmaceutical manufacturing to ensure product safety, purity, quality and regulatory compliance. While

quantitative PCR (qPCR) and IEC-HPLC is widely used for its high sensitivity and accuracy, its cost and complexity can be prohibitive for routine screening in resource-limited settings. This study explores the optimization of agarose gel electrophoresis as an alternative for hcDNA quantification and DNA homogeneity, comparing its performance to qPCR and IEC-HPLC in terms of sensitivity and cost efficiency. By refining parameters such as sample loading, imaging techniques, we demonstrate that agarose gel electrophoresis can reliably detect hcDNA at levels suitable for initial screening, significantly reducing operational costs. Similarly, agarose gel electrophoresis, alongside densitometry, can be utilized to determine plasmid supercoiled percentages comparatively to IEC-HPLC analysis. Our findings provide a practical framework for selecting the appropriate method based on analytical needs and budget constraints, offering valuable insights for optimizing hcDNA quantification and DNA homogeneity processes in biopharmaceutical production. This comparative study highlights the trade-offs between cost and sensitivity, enabling informed decision-making for quality control in biomanufacturing.

1795 Genomic Integrity Analysis of Starting Material using Advanced Cytogenetic Analysis for Donor, Cell Line, and/or Clone Screening and Qualification

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Cell and Gene therapies are transforming the treatment landscape for a growing number of medical conditions. In oncology, chimeric antigen receptor (CAR) T-cell therapies are beginning to have significant impacts in several forms of blood cancer, including leukemia, lymphoma, and multiple myeloma. While challenges still exist, treatment solutions for solid tumors are on the horizon, and genetic conditions such as sickle cell disease and spinal muscular atrophy now have multiple disease-modifying therapeutic options approved by the FDA. Additionally, many other therapies are in development for a variety of congenital and/or rare diseases.

When producing a biologics-based therapy from any starting material, donor cells, engineered cells, cell lines, or clones, maintaining the integrity of the genome throughout engineering, editing, and expansion processes is critical. Minimizing new risks to the patients receiving these treatments requires the cellular drug products and the patients receiving these treatments to be monitored for induced chromosomal mutations, clonal outgrowth, and genomic instability. Karyotyping is a classic cytogenetic method for imaging the structure of metaphase chromosomes, typically applied to measure the high frequency structural variation associated with congenital genetic diseases or characteristic of blood cancers.

Here we show how Kromatid's KROMASURE™ whole genome karyotyping assays provide unbiased chromosomal rearrangement and aneuploidy detection with low frequency measurement of events that occur during editing and expansion. These technologies provide advanced cytogenetic studies beyond classical 20-cell analysis with up to 10x the number of cells analyzed and can be used to compliment other methods, providing orthogonal validation. Analysis is performed on a matched sample pair: the pre-editing donor or wild-type cells and the final therapeutic product after editing and any required expansion. The structural variant report provides a "before" and "after" comparison, measuring the differences between edit-associated, engineering-associated, or expansion-associated structural variants and shows the karyotypic variation from baseline levels present in the cellular starting material. Importantly, the risk associated with instability, or a specific variant is only related to the identity of the variant, not the cause or the source. KROMASURE Genomic Integrity K-Banding and KROMASURE Screen provide measurements of structural variation in populations of engineered cells, whether the source of the chromosomal aberration is an edit, the result of impaired DNA damage repair capacity, or is simply present in the donor cell genome.

1796 End-to-End Production of T Cells on the Cocoon® Platform, Using Buoyancy-Activated Cell Sorting™ for T Cell Isolation from Leukopaks

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Summary: Using the Lonza Cocoon® Platform, an automated upstream process for isolation and manufacturing of T cells directly from leukopaks was developed using Akadeum Life Sciences' Buoyancy-Activated Cell Sorting (BACS™) technology. Introduction: T cell isolation is a crucial upstream process in chimeric antigen receptor-modified T (CAR-T) cell manufacturing, as it selects an enriched population of T cells from the patient's blood starting material. Two common methods for isolating T cells are positive selection and negative selection. Negative selection is used in therapeutic applications to avoid the potential activation or exhaustion of T cells that can occur with positive selection methodologies. Additionally, negative selection helps minimize impurities from non-T cells, improving the overall efficiency of CAR-T cell expansion and therapy effectiveness.

Methods: CD3+ cells were enriched using the Akadeum Human T Cell Leukopak Isolation Kit – GMP Grade (antibodies and buoyant microbubbles). Antibodies were added directly to the leukopak material, followed by automation using the Cocoon® Platform for mixing, transfer of the microbubbles for binding, buoyant separation using gravitational forces and collection of the negatively selected T cells. Post upstream processing, 1×10^8 enriched T cells were seeded into the Cocoon® Cassette for automated activation and expansion using the standard 9-day CAR-T Cocoon® Platform process.

Results: Upstream processing on the Cocoon® Platform using BACS™ technology recovered 68.7±14.9% of CD3+ cells with purities of 91.3±3.0% and viability of 97.1±2.3%. The 1×10^8 cells seeded in the Cocoon® Cassette expanded to $2.67\pm0.53\times10^9$ cells with 96.1±3.5% viability at the end of the automated 9-day CAR-T Cocoon® Platform process.

Conclusion: The Cocoon® Platform in combination with BACS™ negative selection technology can automate isolation, activation, and expansion of T cells with high purity and recovery, as well as final product viability and yields. Donor leukapheresis materials are processed directly from the leukopak, without need for washes to minimize material and handling steps. This process significantly reduces hands-on time involved in manual T cell isolation and expansion, through reliable automation of end-to-end CAR-T therapy manufacturing.

1797 Healthier Cells with Akadeum's Alerion Microbubble Separation System: A Novel Approach to Gentle and Efficient Cell Isolation

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A high-quality cell isolation that preserves cell health and functionality is a critical step in the evolution of chimeric antigen receptor T (CAR T) cell therapy workflows. Isolated T cells undergo significant stress during genetic modification processes, making it crucial for therapeutic efficacy and manufacturing success that the cells are in optimal health prior to modification and their viability and functionality are not compromised during the isolation process. The combination of Akadeum's Alerion™ Microbubble Separation System, BioRise™ consumable, and negative selection Human T Cell Leukopak Isolation Kit provides a novel approach to cell isolation that prioritizes preserved cell health and efficiency.

In this study, the Alerion™ based Human T Cell Leukopak Isolation was evaluated in a head-to-head comparison against magnetic-based method for its ability to gently isolate T cells while also delivering a high yield of pure T cells. Akadeum's unique silica microbubble technology leverages gravity for separation, allowing the T cells to remain untouched throughout the separation workflow, which is proposed to minimize cellular stress. Results demonstrated that the T cell isolation workflow on the Alerion™ consistently outperformed traditional magnetic isolation methods in preserving T cell health and maintaining highly functional cells.

The Alerion™ is a scalable semi-automated instrument that provides a closed system capable of isolating billions of T cells in less than one hour. This allows more cells to be processed in less time, leading to potential cost savings and higher productivity. By preserving the natural state of T cells, the Alerion™ Microbubble System, BioRise consumable, and Akadeum's negative selection Human T Cell Leukopak Isolation Kit demonstrates advantages in cellular survival that could have major impacts on the efficacy of CAR T cell manufacturing, providing the potential for better therapies.

1798 Peripheral Blood Mononuclear Cell (PBMC) Isolation Using Cell Fractionation Filters

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Leukapheresis collections, the standard starting material for CAR-T cell therapies, provide sufficient cell numbers but require specialized equipment and long collection times, increasing the overall manufacturing costs and timelines. In contrast, isolating T cells from whole blood offers a safer and more cost-effective cell procurement alternative, enhancing the overall patient experience. However, whole blood contains 10 to 100 times more red blood cells (RBCs) and platelets than leukapheresis material. These cell impurities interfere with standard T cell labeling and enrichment operations and should be removed via a peripheral blood mononuclear cell (PBMC) isolation step prior to processing. Traditional PBMC isolation methods, such as manual or automated Ficoll gradient separation, often result in low recovery, highlighting the need for improved techniques to make whole blood a viable starting material for CAR-T manufacturing.

In this study, we explored the use of cell fractionation filters with pore sizes ranging from 5 to 9 µm, as a novel method for PBMC isolation. These filters separate the larger white blood cells (WBCs) from smaller RBCs and platelets via size exclusion. Using whole blood from two healthy donors, we identified the optimal filter pore size for RBC and WBC separation, achieving 80% CD3+ T cell recovery—a 2-fold improvement over Ficoll gradient separation. We also demonstrated >99% RBC and platelet depletion, 20% granulocyte depletion, and maintained high cell viability (>94%) in the enriched PBMCs. Additionally, we assessed T cell purity and recovery after magnetic enrichment, both with and without prior PBMC enrichment. Finally, we proposed a closed-system T cell enrichment workflow incorporating cell fractionation filters. This workflow facilitates the rapid and scalable production of CAR-T therapies directly from whole blood, improving their accessibility and affordability.

1799 Rapid Isolation of Immune Cells for Cellular Therapeutics Using Multiple Antigen Criteria

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Introduction. Cell therapies have emerged as highly promising treatments for blood-borne cancers, solid tumors and diseases resulting from viral infection. However, CAR-T cell generation is a labor-intensive, rigorous, and costly procedure, and progress towards optimizing cell selection and manufacturing has been slow. The process requires highly trained personnel and/or equipment with a large footprint and exhibits minimal flexibility to suit patient needs and facility scheduling. Moreover, current methods are based on antibodies - which are expensive and time-consuming to produce, exhibit batch-to-batch variability, can have a stability of less than three months in cGMP settings, and may come with a risk of immunogenicity. Centrifugation is often used in this preparatory step, and it has been shown to be associated with cellular stress and cell damage. Finally, the most common cell isolation strategies are based on magnetic nanobeads, which can cause DNA damage through reactive oxygen species generation. We wish to report the development of a closed and automated cell isolation device and cell therapy manufacturing device that significantly decreases the time and cost of cell isolation from both Leukopak and peripheral blood. The technology does not use centrifugation, magnetic beads, or antibodies, and produces intact immune cells isolated using one or more surface marker criteria (double- or triple-positive isolation). Enrichment/depletion can be performed in parallel, minimizing cellular stress during the process of cellular isolation.

Methods. We compared CD8+ CAR-T cell manufacturing performed using the platform against the current leading platform for cell therapy manufacturing. We have demonstrated the flexibility of this approach to isolate double-positive CD8+/CD62L+ T Cell Helper cells.

Results. Cell therapy manufacturing runs performed on the device resulted in a CD8+ CAR-T product with better cell viability, better transduction efficiency, better expansion rate, reduced exhaustion markers, and comparable purity and yield when compared with the leading cell therapy manufacturing device. In addition, the device does not require platelet washing prior to the manufacturing process - reducing the cell isolation process to less than one hour. In the case of frozen starting material, a simple dilution of the cryopreservant agent is all that is required. Small-scale, double-positive isolation of CD8+/CD62L+ T Cell Helper cells (**Figure 1**) provided about 70 million target cells with a purity greater than 95%, viability greater than 99% and a recovery rate greater than 70% in less than 90 minutes. Further developments are undergoing to increase the cell yield to 1 billion target cells.

Conclusion. The cell isolation device provides research centers and manufacturing facilities with a closed, scalable and automated system that provides intact target cells with high viability based on multiple surface antigen selection criteria.

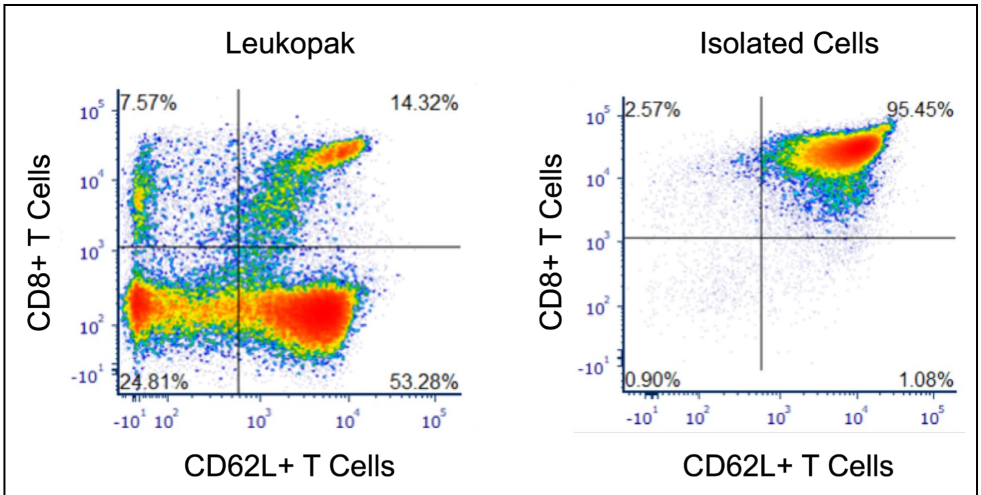

Figure 1: Double-positive isolation of T Cell Helper cells from Leukopak with high purity and yield. Two cell isolation modules were daisy-chained together, with the first module enriching for CD8+ T cells and the second module performing the second selection criteria based on the CD62L surface antigen.

1800 Comparison of Isolation Methods of CD3+ T Cells from Leukapheresis

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Method: Healthy donor leukapheresis and whole blood samples were used as starting materials for T cell isolation. Three methods were compared: two rapid isolation processes and a conventional GMP-compliant isolation method. The rapid isolation methods were tested in both open and closed systems. Following isolation, T cells were cultured and transduced with a lentiviral vector encoding a CAR. CD3+ cell recovery, viability, phenotype, purity, transduction efficiency, and expansion capacity were analyzed. Process efficiency, material cost reduction, and isolation time were also assessed for all methods.

Results: The rapid T cell isolation methods demonstrated notable efficiencies. Post-selection, CD3+ frequencies increased from 56% to 83% (open system) and from 58% to 96% (closed system). CD4/CD8 ratios remained stable across all methods, though donor variability was observed with average viability consistently measured at 98%.

Unwanted cell populations, including CD14+, CD56+, and CD19+ cells, were significantly reduced. CD14+ cells decreased from 24% to 4% (open system) and from 27% to 0% (closed system). Similar reductions were observed for CD56+ and CD19+ populations. T cells isolated using the rapid methods exhibited proliferation comparable to cells isolated via the conventional 5-hour process. The transduction efficiency is equivalent to the conventional GMP compliance process. The closed-system rapid isolation method, which is fully GMP-compliant, achieved similar or superior cell quality while reducing material costs by 52% and processing time to 2.5 hours.

Conclusion: This study highlights a new rapid T cell isolation process suitable for GMP-compliant CAR-T manufacturing. Compared to the conventional GMP-compliant isolation process, the rapid method offered significant advantages, including a 50% reduction in labor, over 50% reduction in total isolation time, material cost savings of up to 52%. Most importantly, the rapid process ensured the delivery of CAR transgenes to target cell types such as T cells, thereby mitigating the risk of transducing malignant cells from starting materials. These findings support the use of new rapid cell isolation process as a viable alternative for automated, closed-system CAR-T cell manufacturing, offering enhanced efficiency, cost savings, and scalability for clinical applications.

1801 Novel Automatic Application for Enrichment of CD34$^+$ Hematopoietic Stem and Progenitor Cells from Apheresis Products

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Enrichment of CD34$^+$ cells from leukaphereses samples has been employed for decades as a part of curative treatments for hematologic malignancies in the context of allogeneic hematopoietic stem cell transplantation (HSCT) to mitigate graft vs host disease (GVHD). Accordingly, CD34$^+$ enrichment represents the first and essential process step for the eventual genetic modification in the HSC gene therapy field. In addition, interventions termed as a “stem cell boost” for both HSCT and CAR-T cell therapy, have been introduced as a measure to manage cytopenia. Therefore, the need to advance the CD34 enrichment process still constitutes a major contributing step in manufacturing and constant improvements are mandatory to further advance cell and gene therapies. To this end, we developed two new, highly efficient streamlined processes to facilitate administration of HSCs in differing therapeutic scenarios without compromising the number and purity of transplantable CD34 cells.

Based on MACS Technology the CliniMACS Prodigy HSC Enrichment System (GMP) and the LP-34-(320) (CE) System, enable automatic production of an injectable CD34-enriched cell population which is also T cell depleted by virtue of the processing procedure. Central components of the new application, which utilizes tubing set TS 320, include a newly developed platelet removal step, a white blood cell (WBC)-adjusted separation and the possibility of product release at a requested time point because the process allows storage of the labeled cells for up to 16h. The process is sufficient for the enrichment of $1.2x10^9$ CD34$^+$ cells from up to $120x10^9$ total WBCs. To facilitate analysis during manufacturing, a new fluorescent flow analysis protocol suitable for analysis of the very low percentage of remaining T cells in the purified product and a low percentage of CD34 cells in the starting material was additionally developed.

In house evaluation runs (n=24) using mobilized leukapheresis products resulted in a mean depletion of 4.7 log (range 4.2 – 5.4) for T cells, a mean platelet depletion of 2.8 log (range 2.0 – 3.7), a mean B cell depletion of 3.2 log (range 2.0 –3.8), and a mean purity of 90.9% (range 82.7 – 97) in the CD34-enriched product. Viability of CD34 cells in the target product was above 99%, mean yield was 65.2% and mean WBC recovery was 85.4%. The process duration is approximately within 3.0 – 5.5 hours, depending on the cell numbers, excluding the device set up and the optional in-process storage. The comparability to the existing CliniMACS Plus System was also addressed and will be presented.

Conclusively, the novel application is an automated process in a closed fluidic system with minimal user interaction and flexible time control that supports the need for individual cell manufacturing. The highly purified $CD34^+$ stem cells can be used either as an injectable stem cell graft or as a starting product for subsequent stem cell engineering approaches.

1802 From Fresh to Frozen and Everything in Between: Revolutionizing WBC Isolation from Leukopak and Whole Blood with the Rotea Counterflow Centrifugation System

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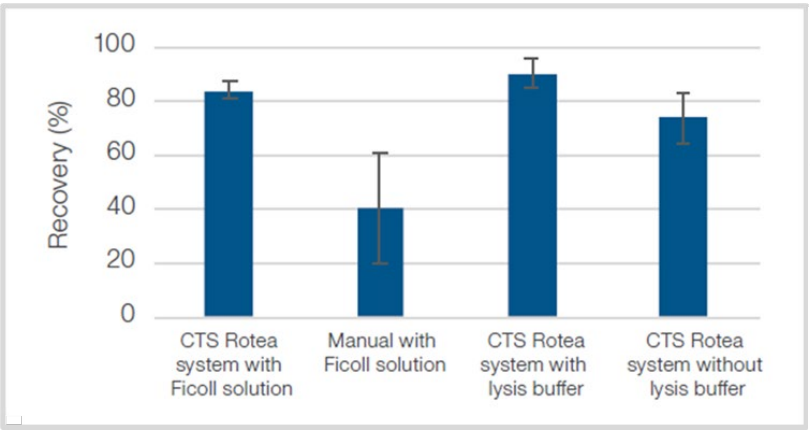
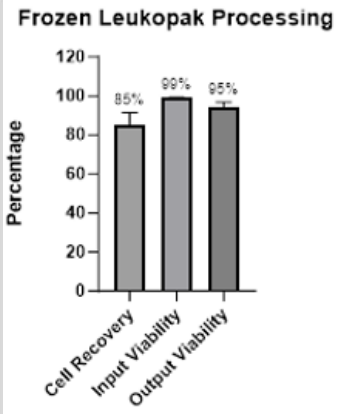
Aim and background: The cell therapy market today comprises numerous companies and academic institutions utilizing various starting materials to produce final cell therapy products. As the cell therapy market continues to grow rapidly, the demand for flexible and efficient processing instruments is increasingly critical. The Rotea Counterflow Centrifugation System is a versatile cell processing instrument capable of isolating white blood cells (WBCs) from multiple sources, including whole blood, fresh leukopak, and frozen leukopak, with minimal viability loss and exceptional cell recovery.

Methods & Results: The counterflow centrifugation system was used to isolate the WBCs using the 3 different source material with multiple donors to account for the donor to donor variation. The cell recovery and viability pre and post processing was analyzed using an automated counting instrument and the phenotype pre and post processing was measured using flow cytometry. Cells post isolation were also cultured to make sure of no adverse effect in the expansion process. For fresh leukopak, the system offers three distinct methods to isolate WBCs, employing different reagents. These methods cater to customers using legacy reagents like Ficoll and introduce new approaches with ACK lysis buffer for RBC lysis and WBC isolation, achieving a cell recovery rate of 91%. Phenotypic analysis before and after processing indicates no significant differences. For users preferring frozen leukopaks due to their convenience, the system provides an efficient option for WBC isolation, achieving an 85% cell recovery rate without significantly impacting viability. The system also supports whole blood processing, appealing to those seeking time, cost, and resource efficiency. This method yields a 70% cell recovery rate with minimal RBC carryover and no significant viability impact. Phenotypic data confirm no notable differences pre- and post-processing. The process can also be customized to enrich a specific set of cell type as well.

Conclusion: The adaptable and modular design of the Rotea Counterflow Centrifugation System makes it an invaluable tool for laboratories aiming to optimize WBC isolation processes. As demand for advanced cell therapy solutions increases, the need for such versatile and efficient instruments will become even more pronounced, ensuring high-quality outcomes in the evolving cell therapy landscape.





1803 Novel Detection Method for Residual Beads in CAR T-Cell Manufacturing Using Imaging Flow Cytometry

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Gene-modified cell therapies, such as Chimeric Antigen Receptor T-cell (CAR-T) therapy, have revolutionized cancer treatment and show potential for treating genetic disorders and autoimmune diseases. The manufacturing process involves isolating, activating, and expanding human T cells, for which CD3/CD28 Dynabeads offer a streamlined solution. These beads have been used in over 200 clinical trials; however, since they are non-biodegradable, their removal from the final product is crucial. Newer systems with detachable beads still consider residual beads as contaminants, with the FDA requiring fewer than 100 beads per 3 million cells in the final product. Consequently, a precise and accurate assay for detecting and quantifying residual beads is essential for product release testing. Current methods are either labor-intensive, have low throughput, or require specialized equipment. This study introduces a novel imaging flow cytometry-based assay designed to improve quality control and reliability in product release testing. The study employed the Attune CytPix flow cytometer, which combines traditional cytometry with high-content imaging, making it ideal for detecting rare events like residual beads in cell products. Over 300,000 images per sample were captured and analyzed using the instrument's software to assess object and pixel attributes. Twenty-six image analysis parameters were explored in various combinations to determine the optimal gating and analysis strategy for distinguishing cells and beads. The analytical procedure was validated according to ICHQ2R2 guidelines to ensure consistency, accuracy, and reproducibility. The method proved reproducible across different users, instruments, and labs, with a lower detection limit of 20 beads and a lower quantification limit of 20 beads per million cells. This novel assay offers a reliable alternative to existing residual bead detection methods and can be implemented on a platform commonly available in cell therapy manufacturing facilities.

1804 Method Development and Scale Prediction for Separation Systems to Solve Challenges in Allogeneic Scale-up

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There are many challenges to scalability and continuous processing for allogeneic therapies that will need to be addressed to reduce production costs and ensure stable supply. One such consideration for both the production of viral vectors and for large scale cell therapies is the high weight % of solids in concentrated and process intensified bioreactors. As the titers increase, it is critical to reduce hold-up and utilize smaller surface area devices, achieving high flux and throughput to keep processing times short and eliminate loss. This work presents the application of established filtration methods to predictively model the throughput and hold-up per surface area on different nominal porosity separation systems and compares them to other methods. This also presents a process for establishing surrogate material to establish boundary conditions for the devices ahead of approaching cell separation by trial and error.

1805 Streamlined Ex-Vivo Engineering of Human T Cells: A Single-Step Approach to Activation and Lentiviral Transduction

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Efficient T cell engineering is essential for the success of CAR T cell therapy. Conventional methods—encompassing T cell isolation, activation, and transduction—are often intricate and time-consuming. They typically require materials from multiple suppliers and involve separate activation steps using antibody-coated beads or soluble antibody complexes, followed by transduction processes that frequently depend on spinoculation with

enhancers such as Polybrene or LentiBOOST. These traditional approaches demand considerable time and labor while often exhibiting variability and inefficiencies that can negatively affect cell yield and therapeutic effectiveness. We present an innovative approach to enhance the ex-vivo engineering of human T cells by integrating cell activation and efficient lentiviral transduction into a single step, reducing time and resource requirements while maintaining high performance. Our approach leverages a lyophilized, GMP-grade alginate-based microfluidic sponge embedded with anti-CD3 and anti-CD28 antibodies and interleukin-2 for efficient activation of T cells. The one-step transduction is performed by simply applying a mixture of recombinant virus and unstimulated T cells or PBMCs to the Sponge, followed by incubation at 37°C. The hygroscopic nature and 20-300 nm pore size of the sponge help to spatially colocalize cells and virus within an exceedingly small space, facilitating efficient transduction as well as ensuring uniform exposure to activation signals. The release of activated and transduced cells is facilitated by a chelating release buffer that quickly depolymerizes the sponge, ensuring the rapid recovery of high-quality transduced cells with minimal handling. This design simplifies the workflow compared to spinoculation with Polybrene, reducing the process by 9 steps compared to traditional methods, while enhancing transduction efficiency via a centrifugation and enhancer-free microfluidics approach. The reduced number of handling steps also minimize contamination risks and variability between batches. Our data across multiple donors and operators consistently indicate strong activation and transduction efficiencies (%CV <15%; n=54), with no adverse effects on growth rates, cell phenotypes, or exhaustion marker expression compared to standard protocols. The reproducibility of this new method underscores the robustness of our approach. Furthermore, our ex-vivo lentiviral T cell transduction sponge is user-friendly and scalable, accommodating cell numbers ranging from 2e6 to 1e7 per sponge, making it suitable for both research and preclinical applications. By providing a streamlined and user-friendly system for T cell engineering, we strive to drive progress in T cell therapies and expand their availability to a wider range of patients.

1806 Maximizing Safety and Efficacy in Hematopoietic Stem Cell Gene Therapy

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Although new treatments for hematopoietic stem cell (HSC) gene therapy have been recently approved for several diseases such as beta-thalassemia (Zynteglo), sickle cell disease (Lyfgenia and Casgevy), and adrenoleukodystrophy (Skysona), a certain number of challenges still remain such as the high costs and scalability associated with these therapies, as well as the increased safety concerns regarding the related leukemic events. These indicate a need for improved and standardized manufacturing steps allowing for large scale implementation of gene therapy interventions to a degree that addresses the population suffering from the long-term sequalae of these diseases. Automation is the key to achieve these goals and, to this end, the CliniMACS Prodigy as an automated, closed, GMP-compliant platform for HSC transduction, is an ideal candidate to streamline cell processing. We had previously shown (https://doi.org/10.1182/blood-2019-128780) that lentiviral transduction (LV) is facilitated on the CliniMACS Prodigy compared to manual steps when a VSVG pseudotyped LV expressing GFP was utilized. In the current study, we extended the same rationale by employing LVs expressing CAR constructs, VSVG- or BaEV (Baboon Endogenous retrovirus)- pseudotyped, to compare transductions performed on the CliniMACS Prodigy vs the open manual steps. We also made a slight modification to our original transduction medium which now contains only the “basic” necessary cytokines such as SCT, FLT3 and TPO. With the new modifications we overall observed comparable cell recovery and viability between the manual and automated conditions, but still superior performance of the automated platform in terms of transduction efficiency. Flow cytometric analysis for transgene expression using the VSVG pseudotyped LVs with an MOI of 30 showed 39% vs 20% expression on the Prodigy vs the manual process without a significant difference in vector copies per genome. Interestingly, our analysis for the BaEV pseudotyped vectors demonstrated superior transduction efficiency with a very low MOI (0-5) in the presence of vectofusin. More specifically, by using the open manual process and an MOI of 2 and an MOI of 0.75, we saw 40% and 20% transgene expression, respectively, which is unprecedent for HSC transductions under these minimal MOIs. Even under these conditions of minimal MOI, the automated platform exhibited higher transduction efficiency vs the manual steps. *In vivo* experiments using NSG mice also demonstrated better engraftment for the $CD34^+$ cells processed on the CliniMACS Prodigy under the “basic” transduction conditions compared to the

manually cultivated $CD34^+$ cells and also compared vs the "gold standard" i.e. uncultivated and unmanipulated $CD34^+$ cells directly after enrichment. Better engraftment was also corroborated by the stable number of CFUs generated by the cells processed on the CliniMACS Prodigy vs cells processed manually which showed a clear trend towards decreasing numbers of CFUs. Based on these results, we propose a combined strategy of transduction enhancers and the use of Baboon envelope pseudotyped LV on the automated platform to achieve significantly higher transduction efficiencies and enhanced engraftment. Experiments to investigate the mechanisms involved in better performance of LV transduction on the automated platform, including detailed molecular analysis of gene expression pathways by Next Generation Sequencing (NGS), are ongoing.

1807 Optimizing CRISPR/Cas-mediated CAR Knock-in in Primary Human T Cells Using MaxCyte® Electroporation

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In recent years, chimeric antigen receptor (CAR) T cells have emerged as a leading treatment for various hematological cancers. Despite demonstrating great promise, there are several challenges that limit CAR T cell therapies including concerns regarding efficacy, safety and manufacturability. To address these, groups have turned to non-viral engineering of CAR T cells using CRISPR gene editing. In addition to reduced immunogenicity and manufacturing costs, this technology enables precise knock-in of tumor-targeting receptors and knockout of genes responsible for rejection, toxicity and immunosuppression. To CRISPR engineer CAR T cells, Cas nucleases, guide RNA (gRNA) and homology-directed repair (HDR) template DNA must be efficiently delivered into T cells without jeopardizing their viability or functionality. With this in mind, we sought to generate optimized workflows that enable CRISPR-mediated gene editing in primary human T cells using MaxCyte® electroporation. To this end, we first identified optimal electroporation protocols and concentrations of sgRNA, Cas9 and HDR template required to knock-in *GFP* at the *TRAC* locus. Next, we compared different repair template designs, including the addition of Cas9 targeting sequences (CTS). We also investigated multiple HDR template DNA formats, including linear double-stranded DNA, single-stranded DNA, conventional plasmids and Nanoplasmids™. In addition, we explored the use of small molecule enhancers and identified several that further improved editing efficiencies. These findings were then used to engineer CD19-targeting CAR T cells. With activated T cells from healthy donors, we could achieve CAR expression levels of greater than 70%. In addition to efficient and reproducible CAR knock-in in T cells from multiple donors, cells engineered using this workflow were viable and retained the ability to expand and eradicate CD19-expressing target cells. Together, these demonstrate the capability of MaxCyte's clinically-validated ExPERT™ electroporation technology to enable non-viral engineering of CAR T cells using the CRISPR/Cas system.

1808 A Versatile Platform for Cell Therapy Cytotoxicity Assays Using mRNA Transfection

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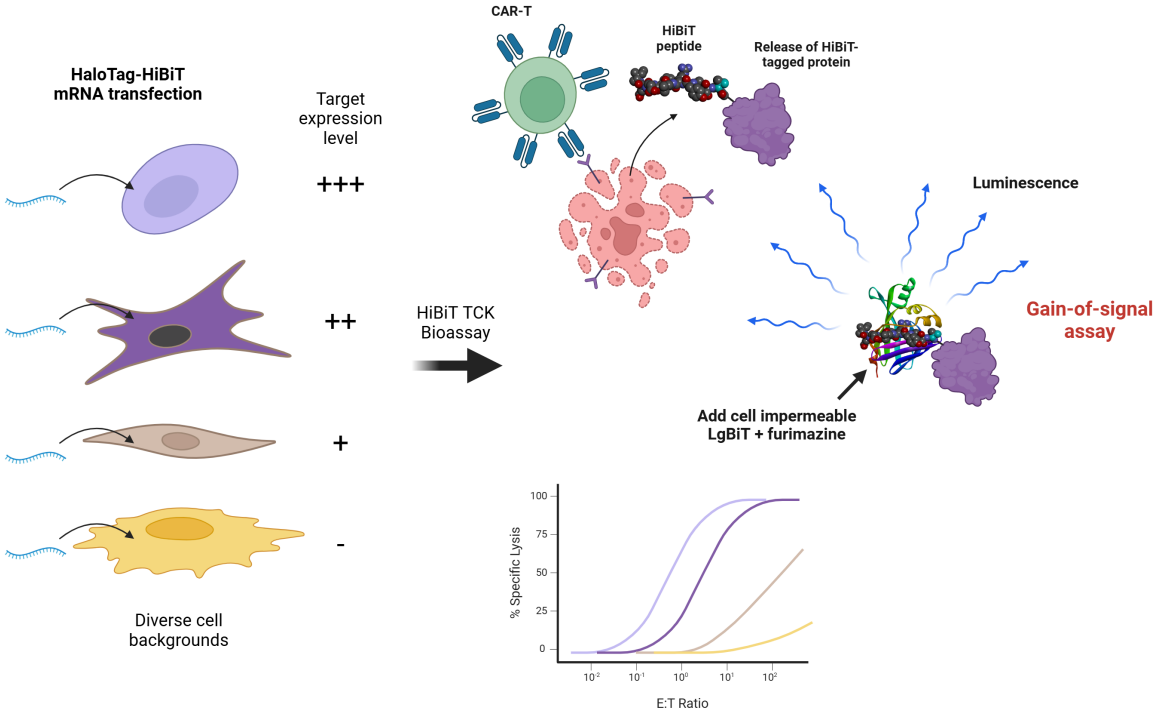
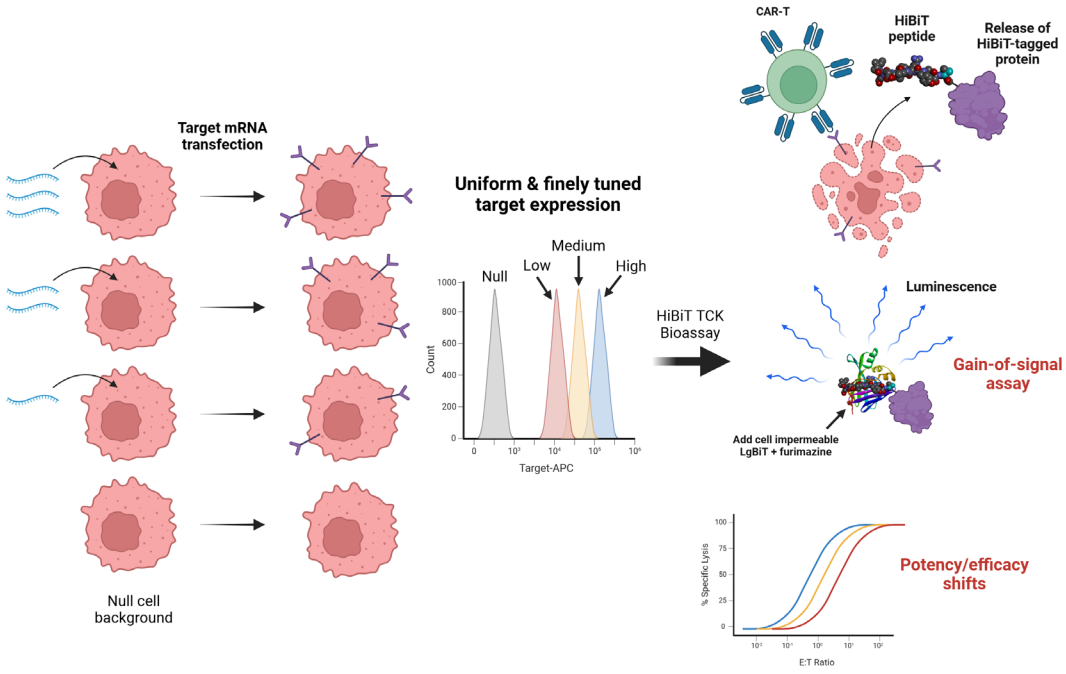
The HiBiT Target Cell Killing Bioassay (HiBiT TCK Bioassay) is a sensitive and selective method for quantifying target cell lysis in coculture experiments with effector cells. In this assay, target cells engineered to express a HiBiT-tagged protein are cocultured with effector cells, where target cell death promotes release of the HiBiT-tagged protein into the extracellular medium. Once there, HiBiT can bind to cell-impermeable LgBiT to generate a bright, luminescent enzyme in the presence of furimazine substrate. In this manner, a non-lytic detection reagent containing LgBiT and furimazine substrate can be used to quantify target cell death in a gain-of-signal assay format with a cumulative readout. To date, this approach has been used exclusively with stable expression of the HiBiT-tagged protein, requiring time and effort to make stable clones.

To expand beyond the use of stable clones, we developed a novel mRNA transfection reagent that enables rapid and uniform expression of HaloTag-HiBiT in a wide range of target cell types. This approach dramatically reduces the

workload for creating HiBiT target cells, allowing users to readily label target cell type(s) of interest. We demonstrate labeling of a variety of target cell types and their use in the HiBiT TCK Bioassay. Additionally, we demonstrate the use of mRNA transfection to achieve uniform and highly tunable expression of target receptors in cells stably expressing a HiBiT-tagged protein, allowing drug potency/efficacy testing across a broad range of receptor expression levels in a single cell type.





1809 Establishment of The Inducible AAV Production Cell Line 293RSS2.0

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The production of adeno-associated virus (AAV) in mammalian cells predominantly relies on the transient triple-plasmids transfection into HEK293-based cells. Four of seven EMA/FDA-approved AAV gene therapy products employ this production approach, capitalizing on its inherent assurance of product quality. Nevertheless, owing to the expenses associated with the transfection reagent and the production of three plasmids, the overall cost of transfection-based manufacturing proves to be high. Conversely, the constrained stability exhibited by the transfection complex during operational durations, coupled with the challenges associated with achieving prompt yet gentle mixing in a bioreactor, poses impediments to the scaling-up of production volumes beyond 500 liters for transfection-based AAV production. The cumulative drawbacks underscore the pressing necessity for the development of a production cell line that can seamlessly integrate all essential elements for AAV production. In our previous work, we have constructed a prototype production cell line, 293RSS1.0, characterized by inducible expression of AAV Rep and Cap proteins, which demonstrated promising potential for inducible AAV vector packaging. A new generation of stable production cell line, 293RSS2.0, was successfully developed with the stable integration of inducible Rep, Cap, and Helper expression cassettes, which only need the GOI plasmid transfection for AAV production.

In 293RSS2.0, further refining the expression ratio between Rep78 and Rep52 was achieved by engineering the inducible Rep expression cassette, which exhibited over a >10-fold enhancement in AAV production yield compared to 293RSS1.0. Lingyi-patented inducible Cap expression cassette and inducible Helper expression cassette were both incorporated in 293RSS2.0 cell line. This 293RSS2.0 production cell line consolidates all essential components necessary for AAV vector packaging, except for the gene of interest (GOI). Efficient AAV vector packaging was achieved via transfection with a single GOI plasmid in conjunction with a small-molecule inducer. In the absence of the inducer, the AAV packaging rate remains exceedingly low, consistent with Western blot results indicating minimal leakage of the essential components required for AAV packaging. The bioactivity of the vector generated through this innovative system was evaluated, demonstrating comparable bioactivity to that of the vector produced via the conventional triple-transfection method.

In conclusion, the 293RSS2.0 cell line facilitates the inducible expression of various components necessary for AAV vector packaging. The comparable yield and quality of AAV production suggest that this stable cell line-based system has the potential to serve as a transformative platform for future AAV vector manufacturing.

1810 The First Coacervate-Based Delivery System for CAR-T Cell Engineering and Manufacturing

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Chimeric antigen receptor (CAR) T cells have revolutionized the treatment of hematological malignancies, but efficient and scalable engineering of primary cells remains a significant challenge in CAR-T cell manufacturing. Coacervates are condensed droplets containing proteins or nucleic acids formed via liquid-liquid phase separation. Here, we report the identification of a mammalian endogenous protein capable of forming coacervates with nucleic acids. These coacervates exhibit an RNA encapsulation capacity 1000-fold greater than lipid nanoparticles (LNPs), efficient cellular uptake, robust cytoplasmic release of nucleic acids, and broad compatibility with diverse primary immune cell types, including T cells, NK cells, and hematopoietic stem cells (HSCs). Building on these properties, we developed the first coacervate-based system for gene delivery (EASY) engineered using human and mouse proteins (PCT/CN2024/124967). EASY supports a wide range of genetic cargoes for gene overexpression, knockout, and targeted integration.

Using EASY, we achieved >90% transfection efficiency of mRNA encoding GFP in primary human T lymphocytes while maintaining 95% cell viability. GFP expression was sustained in 64% of cells for up to 12 days post-transfection, underscoring the platform's capacity for prolonged expression. Using EASY, we successfully generated mRNA-based anti-CD19 CAR-T cells that eliminated tumor growth in NSG mice xenografted with CD19+ Raji cells after a single dose (5×10^6 CAR-T cells). Furthermore, EASY facilitates efficient gene knockout by co-delivering Cas9 mRNA and sgRNAs. For instance, >80% knockout efficiency was achieved in the TRAC gene in primary T cells, enabling precise CAR-T engineering.

To enable gene-specific integration for CAR-T manufacturing, EASY was employed to co-deliver Cas9 mRNA, sgRNA, and single-stranded DNA (ssDNA) templates for homology-directed repair (HDR). This approach achieved >30% knock-in efficiency of an anti-CD19 CAR cassette at the PD locus, generating CAR-T cells with sustained CAR expression and disrupted PD1 function. These integrated CAR-T cells demonstrated potent anti-tumor activity in NSG mice xenografted with CD19+ Raji cells, highlighting their therapeutic potential.

EASY's simplicity, adaptability, and scalability make it ideally suited for clinical applications. The system has been successfully scaled up to deliver mRNA into over 10^9 primary T cells with >90% efficiency, demonstrating its feasibility for large-scale CAR-T manufacturing. Its straightforward preparation and high-throughput transfection capability further distinguish it as a transformative tool for advancing CAR-T engineering. In conclusion, EASY offers an innovative, non-viral, LNP-free, and electroporation-free platform for efficient and scalable CAR-T cell engineering. Its high efficiency, minimal toxicity, and compatibility with diverse genetic modifications position EASY as a next-generation solution for CAR-T manufacturing, unlocking new possibilities for cell therapy development.

1811 Development of a Quantitative Screen to Assess AAV On/Off-Target Binding and Transduction

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Adeno-Associated Viruses (AAV) are a leading vector in gene therapy, with three FDA therapies approved over the last 5 years (1). Continued interest in AAV's shows the promise of this field, however issues including immunogenicity and large-scale manufacturing costs remain limiting factors for advancing AAV-based gene therapies (2,3). Here we describe a novel flow cytometry based dual screening capability for AAV's as a downstream assessment of targets identified using the Charles River Retrogenix® Cell Microarray platform.

Data from the Retrogenix® Cell Microarray technology, consisting of >6,500 full length human cell surface, secreted, tethered secreted, and heterodimeric vectors, and more recently non-human (Cynomolgus) protein targets, each over-expressed in HEK293 cells, is already used extensively in regulatory BLA and IND submissions. To provide further understanding for AAV profiling (4), flow cytometry testing has been developed. This sensitive, multiparametric and quantitative method provides a powerful tool for gene therapy by enabling detection of AAV receptor binding, transduction efficiency, and expression of encapsulated reporter genes in live cells.

Wild Type AAV9 controls were used to develop a flow cytometry binding assay to the known KIAA0319L AAV receptor, along with off targets previously identified using the Retrogenix® platform. In addition, AAV internalization (transduction) methodology was developed by measuring reporter gene expression. To avoid underestimating functional effects of low-level transgene expression, this was recorded as % positive cells, a method typically used in gene therapy (5,6).

Complimenting Retrogenix® Cell Microarray data using an orthologous platform provides a comprehensive insight into primary receptors and potential adverse event liabilities of AAV's. With the recent addition of non-human plasma membrane proteins with 90% human homology in the extracellular domain, our approach of assessing AAV

activity and specificity at these overexpressed proteins can help de-risk therapeutic candidates, aid in-vivo model selection, and provide data to support regulatory submissions.

1812 A Kinetic Perfusion Assay to Measure Gene Switch Responses *in vitro*

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Introduction: Synthetic biology has made great strides toward approaching user-controlled gene expression by designing complex signaling systems within one genetic structure. These "gene switches" include the use of inducible promoters, synthetic receptors, and feedback loops to improve the specificity and sensitivity of transgene expression. Yet, these gene switches have transgene dynamics that have short timescales (~ minutes – hours) making them difficult to monitor using conventional cell culture approaches. Here, we present a flow assay system (Fig. 1A) to study the kinetics of gene switches with automated supernatant sample collection by integrated fractionation. Specifically, we tested a small library of inducible promoters that are switched on by cytokines to drive the release of a secreted biomarker (Fig. 1B) and characterized kinetic parameters by curve-fitting experimental data to math models of gene expression.

Methods: A continuous flow system was assembled to perfuse an in vitro cell culture and collect fractionated media over 24 hours. HEK cell reporter lines were stably engineered with different inducible promoter systems that encoded for specific transcription factor response elements (NFkB, STAT3, and AP-1). The inducible gene switches were upstream of a secreted biomarker, Gaussia Luciferase (GLuc), which was measured in fractionated supernatants using a bioluminescence assay. An in vitro stimulation study was performed by pulsing a cytokine-supplemented media followed by 24-hours of continuous flow of cell media to measure GLuc secretion dynamics. Complex media studies were also conducted using combinations of cytokines as well as supernatants harvested from a mixed lymphocyte reaction (MLR) assay to model an immune response. GLuc data was plotted against time to extract gene switch parameters by fitting data to mathematical expressions that model switching phenomena.

Results: Transgene induction and decay of a representative gene switch is shown to exemplify the type of kinetic data gathered using this perfusion system (Fig. 1C). In this example, a short pulse of TNF-α activated the reporter line resulting in a transient increase of GLuc followed by non-linear reduction in GLuc over time. The dose-response curve for the NFkB cell line was normalized to a non-engineered control and an activation curve was plotted (Fig. 1C). From the data provided by this dynamic system, we can extract several parameters (AUC, $t_{1/2}$, C_{max}, k_{on}, and k_{off} rates) of the gene sensors. Results using different inducible promoters, stimulated by single, combinations, or immune cell-derived cytokines were parametrically compared to learn about gene switch response dynamics under more complex conditions.

Conclusions: This flow-based assay system is designed for ease of use to enable kinetic sample collection in a relevant time scale for the study of gene switches. Parametric analysis of this kinetic data becomes useful in comparing responses for different genetic designs to see which is suitable for a given application. The characterization of dose-response relationships, on/off rates for a gene switch, and how complex media conditions can activate a given gene switch can add important insight for translating these technologies for cell and gene therapy.

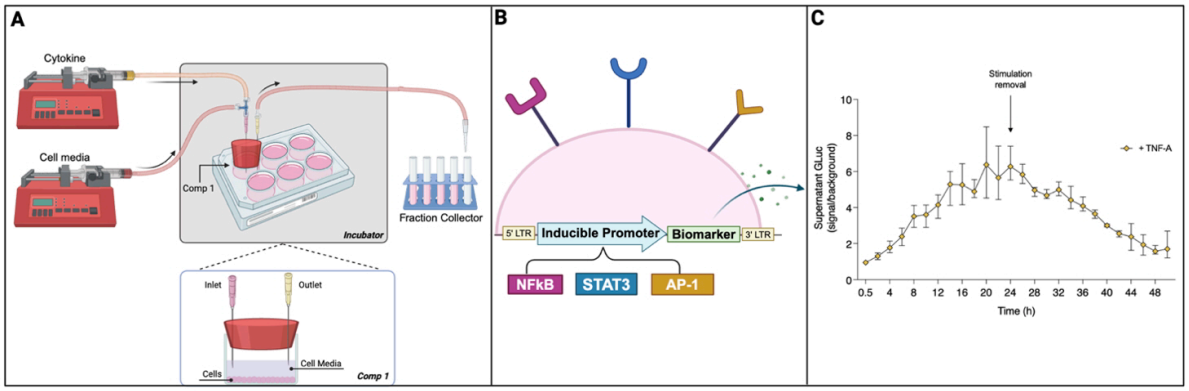

Figure 1. Perfusion assay system to study temporal activity of gene switches. (A) Perfusion bioreactor system with automated supernatant collection for cell secretion dynamic studies. (B) Schematic of inducible immunosensor designs where gene switches are activated to improve specificity and sensitivity of transgene expression. (C) Study of inducible response element (NFkB) activation dynamics upon continuous stimulation with cytokine supplemented media for 24 hours. Stimulation was stopped in the last 24 hours. Biomarker secretion (GLuc) was measured via bioluminescence assay in all fractions collected throughout the study.

1813 Rapid Mycoplasma Detection Techniques for Facilitating Quality in Low-Volume T Cell Therapies

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The increase in demand for CAR T-based immunotherapies has necessitated improvements in the analytical workflow to enhance patient access while maintaining safety and efficacy. Particularly relevant for cell therapy manufacturers is the potential challenge of limited sample availability. To address this challenge this study delves into the sensitivity and effectiveness of a qPCR-based assay for detecting mycoplasma contamination in T-cell cultures, which are pivotal for the development of cell-based therapies. The research specifically targets the assay's performance in low-volume T-cell samples, typically ranging from 1 to 3 mL.

Methods: The method used in this study involved the preparation and analysis of low-volume T-cell samples to detect mycoplasma contamination using the Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection assay, which is PCR-based. Initial sample volumes of 1, 2, and 3 mL were processed. The procedure included spiking DNA templates into the samples, followed by cell lysis, DNA binding, washing, and elution with either Applied Biosystems™ PrepSEQ™ Manual or Automated Express extraction workflow followed by alcohol precipitation steps to concentrate the final eluate. The DNA was then subjected to qPCR analysis using specific reagents and conditions to determine the presence of mycoplasma. Both automated and manual extraction workflows were evaluated to optimize the detection sensitivity and enable reliable results across various sample volumes.

Results: The study demonstrated the high sensitivity of the MycoSEQ Plus assay in detecting mycoplasma contamination in low-volume T-cell samples across various matrices. The detection rates for key mycoplasma species (M. arginini, M. salivarium, M. fermentans, M. orale, and M. pneumoniae) spiked into different media were consistent and reliable, with all tested species detected at a 100% rate at the specified LOD. The comprehensive workflow combining the Applied Biosystems™ PrepSEQ™ Manual or Automated Express extraction, followed by alcohol precipitation, proved effective in concentrating the DNA for accurate qPCR analysis. Both manual and automated workflows seamlessly integrated with the Applied Biosystems™ AccuSEQ™ analytical software, helping to ensure robust and reproducible results while helping to achieve 21 CFR Part 11 compliance.

Conclusions: The MycoSEQ Plus Mycoplasma Detection Kit provides an effective and sensitive solution for the detection of mycoplasma in low-volume T-cell samples. Its successful application in different sample matrices underscores its utility in quality assurance, quality control, and process development for cell therapy manufacturing. By offering reliable detection in lower sample volumes, it expands the usability of the MycoSEQ Plus assay, particularly for cell and gene therapy applications where sample volume is often limited.

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1814 Assessment of Adeno-Associated Virus (AAV) Purity by Capillary Electrophoresis-Based Western

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In the development of AAV-based gene therapies, it is important to obtain a drug product with high purity and minimal levels of impurities. In this study, we present two CE-Western assays for the analytical assessment of AAV, focusing on quantifying the relative stoichiometry of viral proteins (VP) and measuring residual baculovirus protein impurities, specifically GP64. Various purified samples from diverse AAV serotypes were analyzed to determine their VP ratio or GP64 levels. The ratio of VP3/VP1 in rAAV samples was correlated with biological activity, and the clearance of GP64 from the manufacturing process was demonstrated. The results were further supported by LC-MS analyses. We demonstrated that CE-Western can be used as a high-throughput platform to assess the identity, composition, and purity of rAAV drug products.

1815 Seroprevalence, Correlation, and Clinical Relevance of Total and Neutralizing Anti-AAV Antibodies and Assay Formats

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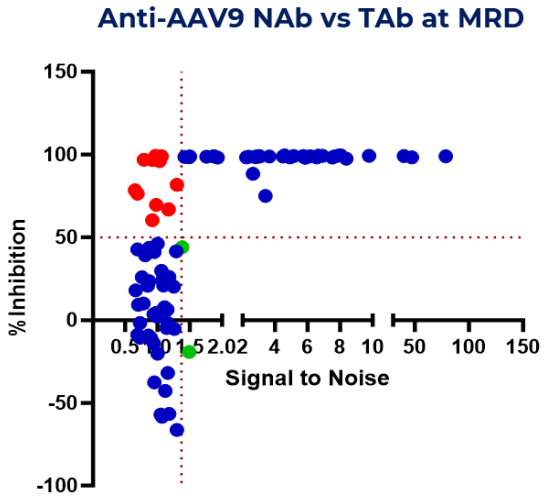
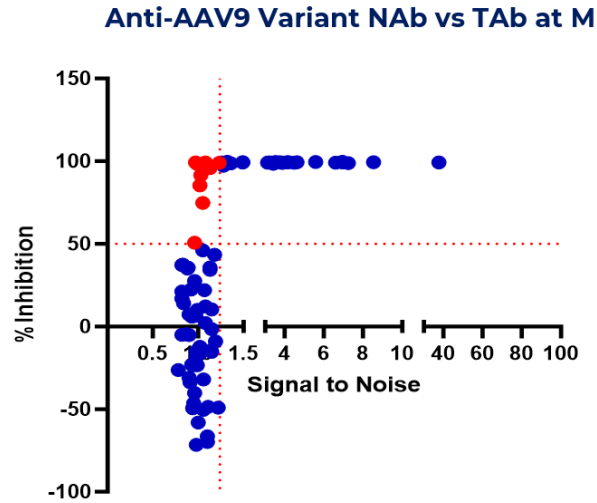
AAV based gene therapies provide great promise for curative treatment of many genetics-based diseases but are limited in practice due to existing anti-AAV antibodies, for which seroprevalence is high and is also dependent on age, race, and disease populations. These pre-existing antibodies can inhibit the transduction of AAVs carrying therapeutic transgenes, and subjects who have them are typically excluded from participating in clinical trials due to safety and efficacy concerns. Therefore, a robust and translatable bioanalytical method that measures and characterizes these antibodies must be employed during screening periods as an exclusion/inclusion criterion. There is currently no formal guidance on what method to use, and therefore, different assay formats have been applied, including cell-based transduction inhibition assays (neutralizing antibody assays; NAb) and total antibody-based ligand binding assays (TAb). We investigated the seroprevalence of anti-AAV antibodies in healthy donors for two different capsids, AAV9 and a variant of AAV9, using TAb and NAb assays to help facilitate the decision for which assay format would be the most appropriate for clinical trial inclusion and exclusion. Among 200 serum samples tested in the TAb assays, 45% and 39% were positive respectively, for anti-AAV9 and the AAV9 variant. TAbs with titers ranging 50 (the MRD or minimum required dilution) to 51,200-fold. 100 samples were tested in the NAb assays resulting in 55% and 50% being NAb positive, respectively, for AAV9 and the AAV9 variant with titers ranging from 5 (the MRD) to >40-fold. We observed a strong correlation between AAV9 and the AAV9 variant TAb positive samples (R^2=0.62). The seroprevalence of AAV9 antibodies that we observed aligns with relevant literature using robust qualified assays and cut-points. We also observed a strong correlation between positive and negative TAb samples with their characterized NAb responses for both capsids with an overall 10% discordance seen between TAb and NAb. We performed isotyping for IgG and IgM to explore the contribution of antibody-related affinity or avidity binding differences between the bridging and cell-based formats and did not observe an IgM related contribution to the discordance. TAb seroprevalence being 10% lower than NAb seroprevalence for both capsids is most likely due to non-antibody related factors and other assay format differences that requires

further investigation. For clinical trial enrollment expectations, a seroprevalence rate of 45% for AAV9 TAbs with a goal to enroll 50 subjects would require recruiting at least 111 subjects for screening.

In summary, the overall concordance data and assessment supports using the TAb assay format for patient screening and future companion diagnostic development for AAV gene therapy programs using AAV9 and closely related capsids.



	Number
TAb - NAb -	43
TAb - NAb +	13
TAb + NAb -	2
TAb + NAb +	42



	Number
TAb - NAb -	50
TAb - NAb +	9
TAb + NAb -	0
TAb + NAb +	41

1816 Novel Bioluminescent Assays for Detecting Anti-AAV Neutralizing and Total Antibodies in Serum Samples

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Adeno-associated virus (AAV)-based gene therapies have shown great promise for treating a range of genetic disorders. However, pre-existing or therapy-induced anti-AAV antibodies pose significant challenges, impacting therapy efficacy and patient eligibility. Existing methods for detecting anti-AAV antibodies often require extensive hands-on time and specialized instrumentation, limiting their utility in clinical and preclinical settings.

This study aimed to develop and validate multiple novel assays capable of detecting anti-AAV Neutralizing Antibodies (NAbs) and Total Antibodies (TAbs) in serum samples, while offering enhanced sensitivity, specificity, and ease of use.

The first approach, a cell-based AAV transduction inhibition (TI) assay, leverages the superior brightness of NanoLuc Luciferase to detect NAbs or other factors influencing AAV transduction. These assays were optimized for AAV serotypes AAV2, AAV8, and AAV9, with low multiplicity of infection (MOI ≥ 100) and low minimum required dilution (MRD 1:3), maximizing the detection of low-titer samples. At higher MOIs (1,000–10,000), total assay runtimes can be reduced to as little as six hours, enabling completion of TI experiments in a single day. Compared head-to-head with firefly luciferase as the reporter, NanoLuc was found to be >700-fold brighter, significantly enhancing assay sensitivity. Furthermore, the use of thaw-and-use cells was validated to increase assay robustness, further streamlining the workflow.

The second approach focuses on the detection of TAbs through proof-of-concept immunoassays using various forms of split NanoLuc. These include both solid and solution phase approaches. The solution phase method employs a completely homogeneous, no-wash protocol that can be completed in as little as two hours. Further characterization and validation are ongoing to establish assay performance and utility.

Together, these assays provide novel approaches for detecting anti-AAV NAbs and TAbs in serum samples. Their robust performance and user-friendly formats address key limitations of existing methods, facilitating improved patient screening and monitoring in gene therapy programs. Future studies will focus on expanding the assays to additional AAV serotypes and validating their utility in diverse settings.

1817 Anti-Pan AAV – New Versatile Antibody for the Detection of Various AAV Serotypes Including Novel Capsids

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Recombinant adeno-associated virus (AAV) vectors have become leading tools for viral gene therapy. However, some biological mechanisms are still not completely understood. Different AAV serotypes are employed in gene therapy development, each offering unique tissue tropism depending on the specific serotype. Additionally, the field is witnessing a surge in the development of novel, engineered AAV serotypes with optimized properties to address diverse therapeutic needs. In this context, antibodies capable of specifically detecting conformational epitopes on AAV capsids—thereby recognizing only intact and fully assembled particles—are indispensable for the accurate quantification of AAV titers. These specialized antibodies are essential for establishing stringent quality control standards, ensuring robust and precise evaluation of AAV gene therapy products. By enabling accurate titer determination, these antibodies contribute significantly to the therapeutic efficacy and safety of AAV-based gene therapies.

PROGEN offers an extensive portfolio of AAV antibodies tailored to bind the specific serotypes. However, certain research applications can benefit from using a single antibody that recognizes a broad spectrum of serotypes. Additionally, with the increasing use of novel capsid variants and shuffled vectors, commercially available antibodies often fall short. To address this need, we have developed a new recombinant antibody that exhibits robust binding reactivity to intact particles of various AAV serotypes.

Here we present comprehensive characterization data for the new anti-pan AAV antibody. We introduce several variants of this antibody and demonstrate its cross-reactivity and high sensitivity against multiple AAV serotypes, including new variants and shuffled vectors. We provide data supporting its applicability across a range of research needs. Specifically, we illustrate its effectiveness as a neutralizing antibody, its compatibility with ELISA test

systems, and its utility in fluorescence-based applications requiring labelled antibodies. Due to this multifaceted usability, the anti-pan AAV antibody displays a valuable tool in AAV research and therapeutic development.

1818 Developing a methodology for seropositivity assessment through sensitive anti-AAV total antibodies ELISA

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[1]Svar Life Science, Malmo, Sweden, [2]Svar Life Science, Malmö, Skane lan, Sweden, [3]Svar Life Science, Malmö, Sweden, [4]Svar Life Science, Cambridge, MA
**Corresponding Author*

Adeno-associated virus (AAV) vectors are at the forefront of gene therapy, offering a safe and effective means of delivering therapeutic genes. However, the prevalence of pre-existing anti-AAV antibodies in the human population poses a significant challenge to the widespread application of this technology. These antibodies, particularly total binding antibodies (TAbs), can impact both the efficacy and safety of AAV-based therapies. Therefore, accurate and sensitive methods for detecting and quantifying anti-AAV TAbs are crucial for patient selection, dose optimization, and risk assessment in clinical trials.

This study evaluated the seroprevalence of anti-AAV TAbs in a cohort of 100+ human serum samples from healthy donors, focusing on AAV2 and AAV9 as examples of commonly used serotypes, using a highly sensitive, standardized ELISA platform. Our findings reveal a significant proportion of individuals with detectable TAbs against both AAV2 and AAV9, aligning with seroprevalence data reported in the literature and confirming the widespread pre-existing immunity to these serotypes. Our findings reveal a significant proportion of individuals with detectable TAbs against both AAV2 and AAV9, aligning with seroprevalence data reported in the literature and confirming the widespread pre-existing immunity to these serotypes.

The developed ELISA demonstrates excellent sensitivity and specificity, enabling the reliable quantification of anti-AAV TAbs even at low levels. The assay's performance characteristics, including intraassay precision of <5%, ensure robust and reproducible results. Data analysis revealed a broad range of TAb titers across the cohort, highlighting the variability in individual immune responses to AAV.

These findings underscore the importance of comprehensive TAb assessment in the context of AAV gene therapy. The high prevalence of anti-AAV antibodies, coupled with the observed serotype cross-reactivity, necessitates careful consideration during patient screening and treatment planning. The evaluated ELISA platform provides a valuable tool for characterizing the humoral immune response to AAV, and it is adaptable for the evaluation of other AAV serotypes, contributing to the development of safer and more effective gene therapies. This assay has the potential to guide clinical decision-making and improve outcomes for patients receiving AAV-based treatments.

1819 A Novel Tool for Complement Activation Profiling in AAV Gene Therapy

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Gene therapy utilizing adeno-associated virus (AAV) vectors demonstrates excellent safety profiles and remarkable clinical efficacy in treating diverse diseases. Despite its relative safety, evidence from clinical trials has shown that AAV can trigger severe life-threatening inflammatory responses, especially when administered intravenously at high doses. Many of these adverse reactions are associated with the secondary activation of the complement system, leading to complications such as acute kidney injury due to atypical hemolytic uremic syndrome–like (aHUS-like) complement activation, thrombotic microangiopathy (TMA), thrombocytopenia, and immune-mediated myocardial inflammation. Though AAV-mediated complement activation is a significant safety concern, we still lack understanding of the activation mechanism and a predictive method to prevent or mitigate activation in administered

patients. In this context, our research has been focused on the development and evaluation of an ELISA-based AAV-mediated Complement Activation assay. The technique combines a serotype specific AAV-coated plate with a tailored buffer composition, selectively allowing evaluation of the distinct classical, lectin, or alternative complement system pathways. The assay detects de novo-generated terminal complement complex (TCC) deposition, making it a robust tool for testing and predicting complement reactions to AAV in analyzed sera.

Utilizing this assay, we demonstrated a clear dose-response relationship between complement activation and different anti-AAV antibody sources, including both IgG and IgM. Experiments using C3- and Factor H-depleted sera confirmed the specificity of complement responses and highlighted a potential role for the alternative pathway in AAV-mediated complement activation. Moreover, the assay effectively captured individual complement system characteristics in response to AAV, independent of baseline complement activity within the sample, demonstrating that it measures the formation of newly generated complement components specifically induced by AAV. Human sera from 100 healthy donors were screened for complement activation in response to AAV2 and AAV9, revealing variability in individual responses. Importantly, the AAV-COMPL assay demonstrated high intra-assay and inter-assay precision for both AAV2 and AAV9.

Given the complexity of serum interactions with anti-AAV antibodies and the complement system, our assay holds promise in shedding light on pivotal immune interactions and offers valuable insights to enhance the safety and efficacy of AAV-based gene therapies.

1820 Development of a Novel Luminescent Assay for Sensitive and Specific Quantitation of Double-stranded RNA

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mRNA-based therapeutics have demonstrated clinical efficacy, as evidenced by the success of mRNA vaccines for SARS-CoV-2. Typically, mRNA drug substances are produced via in vitro transcription (IVT). A common byproduct and contaminant of IVT is double-stranded RNA (dsRNA), which is highly immunogenic and can induce inflammation, cell death, and inhibition of protein translation, including the therapeutic mRNA's encoded protein. Consequently, accurate measurement of dsRNA in IVT products is critical to ensuring their safety and efficacy. Current methods, such as dot blot and ELISA, for detecting dsRNA are limited in sensitivity and lack quantitative capability. To address these challenges, we have developed a novel luminescent assay for the sensitive and specific quantitation of dsRNA in IVT products. Unlike traditional assays, this method does not rely on dsRNA-binding antibodies. Instead, it utilizes the two components of the NanoBiT® split luciferase system, which are genetically fused to dsRNA-binding domains. In the presence of dsRNA, these constructs bind via the dsRNA-binding domains, enabling dimerization and reconstitution of functional NanoBiT® luciferase. The resulting luminescence is directly proportional to the amount of dsRNA in the sample. This assay detects dsRNA fragments ≥30 base pairs in length and is independent of their size and sequence. It does not cross-react with single-stranded RNA or DNA and is highly sensitive, with a limit of quantitation of 1 pg/ml. The assay is designed in a convenient "add-mix-read" format, requiring no wash steps or media transfers. It exhibits robust, sensitive, and reproducible performance, making it a groundbreaking tool for the specific detection of dsRNA contaminants in complex mixtures.

1821 A Novel Next-Gen Sequencing Library Preparation Method To Sequence Low-Quality (Fragmented/ Degraded) With Low (*Pico*-Grams) Quantity Of DNA/RNA Templates For Better Diagnostics, Biomarker Discovery, And Research.

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[1]*University of Minnesota, Circle Pines, MN,* [2]*University of Minnesota, Minneapolis, Minnesota,* [3]*Unviersity of Minneosta, Minneapolis, MN*
**Corresponding Author*

***Background and limitations of current technologies*:** Next-generation sequencing (NGS) of RNA and DNA is a method of high-throughput sequencing of biological samples. There are several well-established methods in published literature and also commercially available in the form of products/kits dedicated to sequencing total RNA, DNA, exome, small RNAs, and selected panels of gene/ loci to address specific issues. However, it has been a great challenge to sequence highly fragmented and degraded DNA/RNA due to the limitations of library preparation methods. This affects the sequencing of clinical samples, biological research materials, liquid biopsy, FFPE tissue, exosomes, forensics, cell-free RNA/DNA, and archeology, among several other types of materials. A large fraction of archived and even fresh samples do not generate high-quality data, which cripples basic research, biomarker discovery, and diagnostics. Currently, it is also impossible to sequence small RNAs alone or small and long RNAs together with pico-gram quantities of total RNA template.

Methods and results: We have addressed these limitations by developing a novel method to prepare NGS libraries from pico-gram quantities of total RNA templates. Our methods could efficiently capture RNA of all sizes (20bp and above, including small and long RNAs) simultaneously and detect 2-10 times more unique transcripts at a similar sequencing depth than the current state-of-the-art methods. We could capture 20,000 to 100,000 more transcripts than an established method in a proof-of-concept study. We could capture small (as small as 20 bases and above) and long RNAs in the same library using very low (pico-grams) quantities of total RNA.

Applications and broader impacts: Our method is applicable to highly fragmented and degraded samples like Clinical samples, FFPE tissue, cell-free RNA/DNA, liquid biopsies, archeological samples, and also normal biological samples. The variations and errors observed in NGS data due to differential degradation between samples are negligible in our method. This helps generate consistent and high-quality sequencing data. We could generate 2-10 times more high-quality data with as little as 500pg of total RNA, which is close to the total RNA obtained by a single cell and hence applies to single-cell sequencing. The method is compatible with automation as well. Due to its high efficiency and sensitivity, we think our method will enable better diagnostics, biomarker discovery, and research.

1822 Development of a Novel High-Throughput Determination of Infectious Virus Titers by Kinetic Measurement of Infection-Induced Changes in Cell Morphology

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Infectivity assays are the key analytical technology for development and manufacturing of virus-based therapeutics. We have recently developed a novel assay format that utilizes label-free bright field images to determine the kinetics of infection-dependent changes in cell morphology. This so-called kinetic infectious virus titer (KIT) assay is, provides results within ~24 hours post-infection due to its sensitive readout method. Compared to traditional infectivity assays like TCID50 or plaque assay our cumulated analysis of kinetic data by a fit model results allows a precision of CV < 20% based on only six wells per sample on a 96-well plate. The KIT assay allows for a high throughput with ~400 samples processed by a single operator per week. This assay format is a potent solution to overcome the analytical bottleneck in infectivity assays limiting significance of e.g. process-optimizations within the pharmaceutical industry or routine analytics in academic research.

Here we provide insights into the assay optimization via our functional Design of Experiment approach and the introduction of ready-to-assay cells both contributing to increase the precision of the KIT assay even further.

1823 Enhancing Robustness in rAAV Characterization with Optimized Workflows and PEA Technology

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[1]Thermo Fisher Scientific, South San Francisco, CA, [2]Thermo Fisher Scientific, El Cerrito, CA

Recombinant adeno-associated viruses (rAAV) are essential vectors in researching solutions for gene and cell therapies due to their low immunogenicity, broad tissue tropism, and durable gene transfer capabilities. Precise dosing of these vectors requires rigorous quantification of viral titers throughout development, scale-up, and product release phases. The genomic titer (GC/mL), viral particle titer (vp/mL), and content capsid ratio are critical attributes for characterizing rAAV products. Achieving robust and reproducible results is critical, particularly in workflows susceptible to variability due to viral aggregation and capsid instability during the process.

We've optimized our reagents and workflow, improving capsid stability and minimizing aggregation, while increasing workflow robustness by transitioning from Proximity Ligation Assay (PLA) to Proximity Extension Assay (PEA) technology. While PLA relies on temperature sensitive enzyme, ligase, to generate a DNA template from viral capsids, PEA uses a direct oligo extension mechanism by a temperature stable polymerase, reducing variability in the workflow.

Different rAAV serotypes were tested using PEA vs PLA workflow on the QuantStudio™ Absolute Q™ digital PCR system. Results demonstrated significant improvements of the PEA workflow, increasing replicability and robustness. Capsid content ratios measured using PEA-dPCR aligned closely with results obtained from orthogonal methods and exhibited lower inter-assay variability.

In summary, we present an enhanced workflow that addresses critical bottlenecks in rAAV characterization. By mitigating aggregation and capsid instability and leveraging the stability advantages of PEA technology, we have developed a more robust and reliable process for measuring rAAV genomic titer, viral particle titer, and capsid content ratio. This improved approach has been validated across diverse rAAV samples, including different serotypes, vendors, and in-process materials, supporting the production of high-quality rAAV vectors in researching solutions for therapeutic applications, facilitating precise and consistent results.

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1824 Automated co-detection of small RNAs, mRNAs and proteins with a new RNAscope™ assay on the Roche DISCOVERY™ ULTRA using translucent chromogens

Renzo Adilardi[1], Manvir Sambhi[1], Emerald Doolittle[1], Sonali Deshpande[2], Anushka Dikshit[3], Henry Lamparski[1], Maithreyan Srinivasan[4]
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Advances in precision medicine utilizing antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) as therapeutic platforms have brought promising solutions for various neurodegenerative/neuromuscular disorders and rare diseases. Currently, 20 oligonucleotide drug products have been commercially approved by the FDA and EMA, and many more are in clinical phase I-III trials. The FDA has issued recommendations to generate nonclinical biodistribution (BD) data for gene therapy products to evaluate and interpret nonclinical pharmacology and toxicology findings before initiating human clinical trials. *In situ* hybridization (ISH) and immunohistochemistry (IHC) are increasingly used to spatially visualize the delivered therapeutic, target gene, transgene, and/or cell markers and complement information gathered from molecular technologies such as quantitative polymerase chain reaction (qPCR) and digital PCR. Direct visualization of oligonucleotides can also monitor the risk of off-target events by studying BD of potential therapies in various organs.
To expand our current RNAscope™ capabilities on the Roche DISCOVERY™ ULTRA System, we developed a new ISH assay enabling the detection of ASOs, siRNAs, microRNAs (miRNAs), and other small RNAs that are 17-50 bases. This fully automated assay allows the co-detection of small RNAs and mRNAs and can be combined with IHC to detect protein targets on the same formalin-fixed paraffin-embedded tissue section. This workflow includes harmonized cell conditioning for an efficient combined RNA and antigen retrieval and the new protease-free pretreatment reagent VS PretreatPro, allowing for RNA detection without disrupting protease-sensitive epitopes. Visualization of multiple RNA species and protein targets at the single-cell level is achieved by combining different HRP and AP-based detection, leveraging Roche's translucent chromogens.
For proof-of-concept, we used this new automated assay to investigate the expression profile of miRNAs in the

mouse brain and combine them with RNA and protein markers of specific neural cell populations for spatial and morphological context. Detection of *RNU6* and *Snord85* miRNAs was combined with *Tmem119* mRNA for the identification of microglial cells by ISH, while sequentially detecting NeuN for mature neurons, GFAP for astrocytes, and Olig2 for oligodendrocytes by IHC. This new automated ISH assay showed high sensitivity and specificity for the detection of small RNAs with different expression profiles, and subcellular resolution in intact tissue context. This novel assay will be particularly valuable for the study of ASO/siRNAs delivery, biodistribution, stability, and expression profile of their associated RNA targets for the development of new oligonucleotide therapeutics.

1825 Strategies for Overcoming Analytical Challenges in Complex Upstream AAV Samples

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Robust high-throughput analytics that accurately measure viral vector product quality in complex matrices are essential for the development of well characterized upstream processes. Upstream AAV material, particularly from HEK293 cells prior to clarification, often contains a complex mixture of cell debris, DNA, and other process impurities that can interfere with standard analytics. Technologies that facilitate early and precise product characterization in these upstream matrices are critical for making informed decisions about process performance. In this study, we evaluate analytical technologies with a goal of enhancing throughput, improving operational efficiencies, and accelerating process characterization in upstream unit operations. To compare technology performance and usability we leveraged upstream samples from various AAV serotypes produced in HEK293 and insect cells to assess Digital PCR for genomic titer and Mass Photometry for determining empty/full (E/F) capsid ratio. Finally, we present data demonstrating how rapid, cost-effective, process-agnostic analytical methods can be implemented to define, monitor, and control upstream AAV processes effectively.

1826 Massively parallel sequencing-based method to detect misuse of gene transfer technologies in sport

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With gene and cell therapies continuing their rapid evolution into the clinic, there is a growing danger that the developed technologies might be misused. Gene doping has the potential to unfairly improve athletic performance in sport and the anti-doping community has been focusing efforts on developing tests for its detection. Polymerase chain reaction (PCR)-based methods have already been developed by us and others to detect several doping genes in athletes blood samples. Each PCR assay targets a unique sequence which correspond to exon-exon junction in the intronless cDNA of the doping gene and not present in a natural gene. Nonetheless, a quest for methods that utilise advanced molecular technologies continues.

In this project we designed, evaluated and tested the protocol for a targeted massively parallel sequencing (MPS)-based method for simultaneous detection of erythropoietin, follistatin, growth hormone 1, growth hormone releasing hormone and insulin-like growth factor 1. These genes are likely candidates for gene doping due to their potential to increase athletes' endurance or muscle size and strength. Kits, reagents and protocols for each step were evaluated and optimised with the aim of achieving high reliability and sensitivity, operational ease and reduced cost and turn-around time of a future test.

To mimic DNA extracts from athletes' blood samples we used solutions of doping genes and genomic DNA. Target enrichment was achieved by simplex PCR designed to amplify a large portion of each transgene covering several exon-exon junctions. Enriched materials were purified, mixed and subjected to library preparation and sequencing. We also designed, produced and tested a Control material that is suitable for use in positive controls in the test. Its

unique design enables sequence discrimination from the transgenes and minimises a chance of false positive test results in case it inadvertently contaminates samples during the test. A tailored bioinformatics pipeline with stepwise analysis was developed that reliably distinguishes doping genes from the corresponding endogenous genes and from the Control. Using simulated DNA extracts from blood samples, we showed that MPS-based approach can detect down to five copies of doping genes in a background of genomic DNA similar in quantity to that in one milliliter of blood.

The developed MPS method for detecting gene doping was shown to be reliable and sensitive. Compared to PCR, the MPS approach potentially has higher likelihood of transgene detection as it targets multiple splice sites simultaneously and allows greater flexibility in choosing regions used in target enrichment. The MPS multiplexing capability will allow simultaneous analysis of many samples and genes, potentially reducing test's cost and turnaround time.

The developed approach could also be considered in legitimate applications of gene transfer technologies, for example, in vector biodistribution, persistence and shedding analyses or transgene integration studies.

1827 Confirmation of Lentiviral Vector Identity Using Short-Read Next-Generation Sequencing

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Lentiviral vectors (LVVs) are valuable tools in gene therapy and biomedical research, enabling efficient and stable delivery of genetic material to target cells. Ensuring the identity and integrity of these vectors is critical for regulatory compliance, therapeutic safety, and experimental reproducibility. In addition, prior confirmation of the genetic fidelity of the lentiviral drug substance is crucial for downstream applications such as integration site analysis in transduced cells. Traditional methods for viral vector characterization and quality control, such as restriction enzyme mapping and Sanger sequencing, often fall short in providing comprehensive sequence verification and detecting low-frequency or compound variants. Here, we present a robust and scalable approach using short-read next-generation sequencing (NGS) to confirm the identity of LVVs.

In this study, we developed and verified an NGS-based workflow for the analysis of LVV genomes. The workflow includes optimized protocols for extraction and purification of encapsidated RNA, cDNA synthesis, library preparation with optional polymerase chain reaction (PCR) amplification, and bioinformatics pipelines for alignment, variant calling, and quality control. We successfully applied this method to lentiviral preparations encoding therapeutic cargo, achieving high coverage and accurate mapping of vector sequences. Key findings include the complete and unambiguous characterization of LVV identity through full-genome sequence analysis. We detected single-nucleotide variants (SNVs) and indels occurring at frequencies as low as 5%, providing insights into the fidelity of vector productions.

Our NGS-based approach demonstrates significant advantages over traditional techniques, offering superior sensitivity, specificity, and throughput. The assay provides a powerful tool for vector quality control as well as support for research applications, aligning with the increasing demands for gene and cell therapy development. This methodology establishes a new standard for lentiviral vector identity confirmation.

1828 Optimizing AAV Sample Preparation Workflow for Short and Long Read NGS for AAV Characterization.

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The potential safety, efficacy, and quality of gene therapy products such as recombinant adeno-associated virus (rAAV) vectors are ensured by their thorough characterization using well-developed analytical techniques. The next-generation sequencing (NGS) has become a preferred analytical tool for this purpose. NGS plays a crucial role in the design and clinical development of rAAV vectors by enabling the evaluation of key quality attributes, including genome heterogeneity, purity, and identity. Short-read (e.g., Illumina) and long-read (e.g., Oxford Nanopore, PacBio SMRT) sequencing technologies are applied to meet specific characterization requirements.

A critical step for reliable NGS results is optimizing rAAV vector DNA sample preparation. However, this process is often hindered by challenges such as vector type (e.g., single-stranded vs. double-stranded), impurities, and low vector genome titers. To address these issues, we developed an optimized rAAV vector sample preparation method that can be used for both short-read and long-read sequencing partly based on a previously established method (Lecomte E et al., Methods Mol Biol. 2019;1950:85-106.).

These improvements facilitate precise characterization of critical vector attributes, such as determining the percentage of complete versus partial capsids, classifying different rAAV genome configurations, and detecting residual contaminants using Oxford Nanopore sequencing. The method also confirms vector identity and detects INDELs and SNPs through Illumina short-read sequencing. Furthermore, genome heterogeneity results obtained via Oxford Nanopore sequencing are validated against an orthogonal method, ensuring robustness and reliability.

1829 Development of a Novel Scale-Down Model for High-Throughput Clone Selection for Adeno-Associated Virus (AAV) Production

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Producing Adeno-Associated Virus (AAV) in stable producer cell lines has advanced significantly, with high cell density cultures and perfusion systems becoming more prevalent for large-scale production. However, high cell density culture and perfusion at the early stage of cell line development presents unique challenges due to the difference in scale and operational requirements. Traditional high throughput clone screening method relies on batch or fed batch culture where relative performance of clones being screened is not representative of the relative performance of clones in a perfusion process. To effectively select cell clones with optimal performances for intensified perfusion systems, suitable scale-down models are required. These models should enable high-throughput experiments with minimal raw material requirements. Herein we report the progress that has been made to enable high throughput selection of clones in a much more representative procedure which enable cell culture performance much more similar to a perfusion process as compared to the traditional cone screening method.

AAV production for various clones was evaluated using the automated microbioreactor system (AMBR15), which involves utilizing a scale-down mock perfusion system with cell sedimentation as the method for cell retention. To establish a baseline for the AMBR15 model, we conducted experiments to examine the impact of multiple critical factors, including sedimentation time, cell density, and various strategies for mimicking perfusion conditions. As a result, the mock perfusion process in Ambr15 performed well compared to actual perfusion process in AMBR250. Notably, under high cell density culture condition, mock perfusion process in AMBR15 maintained the AAV productivity and product quality in terms of ratio of full/empty/partial particles. Whereas batch processes exhibited a significant reduction in AAV production with high seeding density. This suggests perfusion strongly supports AAV production under these conditions. Next, we validated the mock perfusion process as a tool for clone screening with multiple programs and clones. Studies demonstrated mock process in AMBR15 showed stronger correlation with actual perfusion in AMBR250 compared to batch process.

Overall, our process in AMBR15 system with mock perfusion provides a more representative high-throughput model for clone selection for AAV production than the previously used batch process. This approach not only streamlines the cell line development process but also enhances its accuracy by closely mimicking the conditions of perfusion at the production scale.

1830 Automated solutions for characterization and release of ATMPs

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In the field of gene therapy, an efficient delivery of the therapeutic gene and a proper gene expression are crucial for achieving clinically relevant effects. Lentiviral vectors (LVV) are effective transfer system for the introduction of transgenes into target human primary cells, such as CD34+ cells. AGC Biologics has developed and optimized a manufacturing process for LVV transduction of primary cell lines, that includes a comprehensive set of in-house developed tests for the characterization of the Drug Substance (DS) and the release of the final Drug Product (DP). Some of these tests have been automated based on Hamilton automation solutions.

For the release of the DP, two methods are in place at AGC Biologics to assess the efficacy of the transfer of the vector: the quantification of the mean number of the provirus integrated into the host genome (VCN) and the transduction efficiency (TE). Both methods have a Real Time PCR assay readout and have been automated to increase the throughput, consistently reduce timelines, operator effort and volume dedicate to testing.

The Hamilton's automated liquid handler for qPCR execution is integrated with a variety of devices, including a Real Time qPCR thermal cycler and a plate sealer. This integrated system allows a complete automatic flow from reagents and sample preparation to assay execution and obtaining of raw data. For VCN determination, the lysate of transduced cells is directly processed by qPCR, while the TE includes the harvest and lysis of colonies derived from transduced CD34+ cells, previously seeded on a methylcellulose substrate. A fixed number of picked colonies are analysed and the TE is determined as the percentage of transduced colonies.

Both methods were validated before being transferred to automation, and the automated performance has been optimized to be aligned to the manual execution, as demonstrated by a repeatability ≤ 14% and inter-assay variability ≤ 19%. A maximum percent difference of 4% between automation and manual results for TE was observed, while for VCN the qPCR positive control results were monitored over 2 years showing no significant difference between automation and manual, confirming the consistency of the automated system performance.

Moreover, at AGC Biologics different ELISA assays are executed for the verification of the removal of impurities during the transduction process (e.g. cytokines, Host Cell Proteins (HCP)). Among them, the ELISA for 293T HCP has already been transferred to automation. In this case, the automated system was designed with all devices needed to perform each step of ELISA assay and was conceived with a different requirement, to allow for the flexibility to add other assays to be run together to share the same sample aliquot, always having a complete traceable flow of the tested samples up to the final result. The automated method assures a comparable performance with the manual method ($p \geq 0.26$) and has been qualified according to guidelines with both repeatability and intermediate precision ≤ 20%.

These automated systems are CFR21 compliant that enable a two-fold increase in throughput, reducing timelines, and significantly enhance laboratory capacity during process characterization or DP release.

1831 A Solid-state Nanopore Mesh Device for Quantification and Quality Control of Carrier Particles Used in Gene Therapy

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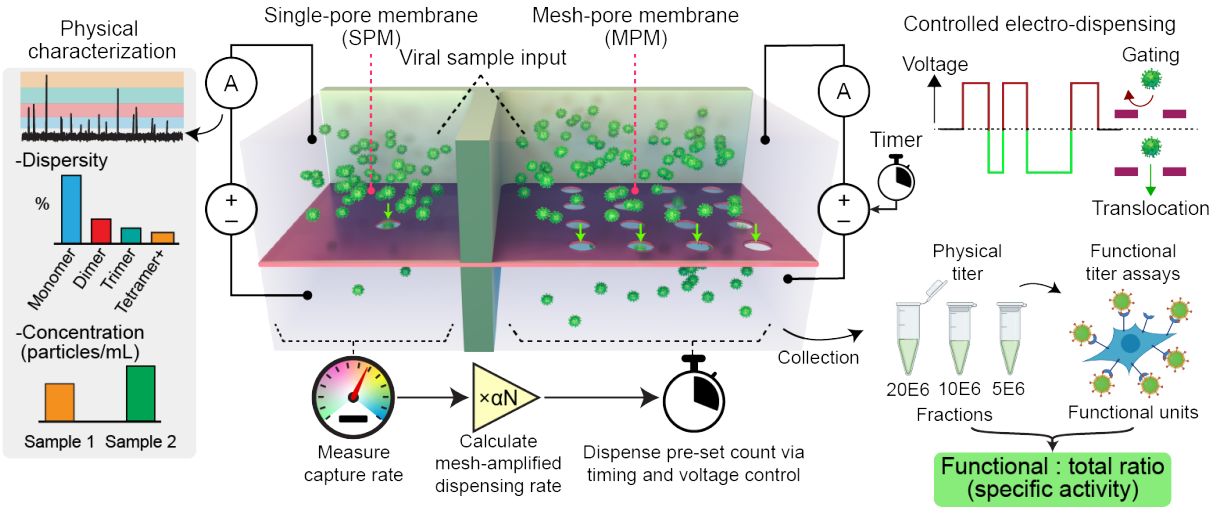
Viral vectors for gene therapy remain challenging to measure and control for quality. Comprehensive assessment of concentration, aggregation, and functional activity often relies on separate, uncoordinated methods, and there is no consensus on how to unite these factors in a single quality measure. Here, we present a quantification pipeline that integrates functional assays with physical particle properties, providing a more complete measure of specific activity. Our approach uses solid-state nanopore sensors—a tiny hole in a thin membrane separating two electrolyte baths. As carrier particles pass through the pore, they disrupt the ionic current, creating blockade events. The amplitude of these events indicates particle size, while their frequency reflects particle concentration and charge. This setup lets us measure concentration, size distribution, and aggregation at single-particle resolution. Next, through robotic and electronic automation, we electro-dispense and collect pre-programmed quantities of particles through a nanopore mesh built into the same device, then use these fractions in a functional titering assay (e.g., qPCR or tissue culture transduction). By combining the physical data from the nanopore sensor with the functional data from the assay, we obtain robust and translatable measures of specific activity. We verified this pipeline using protein-coated fluorescent nanoparticles and validated it with AAV vector samples, where the dispensed AAV particles were further characterized with qPCR for genome counts. Given the tunability of nanopore sensor dimensions and surface properties, this system can be used to characterize a broad range of viral and non-viral carrier particles. Our method provides a unified alternative to multiple, fragmented titration assays, improving measurement consistency in gene therapy process development and manufacturing quality control.

Figure 1. Solid-state nanopore pipeline for characterization of gene therapy carrier particles (viral or non-viral vectors). First, physical properties and concentration of particles are measured with single-particle resolution using single-pore membranes. Then, a pre-programmed quantity of particles is electro-dispensed through mesh-pore membranes. These fractions are collected and further analyzed in functional assays (tranduction, genome count, integrity), and the combined analysis provides standardized metric of quality for the particles.



1832 Methodological validation of sedimentation velocity analytical ultracentrifugation method for Adeno-Associated Virus and collaborative calibration of system suitability substance

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In recent years, the development of in vivo gene therapy has been in full swing. Currently, there are 9 AAV drugs on the market worldwide. The field of gene therapy is undergoing tremendous development! According to statistics, there are about 350 million patients with rare diseases in the world and 20 million in China, of which 80% are related to genetics. The rapid launch of gene therapy drugs has brought great benefits to the treatment of patients with rare diseases in the world! The AUC-SV method used to detect the empty shell rate of AAV products is known as the gold standard for product impurity detection. 1. For the first time in this study, the AUC-SV method is

systematically used for systematic methodological verification of AAV samples. 2. This study provides the basis for follow-up work, establishes the world's first System Suitability Substance, and provides a new weapon to solve the key attribute problems of gene therapy drugs. 3. Our work can provide valuable reference for AAV methodology validation of gene therapy enterprises, solve the pain points of product impurity detection, and promote the vigorous development of gene therapy industry. We performed the systematic methodological validation of quantifying the AAV empty and full capsid ratio. This includes specificity, accuracy, precision, linearity, and parameter variables involving the sedimentation velocity analytical ultracentrifugation (SV-AUC) method. Specifically, SV-AUC differentiated among the empty, partial, full, and High Sedimentation Coefficient Substance (HSCS) AAV particles while evaluating their sedimentation heterogeneity. The intermediate precision analysis of HE (high percentage of empty capsid) and HF(high percentage of full capsid) samples revealed that the specific species percentage, such as empty or full, was more significant than 50%. Moreover, the RSD (relative standard deviation) could be within 5%. Even for empty or partial less than 15%, the RSD could be within 10%. The accuracy recovery rates of empty capsid were between 103.9% and 108.7% across three different mixtures. When the measured percentage of specific species was more significant than 14%, the recovery rate was between 77.9% and 106.6%. Linearity analysis revealed an excellent linear correlation between the empty, partial, and full in the HE samples. The AAV samples with as low as 7.4×1011 cp/mL AAV could be accurately quantified with SV-AUC. The parameter variable analyses revealed that variations in cell alignment significantly affected the overall results. Still, the detection wavelength of 235nm slightly influenced the empty, partial, and full percentages. Minor detection wavelength changes showed no impact on the sedimentation coefficient of these species. However, the temperature affected the measured sedimentation coefficient. These results validated the SV-AUC method to quantify AAV. Based on the above results, collaborate study was performed to calibrate the prepared empty capsid AAV, precision analysis of 24 replicates recorded a sedimentation coefficient of 61.3S ± 0.3S, with an RSD of 0.6%, and a percentage of empty capsid AAV content of 95.0% ± 2.6%, with an RSD of 2.7%. The empty capsid system suitability substance could be used as an external reference to measure the performance of the instrument（published on Human Gene Therapy). Next, we will expand the application of this standard to AEX, CE and SEC-MALS. In addition, we also established AAV particle count sta

1833 Development of a Novel Automated Loading Approach Which Significantly Reduces Processing Time for Enriching Full AAV Capsids Using Ultracentrifugation.

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The use of recombinant AAV (rAAV) as a targeted delivery vehicle is increasingly showing promising results for treating rare and common genetic disorders. As the need to manufacture more material at larger scales grows, several challenges for scaling the full enrichment step (enriching for capsids containing the therapeutic gene) persist. Currently, the most widely practiced unit operation for full capsid enrichment is ultracentrifugation using a cesium chloride (CsCl) density gradient. An ultracentrifuge compatible tube is filled with a high concentration of cesium chloride and bulk AAV material containing empty, partial, and full capsids. The tubes are spun at a high speed for an extended duration forming a density gradient with the cesium chloride. The different populations of capsids migrate to specific locations in the tube based on their buoyant densities. Due to the long processing times, scaling ultracentrifugation to larger production trains (>1000L) is not feasible. In this work, we explore using an automated gradient formation tool, OptiMATE Gradient Maker$ (Beckman Coulter®), which establishes a linear CsCl density gradient prior to spinning. The tool varies the amount of bulk rAAV material and concentrated CsCl stock solution added to the tube during the filling process. We explore several process parameters including density gradient profile, centrifuge run duration, g-force, and spin temperature to identify the optimal conditions for enriching full capsids. As a result of the gradient loading (pre-formed gradient) and optimization studies, the spin time was reduced considerably. Based on product recovery measured by ddPCR, aggregation by HPLC-SEC, purity by CE-SDS, and purity by analytical ultracentrifugation (AUC), we were able to reduce the processing time 10-fold while maintaining the same product attribute profile when compared to typical run conditions.

$ Product in development. Performance characteristics have not been validated.

1834 Purifying AAV Free of Endonuclease Treatment & Pre-Capture TFF

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Long residence time is necessary when using bead based resin in the purification process due to its low pressure resistance and diffusion effect of the flow within the mesh pore of the matrix. Constrained by the slow flow rate, low culture titer and large culture volume, purification of Adeno-associated virus(AAV) using bead based affinity resin normally require a significantly long time to process. To shorten the loading time, most company pack a super larger columns, resulting not only an oversized column volume, but also a compromised utility of binding capacity of the resin.

Here we present an alternative solution by introducing ACC(affinity convective chromatography). The performance of ACC is independent of flow rate and feed concentration due to its large pore size(2μm-5μm) which eliminates diffusion in the mass transfer mechanism. The large pore size guarantees that the target molecule will flow through the matrix and be captured by the affinity ligand without entrapment, resulting in a high yield(>90%).

Rather than employing endonuclease to cleavage host cell DNA prior to depth filtration, Wuxi Extpure have developed a novel AAV purification process PAEFT which is free of endonuclease treatment and Pre-Capture TFF by utilizing ACC . The process details are as below:

Step 1: depth filtration
Step 2: affinity capture
Step 3: AEX1
Step 4: AEX2

This novel process deviates from conventional bead based chromatography in two distinct aspects: firstly, the absence of endonuclease treatment leads to a substantial reduction in expenses. For instance, in a 500-liter bioreactor AAV production process, the cost of endonuclease is approximately $100,000. The elimination of endonuclease introduction in the process renders the necessity for nuclease residue detection, thus simplifying the QC process and reducing operational expenses. Secondly, a pre-capture TFF free process leads to cost reduction by eliminating the use of membrane and yield enhancement through the reduction of processing steps.

1835 Size Exclusion Chromatography of messenger Ribonucleic acid, Adeno-Associated Virus and Virus-Like Particle Analysis using Newly Developed Wide Pore Size Exclusion Chromatography Columns

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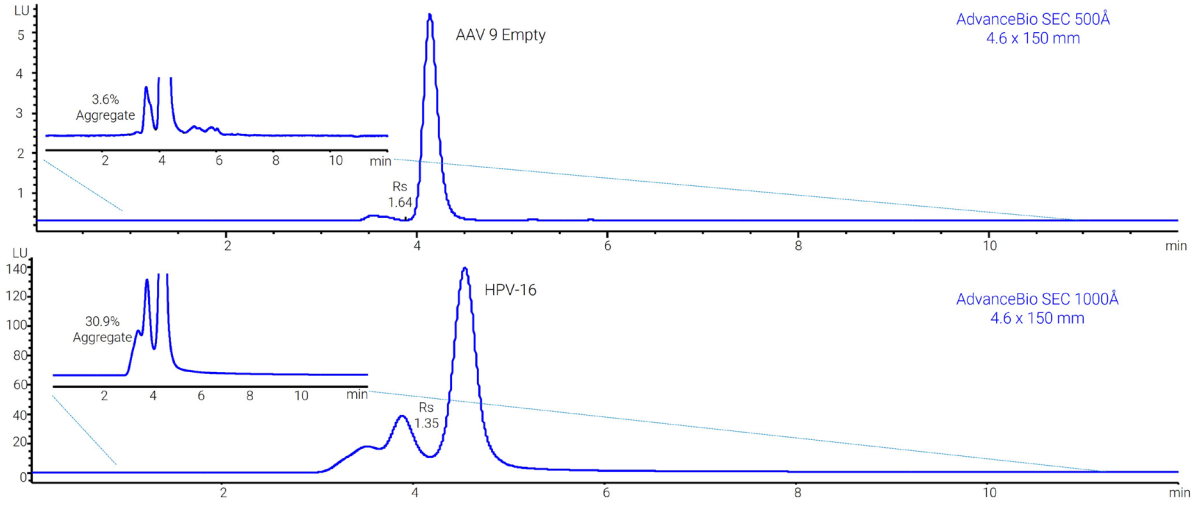
High-resolution SEC HPLC is essential for aggregate analysis of large biotherapeutic molecules like mRNA and viral vectors to ensure their quality and safety. Aggregates are product-related impurities, and their identification and quantification are considered as critical quality attributes. It is vital to choose a SEC column with an appropriate pore size, higher pore volume and consistent pore size distribution to provide a baseline separation between the analyte monomer and its sub-micron size aggregates.

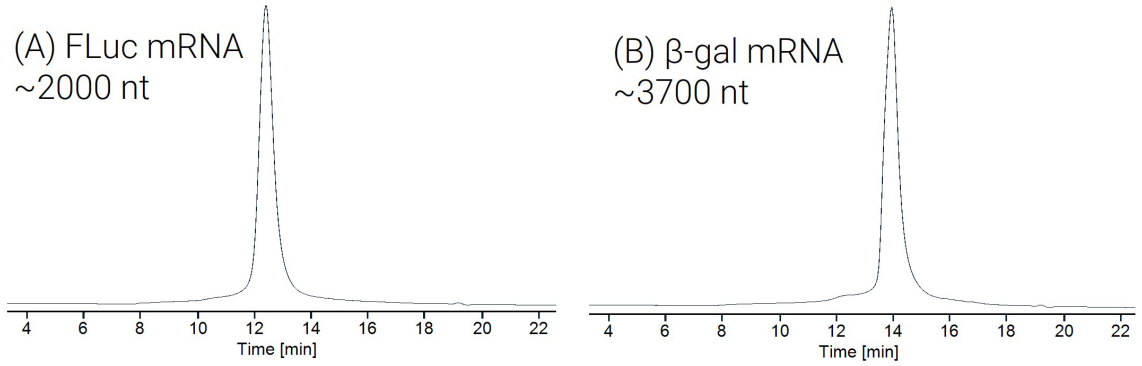
Here, we demonstrate the performance of two novel 2.7 μm SEC columns with 500Å and 1000Å pores that were designed specifically for robust, high-performance analysis of adeno-associated viruses (AAV), messenger Ribonucleic acid (mRNA), single and double stranded oligonucleotides, LNP and virus-like particles (VLP) such as HPV-16. Different molecular weight standards of polyethylene glycol (PEG) and polyethylene oxide (PEO) were

also studied on various columns to understand the effects of column pore size and pore size distribution on separation of variously sized analytes.

Both newly developed SEC columns exhibit excellent baseline resolution between monomer species and their aggregates with a short runtime (< 6 min) for all tested AAV serotypes and VLP test samples (Fig-1). The column also demonstrates excellent performance when tested at different flow rates ranging from 0.1 mL/min to 0.7 mL/min and capturing similar amount of aggregate and monomer species while maintaining the baseline resolution between them. The column showed good compatibility with MALS detector and demonstrated that the biophysical characterization of larger biomolecules like mRNA sample with >4,000 nucleotide in length is easily achievable. The columns demonstrated superior resolution and recovery when compared to columns from other vendors. While SEC is used to estimate analyte molecular weight, a series of double stranded and single stranded oligonucleotide standards, as well as study of a variety of mRNA samples, illustrates the complexity of single stranded oligonucleotide samples. Under ambient or slightly elevated temperatures double stranded oligonucleotides are much more elongated than single strand, requiring large pore sizes for similar length strands. Additionally, mRNA folding in solution may be such that longer mRNA sequences are more compact and elute later, such as the 3700 nt β-gal mRNA eluting later than ~2000 nt FLuc mRNA (Fig-2). Light scattering data confirms relative size in solution of different samples.

In conclusion, the newly developed SEC columns offer fast separation with ultra-high resolution for aggregate analysis & biophysical characterization of larger biotherapeutic molecules.





Despite its shorter sequence, Fluc mRNA (A) elutes earlier than β-gal mRNA (B), indicating it is more elongated in solution

1836 High Throughput Analysis of CRISPR sgRNA using Ultrashort Ion Pairing Reversed Phase Column Chromatography and High Resolution Mass Spectrometry

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With the first FDA approved CRISPR based therapeutics, high throughput (HT) and reliable analytical methods are more important than ever. Given the inherent cost and time associated with therapeutic development, analytical methods that are faster while providing a wealth of information are desirable. CRISPR gene editing requires two components, a CRISPR Associated Protein (Cas) and a single guide RNA (sgRNA), which together form an ribonucleoprotein (RNP) complex. These RNPs are used to edit genomes by targeting sequences of DNA, making a single strand cut, and inserting a new sequence. Single guide RNA molecules consist of two separate regions. The first ~ 20 nt segment is specific to the gene of interest, designated as crispr-RNA (crRNA). The remainder of the sgRNA sequence is associated with binding the sgRNA to the Cas protein (tracrRNA). It is imperative that sgRNA is pure and that the sequence accurately targets the gene of interest to avoid off-target effects such as DNA mutations or undesired immune response. In addition to purity and sequence confirmation, the ability to investigate the native conformers and charge heterogeneity of sgRNA provides another layer of knowledge about how sgRNA behaves in native conditions.

Methods for analyzing RNA by ion-pairing reversed-phase liquid chromatography (IP-RPLC) with high-resolution mass spectrometry (HRMS) exist but tend to use analytical columns that are ≥ 100 mm and require ≥ 30 min run times. To improve throughput, we developed an IP-RPLC-HRMS method by scaling a published 30-min method to a HT 5-min method. We utilized hexafluoroisopropanol (HFIP) and N,N-diisopropylethylamine (DIPEA) as ion-pairing agents and aimed to reduce analysis time while maintaining an effective chromatographic separation. The method was directly scaled from a 100 mm column to the 20 mm column, followed by scaling equilibration times, and finally optimizing the retention window and focused gradient programming of the method.

Additionally, this method was utilized to analyze sgRNA fractions collected via our published method utilizing directly coupled strong anion / strong cation exchange chromatography (IEX). Single guide RNAs produced with different purification techniques (such as desalting or RPLC) were injected onto the coupled IEX columns where substantial charge heterogeneity was observed. Fractions of these IEX-separated peaks were analyzed by the 5-min IP-RPLC-MS method to investigate differences in charge heterogeneity.

Utilizing ultrashort 20-mm columns, we achieved HT separations with a run time of 5 min per sample. This reduced analysis time not only aids in faster decision making in production and drug development, but also provides a 6X reduction in reagents used. We analyzed sgRNA of differing purity, obtaining accurate mass data for the samples with mass errors ranging from 0.1 to 2 Da (~2 to 50 ppm). Samples were stressed under thermal and acidic degradation conditions prior to analysis to demonstrate method suitability for rapid purity determination. These techniques are viable options for precise stability indicating assays.

Fractions of the heterogeneous sgRNA collected by IEX and analyzed using this 5-min IP-RPLC/HRMS method, revealed that heterogeneous peaks observed in sgRNA samples by IEX were identical in IP-RPLC-HRMS. Our results show the feasibility of this novel application for HT IP-RPLC-HRMS analysis of sgRNA and give insight into sgRNA conformational heterogeneity.

1837 Dual Cation Exchange coupled with Multi-Modal Chromatography Allows the Production of High-Purity and Low residuals cGMP-Grade Cas9 Nuclease for Cell Therapy Applications

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RNA-guided CRISPR-Cas9 genome editing that exploits the bacterial CRISPR-Cas system to target and edit genome at specific double strand sequence with high precision has opened endless possibilities for cell therapy. The use of CRIPSR-Cas9 system allows specific permanent correction of deleterious base mutations or modifying genes responsible for diseases. The applications of such gene editing tools nucleases are ever-expanding and were recently validated with the US FDA approval of Casgevy, the first gene editing therapy for the treatment of sickle cell disease. This milestone, along with the growing number of gene-editing programs, accentuates the need for scalable, cost-effective enzyme production and purification processes. Previously, we reported the purification of our Histidine Tag-less 160KDa Cas9 employing an optimized homogenization matrix coupled with a size-exclusion chromatography (SEC) polishing step.
In this report, we present a second-generation purification process that significantly improves production efficiency and product quality. This process replaces the labor-intensive and scale-limited SEC-based process with a sequential dual cation exchange chromatography (CEX) followed by multimodal and anion exchange polishing columns, achieving higher product purity and yield, at a fraction of the time and cost. Limitations of the SEC-based first-generation process is presented side-by-side with this second-generation process. The final product consistently showed a purity greater than 95%, less than 20 ng/mg residual host cell protein, less than 1% (w/w) residual host cell DNA, less tha 20 EU/mg endotoxin and low level of residual RNase and DNase. The second-generation process using cGMP compatible resins, chemicals, and supplies supports gram-scale production of cGMP-grade Cas9. In addition, this purification approach could be applied to similar nucleases or other proteins with comparable physicochemical properties.

1838 High-Throughput Developability Assessment of AAV Drug Products: A Predictive Approach

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Adeno-associated virus (AAV) vectors have demonstrated immense promise for the treatment of genetic diseases, but their successful translation to the clinic requires a robust, developable vector candidate. Here we demonstrate a high-throughput approach to rapidly evaluate and differentiate AAV vector candidates and formulations based on their physical and chemical stability. A suite of predictive techniques was assessed including forced precipitation, forced denaturation, and temperature ramping, to assess colloidal, conformational, and thermal stability, respectively.

We demonstrate the feasibility of predictive approaches for AAV vectors under various conditions in a low-volume, high-throughput manner. Such workflows can be used to prioritize promising candidates for further development. This approach has the potential to significantly accelerate the development of stable and efficacious AAV-based gene therapies.

1839 Upgrading Our Toolbox: New Analytical Tools to Support rAAV Vector Bioprocess Development and Product Understanding

Marco Thomann, Roche, Penzberg, Bayern, Germany

The presentation will give an overview of the current standard analytical panel used during development of rAAV vectors. The main focus will be on new analytical tools for rAAV. Analytical techniques like mass spectrometry, mass photometry, fragment analyzer and next-generation sequencing will be discussed in more detail including opportunities and challenges. Using multiple case study data, the new methods will be compared to current standard methods (e.g. AUC, PCR, SDS-PAGE) and their benefit for using them during process development will be explained. In detail, comparison of full-empty levels across four different methods will be presented (Linearity, Accuracy, Repeatability,…). Furthermore, characterization of rAAV vectors using NGS, fragment analyzer, dPCR

and mass photometry will be presented as an example. The new methods add value to the existing analytical tool box, but also expectations need to be managed, e.g. limitations / complex interpretation of data and potential validation of such methods as well as use for GMP release of rAAV.

1840 Next Generation digital PCR Technology for Sterility Testing of Cell and Gene Therapy Products

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The bio-pharmaceutical world has moved drastically towards short-lived personalized cell and gene therapy products in recent years. With the new quality control requirements, several new rapid microbial detection methods have been developed. USP <1071> states, “The ability to detect contamination, in real-time, prior to the administration of the short life product may be considered more important than detection of a single colony-forming unit (CFU) in the product.” However, the new methods strive to detect the holy grail of 1 CFU. Will this ever be possible, or is it even necessary? In this poster, we will present the first digital (droplet) PCR based approach for sterility testing which is intended for testing of cell and gene therapy products, elucidate the critical steps, and highlight the many benefits of this approach. We will address the handling of background signals of a bacteria digital (droplet) PCR experiment and explain how to clearly differentiate between the background and real positive signals. We will demonstrate how the groundbreaking digital (droplet) PCR technology allows a new level of precision in rapid sterility testing. We propose an open discussion on this advanced method and its potential for QC testing and release.

1841 Using Next Generation Sequencing to Test Raw Materials used in Biologics Manufacturing

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When using raw materials in the manufacturing of biological products and vaccines, there is a risk associated with introducing exogenous sequences attributed to these materials into the test system. Next Generation Sequencing (NGS) testing is recommended at multiple steps in the cell-culture process to look for adventitious viruses but can also be performed on raw materials introduced into the manufacturing process. A popular raw material associated with cell culture is Fetal Bovine Serum (FBS). This serum is an animal derived material which provides additional proteins and growth factors to support cell growth. By using NGS for adventitious virus detection in raw materials, this can help ensure a more comprehensive profile of exogeneous sequences being introduced into the process. The profiles of these sequences can also be used to identify any source of downstream contamination if it were to occur.

In this study, we tested FBS for the presence of contaminating influenza sequences specifically H5N1, due to the increased detection in US herds. Due to the nature of FBS, the serum is full of various components that are extremely beneficial to cell-culture but have the potential to cloud NGS based assays. To reduce the background noise contributed from FBS (i.e. cell-based nucleic acids), an endonuclease treatment such as Benzonase® endonuclease has been proven to remove extraneous nucleic acids. Benzonase® endonuclease removes these residual nucleic acids while leaving intact viruses undisturbed, which enriches the test sample for detection of encapsidated viral sequences.

The study was designed to detect contamination of raw materials and determine if a Benzonase® endonuclease pre-extraction treatment would be beneficial in the detection of influenza contaminants alone. To assess the feasibility of this, FBS lots were processed through our GMP-validated NGS workflow. Prior to extraction, an aliquot of FBS was spiked in with the Center for Biologics Evaluation and Research (CBER) Next Generation Sequencing Virus Reagents virus panel. The representative panel includes five viruses selected for different sizes, genomic characteristics, and chemical resistance and are representative of potential contaminating viruses. One spiked and

one unspiked sample would undergo the Benzonase® endonuclease pre-extraction treatment to look while another set would not undergo the pre-extraction pre-treatment. After the optional treatment, all samples underwent a total nucleic extraction, cDNA conversion and sequence compatible DNA libraries were generated. Sequencing was performed using the Illumina NextSeq2000 and data analysis was performed using proprietary bioinformatics software. A significant reduction in the background sequences was observed when the FBS was treated with Benzonase® endonuclease prior to extraction. For the spiked samples, the Benzonase® endonuclease showed a significant improvement in the detection of the viruses. All five viruses were identified in the treated sample, while in the untreated sample, only two of the five viruses were detected at high concentrations. The detection of the two viruses across the board improved by at least 100X using Benzonase® endonuclease pretreatment. This study demonstrated that the use of our validated NGS method is amenable to adventitious virus detection for raw materials and that the use of a Benzonase® endonuclease could improve the sensitivity of the assay.

1842 Extraction-free ddPCR quantification of host-cell derived E1A impurities in rAAV samples

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Packaged residual DNA impurities in recombinant adeno-associated virus (rAAV) products arise during manufacturing and pose a risk to the patient safety, product efficacy and persistence of gene therapy. In production processes using HEK293 cells, the integrated adenoviral E1A gene is a potential contaminant from host cell DNA with cell-transforming potential. Therefore, the level of contaminating E1A is a critical quality attribute for AAV produced in HEK293.

Ascend has developed and qualified a ddPCR for the absolute quantification of E1A copy numbers in rAAV batches using sequence-specific primers and probe. While most currently used release methods require DNA extraction to achieve the required sensitivity, the ddPCR method developed and presented here enables analysis without any sample treatment or DNA extraction. Thus, our E1A ddPCR method avoids experimental errors due to insufficient or poor DNA extraction and saves sample volume and time while maintaining high sensitivity. It was developed for purified rAAV samples such as drug substance and drug products, allowing very low sample dilutions without matrix interference.

Method qualification demonstrated high precision, accuracy and sensitivity, enabling product-specific validation with very limited effort and application as a robust and accurate drug substance and drug product release assay in accordance with regulatory expectations.

1843 Rapid and Versatile Analysis of AAV Samples Using Lateral Flow Assays: Applications in Gene Therapy Development

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Adeno-associated viruses (AAV) represent the most commonly used viral vector for the delivery of therapeutic transgenes in academia and industry. However, the gene therapy community is facing major challenges like the high demand for effective and reliable analytical AAV tools for gene therapy manufacturing and development.

Though the use of orthogonal methods is recommended for comprehensive characterization, rapid testing of individual and crude AAV samples during the development and manufacturing process is often difficult. Most precise analytical AAV tools are not convenient for single sample measurements and/or require highly purified samples.

Our new AAV lateral flow assays can easily be used for a broad range of applications, helping to facilitate and accelerate AAV-based gene therapy.

Here, we show potential applications of the AAV lateral flow assay for serotypes AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9, i.e. (1.) comparing the capsid titer of two unknown samples, (2.) estimating the titer using a reference standard, (3.) spot landing in the optimal dynamic range and (4.) testing antibody binding to variant AAV serotypes.

1844 A Novel AAV TCID50 Testing Method Using Replication-Deficient Helper Adenovirus and Inducible 293 Cell

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Background: The TCID50 value of adeno-associated virus (AAV) is commonly used to evaluate the infectivity of AAV products in vitro. It serves as a critical testing parameter in the research, production, and quality control of AAV. However, the traditional TCID50 assay involves the addition of wild-type adenovirus, which presents significant challenges to the stability and safety of the test. To address this, we have developed a novel YM-TCID50 testing method. This method incorporates a replication-defective adenoviral vector with E2 mutation to prevent virus replication and cell lysis during the assay and employs a Cre-inducible AAV Rep expression 293 cell line to mitigate the cytotoxic effects of Rep.

Methods:
(1) Constructing a replication-deficient adenoviral vector that can only amplify in specially engineered 293 cells.
(2) Establishing a 293 cell line, 293RC24, with Cre-induced AAV Rep expression to evaluate AAV replication using a luciferase assay.
(3) AAV TCID50 testing was performed using 293RC24 and the replication-deficient AdV, with the traditional HeLaRC32 serving as a control.

Results:
(1) The replication-defective adenovirus was efficiently amplified in our specially engineered 293 cells, achieving a titer of 2.43×10^9 PFU/mL.
(2) AAV replication in 293RC24 cells is 24.6 times and 35.9 times higher than that in 293A cells, respectively.
(3) The AAV TCID50 assay using the 293RC24 cell line is more stable than the traditional HeLaRC32 method due to the non-lytic nature of the replication-deficient AdV.

Conclusion: We have developed a more stable and safer AAV TCID50 analytical method that integrates an inducible Rep 293 cell line (293RC24) and a replication-deficient adenoviral helper virus.

1845 Phase 1 Study of GEN2, A Personalizing Gene Therapy Vector, in Adult Patients with Locally Advanced or Metastatic Solid Tumor Malignancies

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Drug candidates based on oncolytic viruses containing viral thymidine kinase (vTK) or GM-CSF encoding payloads and replication-deficient adenovectors containing a vTK payload have demonstrated encouraging results in skin, bladder, prostate, pancreatic, and lung cancers when administered intratumorally (IT) or intralesionally (IL). However, there are practical limitations to IT or IL administration, including the number of lesions that can be

injected and the invasive nature of injections into non-cutaneous lesions. Therefore, we are investigating GEN2, an intravenously (IV) administered non-replicating gene transfer vector that encodes for both an optimized prodrug activated enzyme (enhanced viral thymidine kinase; HSV-eTK) and the immunostimulatory cytokine GM-CSF. When transduced into a tumor cell and in the presence of the oral prodrug ganciclovir (VGCV), HSV-eTK triggers cancer cell death by DNA chain termination and subsequent release of the full complement of unique tumor antigens including neoantigens (NA). The local secretion of hGM-CSF augments and facilitates an immunotherapeutic effect by enhancing NA presentation to local immune effector cells. IV infusion of GEN2 to forty-eight (48) late-stage cancer patients with hepatocellular cancer (HCC) or cancers metastatic to the liver was well-tolerated with minimal toxicity, with 10 of 48 patients on study > 6 months in an Asian phase 1 clinical trial. A Recommended Phase 2 Dose (RP2D) was not identified. This is an open label, US Phase 1 study in adult patients with locally advanced or metastatic solid tumors to determine the recommended Phase 2 dose (RP2D) for GEN2 by intravenous (IV) infusion. Secondary objectives include immunogenicity, PK and preliminary anti-tumor activity. Exploratory endpoints include assessment of replications competent retrovirus (RCR) in peripheral blood, changes in cytokines and circulating immune cells, and tissue changes in paired biopsies. Dose escalations were initially in semi-log increments in single patient cohorts; after the third dose increase, the maximal additional dose increases are 100%. As of January 20, 2024, 8 patients have been treated at 4 dose levels starting at 3.4E+6TU/kg and reaching 1E +8 TU/kg. Treatment duration ranges from 1 to 9+ weeks. Median age was 60, 4 patients were females; patients had a median of 3 prior lines of therapy (range 3-5). Treatment related adverse events (TRAEs) were all grade 1-2, and included diarrhea (n = 2), nausea (n = 1), vomiting (n = 1), chills (n = 1), and hyperhidrosis (n = 1). Dose Limiting Toxicity has not yet been observed. No patient discontinued treatment due to a related AE. PK data demonstrate an elimination half-life between 0.28 to 3.04 hours; mean clearance 2010-3780 mL/hr/kg and mean Vss 986 - 2030 mL/kg. Safety review for Dose Level 4 is ongoing. RCR/vector persistence has not been observed over this dose range. No objective responses have been observed to date. Batched analysis of correlative data is in progress. GEN2 has been well tolerated early in dose escalation without occurrence of DLTs; further clinical activity including PK results, response assessment, and pharmacodynamic data will clarify determination of a RP2D. A parallel cohort for intratumoral injection is on-going in cutaneous malignancies. Once the IV RP2D is identified, the protocol will open three expansion arms: hepatocellular carcinoma, HR+ breast and cutaneous malignancies (NCT06391918).

1846 Analysis of Clinical Benefit for a Phase 2 Basket Study Using DNG64 Cyclin G1 Inhibitor Gene Vector + Immunotherapy for CCNG1 Oncogene Expressing Tumors Predicts Successful Efficacy Endpoints (NCT04091295)

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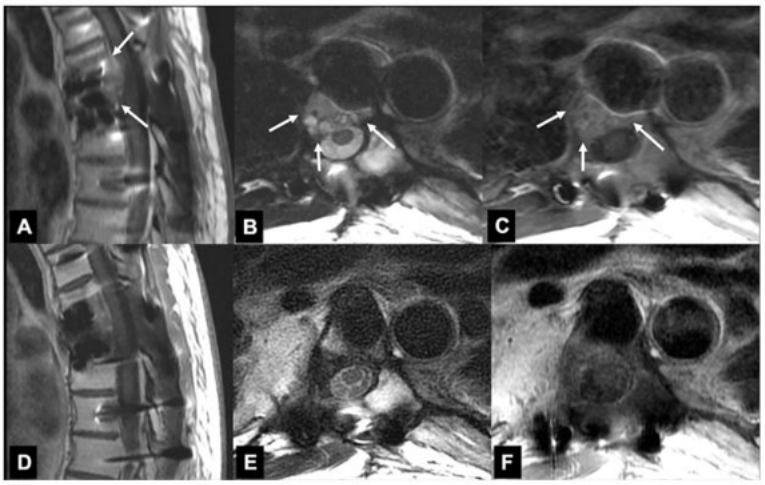
Background: DNG64, a tumor targeted retroviral vector encoding a Cyclin G1 inhibitor construct, not only induces apoptosis in cancer cells and tumor vasculature but also primes the tumor microenvironment for enhanced immune cell and/or drug entry by killing tumor associated fibroblasts, thus, reducing stroma production. Expanded access for DNG64 is currently open for advanced sarcoma, pancreatic cancer, and breast cancer (NCT04091295). In this patient population, the Simon 2-Stage design criteria was implemented to determine the potential of DNG64 + immunotherapy treatment to meet efficacy endpoints in a planned phase 2 basket study for CCNG1 expressing tumors.

Methods: Endpoints: (1) Clinical benefit rate or CBR (CR, PR, SD]) at month 4 by CT/MRI by RECIST v1.1 (2) Overall response (ORR); (3) Correlation between CCNG1 expression and CBR. Up to 40 patients with advanced sarcoma, pancreatic cancer or breast cancer will be treated with DNG64 IV (1.7 x 10e10 RV copies 3x a week x 3

weeks/month) plus FDA approved drug (DNG64+ regimen). Archived tumor specimens from all patients will be analyzed for CCNG1 expression level using RNA sequencing. CCNG1 levels will be stratified as: Low = <17%, Medium-Low = 17- 49%, Medium-High = 50-83%, or High = >83%. A Simon 2-stage design with Type I error rate = 0.1 and power = 0.8 will be used, accordingly, a CBR ≥ 30% would warrant continuing the planned Phase 2 basket study.

Results: All patients had previously failed standard chemotherapy. Eight patients with advanced sarcoma (n=5), pancreatic cancer (n=2), and breast cancer (n=1), were treated with the DNGG64+ regimen. Three patients received DNG64, SNK01 (autologous enhanced natural killer cells) and a PD1 inhibitor. All tumors showed enhanced CCNG1 expression. Four of 8 (50%) patients had PR; 6/8 (75%) patients had PR or SD at month 4; 2/3 (66%) patients treated with DNG64, SNK01 and a PD1 inhibitor had PR. In an analysis of patient tumors by CCNG1 expression level, High CCNG1 expressors (n=3) had a 33% (1/3) CBR, Medium-High expressors (n=3) had a 100% (3/3) CBR, and Medium-Low expressors (n=2) had a 100% (2/2) CBR. By tumor type, sarcoma patients had 60% (3/5) CBR, pancreatic cancer patients had 100% (2/2) CBR, and breast cancer patients had 100% (1/1) CBR. One subject with recurrent osteosarcoma of T4-T5 spine and High (89%) CCNG1 tumor expression had a 65% decrease in tumor volume when DNG64 was given with proton therapy.

Conclusions: Taken together, the data analyses indicate that: (1) All tested tumors show enhanced CCNG1 oncogene expression, reinforcing the use of intravenous DNG64 Cyclin G1 inhibitor therapy for advanced sarcoma, pancreatic and breast cancer; (2) An Overall Response Rate of 50% for all groups, a 66% ORR for DNG64, SNK01 and PD1 inhibitor group, and a clinical benefit rate of 75% for all groups, when categorized by both tumor CCNG1 expression level and cancer type, meet the Simon's 2-stage design threshold clinical benefit rate of 30%, thereby qualifying all groups for further studies of efficacy and safety in a randomized Phase 2 basket study using combination regimens of DNG64 + immunotherapy.



Description of MRI Image:

Sagittal T1-postcontrast, axial T2 and axial T1 postcontrast MR images of thoracic spine are shown before (A, B, C) and after (D, E, F) treatment with proton therapy and concurrently with DNG64, a tumor targeted gene therapy bearing a cytocidal *CCNG1* inhibitor gene. There is a 16 x 14 x 25 mm mass in the ventral epidural space at T5-T6 level with heterogenous T2 hyperintense signal (arrows on B) and postcontrast enhancement (arrows on A and C). MR images obtained 2 months after proton therapy and DNG64 show significant decrease in the size (10 x 14 x 14 mm) and degree of T2 signal and postcontrast enhancement suggestive of treatment response. Note: Mechanistically, intravenously administered DNG64 nanoparticles seek out and accumulate in injured tissues (i.e., irradiated tissues in this case) favoring enhanced efficacy of tumor kill with proton therapy in a subject with osteosarcoma, a notoriously radioresistant type of sarcoma. Phase 2 studies are planned using concurrent administration of DNG64 with proton therapy.

1847 Interim Analysis of a Phase 1/2a Study of ICM-203 AAV Gene Therapy for Osteoarthritis

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Introduction: An unblinded interim analysis of the low dose cohort of the first-in-human phase 1/2a study of ICM-203, a recombinant adeno-associated viral (AAV) vector designed to express a truncated form of human Nkx3.2, a transcription factor which plays an important role in both chondrocyte and synoviocyte activity, was conducted to obtain a preliminary assessment of the safety, immunogenicity, and biological activity of ICM-203.

Methods: In the low dose cohort of this phase 1/2a, double-blind, placebo-controlled, dose escalation study (NCT04875754), 8 subjects with Kellgren-Lawrence grade 3 osteoarthritis (OA) of the knee were randomized to receive a single intra-articular injection of ICM-203 or placebo in a 3:1 ratio. The primary safety endpoint was safety and tolerability of ICM-203 through assessment of treatment-emergent adverse events (TEAEs). Immunogenicity endpoints included measuring serum neutralizing antibody (NAb) titers and T-cell responses to ICM-203's AAV capsid. As efficacy endpoints, changes in knee pain and function were assessed using the Knee Injury and Osteoarthritis Outcome Score (KOOS) pain subscale and KOOS activities of daily living (ADL) subscale, respectively; these KOOS scores were then used to calculate Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores. Imaging endpoints included Magnetic Resonance Imaging (MRI) Osteoarthritis Knee Scores (MOAKS), focusing on bone marrow lesions (BML), synovitis, articular cartilage damage, and osteophytes, as well as minimum joint space width (mJSW) as measured by knee radiograph.

Results: Of 11 screened subjects, 8 qualified and received a single intra-articular injection of ICM-203 (N=6) or placebo (N=2); all subjects completed 52 weeks of follow-up. Subject age ranged from 56 to 73 years; body mass index (BMI) ranged from 24.6 to 38.6 kg/m^2. No significant concerns about safety or tolerability arose. The most common treatment-related TEAE was mild to moderate arthralgia, which occurred in 3 of 6 ICM-203 subjects and 1 of 2 placebo subjects. At baseline, 3 ICM-203 subjects had positive NAb responses to AAV capsid; no subjects had significant T-cell responses. All 6 ICM-203 subjects developed both a humoral and cellular response against AAV capsid, whereas neither placebo subject did. ICM-203 subjects with negative NAb at baseline (N=3) demonstrated greater improvement over placebo subjects (N=2) in KOOS pain (1.3-fold), KOOS ADL (2.3-fold), WOMAC (2.7-fold), as well as in imaging endpoints, including MOAKS BML (2.7-fold) and mJSW (2.0-fold). In contrast, ICM-203 subjects with positive NAb at baseline (N=3) showed no sign of efficacy in these endpoints. Improvement in synovitis was observed in 2 of 6 ICM-203 subjects (1 with positive NAb and 1 with negative NAb), whereas no improvement was observed in either placebo subject. For articular cartilage and osteophytes, no significant changes were observed in any subject between baseline and week 52.

Conclusions: Intra-articular injections of ICM-203 were safe and well tolerated. ICM-203 appeared to show greater therapeutic activity over placebo in subjects with negative NAb at baseline. Current findings indicate ICM-203 may demonstrate potential as a disease-modifying osteoarthritis drug (DMOAD), between reducing osteoarthritis symptoms, ameliorating structural joint damage, and alleviating synovial inflammation. Investigation of higher doses of ICM-203 is in progress.

1848 Design of the First Clinical Trial of AAV Gene Therapy in Immuno-Oncology (SENTRY-ALL): A Single Dose CD3/CD19 Bi-Specific T-Cell Engager for the Treatment of CD19+ B-Cell Acute Lymphoblastic Leukemia

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VNX-101 is an investigational adeno-associated virus (AAV) gene therapy developed to express a secreted anti-CD19/anti-CD3 scFv diabody (termed GP101). GP101 binds both cluster of differentiation (CD)19 and CD3, inducing T-cells to kill both benign and malignant B-cells. Following a single dose intravenous (IV) infusion, the vector induces the liver and key tissues to continuously secrete GP101 into the bloodstream, resulting in long-term, continuous and consistent serum levels of GP101. Potential advantages of VNX-101 over autologous CAR-T therapy include it is off-the-shelf, provides a gentle onset of action, does not require lymphodepletion chemotherapy, engages all T-cells continuously (including those freshly produced from the bone marrow), and utilizes highly efficient signaling through the native T-cell receptor. Reduction in CD19+ leukemia or lymphoma cells and prolonged survival were observed in NSG mice following VNX-101 administration. Safety, PK, and biodistribution were further established in toxicology and larger species (baboon) studies. Based on these findings, FDA IND clearance was received to conduct a Phase 1/2 clinical trial of VNX-101 in patients with relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL), representing the first FDA approval of an AAV cancer clinical trial.

SENTRY-ALL is a phase 1/2, multicenter, open-label trial in patients with relapsed or refractory CD19+ B-cell ALL (NCT06533579). The key inclusion criterium is CD19+ bone marrow blasts via flow cytometry <50% (baseline or after cytoreductive therapy). Prior blinatumomab and/or CD19 CAR T-cell treatment is allowed. AAVrh74 total antibody titer is required to be <1:400.

In this 2-part study, dose-finding data from Part 1 of the study (n~12 patients) will be used to determine the dose for Part 2. Part 1 is a dose-finding PK study in adults ≥18 years old designed to determine the minimal dose that achieves target PK serum levels of GP101 at steady state (8-week timepoint) without dose-limited toxicities, defined as the recommended Part 2 dose. Part 2 (n~14) will be opened following data safety monitoring board review of Part 1 data and is designed to determine the safety and pharmacokinetics (PK) of VNX-101 at the recommended dose in a broader array of subjects including those of a lower age, ≥13 years old. Patients will be followed for safety and efficacy up to 5 years post VNX-101 dosing. Long-term follow-up assessments for safety will be conducted for 6 to 15 years post VNX-101 dosing. Primary endpoints are safety and to determine the RP2D. Secondary endpoints include change in B-cell counts, immunoglobulin levels, MRD/hematologic response, progression free survival, and disease-free survival. Exploratory endpoints include GP101 PK and vector shedding. The first patient is anticipated to be dosed in Q1 2025. Up to 26 patients will be enrolled across centers in the United States.

VNX-101 has the potential for improved safety, efficacy, and accessibility over current CD19-directed therapies. This trial aims to provide the first clinical experience in the use of AAV gene therapy as a new paradigm for cancer immunotherapy.

1849 A Randomized, Double-Blind, Placebo-Controlled Dose-Ranging Phase 1b Study Evaluating Safety, Tolerability, and Pharmacodynamics of a Local AAV-Mediated Anti-Interluekin-1 Gene Therapy in Subjects With Knee Osteoarthritis: 6-Month Interim Results

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Knee osteoarthritis (OA) is a chronic, painful, and degenerative disease causing disability. It affects millions of people but has no treatment to safely reduce pain and reverse joint damage. Interleukin-1 (IL-1) is an inflammatory and catabolic cytokine that is a major contributor to the pathophysiology of OA, including pain and tissue erosion. Previous attempts with small molecule drugs and biologics targeting IL-1 have failed to show sustained efficacy in affected joints due to inadequate delivery and rapid clearance. Genascence is developing a gene therapy, GNSC-001 to confer local, long-term IL-1 inhibition after a single intra-articular (IA) injection to the affected knee. GNSC-001 is a recombinant adeno-associated virus (rAAV) that encodes human interleukin-1 receptor antagonist (IL-1Ra), a potent inhibitor of IL-1. Following a small open-label, dose-escalation Phase I study, a larger randomized, double-blind, placebo-controlled, dose-ranging Phase 1b trial of GNSC-001 in knee OA subjects was initiated. The primary objective was to evaluate the safety and tolerability of GNSC-001 administered as a single IA injection. A key secondary objective was to evaluate long-term expression of transgenic IL-1Ra in synovial fluid (SF). Another secondary objective was to understand the role of transient immune conditioning (IC) regimens on effectiveness of the gene therapy. Six-month interim results are reported.

Consented subjects (n=53) aged 40-75 years with moderate to severe knee OA, Kellgren Lawrence (KL) Grade 2-3, and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) target knee pain ≥ 20/50 were randomized into 5 groups in 1:1:1:1:1 ratio and received an ultrasound-guided IA injection of: Group 1) GNSC-001 1×10^{12} vg; Group 2) GNSC-001 1×10^{12} vg + IC- oral; Group 3) GNSC-001 1×10^{13} vg; Group 4) GNSC-001 1×10^{13} vg + IC- oral; or Group 5) Placebo (formulation buffer) on Day 1. IC in Groups 2 and 4 was a short course of 60 mg oral prednisone for 7 days, starting Day -1 and tapering for 6 weeks. The trial was expanded to include a non-randomized open-label arm- Group 6 (n=14) that received GNSC-001 1×10^{13} vg with a local steroid injection plus an abbreviated course of oral steroids (IC- IA+oral); specifically, 80 mg IA methylprednisolone, and 60 mg oral prednisone on Day -1 for 3 days. Study visits included Months 1, 3, 6, & 12 (+ 48 months safety follow up).

Follow-up though 6 months showed that there were no deaths or safety-related study withdrawals. Two treatment-unrelated serious adverse events (SAEs) were reported. Treatment-Emergent Adverse Events (TEAEs) were balanced in Groups 1-5, trending higher in Group 6. Target knee AEs (TKAEs) across Groups 1-6 were mostly Grade 1-2 (mild to moderate), with one Grade 3 (severe), which was a recurrent knee effusion in Group 6. TKAEs were generally arthralgia, swelling, effusion, and stiffness. All GNSC-001-treated groups resulted in elevated SF IL-1Ra levels compared to baseline and Placebo. Higher IL-1Ra in SF was associated with IC and a dose-reponse was observed between the 1×10^{12} vg and 1×10^{13} vg groups with IC through the 6-month visit.

The 6-month interim results suggest that this novel treatment is safe, well-tolerated, and capable of yielding and sustaining mean expression of IL-1Ra to target levels in multiple groups of the study. GNSC-001 is thus of potential therapeutic benefit in knee OA, a previously undruggable disease, and warrants further development.

1850 Patients perspective on their cross-border personalized treatment experience

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Background: Personalised medicine is often only accessible through specialized centres, which for patients with rare diseases are often established in different countries than the patient's country of residence. Attending specialists and affected families can seek effective treatment solutions including Advanced Therapy Medicinal Products (ATMPs) and cross-border options, which can be accessed through various routes (S2 in EU, individual requests, clinical studies, compassionate use etc). This phenomenon is a subgroup of medical tourism, which is not driven by convenience, but only by efficacy.

Methods: We used a questionnaire to interview 12 parents of minor patients who applied for cross-border treatment for specific, personalized treatments that were not available in the country of origin. A total of 5 families from Slovakia and 2 from the Czech Republic were interviewed and provided feedback, who planned treatment in

Germany, Netherlands and Italy. Some families stayed more than 3 months abroad. We have focused on the challenges of travelling abroad for treatment.

Results: All families recommended cross-border treatment. Parents decided to pursue the cross-border treatment of their children based on its efficacy, even though three families also considered less effective treatment available in the country of residence. The health insurance (HI) coverage of treatment was an important aspect, as well as coverage for travel and stay. While speaking at least one foreign language, language limitations in communication with healthcare professionals (HCP) was one of the main concerns, followed by concerns about organization and financial aspects of treatment. The main source of information about treatment options was provided by an attending specialist, closely followed by Internet search and recommendations from families facing similar challenges. Communication with foreign HCP was evaluated as effective, but it was not perceived as effective with administrative personnel nor HI. All considered it important to accompany the child as a whole family.

Conclusions: Although the ATMP treatment option was highly recommended by all, the communication was the main concern and challenge. Attending physicians have a critical role in initiation of cross-border treatment. The Internet search, networking with families with similar experiences and learning the language are highly recommended by the respondents to facilitate treatment.

1851 The Kids Advanced Therapeutics Program – Responding to the Need for Systems Readiness in the Gene Therapy Translation Pathway

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Health systems readiness is an imperative aspect of gene therapy clinical trials and translation. The Sydney Children's Hospitals Network (SCHN), Australia's largest paediatric health service, was the first site in Australia to become approved for therapeutic delivery of onasemnogene abeparvovec in spinal muscular atrophy and the first site in the world to deliver gene therapy for Duchenne muscular dystrophy to boys under 4 years. SCHN has responded to this rapidly evolving pipeline by pioneering an innovative enabling platform, the Kids Advanced Therapeutics (KAT) Program, to provide multidisciplinary and disease agnostic frameworks that embed gene therapy trials in existing health systems.

The KAT Program prioritises governance, safety and equity through multiple initiatives that aim to embed gene therapy, as well as CAR-T cell, phage therapy and other advanced therapeutics and in sustainable models of care. A Trial Evaluation Flying Squad (TEFS) enabled investigators, clinicians and administrators to access broad expertise in selecting and preparing for gene therapy trials amid a rapidly changing landscape. A Gene Therapy Multi-Disciplinary Team (MDT) was established to provide consultative services to patients admitted for gene therapy delivery and support optimal management of adverse events including thrombotic microangiopathy, immune mediated myositis and complications from iatrogenic immunosuppression. An AAV Gene Therapy Multi-Disciplinary Education Program provided fit for purpose education modules that responded to areas of need across clinical care and safety, as well as ethics and responsibility.

An ethical model of patient selection for competitive recruitment was devised and piloted following input from clinicians and clinical ethicists. Patient facing materials that provide plain language explanations of gene therapy have been co-designed and disseminated to consumers.

During the last 5 years, systems readiness activities facilitated by the KAT Program have supported expansion of gene therapy activity at SCHN and significantly improved workforce confidence, with the number of clinical staff who felt 'not at all confident' caring for patients with AAV falling 68% to 14% after accessing KAT education resources. SCHN has now expanded its gene therapy clinical trial portfolio to include Rett syndrome, inherited deafness and haemophilia. The KAT Program is supporting capacity building at other Australian tertiary hospitals

and leading interstate advocacy for nationwide systems and infrastructure. There is now a national system for interstate referrals for neuromuscular gene therapy clinical trials in Australia. (1)

Australia's vast geographic landscape position it to be an exemplar for delivering specialised therapies despite physical distance, with an emphasis on agile clinical systems and equity of access. Learnings from the KAT program remain relevant beyond Australia and discussion of these experiences and insights may provide opportunities for partnership and planning across broader jurisdictions and sectors.

References:
Lorentzos MS, Metz D, Moore AS, Fawcett LK, Bray P, Attwood L, et al. Providing Australian children and adolescents with equitable access to new and emerging therapies through clinical trials: a call to action. Med J Aust. 2024;220(3):121-5.

1852 Over a Decade of Gene Therapy Trials: Evolution from 2010 to 2025

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The field of gene therapy is evolving rapidly, offering transformative treatment opportunities for various conditions, from inherited genetic disorders to complex malignancies. Since the first successful gene therapy in 1990, significant strides have been made, leading to the regulatory approval of multiple therapies in recent years. As an update to our previous review [1], this abstract reflects the remarkable growth of clinical trials and the expanding therapeutic landscape.

We reviewed clinical trials information on Clinicaltrials.gov from 2010 to January 2025. The search results were initially screened based on their titles, and any unrelated entries were excluded. Data extraction was then conducted on the remaining clinical trials. The final dataset included the following key parameters: study registration details (start year and country), study timelines (start and end dates), type of vectors used, engineered cells or targeted tissues, approach to gene therapy (ex-vivo or in-vivo), clinical trial phase, disease category (cancer, genetic disorders, infectious diseases, and others) along with their respective subcategories, and the specific condition for which gene therapy was applied.

Among 2,987 clinical trials, lentiviral vectors accounted for the largest share (29%), followed by adenoviral vectors (15%), retroviral vectors (10%), and AAV (9%). Among non-viral vectors, RNA-based approaches accounted for the largest share (14%), followed by plasmids (7%), DNA (3%), and gene-editing tools (1%) (Figure 2A). In recent years, gene editing technologies, such as CRISPR, have entered clinical trials. Lentiviral vectors exhibited a marked increase post-2019, peaking from 2021 to 2024, while adenoviral and AAV vectors showed relatively consistent usage. The rise of lentiviral vectors has coincided with the advancement of CAR T cell therapies. We have witnessed an increasing number of CAR T cell clinical trials for autoimmune diseases in recent years (Figure 2B). Over the past decade, in-vivo approaches were initially dominant, but ex-vivo methods have steadily risen, surpassing in-vivo trials in recent years. Ex-vivo trials increased from 33 (36.7% of 2010 trials) in 2010 to 194 (68.1% of 2022 trials) in 2022, while in-vivo trials peaked at 134 (52.1% of 2021 trials) in 2021 before declining (Figure 2C). Among ex-vivo therapies, T cell-based therapies dominate (85%), followed by HSC (6%). In in-vivo therapies, tumors represent the largest target (mainly in trials using oncolytic viruses), followed by the immune system (mainly in vaccine trials), eye, and CNS (Figure 2D). While, cancer trials have the highest number of studies in phases 1 and 2, significantly outnumbering other disease categories, they did not considerably proceed to later stages. On the other hand, genetic disease trials are relatively low in phase 1 but show notable advancement in phases 2 and 3. Infection-related trials also exhibit a similar pattern, with a moderate progression in phases 2 and 3 (Figure 2E).

Analyzing strategic trends in successful clinical trials provides valuable insights for future research, study optimization, and policy development. Understanding these trends can help improve therapeutic approaches, regulatory frameworks, and the translation of gene therapies into clinical practice.

1.Arabi F, Mansouri V, Ahmadbeigi N. Gene therapy clinical trials, where do we go? An overview. Biomedicine & Pharmacotherapy. 2022;153:113324.

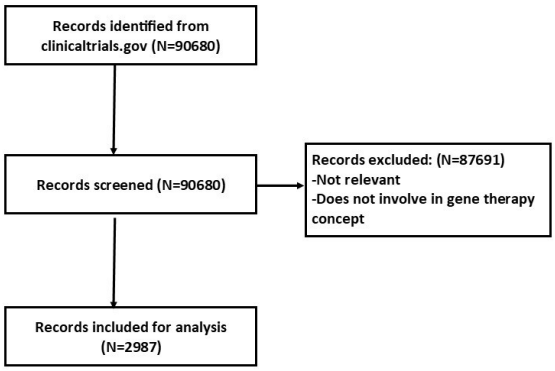


Figure 1. The flowchart diagram of included clinical trials in the overview. The search strategy used in this review was as follows: "Gene therapy" OR "Gene delivery" OR "Gene transfer" OR "Gene editing" OR "Gene addition" OR "Gene augmentation" OR "Gene repair" OR "Gene edition" OR "Gene replacement" OR "Gene" OR "Genetic" OR "Gene-based" OR "Gene modification" OR "Genetic therapy" OR "CAR T cell" OR "Chimeric antigen receptor" OR "CRISPR" OR "DC" OR "Engineered" OR "Viral vector" OR "Non-viral vector" OR "Lentivirus" OR "Adenovirus" OR "Lenti" OR "Retro" OR "Adeno" OR "AAV" OR "HSV" OR "Micro RNA" OR "miR" OR "miRNA" OR "siRNA" OR "T cell" OR "TCR" OR "TALEN" OR "Zinc finger" OR "ASO" OR "antisense oligonucleotide" OR "antisense".

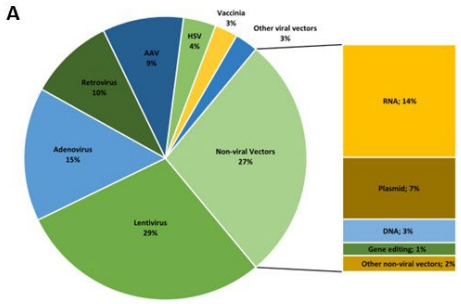
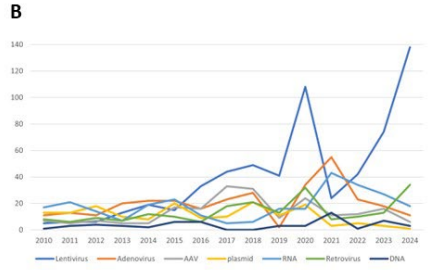
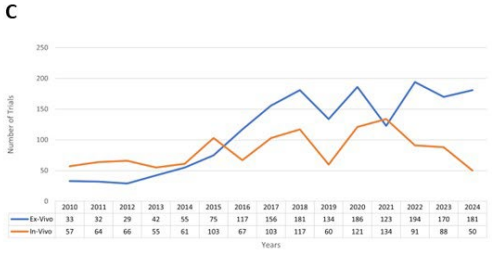
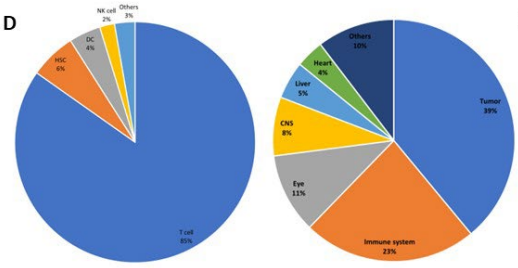
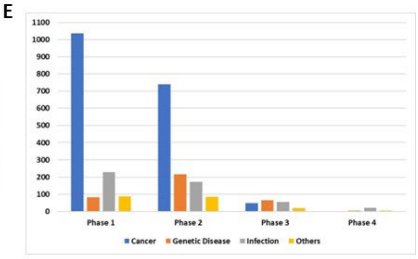


Figure 2. Gene therapy clinical trials' trends from 2010 to 2025 (A) The distribution of different types of vectors including viral and non-viral vectors. (B) The time trends of clinical trials using different vectors. (C) The time trends of clinical trials using in-vivo and ex-vivo gene therapies. (D) The distribution of each type of cell engineered in ex-vivo trials (left) and each type of tissue targeted in in-vivo trials (right) (E) The distribution of clinical trials across phase and disease groups.









1853 Ethical, legal, and social issues (ELSI) in human somatic gene therapy clinical research: A Scoping Review

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Dozens of gene therapies (GTs) have received marketing authorization, and hundreds more are in various research phases. Still, there are many ethical, legal, and social implications (ELSI) involved with the clinical development of this relatively new therapeutic class. To characterize ELSI arising in the context of human somatic GT clinical research, we conducted a systematic scoping review of the literature. Articles were eligible if they were written in English and discussed ELSI in the context of human somatic GT clinical research.179 articles, including empirical research papers, reviews, commentaries, news articles, and blog posts published from 2019-2024, met eligibility criteria. Relevant text was extracted from these articles and thematic analysis was employed to organize and synthesize findings. 24 themes were identified, leading to the generation of 5 high-level themes: 1) assessment of risks and benefits is scientifically and ethically challenging, 2) communication with and engagement of the patient community is crucial, 3) access and justice issues are heightened, 4) ethical GT trial design requires thoughtful consideration, and 5) resource allocation in GT research has ethical implications and is impacted by financial and regulatory considerations. Potential approaches to these challenges and gaps in the literature are explored and discussed.

1854 Safety and efficacy results from a phase I/II open label gene therapy trial for Spastic Paraplegia 50 (NCT05518188).

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Background: Spastic Paraplegia 50 (SPG50) is an ultra-rare, childhood-onset neurological disorder caused by bi-allelic loss-of-function variants in *AP4M1*. Affected individuals present with global developmental delay, intellectual disability, and progressive motor impairment due to spasticity. Currently, no therapies exist to halt disease progression. To address this unmet need, an AAV9-mediated *AP4M1* gene replacement therapy was developed and evaluated in preclinical models. Efficacy studies showed functional improvement in both *in vitro* and *in vivo* models, while safety assessments in rats and non-human primates indicated minimal toxicity concerns. These findings support the translational potential of AAV9/*AP4M1* as a therapeutic approach for SPG50. The first patient was treated in a phase I open label trial in Canada in 2022.

Methods: In 2023, a phase I/II open label trial was initiated at UT Southwestern Medical Center enrolling 4 patients with SPG50 to determine the safety and tolerability of AAV9/*AP4M1* (primary outcome) and explore efficacy (secondary outcome). The trial utilizes a lumbar intrathecal route of administration to deliver AAV9/*AP4M1* at 1E15 vg per patient. All participants were <10 years of age at treatment, one of which being the youngest we are aware of to receive a lumbar intrathecal gene therapy (treated at 5.5 months of age). An immunomodulatory regimen with corticosteroids, sirolimus, and optional tacrolimus (for CRIM- patients) was implemented to allow inclusion of both CRIM- and CRIM+ patients. The trial will follow participants for 5 years post dosing. We present data through the 2nd year follow-up visit of the first participant.

Results and Conclusions: Safety was assessed by physical and neurological exams, laboratory studies, cerebrospinal fluid analysis, contrast-enhanced brain/spinal cord imaging, and nerve conduction studies. Adverse events were reported in all participants, with expected treatment emergent adverse events in 2 participants that included fever, vomiting, and weight loss soon after dosing which resolved quickly. Neutropenia and

thrombocytopenia were observed in 2 participants at 7 days post dosing but resolved by day 14. No concerns regarding liver function were observed and cerebrospinal fluid tests did not indicate any inflammation. Additionally, nerve conduction studies did not show any evidence to suggest dorsal root ganglion toxicity. Together, AAV9/*AP4M1* was well tolerated among all participants. Efficacy was explored via spasticity, motor function, cognition, and quality of life assessments. The developmental quotient improved for 2 and declined for 1 participant on the cognitive domain, remained stable for 2 and declined for 1 on the receptive communication domain, improved for 1 and remained stable for 2 on the expressive communication domain, and remained stable for 2 and declined for 1 on the fine motor domain of the Bayley Scales of Infant and Toddler Development III. Tardieu scale scores for two muscle groups in 1 participant increased, otherwise scores were stable. Modified Ashworth scale scores were stable for 1 and showed mixed results for 2 participants. SPRS and SPATAX-EUROSPA scores were stable for all participants. In 4/5 domains of the GMFM, scores all trended upwards. The 5th domain, walking/running/jumping, remained stable for all participants. Follow-up visits and comparison to disease natural history will be critical to determine the efficacy of AAV9/*AP4M1*.

1855 Deconstructing FDA's Expedited Program Determinations: Analyzing how the Agency may Set the Bar for RMAT Designation

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Regenerative Medicine Advanced Therapy (RMAT) is an expedited program designation intended to accelerate development of cell and gene therapies (CGTs) that provides similar benefits for drug developers compared to those associated with Breakthrough Therapy (BT) designation.[i] FDA's Office of Therapeutic Products (OTP) publishes annual statistics on the number of RMAT designations.[ii] However, FDA doesn't make public the data that were provided to support an RMAT request and how FDA weighed the evidence in making their decision, often leaving developers to wonder how high FDA sets the efficacy bar for granting RMAT designation. Parexel explored the relationship between the early-phase data from press releases around the time of RMAT designation and pivotal data from FDA review memos for marketing applications for two cases of RMAT-designated gene therapies, VYJUVEK (beremagene geperpavec) and BEQVEZ (fidanacogene elaparvovec-dzkt), that were approved in 2023 and 2024.

For VYJUVEK, a topical gene therapy indicated to treat dystrophic epidermolysis bullosa, the endpoint of interest is wound healing. The data around the time of RMAT were: 7 of 8 (87.5%) treated wounds in the Phase 1 and 2 studies healed completely versus zero (0%) of the placebo-treated wounds;[iii] the pivotal study showed that 20 of 31 (64.5%) treated wounds achieved complete healing versus 8 of 31 (25.8%) placebo-treated wounds.[iv] For BEQVEZ, a gene therapy indicated to treat Hemophilia B, the endpoint of interest is reduced bleeding. In the study supporting RMAT designation, the mean ABR (annual bleed rate) data showed 0.2 bleeds/year post-product administration, compared to 8.9 bleeds/year before product administration.[v] In the pivotal study, the ABR was 2.5 bleeds/year post-treatment, compared to 4.5 bleeds/year before product administration.[vi]

Our analysis found that the point estimates for the treatment effect were larger in the RMAT-supporting studies than in the pivotal studies that supported licensure. No definitive conclusions can be made about FDA evidentiary thresholds for RMAT, due to inherent limitations of this two-product evaluation. However, Parexel's analysis identified factors such as small sample size and short follow-up times, which are common limitations of early-phase CGT studies that can reduce precision in estimating the true treatment effect size. Our analysis suggests that in situations similar to the two presented above, FDA's bar for RMAT can be at least as high, or higher, than that for approval. Conducting analyses, such as the one listed above, that contextualize the RMAT-designation supporting data within the totality of publicly disclosed information for that development program, can help inform developers in making decisions about the amount of evidence and timing for submission of RMAT designation requests.

[i] FDA Guidance for Industry: Expedited Programs for Regenerative Medicine Therapies for Serious Conditions https://www.fda.gov/media/120267/download

[ii] Regenerative Medicine Advanced Therapy Designation | FDA

[iii] https://ir.krystalbio.com/news-releases/news-release-details/krystal-biotech-announces-positive-results-phase-2-clinical

[iv] Clinical Review Memo - VYJUVEK

[v] https://www.globenewswire.com/news-release/2018/05/22/1509680/0/en/Spark-Therapeutics-and-Pfizer-Announce-Data-from-15-Participants-with-Hemophilia-B-Showing-Persistent-and-Sustained-Factor-IX-Levels-with-No-Serious-Adverse-Events.html

[vi] https://www.fda.gov/media/178737/download

1856 Accelerated Regulatory Approvals in Cell and Gene Therapy

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Although there is an increasing number of cell and gene therapy (CGT) products entering the clinical development stage, the rate of regulatory approvals has to increase substantially to enable adequate access to patients suffering from thousands of diseases without any treatment option available today, and the approval timelines need to be significantly shortened. The average time from drug development to final approval takes 8 yrs or more. [1] Further, in slowly progressing diseases, it takes multiple years to observe treatment effects as compared to natural history. According to the FDA website, there has been 188 new Investigational New Drug (IND's) and Investigational Device Exemption (IDE) and 22 biological license application (BLA) in the year 2024. [2] Therefore, there is heightened interest in pursuing accelerated approval pathways using biomarkers/endpoints at an earlier point than what would be needed for final approval.

As emphasized by CBER Director, Dr. Peter Marks, accelerated approvals will be a new norm in the CGT space, enabling this regulatory pathway to bring life-altering therapies to the market sooner. FDA has recently issued a draft guidance in December 2024[3] to further guide the industry on accelerated approval

Sponsors working in the CGT space have leveraged FDA's accelerated pathways to expedite the development and review of therapies targeting serious conditions. The accelerated approval, in appropriate cases, provides a pathway for the approval of drugs for serious conditions to fill unmet medical needs based on a surrogate endpoint or an intermediate clinical endpoint that is reasonably likely to predict an effect on irreversible morbidity or mortality or other clinical benefit.[4]

Over the past few years, several CGT products have gained accelerated approvals based on biomarkers or surrogate endpoints that demonstrated clinical benefit. This presentation will spotlight these products, focusing on each of the disease-specific biomarkers /surrogate endpoint analyses that supported their approval. Additionally, the presentation will detail insights into the clinical designs and evidence of the clinical benefits of these CGT products. All the data will be extracted from the information available publicly from the FDA and the sponsors' websites.

References:

- https://patienteducation.asgct.org/gene-therapy-101/clinical-trials/clinical-trials-process
- https://www.fda.gov/about-fda/fda-track-agency-wide-program-performance/fda-track-center-biologics-evaluation-and-research-goal-1
- Expedited Program for Serious Conditions — Accelerated Approval of Drugs and Biologics- Draft Guidance for Industry-December 2024
- Food and Drug Administration, Final Rule, "New Drug, Antibiotic, and Biological Drug Product Regulations; Accelerated Approval" (57 FR 58942, December 11, 1992) (21 CFR parts 314 and 601) and Food and Drug Administration, Proposed Rule, "New Drug, Antibiotic, and Biological Drug Product Regulations; Accelerated Approval" (57 FR 13234, April 15, 1992).

1857 Trade Secret Protections in Early Stage Gene Therapy Research: Patient Harm Through Limited Transparency?

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Early Gene Therapy (GT) trials generally proceed following a rubric established decades ago for small molecule development which includes privileges for protection of trade secrets. While largely methodologically sound, several facets of that traditional early development rubric are problematic in the context of GTs. Unlike small molecules, GTs cannot be re-dosed, they require immunosuppression, and cannot be turned up, turned down, or turned off, each of which can influence proper scientific and ethical trial design. Similarly, because most GTs rely on a viral vector-based delivery system, they are far more related to each other, creating greater cross-trial relevance than the myriad of small molecules in development across thousands of disparate diseases. This similarity intensifies the need for more transparency in early GT development for clinical investigators, ethics committees, and data safety monitoring committees. Any GT investigations using the same or similar vector serotypes, routes of administration, vector genome doses, immunosuppression, and rescue methods for acute reactions should they occur, have relationships to each other, regardless of target indication. Strict trade secrecy protections established during an era dominated by small molecule development which prohibit regulatory agencies from sharing potentially relevant safety or other details are obsolete and dangerous in the context of these greater between-program similarities. A precedent for rapid and wholesale adjustment of first-in-human trials exists, as the requirement for sentinel patient dosing emerged from the Northwick Park/Theralizumab incident in 2006. Although rare, similar acute systemic and fatal reactions have occurred in early-phase GT clinical trials as recently as 2024. It is prudent to re-examine these outdated restrictions on data sharing to allow regulators the latitude to disclose more information about adverse events across relevant GT trials so that untoward outcomes may be reduced or avoided. Several suggestions for achieving greater data sharing, transparency, and reduction of barriers to safety information for early GT clinical investigators and key stakeholders will be presented.

1858 Efficacy and Safety of BRL-101, CRISPR-Cas9-mediated Gene Editing of The BCL11A Enhancer in Transfusion-dependent β-thalassemia and Severe Sickle Cell Disease

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BACKGROUND: BRL-101 is a nonviral cell therapy designed to reactivate fetal hemoglobin synthesis by means of editing of autologous $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) to disrupt the GATA1-binding site at the +58 BCL11A erythroid enhancer.

METHODS: In 3 single-arm, open-label, single-dose clinical trials (NCT05577312, NCT04211480 and NCT04205435) for transfusion dependent Thalassemia (TDT) and 1 trial (NCT06300723) for sickle cell disease (SCD), 15 TDT patients who received transfusions of >100 ml/kg of pRBC per year and 1 SCD patient who had a history of 3 vaso-occlusive episodes per year were enrolled respectively (Tab.1). Mobilized autologous $CD34^+$ cells from the 16 patients were edited with CRISPR-Cas9 RNP at the +58 erythroid specific enhancer region of the BCL11A gene. Before BRL-101 infusion, all patients underwent myeloablative conditioning with busulfan. Subsequently, we monitored adverse events (AEs), neutrophil and platelet engraftment, transfusion independence (TI, defined as 60-Day washout period after last red-cell transfusion, no routine transfusions, total Hb continued ≥ 9.0 g/dL) for TDT patients and hemoglobin response (defined as having met the following criteria for at least 6 months: no transfusion and no hydroxyurea use, HbF ≥ 30% Hb with a concurrent Hb increase from baseline ≥ 3.0 g/dL or Hb ≥ 10.0 g/dL without transfusion) for SCD patient.

RESULTS: As of January 16, 2025, the median follow-up was 23.3 (range: 16.8-56.6) months for TDT patients and the SCD patient was followed for 83 days. All 15 TDT patients were transfusion-free and achieved TI. The median time to last pRBC transfusion was 29 (range: 12-116) days and to transfusion-independent was 89 (range: 55-202) days respectively after BRL-101 infusion. The median duration of TI and transfusion-free were 20.6 (range: 10.1-54.0) months and 22.6 (range:14.2-56.0) months respectively (Fig.1). Mean (SD) of total Hb and HbF levels

increased as early as month 2 after BRL-101 infusion and continued to increase to 12.4 (2.2) g/dL and 11.7 (2.4) g/dL respectively at month 6, then maintained thereafter (Fig.2). The proportion of HbF-expressing red blood cells in peripheral blood had reached 96.3% at month 5 and then continued to rise and remained around 98.0-99.0% (Fig.3). The baseline hemoglobin level of the SCD patient was 7.3 g/dL, which increased to 12.8 g/dL at month 2 and 12.2 g/dL at month 3 without transfusion. At baseline, the fetal hemoglobin level was 4.6% and the sickle hemoglobin level was 39.5%. At month 3, the fetal hemoglobin level rose to 64.1% and the sickle hemoglobin level was 32.9% (Fig. 4). As data cut-off, 15 TDT patients occurred 925 adverse events. Among them, 88.4% were ≤ grade 2. 4 patients experienced 5 serious AEs, including decreased platelet count, shock, febrile infection, soft tissue infection, and veno-occlusive liver disease. Only decreased platelet counts related to BRL-101, the others were due to busulfan treatment. All these serious AEs were resolved (Tab.2 and Tab.3). The SCD patient experienced 73 AEs, of which 91.7% were ≤ grade 2 and no serious AEs occurred (Fig. 5).No study withdrawals or deaths occurred in all studies.

CONCLUSIONS: Whether TDT patients or SCD patient, one-time infusion of BRL-101 provided clinically meaningful increases in total Hb and HbF, the safety profile of BRL-101 was manageable, resulted in durable efficacy and improved quality of life for patients with TDT.

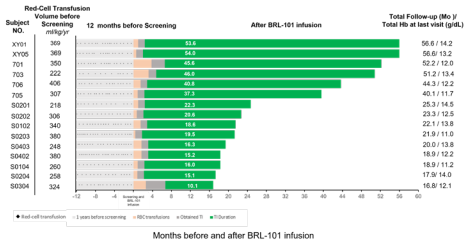

Figure 1. Transfusion status after BRL-101 infusion

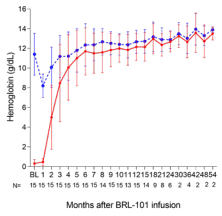

Figure 2. Changes in total and fetal hemoglobin levels after BRL-101 infusion

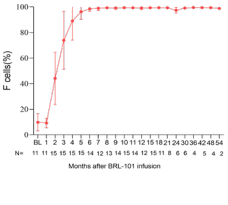

Figure 3. Percentages of F cells after BRL-101 infusion

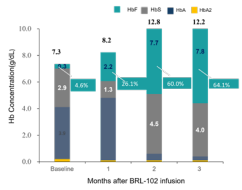

Figure 4. Changes in HbF and HbS levels after BRL-101 infusion

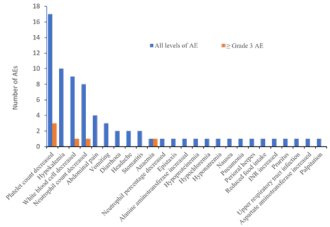

Figure 5. Occurrence of adverse events in the SCD patient

Table 1. Patient Baseline and Treatment Characteristics

	Patient characteristics		Treatment characteristics	
TDT	**Genotype**, n	β^0 / β^0 8 β^0 / β^+ 3 β^+ / β^+ 3 β^0 / β^E 1	**Drug product cell dose,** CD34+ cells × 10^6/kg	**Median** (range) 8.63 (3.9, 14.8)
	Gender, Female/Male, n	7/8	**Neutrophil engraftment** Study Day	19.0 (14, 29)
	Age at consent, years	11.5 (6.0, 25.0)	**Platelet engraftment** Study Day	41.0 (21, 165)
	Pre-study pRBC transfusions ml/kg/year, median (range)	322.3 (218, 406)	**Duration of follow-up,** Months	23.3 (16.8, 56.6)
SCD	**Genotype**	β^s / β^s	**Drug product cell dose,** CD34+ cells × 10^6/kg	9.6
	Gender	male	**Neutrophil engraftment** Study Day	17
	Age at consent, years	10	**Platelet engraftment** Study Day	35
	Annualized rate of severe VOCs in the 2 years prior to enrollment (events/year)	3	**Duration of follow-up,** Days	83
	Clinical manifestations of severe VOCs	Joints swollen and painful , ostealgia		

Table 2. Safety profile of BRL-101

Post-BRL-101 AEs Overview	n=15
Patients with any AEs, n(%)	15 (100%)
Patients with AEs related to BRL-101, n(%)	6 (40.0%)
Patients with SAEs, n(%)	4 (26.7%)
Patients with SAEs related to BRL-101, n(%)	1 (6.7%)
Patients with AEs Grade 3/4, n(%)	15 (100%)
Patients with AEs leading to death, n(%)	0

Table 3. Patients with AEs Grade 3/4 and SAE Sorted by SOC and PT

AEs Grade 3/4 and SAE	n=15	AEs Grade 3/4 and SAE	n=15
Investigations		**Vascular disorders**	
White blood cell count decreased	15 (100%)	Shock*	1 (6.7%)
Platelet count decreased*	15 (100%)	**Metabolism and nutrition disorders**	
Neutrophil count decreased	15 (100%)	Hypokalaemia	4 (26.7%)
Neutrophil percentage decreased	3 (20.0%)	**Respiratory, thoracic and mediastinal disorders**	
Lymphocyte count decreased	2 (13.0%)	Unilateral pleural effusion	1 (6.7%)
Alanine aminotransferase increased	1 (6.7%)	**Vascular disorders**	
Erythrocyte sedimentation increased	1 (6.7%)	Epistaxis	1 (6.7%)
Blood and lymphatic system disorders		**Infections and infestations**	
Febrile neutropenia	3 (20.0%)	Febrile infection*	1 (6.7%)
Anemia	2 (13.3%)	Pneumonia	2 (13.3%)
Gastrointestinal disorders		Infections	1 (6.7%)
Stomatitis	4 (26.7%)	Sepsis	1 (6.7%)
Oral bacterial infection	2 (13.3%)	Upper respiratory tract infection*	1 (6.7%)
Tongue thrust	1 (6.7%)	**Skin and subcutaneous tissue disorders**	
Upper gastrointestinal haemorrhage	1 (6.7%)	Soft tissue infection*	1 (6.7%)
Lower gastrointestinal haemorrhage	1 (6.7%)	**Hepatobiliary disorders**	
Psychiatric disorders		Veno-occlusive liver disease*	1 (6.7%)
Dysphoria	1 (6.7%)		
Reproductive system and breast disorders			
Heavy menstrual bleeding	1 (6.7%)		

** Including SAEs (all resolved).*

1859 Preclinical Development of CD33-Null Hematopoietic Stem and Progenitor Cells for Enhanced CD33-Directed Immunotherapy in Acute Myeloid Leukemia

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**Corresponding Author*

Gemtuzumab ozogamicin (GO), a CD33-targeting antibody–drug conjugate, has demonstrated clinical efficacy in acute myeloid leukemia (AML). However, its broader application is hampered by on-target, off-tumor toxicity against CD33-positive hematopoietic stem and progenitor cells (HSPCs), leading to severe hematologic side effects. To overcome this limitation, we are advancing toward a Phase I clinical trial (GALAXY-33, NCT05662904) that assesses the safety and efficacy of donor-derived, CRISPR/Cas9-edited HSPCs with a targeted disruption of the CD33 gene in patients with AML relapse after allogeneic stem cell transplantation. This approach aims to allow high-dose GO administration without compromising the normal hematopoiesis.

In our preclinical studies, we designed CRISPR/Cas9 ribonucleoprotein (RNP) complexes targeting exon 3 of CD33. RNPs were electroporated into primary human HSPCs using the MaxCyte ExPERT® platform to maximize transfection efficiency and preserve cell viability. Post-editing analyses via Inference of CRISPR Edits (ICE) and flow cytometry revealed editing rates of up to 87%. To ensure the highest level of genomic integrity, off-target effects were evaluated using CAST-Seq and Amp-Seq, revealing only non-relevant off-target edits. Functionally, CD33-null HSPCs remained robustly resistant to clinically relevant GO doses while retaining normal growth and viability (Liu et al., Int J Cancer 2021).

Ongoing investigations include a comprehensive evaluation of safety, incorporating genotoxicity profiling, immune function assays, and long-term engraftment studies *in vivo*. Additionally, preclinical proof-of-concept studies will further validate the protective effect of CD33-null HSPC transplantation against GO-induced toxicity.

By employing a CRISPR/Cas9-based gene editing strategy in the setting of allogeneic stem cell transplantation, the GALAXY-33 trial seeks to harness CD33-directed immunotherapy while mitigating its hematologic toxicity. This strategy holds promise for enhancing the therapeutic index of CD33-directed immunotherapy, ultimately improving survival and quality of life in patients with AML.

1860 Safety and Efficacy of Umbilical Cord-Derived Mesenchymal Stem Cells (TH-SC01) in Patients with Perianal Fistula in Crohn's Disease: Phase 1 and 2 Clinical Trials

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Objectives: TH-SC01 is an umbilical mesenchymal stem cell product manufactured in a GMP facility. We evaluated the safety and efficacy of intralesional administration of TH-SC01 allogeneic umbilical cord-derived mesenchymal stem cells (TH-SC01) in patients with perianal fistula in Crohn's disease (CD).

Methods: The phase 1 trial was an open-label, single-arm study (from 2022, through 2024). The phase 2 trial was an randomized placebo-controlled, double-blinded study (from 2023, through 2025). Adult patients with treatment-refractory CD perianal fistulas (pfCD) were enrolled.

Main Outcomes and Measures: The primary objectives of phase 1 were to evaluate dose-limiting toxic effects and establish RP2D. The primary objective of phase 2 was to assess combined remission and safety at 24 weeks. Patients received a single intralesional injection of TH-SC01 cells. Combined remission (CM) was defined as an absence of suppuration through an external orifice, complete re-epithelization, and closure of external orifice and absence of collections larger than 2 cm measured by magnetic resonance imaging (MRI) at 24 weeks after cell administration.

Results: Nine in phase 1 (median age, 27 years [range, 18-47 years]) and 40 patients in phase 2 (median age, 26.5 years [range, 18-54 years]) were enrolled. The recommended RP2D was 120 million cells. In the phase 2 population, CM at 24 weeks was 50% (95% CI, 14.2%-65.8%) in the TH-SC01 group and 10% in the placebo group. Significant improvement in Perianal Crohn Disease Activity Index, Pelvic MRI-Based Score, Crohn Disease Activity Index, and quality of life score were observed at 24 weeks in patients who received TH-SC01 cells. No serious adverse events occurred in both phases 1 and 2 trials.

Conclusion: Intralesional administration of 120 million TH-SC01 cells might be safe and effective for treatment-refractory pfCD. These findings warrant further investigation in an ongoing randomised phase 3 trial.

1861 Evaluation of Salt-Tolerant Endonucleases for Lentiviral Vector Purification

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Lentiviral vectors (LV) are among the most widely used vehicles for delivering genetic material in cell and gene therapy. The success of LVs in clinical trials and the market approval of several LV-based products has driven an increase in both LV manufacturing demand and the need for higher-quality LV products. The downstream purification of LV suspension culture typically starts with an endonuclease treatment, followed by a clarification step. The endonuclease treatment, carried out in the bioreactor, is designed to digest both free and chromatin-associated DNA. Residual DNA poses several challenges to the clarification step by increasing solution viscosity, causing potential filter fouling, prolonging processing time and elevating overall filter pressure differential. Moreover, residual DNA can form hydrodynamically large complexes with viral particles, which are difficult to remove downstream. These complexes may lead to aggregation and potential product recovery losses during the purification process. Despite its importance, traditional endonuclease treatment in LV cultures often exhibits low efficiency and a second endonuclease step is required to achieve higher log reduction values of residuals and host cell DNA levels that meet regulatory requirements. This low process efficiency is primarily due to the low activity of endonucleases under the physiological conditions present in LV cultures. Recently, commercially available salt-tolerant endonucleases, such as M-SAN® HQ and Denarase® High Salt, have shown promise in overcoming these challenges.

In this study, we compared the performance of two salt-tolerant endonucleases (M-SAN® HQ and Denarase® High Salt) at three different concentrations in the digestion of DNA from LV suspension cultures, against a non-salt tolerant enzyme. The effect of sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) to optimize conditions for each endonuclease was also evaluated. Additionally, two design of experiments (DoE) were performed with M-SAN® HQ to investigate the effects of endonuclease concentrations and its co-factor $MgCl_2$ on DNA digestion. All

experiments utilized freshly generated material from a 7L bioreactor. The LV culture was sampled in 250mL shake flasks and incubated with the endonuclease at 37°C for 1-, 2.5-, and 4-hours. The stability of the lentiviral vector in the presence of the different endonuclease and $MgCl_2$ concentrations was accessed by functional titer. All endonucleases tested decrease the DNA content of the LV culture as measured by PicoGreen™ and hcDNA 18s-S (qPCR). Both salt-tolerant endonucleases outperformed the industry-standard endonuclease, even at lower endonuclease concentrations, with M-SAN® HQ showing the best overall host cell DNA digestion. Furthermore, DNA digestion with M-SAN® HQ improved with increased $MgCl_2$ concentration. The combined DoE model predicts Log10 reduction values of at least 1.0 for plasmid DNA, 0.8 for hcDNA18s-S, and 0.5 for total DNA after 1 hour of incubation at 37°C, demonstrating the potential of M-SAN HQ to enhance the efficiency of DNA digestion in LV manufacturing.

1862 High Salt, High Impact: An Endonuclease Engineered for High-Efficiency DNA Removal in Viral Vector Manufacturing

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In the manufacturing of viral vectors for cell and gene therapy and vaccine applications, ensuring the safety and efficacy of the final therapeutic product is of utmost importance. DNA contaminants resulting from lysis of genetically transformed cells used for virus production pose the potential threat of being immuno- or oncogenic.[1,2] Therefore, the FDA has set acceptable limits for the concentration (>10 ng/dose) and size (>200 base pairs) of residual host cell and plasmid DNA in the final drug product.[3,4] Endonucleases, which can break down all forms of nucleic acids into smaller fragments without affecting viral particle integrity, are used to meet this regulatory requirements. Currently, endonucleases from *Serratia marcescens*, are the industry standard for enzymatic nucleic acid removal. The use of these enzymes not only enhances the safety profile of the viral vector-based therapeutics but also improves overall process efficiency by preventing the formation of aggregates and viscosity caused by presence of extracellular DNA. This work presents the performance a novel salt-tolerant enzyme, an engineered variant of the wild-type *Serratia marcescens* endonuclease specifically designed for efficient DNA removal at elevated salt concentrations and process-relevant pH conditions offering flexibility in viral vector purification processes.

[1] Sheng-Fowler L, Lewis AM, Peden K. Issues associated with residual cell-substrate DNA in viral vaccines. Biologicals. 2009;37(3):190-195. doi:10.1016/j.biologicals.2009.02.015
[2] Singh SK. Impact of Product-Related Factors on Immunogenicity of Biotherapeutics. J Pharm Sci. 2011;100(2):354-387. doi:10.1002/jps.22276
[3] US Food and Drug Administration; Rockville, MD: 2010. Guidance for industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications.
[4] US Food and Drug Administration; Rockville, MD: 2020. Guidance for industry: Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications.

1863 Characterization of 27 Primate and Non-Primate rAAV/BoV Vectors *in vitro*

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Over the last decades, adeno-associated virus (AAV) has become a predominant vector for therapeutic gene transfer in humans. Still, an ever wider use of AAV is often hampered by its restricted cargo capacity of ~5 kb of DNA. Notably, Bocaparvovirus (BoV), another member of the family *Parvoviridae,* has the potential to overcome this problem by enabling the cross-packaging of AAV vector DNA with a size of up to 6 kb. This, in turn, permits the

encapsidation of larger transgenes or regulatory elements, while concurrently allowing to benefit from other unique features of BoV such as their distinct tropism and immunogenicity.

To date, we and others have shown that the human (H)BoV1 capsid efficiently transduces not only primary human airway epithelial (pHAE) cells *in vitro* but also ferret lung *in vivo*. Moreover, our group reported that HBoV1-4 as well as GBoV (gorilla variant) transduce various other primary cells and cell lines efficiently. Together, the extended packaging capacity and the robust transduction by the five previously characterized isolates makes BoV highly interesting and relevant for use in gene therapy, and it therefore motivates continued research into this unique vector system, especially the study of other naturally occurring BoV variants.

To this end, we systematically compared the homology of published BoV sequences before selecting the phylogenetically most distant 27 representative candidates covering the full diversity of known bocaviruses among more than 600 sequences analyzed for experimental investigation. Following the synthesis and cloning of all respective BoV capsid genes into helper plasmids, we discovered marked differences in viral vector production efficiency depending on the viral isolate. In most cases, the primate candidates yielded higher vector titers than the non-primate variants. Curious exceptions were three human BoV variants that yielded only ~1×10^{-2} vector genomes (vg) per cell, which is comparable to the upper titer range of the non-primate candidates. Vice versa, one non-primate isolate was distinguished by its higher titer compared to the other non-primate variants, namely, BoV from bats, which yielded ~3×10^{1} vg/cell similar to most primate isolates.

For further characterization, we studied the *in vitro* transduction profile of the new BoV vector variants expressing a *Gaussia* luciferase in the presence of a proteasome inhibitor in Huh7 cells. Also here, we noted striking differences depending on the viral isolate that ranged over two orders of magnitude for the primate candidates (from 2.4×10^{2} to 2.0×10^{4} relative luminescence units (RLU)). Interestingly, the bat variant outperformed the benchmark GBoV as well as all other primate variants with 6.8×10^{4} RLU, even though ten times less viral particles were used, making it particularly relevant for continued development as a gene therapy vector.

In current work, we are pursuing the hypothesis that vector titers can be further improved by harnessing the cognate non-structural proteins for genome packaging, rather than the HBoV1 counterpart that is routinely employed in present helper plasmids. Concurrently, we study the tropism, as well as the role of specific amino acids and domains of the different viral capsids, that may explain the distinct transduction properties, including cell attachment and post-entry steps, and whose understandings in turn will foster the rational engineering of these promising vectors.

1864 The Challenge of Comparability: The benefit of common release standards for *Serratia marcescens* endonucleases.

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In the manufacturing of viral vectors for cell and gene therapies and vaccines, the removal of residual DNA and RNA from host cells and plasmids is critical to meet regulatory requirements[1] and streamline vector purification. For this purpose, endonucleases are commonly added to enzymatically cleave polynucleotide chains into smaller fragments of around 3-5 base pairs. Endonucleases derived from *Serratia marcescens* are the current industry standards due to their efficient activity on diverse DNA/RNA substrates. Endonucleases from different suppliers are identical in protein sequence and could be applied interchangeably, with the enzyme activity being a key parameter in this context. However, variations in the release testing of enzyme activity in units (U) between vendors make direct comparison of Certificates of Analysis (CoAs) challenging. Despite their widespread use in GMP manufacturing for clinical trials and licensed drugs, there is no pharmacopoeia monograph outlining specifications, release tests and reference standards for *S. marcescens* endonucleases.[2] However, comparability of endonucleases is essential to secure supply chains, including dual sourcing strategies. This study uses the endonuclease activity release test developed by c-LEcta to compare activities two *S. marcescens* nucleases from different suppliers. Applying the same activity release assay test protocol to both enzymes, it is evaluated how these results correspond to the respective product's CoA values.

[1] US Food and Drug Administration. Chemistry, Manufacturing, and Control (CMC) Information for Human Gene

Therapy Investigational New Drug Applications (INDs).; 2020
[2] BioPhorum Operations Group Ltd; D. Blake; A. Schaefer, A. Witz, N. Preyat, R. Hodges & V. Mwanza; A proposal to align release standards for endonucleases used in nucleic acid removal; 2023. https://doi.org/10.46220/2023CGT002

1865 A New Approach to Designing Synthetic Affinity Ligands for Enhanced Viral Vector Purification

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Introduction: Patient access to cell and gene therapy products is hindered by low recovery, high-cost processes, that limit the commercial viability of these therapies. Development of novel capture reagents is key to addressing these challenges in viral vector purification and analytics. We introduce innovative chromatography solutions utilising rationally designed synthetic polymers as pseudo-affinity ligands for gentle capture and release of viral particles and analytical application. We demonstrate how our approach to reagent design, coupled with the optimisation of bind and elute conditions, ensures high recovery of functional particles.

Methods: We present our rational approach to ligand design targeting chosen surface epitopes of the VSVG envelope protein of lentiviral vectors, aided by in-silico predictive molecular modelling to determine optimum synthetic ligand composition to achieve the desired affinity. After confirmation of monomeric ligand components through in-vitro HPLC testing, synthetic affinity ligands were polymerised using molecular imprinting for synthesising high affinity nanoparticles, suited for analytical applications, or tuned affinity smart polymers, suited for purification application. Polymer characterisation was performed across several manufacturing scales, using tailored analytical techniques, to demonstrate repeatability and reproducibility. The performance of molecularly imprinted polymers was assessed using SPR (Surface Plasmon Resonance) and BLI (Bio-Layer Interferometry). Smart polymer performance and optimisation was performed through immobilisation on filters, resins and monoliths commonly used for purification of viral vector. Optimisation of smart polymer composition was focused on ensuring high recovery of functional particles, by utilising gentle elution conditions. Binding and elution events on these solid supports was visualised using fluorescence and assessed off-line using FIDA (Flow Induced Dispersion Analysis). Toxicity of smart polymers was also assessed using a cell-based assay, to understand the potential impact of leached ligands on patient safety.

Results: Our results demonstrate that the rational ligand design approach resulted in a 38% hit rate out of 50 molecularly imprinted polymers designed against 5 target surface epitopes. Molecularly imprinted polymers showed a dose dependent response to lentiviral vector in the range of 10^5 – 10^8 TU/mL on the BLI platform. Our results also demonstrate our ability to tune smart polymer composition to control affinity, resulting in elution events in physiological buffer conditions. Scale up experiments demonstrated our ability to maintain consistent polymer composition and size at larger scale, with percentage coefficients of variation as low as 5%. Toxicity assessment showed no detrimental impact on cell viability after prolonged incubation in presence of smart polymer, at concentrations greater than expected in application.

Conclusions: Our innovative approach to affinity ligand production, utilising rationally designed synthetic polymers, offers a transformative solution for improving the efficiency and recovery and quantification of functional viral particles.

1866 State-of-the-Art Mass Spectrometry Characterisation of Adeno-associated Virus (AAV) by Intact Mass, Peptide Mapping, Host Cell Protein and Metabolomics Analysis

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Characterisation strategies for AAV gene therapy products have significant challenges due to their large molecular size, structural complexity, heterogeneity, and relative novelty but are essential to support CMC requirements. As with other therapeutic modalities, there is an increasing expectation in terms of the depth of analytical data as methodologies and technologies deepen our understanding of the molecules of interest. A natural starting point is to deploy, and assess, currently available analytical techniques whilst mitigating for the complexity and nuance inherent in the viral vector modalities. To this end, Pharmaron have deployed advanced mass spectrometric techniques to provide deep insights into both product and process – characterising viral capsid critical quality attributes (CQAs) using an intact mass and peptide mapping approach, assessing manufacturing efficiency through LC-MS metabolite analysis, and interrogating the host cell protein profile through application of proteomic analysis. Combined, these state-of-the-art techniques help support AAV product development and data packages for their customers' regulatory submissions.

In the following work two separate AAV serotypes, manufactured on site at Pharmaron Liverpool, were tested. Analysis of the viral proteins was attained by first denaturing the capsid into its constituent VP1, VP2, and VP3 subunits follow by column concentration and subsequent gradient elution/MS analysis. Identities for VP1 to 3 proteins were confirmed with a mass accuracy ranging from 1-35ppm. VP1 and VP2 were identified as predominantly acetylated, whereas VP2 did not show any significant level of acetylation. A clipped variant of VP3 was also identified.

Peptide mapping was performed, digesting the sample material using orthogonal proteases prior to LC-MS analysis. Sequence coverage ≥98% was obtained across all viral proteins, and robust relative quantification of post translational modifications including acetylation, oxidation, and deamidation was achieved (<20% RSD observed for majority of modification sites). Peptide mapping data was then interrogated, leading to the identification of a clipped site of the truncated VP3 protein observed during the intact mass analysis.

Host cell proteins (HCPs) are a major class of process impurities, which require monitoring and reporting to ensure product safety and quality. Early indications of immunogenicity linked with AAV infection/ treatment in human patients may have been exacerbated by the presence of these process related impurities. Compared to an ELISA approach, mass spectrometric analysis can provide a much deeper understanding of the HCP profile of a material and help identify specific proteins of immunological significance, allowing for better monitoring and process related control/removal strategies. Pharmaron demonstrate their expertise on how a mass spectrometric approach, enhanced through the use of cutting-edge instrumentation and nano-LC separation – can provide extensive information on AAV related HCP profiles, with sensitivity to detect proteins down to low ng/mg (HCP:AAV) levels.

1867 Streamlining Downstream Lentiviral Vector Production: A Novel IEX Platform for Improved Recovery, Infectivity, and Purity

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Lentiviral vectors (LVs) hold immense promise for gene therapy applications; however, current LV production and purification are limited by low process recovery of infectious units (10-15%) after downstream processes. Contributing factors include high concentrations of residual impurities, such as host cell protein (HCP), and lengthy processing times and poor LV stability. Traditional IEX bead-based resins often lack the optimized bead porosity for large viral vectors like LV. This can lead to both optimization challenges and the need for large bed volumes resulting in longer processing times. Despite extensive optimization, current methods (bead-based resins and membrane adsorbers) yield suboptimal infectious LV recoveries (10-30%) and further downstream processing often result in an overall recovery of 10-15%, highlighting the need for improved purification strategies.

To address this challenge, SK Pharmteco Cell and Gene Therapy North America (SKPT CGT NA) has developed and optimized an innovative IEX membrane adsorber capture step. Compared to traditional methods, our optimized process achieves a more than a 2-fold increase in infectious unit titer recovery compared, while reducing processing

time to approximately 1 hour while maintaining a greater than 98% HCP clearance. The reduced processing time enables a 1-day purification process, particularly beneficial for ex vivo applications.

We showcase the development and scale-up of this IEX capture step from benchtop to 50 L production. This streamlined approach simplifies LV capture, enabling rapid and cost-effective production of LV while maintaining high product quality.

1868 Advancements in Lentiviral Purification: From High Throughput Screening to Scalable Solutions

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Lentiviruses are commonly used in cell and gene therapy due to large insert capacity and capability to integrate in both dividing and non-dividing cells. The use of LVs, and number of gene therapies involving them, have steadily increased over the years, with focus being mostly on ex vivo therapies. Recently, however, there has also been a surge of interest for in vivo lentiviral treatments, leading to several planned human clinical trials. With in vivo applications, requirements for purity are much higher than ex vivo. As such, the need for better downstream processes, with increased impurity clearance and infective LV recovery, has become evident. With chromatographic step being essential for scalable and cost-effective downstream process, we decided to test monolithic columns for purification of lentivirus.

Good initial results have been achieved on CIMmultus QA. To further improve the recovery of the downstream process, we decided to determine the factors and conditions, which contribute the most to downstream recovery, by using Design of Experiments (DoE) approach. To increase throughput of the DoE studies, we used CIM QA 0.05 mL Monolithic 96-well Plates (6 µm), which significantly speed up the testing process, as well as decrease variables which occur from day-to-day work. From the factors tested in the DoE, results indicated starting NaCl concentration in lentiviral load has the most profound effect on lentiviral recovery, with increased NaCl concentration leading to better downstream recovery. Using DoE and high throughput testing with plates, we could improve the infective lentiviral recovery from 20% up to 90%. The process was then successfully transferred to 1mL QA (6 µm) column, using conditions, which can be scaled up to manufacturing columns. On 1mL column, 70% average infectious recovery was achieved, with over 2000-fold decrease in host-cell protein (HCP) content. Final average HCP and hcDNA contents were calculated to be 490 ng of HCP and 230ng of hcDNA per dose of 10^9TU. Additionally, particle composition analysis demonstrated removal of incomplete particles during chromatography and enrichment of complete lentiviral particles, from 34% in clarified harvest, to 51%, thus increasing potency (ratio infective : non-infective particles) by 51%. When tested for capacity it was determined to be 3E+12 vp/mL of QA monolith (6 µm).

1869 Hedge Your Bets in Genetic Roulette with cliniVec: Accelerating Cell and Gene Therapy Development Through Optimized Vector Design and Characterization

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As the cell and gene therapy (CGT) field expands, the number of therapeutic candidates entering preclinical and clinical pipelines is increasing rapidly. This surge has brought unique challenges related to manufacturing, characterization, and regulatory requirements for vectors used in gene delivery. Key complications include bottlenecks in production scalability, variability in therapeutic efficacy, and the need for enhanced safety and quality

characterization of vectors. It is now understood that a large portion of Chemistry, Manufacturing, and Controls (CMC) issues come from poor manufacturability challenges as a result of inferiorly designed vectors obtained from a variety of sources. These are often legacy constructs that have never been stress-tested or have been redesigned with cloning speed and not manufacturing quality in mind.

This presentation explores developer feedback and how we can facilitate innovative approaches to addressing these challenges by focusing on expanding candidate selection with emphasis on vector design optimization, starting at the candidate selection stage which is way upstream of the clinical construct selection.

By examining how specific intrinsic design elements influence efficacy, manufacturability, and safety, researchers can streamline the transition from early research to clinical application. Furthermore, the importance of deeper vector characterization, even when not explicitly required by regulatory agencies, is emphasized as a strategy to improve outcomes in CGT development and reduce the valley of death.

Drawing from insights in vector construction and characterization (>1 million vectors designed) over the past decade, VectorBuilder highlights key considerations for designing vectors that meet the complex requirements of preclinical and clinical studies. The cliniVec approach aims to provide a comprehensive framework for advancing CGT development through enhanced design and characterisation strategies that prioritize safety, performance, and scalability throughout the entire lifecycle of any CGT viral vector.

1870 Host and viral (non-viral genomic) DNA/RNA contamination in viral particles produced for clinical applications under GLP/GMP and profiling of RNA/DNA packaged inside viral particles.

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Background: Viruses are increasingly being used as vectors for delivering therapeutic cargo. Often, viruses themselves are used as therapeutic agents for, e.g., oncolytic viruses. In all such cases, efficient amplification and purification of viral particles is an essential step. It includes designing, producing, testing, and validating viral particles that meet the standards of GLP, GMP, and standards of impurities. Viral particle assembly and packaging in cells is an organized process. The process differs between different viruses based on whether they are enveloped, the type of genome, the structure of the protein capsids, etc. There are several reports that mention host and viral RNAs (non-genomic) get packaged in mature and empty virus particles, which may have functional importance to the virus infection cycle in general. It is not completely clear if all the host and viral non-genomic RNAs found in viruses are of functional importance or if they get packaged randomly. Most importantly, the experiments used in several such reports were not stringent enough to rule out the possibility of such RNAs bound outside the viral particles rather than getting packaged inside. So, at least in some viruses, it still remains to be investigated if host and viral RNAs get packaged inside viral particles at all using very stringent and efficient methods. For clinical applications, such impurities may affect the quality of the viruses (prepared under GLP and GMP) and may lead to many unwanted and untargeted effects on patients. Several reports suggest that some host/viral RNA contamination is unavoidable in adenoviruses and AAVs. The standards for testing such contaminants and how much contamination is acceptable are not very clear and are still being debated.

Material and methods: We tested viruses (AdV and AAVs) prepared with current state-of-the-art methods and standards and observed significant host and viral DNA/RNA contamination. Viruses were prepared using ultracentrifugation. We deployed robust DNA/RNA extraction methods and next-gen sequencing methods to identify if there are RNAs and to determine the sequences, if any, are present. We used current state-of-the-art methods available through our genomics core facilities and also our novel NGS library prep methods, which are more sensitive than others.

Results: We could identify most of such contamination is only from outside of the viral particles rather than inside. We could not detect any of the transcripts (host / viral) reported earlier to be packaged inside viral particles, and also, no new transcripts were detected. This is contradictory to several earlier reports. We also devised novel methods to remove DNA/RNA contaminants from AdV particles, which seem to be mostly from outside. We hope our study and recommendations can be implemented to prepare better-quality viral particles with no contamination of nucleic acids from hosts. This will help in meeting zero contamination levels for clinical applications and manufacturing.

1871 Enhanced Lentiviral Vector Characterization Using Digital PCR: Genome Titer, VCN, and RCL Detection

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The advancement of cell and gene therapies is essential for treating a variety of diseases. Lentiviral vectors (LVVs) have become a key technology for delivering therapeutic genes into cells. Ensuring the safety, efficacy, and consistency of LVs requires thorough characterization. One important aspect is accurately quantifying viral vector genome titers and vector copy number (VCN), which indicates the number of transgene copies in the target cell genome. The effectiveness of viral transduction depends on factors such as viral dose and target cell type, making precise VCN measurement crucial for optimizing viral doses in therapy applications.

After extracting genomic DNA from transduced cells, the number of lentiviral gene copies is compared to a reference gene. Traditional methods often use qPCR, which requires external standard curves. Digital PCR (dPCR) offers absolute quantification with high precision and tolerance for inhibitors, eliminating the need for standards. We propose a rapid dPCR method to determine VCNs, providing insights into transgene stability and expression. Consistent VCNs are vital for maintaining uniform product quality in biopharmaceutical production.

Lentiviral quantitation is another critical quality attribute in viral vector manufacturing for cell therapy development. Digital PCR is a powerful tool for absolute quantification of LVVs, addressing the limitations of qPCR with its precision, accuracy, specificity, and reproducibility.

A significant safety concern with lentiviral systems is preventing the creation of replication-competent lentivirus (RCL) through recombination. Regulatory bodies like the FDA and EMA require comprehensive RCL testing in retroviral vector products. We demonstrate a streamlined dPCR workflow for quantifying lentiviral genome titers, VCN, and RCL detection, enhancing characterization with greater precision, accuracy, and robustness.

1872 Importance of AAV sample preparation for gc/ml titer determination using PCR.

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Determining the gc/ml titer of an AAV sample is not only important for process development but is even more critical for the administration of a therapeutic AAV dose to a patient's body. AAV is known to be a challenging sample for PCR amplification due to several factors, including the template being hidden inside the capsid, the ITR hairpin regions surrounding the packed genome, and small DNA fragments that may be bound to the capsid and potentially amplified. This poster focuses on a comprehensive comparison of gc/ml titers obtained from PCR amplifications with and without sample pre-treatments.

1873 A new approach for high-resolution full and empty AAV capsid analysis using a high throughput method for comprehensive AAV evaluation on a single CE platform

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Before regulatory submission, key critical quality attributes (CQAs) for gene treatments delivered by adeno associated virus (AAV) vectors must be thoroughly evaluated to verify their safety and efficacy. Multiple CQAs are currently being characterized using various analytical systems. Without significant improvement, these techniques have drawbacks or cannot be utilized to examine all serotypes. This poster outlines a method for performing multiple CQA analyses of AAVs at high throughput on the multi capillary BioPhase 8800 system. A comprehensive analysis of all key CQAs such as nucleic acid loading and titer, viral capsid protein profile and ratio, capsid titer and full and empty ratio can be done on a single platform, the BioPhase 8800 system, within a single day. Platform methods on the BioPhase 8800 system with fast process times can shorten development timelines, save costs, streamline the QC process and accelerate time to market.

1874 Enhancing rAAV genome integrity through process development optimization

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Conventional PCR-based methodologies lack the resolution to characterize integrity of rAAV genomes, which can play a role in rAAV potency. To overcome this, we employed long-read sequencing methods and investigated rAAV samples from two early-stage programs. We found that process changes impacted rAAV genome properties. Thus, long read sequencing technologies overcome the limitations of traditional methods to characterize rAAV genome heterogeneity and can be routinely applied to optimize process development and vector properties.

1875 Counting, Sizing, Imaging, and Identifying Viral Vector Product Aggregates at Ultra Low Volume

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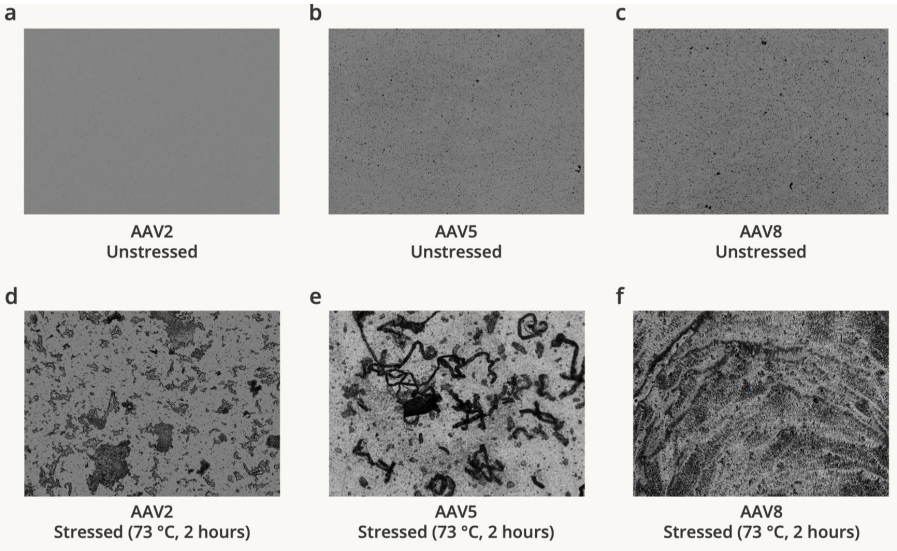
In all biological products, distinguishing aggregated API from other particle types matters for understanding the root cause of product instability. Until now, subvisible particle (SVP) characterization methods have been unreliable, slow, and difficult to use across many workflows. Subvisible particles are critical quality attributes for parenteral therapies. Recent experience with protein biomanufacturing and formulation has provided insights into the challenges of formulating stable biotherapeutics. The inherent complexity of advanced therapy medicinal products (ATMP) highlights new challenges and opportunities, in the formulation and manufacture of cell and gene therapies.

As the field moves towards biomanufacturing ever more complex APIs, novel formulation strategies must keep pace. More traditional formulation strategies may not be applicable to the complex drug formulations associated with the advancement of cell and gene therapies (CGT), as they present unique stability challenges as multi-component systems. A novel ultra-low volume, high-throughput approach with positive particle identification is needed for rapid sub-visible particle and aggregate assessment.

The Aura+ utilises three imaging approaches: backgrounded membrane imaging (BMI), side illumination membrane imaging (SIMI) and fluorescence membrane microscopy (FMM) to provide a full spectrum of particle analysis. The ability to specifically detect, count, and characterize subvisible particles for product quality measurements is applicable across the whole range of therapeutic products, from small molecule to protein, cell, and gene therapy applications.

Here, we show how the combination of BMI, SIMI, and FMM can be used for total particle analysis providing counts, images, sizes, and positive identification of subvisible API aggregates and extrinsic particles in a rapid and low-volume format. We present case studies highlighting the value of a high throughput approach for SVP analysis across the whole spectrum of bio-manufactured products, from protein therapeutic stability to cell line development through manufacturing, viral vector stability, and cell therapy aggregation and product purity.



1876 Tangential Flow Filtration membranes for robust ultrafiltration and diafiltration in Lentiviral vectors downstream processing

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As the lentiviral vectors (LV)-based therapeutics market grows, their applications are moving from localized to systemic disorders, and their use is now commonplace in academic laboratories and industry for both research and clinical gene therapy applications. As such, manufacturing processes must be optimized to ensure scalability, reproducibility, and cost-effectiveness. One crucial aspect of LV production lies in the downstream purification steps, with ultrafiltration and diafiltration (UF|DF) playing central roles in achieving high product concentration (volume reduction) and buffer exchange in addition to the removal of low molecular weight impurities while retaining vector particles.

Hydrosart® high-performance UF|DF membranes have been optimized for biopharmaceutical process applications, which features a broad pH and temperature range. This cellulose-based membrane is extremely hydrophilic, making it non-protein binding and virtually non-fouling. As a result, it has extremely high fluxes. Furthermore, Sartorius offers two different cassette geometries for ultrafiltration and diafiltration operation, the E-screen and ECO format. Compared to the E-screen geometry, the ECO cassette features 26% more surface area per standard cassette width, making it possible to install more area in the cassette holder as well as decreasing feed pump power demand. Consequently, the need for larger systems at scaled-up processing is reduced when operating with the ECO cassette format.

In this study, Hydrosart® 100 and 300 kDa TFF membranes in two different cassette formats were evaluated for their performance in the UF|DF of LV. The identified optimal processing parameters resulted in high LV recoveries, both in particle and infective titer (95 – 100%), while achieving significant removal of DNA (51 – 63%) and proteins (10 – 47%). Higher pumping rates for the E-screen cassette generally resulted in higher permeate flux and shorter process times. The ECO cassettes still allowed LV concentration in a reasonable time and with gentler processing conditions, as well as reduced pump capacity, which is important when operating at larger scales. Overall, excellent reproducibility of the LV UF|DF process was demonstrated, ensuring reliable process performance.

1877 Development of a Fully Closed and Sterile Manufacturing Process for Lentiviral Vectors: Eliminating the Need for Sterile Filtration

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AGC biologics is a leading global CDMO, providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells.
Lentiviral vectors (LVV), produced with transient transfection in HEK293 and HEK293T cells are extensively used for genetic modification in gene therapy for ex-vivo and in-vivo treatments. AGC Biologics developed the ProntoLVV platform for the manufacturing of LVV at different scales in adhesion and suspension, flexible to be adapted to different GOI (Gene Of Interest). The demand for LVV is constantly rising to support advanced clinical phases and commercial applications. In this regard suspension cells-based systems offer a great advantage in scalability vs. traditional adherent systems widely used so far, allowing to design very large scale processes.
While suspension processes significantly boost productivity, optimization of each process step remains essential for both suspension- and adherent-cell based processes. Upstream phases are fine-tuned to maximize vector production, and downstream steps are continuously developed to enhance recovery without compromising vector quality.
Among all downstream steps, the final sterilising filtration has the most significant impact on overall process recovery. Sterilizing-grade membranes pore size is close to that of lentiviral vectors, resulting in low yields and frequent clogging, posing a major challenge to process efficiency. Filtration step recoveries can range from 50% to 30% and – for some particularly difficult LVVs – can get as low as 10%. This represents a major loss of LVVs in the economy of the process.We recently investigated the possibility to remove the final sterilising filtration in our LVV manufacturing platform, by designing a completely closed and sterile process.
To make this strategy viable, every step of the process must be executed in aseptic conditions within a fully closed system. This requires single-use equipment and gamma-irradiated sterile kits that can be connected using sterile connectors. While clarification filters compatible with gamma irradiation are readily available, the DSP chromatographic steps represent a major hurdle for sterile process. Standard IEX and SEC resin-packed column are not supplied as sterile products. Due to these considerations, we present our results on Cytiva Mustang Q membranes, a known chromatographic membrane used for the purification of LV vectors, that are available in a format that can be autoclaved. Development work was conducted to optimize the binding and elution conditions of LVVs to the Q-membrane, and dedicated single-use, sterile flowkits have been designed to aseptically connect and operate the Mustang Q membrane to standard single use Chromatographic skids (AKTA Ready).
Ultrafiltration/Diafiltration steps can easily be designed to be operated in fully closed and sterile conditions, and TFF media such as hollow fibers are already available as sterile units. The results showed recoveries of the new implemented process similar to standard AGC Pronto platform up to the pre-sterilising filtration step.
Thanks to the fully enclosed, sterile nature of the whole process, the final sterilising filtration can be avoided, resulting in a net increase in the overall process recovery ranging from 30% and up to 70% compared to standard processes.

1878 High-Throughput Flow Cytometry Assay for Quantifying Infectivity of AAV, Adenovirus, and Lentivirus Vectors in Gene Therapy Applications

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The efficient delivery of genetic material to target cells is critical in gene therapy, with viral vectors such as Adeno-associated virus (AAV), Adenovirus (Ad), and Lentivirus (LV) being essential to this process. Assessing the infectivity of these vectors is vital for optimizing gene delivery strategies. Traditional methods for evaluating vector infectivity are often time-consuming, qualitative, or lack the sensitivity required for precise and quantitative measurements. Flow cytometry provides a rapid, high-throughput platform that can quantify infectivity titers and percentage transduction with high sensitivity and specificity.

In the present study design, flow cytometry was used to assess the infectivity of in-process and final drug products produced using AAV, Adenovirus, and Lentivirus vector platforms, by utilizing reporter genes or surface markers to quantify transduction efficiency in target cells. The flow cytometry assay demonstrated high sensitivity and reproducibility over traditional cell-based assays such as FFU, PFU, and transduction unit assay, enabling the analysis of large sample sizes and the precise determination of infectious titers. Selected HEK293 suspension cells, Jurkat cells, or HeLa cells were transduced with samples containing AAV, Adenovirus, or Lentivirus vectors, respectively, and a time-course analysis was conducted up to 48 hours. Infectivity was measured by quantifying the number of transduced cells expressing the gene of interest (GOI), and dose-response curves were generated to evaluate the functional efficiency.

The future aspect for this method development involves exploring different cell lines for infectivity-based analyses, therefore assessing how viral vectors interact with and transduce distinct cell types and thereby providing insights into tropism, efficacy, and cytotoxicity. By overcoming the constraints of traditional assays and providing superior specificity, accuracy, and precision, flow cytometry holds the promise of delivering a robust and time-saving approach to measure the functional titer of viral vector samples.

1879 STRATEGY FOR IMPROVING THE RESCUE, REPLICATION, AND PACKAGING OF GENERATION Z (GenZ) SINGLE-STRANDED AAV VECTORS

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We have previously reported the development of a generation Z (GenZ) ssAAV DNA genome in which the D-sequences in the AAV inverted terminal repeats (ITRs) were replaced with a sequence obtained from a random nucleotide library (*Mol. Ther*., 31: 234-235, 2023). The GenZ ssAAV genome overcomes the rate-limiting step of the viral second-strand DNA synthesis, and mediates significantly higher levels of transgene expression. Thus, it behaves more like a scAAV genome, but without the size-limitation. However, we observed that the efficiency of rescue and replication and packaging of the GenZ AAV genome from recombinant plasmids were significantly reduced, and depending on the AAV serotype capsid, ranged between ~10% to ~60% that of the AAV genome containing the D-sequence (*Mol. Ther*., 32: 141-142, 2024). This was not entirely unexpected, given the absolute requirement of the D-sequence for the optimal rescue, replication, and packaging of the AAV genome (*J. Virol.*, 71: 3077-3082, 1997; *Mol. Ther.*, 9: 2628-2630, 2021). We have previously reported that the proximal 10 nucleotides (D10), but not the proximal 5 nucleotides (D5), in the D-sequence are necessary and sufficient to mediate each of these functions (*J. Virol.*, 71: 3077-3082, 1997). In our current studies, we revisited the role of D5, D6, D7, and D8 nucleotides (D8, D9, and D10 nucleotides are identical in the D-sequence) in rescue, replication, and packaging of the AAV genome. Four different recombinant AAV genomes expressing a firefly luciferase-enhanced yellow fluorescence (FLuc-EYFP) fusion reporter gene flanked by D5, D6, D7, and D8 sequences. AAV genome rescue and replication analyses (**Figure 1A**) revealed that, consistent with our previously published data, whereas the D5 sequences were insufficient for successful rescue and replication, addition of 1 nucleotide (D6) also failed to mediate successful rescue and replication. Interestingly, addition of 1 additional nucleotide (D7) restored successful rescue and replication, similar to the wild-type D-sequence (D8). Furthermore, the D7 sequences also allowed successful packaging, followed by transduction to nearly the same extent as that by the conventional AAV vectors (**Figure 1B**). Restoration of the D7-sequence in the GenZ genome is likely to overcome the major limitation of the low-titers of GenZ vectors, which has important implications in achieving high-efficiency transgene expression from ssAAV vectors, such as for human clotting factor VIII (hFVIII) for gene therapy of hemophilia A.

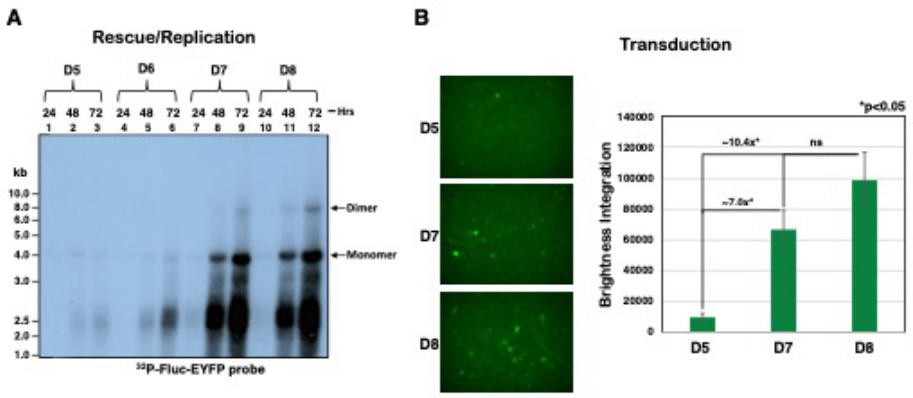

Figure 1: (A) Southern blot analysis for rescue and replication of the AAV genomes containing D5, D6, D7, and D8-sequences. **(B)** Transduction efficiencies of recombinant ssAAV2-FLuc-EYFP vectors containing D5, D7, or D8 sequences that were used to transduce HeLa cells in triplicates at $3x10^3$ vgs/cell. Transgene expression was visualized 72 hrs post-transduction and quantitated using a Keyence microscope. ns=not significant.

1880 A Novel AAV Vector Design for Reducing Cross-Packaged ITR Promoter Activity

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**Corresponding Author*

Adeno-associated virus (AAV) vectors are viable tools for delivering therapeutic drugs to treat various diseases. Nevertheless, during their clinical development, it is essential to address impurities present in even highly purified AAV preparations. Impurities in the AAV packaging production include DNA, proteins, and empty capsids. Most of the DNA impurities, consist of viral plasmids and host cell genomes, can be digested by subsequent purification steps. But non-GOI DNA components encapsulated within the AAV cannot be efficiently eliminated. Non-GOI DNA contain bacterial elements in the plasmid backbone, involving antibiotic genes, bacterial replicons, and potentially open reading frame (ORF) sequences. These ITR guided mis-packaging contaminants are commonly referred to as cross-packaging. ITR is an essential component for viral packaging and replication and possess promoter activity. Adjacent plasmid backbone sequence can be driven for transcription and potential expression of unknown toxic proteins by ITR, which may both incite immune responses and bring safety risk for AAV products. The polyA signal is a DNA sequence that exerts a transcriptional termination effect and guides the polyA tail to attach to the post transcriptional mRNA. To mitigate the ITR-driven transcripts of cross-packaged AAV, we introduced polyA signal sequences (PolyA SS) proximate to ITR and developed a novel viral plasmid for AAV production. In addition, 3x overlapped stop codons (corresponding to +1/+2/+3 reading frames) was incorporated prior to the polyA SS to restrict possible translation from the unexpected transcripts, and these modifications are termed translation and transcription termination elements (TTTE). We evaluated the effects of TTTE on the transcription of the plasmid backbone *in vitro*. HEK293T were transduced with viral plasmids with the additional TTTE at upstream of the 5' ITR or downstream of the 3' ITR or both. Compared to parental plasmid, the insertion of TTTE at upstream of the 5' ITR or downstream of the 3' ITR reduced the backbone transcription products by 38% or 18%, and by about 87% when both insertions were applied. To confirm the TTTE effects *in vivo*, AAV were packaged through triple transfection system and administered into the tibialis anterior muscle of B6 mice. The modified and unmodified products were injected in the left and right legs respectively. Two weeks post injection, muscles were collected and analyzed for backbone expression. In vivo experiments demonstrated that TTTE modifications significantly reduced backbone transcription by more than 99%. These findings indicated that the introduction of TTTE into the upstream of 5'ITR and 3'ITR of AAV packaging plasmid can effectively inhibit the ITR-driven backbone transcription, which facilitates to increase the safety of AAV products.

1881 Adeno-Associated Virus characterization using Nanopore long read sequencing and analysis

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Recombinant adeno-associated virus (rAAV) has become a leading vector for *in vivo* gene delivery, with numerous clinical trials exploring its use for treating a range of human diseases. The quality of the AAV product is crucial for the success of gene therapy, and thorough understanding of the viral genome sequence is becoming essential for effective quality control. During AAV production, non-targeted sequences may be inadvertently packaged alongside the gene of interest (GOI). To ensure the safety of the final product, it is also critical to evaluate and characterize these non-GOI sequences.

In this study, we evaluate and develop optimized Nanopore long-read sequencing workflow to analyze our AAV viral genome produced from our triple transfection platform. This method confirms the correct gene of interest (GOI) sequence for identity, its integrity and provides insights into the impurity profile of the AAV product. Specifically, we assess the presence of mis-packaged sequences from the host cell genome or vector backbone used in a triple transfection platform. Nanopore sequencing offers a comprehensive view of the AAV genome and delivers robust, informative analysis of both in-process and purified products.

Our Nanopore sequencing results demonstrate a strong correlation with our recently developed 3D digital PCR (dPCR) assay, including 3D linkage analysis, further validating its utility in assessing genome integrity. This fast, cost-effective sequencing approach can complement high-dimensional quantitative digital PCR methods, offering a powerful tool to advance rAAV-based gene therapies for treating human diseases.

1882 Evaluating Plasmid Architectures for Improved rAAV Production in HEK293s

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Gene therapies employing recombinant adeno-associated virus (rAAV) are a leading approach to treat, and even cure, otherwise intractable genetic diseases. A key technical challenge for rAAV-based therapies is the high cost of production, which is partially driven by inherent limitations of any chosen production platform. For example, suspension HEK293-based processes generate rAAV by transiently transfecting HEK293s with three plasmids that encode: 1) the AAV *rep* and *cap* genes (Rep/Cap), 2) the adenoviral "helper" genes necessary for rAAV production, and 3) the gene of interest (GOI), which may be a model or therapeutic cargo. Key advantages of this process are fast prototyping speeds and high potency of the manufactured rAAV. Compared to other processes however, HEK293 systems historically demonstrate low viral production (i.e., titers), have poor loading of genomes into capsids (i.e., low percentages of genome-containing capsids), and require multiple expensive GMP-grade plasmids for large scale rAAV production. Upstream process improvement is needed.

Here, we explored two avenues for improving rAAV yield, product quality, and cost using a commercially-available suspension HEK293 cell line and a model NanoLuciferase GOI. First, we removed extraneous components from a commonly used adenoviral helper plasmid to reduce the plasmid's size by up to 35%. Our modifications enhanced rAAV production by up to 68% as determined by vector genome count via nanoplate digital PCR (ndPCR) and up to 55% by *in vitro* cell-based transduction assays. Additionally, we evaluated three Rep/Cap plasmid architectures head-to-head and identified designs that improved rAAV production by greater than 3-fold for two different serotypes tested via *in vitro* transduction assays. While we are currently evaluating additional designs, these two strategies to date have boosted vector genome yield in excess of 10^{11} copies / mL using a routine shake flask culture process. Next steps include product quality assessments and extension of these studies to additional serotypes. In sum, our studies show that plasmid architecture remains a fruitful area for improving rAAV manufacturing methods,

and we anticipate that the improvements we have identified will be applicable broadly across rAAV serotypes and functional cargos.

1883 Differential Effects of AAV GOI Plasmid Input on the Yield and Impurity Profiles of Single-Stranded Versus Self-Complementary AAV

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Recombinant adeno-associated viral vectors (rAAV) show great promise as gene delivery vectors to treat various diseases. The most commonly used method of rAAV production is triple plasmid transfection which produces rAAV drug products that contain antibiotic-resistance and bacterial DNA impurities from packaged plasmid backbones. Reports indicate residual plasmid DNA levels can exceed 8% of the rAAV product, raising patient safety concerns and highlighting the importance of monitoring and understanding the parameters that can influence these residual values[1,2]. Liu et al. (2024) have reported successfully decreasing plasmid backbone impurity packaging via a low-cis triple transfection method in both single-stranded (ssAAV) and self-complimentary rAAV (scAAV); however, this method is production system dependent and not tested at bioreactor scale[3]. Our study extended this work by testing whether reducing the relative ratio of GOI plasmid in triple transfection rAAV preparations could decrease backbone packaging across three scAAV and two ssAAV constructs produced in HEK293 cells using shake flask and AMBR250 bioreactors. Vector titer and backbone impurity levels of affinity-purified intermediates were measured using ddPCR against GOI and kanamycin resistance sequences, respectively. Our data demonstrate that reducing GOI plasmid input affects residual plasmid DNA impurities and the yield of ssAAV and scAAV differently. Reducing the GOI plasmid content by 50% significantly lowered the backbone impurity levels without affecting the titer in ssAAV production. Conversely, reducing the GOI plasmid content by 50% in scAAV production did not decrease the backbone impurity and, in some cases, reduced the yield of the scAAV vector. Reducing the GOI plasmid content by 90% decreased the titer for both ss and scAAV and had inconsistent effects on backbone impurities. The results of this study offer process development tools to produce safer and more efficient recombinant single-stranded AAV vectors for clinical applications. Our study also highlights the need to monitor residual plasmid DNA impurities during process optimization, noting that the configuration of GOI plasmids (sc vs ssAAV) affects these values.

References

- Wright, J. F. (2014). Product-Related Impurities in Clinical-Grade Recombinant AAV Vectors: Characterization and Risk Assessment. Biomedicines, 2(1), 80-97. https://doi.org/10.3390/biomedicines2010080
- Brimble, M. A., Cheng, P. H., Winston, S. M., Reeves, I. L., Souquette, A., Spence, Y., ... & Davidoff, A. M. (2022). Preventing packaging of translatable P5-associated DNA contaminants in recombinant AAV vector preps. Molecular Therapy-Methods & Clinical Development, 24, 280-291.
- Liu, H., Zhang, Y., Yip, M., Ren, L., Liang, J., Chen, X., Liu, N., Du, A., Wang, J., Chang, H., Oh, H., Zhou, C., Xing, R., Xu, M., Guo, P., Gessler, D., Xie, J., Tai, P. W. L., Gao, G., & Wang, D. (2024). Producing high-quantity and high-quality recombinant adeno-associated virus by low-cis triple transfection. *Molecular therapy. Methods & clinical development*, *32*(2), 101230. https://doi.org/10.1016/j.omtm.2024.101230

1884 Long-Read Sequencing Identifies Novel Class of Contaminants in Adeno-associated Viruses

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Efficacious and safe adeno-associated virus (AAV) gene therapies require stringent control of vector genome composition. Long-read sequencing, in which continuous kilobases of DNA can be assessed, has been applied to read out the entire genomic composition of AAV particles. It is known that AAVs with secondary structures and high homology as conferred by microRNA or guide RNA constructs can lead to truncations and contaminating species called snapback genomes. Additionally, previous work has shown that an undersized backbone can lead to increased levels of reverse packaging, wherein the backbone region is encapsidated and included in the final AAV product. With long-read sequencing we were able to discover and identify contaminating genomic species in our AAV preps. Surprisingly, the identity of this species demonstrates a unique mechanism of replication with the plasmid backbone outside of the ITRs.

AAV vectors were produced in adherent HEK293T cultures using PEI mediated triple transfection and purified with either iodixanol ultracentrifugation or affinity chromatography. During the quality control of AAV vectors, alkaline gels were run to assess vector packaging and genome integrity. From these gels, the presence of an unexpected band at ~1900 bp was observed in multiple AAV constructs. Due to the consistent appearance of this band in constructs with different transgenes, we further investigated the origin of this conserved band. To identify the contamination band, we assayed our AAV constructs with PacBio Single Molecule Real Time (SMRT) sequencing. We observed that ~12% of reads aligned to the correct length of the aberrant band. De novo assembly of these reads identified a snapback genome, a self-complementary AAV (scAAV) containing a polyA sequence and our AAV genome between the SV40 and the ITR. The formation of this snapback genome is facilitated by the presence of identical SV40 polyA sequences within the transgene to terminate transcription and another SV40 sequence in the backbone. The homology of these regions is sufficient to induce recombination resulting in a significant level of the SV40-snapback genome present in the purified AAV prep. To determine the impact of the external SV40 sequence element, parallel constructs were produced including or removing the backbone polyA. These vectors were analyzed by SMRT sequencing and alkaline gel and confirmed that removal of the external SV40 sequence prevents the snapback genome contaminant. The transduction efficiency of these constructs was tested both in vitro and in vivo. In HEK293 cells, there was no difference in transduction between constructs with or without the external SV40. Mice injected retro-orbitally (n=3) with vectors excluding the external SV40 resulted in decreased liver transduction by 34%.

Here we described the identification through long-read sequencing and analysis of a novel contaminant in AAV vectors that compose a significant sub-species in our final AAV product; however, the mechanism by which SV40-snapback genomes improve transduction in vivo remains unclear. These findings provide further evidence that the composition of the backbone region plays a critical role in packaging and efficiency. Optimization of these features is a crucial step along the path to clinical readiness as a homogenous final clinical product is essential for the safe and effective treatment of patients.

1885 Characterization of the AAV production kinetics from a recombinant baculovirus and insect cell platform

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Recombinant adeno-associated virus (rAAV) has emerged as a leading viral vector for gene therapy due to its strong safety profile and the ability to support long-term gene expression. To meet the growing demand of pre-clinical and clinical-grade rAAV vectors, many studies have been conducted to overcome the bottleneck of high manufacturing cost and low production yields for rAAV-based gene therapy. Recombinant baculovirus (rBV) and Sf9 insect cell system is one of the main rAAV production platforms exploited by academia and industry. Here, we study the production kinetics of two AAV serotypes carrying GFP gene (AAV9-GFP and AAVPVL-GFP) in crude lysate, cell pellet fraction, and supernatant fraction from the rBV/Sf9 platform over a prolonged 6-day process. AAVs were collected from each fraction throughout the 6 days and were titered by ddPCR. For both serotypes, the cell pellet sample collected on day 3 exhibited the highest titer among all the six cell pellet samples, while the supernatant sample obtained on day 6 displayed the highest titer among all six supernatant samples. Total AAV yields from crude lysate (cell pellet + supernatant) maintained at the same level from day 3 to day 6. Western blot was performed to detect the production level of AAV Cap and Rep proteins in Sf9 cells, as these proteins directly affect the yields and quality of rAAV. A high-throughput affinity purification process was utilized to purify rAAVs from both the day 3 cell pellet and day 6 supernatant samples. The purified rAAVs were titered by ddPCR and the capsid purity was assessed by CE-SDS. To evaluate the potency of the purified rAAVs, we performed in vitro transduction in HEK293 cells followed by flow cytometry analysis of the GFP signal. Our results revealed that rAAVs from the day 3 cell pellet had higher VP1 contents and higher GFP expression levels compared to the rAAVs from day 6 supernatant. Based on our historical data, a suboptimal viral capsid protein VP ratio is associated with poor rAAV quality. Our current process of harvesting at day 3 predominantly produces intracellular rAAVs that exhibit higher quality. A 6-day process may be operationally easier since most rAAVs are released into the supernatant, simplifying the harvesting step by only requiring the concentration of rAAVs from supernatant. This helps avoid contamination of host cell DNA and protein during the cell lysis step. However, it results in lower rAAV quality. Taken together, this work highlights the importance of process control, as it significantly impacts the quality of final rAAV product. When comparing AAV production platforms, it is crucial to optimize the process for each platform before conducting the study.

1886 Balance is Key: Optimization of Rep Protein Levels for Superior rAAV Production.

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Recombinant adeno-associated viruses (rAAV) are the leading viral vectors in clinical trials, typically manufactured with transiently transfected HEK293 cells. A critical part of the optimization process is to establish genetic constructs and methods which are optimal for the cell line to produce high yield functional titers. As shown by us last year, the interplay between the specific HEK cell line and AAV plasmids is greatly impacting the titer. Hence, it is mandatory to understand the effect of rep expression on a mechanistic level. Accordingly, we dissected the rep wildtype system to better understand baseline levels, the interplay of rep ratios, and split of rep from cap. We performed Western Blot and detailed mRNA ddPCR analysis to characterize the expression of the four rep proteins in various expression setups from wildtype, split construct ribosome skipping to co-transfection and correlated them with vg, cp and TU yields to develop a hypothesis for optimal rep expression strategies. Luciferase experiments complemented the expression analysis as well as tests with a wide range of transcriptional activity from established and novel promoters and very diverse rep ratios.

This allowed us to develop a working model about an optimal long Rep (lRep) : short Rep (sRep) ratio. Combinations which showed poor rAAV titer could be rescued accordingly. Impact of accessory AAV proteins was tested as well as 3’ UTR modifications. In summary, simplification of the rep expression/regulatory feedback loops was possible with identification of optimal baseline levels for specific lRep and sRep protein isoforms facilitating highly efficient rAAV production processes. Interestingly, wildtype results and rAAV data displayed very different pictures, further elucidating the complexity of the rAAV replication process.

1887 Encapsulation of Adeno-Associated Virus-2.5 in Extracellular Vesicles Improves Transduction Efficiency to Human Chondrocytes *in vitro*

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INTRODUCTION: Extracellular vesicles (EVs) are small, lipid-bound vesicles ranging in size from 30 nm to 1 µm, released by cells into the extracellular space.[1] They play a significant role in intercellular communication via paracrine signaling. Beyond their natural biological functions, EVs have emerged as promising vehicles for delivery of therapeutic agents, including adeno-associated viruses (AAVs).[2] EVs possess a highly complex bilayer structure that incorporates numerous proteins, lipids, carbohydrates, and surface-bound molecules, in addition to their internal cargo. This intricate composition protects internal loads from degradation and immune recognition as well as extending systemic retention, thereby addressing key barriers to clinical translation, such as short-lived delivery, neutralization by the immune system, and undesirable side effects. Due to their nanoscale size, EVs are capable of penetrating deep into tissues. Extracellular vesicle-associated AAV (EV-AAV) has several advantageous properties, including high transduction efficacy, reduced immunogenicity, prolonged transgene expression, and the ability to evade neutralizing antibodies.[3,4,5] This study explores the use of EV-AAV2.5 for green fluorescent protein (GFP) gene delivery to human chondrocytes, with a focus on their characterization and efficacy *in vitro*.

METHODS: Naked AAV2.5-GFP and EV-AAV2.5-GFP were prepared following the method described previously.[3] The titers of both vectors were quantified using quantitative real-time PCR. Naked AAV2.5-GFP and EV-AAV2.5-GFP complexes were characterized via nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). TEM was employed to confirm the structural integrity of EVs and the encapsulation of AAV within EVs. To evaluate transduction efficiency, HEK293 cells, and human chondrocytes were cultured in monolayers and transduced with either naked AAV2.5-GFP or EV-AAV2.5-GFP of 5,000, 10,000, and 20,000 viral genomes (vg) per cell. GFP expression was visualized using fluorescence microscopy and quantified using a fluorescence reader.

RESULTS: TEM analysis confirmed the proper EV structure and successful encapsulation of AAV2.5 within EVs (Figure 1A). NTA revealed a size distribution of 152.6 nm for EV-AAV2.5, indicating successful complex formation (Figure 1B). HEK293 cells were transduced with both naked AAV2.5-GFP and EV-AAV2.5-GFP (Figure 1C). EV-AAV2.5 demonstrated a higher transduction efficiency compared to naked AAV2.5-GFP (Figure 1D). Similarly, human chondrocytes were transduced with naked AAV2.5-GFP and EV-AAV2.5-GFP (Figure 2A). GFP expression from naked AAV2.5 and EV-AAV2.5 was quantified, revealing comparable GFP expression levels for EV-AAV2.5 relative to naked AAV2.5-GFP (Figure 2B).

DISCUSSION: TEM and NTA characterization confirmed the successful formation of EV-AAV complexes. EV-AAV2.5 demonstrated enhanced transduction efficiency in HEK293 cells. Furthermore, human chondrocytes exhibited robust transduction with EV-AAV2.5. Future studies will focus on evaluating the impact of neutralizing antibodies in knee joints *in vivo*.

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REFERENCES:
Duan et al. Nanoscale 2021. 2. Maguire et al. Mol Ther 2023. 3. Maguire et al. Mol Ther 2012. 4. Li et al. Circulation 2023. 5. Molina et al. Mol Ther Clin&Dev 2024.

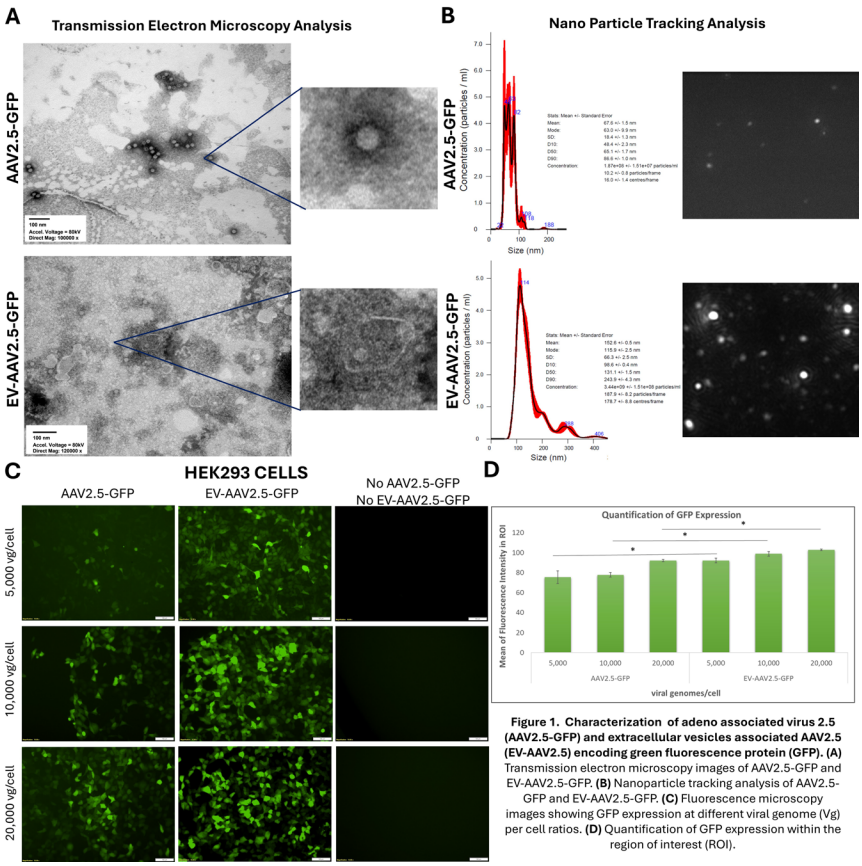

Figure 1. Characterization of adeno associated virus 2.5 (AAV2.5-GFP) and extracellular vesicles associated AAV2.5 (EV-AAV2.5) encoding green fluorescence protein (GFP). (A) Transmission electron microscopy images of AAV2.5-GFP and EV-AAV2.5-GFP. **(B)** Nanoparticle tracking analysis of AAV2.5-GFP and EV-AAV2.5-GFP. **(C)** Fluorescence microscopy images showing GFP expression at different viral genome (Vg) per cell ratios. **(D)** Quantification of GFP expression within the region of interest (ROI).

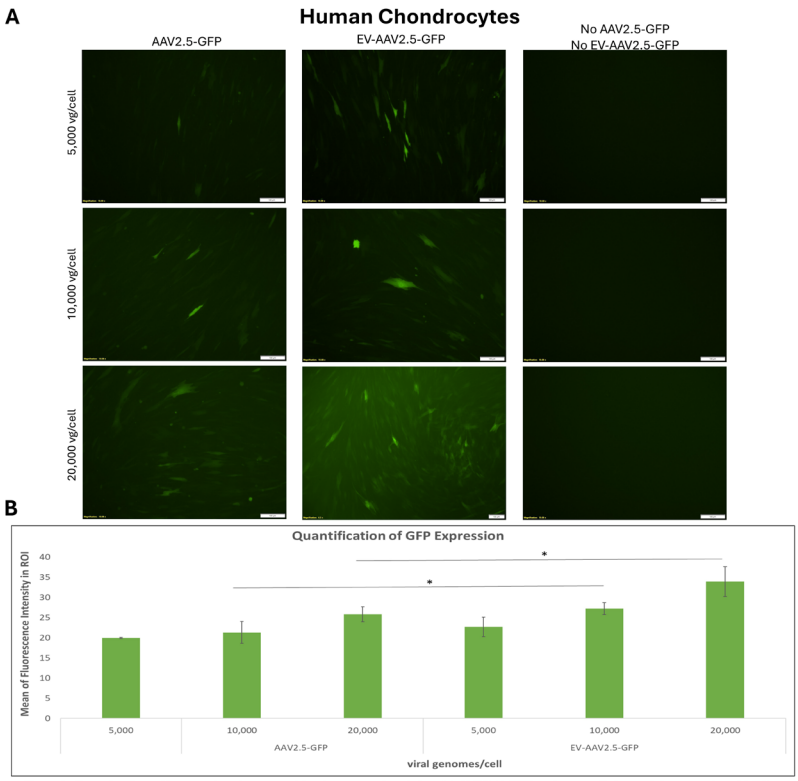

Figure 2. Transduction of human chondrocytes with adeno associated virus 2.5 (AAV2.5-GFP) and extracellular vesicles associated AAV2.5 (EV-AAV2.5) encoding green fluorescence protein (GFP). (A) Fluorescence microscopy images showing GFP expression (Vg/cell: viral genomes/cell). **(B)** Quantification of GFP expression within the region of interest (ROI).

1888 Serotype-Dependent Optimization of Formulation for AAV Vectors Based on Stability against Aggregation

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Adeno-associated virus (AAV) vectors are a major platform of *in vivo* gene therapy due to their safety and long-term gene expression capabilities. AAV vectors can efficiently target specific tissues or organs depending on the serotype. Considering the practical use of AAV vectors as a pharmaceutical drug, the optimization of formulation for different serotypes of AAV vectors could be one of the keys to the successful development of AAV therapeutics. However, the stability of AAV vectors against aggregation has been considered to be limited. For example, AAV2 vectors tend to form aggregates especially at low salt concentration like 50 mM NaCl [1]. Requirement of high NaCl addition exceeding 200 mM limits the range of feasible formulation screenings. In addition, the low stability against aggregation results in a decrease in vector transduction efficiency. Moreover, aggregation of AAV vectors may trigger immunogenic responses, further limiting their clinical application.
As introduced above, previous studies have reported that high ionic strength in AAV2 vector formulations is important for maintaining the stability against aggregation. However, these findings were mainly based on a limited number of serotypes and formulation conditions, and the applicability to other serotypes has not been sufficiently investigated. Moreover, factors such as pH and buffer composition, which may also play a role in maintaining stability against aggregation, have not been comprehensively studied. Therefore, it is essential to design formulations that consider the characteristics of each AAV serotype when developing AAV-based therapeutics.
In this study, we investigated and compared the effects of pH, ionic strength, and buffer types on stability against aggregation across three serotypes of AAV vectors (AAV2, AAV5, and AAV8) to identify optimal formulations. Consistent with the previous report, our results of AAV2 confirmed that high ionic strength is necessary to maintain the stability. In contrast, for AAV5 and AAV8, high ionic strength did not necessarily improve stability against aggregation, and the optimal ionic strength was crucial for stability against aggregation. The responses to pH and buffer composition also varied among serotypes. Furthermore, the 5 cycles freeze-thaw (5xF/T) stability and storage stability at 4ºC of AAV2, AAV5, and AAV8 were evaluated. AAV2 exhibited lower 5xF/T stability compared to the other serotypes. Subsequently, the potential of additives to enhance the stability of AAV2 was investigated, and it was proposed that the addition of sugar could improve its stability. These findings contribute to the serotype-dependent optimization of AAV vector formulations and provide valuable insights for the development of gene therapy products.
[1] F. Wright *et al*., "Identification of factors that contribute to recombinant AAV2 particle aggregation and methods to prevent its occurrence during vector purification and formulation", *Mol. Ther.,* vol. 12, no. 1, pp. 171-178, July. 2005, doi: 10.1016/j.ymthe.2005.02.021

1889 The Impact of Individual Helper Genes on AAV Productivity

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The typical helper plasmid used for adeno-associated virus (AAV) manufacturing contains Adenovirus genes essential for AAV replication and gene expression: VA RNA, E2a and E4. The E2a gene produces DNA-binding protein (DBP), however it has recently become clear that a fourth gene, L4 22/33k, encoded on the opposite strand of the E2a gene, plays a critical role in AAV production. Furthermore, VA RNA encodes two individual RNA species, VA RNAI and VA RNAII, and the E4 gene contains a number of unique open reading frames (orfs). In the pursuit to optimize the helper sequence, each helper gene was split into four individual plasmids: VA RNA, DBP, E4 and L4 22/33k.

It was determined that E4orf6 alone was sufficient, but the addition of E4orf6/7 was optimal for AAV productivity. Similarly, L4 22k expression alone was sufficient but addition of L4 33k was beneficial. Lastly, VA RNAI was the major contributor to VA RNA function and VA RNAII plays a minor role.

Removal of each of the four helper gene plasmids had a unique impact on vector genome (VG) and capsid productivity. A lack of DBP was the most impactful at decreasing genome productivity (86x) while VA RNA was the least impactful (5x). 22K was the most effective at decreasing capsid productivity (30x) while Orf6/7 showed minimal impact on capsid productivity. The absence of DBP and Orf6/7 independently resulted in stronger 22K expression shown by western blot. Furthermore, the input of each helper plasmid was varied to identify the optimal level of each component.

These studies led to a better understanding of how the individual helper genes impact AAV production. This allows for the elimination of unnecessary portions of the helper sequence and provides insight to enable an engineered helper plasmid that produces the desirable expression of each component to facilitate optimal AAV production.

1890 Expedited Quantification of AAV Titers Using a Single-Wash ELISA Assay

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Adeno-associated viruses (AAVs) serve as essential vectors in gene therapy and biomedical research, requiring precise and efficient titer quantification methods to ensure experimental consistency and therapeutic success. This study presents optimized single-wash Enzyme-Linked Immunosorbent Assay (ELISA) assays for rapid, reproducible quantitation of AAV titers, for serotypes 2, 8, and 9, respectively.

The single-wash protocol is a key attribute of these assays, which minimizes hands-on time and procedural complexity while preserving analytical rigor. The streamlined workflow is completed within 90 minutes, delivering significant time savings compared to conventional methods, enabling compatibility with high-throughput automation systems. Validation studies demonstrated a high degree of concordance with quantitative PCR (qPCR) titer data, underscoring its reliability.

The assays employ highly specific antibodies detecting AAV capsid proteins for accurate viral particle-capture. A horseradish peroxidase (HRP)-conjugated AAV detection antibody allows for signal generation through enzymatic activity. Empty AAV particles of the respective serotypes with known titers are used to create the standard curve to be used for quantitation. This approach achieves a linear detection range of 10^7 to 10^9 vp/ml and allows for a limit of detection (LOD) of 1.10×10^7 vp/ml and a limit of quantitation (LOQ) of 3.63×10^7 vp/ml. The assays provide a high level of consistency, with inter-assay and intra-assay coefficients of variation (CV) below 20% and 10%, respectively.

These single-wash assays offer a rapid, sensitive, and reproducible method for AAV titer quantification. Its optimized protocol and validated accuracy make it a valuable tool for advancing AAV-based gene therapy research and production, addressing the field's growing demand for efficient and reliable methodologies.

1891 Correlation between Deamidation and Structure of Adeno-Associated Virus Vectors

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As one of the most used platforms for gene therapy, recombinant adeno-associated virus (rAAV) vector has been developed and several critical quality attributes (CQAs) of rAAV are directly or indirectly related to the therapeutic product. Recently, chemical modifications of rAAV, one of the potential CQAs, have been studied to reveal the

relation to the safety and efficacy of the vector. Among the chemical modifications, previous studies reported that the deamidation of certain amino acids was related to the reduced transgene expression or potential cause of immunogenicity.
In this study, a workflow of analytical anion exchange chromatography (AEX) was established to monitor the deamidation level of rAAV, which was confirmed by peptide mapping using liquid chromatography tandem mass spectrometry (LC-MS/MS). As a result, we identified at least 2 groups of empty particle (EP) with different behaviors in AEX from the same batch of in-house produced rAAV.
Accelerating storage test was also carried out, with multiple biophysical methods applied to characterize the peaks in AEX of accelerated samples, and led to further identification of the correlation between deamidation and structure of rAAV particles. This study will provide important information that can be used for analytical development of rAAV characterizations by AEX as well as for the process development using preparative AEX.

1892 Advancing Gene Therapy: Stabilization of Viral Formulations with Recombumin® Human Albumin

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Viral Vaccines, and Viral Vector preparations, such as Adeno-associated viruses (AAVs), in gene therapy face a major challenge: maintaining stability in clinical settings because degradation above -80°C compromises biological activity. Our high-quality recombinant human albumin (rHA) Recombumin® effectively addresses this issue by stabilizing AAVs, preserving their integrity during storage, transport, and administration.

As a multifunctional excipient, albumin offers benefits such as preventing surface adsorption, minimizing aggregation, protecting against mechanical stress, and acting as a potent antioxidant. Recombumin® distinguishes itself from serum-derived albumin and other commercially available rHA's with its high purity and low aggregation profile, ensuring reliable viral stabilization by maintaining structural integrity and preventing degradation. This enables more flexible storage options, extends product shelf life, and reduces waste.

The use of high-quality Recombumin® in combination with specific anions and excipients in an AAV formulation, yields enhanced AAV stability, which should lead to more effective gene therapy treatments. Furthermore, we expect the enhanced stability to result in higher intact viral titers and reduced dosing requirements, thereby significantly lowering the costs of developing and delivering viral-based therapies and increasing their accessibility.

Insights into the role of Recombumin® in AAV formulations will be showcased, highlighting its potential to transform the field of gene therapy.

1893 Human iPSC-derived Cells as a Platform to Determine AAV Transduction Efficiency

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Introduction: In vitro cellular models are a centerpiece for understanding, modifying, and quantifying the complexity of gene delivery. Induced pluripotent stem cells (iPSC) provide the ability to interrogate biologically relevant cell types *in vitro*. These cell types are compatible with various gene targeting approaches, particularly adeno-associated viral (AAV) vectors. Defining AAV transduction permissibility across different iPSC-derived cell types is a necessity. Quantifying the effects of viral vector serotype, multiplicity of infection (MOI), promoter, media, and assay timing in a human-focused model system is possible with iPSC-derived cells. These specialized cell types offer a major advancement for AAV studies of gene delivery.

Methods: Commercially available cryopreserved human iPSC-derived cell types from FUJIFILM Cellular Dynamics, Inc. (FCDI), culture media and supplements were used according to manufacturer's recommendations. Cells used in this study included: iCell Cardiomyocytes[2], 01434, iCell Hepatocytes 2.0, 01434, iCell Retinal Pigment Epithelial Cells, 01279, iCell Astrocytes, 01434, iCell Astrocytes 2.0, 01279, iCell Pericytes, 01279, iCell Brain Microvascular Endothelial Cells, 01279, iCell Motor Neurons, 01279, and iCell Sensory Neurons, 01279. With Promega, a NanoLuc®-HaloTag® Dual Reporter System for AAV vectors was generated. AAV viruses were manufactured by VectorBuilder. Serotypes examined were AAV1, 2, 3, 4, 5, 6, 7, 8, 9, rh10, and PHP.eB, built with a CMV or SYN1 promoter to drive gene expression. The panel of AAV reagents was used to transduce cells at MOI ranging from 10 to 10^6. If transduction was successful, the expressed NanoLuc® luciferase produced a luminescent signal in the presence of substrate with signal intensity proportional to AAV transduction efficiency. AAV-positive cells could be quantified by imaging or flow cytometry when cells were labeled with Janelia Fluor® HaloTag® 646 Ligand post-transduction. Kinetic assays were performed over 48-72 hours on a BMG Labtech CLARIOstar. In collaboration with FUJIFILM Diosynth Biotechnologies (FDBT), the transfection efficiency of AAV serotypes 1, 2, 4, 5, 6, 8, and 9 was evaluated and CAG and CMV promoters driving GFP was compared. AAV viruses for this part of the study were manufactured at Vector BioLabs. Cells were cultured in 96-well plates with empty AAV vector viruses as negative controls and BacMam GFP as positive transduction control where appropriate. MOI of 100K was the maximum concentration of virus tested. The Celigo S Image Cytometer from Nexcelom and a CytoFLEX flow cytometer from Beckman Coulter were used to quantify GFP expression in different cell types.

Conclusions: As a model to evaluate AAV serotype transduction efficiency, iCell products are a great preclinical system. Cost effective and systematic evaluation of recombinant AAV efficiency variables for any given cell type is recommended. Commercially sourced cryopreserved cells are an ideal reagent for use in these studies because of their scale of manufacture and functional consistency. AAV material handling and storage is critically important and can significantly affect transduction efficiency. Lastly, combining iPSC-derived cells and AAV technology to dissect cellular diseases *in vitro* has the potential to offer a robust method for potency or material release.

1894 Enhanced Gene Transfer to Brain Parenchyma via Next-Generation AAV5 Capsids

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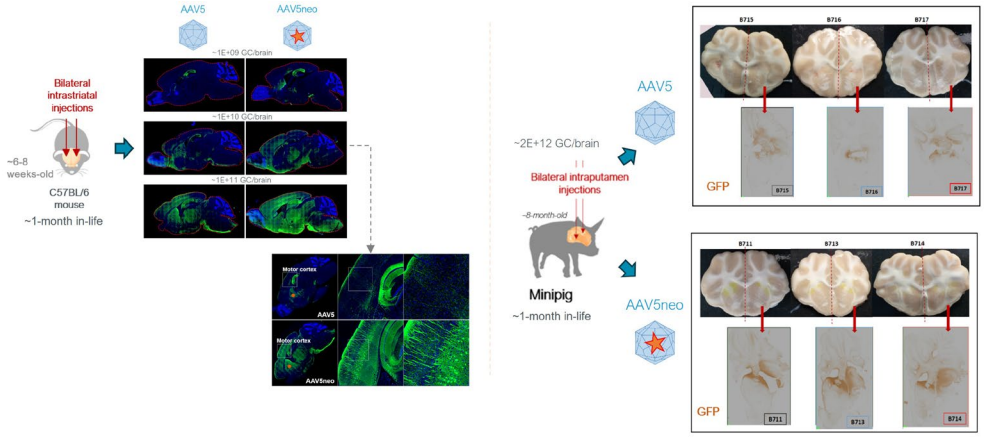
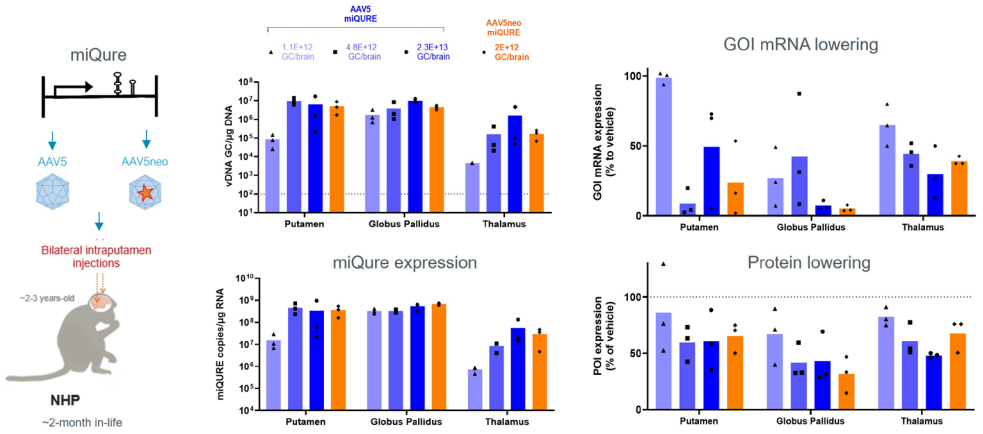
Gene therapies for brain disorders hold great promise but face significant challenges in efficiently accessing the brain parenchyma due to inherent physiological barriers. To overcome these obstacles, intracranial delivery has emerged as a preferred approach to enable precise targeting of brain regions while minimizing therapeutic material loss.

Adeno-associated virus (AAV) vectors are pivotal in intracranial gene delivery, offering safe and long-lasting transgene expression after a single administration. Among natural AAV capsid serotypes, AAV5 stands out for its strong safety profile, low immunogenicity, and proven efficacy in clinical trials, establishing it as a leading choice for therapeutic gene delivery in brain disorders.

Building on AAV5's strengths, a novel AAV5-based capsid variant has been developed and thoroughly characterized. This next-generation capsid, designed to overcome innate physiological barriers, demonstrates enhanced transduction efficiency and broader spread in rodent, minipig, and non-human primate (NHP) models. In NHP studies, it achieves potent therapeutic transgene delivery at significantly lower doses than the original AAV5.

These findings underscore the transformative potential of this advanced capsid for central nervous system gene therapy, paving the way for safer, more effective treatments for brain disorders.





1895 Rewriting Tropism: Engineered AAV-Kingfisher Capsids show Superior Oligodendrocyte Targeting and Spread

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Gene therapy for oligodendrocytes shows great promise in treating demyelinating disorders such as multiple sclerosis and leukodystrophies. However, efficient and targeted delivery of therapeutic genes to these specialized glial cells remains a significant challenge due to the complexity of the central nervous system (CNS) and the need for precision in targeting specific cell types. rAAVen Therapeutics has developed an innovative solution with the rAAptr (rational AAV peptide research) platform, which utilizes the rational design of adeno-associated virus (AAV) capsids to create tailored gene delivery vectors for cell-specific applications. The rAAptr platform employs a rationally designed library of peptide inserts within the AAV capsid, generating a vast array of AAV variants. This approach enables the identification of highly efficient AAV variants in a single round of screening, significantly reducing the number of animals required compared to traditional directed evolution methods. These variants are subsequently screened for cell type-specific transduction by sequencing expressed DNA barcodes from the viral transgene, enabling the identification of AAV capsids that are optimally suited for targeting specific cell types within the brain.
In this study, we applied the rAAptr platform to develop AAV capsids specifically targeting oligodendrocytes. By

designing a library enriched for oligodendrocyte-targeting peptides, we observed a significant shift in transduction profiles, favoring the white matter regions of the brain where oligodendrocytes are predominantly located. Additionally, we identified capsid variants capable of dispersing from the ventricular system to adjacent brain areas, which is crucial for widespread gene delivery within the CNS. To pinpoint variants with a high affinity for oligodendrocytes, we utilized fluorescence-activated cell sorting (FACS) to separate oligodendrocytes from non-oligodendrocytes, followed by sequencing of the viral barcodes. With this approach, we could identify and validate AAV capsids with robust oligodendrocyte specificity. Among these, novel Kingfisher variants 10/28/193/249/D2 demonstrated superior performance compared to parental AAV2 and Olig001, the current best-in-class oligodendrocyte-targeting AAV. Kingfisher variants demonstrated enhanced tropism for oligodendrocytes, along with the ability to spread and effectively transduce cells in white matter regions of the brain, both after direct brain injection and following intracerebroventricular (ICV) delivery.
Our findings underscore the efficacy of the rAAptr platform in designing AAV vectors with precise cell type specificity. The identification of AAV-Kingfisher variants represents a significant advancement in the development of more effective and targeted gene therapies for oligodendrocyte-associated disorders. This research not only provides a pathway for treating demyelinating diseases but also highlights the potential of rational design approaches in creating next-generation gene therapy vectors tailored for specific therapeutic needs, all while minimizing the use of animal models.

1896 Characterization of Receptor-Targeted Blood-Brain Barrier Penetrant AAV Capsids

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Blood-brain barrier penetrant adeno-associated virus (AAV) capsids have the potential to transform the treatment of neurological disorders by enabling efficient intravenous delivery of genomic medicines to the central nervous system. We have previously described SIFTER, an *in vivo* library screening platform for the identification of AAV capsids that mediate widespread central nervous system (CNS) delivery. We have conducted SIFTER screening campaigns in non-human primates to identify engineered capsids that target the CNS after intravascular (STAC-BBB) or cerebrospinal fluid (STAC-102 and STAC-103) administration. A complementary strategy for engineering AAV capsids is to conduct *in vitro* screens for capsids that interact with receptors on the human blood-brain barrier. Here, we report the characterization of three receptor-targeted AAV capsid families. Each of these capsid families targets a distinct human blood-brain barrier receptor. These receptor-targeted capsid families are unrelated to STAC-BBB, but likewise have the potential to mediate widespread CNS delivery. Multiple lead capsids were selected from each of the three families for further characterization as individual capsids. The capsids were additionally tested in a barcoded pool format with dual ranking of expression from the neuron specific human synapsin 1 and ubiquitous human U6 snRNA promoters. Lead capsids targeting one of the receptors exhibited enhanced transduction in cells expressing the human, macaque, or mouse receptor orthologs. A subset of capsids targeting the other two receptors exhibited cross-species enhancement of transduction in cells expressing the human or macaque receptor orthologs. In the progression from preclinical to clinical testing, cross-reactive receptor engagement in primates is a critical translational consideration. In addition to evaluating capsid potency, capsid manufacturing yield in suspension HEK293 cells was benchmarked against the parental AAV serotype. These receptor-targeted capsids are being evaluated in vivo to assess BBB crossing efficiency and tropism for CNS cell types.

1897 Harnessing Human Brain-Specific Carbonic Anhydrase IV for Targeted AAV Gene Delivery

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Gene therapy delivery to the brain faces significant challenges, particularly penetration of the blood-brain barrier (BBB). While clinically approved adeno-associated virus (AAV) therapies using natural serotypes show promise, their applications remain limited due to poor targeting specificity, high dosing requirements, and associated safety concerns. Although directed evolution has produced enhanced AAV capsids in animal models, inter-species differences complicate translation to human therapies.

Recent in-vitro efforts have engineered AAVs to target specific BBB transcytosis receptors, including murine-specific LY6A (Huang et al., PLOS Biology, 2023) and the widely-expressed TfR1 for human brain gene therapy (Huang et al., Science, 2024). Our work advances this paradigm by targeting the human BBB receptor carbonic anhydrase IV (CA4), which exhibits a more central nervous system (CNS)-focused expression pattern than previously established BBB receptors.

Through in vitro pulldown and cell transduction screening, we identified a pool of human CA4-targeting AAVs. While some AAV variants demonstrated strong CA4 affinity in SPR assays and CA4-enhanced cell infectivity, they did not show significantly improved BBB crossing, as evaluated in transwell models and human CA4-expressing mice.

To overcome this challenge, we further developed a "humanized" mouse model for final candidate selection. This approach uses mouse brain endothelial cell-specific AAVs to introduce human BBB receptors—a more flexible and rapid approach than developing transgenic mice. By utilizing AAV2-based vectors for model development, we enabled subsequent AAV9-based library screening with minimal neutralizing antibody cross-reactivity. Importantly, our approach preserves normal immune competency, unlike immune-deficient mouse models which cannot properly replicate AAV-immune system interactions during brain transduction. Using this workflow, we successfully identified AAV variants with efficient BBB transcytosis and robust brain transduction from our pool of human CA4 binders.

Overall, our work advances our understanding of the complex relationship between BBB receptor binding and efficient barrier crossing. By employing a mechanism-driven approach to engineering that combines selections in receptor binding assays and “humanized” mouse models, we identified AAVs that effectively engage a human BBB transcytosis system. This strategy offers a clear path toward developing more effective and clinically relevant gene therapies targeting the human brain.

1898 Engineering AAV vectors with TfR1-targeting domains for CNS gene delivery in cynomolgus macaques and humanized mice

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The development of adeno-associated virus (AAV) vectors capable of efficiently crossing the blood-brain barrier (BBB) and delivering genes to the central nervous system (CNS) would expand therapies for neurological diseases. AAV vectors that can cross the BBB in non-human primate (NHP) and relevant humanized mouse models have been reported. However, it remains challenging to identify capsids with comparable CNS transduction in multiple animal models due to cross-species differences in cognate BBB receptors mediating transcytosis of AAVs across the brain endothelium. To de-risk CNS-targeted gene therapies using engineered novel capsids, one of our current strategies requires that vector candidates demonstrate adequate CNS transduction and safety profiles in both NHPs and humanized mice.

To engineer CNS vectors with species cross-reactivity, we designed AAV capsids displaying targeting domains able to bind to both human and cyno orthologs of transferrin receptor 1 (TfR1), a clinically validated receptor that mediates transcytosis across the BBB. We previously reported that several AAV capsids engineered using this strategy demonstrate enhanced CNS transduction over AAV9 in humanized TfR1 knock-in (hTfR1 KI) mouse screening and NHP screening. In addition, lead capsids were individually evaluated in hTfR1 KI mice, with one top candidate showing robust BBB penetration and neuronal transduction.

Analysis of the first-generation results revealed an inverse relationship between TfR1 binding affinity and neuronal transduction, informing the design of our second-generation library. This library incorporated targeting domains with diverse TfR1 binding affinities and epitopes, alongside modified first-generation leads informed by cryo-EM structures. Following barcoded capsid library screening in hTfR1 KI mice, lead capsids from first- and second-generation libraries were individually evaluated with a therapeutic transgene in NHPs and hTfR1 KI mice. These vectors show widespread and enhanced CNS transduction compared to AAV9 in both models.

This work establishes a platform for developing TfR1-targeted AAV vectors with cross-species BBB penetration and CNS transduction capabilities. Our current leads show enhanced performance relative to AAV9 in both NHPs and hTfR1 KI mice. We will continue to characterize these capsids in future work, leveraging our initial success, the modular nature of this targeting approach, and our increasing knowledge of capsid design parameters.

1899 Blood-Brain Barrier Crossing AAV Vectors Targeting the Transferrin Receptor Engineered Using Two Different Approaches

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Naturally occurring AAV serotypes have very low brain penetration when administered intravenously due to the blood-brain barrier (BBB). In this work we employed either designed ankyrin repeat proteins (DARPins) or short peptides to engineer AAV vectors that bind human transferrin receptor 1 (hTfR1). TfR1 is expressed at high levels in the brain vasculature and has been shown to effectively shuttle macromolecules bound to it across the BBB and into the brain parenchyma.

In the first approach, we undertook a ribosome display selection campaign to identify Athebody® platform DARPins that bind to hTfR1 with a wide range of affinities. We optimized the DARPin insertion site in AAV9 VR-IV and generated vectors displaying up to 12 DARPin copies per capsid. In the second approach, we used the REGENXBIO NAVIGATE directed evolution platform to identify a capsid with an hTfR1-binding peptide insertion. Capsid binding to hTfR1 was confirmed via an Octet BLI binding assay and transduction of HEK293-hTfR1 cells. Both methods produced AAV titers similar to the control capsid.

We then intravenously administered the DARPin and peptide insertion vectors to transgenic mice expressing hTfR1. Both vector types robustly transduced the brain and spinal cord resulting in greater than 28-fold higher vector genome copies (vg) per cell and greater than 44-fold higher mRNA transcripts in the brain vs control. In the liver, there was greater than 3-fold reduced vg/cell vs control for the DARPin and peptide insertion vectors. Histological analysis revealed that both vectors efficiently transduced regions throughout the entire brain, with the highest transgene expression observed in the thalamus and striatum. In areas of cortex, the two vectors showed prominent differences in cell type transduction, with the DARPin vector exhibiting neuronal tropism and the peptide vector exhibiting tropism for astrocytes in this humanized mouse model.

Both hTfR1 targeting strategies are effective for crossing the BBB and show promise for CNS gene therapy applications. Further work is needed to characterize BBB crossing and cell-type tropism in a large animal model and manufacturability at a larger scale.

1900 Expanding Access to Gene Therapies for Neurometabolic Disorders: A Platform Approach Using TfR1 CapX

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Introduction:
Neurometabolic disorders represent a diverse group of conditions with severe CNS manifestations, many of which are amenable to gene therapy. However, effective AAV-based CNS delivery remains a major translational challenge, particularly for large or systemically progressive disorders requiring broad brain distribution. TfR1 CapX, an engineered transferrin receptor-targeting AAV capsid, has demonstrated high-efficiency BBB penetration and robust CNS transduction after intravenous administration, positioning it as a versatile platform for neurometabolic gene therapies.

Finalized and draft FDA guidance as well as INTERACT feedback that Apertura Gene Therapy has recently received support the use of alternative models when scientifically justified, particularly in cases where non-human primate (NHP) studies lack pharmacological relevance. Based on our regulatory experience in genetic epilepsies, preclinical data, and direct FDA engagement, we outline a streamlined pathway for developing neurometabolic gene therapies with TfR1 CapX—one that does not require NHP studies.

Methods:
We leveraged preclinical biodistribution, transduction, and therapeutic efficacy data to assess TfR1 CapX's suitability for neurometabolic disorders. Key regulatory precedents and direct INTERACT feedback were analyzed to define a translationally viable, NHP-free approach.

Results:
TfR1 CapX achieves widespread CNS transduction following intravenous administration in human transferrin receptor knock-in (B-hTfR1) mice, a commercially available model that provides robust pharmacokinetic and safety data. INTERACT feedback confirmed the pharmacological irrelevance of NHPs, as TfR1 CapX does not efficiently bind NHP transferrin receptors. These findings, aligned with prior regulatory decisions, eliminate the need for large-scale NHP toxicology studies and significantly reduce costs and timelines.

Conclusion:
TfR1 CapX represents a scalable and regulatory-aligned platform for neurometabolic gene therapies, allowing for efficient clinical translation without reliance on NHP models. With a clear FDA-supported preclinical pathway, robust CNS delivery, and established translational feasibility, TfR1 CapX is well-positioned to advance next-generation gene therapies for neurometabolic disorders.

1901 Novel AAV capsids that bind human transferrin receptor (TFRC) demonstrate widespread and preferential CNS tropism in TFRC-KI mice after low dose systemic dosing

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Adeno-associated viral (AAV) vectors capable of delivering genetic cargo across the blood-brain barrier (BBB) can enable drug development for diseases of the central nervous system (CNS). An approach to potentially de-risk translatability of potential CNS AAVs is to identify capsids that engage receptors with known ability for enabling human BBB penetration, such as the transferrin receptor (TFRC). We developed a high throughput screening platform to identify capsids that can bind human TFRC protein in vitro and penetrate the BBB via TFRC in vivo. We first screened a library of AAV9 capsids with random 7mer peptide insertions for binding the human TFRC extracellular domain immobilized on beads. Clustering these binding hits identified multiple conserved sequence motifs. A deep learning generative AI framework was applied to the binding data to predict additional TFRC hit capsids. A library that combined the observed binding hits with the AI-predicted TFRC hits was used to dose transgenic mice harboring TFRC receptor on the BBB (TFRC-KI) intravenously. Brain samples from these mice revealed a large number of hit motif groups with in vivo activity. Interestingly, the best performing capsids were AI-predicted hits. While multiple capsids demonstrated >100-fold higher RNA expression in the CNS vs AAV9, three capsids with favorable tropism and manufacturability attributes were individually packaged with reporter cargo for dosing TFRC-KI mice at 2.5e13 vg/kg. We observed 20- to 70-fold higher vector genomes and 140- to 270-fold higher mRNA expression, compared to AAV9. These expression levels translated to 90% neuronal transduction across cortical and deep brain regions. In addition to enhanced CNS tropism, one of our TFRC-capsids demonstrated 10-fold lower vector genome levels in the liver. Based on these data, we set out to determine the dose-to-biodistribution relationship for our lead capsids across a 100-fold dose-range within TFRC-KI mouse CNS and non-CNS compartments. Comparison of in vivo potency between our first generation TFRC-capsids vs published comparators is being analyzed and will be shared. Mechanistic characterization of our lead capsids, as well as functional data in a human BBB model system, will also be presented. In summary, we will present our strategy to identify and validate AAVs that engage human TFRC and thus have higher potential for translatability to neurological applications in the clinic.

1902 Second Generation Human Transferrin Receptor Targeted AAV Capsids With Enhanced CNS Tropisms and Liver Detargeting In Vivo

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Numerous genetic diseases that affect the central nervous system (CNS) require gene therapies that are efficiently delivered to cells throughout the CNS. Adeno-associated viruses (AAV) have been engineered to deliver genes CNS-wide after systemic injection. However, several engineered AAVs use mechanisms of action that are unknown or not well validated for transcytosis across the BBB. Therefore, we engineered AAV9 to bind human Transferrin Receptor (TfR1), a protein that is highly expressed on the blood-brain barrier (BBB) and well validated for receptor-mediated transcytosis of biologics in humans. The first generation capsid, BI-hTFR1, is actively transported across the BBB and provides CNS-wide transduction in humanized TFRC knock-in mice (Huang & Chan et al., Science, 2024). We developed BI-hTFR1v2, a next generation capsid with improved CNS tropism and reduced liver transduction. When intravenously injected at 2e12 vg/kg into TFRC KI mice, BI-hTFR1v2 demonstrated 2-fold and 175-fold increased AAV genome biodistribution to the brain, alongside 4.5-fold and 3-fold reduced biodistribution to the liver compared to BI-hTFR1 and AAV9, respectively. Remarkably, the enhanced distribution to the CNS resulted in 8-fold and 1300-fold increases in luciferase reporter signal in whole brain homogenates as compared to BI-hTFR1 or AAV9, respectively. Despite the low dose, BI-hTFR1v2 transduced 80% and 55% of neurons in the cortex and striatum, respectively. In parallel studies, we have generated a version of BI-hTFR1v2, BI-hTFR1v2 NAbE, that is capable of evading neutralization by more than 40% of the individual human donor sera that neutralized AAV9 and AAV5 while maintaining the enhanced CNS tropism of BI-hTFR1v2. These capsids can be produced at yields comparable to AAV9 and purified using commonly used affinity resins. They are currently being advanced toward the clinic as part of an SCGE-funded epigenetic editing therapy for prion disease (Neumann et al., Science 2024). Our work establishes these human TfR1-targeting capsids as promising vectors for human CNS gene therapy.

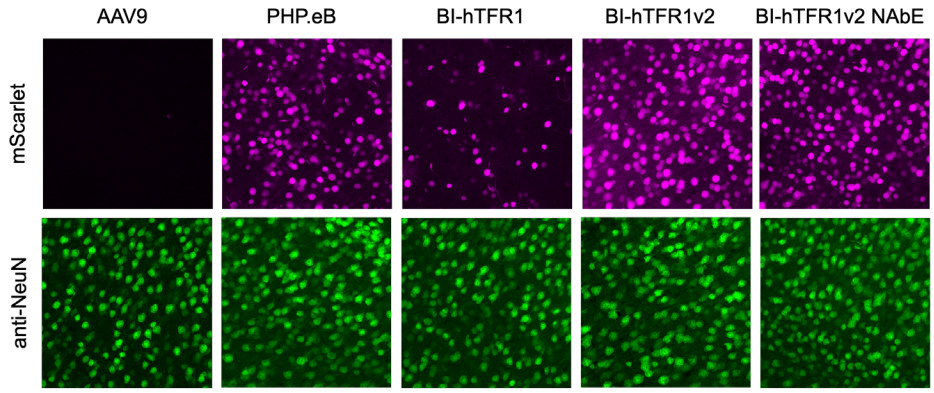

Figure 1. TfR1-targeted AAV capsids achieve efficient CNS-wide transgene expression in human *TFRC* knock-in mice. AAV9, PHP.eB, BI-hTFR1, BI-hTFR1.2 or BI-hTFR1v2 NAbE were intravenously injected into adult *TFRC* knock-in mice at 2.0e12 vg/kg. Representative image of the native mScarlet fluorescence in brain sections stained with anti-NeuN in cortex.

1903 Combining In Vivo and In Vitro Screening Approaches for Identification of BBB-Crossing Capsids Across Species

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In recent years, recombinant adeno-associated virus (AAV) vectors have become a pivotal delivery vehicle for in vivo gene therapies. Despite emerging clinical success across multiple therapeutic areas with vectors with increased efficiency and / or specificity for various tissues, targeting the central nervous system (CNS) remains a challenge. The blood-brain-barrier (BBB) has been found to significantly hinder efficient gene transfer into the CNS. Overcoming delivery hurdles like the BBB, will require a combination of identifying novel AAV capsid variants, determining optimal routes of administration (RoA) and understanding the mechanisms underlying cross-species differences.

Here we describe the implementation of a BBB crossing AAV capsid variant screening pipeline, in which specific capsids are uniquely barcoded for the detection and quantification of physical and functional transduction in a high throughput manner. Applying a capsid pool enables us to accurately screen multiple variants in a single experiment more rapidly compared to traditional directed evolution approaches. This approach allows us to generate a quantitative biodistribution for the different AAV capsid variants based on association with their respective barcode. This pooling approach is cost and time efficient, with the additional benefit of a reduction in the number of animals required to generate reliable datasets. Utilizing this pipeline, we have directly compared the performance of natural serotypes and benchmarks in a single pool, in mouse versus non-human primate (NHP) and using two routes of administration: systemic intravenous delivery (IV) and intra-cisterna magna (ICM) injections.

Further, to evaluate the translatability between in vitro and in vivo for crossing the BBB, we utilized the two-channel Emulate chip platform. Here, we have established a comprehensive, microfluidic model of the neurovascular unit comprising four neural cell types in the top channel and brain microvascular endothelial cells in the bottom channel to mimic the human BBB. Analyses of different parameters for characterization of the BBB model, including barrier function and immunofluorescence staining, have been implemented. After application of

our AAV pool to the BBB chip model, we observed robust transduction with variants known for animal CNS-tropism and their ability to cross the BBB.

In summary, the integration of in vivo and in vitro screenings across different species may facilitate the identification of capsids that can cross the BBB and enhances the potential for translation to human studies.

1904 Engineered AAV capsids that target a novel human brain endothelial receptor achieve robust transduction in non-human primate central nervous system after intravenous dosing

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Engineered adeno-associated viruses (AAV) can deliver genetic cargo to central nervous system (CNS) by crossing the blood-brain barrier (BBB) after intravenous dosing in preclinical animal models. However, the therapeutic utility of these engineered capsids has been complicated by a lack of cross-species tropism. To address this risk, we identified engineered capsids that bind both the human and non-human primate (NHP) orthologs of a novel protein (referred to as Receptor-Y) that is highly expressed in brain endothelium of the mouse, NHP, and human. We first screened a library of AAV9 capsids with random 7mer peptide insertions for binding the extracellular domain of human or NHP Receptor-Y immobilized on beads. Clustering these binding hits identified many conserved sequence motifs. A library of ~13K Gen1 binding hits was screened in NHPs using intravenous dosing and revealed that a minority of binding hits were detectable in the CNS. A Gen2 library of ~50K capsids was generated using SAR of the Gen1 in vivo active sequence motif families and then used for a second round of screening in NHPs. In comparison to parental AAV9, several Gen2 capsids achieved >1000-fold mRNA expression across NHP cortical and deep-brain regions. Mechanistic characterization of our lead Gen2 capsids will be presented. In summary, we will present a capsid development strategy with a higher potential for translatability to neurological diseases in the clinic due to cross-species binding of a novel brain endothelial receptor.

1905 Optimized CNS-targeting AAV Capsids with NHP and Human Cross-reactivity

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Overcoming the blood-brain barrier (BBB) represents the most promising approach for achieving widespread therapeutic gene delivery to the central nervous system (CNS). Directed evolution of peptide insertion AAV libraries has produced a variety of novel capsids capable of crossing the BBB and transducing CNS cells in mice and non-human primates (NHPs). Using this method, we previously developed our Generation A (GenA) CNS-targeting AAV capsids, which demonstrated safe and enhanced CNS gene delivery compared to AAV9 following systemic delivery in NHP.

Here, we focus on establishing human translatability as well as cross-species activity optimization of this capsid family. The key to unraveling the uncertainty around human-translatability lies in identifying cognate BBB receptors

followed by functional comparison of capsid activities against receptor homologs. To this end, we identified 'Receptor X', a membrane protein expressed in brain endothelial cells, as the key receptor mediating transcytosis of GenA capsids. The lead GenA capsid binds to both human and cynomolgus Receptor X homologs.

To identify additional CNS targeting capsids that specifically bind to Receptor X, we designed a unique targeted optimization library based on binding to Receptor X. We then screened this library and identified several Generation B (GenB) capsids that transduce CNS in both NHP and humanized mice. These GenB capsids demonstrate widespread and enhanced CNS transduction compared to AAV9.

In summary, our work highlights the potential of directed evolution and targeted optimization to develop AAV capsids with improved human translatability and cross-species activity. The identified GenB capsids represent another group of promising candidates for CNS-targeted gene therapy, with significant implications for future clinical applications.

1906 AAV-ShDs: Liver-detargeted AAV Capsids with Strong Cross-species Lung Tropism and High BBB Activity in Non-human Primate and In Vitro Human Models

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The safety and efficacy of gene therapies rely on targeted transgene expression while minimizing off-target effects. The adeno-associated virus-9 (AAV9) serotype is widely used for its strong gene expression across multiple organs, including liver, brain, lung, heart, muscle, and kidney. Utilizing our ATHENA capsid engineering platforms, we selected and evolved AAV capsids, and discovered novel variants with high DNA enrichment in lung, termed AAV-ShDs, which also significantly detargeted liver and other major organs in C57BL/6J and B6C3 mice. The lead capsid, AAV-ShD, showed approximately 500-fold higher DNA levels in lung of C57BL/6J mice compared to AAV9. AAV-ShDs also demonstrated markedly higher RNA enrichment in the lung of B6C3 mice compared to AAV9. The AAV-ShD family specifically targets alveolar type II (AT-II) cells in lung, with VR-IV and VR-VIII regions contributing to lung tropism. To assess translational potential, we conducted similar screenings in non-human primates (Cynomolgus macaques) and identified the same AAV-ShD capsids with high lung enrichment. These capsids showed over 100-fold DNA enrichment and about 22-fold RNA enrichment in lung of macaques compared to AAV9. Additionally, AAV-ShDs efficiently traverse the blood-brain barrier (BBB) in macaque, and AAV-ShDs achieved 385-fold higher DNA level and 8-fold higher RNA level compared to AAV9, based on barcoded next-generation sequencing analysis. In the spinal cord, DNA level was 25-fold higher, and RNA level was 28-fold higher with AAV-ShDs compared to AAV9. In a cellular transwell model, AAV-ShD exhibited enhanced BBB penetration, leading to efficient transduction of human microglia, astrocytes, and neurons. Furthermore, AAV-ShDs successfully delivered transgenes to cultured primary human brain microvascular endothelial cells, pericytes, hippocampal astrocytes, and cortex microglia. Notably, in microglia-containing cerebral organoids derived from human iPSCs, AAV-ShDs demonstrated higher transduction activity than AAV9 in various neural cell types. In conclusion, we identified a novel set of AAV-ShD capsids capable of cross-species lung targeting. Moreover, AAV-ShDs crossed the BBB and efficiently transduced neural cells in both non-human primates and in vitro human cell models. The high DNA levels observed in AAV-ShD-transduced tissues/cells suggest potential for long-term transgene expression at lower dosages, making these capsids promising templates for further engineering and clinical applications.

1907 Cross-species Compatibility of Novel AAV-R2e-MAC to Cross Blood-brain Barrier and Transduce Myeloid Cells in the Central Nervous System

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Myeloid cells, including resident microglia and central nervous system (CNS)-associated macrophages, play crucial roles in both healthy and diseased conditions within the CNS. Gene therapy targeting myeloid cells holds significant promise for treating CNS diseases and injuries. Extensive research has identified AAV9, AAV8, and AAVrh10, along with their derivatives, as capable of crossing the blood-brain barrier (BBB) in both neonatal and adult animals. These serotypes, classified as AAV-B, are known for their high efficiency in transducing neurons, astrocytes, oligodendrocytes, and blood vessels. However, these serotypes exhibit limited or no ability to transduce brain myeloid cells in vivo. Conversely, several AAV serotypes, such as AAV1, AAV2, AAV5, and AAV6, have demonstrated potent tropism for myeloid cells (AAV-M). To develop novel AAV serotypes (AAV-BM) that combine the BBB-crossing ability of AAV-B with the myeloid cell transduction capability of AAV-M, we performed epitope insertional hybrid screening. This involved transferring a BBB-crossing epitope from AAV-B to the variable region VIII of the AAV-M capsid. Among the eight AAV-M serotypes validated in our preliminary studies, we selected AAV2 for initial screening due to its high transduction efficiency in human, monkey, and mouse primary microglia, well-documented study history, and natural binding to heparan sulfate proteoglycans (HSPGs) on many cell types. AAV-CAP-MAC, AAV-9P31, AAV-PhP.eB, AAV-cc47, AAV-CPP16, AAV-CPP21, AAV-Pal1, and AAV-MDV1A were chosen for the first trial due to their efficiency in crossing the BBB at relatively low dosages in multiple species. This trial identified R2-MAC as the primary AAV-BM, derived from AAV-CAP-MAC and natural AAV2. By introducing quadruple tyrosine-mutants (Y272F, Y444F, Y500F and Y737F) and one threonine mutant (T491V) to the R2-MAC capsid, we created an enhanced version, R2e-MAC, which showed higher transduction efficiency in cultured microglia. To test R2e-MAC, we packaged CMV-EGFP using the AAVone system and administered doses ranging from 1×10^{10} to 5×10^{12} genome copies (GC) per mouse intravenously (IV). R2e-MAC demonstrated dose-dependent BBB crossing and neural cell transduction across various mouse strains, including C57BL/6, BALB/c, and FVB/N. The capsid also proved effective in rhesus macaques (3×10^{13} GC/kg, IV) and human organoid BBB models. Both male and female C57BL/6 mice showed comparable transduction efficiency, while lipopolysaccharide (LPS) treatment robustly enhanced neural cell transduction. Bioinformatics analysis of single-cell RNA sequencing from fluorescence-activated cell sorting (FACS)-sorted EGFP-positive cells of the C57BL/6 mouse brains ($5x10^{12}$ GC, IV) revealed 30% myeloid cells, 64% endothelial cells, 3.9% astrocytes, 0.6% mature neurons, and 1.4% oligodendrocytes. Hybridization Chain Reaction RNA Fluorescence In Situ Hybridization (HCR-RNA-FISH) validated the transduction in TMEM119 and HexB-positive microglia. In conclusion, the hybrid AAV-R2e-MAC capsid outperforms the parent AAV-CAP-MAC in BBB crossing and AAV2 in myeloid cell transduction across different species, offering a potential gene therapy approach for CNS diseases and injuries.

1908 Efficient Neuronal Transduction of AAV2-derived CereAAV.YN Vector in Cynomolgus Macaque Brain without Liver Transduction by Systemic Injection.

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Recombinant adeno-associated virus vectors (AAVs) have been widely used as vehicles to deliver gene of interest for both research and therapeutic purposes. Zolgensma a treatment for spinal muscular atrophy, was approved by the US Food and Drug Administration and numerous pre-clinical and clinical studies have been conducted using AAV2 and AAV9 serotypes to target brain diseases via local and systemic administration. In particular, AAV9 have been frequently used for gene delivery into the brain including central nervous system (CNS), and several AAV9 derivatives such as AAV-PHP.eB, have been identified to transduce CNS efficiently. On the other hand, it has been reported that an AAV2 variant, designated AAV-BR1 efficiently transduced the Blood Brain Barrier (BBB) - associated endothelial cells by systemic administration. However, there is still no AAV2 variant reported to

transduce CNS across the BBB efficiently. To overcome the limitations of AAV2, we addressed to develop a novel brain-targeting AAV vectors based on AAV2 serotype. To date, we have developed AAV2 capsid mutant CereAAV.O, which has shown efficient gene transfer to the brain by intravenous administration in mice and non-human primate models. Furthermore, we have identified a novel CereAAV mutant (designated as CereAAV.YN) that significantly increases the transduction efficiency to the mouse brain compared to CereAAV.O and AAV9.

In this study, we have evaluated the gene transduction capability of CereAAV.YN vector in non-human primate model (Cynomolgus macaque). The CereAAV.YN vector carrying AcGFP gene as a reporter under the CAG promoter control was injected systemically into two male Cynomologus macaques at a dose of 1e13 vg/kg. After 28 days, various tissues were collected and the gene transduction was evaluated by immunofluorescence assay. As a result, significant AcGFP expression was observed in the brain, spinal cord and heart, but not in the liver. Furthermore, immunostaining analysis showed that CereAAV.YN vector was highly transduced into neurons in the monkey brain, but not into astrocyte, oligodendrocyte, or microglia, similar to previous study in a rodent model. The neuronal transduction of the CereAAV.YN vector in the cerebral cortex occurred in more than 70%-90% of neurons in the various region of the brain. Moreover, the AcGFP expression in the cerebral cortex was higher than in internal brain regions, such as the thalamus and putamen. We also evaluated the toxicity of the CereAAV.YN vector systemic injection. To this end, serum alanine aminotransferase (ALT) activity was measured as a marker of liver injury in this study. The ALT activity was transiently increased in response to AAV injection in both macaques on day 3 after injection, but decreased to normal level on day 7. In addition, the ALT activity was transiently elevated again on day 14 in only one macaque and then decreased to normal level on day 21. Finally, the serum ALT activity showed normal level on day28 in both macaques and liver abnormalities were not observed in necropsy samples. These results indicated that CereAAV.YN vector is a promising tool for both research and clinical studies targeting brain diseases without any liver failure. Currently, further studies are underway to reveal the mechanism of gene delivery across the blood brain barrier by CereAAV vectors.

1909 Fitness Maturation of STAC-BBB Yields Second-Generation Capsid Variants with Enhanced Delivery to the Central Nervous System

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Engineered Adeno-associated virus (AAV) capsids that cross the blood-brain barrier (BBB) have the potential to enable transformative genomic medicines for a variety of neurological disorders. We previously described the identification and characterization of STAC-BBB, a blood-brain barrier penetrant AAV capsid that exhibits widespread neuronal transduction throughout the central nervous system (CNS) and is de-targeted from the liver following intravenous (IV) injection. Here we describe the fitness maturation of STAC-BBB and the identification of second-generation capsid variants with enhanced CNS delivery relative to STAC-BBB. We first generated barcoded capsid libraries that systematically explore the STAC-BBB sequence landscape. These libraries were administered intravenously to non-human primates and next-generation sequencing analysis was conducted to assess the relative level of neuronal and ubiquitous mRNA expression mediated by each capsid variant. Expression levels were analyzed in whole brain slices, punches from key CNS regions, spinal cord, dorsal root ganglia, and peripheral tissues. We additionally compared the level of barcoded mRNA expression versus DNA vector genome delivery within these tissues. Second-generation STAC-BBB variants were identified that exhibit enhanced delivery to CNS tissues, reduced off-target tissue biodistribution, and demonstrated the ability to engage both the non-human primate and human orthologs of the putative STAC-BBB receptor in vitro. In addition to second-generation capsids that exhibit higher neuronal expression compared to STAC-BBB, we also identified variants that exhibit an apparent shift in cellular tropism. These capsids exhibit much higher ubiquitous expression compared to neuronal expression throughout the CNS. The production yield in suspension HEK293 cells was also assessed in preparation for evaluation of individual capsids in vivo. These second-generation STAC-BBB variants have the potential to further improve CNS delivery after intravenous administration of genomic medicines for neurological disorders.

1910 Machine Learning-Driven Engineering of AAV Capsids for Enhanced Blood-Brain Barrier Penetration and Optimized Tissue Specificity

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Adeno-associated viruses (AAVs) are widely utilized as gene therapy vectors due to their safety and long-term gene expression capabilities. However, optimizing AAV capsids for targeted tissue specificity while maintaining high manufacturability remains a key challenge. In this study, we applied machine learning-driven AAV engineering to develop novel AAV capsid variants with enhanced blood-brain barrier (BBB) penetration and improved off-target minimization.

The machine learning approach enabled us to collect high-quality data on packaging efficiency, which was then incorporated into the engineering process. The newly designed AAV variants demonstrated a remarkable increase in BBB penetration efficiency compared to AAV9, as confirmed through in vivo mouse experiments. Moreover, these optimized vectors exhibited excellent tissue specificity, with minimal to undetectable presence in non-target tissues, including the liver, heart, and peripheral nervous system. This specificity ensures a significantly reduced risk of off-target effects, making these AAVs highly suitable for neurological applications.By integrating machine learning-driven design with experimental validation, this study presents a scalable framework for developing next-generation AAV vectors with unparalleled BBB penetration, optimized tissue targeting, and high manufacturability. Our findings highlight the potential of ML-guided AAV capsid engineering to revolutionize gene therapy by overcoming existing delivery limitations and enhancing therapeutic efficacy.

1911 Machine-Learning for AAV9 Mutant-Capsid Screening for both Production and ALPL-Mediated Transduction Efficiency

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We recently identified AAV-capsid variants that show high CNS transduction in rodents and primates, relying on ALPL as their primary surface receptor for transport across the blood-brain barrier. These gene delivery vectors hold significant potential to treat a broad range of CNS disorders. However, their widespread application is constrained by the prevalence of anti-AAV antibodies in a fraction of the human population. This prompted us to further engineer our vectors by removing major antigenic sites on the capsid surface. In so doing, however, two potential problems arose. Data generated from random mutations in a highly constrained AAV9 antigenic area showed that less than 10% of variants assembled into capsids, and of these only a small fraction retained the capacity to infect ALPL-expressing cells in vitro. To mitigate these problems, we took a machine-learning-based (ML) approach. We developed ML models to discriminate between helpful and harmful mutations in terms of both capsid stability ($r = 0.95$) and ALPL-receptor engagement (AUC=0.99), and we then used these models to build a focused AAV capsid library pre-optimized for both capsid stability and ALPL-mediated transduction. Also, to spread the library as evenly as possible across the search space, a minimum hamming distance constraint was applied. The resulting library was then used in an in vivo screen to identify variants with increased antibody evasion. The ML-optimized capsid library, when tested in mice in the absence and presence of human intravenous immunoglobulin, yielded capsids with substantially improved both brain transduction and antibody evasion properties. Our data demonstrate that ML can be successfully used to pre-optimize capsid libraries to perform directed evolution of highly constrained capsid regions.

1912 Rapid Discovery of Novel AAV Capsids Using the BRAIN-X Insect Cell Library and Q-CAST

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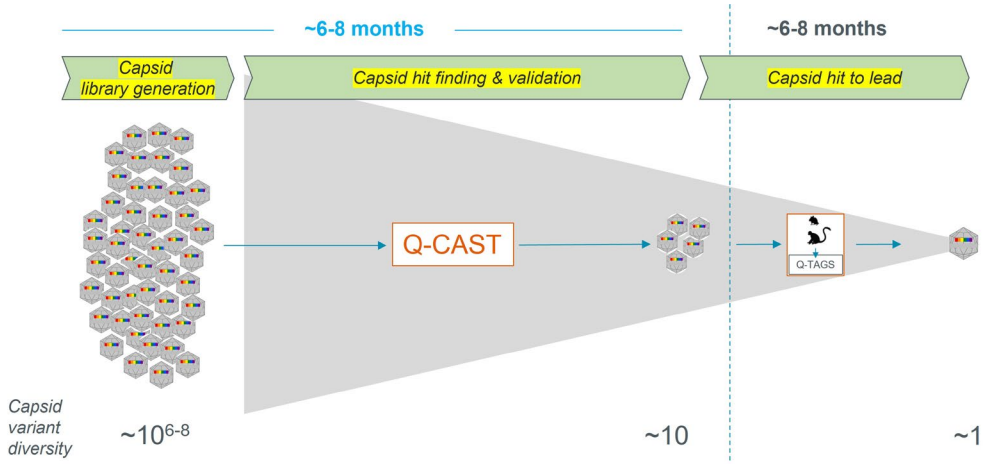
Capsid engineering is critical for advancing the efficacy of gene therapies by developing novel adeno-associated virus (AAV) capsids. Traditional capsid engineering methods, which rely on mammalian cells, face limitations such as cross-packaging artifacts and the high DNA input required per cell. Additionally, mammalian-derived AAV capsids often need further adaptation for efficient expression in insect cell-based production platforms.

To address these challenges, we present the *BRAIN-X capsid library*, a novel methodology leveraging insect cells. This approach significantly reduces the DNA requirement per cell while maintaining high AAV capsid library titers. Libraries generated with the proprietary BRAIN-X technology are deep, diverse, and exhibit minimal cross-packaging artifacts, enhancing the robustness and efficiency of capsid engineering efforts.

To accelerate the discovery of novel capsids, the BRAIN-X capsid library has been integrated with the *Q-CAST (Quick Capsid-library Assessment and Screening in Targeted-evolution)* platform. This combination facilitates the rapid identification of AAV capsids with the potential to cross the blood-brain barrier (BBB). In a short timeframe, multiple discovery campaigns utilizing this integrated platform have yielded several promising BBB-crossing capsid hits with putative consensus sequences.

This study underscores the transformative potential of the combined BRAIN-X and Q-CAST methodologies for rapid and cost-effective AAV capsid development. By significantly reducing costs and timelines, these innovative tools offer valuable advancements for gene therapy research and therapeutic applications.



1913 Enhancing AAV2 Capsid Engineering Through Co-Evolutionary Coupling and Predictive Modelling

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Adeno-associated virus (AAV) has emerged as a promising tool in gene therapy due to its safety, long-lasting transgene expression, ability to infect both dividing and non-dividing cells, and wide tissue targeting. Its success is reflected in the growing number of approved therapies and ongoing clinical trials. However, challenges including efficacy, stability and low yield still need to be addressed to improve AAV-based therapies.

Current methods for AAV engineering rely on directed evolution and rational design. Directed evolution involves creating a library and screening capsids for better traits. While effective, it is often inefficient because most single amino acid changes have no effect or are harmful, with only a small percentage showing improvements. Rational design aims to predict useful mutations based on protein structure and function, but it is limited by gaps in our understanding of how these changes affect AAV.

To tackle these issues, we are using a combined approach of co-evolutionary coupling and consensus-based design. Co-evolutionary coupling helps identify groups of amino acids that evolve together and are important for capsid properties. Replacing these residues with their most common natural versions (consensus residues) can reduce the risk of harmful changes. This method has been successfully used to improve enzyme stability and function, like in phytases and transketolase.

Two batches of AAV2 mutants were designed by identifying co-evolutionary coupling amino acids based on the VP1 and VP3. Those sites are substituted by their consensus and second consensus amino acids. We are applying this strategy to create and analyse 105 AAV2 mutants, aiming to design capsids with better infectivity, stability, and yield. The first screening was done using the crude lysate. The stability assay was carried out using heat shock at 60 degrees Celsius for 20 minutes. Based on the performance of the mutants compared to the wildtype AAV2, 21 mutants were selected for further analysis. These selected mutants were purified and placed in a formulation buffer for AAV2 for further testing. Consistency of their performance in the crude lysate compared to in the formulation buffer was tested.

The results were grouped into clusters to identify shared features that may contribute to their viability. Additional mutants will be designed and produced to validate these findings. Ultimately, the overall results will be used to train a model capable of predicting mutations that enhance capsid properties, facilitating the development of safer and more efficient gene therapy vectors.

1914 A Novel Conjugation Technology for Enhanced Specificity and Potency in AAV Gene Therapy

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Adeno-associated virus (AAV)-based gene therapy has emerged as a powerful therapeutic modality for treating a range of genetic disorders. However, key challenges, including limited tissue specificity and suboptimal therapeutic potency, have led to toxicity and hinder its broad clinical application. To address these limitations, we have developed a novel technology that enables precise targeting of AAV vectors. By leveraging sortase enzyme-mediated conjugation, we can attach targeting moieties directly to the AAV capsid, enhancing its ability to engage specific human cellular receptors.

This modular AAV retargeting platform has shown compatibility with several serotypes and diverse targeting ligands. *In vitro* studies demonstrated that this approach significantly improves transduction specificity. Furthermore, *in vivo* evaluation leveraging receptor-mediated transcytosis with human transferrin receptor targeting groups revealed enhanced delivery to the central nervous system (CNS). Our platform achieved widespread distribution within the CNS, including deep brain structures, increased neurotropism, and effective liver detargeting.

Our findings suggest that this versatile and potent platform has strong potential to advance the development of AAV-based gene therapies, offering improved targeting specificity and therapeutic outcomes.

1915 The generation of a versatile AAV engineering platform via the separated capsid protein expression

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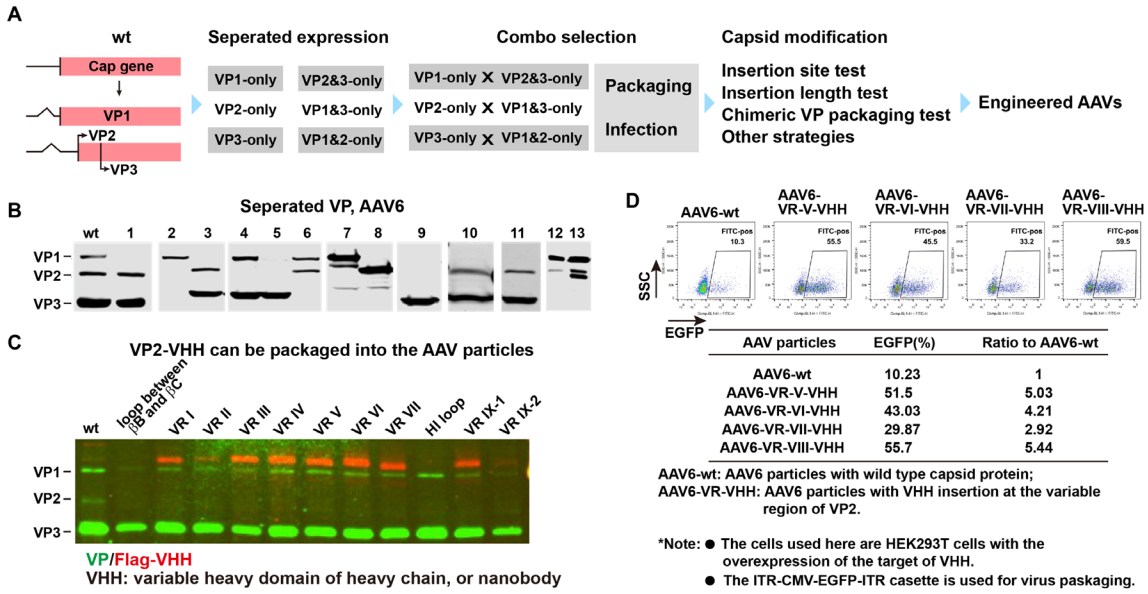
Adeno-associated virus (AAV)-based gene therapy offers great potential for treating genetic diseases by transferring the therapeutic genes toward proper organs or cells. However, the widely used AAV serotypes in AAV-based therapies face several drawbacks: the off-targeting effect and the pre-existing neutralizing AAV antibodies in patients. As a result, higher doses of AAV vectors are required to ensure efficient delivery of therapeutic genes, which not only raises safety concerns but also increases the cost of AAV-based therapies. The targeting efficacy and immunogenicity of AAVs are mediated by capsids, which are composed of three types of capsid proteins: VP1, VP2, and VP3. The three capsid proteins share a common C-terminus, and VP2 and VP3 can be regarded as the truncated forms of VP1 and VP2 respectively. The N- terminus of VP1 and VP2 offers them additional functions such as the infectivity. VP engineering is the promising approach to overcome the off-targeting and naturalization challenges of AAV vectors. However, the conventional engineering strategy changes all the capsids simultaneously for all three capsid proteins are coded by the same genes, which reduces the packaging or transduction efficacy. We hypothesize that selective engineering of specific capsid proteins—where only the necessary capsid protein(s) are modified, rather than all—might provide a viable solution to these problems. We designed the separated capsid protein expression plasmids to express individual or specific combinations of capsid proteins (VP1-only, VP2-only, VP3-only, VP1&2-only, VP2&3-only and VP1&3-only protein) for AAV2, AAV5, AAV6, AAV8, and AAV9 by combining start codon mutation, missense mutation, nonsense mutation, and independent expression with truncated coding sequences. By comparing the packaging and transfection efficacy of all combinations of plasmids, the combos with little reduced packaging and transfection efficacy were selected. Further, we tested the effect of protein (such as peptide, antibody or large binders) insertion on the AAV packaging in all the flexible loops located on the surface of AAV of each VP. Our data demonstrate that different VP tolerated distinct types of insertions in their flexible loops, which can be used for the rational design of AAVs with specific functions. The transfection efficacy was tested by inserting a variable heavy domain of heavy chain (VHH or nanobody) in the flexible loops of AAV6. All variable regions (VRs) are tolerant for VHH insertion, while VHH inserted into the loop between βB and βC or the H1 loop cannot be packaged into the AAV virion. The transfection efficacy of AAV6 with VHH inserted at the VR-V, VR-VI, VR-VII and VR-VIII in VP2 were tested, and VHH-coated AAV6 showed 2.9 to 5.5-fold enhanced transfection efficacy in antigen-expressed HEK293T cells than wild-type AAV6. In summary, we generated the versatile AAV engineering platform via the separated capsid expression for AAV2, AAV5, AAV6, AAV8, and AAV9 with the following advantages: 1, the possibility for the engineering of desired VP(s) without affecting other capsid proteins; 2, the capacity for packaging chimeric AAV particles with the VPs that have different modifications; 3, reduced packaging loss for AAVs using capsids with large protein insertions. This platform is expected to promote AAV engineering and facilitate the development of AAV-based therapies with enhanced safety and reduced costs.



AAV particles	EGFP(%)	Ratio to AAV6-wt
AAV6-wt	10.23	1
AAV6-VR-V-VHH	51.5	5.03
AAV6-VR-VI-VHH	43.03	4.21
AAV6-VR-VII-VHH	29.87	2.92
AAV6-VR-VIII-VHH	55.7	5.44

AAV6-wt: AAV6 particles with wild type capsid protein;
AAV6-VR-VHH: AAV6 particles with VHH insertion at the variable region of VP2.

*Note: ● The cells used here are HEK293T cells with the overexpression of the target of VHH.
● The ITR-CMV-EGFP-ITR casette is used for virus paskaging.

Figure legend:
A, the schematic diagram of the AAV engineering platform via the separated capsid expression. B, western blot analysis of VP expression in each plasmid. The AAV6 were used as an example. The number above each lane indicates the different format of seperated VPs. C, western blot analysis of the incorporation of VP2-VHH into the AAV particles. The sites in which the VHH inserted into VP2 are shown above each lane. D, flow cytometry analysis shows the enhanced infection of AAV6-VHH to HEK293T cells overexpressed with the target of VHH.

1916 Functional, Electrophysiological, and Histopathological Improvements in a Mouse model of CMT2A Following AAVrh74.tMCK.NT-3 Surrogate Gene Therapy

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Introduction and Aim: Charcot–Marie–Tooth disease (CMT) is a group of heterogeneous inherited polyneuropathies. Mutations in Mitofusin 2 (MFN2) gene are associated with the most common axonal CMT subtype, CMT2A. Neurotrophin 3 (NT-3) is an autocrine factor supporting Schwann cell survival and differentiation and stimulates axon regeneration and myelination. NT-3 also has a role in the maintenance of neuromuscular junction (NMJ) integrity and mitochondria biogenesis. Previously, we showed the efficacy of AAV1.tMCK.NT-3 gene therapy via intramuscular (IM) delivery in mouse models for CMT1A, CMTX1, CMT2D and CMT4C. In this study, we assessed the efficacy of NT-3 gene therapy using the AAVrh74 serotype in the Mfn2+/− mouse model for CMT2A.

Methods: Eight-month-old Mfn2+/− mice received 3x1011 vg dose of AAVrh74.tMCK.NT-3 via IM injection (NT-3 cohort). Age- and sex-matched Mfn2+/− mice received Ringer's lactate as untreated (UT) controls (n=12 for both cohorts with equal sex distribution). Mice were sacrificed six months post gene delivery. Rotarod and grip strength functional tests and electrophysiological measurements were performed at baseline, four months post-treatment and endpoint. Peripheral nerves, skeletal muscles, and innervation status of NMJs were also assessed with quantitative histological experiments (NT-3, n=7; and UT, n=6 have reached endpoint).

Results: Rotarod and grip strength functional assays showed that NT-3 treated mice performed significantly better than the UT mice by 63.7% and 26.3%, respectively at endpoint (rotarod, NT-3: 27.29 ± 1.6 s vs UT: 16.67 ± 1.0 s, p=0.0002; grip strength, NT-3: 0.111 ± 0.01 vs UT: 0.088 ± 0.01, p=0.027). Compound muscle action potential (CMAP) tests indicated significantly higher values by 13.6% for the treated group at the endpoint (NT-3: 49.07 ± 4.35 vs UT: 43.20 ± 4.31 mV, p=0.033). Serum NT-3 levels detected by ELISA reached therapeutic levels in the

treated cohort (5.39 ± 0.37 ng/ml). We observed a significant increase in the percent of innervated NMJs in the treated mice compared to UT counterparts in the lumbrical muscles (NT-3: 57.58 ± 5.77% vs UT: 22.81 ± 5.76%, p=0.020). Quantitative histological analysis on gastrocnemius (GAS) and tibialis anterior (TA) muscles showed that fiber size distribution shifted significantly towards larger diameter subgroups following NT-3 gene therapy in both muscles analyzed. On the SDH-stained muscle sections of Mfn2+/− mice, we observed an increase in the fibers with abnormal mitochondria content and distribution. Our measurements in GAS and TA muscles showed that number of abnormal fibers significantly reduced in the NT-3 mice compared to UT counterparts, in both muscles (GAS, NT-3: 6.79 ± 1.04 number/mm2 vs UT: 21.41 ± 3.80 number/mm2, p=0.004; TA, NT-3: 5.57 ± 0.58 number/mm2 vs UT: 12.53 ± 1.68 number/mm2, p=0.003). We also observed a significant increase in the fiber size diameter of slow-twitch oxidative fibers in TA muscle from the NT-3 cohort (NT-3: 34.65 ± 1.80 μm vs UT: 28.73 ± 1.70 μm, p=0.038).

Conclusion: IM delivery of AAVrh74.tMCK.NT-3 to the Mfn2+/- mouse model for CMT2A resulted in therapeutic serum NT-3 levels and functional, electrophysiological, and histological improvements. With this study, we show that the AAV.NT-3 gene therapy is efficacious in a mouse model for another CMT subtype, providing further support for the translational application of this surrogate gene therapy approach to large groups of CMT patients.

1917 Targeting Oligodendrocytes in Neonatal Wildtype C57BL/6 Mice

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Leukodystrophies are a heterogeneous group of rare, progressive genetic disorders primarily affecting the white matter of the central nervous system, with gene therapy emerging as a potential treatment avenue. To assess oligodendrocyte-specific transduction, we conducted a capsid comparison study testing two clinically applicable adeno-associated viral (AAV) capsids, AAV9 and Olig001. We expressed GFP under transcriptional control of CAG, a ubiquitous promoter. AAV9.CAG.GFP and Olig001.CAG.GFP were delivered at 1×10^{11} vg/ pup by intracerebroventricular (ICV) injection into neonatal P1 wildtype C57BL/6 mice. After 4 weeks, five brain regions of interest were collected- cortex, forebrain, midbrain, hindbrain and cerebellum- and were analysed for GFP expression by western blot, quantitative PCR (qPCR), and immunohistochemistry. Western blotting confirmed expression throughout the brain with both capsids with regional differences. AAV9 expressed significantly higher in the cortex, whilst Olig001 expression was highest in the hindbrain and cerebellum. The GFP immunohistochemistry of sagittal brain sections showed widespread brain transduction achieved by both capsids with mixed cell type transduction. The morphology of GFP+ cells revealed that AAV9 is predominantly neuronal, whereas Olig001 exhibits a more mixed neuronal and glial morphology. The % GFP+ cells that were Olig2+ in the corpus callosum was higher in Olig001 (71.43%) compared to AAV9 (59.46%), reflecting increased oligodendrocyte targeting. Future studies to increase oligodendrocyte targeting include using a selective promoter and Olig001 capsid towards leukodystrophy gene therapy application.

1918 Human Frataxin Gene Transfer to the Cerebellar Dentate Nucleus Improves Motor Coordination in a Mouse Model of Friedreich Ataxia

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Friedreich ataxia (FA) is a multisystemic genetic disorder that stems from a trinucleotide GAA repeat expansion in the first intron of the frataxin (*FXN*) gene, which results in transcriptional silencing and subsequent loss of function of the frataxin protein. This decreased frataxin expression results in a wide array of clinical symptoms that includes,

but it is not limited to, peripheral neuropathy, proprioceptive abnormalities, dysarthria, diabetes, hypertrophic cardiomyopathy, and most notably ataxia. Specifically, the ataxia that manifests in FA patients is thought to, at least in part, stem from the degeneration and loss of large principal excitatory neurons in the cerebellar dentate nucleus (DN). As such, we hypothesized that restoring frataxin expression in the DN by direct intraparenchymal injection can slow/reverse disease progression in a mouse model of FA neuropathology. Specifically, the PV-Cre::$FXN^{flox/null}$ (FA-PV) mouse model was utilized for these studies. These mice were genetically engineered to fully knock out *FXN* in parvalbumin-expressing neurons, while all other neuronal subsets express heterozygous levels of *FXN*. While these mice genotypically differ from patients with FA, which suffer from a global *FXN* knockdown in all cells, they recapitulate some aspects of the ataxic phenotype that is observed in patients. Our therapeutic approach consisted of designing an AAVrh10-serotyped vector that expresses the human *FXN* gene (*hFXN*) under the control of the SYN1 promoter, which would preferentially restrict vector expression to neurons (AAVrh10-SYN1-hFXN). A concentration-dependent increase in frataxin protein expression was confirmed in SK-N-SH cells. To assess the functional efficacy of our vector after cerebellar intraparenchymal injections, rotarod testing, parallel bar floor grid tests, beam walk assessments, and automated gait evaluations were performed at 11-12 weeks post-dosing. At 12-weeks post-dosing, FA-PV mice treated with 3.0E+8 and 1.0E+9 vector genomes (vg)/brain showed a significant improvement in rotarod performance over mice that were only treated with vehicle ($p = 0.015$ and $p = 0.037$). Additionally, at 11-weeks of age, FA-PV mice treated with 1.0E+9 vg/brain had decreased ataxia ratios compared to vehicle-treated FA-PV mice ($p = 0.011$). Altogether, for the first time, we demonstrated that direct targeting of the DN slows disease progression in FA-PV mice. This study highlights the importance of the DN in FA disease progression, and how restoration of *FXN* expression in that brain region can potentially modify the disease.

1919 Comparative analysis of cell-specific promoters in AAV9-mediated gene therapy targeting the central nervous system.

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Advancements in adeno-associated virus (AAV)-mediated gene therapy have highlighted the need for precise transgene expression to maximize therapeutic efficacy while minimizing off-target effects. In this study, we systematically evaluated a panel of ubiquitous and cell-specific promoters delivered via single-stranded AAV9 (ssAAV9) vectors to achieve targeted enhanced green fluorescent protein (eGFP) expression across the mouse central nervous system (CNS). Our findings provide critical insights into promoter-driven gene expression patterns, aiding the development of refined strategies for neurological gene therapy applications. Ubiquitous promoters, such as the full-length cytomegalovirus major immediate-early enhancer combined with the chicken beta-actin promoter (CAG), demonstrated extensive eGFP expression across multiple brain regions and various neural cell types, reinforcing their suitability for applications requiring widespread transgene distribution. However, the use of cell-specific promoters allowed for more precise targeting, which is essential for reducing potential off-target effects and optimizing therapeutic impact. Astrocyte-specific promoters, particularly variants of Glial Fibrillary Acidic Protein (GFAP), exhibited distinct expression patterns. The novel gfaABCD1405 (gfa1405) promoter and the gfaABC(1)D variant both yielded robust astrocyte-selective expression, with notable localization in the neocortex and hippocampus. Our comparative analysis suggests that gfa1405 provides superior transduction efficiency while maintaining stringent astrocyte specificity, offering an improved tool for astrocyte-targeted therapies. Neuronal targeting was achieved using a truncated Mecp2 promoter (p546), which demonstrated high specificity for neuronal populations within the neocortex and hippocampus. The distinct spatial expression pattern of p546, with strong neuronal soma labeling and detectable signals in neuronal projections, underscores its potential for therapeutic strategies addressing neuronal dysfunction in CNS disorders. Oligodendrocyte-specific promoters, including myelin-associated glycoprotein (MAG) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), produced unique distribution profiles. While MAG-driven eGFP expression was largely restricted to the corpus callosum, CNP-mediated expression extended across a broader range, suggesting differential utility for targeting oligodendrocyte-related pathologies. These findings emphasize the need for precise promoter selection based on disease-specific targeting requirements. Our study provides a comprehensive evaluation of AAV9-delivered promoters and highlights their potential for achieving targeted gene expression in distinct CNS cell populations. These insights

pave the way for more refined gene therapy approaches aimed at treating neurodevelopmental and neurodegenerative disorders. By reducing off-target expression and enhancing therapeutic specificity, promoter optimization plays a crucial role in advancing clinical translation of gene therapy strategies. Future research will focus on refining these promoter-driven vectors, expanding their applicability across various CNS disease models, and integrating them into preclinical and translational pipelines.

1920 Targeting misfolded mutant HTT protein with vectorized antibodies for the treatment of Huntington Disease

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Protein misfolding is a pathogenic feature of multiple neurodegenerative diseases, including Huntington Disease (HD). As the pathogenic mechanisms usually include both a loss of function and a gain of toxicity by misfolded and aggregated proteins, strategies are required to specifically target the toxic protein species whilst preserving the function of the native monomers.

In HD, the CAG repeat expansion in the Huntingtin (HTT) gene results in the translation of a mutant protein with an expansion of glutamines (polyQ). In neuronal cells, the mutant HTT protein forms soluble oligomers and insoluble inclusions which induce toxicity and ultimately cell death.

VectorY has developed vectorized antibodies, called VecTabs®, to target and reduce mutant HTT protein species. We screened and identified intracellularly expressed VecTab candidates that reduce mutant HTT protein aggregates in different in *vitro* models. Moreover, VecTabs delivered by adeno-associated virus (AAV), were also tested in a mouse model of HD, resulting in the reduction of mutant HTT protein aggregates in the striatum.

Our data support the further development of a gene therapy for the treatment of HD. In addition, this concept forms the basis of similar approaches in other neurodegenerative diseases caused by the formation of misfolded protein aggregates that cause cellular toxicity.

1921 Focused ultrasounds enhance efficacy of AAV transduction via intracerebroventricular administration in mice

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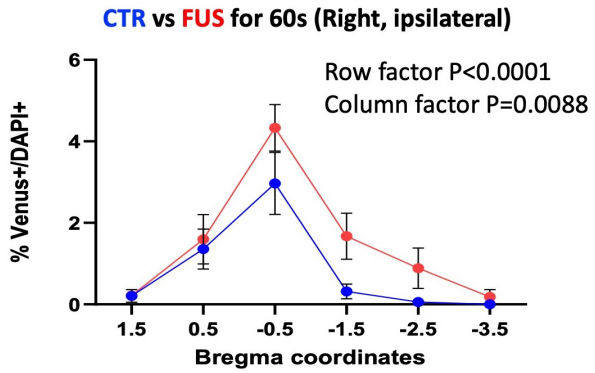
Systemic delivery of AAV vectors targeting the central nervous system has the potential to solve many neurodevelopmental disorders, yet it is made difficult by the filtering effect of the blood-brain barrier and systemic complications. To overcome this limitation, we attempted to inject a Venus-expressing, oligodendrocyte-targeting AAV9 viral vector in the ventricles together with lipid microbubbles and subjected them to focused ultrasound; the resulting mechanical stimulation on the brain ventricles presumably opens small, temporary gaps from which vector particles can leak and spread. Our findings indicate that focused ultrasound can increase viral vector diffusion across both the anteroposterior and left-right axes without influencing cell tropism; significant effects were found with 60 and 90 seconds exposure time, but no effects were observed with longer intervals. Taken together, these results highlight a new strategy for the safe and effective delivery of viral vectors, and offer new perspectives for the development and application of gene therapies for central nervous system diseases.



1922 Comparative Evaluation of Intranasal and Intravenous AAV Delivery for Safe and Targeted CNS Gene Therapy

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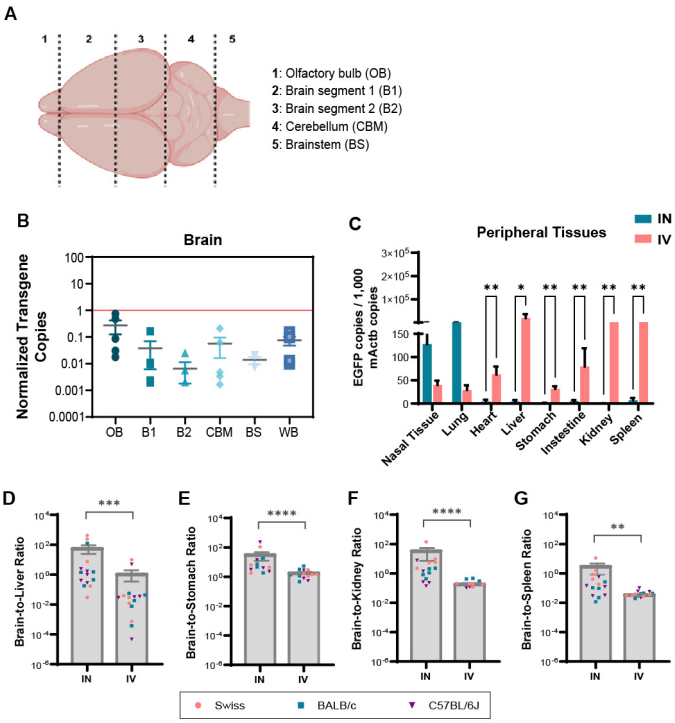
Introduction: Adeno-associated viruses (AAVs) hold significant promise for gene therapy targeting the central nervous system (CNS). However, current delivery methods are limited by their associated procedural risks and off-target effects. Intranasal (IN) delivery offers a transformative, noninvasive alternative by bypassing the blood-brain barrier (BBB) while minimizing systemic exposure. This study systematically evaluates the delivery efficiency and biodistribution of AAV in the brain and peripheral organs following IN administration, assessing its potential for safe and targeted delivery to the CNS compared to the conventional intravenous (IV) route.

Methods: AAV encoding the enhanced green fluorescence protein (EGFP) reporter gene was administered intranasally or intravenously to Swiss, BALB/c, and C57BL/6J mice, enabling evaluation across diverse genetic backgrounds to account for biological variability. Brain and peripheral tissues were collected four weeks post-delivery, and quantification of transgene copies and protein expression levels was performed using droplet digital PCR (ddPCR) and ELISA, respectively.

Results: IN delivery resulted in localized brain transduction, predominantly in the olfactory bulb, while IV injection led to widespread transduction across the brain. Although IN delivery achieved lower overall transgene expression and EGFP protein levels in the brain—approximately 15% and 9% of levels observed with IV injection, respectively—IN delivery significantly reduced systemic exposure, up to 1.3×10^4-fold, in peripheral organs compared to IV injection. Furthermore, IN delivery demonstrated superior brain-to-peripheral transduction ratios for major peripheral organs, including the liver, kidney, spleen, and stomach, highlighting its potential to reduce systemic impact.

Conclusion: These findings suggest that IN delivery offers a viable, noninvasive approach for brain-targeted gene delivery, offering minimized off-target exposure—a critical factor for improving the safety of CNS-targeted therapies.



(A) Schematic illustrating the mouse brain dissection method used to evaluate AAV biodistribution. **(B)** Brain-targeting efficiency of intranasal (IN) AAV delivery relative to intravenous (IV) delivery (red horizontal line) in Swiss mouse brain segments. **(C)** EGFP transgene levels in peripheral tissues of Swiss mice following IN (blue) and IV (red) AAV delivery. **(D-G)** Brain-to-peripheral transgene ratios for the liver (D), stomach (E), kidney (F), and spleen (G), pooled across all tested mouse strains.

1923 Preclinical development of AAV mediated gene editing for SOD1-ALS using a Type V CRISPR-Cas enzyme

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease leading to paralysis and death typically 3-5 years post disease onset. Missense mutations in superoxide dismutase 1 (SOD1) gene cause ALS via a toxic gain of function of SOD1 protein. QALSODY, a clinically validated antisense oligonucleotide dosed once a month via intrathecal injections, is approved for use in SOD1-ALS patients in the US and EU. By lowering SOD1 mRNA, QALSODY reduces protein levels leading to a ~55% decrease in neurofilament-light chain, a marker for neurodegeneration in ALS patients. These results validate the lowering of SOD1 as an attractive target for ALS treatment. In this study, we describe the preclinical development of a one-time treatment for SOD1-ALS by using an AAV vector to carry our proprietary Type V CRISPR enzyme to target the SOD1 gene and efficiently lower SOD1 protein levels.

Large indels (~10bp) generated by Type V enzymes (nucleases) favor targeting across the entire gene to destabilize the protein compared with Type II enzymes that generate small indels and may not lower protein abundance. Using bioinformatics and HEK293T cells, we initially screened ~250 different combinations of guides and nucleases to identify a stratified pool that provides substantial editing. Top nuclease-guide combinations were packaged into AAV and tested in human motor neurons, the primary disease affected cell type. Here, we observed up to 95% indels and subsequently up to a 90% reduction of SOD1 protein with various guides along with a longitudinal

increase in indel formation and subsequent protein reduction. Based on these data, we built our AAV construct comprised of 1) a Pol II promoter driving expression of the Type V nuclease in both neurons and astrocytes, cell types with SOD1 aggregates in patients; 2) a Pol III promoter driving expression of the guide RNA and 3) regulatory elements for spatial control of nuclease expression. Our top five constructs were tested in the high-copy hG93A-BAC mouse model (the gold standard for SOD1-ALS *in vivo* pharmacology) and demonstrated indel formation and subsequent significant lowering of SOD1 protein. In addition, we also evaluated the efficacy of the guides in rescuing survival and grip strength in the transgenic mice and selected our lead guides. Based on preclinical efficacy of the guides lowering SOD1 protein, we also performed rhAMP Seq analysis to assay any off-targets predicted in silico. Three guides demonstrated low off-target liability. Overall, these findings strongly support the continued development of Type V CRISPR-Cas enzymes for the potential treatment of CNS diseases.

1924 Preclinical Mouse Pharmacology Studies Evaluating the Biodistribution and Pharmacodynamics of LTS-101, a Novel Gene Therapy Candidate for CLN2 Batten Disease

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Introduction: Latus Bio, Inc. is developing LTS-101, a gene therapy for the treatment of neuronal ceroid lipofuscinosis Type 2 (CLN2), a form of Batten disease caused by deficiency in the tripeptidyl peptidase 1 (TPP1) protein, which causes lysosomal dysfunction and neurodegeneration. LTS-101 is an intracerebroventricular (ICV)-delivered gene therapy utilizing a novel capsid, AAV-Ep+, engineered to express the *hTPP1* transgene. AAV-Ep+ transduces ependymal cells lining the cerebral ventricles, resulting in secretion of human TPP1 into the CSF. AAV-Ep+ was identified by a capsid screening campaign in multiple non-human primate (NHP) species as a potent transducer of ependymal cells and neurons. Studies were conducted wherein LTS-101 was introduced ICV to a mouse model of CLN2 disease to evaluate vector biodistribution, TPP1 production and biodistribution, and efficacy in alleviating well-characterized phenotypes such as tremor, reduced survival, and the accumulation of the pharmacodynamic marker of lysosomal dysfunction, lipofuscin. This work identified doses whereby transgene expression was robust within target CNS regions, with resultant decreased lipofuscin accumulation, increased survival, and decreased tremor. Notably, there was minimal biodistribution in peripheral tissues.

Results: LTS-101 was administered ICV at four doses to $CLN2^{-/-}$ mice to identify a minimally efficacious dose. Bilateral ICV administration of LTS-101 demonstrated high transduction in the brain, therapeutic expression levels of TPP1, and pharmacodynamic activity of the transgene. LTS-101 was distributed broadly throughout the brain, with 1-10 vg/dg at the high dose, and low levels in peripheral tissues such as the heart and liver, with <0.01 vg/dg. The hTPP1 protein produced by LTS-101 was catalytically active in brain tissues and reduced lipofuscin accumulation by approximately 50% in hippocampus and somatosensory cortex. LTS-101 treatment also decreased tremor amplitudes by 68% and prolonged average lifespan by 20%.

Conclusion: Preclinical studies in $CLN2^{-/-}$ mice dosed with LTS-101 provide proof-of-concept data in support of a potential therapeutic candidate for CLN2 disease. Definitive mouse pharmacology and NHP GLP toxicology studies are ongoing. LTS-101 is a promising gene therapy candidate with the potential to treat CLN2 Batten disease patients with a one-time ICV administration providing stable, long-term expression of TPP1 for broad distribution throughout the CNS.

1925 Developing an AAV-based Gene Therapy for Chronic Pain Through Identification of Potent and Selective Artificial miRNA Candidates to Knockdown *SCN9A*

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Lucey[1], Camille Artur[2], Elizabeth Hallam[1], Winnie Lin[3], Puja Dhanota[1], Serena Liu[1], Alex Diaz de Arce[2], Sirika Pillay[1], Annie Tanenhaus[1], Raghavendra Hosur[1], Archana Belle[3], Suresh Poda[2], Stephanie Tagliatela[3]
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Chronic pain impacts millions of people globally, with many types resulting from the aberrant firing of nociceptive sensory neurons. *SCN9A* encodes the voltage-gated sodium channel $Na_V1.7$, a critical mediator of pain signaling within the peripheral nervous system. Variants in *SCN9A*—resulting in either loss-of-function (LOF) or gain-of-function (GOF)—are directly linked to pain insensitivity or hypersensitivity in known genetic disorders. However, small molecule inhibitors targeting $Na_V1.7$ have faced clinical setbacks due to issues with selectivity (especially the potential for off-target effects on other Na_V channels, such as $Na_V1.5$), inadequate target engagement, and dose-limiting adverse events. To overcome these challenges, we hypothesized that blocking $Na_V1.7$ signaling via a robust and selective AAV-mediated mRNA knockdown approach would result in long-term reduction in pain following a single intrathecal (IT) administration. Using Encoded's vector engineering platform, we developed vectorized miRNA candidates with optimized potency and selectivity. In vitro screening in HEK293 cells and iPSC-derived neurons identified several miRNA candidates capable of potently and selectively knocking down *SCN9A* without affecting other Na_V channels. Whole transcriptomic RNA sequencing further demonstrated a minimal off-target profile for these candidates. Top candidates were validated in rodent models. IT delivery of lead candidates to post-natal day 1 (P1) mouse pups resulted in potent (up to 95%) and dose-dependent knockdown of *Scn9a* mRNA and $Na_V1.7$ protein in the dorsal root ganglia (DRG). Furthermore, when delivered IT to adult rats, the top candidates successfully rescued mechanical and thermal allodynia induced by spared nerve injury (SNI), with the rescue sustained for over 8 weeks post-dosing. Taken together, these results highlight the potential of a vectorized miRNA-based approach as a highly selective and durable strategy for knocking down *SCN9A*. By addressing the limitations of small molecule, this gene therapy approach represents a promising non-opioid strategy for treating chronic pain conditions.

1926 Gene Therapy for ZNF526 Deficiency Using Endogenous Regulatory Elements

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Neurodevelopmental disorders (NDDs), including autism, attention-deficit/hyperactivity disorder (ADHD), and intellectual disabilities (IDs), are lifelong conditions that disrupt brain development and function. One of the key mechanisms involved in the control of developmental processes is transcriptional regulation of gene expression. Through the activation and repression of target genes, transcription factors (TFs) determine cell fate and orchestrate development at the tissue, organ, and organismal levels. Among the numerous TF families, zinc finger proteins (ZNFs) are particularly notable. Over the past few years, numerous pathogenic variants in zinc finger proteins have been identified in individuals with intellectual disability (ID). The current lack of understanding the disease pathomechanism, coupled with the absence of animal models that accurately recapitulate human disease phenotypes, presents a significant challenge.

ZNF526 deficiency (OMIM: 614387), a severe NDD caused by autosomal recessive frameshift variants in the ZNF526 gene, which encodes a ubiquitous C_2H_2-ZNF protein. This condition is characterized by prenatal-onset microcephaly, developmental delay, bilateral cataracts, hypertonic-dystonic movements, profound psychomotor delay, severe intellectual disability, epilepsy, simplified gyration, and thinning of the corpus callosum. Currently, there is no cure for ZNF526 deficiency. However, rAAV-directed gene therapy holds promise as a potential treatment option Here, ZNF526 deficiency will serve as a powerful tool to evaluate potential therapeutic strategies and unravel the cellular and molecular mechanisms underlying ZNF-related NDDs. One of the key hurdles in developing rAAV-based gene therapies for central nervous system (CNS) disorders is minimizing off-target effects and their associated toxicity. To address this, our goal is to develop a rAAV9-mediated ZNF526 gene replacement therapy regulated by the endogenous ZNF526 gene regulatory elements to precisely control transcription, ensuring physiologically relevant gene expression and regulation for therapeutic applications. We designed four distinct

regulatory cassettes, each containing specific ZNF526 promoter-derived elements driving green fluorescent protein (GFP) expression and evaluated their functionality *in vitro* using human embryonic kidney cells (HEK293), alongside the well-known ubiquitous CB6 promoter as a reference for comparison. We determined that each regulatory cassette expressed varying degrees of GFP. Our results indicate that regulatory elements within the ZNF526 promoter play a critical role in modulating gene expression. These constructs are currently under *in vivo* evaluation using rAAV9 at postnatal day (P)1 and P10.

The use of these regulatory cassettes and dual-injection timepoints will enable the evaluation of spatiotemporal gene expression. Furthermore, this approach will allow for a comparative analysis of ubiquitous versus targeted gene expression, providing valuable insights for optimizing ZNF526 gene therapy.

1927 A codon-optimised human sequence of human *MC4R* regulates cAMP levels and normalise weight in *Mc4r* mutant obese males and females when delivered with as a self-complementary AAV9 (scAAV9) with an intracerebroventricular delivery.

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Melanocortin 4 receptor (*MC4R*) mutations are the most common cause of human monogenic obesity. Patients with MC4R mutations suffer from hyperphagia, severe obesity and hyperinsulinemia. Impact of the different type of MC4R mutation depends on how they reduce MC4R expression and the impact they have on the production of cyclic AMP (cAMP). Mutations in heterozygosity, with partial MC4R functionality are the most frequent, but homozygous mutations and double heterozygous account for 25% of MC4R mutations. There are approximately 50,000 patients with complete *MC4R* loss of function (LoF) and BMI>40 in the United States, European Union and Middle East. Mouse Mc4r have a slightly different sensitivity than the human MC4R, but all null-Mc4r mouse lines recapitulate the most severe human phenotype.

Here we developed a series of new codon-optimised constructs of the human MC4R. We aimed to develop new Adeno-associated vectors (AAV) vectors, therefore we cloned our new constructs to express the transgene as a single strand (ssDNA) or a self-complementary (scDNA) DNAs once packaged into the AAVs. All constructs were under the regulation of the CAG promoter. We tested expression after transfection in human derived HEK293T cells and saw that the codon-optimised self-complementary sequences were able to express the *MC4R* transgene activity more than 5 times more than the wild-type sequences. After packaging the expression ability of the codon-optimised MC4R construct, HEK293 transductions, the AAV9-CAG-hCOMC4R vectors resulted in the high stable upregulation of hMC4R transgene at mRNA and protein levels compared with the AAV9-CAG-hWTMC4R wild-type and single-strand sequences, from the relative 1644 fold change to AAV9-CAG-hCOMC4R to $1.8x10^8$ fold change of the codon optimised AAV9-CAG-hCOMC4RGA. MC4R protein levels were also increased in the all our constructs . We then tested the ability the transgene to regulate levels of cAMP levels after *in vitro* transduction. We observed a clear dose response of the cAMP activation with all constructs indicating a functional MC4R.

To test *in vivo* efficacy, we prepared AAV2 and AAV9 vectors with our the best scAAV MC4R sequence, with better expression and cAMP activity. We dosed *Mc4r*^{tm1Lowl} mutant mice (Jax lab strain B6.129S4-*Mc4r*$^{tm1Lowl/J}$, Strain #032518) and wild-type, males and females, at P7 after birth with a single intracerebroventricular dose of $1x10^{13}$ viral genomes and followed their weight gain. *Mc4r* mutant treated mice followed normal weight gain pattern till week 7 when then, instead to become obese as their littermates, they followed the same weight gain pattern than the WT untreated mice and never become obese. *Mc4r* mutant female treated with AAV9.COMC4R also followed the same WT trend but with a higher variability. WT treated with AAV9.COMC4R or all vehicle (PBS) treated controls (WT or *Mc4r*) had the same pattern than untreated controls. Surprisingly when the same dosing was repeated with an AAV2 capsid no recue of the phenotype was observed (Figure 1).

In summary, we describe here a new human sequence and AAV9 vector (we called AXV-201) that can rescue the weight gain phenotype and could prove to be a powerful treatment for MC4R patients with severe obesity and very high BMIs.

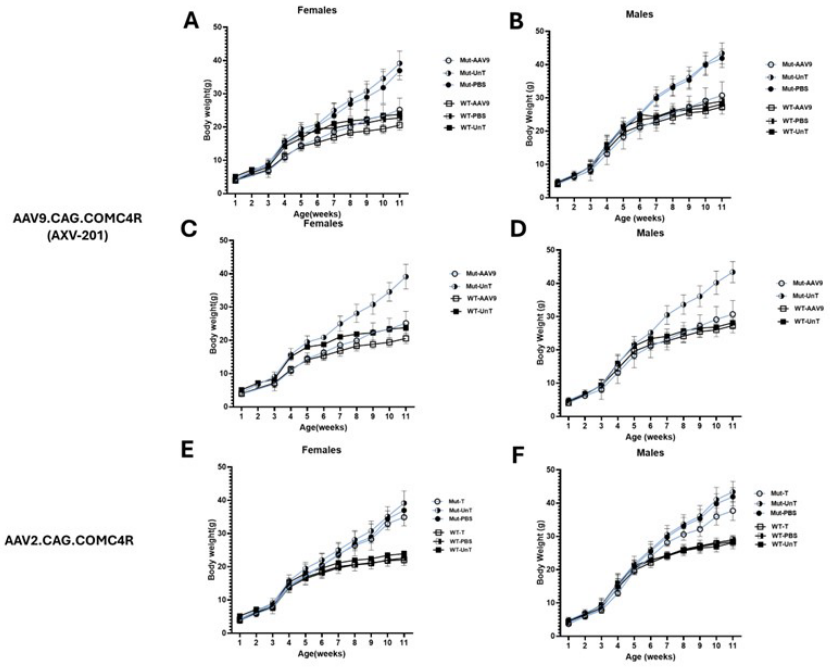

Figure 2. Dosing of Mc4R mutant mice **A-D** AAV9.CAG.COMC4R (AXV-201) completely normalise the weight gain in male and females **E-F**. The same self complementary codon optimised construct (CAG.COMC4R) failed to rescue the mc4r phenotype when delivered in AAV2 capsid.

1928 Ongoing Preclinical Study of an AAV vaccine in Spontaneous Canine Model of Oral Melanoma: Safety and Efficacy Evaluation

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Companion dogs can spontaneously develop oral melanoma (OM), an aggressive cancer that begins in the mouth's mucosal tissue coursing with metastasis in distant organs. OM is treated through surgical removal of tumor followed by intense radiotherapy mirroring human melanoma onset and treatments. This makes OM diagnosed dogs a great preclinical model to test our multivalent optimized AAV6 anti-melanoma vaccine. This vaccine is composed by a mutated AAV6-S663V to prolong the vectors lifespan by avoiding proteolytic degradation in target cells, ensuring delivery of optimized expression cassette and eliciting the corresponding immune response. Briefly, our AAV6 vaccine is obtained through a step gradient protocol using anion exchange chromatography (HiTrap Q HP column) after iodixanol gradient centrifugation of virus from HEK293 triple transfected cells. Chromatography fractions corresponding to UV detection peak are analyzed by qPCR to evaluate vg titers. After, $1x10^{12}$vg or $3x10^{12}$vg of each antigen are used to create a triple-valent vaccine of low or high dose respectively, that delivers 3 known melanoma antigens, glycoprotein 100 (Gp100), tyrosinase (Tyr) and tyrosinase related protein 1 (TRP-1). Previous work has proven the vaccines effectiveness in melanoma mouse models, effectively promoting protection against lung metastasis. The ongoing preclinical trial for this multivalent AAV vaccine against oral melanoma is reaching 3.5

years. To date, 9 dogs with I-III stage OM have been administered the vaccine, 5 males and 4 females ranging between 6.5 and 14.3 years of age. 6 animals received a $3x10^{12}$ vg/ dog dose while 3 animals received $9x10^{12}$ vg/ dog dose. These animals are followed for up to 9 months to evaluate blood, liver and renal function parameters to determine safety and efficacy of vaccine. Prior to vaccine administration, preexisting neutralizing antibodies (NAb) against AAV6 capsid were determined to meet eligibility criteria of 1:2-1:8 titers for enrolment. After vaccine administration, NAb titers increased 1 to 4 weeks in most dogs, and at later time points started to decline, opening the possibility of vaccine boosting. With blood PBMCs, specific immune reaction against vaccine antigens (gp100, TYR and TRP-1) were analyzed through IFN-γ ELISPOT assay determining $CD8^+$ T-cell activation at different time points, revealing three groups of patients, the early, the late responders, and the non-responders (only one dog). Animals were also monitored through blood biochemistry, and chest radiography to track metastasis in the lungs. Globulins and white blood cell (WBC) count were tracked for a sign of inflammatory responses. Alanine transaminase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (ALS) were evaluated for liver function, as were creatinine and blood-urea-nitrogen (BUN) evaluated for renal function. The values of these parameters suggested no evidence of liver or renal problems related to the vaccine administration. To date 2 dogs are currently disease-free, and 2 dogs lived 738 and 896 days post-vaccination disease-free before being euthanized due to age related problems. This is the first study that attempts the use of an optimized AAV cancer vaccine in a large spontaneous cancer animal model. The preliminary conclusions resulting from our study indicates that our AAV vaccine based on off-shelf tumor antigens is safe in dogs. These results encourage the possibility of expanding AAV based treated diseases.

1929 Cardiac catheter delivery of AAV5 to Goettingen Minipig hearts

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Safely achieving therapeutic expression levels with adeno-associated virus (AAV) gene therapy is a significant challenge for treating the heart. Goettingen minipigs provide a more accurate assessment of the feasibility of achieving an effective and safe dose than rodents due to the significantly larger hearts. Here we developed methods for delivering 3×10^{14} vg AAV5 (~2.3×10^{13} vg/kg in ~13 kg pigs) to Goettingen minipig hearts by retro-coronary sinus infusion (RCSI). We found that optimal placement of the delivery catheter and the delivery flow rate significantly improved the biodistribution of AAV5 in the left ventricle and septum, leading to mRNA expression in ~70% cardiac myocytes. The optimized delivery protocol coincided with minimal changes to blood pressure, ST elevation, and transient serum troponin I levels. In a sperate study, we systemically delivered 6×10^{13} vg/kg AAV5 to n = 2 NHPs that led to mRNA expression in ~26% of cardiac myocytes. We conclude that the optimized RCSI is a safe method to deliver significantly more therapeutic to the left ventricle and septum than systemic delivery, and the methods may be broadly applicable to RCSI delivery.

1930 CGT-003: A novel recombinant parvovirus gene therapy for the treatment of hypertrophic cardiomyopathy resulting from genetic variants of myosin binding protein C3 (MYBPC3)

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Hypertrophic cardiomyopathy (HCM) is the most common form of heart disease, affecting as many as 1 in 200 individuals globally. HCM is characterized by left ventricular (LV) hypertrophy, diastolic dysfunction, heart failure,

and sudden cardiac death. Up to 30% of HCM patients develop atrial fibrillation (AF), which increases risk of death even further. Genetic mutations affecting sarcomeric filaments are the leading cause of HCM because they disrupt contractility and beating of cardiomyocytes. Genetic HCM is inherited in an autosomal dominant pattern and juvenile patients are at a 4-fold greater risk of mortality than adults. Pathogenic mutations in the gene encoding cardiac myosin binding protein C3 (MYBPC3) are the most common cause of HCM and the vast majority of these pathogenic variants result in haploinsufficiency. While medications like beta-blockers and calcium channel inhibitors are available to manage symptoms, there are no approved therapies for genetic forms of HCM. A gene therapy for MYBPC3 is currently in the clinic; however, the utility of this and other rAAVs is hampered by high seroprevalence in the human population, limited cargo capacity, safety and efficacy concerns related to high liver transduction. AAV therapies are limited to only one minor genus (dependoviridae) within the parvovirus family, leaving many potentially better suited vectors unexplored. To overcome these limitations, Carbon Biosciences has developed hybrid parvovirus vectors containing capsids from non-primate-derived autonomous parvoviruses and genomes packaged with AAV2 inverted terminal repeats (ITRs). CGT-003 is a hybrid viral vector featuring a novel recombinant non-AAV parvoviral capsid carrying a 5.5kb genome containing a strong promoter, regulatory elements, and a full-length human MYBPC3 transgene capable of robust and specific protein expression in cardiomyocytes. The vector displays exquisite cardiomyocyte tropism while completely avoiding liver transduction, and still leverages the established regulatory and CMC profiles of AAV. CGT-003 represents a best-in-class treatment for genetic forms of symptomatic hypertrophic cardiomyopathy (HCM), specifically engineered to de-target the liver, thereby enhancing safety. The capsid powering CGT-003 is derived from a virus that has not co-evolved with humans, resulting in an absence of pre-existing neutralizing antibodies. This unique characteristic is expected to streamline patient enrollment and accelerate clinical development by expanding the eligible patient population and minimizing the need for extensive prescreening. In this study, we demonstrate CGT-003-driven expression of human MYBPC3 with appropriate sarcomeric localization in human iPSC-derived cardiomyocytes. Preclinical evaluation shows vector genome deposition in critical regions of the heart for both mice and non-human primates. hMYBPC3 protein was readily detected in murine hearts, while no protein was detected in the liver, highlighting the potential for safe and efficacious MYBPC3 gene replacement.

1931 LTS-101: An Intracerebroventricular Delivered AAV Gene Therapy Using a Novel Capsid Variant for the Treatment of CLN2 Batten Disease

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Introduction: CLN2 disease is a rare, fatal, pediatric neurogenetic disorder resulting from deficiencies in the tripeptidyl peptidase 1 (TPP1) protein, which causes lysosomal dysfunction and progressive neurodegeneration. While bi-monthly infusions of recombinant human TPP1 (cerliponase alfa) to the brain have provided benefit to patients, significant disease burden remains and the therapy requires families relocate to infusion centers for the frequent, hours long, infusions. While ERT slows disease progression, it is not curative and a global survey of patients and caregivers indicated that >90% believe there is a need for alternative treatments such as gene therapies, with greater efficacy and without the need for biweekly administration. Latus Bio, Inc. has developed an AAV-based gene therapy using a novel capsid variant, AAV-Ep+, delivering the hTPP1 transgene. AAV-Ep+ robustly transduces ependymal cells lining CNS ventricular spaces as well as intraparenchymal neurons, allowing for production and secretion of TPP1 protein into the CSF and brain parenchyma. Additionally, AAV-Ep+ exhibits limited transduction of peripheral tissues in mice and multiple non-human primate (NHP) species. Preclinical studies with ICV administration of the AAV-based construct have established proof-of-concept in $CLN2^{-/-}$ mice and identified tolerable doses that provide potentially therapeutically relevant levels of TPP1 in CSF of cynomolgus macaques.

Results: In $CLN2^{-/-}$ mice, bilateral ICV administration of LTS-101 demonstrates high transduction, expression, and engagement. LTS-101 produces TPP1 protein throughout the brain and reduces lysosomal protein accumulation (lipofuscin) up to 58%. In a dose range finding (DRF) study in NHPs, bilateral ICV administration of LTS-101 produces brain-wide TPP1 activity, up to 140 ng/mL TPP1 in the CSF, a favorable safety profile, and low systemic exposure at a total dose of 1.00e12 vg which is a logs-fold lower dose than that of conventional capsids used in

similar clinical gene therapy programs using ICV delivery. LTS-101 is manufacturable reproducibly at 250L scale, producing high quality drug product at suitable concentrations for clinical testing.

Conclusion: Preclinical studies in CLN2$^{-/-}$ mice and NHPs have established proof of concept for LTS-101 as a treatment for CLN2 disease. A definitive mouse pharmacology study and NHP GLP toxicology study are on-going. Clinical development planning is progressing in collaboration with world-leaders in CLN2 disease. LTS-101 is a promising gene therapy candidate with the potential to treat CLN2 Batten disease patients with a one-time ICV administration providing stable, long-term expression of TPP1 while eliminating the caregiver and patient burden of repeated ERT treatments.

1932 AAV Gene Therapy with a Bacterial Glycogen Debranching Enzyme in a Dog Model of GSD IIIa

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Background: Deficiency of glycogen debranching enzyme causes glycogen storage disease type III (GSD III), resulting in pathological glycogen accumulation in both liver and muscle (GSD IIIa) or in only the liver (GSD IIIb). We recently reported an innovative gene therapy approach for GSD III using an AAV9 vector expressing a bacterial debranching enzyme (pullulanase) driven by a unique dual promoter, which consists of an immunotolerizing liver-specific promoter (LSP) and the ubiquitous CMV enhancer/chicken β-actin (CB) promoter. We demonstrated that the LSP-CB dual promoter effectively suppressed transgene-related cytotoxic T lymphocyte responses in adult GSD IIIa mice while allowing persistent pullulanase expression and glycogen reduction in liver, heart, and skeletal muscles. We have expanded this treatment strategy to a dog model for GSD IIIa, which we have characterized previously.

Methods: An AAV vector carrying a codon-optimized CpG-free pullulanase cDNA under the control of the LSP alone (AAV-LSP-Pull) or the LSP-CB dual promoter (AAV-Dual-Pull) was packaged into AAV9. For liver-targeted gene therapy, two 10-month-old GSD IIIa dogs were intravenously injected with AAV9-LSP-Pull. One was treated at a high dose (2.5E+13 vg/kg) and the other at a low dose (5E+12 vg/kg). Dogs received 1 mg/kg prednisone once daily starting 3 days before vector administration, The dose was increased as necessary for liver enzyme elevations which were monitored at least weekly for 12 weeks. Liver and muscle biopsies were performed prior to vector administration and 2, 6 and 12 weeks post-treatment. For neonatal treatment, AAV9-Dual-Pull was intravenously injected into two wild-type dogs on the 2nd day of life (1.4E+14 and 1E+14 vg/kg). They did not receive an immunosuppressive therapy. Skeletal muscle biopsies were performed at 1, 3, 6, and 9 months of age, with final tissue collection at 12 months of age. Blood chemistries were performed throughout the study.

Results: Adult GSD IIIa dogs treated with AAV9-LSP-Pull demonstrated a dose-responsive increase in pullulanase activity and vector genomes in the liver with a subsequent decrease in liver glycogen 2 weeks after treatment. However, loss of this treatment effect was noted 6 weeks after administration. There was also an increase in liver transaminases (ALT and AST) beginning at 24 and 42 days post-treatment in the high-dose and low-dose treated dogs, respectively. Neonatal dogs treated with AAV9-Dual-Pull demonstrated persistent pullulanase activity in skeletal muscle (quadriceps and biceps femoris) over time at biopsies and pullulanase activity was present 12 months after treatment in diaphragm, quadriceps, gastrocnemius, biceps femoris and minimally in heart. Blood chemistries did not show elevations of liver or muscle enzymes demonstrating safe expression of pullulanase throughout the study.

Conclusion: Our data demonstrate the efficacy of AAV9-LSP-Pull-mediated gene therapy in a canine model of GSD IIIa. However, the benefits do not last, likely due to an immune-mediated T-cell response that led to the loss of transgene. When treating dogs as neonates with AAV9-Dual-Pull, pullulanase activity persists within the muscle

throughout the 12 month experiment, demonstrating immune tolerance. Studies are currently being initiated in adult GSD IIIa dogs with the dual promoter that utilizes a high potency MyoAAV capsid, to determine whether pullulanase activity can persist in both liver and muscle.

1933 Optimizing Vector Administration of an AAV Gene Therapy for Surfactant Protein B Deficiency in a Neonatal Pig Model

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Surfactant protein B (SP-B) deficiency is an inherited disorder that leads to respiratory failure shortly after birth due to alveolar collapse. Despite its severity, treatment options remain limited, offering only transient relief without addressing the root cause. Given that SP-B deficiency exclusively affects the lungs and is monogenic in nature, targeted gene therapy to the respiratory tract is a promising treatment option. The novel triple mutant AAV6 capsid, AAV6.2FF, exhibits preferential tropism for the lungs. Earlier preclinical studies in mouse models have demonstrated the corrective potential of AAV6.2FF-SPB; however, clinical translation of this therapy remains a significant challenge. To bridge this gap, we have concentrated our efforts on optimizing lung gene delivery in a neonatal pig model whose lung physiology closely resembles that of human infants. Our optimization protocol involved using two AAV6.2FF vectors, one expressing human placental alkaline phosphatase (hPLAP) and a second expressing secreted alkaline phosphatase (SEAP) to assess vector biodistribution in the lungs across different doses and delivery techniques.

In both studies, AAV vectors were endotracheally delivered to 2-week-old York/Landrace/Duroc mixed piglets using the MADgic laryngo-tracheal mucosal atomization device from Teleflex. In Study 1, piglet #1 and #2 received 5×10^{12} vg/kg of AAV6.2FF-hPLAP and AAV6.2FF-SEAP respectively, and piglet #3 was administered PBS in an inoculum volume of 0.5 mL/kg. In Study 2, piglet #4 was given 1.62×10^{13} vg/kg of AAV6.2FF-hPLAP, piglets #5 and #6 each received 1.73×10^{13} vg/kg of AAV6.2FF-SEAP, and piglet #7 received PBS in an inoculum volume of 0.5mL/kg. Serum samples were collected on days 0, 7, 14, 21, and 28 post-AAV. Piglets were euthanized 28 days post-AAV, and their lungs were harvested and divided into 16 sections, labeled A through P. Additional tissues, including the heart, kidneys, liver, spleen, and trachea, were also collected. Tissue samples were fixed, heat inactivated and stained to detect alkaline phosphatase (AP) reporter gene expression. After AP staining, tissues were imaged, embedded in paraffin, and sectioned for histological analysis to identify transduced cell phenotypes. Serum samples were analyzed with the Phospha-Light™ SEAP Reporter Gene Assay to quantify the levels of AP in the blood over time.

Our findings showed that endotracheal delivery via a laryngo-tracheal mucosal atomization device, especially at a higher AAV dose, efficiently transduces the lungs with no obvious off-target expression in auxiliary tissues.Gross imaging, SEAP expression, and histology collectively demonstrated that increasing the dose in Study 2 significantly enhanced both transgene expression and vector distribution in the lungs. Between the two reporter genes, SEAP was selected for future studies due to our ability to track transgene expression in the blood while retaining the benefit of visually staining tissues to evaluate transgene distribution. Future studies will utilize the higher AAV dose in combination with surfactant to maximize transduction efficiency. These findings demonstrate the ability of AAV6.2FF to transduce the lungs in a large animal model, providing a critical foundation for optimizing dosage and delivery methods, and representing a pivotal step toward advancing the clinical translation of this therapy.

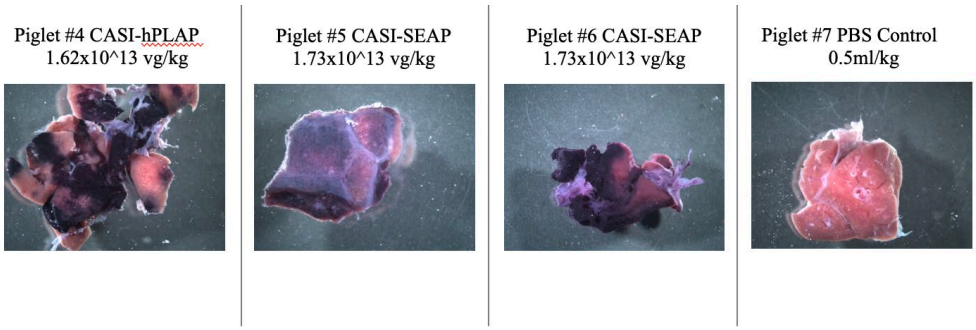

Figure 1: Gross images depicting human placental alkaline phosphatase (hPLAP) or secreted alkaline phosphatase (SEAP) expression in lung section E of piglets 28 days post-AAV administration from Study 2 with elevated doses.

1934 Extending Biolayer Interferometry (BLI) Principles to Develop a Simple Non – Cell Lysis Method for the Detection of Empty vs Full AAV Capsids

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Adeno Associated Virus (AAV) vectors are the leading platform for gene delivery for the treatment of a variety of human diseases. During development, AAV in-process samples often yield mixed populations of particles that include full capsids, partially full capsids and empty capsids amongst others. This heterogeneity during production necessitates that product characterization be efficiently done to establish the empty to full capsid ratios. While there are many techniques that have been deployed to detect these ratios, they are often either expensive or time consuming in addition to many other challenges hence making it difficult for developers to identify an ideal cost-effective technique. Sartorius's AAVX biosensor was developed to enable rapid, real time and high-throughput measurement of AAV capsid titer in samples across the AAV bioprocess workflow enabling quick process optimization, quality checks and increased productivity. In this talk, we propose a method that extends the use of the AAVX biosensors for use in determining empty to full capsid ratios; a method that does not require the lengthy and often inefficient capsid lysis to expose the ssDNA targeted for binding and subsequent detection by some comparative methods.

The principles of the BLI technology is such that binding on the tip of the biosensor results into a molecular layer which increases in thickness as more analyte molecules bind to the surface. The spectral pattern changes as a function of the optical thickness of the molecular layer is what is monitored at the detector. While the AAVX biosensor utilizes an anti-AAV antibody to bind to capsid proteins, the principles of detection of empty Capsids relative to full capsids on BLI capitalizes on the fact that although both bind to the antibody via the proteins on the surface of the capsids, the genomic insert in the full capsid imparts a density difference (Figure 1) between the two that leads to a difference in the binding saturation level when bound to the antibody on the biosensor surface

1935 Characterization and Optimization of AAV Transgene Cassettes Expressing HIV-1 Broadly Neutralizing Antibody 10-1074 for AAV9-Mediated expression in Non-Human Primates

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Background: Successful AAV-expressed antibody therapy for HIV-1 requires high antibody concentrations and reduced immune responses to sustain viral suppression without ART. In an ongoing study, co-administration of AAV9 expressing 10-1074 and PD-L1 led to high plasma concentrations of 10-1074 and low anti-drug antibody (ADA) responses. Based on previous findings that the WPRE enhances transgene expression, we aimed to increase 10-1074 expression to therapeutic concentrations (>50 µg/mL) with PD-L1 co-expression and evaluate the WPRE's impact. Six constructs with varying promoters and introns were packaged into AAV9 for in vitro and in vivo

assessment. The top four cassettes were selected for NHP studies to evaluate expression, ADA responses, and potency.

Methods: Six promoter and intron combinations (CMV, CMV/R, CBA, CASI, CB7, EF1a) expressing 10-1074 were cloned into AAV9 transfer plasmids. Recombinant 10-1074 proteins were expressed from each cassette and purified after transient transfection of HEK293T cells. In-vitro potency was assessed using the TZM-bl neutralization assay. Six groups of NSG mice (N=4) received $2.5x10^{12}$ vg/kg of AAV9 vectors and expression was assessed by gp120 ELISA for eight weeks. Following transgene selection, rhesus macaques were inoculated intramuscularly with AAV9 constructs (CB7, CMV/R, EF1α at $1.5x10^{12}$ vg/kg; CBA w/ WPRE, CBA w/o WPRE at $2.5x10^{12}$ vg/kg) and AAV9 expressing PD-L1 ($2.5x10^{12}$ vg/kg). Serum samples were analyzed over 20 weeks for 10-1074 expression (gp120 ELISA), ADA responses (Fab ELISA), and potency (TZM-bl assay).

Results: In-vitro expressed 10-1074 from transgene variants demonstrated similar potency to the expressed 10-1074 from the unmodified expression plasmid, indicating the retention of its functional neutralizing activity. Expression in mice showed the CMV promoter yielding the lowest average concentration (21 mg/mL) and the CB7 promoter achieving the highest (231 mg/mL). Other constructs from 94 to 109 mg/mL^{-1}. After week 12 in NHPs, 4 out of 5 groups neared or exceeded the therapeutic target (>50 mg/mL). Group 1 (CB7) had the lowest expression (13 mg/mL), while group 4 (CBA w/ WPRE) showed the highest (198 mg/mL), followed by Group 2 (CMV/R, 64 µg/mL), Group 3 (EF1α, 48 µg/mL), and Group 4 (CBA, 82 µg/mL). ADA responses across all groups remained low with group 1 and 2 having the lowest response. Lastly, ex-vivo neutralization assays from isolated sera demonstrated similar neutralizing activity (IC_{50} < 1 µg/mL) across all groups.

Conclusion: Our study demonstrates that AAV9 delivery of 10-1074, combined with PD-L1 expression, effectively achieves sustained antibody expression and potency while minimizing ADA responses, key requirements for successful HIV-1 therapy. The incorporation of WPRE significantly enhanced transgene expression, with the CBA + WPRE construct achieving the highest expression levels in NHPs. These findings highlight the importance of promoter and enhancer selection in optimizing therapeutic antibody expression and support further development of AAV-based strategies for HIV-1 treatment.

1936 A Cis-Regulatory Element Discovery Platform: Machine Guided Design and NHP Validation

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AAV gene therapies hold great potential to treat rare and complex genetic disorders, but are limited by potency and specificity. Promoters can provide control over tissue specificity and transgene expression strength, but most gene therapies utilize only a limited set of promoters, leaving a vast genetic design space for therapeutic optimization. To address this gap, Ginkgo has developed a comprehensive promoter optimization platform by leveraging machine learning and high-throughput screening. Here, we present measurements from ~40,000 short (258bp) core promoters across 18 cell models in vitro using Massively Parallel Reporter Assays (MPRA). This data set identifies subsets of promoter sequences specific to different tissue types and some showing comparable strength to commonly used constitutive promoters. While we have collected a wide consortium of MPRA measurements in several tissue types, it is also of interest to design promoters in tissues that we have not collected data for. To address this, we developed two machine learning approaches to model promoter activity based on availability of MPRA data in target tissues. In data-rich scenarios, we employ a supervised machine learning strategy that directly learns from experimental measurements. We demonstrate its use by training models on more than 330,000 regulatory sequences collected in vivo from mouse liver and skeletal muscle to design tissue-specific promoters. As a proof of concept, we leveraged this pipeline to design strong, fast-onset muscle-specific promoters. For tissues with limited promoter characterization, we propose a machine learning framework that trains on transcriptomic and epigenetic datasets in a wide range of tissue models, enabling computational design of tissue specific promoters when MPRA data is scarce. To validate experimentally and computationally determined promoter hits, an NHP study was initiated with an AAV library of 500 promoters introduced via two different routes of administration. Tissues were harvested and

sequenced to determine promoter specificity and strength across therapeutically relevant targets. We anticipate that this combined MPRA and machine learning based approach will enable identification of novel promoter sequences that translate to therapeutically relevant applications.

1937 Optimizing Plasmid Design to Enhance Safety and Efficiency in rAAV Production

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The AAV2 P5 promoter is commonly employed in the HEK293 cell-based production of recombinant adeno-associated viruses (rAAV) to express Rep78 and Rep68 proteins. While this promoter is known to operate in its original position relative to the Rep gene or downstream of the capsid gene, it sometimes causes the unintended incorporation of plasmid sequences into rAAV virions.

To resolve this, we developed constructs that removed the downstream P5 promoter and upstream backbone elements from the Rep-Cap plasmid. Our results revealed that removing only the downstream P5 promoter did not affect Rep78 expression but prevented the formation of replication-competent AAV (rcAAV). Additionally, removing the upstream backbone elements abolished Rep78 expression, indicating that residual backbone elements, rather than the full P5 promoter downstream of the Cap gene, were driving the non-specific expression of Rep78. Acknowledging the non-specific transcriptional activity of bacterial elements in eukaryotic cells, we redesigned the plasmid to eliminate these backbone elements and the truncated P5 promoter, replacing them with alternative promoters. Among ten tested promoters, one achieved good Rep expression and comparable genome titers to the control.

This study highlights the critical need to optimize plasmid architecture to eliminate non-specific transcriptional activity. By refining plasmid designs, we can ensure the efficacy and safety of gene therapies, mitigating unintended transcriptional consequences.

1938 Fueling Technological and Process Advancements for Increased rAAV Productivity to Meet the Industry's Growing Demand

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The growing demand for large-scale production of gene therapies utilizing recombinant adeno-associated virus (rAAV) vectors is driven by rapid advancement in technology, positive clinical trial results, and the growing number of commercially approved products. A major challenge in rAAV manufacturing lies in developing an optimized, cost-efficient, and scalable process for all rAAV serotypes, including novel capsid designs, to supply both the growing number of programs and large patient populations for these therapies. Forge Biologics has launched a new manufacturing platform called "FUEL™" for AAV gene therapy production, designed to improve process efficiency, productivity and safety by combining its proprietary pEMBR 2.0™ Ad helper plasmid, the Ignition™ cell line, and optimized rep/cap plasmids, all working together for an optimized, efficient and a scalable production process. The synergy between the FUEL™ platform and Forge's tailored upstream and downstream process results in increased rAAV productivity, and therefore reduced manufacturing costs of goods sold (COGS).

Forge's tailored downstream process is designed to support expedited therapeutic rAAV pipeline programs, delivering high yield and high product quality. In our downstream process, crude lysate from the optimized upstream process is clarified using systematically tested depth filters. Following clarification, the rAAV vector is captured using a predetermined resin-buffer combination, based upon our experience with multiple serotypes. Depending on the outcomes, a more customized affinity resin process with specific buffer conditions is incorporated into the process for high recovery and product quality. Subsequent enrichment of the vector material involves

separating full capsids from empty capsids through either cesium chloride ultracentrifugation or anion exchange chromatography. The method of enrichment is determined by program needs and timeline. The final step involves taking the full rAAV capsids through the final formulation process and filling the drug product utilizing state-of-the-art fill/finish processes. This process yields a highly pure rAAV suitable for early preclinical applications, toxicological studies, or clinical use.

The advancements made possible through the development of the FUEL™ platform and optimized process have shown 13x-22x increase in vector productivity compared to a standard process. Forge has systematically tested the rAAV products generated through these advancements on multiple serotypes across multiple scales. This comprehensive approach enables efficient progression through process development and facilitates cGMP manufacturing to achieve optimal yield and reduce process variability. The success of each purification step is verified through stage-appropriate analytical testing, including GOI titer by ddPCR, residual plasmid DNA by ddPCR, product purity by SDS-PAGE or capillary electrophoresis, genome integrity through next-generation sequencing, empty:full particle ratio by analytical ultracentrifugation, potency through qualified cell-based assays, residual host cell DNA, residual host cell protein, endotoxin, and other relevant tests. This presentation will cover key data generated with the FUEL™ platform on rAAV productivity and the product quality attributes of the final drug product.

1939 EPI-321 Development: Strategies to Establish a Scalable and Robust rAAVrh74 Upstream Manufacturing Process from 0.5 L to 1000 L Scale

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EPI-321 is a therapeutic designed to treat facioscapulohumeral muscular dystrophy (FSHD), leveraging the proprietary “GEMS” platform developed by Epicrispr Biotechnologies, that consists of a guide RNA, dead Cas protein, and modulator proteins. These components allow for regulation at the epigenetic level to address the root cause of diseases, without permanently altering DNA. While traditional gene editing therapies are known to cause unwanted cell growth and/or immune responses, EPI-321 provides this embedded layer of safety. Gene therapies for muscular dystrophy have commonly utilized AAVrh74 as their delivery vehicle due to its tropism to muscle cells and demonstrated safety in the muscular dystrophy space. Meeting pre-clinical and commercial demands is challenging when considering the high dosage requirements for systemic administration in adult patients. To minimize the cost of goods, it is imperative to maximize product yield and demonstrate robustness to scaling. Here, we will discuss strategies that aid in creating a commercially viable, scalable, and robust upstream manufacturing process. Improving yield often involves optimizing the transfection step. Two common strategies are to implement engineered plasmids and/or an optimized transfection reagent. By removing non-essential adenoviral components in the Helper plasmid, we observed a 2-fold increase in clarified lysate titer. Next, an engineered RepCap plasmid was evaluated across three transfection reagents. By integrating Helper 2.0, RepCap 2.0 and Transfection Reagent #2 into the EPI-321 manufacturing process, we improved clarified lysate titer by 20-fold at 0.5 L scale. To ensure process scalability, it is advantageous to define strategies early on. The remaining experiments we discuss here characterize critical process parameters for enhanced control of critical quality attributes. Understanding how volume-dependent and volume-independent parameters affect cell culture is necessary to ensure batch-to-batch consistency. Volume-dependent parameters such as agitation and aeration rate are difficult to keep constant with changing vessel geometries. To better characterize agitation rate, we assessed the impact of tip speed on productivity at 5 L scale. While newer cell lines are more resilient to hydrodynamic stress, it is recommended to keep tip speeds as low as possible. In our study, as tip speed approached 1.5 m/s, clarified lysate titer decreased by 25%, suggesting the presence of shear stress. Lastly, a large challenge for process developers is to scale the transfection unit operation. As batch volumes increase, so do transfection complex volumes; however, chemically defined limitations, such as complexation time, remain unchanged. By characterizing complexation time, we can define acceptable ranges to allow flexibility in transfer flow rate(s). To understand how transfer flow rate impacts titer, we assessed different flow regimes (laminar, transitional, turbulent) using Reynolds number. At 5 L scale, a transitional flow rate decreased titer by 35% when compared to laminar flow. To complement this data, a complexation time-course from 15 to 75 minutes was conducted to help define operational limits. The data from these studies informed a robust transfection strategy that helped scale the EPI-321 manufacturing process from bench-scale to 50 L, 200 L and 1000 L scale.

1940 Leveraging Historical Data and Percentile based Modeling for Improved AAV Production Scaling

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Viralgen is a Contract Development and Manufacturing Organization (CDMO) dedicated to the production of adeno-associated viruses (AAVs). The organization employs an innovative and standardized manufacturing platform utilizing Pro10™ cells, which facilitates the production of diverse AAVs and allows scaling from 2L Erlenmeyer flasks to 2000L bioreactors. This universal platform for different AAV products has enabled the generation of data from over 1000 batches produced under the same process across various scales and serotypes, providing a wealth of valuable knowledge based on prior experience.

Scaling from smaller to larger volumes represents a critical factor impacting both CDMOs and their clients, as inaccurate estimations of required capacities to meet product demands can lead to significant and unwanted costs for both parties involved. Historical data from Viralgen over recent years indicates that productivity is scale-dependent, likely due to differences in bioreactor dynamics, geometry, and other operational parameters, which complicate the ideal scaling process. Contrary to the industry's conventional belief in linear scaling, this variability can be manageable if a suitable method is identified to describe the scaling pattern, enabling more accurate productivity predictions.

Currently, the approach for estimating or projecting productivity assumes a linear relationship between scales, applying a 1:1 factor for productivity predictions. However, the data science department at Viralgen has developed a percentile-based model that has successfully reduced prediction errors by 40-50%, depending on the scales for the projection. In this model, productivity between scales is not treated as constant; instead, it is determined based on historical performance metrics, specifically mean values derived from past production data.

The model calculates the percentile of each product relative to already produced scales, providing insights into the product's performance on the platform. This information aids in decision-making regarding whether to optimize the product or process before initiating the scaling process. Once the performance is established, productivity estimates for the next scale are made using a linear regression model that incorporates historical data.

Furthermore, this model will be semi-automatically readjusted with new platform data as it becomes available, with the expectation of further reducing prediction errors by more than 50% compared to the current linear approach. By leveraging historical data and advanced modeling techniques, Viralgen aims to enhance the accuracy of its productivity projections, ultimately improving the efficiency and reliability of AAV production for its clients.

1941 Gaining control of rAAV production: real-time monitoring with Raman Spectroscopy

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During the production of recombinant adeno-associated viruses (rAAVs), slight variations in cell metabolism can affect overall production process in terms of AAV yield and quality. Therefore, close monitoring of offline parameters such as metabolites (Glucose, Lactate, Ammonia and Glutamine) as well as cell density is essential for rAAV vector manufacturing. The application of Raman spectroscopy can help optimize the sampling procedure by providing real-time monitoring of cell growth, nutrient consumption and metabolite accumulation, and may even be able to quantify the rAAV production online. This continuous monitoring will enable faster process optimization, improved error detection and pave the way for process automation via on-demand feeding strategies. To drive innovation in rAAV vector production, this study investigated the use of the ProCellics™ Raman Analyzer to monitor key parameters in real time. The results are used to further optimize our manufacturing platform and integrate emerging market trends.

Raman spectroscopy probes were integrated into four stirred-tank bioreactors (2L and 5L) to collect data for model development. All bioreactor runs leveraged our proprietary EpyQ® two-plasmid system, combined with PEI transfection, to produce AAV2 vectors carrying a 3kb transgene. Different feeding strategies were the only distinguishing feature to create a more representative model. Offline data, including metabolic profiles (BioProfile® FLEX2) and cell density (Vi-CELL BLU analyzer), were collected simultaneously. Capsid titers were determined during lysis using Gyros Immunoassay. These comprehensive data were then used to develop a process-specific analysis model based on the Raman spectral data. The prediction accuracy of this model was subsequently validated in a second round of independent bioreactor runs using the same setup and also adding an AAV9 Capsid serotype to check the generated model robustness.

This successful collaboration between Ascend and Merck has generated a wealth of online data provided by ProCellics™ Raman technology, eventually eliminating the need to generate an excessive number of offline datasets. By enabling online capsid quantification, we can directly proceed with DSP using the appropriate affinity column size, avoiding the time-consuming wait for analytical assay results.

While our current platform has proven to be highly robust and reliable, consistently delivering excellent results, we are constantly seeking to improve our process. This proactive approach ensures that we maintain a competitive advantage, consistently apply best practices, and uphold the highest quality and compliance standards in our AAV gene therapy manufacturing processes.

1942 Optimizing Harvest Time for AAV Full Capsids through Analysis of Production Kinetics in an Industrial System

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The current state of industrial viral vector manufacturing is failing to meet the demands required to deliver life-saving gene therapies to patients. Many existing manufacturing workflows, adapted from suspension-based monoclonal antibody (Mab) processes, result in suboptimal AAV productivity. While the industry has largely focused on suspension systems, adherent processes that harness the natural adaptability and efficiency of HEK293 cells for viral vector production have remained underdeveloped. Today, new process intensification methods allow for a reassessment of the optimal model for AAV manufacturing, with the goal of reducing financial barriers for patients, expanding treatment availability, and alleviating healthcare burdens associated with rare genetic disorders.

VintaProcess is an advanced, intensified adherent manufacturing platform designed to eliminate unnecessary complexity, reduce costs, shorten timelines, minimize operator error, and expedite regulatory approval for early and late-stage clinical assets. The platform uses a fixed-bed bioreactor, ideal for analyzing AAV production kinetics to determine the optimal harvest time and efficient implementation into an industrial process—a challenge for suspension systems. Theoretical evidence suggests that viral DNA replication initiates later than capsid synthesis resulting in empty capsids secreted into the cytosol leading to a high empty-to-full capsid ratio. Inherent to VintaProcess™ is a native barrier that allows effective separation of AAV supernatant from the adherent cells, thus

enabling one to determine the optimal temporal window for full-to-empty capsid enrichment at harvest.

To investigate AAV production kinetics, we conducted experiments analyzing genomic titer and AAV8 capsid production over time. Empty and full capsid production was quantified at specific time intervals, with sampling beginning one-hour post-transfection to 116 hours post-transfection. Additional media exchanges were executed post-transfection to replenish cells with fresh media. Impurity profiles were evaluated to ensure clinical and commercial suitability, including residual plasmid analysis to assess plasmid cellular uptake over time.

Unlike suspension processes, VintaProcess offers a scalable, efficient AAV manufacturing platform that inherently enriches full capsids at harvest. This approach enhances AAV efficacy, reduces run times, and lowers downstream processing burdens by minimizing empty capsids. To advance gene therapy, a paradigm shift is required – one that simplifies AAV manufacturing and fulfills the promise of delivering life-saving treatments to patients.

1943 Enhancing the Upstream Performance of Adeno-Associated Virus (AAV) Vector Manufacturing via Multivariate Data Analysis

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Viralgen as a leading AAV Contract Development and Manufacturing Organization (CDMO) has built a large proprietary data set based on the production of more than 1000 batches produced across multiple scales and constructs using the Pro10 production platform. The goal of our work is to improve the AAV upstream process by identifying with our proprietary data set which parameters exert the most significant impact on titer.

Process variability and high number of process variables create hurdles for the optimization of the manufacturing process. The first results mainly from the nature of biological processes, accuracy of measurements, and manually operated processes; whereas the latter is implied by the hundreds of simultaneous measurements collected for each sample. Traditional univariate or bivariate techniques fail to simultaneously catch the relationships between many variables.

To address the optimization goal with the associated challenges mentioned, dimensionality reduction approaches were applied, and a multivariate data model was developed using data retrieved from over 100 historical batches. The model estimates the relationship between the important process parameters (e.g., pH, metabolite concentration) and product characteristics, with titer as response variable.

Our findings suggest that transfection efficiency decreases as a result of the cell density effect. Specifically, there is a significant negative correlation between vector titer and viable cell density (VCD). Dissolved oxygen levels on the day of the bioreactor inoculation, as well as pH and glucose levels before transfection, along with the transgene size, have also been shown to play crucial roles in batch performance. An experiment was conducted to explore different VCD levels beyond the current process's operational range, confirming the high impact of VCD and aiding to identify its optimal level regarding titer. In addition, it shows that lower viable cell density results in lower pH and higher glucose remnants right before transfection.

To integrate the multivariate analysis in daily operations in Viralgen and to bridge the gap between Data Scientists and process experts, we developed an application which identifies contributing factors to the titer of a historical batch in comparison to others in an automated way. This tool will be utilized by the Manufacturing Sciences & Technology (MSAT), Manufacturing and R&D scientists to review the performance and identify potential process improvements in a continuous fashion.

Future enhancement of the model will include the addition of transfection process information as well as full/empty ratio estimation. In addition, we plan to extend the model to explore mechanistic modeling, incorporating information on metabolic pathways and the virus production process. This will provide a more comprehensive understanding of the interactions and dynamics involved in cell expansion, transfection and virus production.

1944 Streamlining Advanced Therapy Medicinal Product Manufacturing: A Platform Approach for Accelerating Gene Therapy Commercialization at Viralgen

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Viralgen Vector Core, S.L.U. specializes in the manufacturing of Advanced Therapy Medicinal Products (ATMP), specifically adeno-associated viruses (AAVs) for gene therapy applications. Their manufacturing process utilizes a state-of-the-art triple transfection method with a HEK-293-derived suspension cell line (Pro10^{TM} cell line), enabling the production of various AAV serotypes while maintaining consistent quality attributes. The unique nature of gene therapy presents challenges in establishing a cost-effective manufacturing process. Viralgen proposes a platform approach that leverages shared characteristics across different AAV serotypes to streamline operations and reduce variability. This strategy unfolds in three stages: Establishing a platform control strategy; generating experimental data to refine this strategy; and verifying it through specific studies. By adopting this approach, Viralgen aims to enhance the robustness, reliability, and predictability of its manufacturing process, from pilot to clinical to commercial scale, by leveraging process knowledge between different AAV serotypes and various drug products ensures consistency, robustness, process, and provide high-quality output with limited data. Ultimately, this methodology has the potential to address unmet medical needs, shorten development timelines, and improve accessibility and affordability of gene therapies.

1945 Accelerating AAV manufacturing timelines via a novel Rolling Seed Approach

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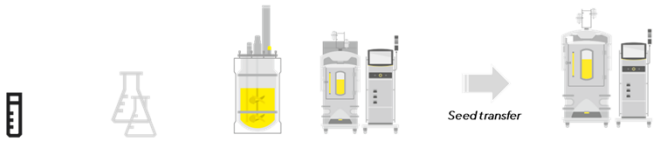
The seed expansion stage is a pivotal phase in AAV production, significantly impacting the overall process duration and efficiency. This study explores three seed expansion strategies: Traditional, Rolling Seed, and a combination of Frozen Seed and Rolling Seed, each offering distinct advantages and challenges. In a 500 L AAV production scenario, the Traditional approach, which takes 22 days, is compared to alternative strategies that can potentially reduce the seed expansion stage to as few as 8 days. These alternative approaches not only shorten the timeline but also enhance process flexibility and enable continuous seed production, facilitating rapid initiation of subsequent AAV batches.

As a proof of concept, multiple AAV batches were produced using the novel Rolling Seed approach. To demonstrate its broad applicability, the novel Rolling Seed method was tested with various suspension HEK293 cells, transfection protocols and bioreactor types, such as stirred tank and rocking motion. Scalability was further demonstrated by expanding the concept from a multiparallel scale-down 250 ml bioreactor system to a 2 L scale. These experiments highlight the Rolling Seed approach's operational flexibility, enabling seamless transitions between production cycles and adaptability to different bioreactor scales and types.

The study was paired with advanced semi-automated analytical techniques, such as the biolayer interferometry (BLI)-based Octet® platform and the Incucyte® Live-Cell Analysis system. As with any emerging process strategy, this study identifies potential bottlenecks that require further characterization and optimization to enhance process efficiencies even further.

Traditional approach



Vessel type	Cryovial	Shaker flasks	Seed bioreactors: 10 L → 50 L STR → 500L	Production bioreactor: 500 L STR
Process volume	Cryoculture	→ up to 0.3 L	→ 1-500 L	→ 500 L
Duration	0 day	~ 7 days	~ 22 days	~ 2-3 days

Viral vector rolling seed



Vessel type	Cryovial	Shaker flasks	Seed bioreactor: 50 L STR	Production bioreactor: 500 L
Process volume	Cryoculture	→ up to 1.2 L	→ 12.5-50 L	→ 500 L
Duration	0 day	~ 10 days	~ 19 days ∞	~ 2-3 days

Frozen seed & Rolling seed



Vessel type	Celsius Pak	Seed bioreactor: 50 L STR	Production bioreactor: 500 L
Process volume	100 ml (5E7 cells/ml)	→ 12.5-50 L	→ 500 L
Duration	0 day	~ 8 days ∞	~ 2-3 days

1946 Optimizing Recombinant AAV Vector Production: Ehnacing Upstream and Downstream Processes for Improved Yield and Quality

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Recombinant adeno-associated virus (rAAV) vectors are one of the most widely used gene therapy delivery vehicles due to their ability to achieve stable and long-term gene expression with minimal immunogenicity and pathogenicity. Since the first FDA approval of Luxturna in 2017, the demand for AAV-based therapeutics has increased significantly. However, existing manufacturing technologies still face challenges in meeting current demands. These challenges are present in both the upstream cell culture process and downstream purification steps. In this study, we aim to bridge the gap between vector manufacturing and demand by identifying key bottlenecks in the HEK293-based rAAV platform and implementing process optimizations to enhance productivity, increase purification yields and product quality.

In the upstream production, we used HEK293 cells for their transfectability and ability to grow in suspension in serum-free media, both crucial for scalable production. However, HEK293 cells show inherent biological variability and transient transfection poses challenges, including cytotoxicity in post-transfected cells due to transfection reagents such as polyethyleneimine (PEI), which leads to batch-to-batch variability. To address these issues, we developed a novel suspension serum-free HEK293 cell population with characteristics comparable to commercial cell lines and additionally improved scalability attributes. Prior to optimization, our cell line achieved a productivity

of 2.5 × 10^{11} vg/mL with a 50% viability and viable cell density of 2.5x10^6 cells/mL at harvest. Through our development, we achieved a 10-fold full capsid titer increase, with 80% viability and a viable cell density of 7x10^6 cells/mL at harvest at shake flask scale. Also, we successfully scaled up transfections to 2 L glass bioreactors, demonstrating the feasibility of large-scale production.

In the downstream process, we focused on improving yields in affinity chromatography, minimizing aggregation, and optimizing ion-exchange chromatography for better full/empty capsid separation. While the affinity step is crucial, low yields often limit efficiency. To address this, we investigated the impact of AAV sample preparation with ultrafiltration and diafiltration (UF/DF), prior to injection into the affinity column. Our results demonstrated that protein clearance with UF/DF process significantly improves AAV yields post-affinity chromatography, with 50% protein clearance leading to a 10% yield increase. Further increasing protein clearance to 90% resulted in an additional 15% AAV recovery, highlighting the critical role of impurity removal. After the affinity process, we optimized buffer composition by testing various excipient and surfactant combinations to enhance monomer stability before ion-exchange chromatography. Finally, we fine-tuned the salt gradients to further optimize the anion-exchange chromatography (AEX) step.

Our study highlights critical processes improvements that enhance AAV vector manufacturing by addressing key challenges in both upstream and downstream workflows. The development of a robust, scalable HEK293 suspension cell line, combined with refined purification strategies, offers a pathway toward more efficient, reproducible, and high-yield AAV production. These optimizations are essential to meet the growing demand for AAV-based gene therapy vectors while maintaining high-quality standards for clinical and commercial applications.

1947 Combining Fixed Bed Bioreactor and Liquid-Phase Affinity Purification Technologies for Next-Generation, Low-Cost Lentiviral Manufacturing

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Manufacturing lentiviral vectors (LVVs) presents significant challenges due to their complex production process and stringent safety requirements. The delicate nature of these viral vectors demands precise conditions for cultivation and multiple, low-efficiency purification steps, often leading to low yields and high costs. Additionally, ensuring consistent quality and functionality while adhering to rigorous regulatory standards adds further complexity to the LVV manufacturing pipeline. Despite these difficulties, LVVs are invaluable tools in gene therapy and biomedical research, as evidenced by the approval of several lentiviral-based therapeutics with significant clinical benefits, albeit with high drug costs impairing broad adoption. Here, we combine flagship technologies from Isolere Bio and Univercells Technologies to remedy the inefficiencies found throughout LVV manufacturing and drive down costs significantly.

Univercells Technologies scale-X™ fixed-bed bioreactor technology has demonstrated high yields of infectious LVV production in batch and perfusion mode from R&D to commercial scale. The structured fixed-bed within the scale-X bioreactor enables high-density cell culture within a small footprint. LVV production with the scale-X bioreactor achieves upstream titers comparable or up to 3-fold higher than standard flask process and up to 10-fold higher than competing bioreactor technologies. Viral molecules produced in scale-X have also shown to contain up to 10x lower host cell protein and DNA levels compared with traditional methods, potentially simplifying clarification and downstream steps.

Isolere Bio's IsoTag™ LV affinity reagent enables non-chromatographic purification of lentiviral vectors with high recoveries and purity, minimal unit operations, and total processing time under 8 hours. Traditional downstream processing requires multiple steps – typically a TFF, AEX, and SEC, with LVV losses seen at each stage. With IsoTag LV that is reduced to a single TFF operation due to the on-command droplet forming technology. LVVs are sequestered into highly pure, reversable droplets and quickly purified using microfiltration via TFF. With this process, LVV is concentrated 100X with 30% functional recovery and > 2 LRV of host cell proteins and DNA.

In this study, for the first time, we combined the two complementary technologies to both produce and purify high quality LVV at half the cost of traditional processes, while decreasing total manufacturing time by one week, and halving the required amount of consumables. Only two pieces of equipment (scale-X bioreactor and TFF) were required to manufacture LVV from start to finish in a proof-of-concept study. We were able to blend these upstream and downstream technologies and add IsoTag LV directly into the scale-X bioreactor, streamlining processing. Importantly, due to minimal purity requirements for the IsoTag process, we were able to remove the nuclease treatment and clarification steps. This did not impact purification performance nor the purity profiles in the final product.

In conclusion, by pairing disruptive upstream and downstream technologies, we demonstrate the possibility of redefining lentiviral vector manufacturing in a simplified, low-cost workflow. We expect this novel approach to facilitate the production of more accessible cell and gene therapies worldwide.

1948 Optimization of AAV Upstream Production for a Low Producing Vector

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Adeno-associated viral vector production is a complex biological and technical process that often involves triple transfection of plasmids into human embryonic kidney (HEK) cells which deliver copies of RepCap, Adenovirus helper genes, and the Gene of Interest (GOI) to generate and package therapeutic AAVs. When the GOI is expressed via a ubiquitous promoter, the protein product of the GOI will be expressed in the HEK cell line following triple transfection, which, depending on the GOI, can lead to cytotoxicity or inhibition of vector production, giving lower-than-expected overall vector yields.

In this study, we present our findings on production optimization of AAV-GOI-1, which originally showed lower-than-expected vector yields in HEK cells. We demonstrate that the lower yields were caused by the function of the GOI-1 protein product. To address this issue, we developed several strategies to improve production yields. These strategies included using biochemical tools to reduce the expression and/or function of GOI-1 during HEK production and employing the Sf9/Baculovirus production platform to avoid GOI-1's expression during vector production. The results from these studies have enabled a potential path forward for vector production of GOI-1 using either the HEK or Sf9 platforms and showcase the strengths of each platform for production of therapeutic AAVs.

1949 Adeno-Associated Viral Vector Production Process Intensification

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Introduction: Intensification of recombinant AAV (rAAV) vector production offers significant potential for manufacturing improvements, including increased product yield and quality and decreased cost of goods. This study reports the results of rAAV process intensification, focusing on high cell density transfection and the application of a production enhancer. Tangential flow depth filtration technology (TFDF, Repligen) was used to enable high cell density transfection, cell culture perfusion, and clarification of bulk harvest. An AAV enhancer (RevIT™, Mirus Bio) was added to improve the rAAV yield.

Methods: The effect of RevIT™ on rAAV production was evaluated with Catalent's® clonal cell line (CAT293) propagated in CTS™ Viral Production Medium (Thermo Fisher). Catalent® Off-the-Shelf (OTS) plasmids for serotypes 1, 2, 5, 6, 8, and rh10 were tested with various transfection reagents. For transfection at high cell density, batch and perfusion modes were compared for rAAV5 and rAAV8 productions in 2-L single-use bioreactors

(Sartorius) using Catalent® OTS plasmids. The concentration of plasmid DNA was normalized to cell density at the time of transfection. Batch mode bioreactors were transfected at viable cell densities of 2.0-3.0´10^6 vc/mL and harvested 3 days post-transfection by centrifugation. Perfusion mode bioreactors, equipped with 30 cm^2 or 50 cm^2 TFDF filters, were operated at 1-1.5´ vessel volume per day (VVD) to achieve 2.5-fold to 3.5-fold higher cell density at transfection compared to batch mode. During production, an AAV-containing conditioned medium was continuously collected as a TFDF permeate fraction. At harvest, cells were lysed in the presence of nuclease using a buffered surfactant. Lysates were clarified by TFDF and/or centrifugation. While a 30 cm^2 TFDF filter size with a surfactant concentration of 0.5% resulted in filter clogging, successful clarification was achieved using a 50 cm^2 TFDF filter and 1% Polysorbate 20.

Results: The addition of RevIT™ in shake flask experiments increased the vector genome and vector particle titers by 2-4 times without affecting vector packaging capacity. In 2-L bioreactors, the assessment of the transfection at a 2.5-3.5-fold higher cell density in perfusion culture demonstrated a 2.5-3.1´ increase in rAAV vector genome and particle yields compared to the traditional batch approach, both with and without the addition of the RevIT™ enhancer. The addition of RevIT™ enhanced vector genome yields more than 2.5 times for AAV5 and 1.7 times for AAV8. The combined effect of high cell density transfection and RevIT™ application on vector genomes was calculated at 8.8 times for AAV5 and 4.4 times for AAV8. The AAV5 vector yield in a 2-L perfusion process achieved 6.45´10^{15} vector genomes per bioreactor. For AAV8, the perfusion process yielded 4.65´10^{15} vector genomes per 2-L bioreactor.

Conclusion: A comparison indicated that a single intensified perfusion process could replace nine batch runs for AAV5 and four for AAV8. This study demonstrates the potential of high cell density transfection combined with RevIT™ application to significantly enhance rAAV production efficiency and scalability.

1950 Comprehensive Evaluation of TruStable™ vs Transient Production Systems for AAV Vector Manufacturing

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While adeno-associated virus (AAV) vectors are predominantly manufactured using transient transfection systems, stable producer cell lines offer significant advantages in consistency, scalability, and process economics through elimination of plasmid-related costs. In this study, we conducted a systematic comparison of AAV vectors produced via the TruStable™ producer platform versus traditional transient transfection using identical constructs. Vector quality attributes including full-to-empty capsid ratios, VP1/2/3 ratios, in-vitro potency, and impurity profiles were evaluated across multiple production runs. The TruStable™ system demonstrated superior process yield, percent full capsids, and batch-to-batch consistency in viral titers while eliminating plasmid-related impurities. Analysis of genome packaging revealed higher percentages of full capsids in the TruStable™ platform (up to ~60% full in cell lysate), while capsid protein composition (VP1/2/3 ratio) remained consistent between systems. Vector potency of terminally purified materials was comparable between platforms, with both systems producing functional particles capable of efficient transgene delivery. This comprehensive characterization demonstrates the clear advantages of the TruStable™ platform for AAV manufacturing, particularly in achieving high quality vectors with improved process yield and consistency, resulting in reduced costs.

1951 Recent Advancements in Enhanced Starting Materials and Process Optimization for Manufacturing High Yield rAAV in Suspension HEK293 Cells in Single-Use Bioreactors

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Recombinant adeno-associated virus (rAAV) is the leading platform for in vivo gene therapy delivery due to its low pathogenicity, ability to target a wide range of tissues (broad tissue tropism), and high efficiency in delivering genetic material to cells. The commonly used platforms to manufacture rAAV are transient plasmid transfection of HEK293 cells, infection of HEK293 (or BHK-21) cells with recombinant herpes simplex virus (rHSV), infection of *Spodoptera frugiperda* (Sf9) with baculovirus expression vectors, and stable producer cell lines with induction of gene expression with doxycycline or Adenovirus infection. A major obstacle in producing rAAV using HEK293 cells in the triple transfection method is the relatively low yield of viral vectors compared to the other production methods. To address this problem, Forge Biologics has recently launched FUEL™ (Foundation for Unleashing Excellence in Life-Changing Therapies) AAV manufacturing platform, which leverages enhanced starting materials (optimized Ad helper and rep/cap) and product-specific process optimization, and this has resulted in remarkable improvements in rAAV vector yields. Upstream process optimization is achieved through transfection design of experiments (DoE) studies by evaluating plasmid ratios, DNA/cell, PEI/DNA ratios, and transfection reagents.

Our platform process utilizes our Ignition™ HEK293 suspension cells that are well-documented for robust transient transfection. The process begins with thawing a cell bank (RCB, WCB, or MCB) and passaging it through seed expansion before going into the production vessels, typically at the 50, 500, or 1000L scale. Once the cells reach the required density for transfection, they are transfected with three plasmids (GOI, rep/cap, pEMBR™ Ad helper) with the help of a transfection reagent. The culture is then monitored for a few days and at harvest, cells are lysed with detergent and nuclease treated prior to clarification. The clarified lysate is loaded on the appropriate affinity column and the eluted product is further polished through CsCl ultracentrifugation or AEX purification, depending on the client's preference. The full capsid product from the polishing step is buffer exchanged into the final formulation and taken through sterile filtration steps for the drug product. This process has been used in over 300+ runs over the last 3 years to produce rAAV gene therapies. Improvements have also been made in plasmid ratio optimizations, addition of transfection enhancers, and affinity optimizations leading to improved titers and improved recovery.

This poster presentation will show data that demonstrate how the FUEL™ platform can improve the manufacturability of rAAV through optimization of the rep/cap and Ad helper (pEMBR 2.0™) design, highlighting its transformative impact on AAV manufacturing efficiency, scalability, and cost of goods sold (COGS). These improvements are often additive and when coupled with the FUEL™ platform, 3-22-fold yield improvements have been achieved.

1952 Optimizing Upstream Process Inputs to Enhance Viral Yields and Ensure Product Quality in Adeno-Associated Virus (AAV) Manufacturing

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Adeno-associated viruses (AAVs) have emerged as a prominent therapeutic treatment for life-threatening genetic diseases due to its tissue specificity, low pathogenicity, and long-term efficacy. Bioprocessing for AAV gene therapies is complex, making development of robust, cost-effective, streamlined processes for large-scale manufacturing challenging. An optimized upstream workflow, that maximizes viral yields and ensures consistent product quality, is a crucial step towards addressing this bottleneck. In this case study, we present our design of experiments (DOE)-based approach to optimizing key upstream process inputs for AAV production, with a focus on HEK lineage, cell culture media, and transfection reagents. Additionally, we will share data that characterizes how the predictive models generated from small-scale DOE optimization studies translate to bench and pilot-scale production systems, with a focus on viral titer and product quality.

1953 Scalable Bioprocessing Strategies for Intensified Viral Vector Production in HEK293 Cell Culture

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Maintaining key culture conditions, such as nutrient supply, mass transfer, and mixing, is essential for achieving consistent cell growth, metabolic behavior, and viral vector production. This presentation explores how intensified cell perfusion systems can work hand in hand with cell culture media development to address these challenges. Our focus for this work is on scale-up strategies that ensure reproducible cell growth and viral vector production across different scales in HEK293 cell cultures.

Join us to discover how to:

- **Enable optimization of nutrient balance**, execute efficient waste management, and achieve consistent and scalable HEK293 cell growth via a specialized HEK293 media perfusion process
- **Intensify viral vector production** by transiently transfecting cells across various perfusion processes and bioreactors
- **Achieve consistent HEK293 cell metabolic profiles** in different cell densities, demonstrating a robust and versatile bioprocess with precise control of bioprocess parameters
- **Reduce operational costs** while enhancing titer yield with the combination of automated control systems and advanced perfusion techniques

1954 Assessment of two HEK293 Cell Line Cloning strategies to improve AAV yield

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Adeno-associated virus (AAV) has proven clinical efficacy in delivering therapeutic payloads, potentially curing diseases with a single dose. However, the low volumetric yield of AAV during manufacturing contributes to the high cost of AAV-based therapies. Currently, most AAV production relies on transient transfection in HEK cells. To increase AAV yield, it is essential to develop more productive cell lines for AAV expression.

We implemented two strategies to identify high producing clones for Voyager's engineered AAV capsids, using different parental HEK cell lines. In the first approach, we started with an internal clonal cell line, which produces $>1 \times 10^{10}$ vg/mL of various novel capsid and transgene combinations. We isolated greater than 700 single-cell clones from VYGR-293, which were seeded into 96-well plates using a single cell sorter device. Of these, more than 200 colonies were grown, transferred to 24-well plates, and cultured in suspension for productivity screening through transient transfection. After several rounds of screening, the top 10 clones were identified, with a lead clone that met our productivity criteria, including improved clumpiness and doubling time. In the second approach, we used an alternate HEK293 non-clonal cell line. A limiting dilution method seeded more than 700 cell groups in 96-well plates. After 14 days, more than 200 cell pools were expanded to 24-well plates, cultured in suspension, and screened for productivity. Following two rounds of screening, the top cell pools were expanded into shake flasks. After additional rounds of screening, a lead cell pool was identified and further adapted to five different media conditions, showing comparable titers to the lead clone from the prior approach. When combined we believe these approaches have the potential to yield a clone that could significantly further enhance AAV production.

1955 High cell density AAV production

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The production process of adeno-associated viruses (AAV) is notably expensive, and reducing production costs has consistently been a challenge for companies involved in AAV manufacturing. One effective strategy for improving productivity in pharmaceutical manufacturing processes that include cell culture is to increase the cell density during cultivation. In the case of antibody production using Chinese hamster ovary (CHO) cells, numerous methods for high-density culture exceeding 10^7 cells/mL have been reported. However, when it comes to AAV production using HEK293 cells, most methods do not surpass the 10^6 order range. This limitation arises because increasing cell density often leads to a decrease in AAV production per cell, resulting in little to no increase, or even a decrease, in overall AAV yield. To address this issue, we have developed a novel approach that combines high cell

density culture with a unique media exchange process which enhances AAV productivity more than threefold. This method is applicable to any transfection reagent or AAV serotype. Therefore, the same materials as in the original process can be used for the high cell density process, and no labor is required for either new residual impurity evaluation or new material management, and related processes. This innovative culture method, which is currently under patent application, will be presented.

1956 Increased AAV Yield: From Screen to Scale Up to Cost Savings Using Viral Sensitizer (VSE™) Technology

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Manufacturing cell and gene therapies (CGTs) is complex and expensive, often resulting in insufficient yields to satisfy commercial demand and high costs that may limit patient access. With an anticipated rise in demand for CGTs in the coming years due to ongoing successful development for new and broader therapeutic applications, it is increasingly crucial to understand the key production cost drivers and explore novel technologies for creating more accessible and affordable therapeutics.

An often underappreciated bottleneck in producing viral vectors, including adeno-associated virus (AAV), are the highly conserved innate cellular defenses present in manufacturing cell platforms. These defenses, triggered during infection, induction or transfection-based manufacturing strategies, can negatively impact both viral vector yield and product quality. Virica's Viral Sensitizers (VSE™) platform offers a novel solution: proprietary small molecule cell enhancers that transiently dampen innate cellular defenses and increase the production of viral vectors across a broad range of cell platforms. VSEs are simple upstream additives, rapidly metabolized by cells with non-detectable residual levels, and can be combined to drive additive enhancements in viral vector yield and significant reductions in manufacturing costs.

Here, we detail the identification and validation of expanded portfolio VSEs specifically for AAV production, using Virica's high-throughput virology platform. We then demonstrate how these novel molecules can be combined with our existing panel of VSEs to drive over a 4-fold enhancement of AAV in the ReiCell-AAV platform developed by our collaborators at ReiThera Srl. This tailored formulation was then validated at a 2L bioreactor scale. Additionally, we highlight a case study in which a third-party client was able to achieve a nearly 4-fold enhancement in AAV yield and a concurrent 2-fold enhancement of full capsids at 5L bioreactor scale, with undetectable residuals, as confirmed by HPLC assay.

Using bioprocess modelling software, we demonstrate the impact of enhanced yield and quality on key metrics such as the cost per patient dose and cost of manufacturing for a hypothetical AAV-based gene therapy. Ultimately, this data underscores the broad applicability of Virica's VSEs as an innovative, economically feasible solution for improved, scalable viral vector production.

1957 Production of large 1e14-1e15 NHP-grade batches of Adeno-associated viruses via fast intensification and parallelized manufacturing

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Adeno-associated viruses (AAV) hold considerable promise as vectors for gene therapy, but large-scale production remains a significant technical hurdle. Conventional strategies predominantly rely on 'platform processes' necessitated by logistical constraints at high volumes and the prolonged cycle times associated with experimentation at large batch size. Here, we demonstrate that large batches of high-quality AAV can be manufactured without

resorting to a traditional scale-up platform. We have developed a rapid process development pipeline that yields robust, high-quality, and high-yield processes tailored to specific capsid–transgene combinations. This is enabled by a combination of high-throughput cell culture screening techniques with innovations in hardware and software tooling and integration. Individual high-efficiency processes can be effectively scaled out, enabling fast transition from process development to manufacturing. Our approach employs standard HEK293 suspension cell culture, triple transfection, scalable downstream chromatography and filtration operations, and available GMP-grade reagents. By leveraging the collective advances in AAV manufacturing over the past decade, we present an integrated, manufacturing-ready methodology with broad applicability across multiple AAV serotypes.

1958 Scalable Production of Recombinant AAV (rAAV) in HEK293 Suspension Cells: From Shake Flask to 10L Bioreactor Scale-Up

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The production of recombinant adeno-associated virus (rAAV) for gene therapy applications requires robust, scalable processes to meet clinical trial drug supply demands. Here, we present the successful scale-up of rAAV production from shake flask to 10L bioreactor using our proprietary HEK293 (PowerSTM-293) suspension. The platform process, developed by ProBio, employs triple plasmids transient transfection with optimized plasmid ratios and DNA/cell parameters to achieve high yields and consistent product quality.

Methods: HEK293 suspension cells were cultured in chemically defined media and transfected at a target viable cell density (VCD) using a combination of helper, serotype-specific, and gene of interest (GOI) plasmids. The process was scaled from small-scale shake flasks to larger-scale bioreactor, with critical process parameters (CPPs) such as pH, dissolved oxygen (DO), temperature, and metabolite levels (e.g., glucose, lactate, pCO2) closely monitored and controlled. Harvested lysates were clarified through depth filtration and purified using affinity and anion exchange chromatography. Product quality was assessed using a in-house developed analytical methods.

Results: The scale-up process demonstrated consistent performance across scales. Process optimizations, including adjustments to plasmid ratios and DNA/cell parameters, contributed to improved yields and reduced cost of goods (COGS). The larger-scale production runs were successfully executed in a GMP-like environment, underscoring the robustness and scalability of ProBio CDMO's platform process.

Conclusion: This study validates the scalability of ProBio CDMO's platform process for rAAV production, achieving consistent product quality and yield across scales. The results highlight the potential for further scale-up to support the development of commercial-ready gene therapies.

1959 Optimized cell culture media for scalable rAAV bioreactor production

Tala Calvi, Cytiva, Westborough, MA

Viral vector manufacturing is an ever-evolving environment often faced with significant challenges. Along with process conditions, cell culture medium selection impacts cell growth culture characteristics such as cell viability and growth rates. Additionally, media selection can play a role in recombinant adeno-associated virus (rAAV) production and overall process scalability. Scalability and process robustness are critical parameters for success in the diverse gene therapy landscape. To address this demand, HyClone™ prime expression medium was developed to support large-scale GMP production of clinically relevant rAAV titers at high cell densities.

Here, we demonstrate the success of HyClone™ prime expression medium in a few well-adopted, commercially available HEK293 cell lines for cell growth and rAAV production (rAAV serotypes 5 and 8). At shake-flask scale, rAAV titers for both serotypes were at or above 6×10^{10} viral genomes per milliliter. The rAAV8 process was scaled up to a 10 L bench-top bioreactor, which showcased the media performance and scalability across several

viral vector production formats. For these results, an unoptimized process was used, which may suggest the media ha even higher potential with other processes.

1960 Optimizing Serum-Free Conditions for Scalable AAV Production in Fixed-Bed Bioreactors

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The clinical and commercial success of gene therapies depends on s**calable, efficient, and cost-effective viral vector manufacturing** platforms capable of meeting the growing demand for high-prevalence indications with substantial dose requirements. A critical enabler of such platforms is the use of **serum-free media** (SFM), which ensures process consistency, eliminates risks associated with animal-derived components, and aligns with regulatory guidance. SFM process should aim to support **high-yield viral vector production** while reducing these **variability and safety concerns**.

In this study, fixed-bed bioreactors were evaluated as a versatile and scalable solution for serum-free AAV-2 production using HEK293 cells. The scale-X™ **fixed-bed bioreactor platform offers a compact, high-productivity design** with demonstrated success in supporting both serum-containing and serum-free processes, making it a promising alternative for scalable viral vector production. Crucially, these fixed-bed systems can help address challenges posed by traditional stirred-tank reactors, such as large operating footprints, limited scalability and the need for suspension-adapted cells.

In the present study, two experimental setups were explored:

- Suspension-adapted HEK293F cells were successfully transitioned to fixed-bed bioreactors under SFM conditions, achieving robust growth and AAV-2 production.
- Adherent HEK293T cells, initially cultured in serum-containing media, were adapted to SFM and maintained efficient growth and AAV-2 production in fixed-bed bioreactors.

These experiments, conducted at small scale, highlighted the system's flexibility and scalability while achieving consistent, high yields comparable to serum-based processes. This study underscores the potential of scale-X fixed-bed bioreactors to overcome key challenges in viral vector manufacturing, offering a **scalable, efficient, and serum-free solution** to meet the growing demands of gene therapy.

1961 Scale-up of an ELEVECTA™ Transient rAAV Production Process in the Xcellerex™ X-platform Single-use Bioreactor

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The delivery of therapeutic genes using recombinant adeno-associated viruses (rAAV) has emerged as a viable treatment option for various diseases, as shown by the approval of multiple rAAV-based *in vivo* gene therapies in recent years. However, the development of scalable, high-titer GMP-compatible production processes remains a significant challenge for the industry.

Previous work has shown that the ELEVECTA™ transient cell line grows in a single-cell suspension, reaches high cell density, and supports high-titer rAAV production for multiple serotypes with significantly reduced levels of hcDNA (including encapsidated) compared to another commercially available HEK293 cell line. To further this work, the previously established process has been scaled to 50 L in the Xcellerex™ X-platform single-use bioreactor. Scalability is shown across 15 mL, 3 L, 10 L, and 50 L scales with comparable cell culture performance, titer, and product quality.

The next-generation Xcellerex X-platform single-use bioreactor, in tandem with the ELEVECTA transient cell line and optimized, GMP-grade HyClone™ prime expression medium, effectively addresses the market demands for scalable, high-titer manufacturing of high-quality rAAV products from R&D through to commercial manufacturing.

1962 Pseudo-Continuous Manufacturing of Adeno-Associated Virus (AAV) Using a Dual-Bioreactor System with Inertial Microfluidic Cell Retention Device

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Adeno-associated virus (AAV)-based gene therapies have enabled transformative treatments for rare genetic disorders by delivering therapeutic genes that correct underlying defects. Since the first FDA approval of AAV therapy (Luxturna) in 2017, substantial efforts have been made to enhance its production. However, low productivity limits accessibility and increases costs. Traditional batch culture-based manufacturing is constrained by a maximum cell density of ~2×10^6 cells/mL, leading to suboptimal yields. Additionally, batch processes require frequent vessel cleaning and sterilization, increasing operational costs. While single-use setups reduce some of these costs, they remain expensive and pose scalability challenges. To address these limitations, we developed a pseudo-continuous AAV production system utilizing two interconnected bioreactors: a culture bioreactor (CB) for high-density cell cultivation and a production bioreactor (PB) for AAV production. The core innovation is an inertial microfluidic cell retention device that facilitates the removal of spent media while maintaining viable cells. Perfusion of fresh media enables high cell densities in CB, from which cells are periodically transferred to PB for triple transfection using PEI-DNA complexes. The cell retention device at PB supports perfusion culture by removing residual PEI-DNA and toxic byproducts while sustaining high-density cell populations. As AAV production occurs in PB, the remaining cells in CB, diluted to 1×10^6 cells/mL after transfer, regrow to ~8×10^6 cells/mL, preparing for the next cycle. After AAV-producing cell harvest from PB, freshly expanded cells from CB are transferred to PB, enabling continuous operation. We demonstrated this system using HEK 293.sus cells cultured in 500 mL mini bioreactors (300 mL working volume). Cells were inoculated in CB at 0.6×10^6 cells/mL and expanded to 10.8×10^6 cells/mL by day 4, at which point 270 mL was transferred to PB, and CB was replenished with fresh media. This process was repeated every four days for 12 days. Transfection in PB was performed using PEI-DNA complexes at a 2:1 ratio, with 2 pg PEI/cell. PB operated without perfusion for 6 hours post-transfection, after which perfusion was initiated to sustain high cell density during AAV production. After four days of production, cells were removed from PB, and newly expanded cells from CB were transferred for the next cycle. AAV titers quantified via ddPCR demonstrated a >2-fold increase compared to standard batch cultures (inoculated at 0.5×10^6 cells/mL and transfected at 2×10^6 cells/mL on Day 2). Since high-density cell culture, transfection efficiency, and sustained cell viability during production are key engineering factors, we anticipate that further optimization of this pseudo-continuous system will significantly enhance AAV yields. Moreover, our microfluidic cell retention device minimizes product loss compared to membrane-based systems, highlighting its potential for broader bioprocessing applications, including monoclonal antibody and lentivirus production.

1963 Downsizing Process for a Large Amount of rAAV Vector Manufacturing by Combination of Perfusion Culture and Transfection to High Densified Cells

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Recently, gene therapies using recombinant AAV (rAAV) vectors have gained regulatory approval and the number of rAAV development pipelines is increasing. Since these include whole-body administered therapeutic targets that require high doses, the need for large supplies of rAAV is expected to expand. Conventional rAAV production process using human embryonic kidney 293 (HEK293) cells in suspension culture and the triple-plasmids transfection with cationic polymer represented by polyethyleneimine (PEI) have been widely employed. However, this method has low productivity and requires a huge bioreactor of thousand liters to obtain a large amount of AAV.

In addition, the further decline in productivity associated with large facilities is also an issue.

Here, we introduce the improvement of rAAV production process fitted to the actual manufacturing. We show a very high AAV titer reaching 2E12 vector genome per milliliter (vg/mL) by combination of high densification of HEK cells in perfusion culture and original gene transfer method into high density cells based on PEI method. This corresponds to approximately 10-fold higher productivity (on a Vg basis) per transfection batch compared to the conventional AAV production method using low-density HEK cells. In this case, the full capsid rate of AAV5 at the completion of the USP process was also improved more than 2-fold from the conventional method. Furthermore, the amount of plasmid was successfully reduced by 20~30% per cell, which is considered to account for the majority of production costs, by optimizing transfection conditions.

We further demonstrated that a stable and continuous supply of HEK293 cells at concentrations of 2-2.5E7 cells/ml for one month can be achieved by repeating approximately 50% volume of cell bleedings and refillings of fresh media once a day and concentration recovery in next 24 hours at the perfusion culture process. In fact, transfection to cells obtained by cell bleeding at Day 1, 8, 15, and 22 after reaching 2E7 cells/mL showed that rAAV productivity could be maintained at about 10-fold higher than that of the conventional PEI triple-transfection method in each experiment. This indicates that this method can provide a stable supply of rAAV equivalent to thousand L scale even in a relatively small-scale culture facility.

1964 N-1 Perfusion Process for Intensification of AAV Production

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Traditionally, production of viral vectors is done by transient transfection where cells are grown in batch mode and transfected with plasmid DNA together with a suitable transfection agent to insert the gene of interest. However, the upstream process is still one of the bottlenecks of adeno-associated virus (AAV) manufacturing usually because of the long and tedious cell expansion steps. These steps are often carried out in multiple shake flasks and seed train bioreactors. To increase the efficacy of viral vector production, perfusion is introduced in the N-1 step to intensify the seed train by means of reducing or even eliminating some of the cell expansion steps.

In this work, we summarize several studies performed to develop a N-1 perfusion process of HEK293 cells to intensify the manufacturing of AAV. Initially, a set of 10 mL-scale tube bioreactor perfusion cultures were conducted to understand the growth characteristics and metabolic rates of HEK293 cells in perfusion mode and to set the foundation for a bench-scale (1.5 L working volume) scale-down model. The process was finally scaled up to 50 L using an Xcellerex™ automated perfusion system (APS). The process was based on our ready-to-use HyClone™ peak expression medium supplemented with glucose and glutamine.

Consistent performance across scales with respect to cell growth and viability was demonstrated with viable cell concentrations of up to 35×10^6 cells/mL at both 1.5 L and 50 L scales. Furthermore, a cell specific perfusion rate (CSPR) value of 80 pL media/cell/day was sufficient for a 7-day perfusion culture, enabling inoculum preparation (N-1) of HEK293 cells for production in bioreactors up to 2000 L. AAV production was showcased by seeding an Xcellerex XDR 10 bioreactor with perfused HEK293 cells and transfecting cells with triple plasmid transfection approach by using PEI MAX transfection reagent.

In conclusion, a N-1 perfusion process was developed for a 10 mL proof-of-concept scale-down model; a bench-scale (1.5 L working volume) scale-down model, and a 50 L scale. Each scale had comparable cell growth, metabolic profiles, and volumetric productivity.

1965 Development of a Perfusion Process for AAV Production Using High-Throughput Bioreactor Systems

Yelena Ilin[1], **Adam Osborne**[1], David Diaz-Vera[2], Ningning Xu[1], Paetrice Jones[1], Jeong Lee
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Reduction of cost of goods (COGs) is essential for AAV gene therapy due to low process yield. For recombinant protein therapeutics, cell culture process intensification is widely implemented to increase titer and reduce COGs. However, intensification of AAV production processes is a challenge due to reduced cell-specific productivities observed at high cell densities. To address this challenge, the Sanofi Genomic Medicine Unit (GMU) CMC process development group has developed a perfusion process to produce AAV at high volumetric and cell-specific titers using a producer cell line. Proof-of-concept was first demonstrated in TubeSpin bioreactors by manually exchanging production media at specific intervals after infection with a helper virus. The process was then successfully transferred to benchtop bioreactors with perfusion enabled using tangential flow filtration through a hollow fiber filter. Further development work was conducted in ambr250 perfusion bioreactors, where process parameters such as pH, perfusion rate, and media composition were explored using a DoE approach. Notably, the ambr250 model yielded consistently higher AAV titers and enhanced quality compared to larger scale. This was partially addressed by adjusting gassing and agitation strategy, however some limitations remain in the use of this model to predict process performance. Through this development work, the volumetric AAV titers were increased three-fold at pilot scale, relative to the legacy batch process.

1966 Enhanced AAV-Vector Production Using a Novel Stable Producer Cell Line and Optimized Perfusion Processes

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The demand for adeno-associated virus (AAV) vectors in gene therapy is rapidly increasing, necessitating scalable and cost-effective production methods. Stable producer cell lines offer significant advantages over traditional triple-transfection methods, including enhanced scalability, reproducibility, and reduced production costs. At the last ASGCT conference, we developed a high-titer HEK293-based producer cell line for AAV vectors with the GAA transgene and an engineered capsid. We now build on that work to introduce a novel producer cell line for a different transgene. This study focuses on process optimization to achieve a six-fold increase in vector titers through the implementation of perfusion processes in ambr15 and 1.5 L bioreactors. Our results demonstrate not only the substantial increase in production yields but also the long-term stability and high quality of the AAV vectors produced. These findings underscore the potential of stable producer cell lines combined with advanced bioprocessing techniques to meet the growing demands of gene therapy applications, offering a robust platform for the production of high-quality AAV vectors.

1967 Process and Quality Considerations for Recombinant Adeno-Associated Virus (rAAV) Manufacturing Platforms

Pouria Motevalian[1], Mintae Park[1], Amrisha Verma[2], Brittany Montaverde[1], Rama Ramaka[1], Abhay Thakur[1], Clifford Froelich[3]
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Recombinant adeno-associated virus (rAAV) is emerging as a key vector for gene therapy, recognized for its favorable safety profile and capacity for long-term gene expression. Despite major strides in the field, current manufacturing practices face persistent challenges related to scalability, product purity, and full/empty capsid separation. In this presentation, we explore three primary rAAV production platforms—transfection, infection, and stable producer cell lines—to provide insights into optimizing yield, reducing costs, and meeting regulatory expectations for clinical and commercial applications.

A comprehensive literature review and comparative analysis were conducted across each platform, encompassing

upstream processes (e.g., seed-train expansion, cell density management, bioreactor design) and downstream operations (e.g., cell lysis, clarification, chromatography, tangential flow filtration, and virus reduction). Emphasis was placed on identifying critical parameters that influence overall productivity and quality, as well as outlining advanced analytical tools for evaluating potency, purity, and safety. Key considerations for media selection, cell-line engineering, and process-control strategies were also discussed.

Our findings underscore the trade-offs inherent in each manufacturing platform. Transfection-based systems offer rapid development but incur high plasmid costs, infection-based systems excel at large-scale production but require careful baculovirus management, and stable producer cell lines deliver consistent yields yet demand considerable effort in cell-line development. Across all platforms, advances in upstream processing (improved culture media and scale-up approaches) and downstream purification (enhanced chromatography resins and filtration technologies) have led to meaningful gains in product purity, higher titers, and a more favorable full-to-empty capsid ratio. Range for all critical quality attributes based on historical batches will be presented.

The accumulated evidence highlights the critical importance of integrated process design and rigorous analytical testing for successful rAAV manufacturing. By selecting the appropriate platform and refining both upstream and downstream operations, researchers and manufacturers can better balance scalability, cost, and regulatory requirements. Ultimately, these improvements will accelerate the transition of rAAV-based therapies from proof-of-concept to patient-ready treatments, expanding the potential impact of gene therapy across diverse clinical indications.

1968 Development of a Serotype-Agnostic AAV Platform for Suspension Cell Culture

Andrew Detzel, SK Pharmteco - US, King of Prussia, PA

Manufacture of adeno-associated virus (AAV)-based gene therapies requires use of either an adherent or a suspension-based process. Both modalities offer their own unique challenges and advantages, however suspension-based processes have been gaining momentum for large-scale manufacturing due to its scalability and increased upstream process yields. We compared our suspension process to an adherent iCellis (200m^2) process and evaluated process yields, recovery from upstream to downstream, and a full analytical panel using our AAV testing platform, including potency, titer, Empty:Full, aggregation, and process-related impurities. We demonstrate that our serotype agnostic suspension platform for AAV production has increased upstream productivity, comparable downstream recoveries, and equivalent critical quality attributes relative to a well-established adherent production platform.

1969 An Optimized Baculovirus Generation Process and Novel ITR-Transgene Design for High Quality AAV Production Using the Insect Cell Production Platform.

Femke Hoeksema[1], Adriaan de Wilde[2], Jelmer Willems[3], Carmen Gallego[3], Giulia De Sabbata[4], Marina Sogorb-Gonzalez[5], Sander Van Deventer[6], Barbara Sanders[7]
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Development of a stable, robust and large scale AAV manufacturing process at low cost of goods is a major hurdle in the Gene Therapy field. The baculovirus/insect cell production system has the potential to overcome these challenges but relies on efficient generation and scale up of intrinsically genetically unstable recombinant baculoviruses. Here, we developed a highly efficient recombinant baculovirus generation process with high rescue efficiency during plaque purification. Furthermore, we engineered a novel molecular ITR-Transgene design to improve baculovirus genetic stability and we identified a small human promoter sequence with desirable sequence characteristics. When incorporated into a recombinant baculovirus, the VectorY ITR-Transgene design provides high genetic transgene stability whilst retaining high AAV production yields, essential for manufacturing scale up. Furthermore, we utilized next-generation sequencing techniques to characterize the AAVs, showing high genome integrity and low contaminating DNA content. Lastly, we show that the AAV batches produced on this production

system exhibit high transduction and transgene expression in human iPSC-derived CNS cell types and *in vivo* in mice and NHP. Process intensification technologies were developed for efficient production and scale-up of these optimized baculoviruses. In conclusion, we present efficient production of stable recombinant baculovirus for high quality AAV production in insect cells.

1970 Development of a Robust and Scalable AAV8 Production Platform: Enhancing Vector Yield and Purity through Optimized Bioprocessing Techniques

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Introduction: Adeno-associated virus (AAV) vectors are pivotal in gene therapy, yet their production faces significant challenges, including scalability, low recovery rates and the need for effective separation of empty and full capsids. This study presents a comprehensive AAV8 production platform developed together with CDMO Matica Biotechnology, addressing these industry pain points through innovative upstream and downstream processes.

Methods: The upstream process was meticulously designed using scalable bioreactor systems up to 50L STR®, specifically optimized for the production of AAV8 vectors. HEK293 cells were cultured under precisely controlled conditions, including temperature, pH, and dissolved oxygen levels, and the transfection protocol was optimized to enhance vector yield, utilizing specific plasmid DNA ratios and FectoVIR-AAV, tailored for AAV8 production. This approach ensured robust vector generation, addressing the critical need for high-efficiency upstream processes in the gene therapy field. The downstream process incorporated advanced filtration and chromatography techniques, including Sartoclear® DL75 depth filter, Sartopore® 2 XLG membrane filter, Hydrosart® TFF cassettes, and CIMmultus® SO3 and PrimaS® monoliths, to enhance virus recovery and purity. The combination of data analytics and screening tools allowed for optimization of process parameters in small scale, and results could be translated to the 50L scale.

Results: The process demonstrated scalability and robustness, with the 50L bioreactor yielding comparable titers to traditional shake flask methods. The downstream platform achieved a good overall process recovery, with a significant reduction in host cell DNA and protein contaminants. Notably, the CIMmultus PrimaS® chromatography step resulted in a three-fold enrichment of full capsids, achieving over 70% full AAV8 particles.

Conclusions: This study successfully addresses key challenges in AAV production, offering a scalable, flexible and efficient platform that ensures high-quality product. The integration of data analytics and innovative screening tools further optimizes process parameters, setting a new standard for AAV manufacturing. This platform not only meets current industry demands but also provides a foundation for future advancements in gene therapy vector production.

1971 Platform Development for Production and Purification of AAV9

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**Corresponding Author*

Introduction: Adeno-associated virus (AAV) vectors are widely utilized in gene therapy due to their ability to target specific organs and tissues with high efficiency. Among various AAV serotypes, AAV9 holds significant therapeutic potential, driving the need for scalable manufacturing processes. This study outlines the development of a robust and scalable method for producing AAV9 using a triple transfection approach with HEK293 suspension cells cultured in serum-free medium. Production and purification parameters were optimized, and the quality of the final product was characterized using advanced analytical techniques.

Methods: AAV9 production was achieved using a chemical based triple transfection strategy with HEK293 cells grown in suspension cultures. The upstream process included transfection, cell lysis, clarification, tangential flow filtration (TFF) for concentration and diafiltration. Purification was performed using affinity and ion-exchange chromatography. Quantification was conducted via qPCR, and characterization was performed using Cryo-TEM (transmission electron microscopy), size-exclusion chromatography with multi-angle light scattering (SEC-MALS), charge detection mass spectrometry (CDMS), and mass photometry.

Results: Optimization of culture media, transfection reagents, plasmid concentrations, and cell density resulted in AAV9 yields ranging from 1E+15 to 1.5E+15 vg/L. Downstream purification was refined through multiple runs, achieving recovery rates of ~80% using two different affinity resins. To address the challenges of separating empty and full capsids, anion-exchange chromatography was employed as a scalable alternative to ultracentrifugation (UC), which requires density-gradient media and presents scalability challenges. The optimized chromatography process yielded ~20% overall recovery with effective enrichment of full capsids. SDS-PAGE/Silver stain analysis confirmed the presence of capsid viral proteins VP1, VP2, and VP3. Mass photometry analysis of in-process samples showed ~22% full capsids, while final samples following Capto Q purification were enriched to ~85% full capsids. Cryo-TEM indicated ~81.6% full capsid content, SEC-MALS measured ~72% full capsids, and CDMS results aligned with these findings. The process was successfully scaled up to 10 liters, consistently demonstrating robust recovery and high-quality AAV9 vector production.

Conclusion: This study presents an optimized, scalable, process for the production and purification of AAV9. The approach achieves high yields and efficient separation of full and empty capsids, offering a viable solution for large-scale AAV manufacturing in pre-clinical and clinical applications.

1972 Development of a Novel High-Yielding Scalable Sf9-Baculovirus Platform to Produce Quality AAV at 200L Scale

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Significant costs, timeline difficulties, and scalability concerns pose challenges to the manufacturing of recombinant AAV (rAAV) with mammalian cell culture utilizing transient plasmid transfection. Alternatively, the Sf9-baculovirus system can address these manufacturing challenges and produce rAAV of comparable yield and quality. We herein present the ability of our Sf9-baculovirus process to produce high titers of high quality AAVrh.10 at the 200L stirred tank bioreactor scale. Extensive process development (PD) allowed for the creation of a low cell density process using an asynchronous infection facilitated by baculovirus-infected insect cells (BIICs). Shake flask experiments were conducted to select for improvements in crude lysate yields and cell productivity, followed by bioreactor optimizations at the 250mL scale and confirmation at 10L. Scalability was then verified up to 200L across multiple programs and subsequently into GMP. Notably, cell culture trends such as viable cell density (VCD) and cell diameter were highly comparable during the scale up from 250mL to 200L (Figure 1). We achieved upstream lysate yields and cell productivities surpassing 2.0E+12 vg/mL and 1.0E+06 vg/cell respectively (Figure 2).

In parallel, we designed a downstream purification platform consisting of depth filtration, tangential flow filtration 1 (TFF1), affinity chromatography, anion exchange chromatography (AEX), TFF2 and sterile filtration. Each step of the downstream process was optimized individually for vector genome recovery and full viral capsid enrichment as

characterized by ddPCR, Mass Photometry, Capsid ELISA, Analytical Ultracentrifugation (AUC) and Waters' Charge Detection Mass Spectrometry. This process reliably produces >1E+15 vg/L of purified AAV with overall vector genome recovery from harvest lysate to bulk drug substance (BDS) >60% and >70% full capsids. Our optimized Sf9-baculovirus process provides a solution to the challenges of HEK293 plasmid-transfection AAV manufacturing by substantially reducing the cost of goods and increasing yield of high quality rAAV at the 200L scale.

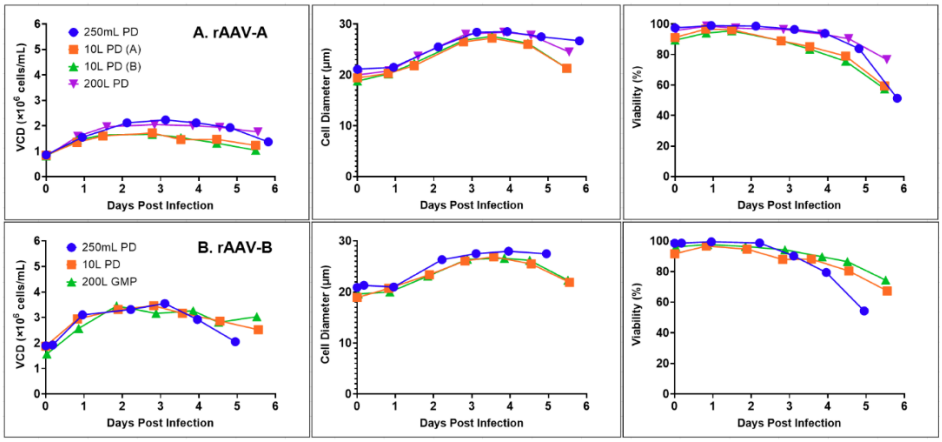

Figure 1: Cell culture behavior following infection by BIICs in 250mL, 10L, and 200L bioreactors.

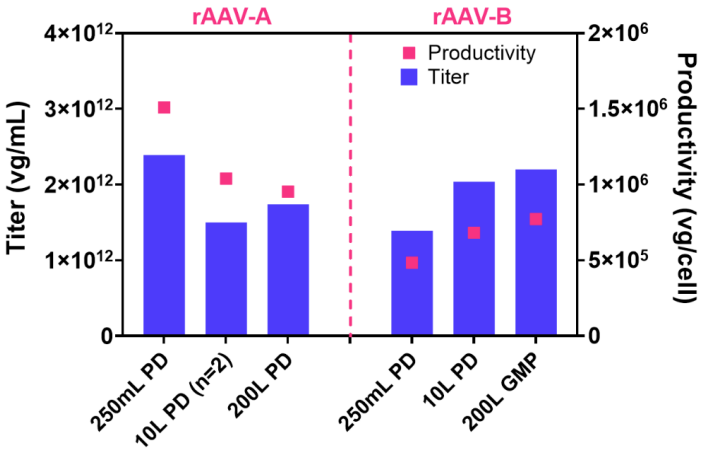

Figure 2: Upstream lysate yields and cell productivities in 250mL, 10L, and 200L bioreactors.

1973 Novel Cell Engineering Platform for High-Yield AAV Production and Improved Manufacturability via Engineered HEK-293 Cells.

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AAV has emerged as a significant therapeutic modality in gene therapy. Challenges such as poor yield and variable product quality persist in the viral vector manufacturing space and we have addressed these problems using our cell engineering technology.

Our cell engineering platform for improved adeno-associated virus (AAV) manufacturing addresses the critical challenges in gene therapy manufacturing, and presents an innovative modality for improving cells for production of viral vector therapeutics. Our platform, yielding cells with improved AAV production and

CQAs, can significantly bolster the efficiency and cost-effectiveness of gene therapy manufacturing, and can accelerate current development timelines.

We used a directed-evolution approach based on repeated cell fusions to shuffle the cell genome, and amplify chromosomes of HEK-293 host cells. Engineered clones enriched for mitochondria phenotypes were isolated, then used as transient-transfection hosts, and for creating stable packaging and producer cell lines. For generation of stable packaging and producer cell lines, we developed a novel inducible system that maximizes the capabilities of the inherent viral production machinery.

Engineered HEK-293 clones grown in suspension culture exhibited up to 15-fold productivity improvement via triple transient transfection for AAV1, AAV2, AAV5, and AAV9 serotypes with capsid titers as high as 10^{17} viral particles/L (vp/L)—at least 10-fold higher than current industrial manufacturing processes. Selection for certain mitochondria phenotypes resulted in a 2-fold improvement in full-to-empty ratio—up to 55% full in crude supernatants. Finally, our engineered stable packaging and producer cell lines achieved capsid titers of up to 10^{16} vp/L.

We demonstrated a multi-modal cell-engineering platform that has significantly improved yield and manufacturability for transient transfection and for stable packaging and producer cell line methods. We further propose a model regarding the role of mitochondria for enhancing capsid percent-full. Taken together, our platform technologies provide solutions for meeting current and future gene therapy manufacturing challenges.

1974 AAVone System Generates over 5E15 VG/L AAV9 Vectors in Suspension HEK 293 Cells

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The high cost of adeno-associated virus (AAV) production is a significant hurdle for its widespread use as therapeutics, despite its relative safety and high efficiency in gene delivery to specific tissues. We previously reported a single-plasmid AAV production system, named AAVone, in which all necessary components for AAV packaging are consolidated into a single plasmid with an approximately 13-kb backbone, achieves two-to-four-fold higher productivity and equal or better AAV quality compared to the traditional triple plasmid transfection. The system requires significantly less total plasmid DNA (pDNA) amount for AAV production compared to triple plasmid transfection. To further optimize the AAVone production system, we here report that by selection of cell culture medium, transfection reagents, transfection enhancers and boosters, productivity of the AAVone system can be further increased more than 5E+15 vg/L of culture using one specific medium combined with a specific transfection reagent and two booster reagents in suspension mammalian cells like VPC2.0. For example, using a pAAVone-AAV9-CMV-EGFP construct (transgene size around 2.8 kb), a titer of 5.8E15 vg/L of culture in the crude cell lysate was achieved. Further productivity evaluation with other AAV serotypes and transgenes, as well as scale-up production and characterization of the AAV vectors produced by this protocol, is ongoing. Taken together, process optimization is crucial for new AAV production systems, including AAVone system. The high productivity of AAVone system with less plasmid amount requirement could significantly reduce the cost of high-quality AAV production, especially for large-scale manufacturing, potentially increase patient accessibility to AAV gene therapy drugs.

1975 Process Optimization of a Baculovirus-Insect Cell Expression System for Scalable rAAV Production

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Large-scale manufacturing of viral vectors can be labor-intensive and costly, particularly when producing materials for therapeutic trials. The baculovirus-insect cell expression system, a well-established platform for vaccine manufacturing, serves as a recognized alternative to HEK-293 platforms for recombinant adeno-associated virus (rAAV) production. This system offers several advantages, including enhanced safety—since baculovirus cannot replicate in mammalian cells—scalability, the use of baculovirus-infected insect cells (BIICs) for streamlined processing, and reduced cost of goods (COGs) at scale. To produce rAAV, the insect cell culture is co-infected with two BIIC banks: one expressing the rep and cap genes (RepCap) and the other expressing the gene of interest. In this study, we present a strategy for development of a scalable and robust upstream process for large-scale AAV production in an insect cell platform. We will also explore how factors such as BIIC ratio, agitation rate, gassing, and cell density at the time of infection can significantly impact process robustness in bioreactors. Finally, we will share data on mid-stream processing methods, including detergent evaluation and endonuclease treatment.

1976 Development of a Robust AAV Upstream Platform Using next generation Raw material, a Comprehensive Design-of-Experiment (DoE) approach & High-Throughput Automated Bioreactors

David Ede[1], Érica Schulze[2], Hakima Flice[3], Christophe Cheve[4], Thomas Jeffrey[5], Paul Giroud[6], Maina Kerbrat[7], Frederique Marme-galloo[7], Timo Schmidberger[8], Noushin Dianat[9], Véronique Blouin[10], Emilie Aubran[11], Oumeya Adjali[12], Laurence Guianvarc'h[13]
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To date, 7 recombinant adeno-associated virus (rAAV)-based gene therapies have received FDA approval, 215 are in clinical trial phase and over 1,000 are currently underway development worldwide. Despite these advances, vector manufacturing remains challenging for therapeutic applications requiring high systemic doses, highlighting the need for new methods to efficiently scale up rAAV production. This will increase rAAV yields and reduce costs, paving the way for more straightforward commercialization. Past efforts to improve rAAV production have focused on optimizing a single variable at a time in non-automated systems (shake flasks), but this approach does not account for the interactions of multiple factors that contribute to vector generation, nor the environment in which the virus is produced.

To address these limitations, we employed a design-of-experiment (DOE) methodology to optimize rAAV2 production in HEK-293 cells cultivated in an automated bioreactor. In the first round of DOE we chose to focus on optimizing bioreactor operating parameters and cell culture media selection. In the second round of DOE, we further fine-tuned cell culture parameters (feeding and booster strategy). In the third and final round of DOE we focused on optimizing the transfection conditions (VCD, DNA amount, plasmid ratio and transfection reagent volume). Using the optimized process derived from these DoE models, we have increased by 73-fold rAAV2 productivity compared to initial conditions in shake flask, reaching above 8.8 ×1011 vg/mL, along with achieving a full particle count above 20 % for rAAV2, which is known to be challenging to produce. These findings underscore the pivotal role in the choice of raw materials, in the use of DoE and in optimizing production processes in automated bioreactors.

1977 Fast-Tracking AAV Process Development Using Innovative and Universal Approaches – The Platform of the Future

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The gene therapy industry is advancing rapidly, with growing emphasis on milestone-driven investment. In this high-pressure environment, companies no longer have the luxury of time or budgets to undertake extensive, product-specific process development from the outset. As such, the future lies in platform approaches that facilitate IND submission within the first year and enable rapid product launch. This presentation will focus on three key AAV production process steps that are notoriously challenging and time-consuming to optimise: transfection, cell culture, and polishing chromatography. We will explore novel strategies and technologies that may offer solutions to streamline these complex processes.

Traditionally, small-scale experiments and Design of Experiments (DoE) approaches are employed to optimise the transfection step. But what if there were a universal solution that could boost AAV productivity and purity? In this talk, we will investigate innovative additives that can be seamlessly integrated into the upstream process, working independently of major industry suppliers. These additives offer a cost-effective alternative to commonly used reagents. We will present promising results from a small-scale screening study of commercially available additives that are suitable for GMP manufacturing and expand on future hopes from the technology.

Increasing upstream yields can also be achieved through alternative strategies. Rather than spending months developing a process from scratch and incrementally improving it by targeting a range of culture parameters, what if perfusion and high-density cultures could bypass the need for such extensive development? We will evaluate the true impact of perfusion on both development timelines and cost of goods considerations, demonstrating how they can accelerate AAV development.

Lastly, we will delve into the intricacies of downstream AAV development, focusing on the challenges of anion exchange chromatography during the polishing step, which is critical for achieving high particle enrichment. This step is widely recognised as both complex and time-consuming to optimise, often requiring numerous studies that, even with Design of Experiment (DoE) approaches, still fail to yield a streamlined process. What if the use of Choline Chloride as an elution salt could dramatically enhance product separation from the outset, in comparison to the commonly used Sodium Chloride, thereby accelerating the entire development process? We will present a data set that has the potential to transform our approach to the polishing step.

We will conclude by introducing our innovative platform of the future, and showcasing our latest partnerships that will help revolutionise AAV process development.

1978 Scaling Up AAV Manufacturing: Expanding AAV-based Therapies Beyond Rare Diseases with Genevoyager Bac/Sf9 System

Xiaobin He[1], Xing Pan[1], He Xiao[2], Yicheng Zhou[2], Liang Du[2], Mengdie Wang[2], Guangmeng Liu[2], Huanhuan Zuo[2]
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Adeno-associated viral (AAV) vectors hold immense promises for gene therapy, yet challenges in production yield, scalability, and stability have limited their broader application. To address these challenges, Genevoyager has developed a proprietary Bac/Sf9 system, offering a scalable and efficient platform capable of producing high-quality AAVs with reduced costs.

The Genevoyager Bac/Sf9 system achieves high yields of 1E+15 to 5E+15 vg/L during scale-up manufacturing. With a consistent recovery rate of ≥30% from 5L to 200L and minimal product loss during each purification step, the platform demonstrates good scalability and efficiency for large-scale production.

In this system, the Baculovirus Expression Vector (BEV) has been optimized to maintain stability over multiple passages and during long-term storage at ≤-80°C for over 24 months. This modification ensures consistent high yields and robust expression levels of AAV batches at commercial scales.

AAVs produced with the Genevoyager Bac/Sf9 system exhibit low aggregation even at high concentration (3E+14 vg/mL). Stability assessments, including ddPCR-GOI and main peak SEC-HPLC, demonstrate that product quality remains stable under 4°C and withstands multiple freeze-thaw cycles.

Moreover, the Bac/Sf9-derived AAVs are free of residual replication-competent AAVs (rcAAVs), exhibit high purity and deliver high expression levels, addressing critical safety and efficacy concerns for gene therapy.

The scalability, robustness, and reliability of the Genevoyager Bac/Sf9 system make it an efficient platform for developing AAV-based therapies not only for rare diseases but also for common conditions such as cardiovascular diseases, cancers, and autoimmune disorders. This system represents a significant advancement in overcoming the production and stability challenges of AAVs, thereby enabling broader access to treatments for a larger patient population.

1979 Strategies to Remove Pre-Existing AAV Antibodies: IgG Enzymatic Degradation vs FcRn inhibition.

Yash Shah[1], Rohini Sheth[1], Layth Awartani[2], Deanna Sedlak[3], Cara Richardson[4], Audry Fernandez[3], Lisa Aimone[3], Dan Kemp[3], Canwen Jiang[5], Simon Lacey[6], Shari Gordon[7]
[1]AskBio, Durham, NC, [2]Askbio, Research Triangle Park, NC, [3]AskBio, Morrisville, NC, [4]AskBio, Durham, [5]AskBio, Durham, North Carolina, [6]AskBio, Philadelphia, PA, [7]Ask Bio, Morrisville, NC

Pre-existing Adeno-associated virus (AAV) antibodies may reduce efficacy of gene therapy by preventing transduction of systemically administered AAV vectors. Moreover, high doses of systemically administered AAV vectors may lead to antibody-mediated complement activation and contribute to immune-related adverse events. Thus, individuals with pre-existing circulating AAV antibodies are often excluded from enrollment in gene therapy trials. To address this issue, there has been significant work in the field to evaluate strategies to reduce the levels of circulating immunoglobulins (IgG) in sera such as plasmapheresis, antibody cleaving enzymes, and more recently FcRN inhibitors. In this study we directly compared an FcRn inhibitor to an IgG cleavage enzyme for their ability to reduce circulating IgG antibodies and enable AAV transduction.

This study utilized a transgenic mouse model (scid FcRn-/- hFcRn (32) Tg mice) wherein immunodeficient mice expressed the human alpha chain FCGRT molecule. To model varying titers of pre-existing antibodies, human intravenous immunoglobulin (IVIG) was given to simulate low level NAb titers and a recombinant, anti-AAV antibody was used to model high NAb titers on day 1. Twenty-four hours later, mice were given either saline, IgG cleavage enzyme, or an FcRn inhibitor via tail vein injection. On day 6 mice were administered 5e10 vg/mouse of an AAV-luciferase vector. Circulating levels of human Ig and AAV specific antibodies were measured weekly and transgene expression monitored by luciferase in-vivo imaging. IVIG and antibody infusion inhibited AAV transduction as seen by reducing luciferase expression in the liver. Mice treated with either IgG cleavage enzymes or the FcRn inhibitor had significantly reduced levels of circulating antibodies. IgG cleavage enzyme treatment fully restored transgene expression comparable to animals without pre-existing AAV antibodies. However, mice treated with the FcRn inhibitor showed a partial restoration with the average luciferase expression significantly less than both the IgG enzyme treated group and the AAV control animals without pre-existing antibodies. These preliminary studies directly compare various strategies to remove pre-existing AAV antibodies and confirm the utility of IgG cleavage enzymes as an approach to potentially expand the pool of patients eligible for systemically administered AAV gene therapy.

1980 Short-Term Repeat Dosing of KJ103 (Ricefidase) Enables Sustained IgG Reduction for Enhanced AAV Gene Therapy

Clara Chang[1], **Yunxia (Erica) Xu[2]**
[1]Baopharma, Taiepi, Taipei, Taiwan, [2]Bao Pharma, Shanghai, Shanghai, China

KJ103 (ricefidase) is a recombinant IgG-degrading enzyme designed to transiently reduce circulating IgG levels, thereby enhancing the efficacy and safety of AAV-based gene therapies. While previous studies have demonstrated its ability to lower IgG and neutralizing antibody (NAb) titers, the feasibility of short-term repeat dosing remains uncertain.

In an on-going Phase 2 study conducted by Bao pharma in China, we present clinical evidence confirming that a second administration of KJ103 within one week of the initial dose is both safe and effective in further reducing IgG levels. We had observed the antibody concentration reduction in 2 consecutive doses within 10 days. This dosing strategy will expand the therapeutic window for different types of AAV vector administration, potentially increasing patient eligibility and improving gene transduction efficiency.

Theoretically, our findings support the incorporation of a repeat-dosing regimen into future protocols of AAV-based gene therapies, providing a novel insight to overcome both pre-existing and treatment-induced anti-AAV immunity.

1981 Clinical Validation of Short-Term Triple Dosing of KJ103 (Ricefidase) for Sustained IgG Reduction in AAV Gene Therapy

Clara Chang[1], **Yunxia (Erica) Xu[2]**
[1]Baopharma, Taiepi, Taipei, Taiwan, [2]Bao Pharma, Shanghai, Shanghai, China

KJ103 (ricefidase) is a recombinant IgG-degrading enzyme designed to transiently lower circulating IgG levels, enhancing efficacy of AAV-based gene therapy. While both single and repeat dosing regimens have shown effectiveness, the impact of more intensive IgG depletion remains unexplored.

In a clinical study conducted by Bao Pharma, we evaluated the safety and efficacy of a short-term triple-dosing regimen of KJ103. Our findings confirm that three consecutive doses within 14 days maintain a favorable safety profile while achieving a deeper and more sustained IgG reduction. This intensified approach may further expand the therapeutic window for AAV vector delivery, mitigating both preexisting and treatment-induced immunity.

These results establish KJ103 as a versatile tool for optimizing AAV-based gene therapy strategies, with potential implications for broader clinical applications.

1982 Rapid Transient Antibody Depletion using Genetically Engineered IgG-Degrading Enzyme allows efficient rAAV9 Gene Delivery in an α-AAV9-Ab$^+$ rabbit model

Surendra Sharma[1], Genevieve Abd[2], Allen Zheng[3], Tierra Bobo[4], Haiyan Fu[5]
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The widespread presence of pre-existing anti-AAV antibodies (αAAV-Abs) in humans poses a critical barrier to the translation of rAAV gene therapy, and only individuals negative for αAAV-Abs are currently eligible for treatment. The IgG degrading enzyme of Streptococci (IdeS) has been demonstrated to specifically cleave IgG of humans, primates, sheep, and rabbit (but not mouse) origin, with the demonstrated potential of depleting pre-existing αAAV-Abs for AAV gene delivery. Using site-directed mutagenesis, we engineered a new recombinant IdeS protein, Ab-cleaver 9.2 (Ab-C9.2), to address the unmet need, enhancing thermal stability and IgG-depleting function. For

testing Ab-C9.2 in vivo, we generated a αAAV9-Abs+ rabbit model by immunizing the animals with an IV injection of rAAV9 vector at 2-5x109vg/kg, resulting in serum levels of αAAV9 neutralizing Abs (αAAV9-NAbs) at 1:200-1:6,400 and αAAV9-IgG at 1:400-1:6,400. 3 months post-immunization, an IV injection of Ab-C9.2 (0.5mg/kg) in animals with αAAV-nAbs ranging from 1:200-1:1,600 led to the rapid reduction of total IgG to undetectable levels and αAAV-nAbs to ≤1:80 within 24hr. Importantly, an IV injection of AAV9-hSGSH vector administered 24hr post Ab-C9.2 treatment led to effective transduction in rabbits with serum αAAV9-nAbs reduced to ≤1:20, comparable to that in rabbits negative for αAAV9-nAbs. However, the clearance of the IV-injected AAV9 vectors were observed in animals with serum αAAV9-nAbs reduced to 1:80. These results indicate that IV Ab-C offers a great tool to overcome the pre-existing αAAV-Abs for the translation of rAAV gene therapy to treat diseases in humans, though it may have limits in depleting pre-existing αAAV9-nAbs under acute Ab conditions. We believe that Ab depletion with Ab-C9.2 may make rAAV gene therapy available to all patients needing administration and re-administration.

1983 Low Pre-existing AAV-Neutralizing Titers Detected Using a Cell-Based Assay Did Not Impact 4D-310 Safety or Efficacy in Fabry Cardiomyopathy Patients

Brian Long[1], Josh Holter[2], Alan Cohen[1], Zachary Sellers[1], Christian Vettermann[1], David Kirn[1], An Song[1]
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Introduction: 4D-310 is an investigational genetic medicine for Fabry disease cardiomyopathy that employs a novel cardiotropic AAV capsid (C102) to deliver a human *GLA* transgene to cardiomyocytes for cell-autonomous alpha-galactosidase (AGA) production. The INGLAXA Phase 1/2 study previously enrolled 6 participants who are followed for up to five years following systemic 4D-310 dose administration.

Methods: Participants were tested for neutralizing antibodies (NAb) specific for the C102 capsid using both a cell-based transduction inhibition assay and an electro-chemiluminescent ligand-binding total anti-drug antibody (ADA) assay.

Results: A retrospective assessment of screening samples revealed that 3 of 6 participants dosed with 4D-310 showed measurable titers of C102 neutralizing factors using the cell-based transduction inhibition assay at baseline, prior to gene therapy dose administration. However, all 6 study participants were negative at baseline for C102 capsid-binding total antibodies as measured in the ligand-binding assay. Participants who were retrospectively found to be positive for pre-existing neutralizing factors, in the absence of clear evidence of capsid-specific antibodies, showed similar or better efficacy outcomes and comparable safety outcomes at Week 52 compared to those who tested negative for neutralizing factors.

Conclusion: Our data underscore that cell-based assays to detect AAV capsid neutralizing factors, in particular assays without a confirmatory Protein A/G/L depletion step, may over-estimate the prevalence of pre-existing AAV neutralizing antibodies. Implementation of ligand-binding total ADA assays for patient screening can help minimize the risk of excluding patients who may otherwise benefit from AAV gene therapy.

1984 Evaluating Neutralizing Antibodies Against AAVs Using a High-Sensitivity LacZ Reporter Assay

Tiffany Pan[1], Venkata Kuppireddy[1], **Irene Song**[2]
[1]PackGene Biotech INC, Houston, TX, [2]Packgene Biotech, Houston, TX

Adeno-associated viruses (AAVs) have become a critical tool for delivering therapeutic genes in gene therapy applications. However, the presence of pre-existing AAV neutralizing antibodies (nAbs) in patients poses a significant risk by reducing the efficacy and safety of AAV-based therapies. Furthermore, the immune response following administration of AAV gene therapy products (DTP) can generate new neutralizing antibodies, complicating long-term treatment outcomes. Therefore, robust methods to measure the levels of nAbs in patients before and after therapy are essential for clinical success.

We have developed a novel LacZ reporter system tailored for multiple AAV serotypes, which enables sensitive and efficient detection of AAV neutralizing antibodies using a cell-based assay. Unlike traditional approaches requiring multi-step protocols and DNA extraction, this single-step method directly quantifies AAV-driven LacZ activity in cell lysates, offering a streamlined workflow with minimal handling and broad dynamic range. The signal remains stable for over one hour, ensuring consistent and reproducible readouts.

This innovative assay offers a powerful and reliable platform for assessing the immunogenicity of AAV-based gene therapies, providing a rapid and streamlined method for detecting neutralizing antibodies. Continued development of such advanced assay platforms is crucial for addressing key challenges in the field, supporting therapeutic research, and ensuring safer and more effective outcomes for patients undergoing AAV-based gene therapy.

3000 Isolation, Quantification, and Cryopreservation of CD34+ Cells from Newborn Cord Blood in Tanzania: Preparing for Gene and Cell Therapy Research

Mohamed Alimohamed[1], Aisha Fikirini[1], Salmaan Karim, Florence Urio[1], Zacharia Mtulo[1], Grace Moshi[1,2], Fadhlun Al-Beity[1], SIANA NKYA[1], Julie Makani[1]
[1]*Muhimbili University of Health and Allied Sciences, Tanzania* [2]*KKH, Singapore*

Introduction
CD34+ cells in cord blood are progenitor stem cells that give rise to all lymphohematopoietic lineages and are crucial for cell-based therapies. The dose and quality of CD34+ cells are vital for successful engraftment in hematopoietic stem cell therapies. This study aimed to validate the efficiency of density gradient centrifugation and magnetic-activated cell sorting (MACS) methods for isolating, purifying, and quantifying CD34+ cells from fresh and frozen cord blood samples in a Tanzanian setting, contributing to the limited knowledge on CD34+ cells from African population.

Materials and Methods
This experimental study included 44 cord blood samples from randomly selected, pregnant women at Muhimbili National Hospital (MNH) and Aga Khan Hospital (AKH). The pregnant women were requested to consent for the cord blood to be collected after they gave birth. Cord blood was collected by qualified nurses and doctors. Sickle cell status of the newborns was determined from the cord blood using a rapid, competitive lateral flow assay (HemoTypeSC TM, Silver Lake Research, USA). Mononuclear cells were isolated by density gradient centrifugation (Ficoll-Paque TM PLUS media, Cytiva, Sweden). A single-platform flow cytometry (BD FACSCanto™ II Clinical Flow Cytometry System - BD Biosciences, USA) assay was used to obtain the absolute count of enriched CD34+ cells with the CD34 MicroBead Kit human 2x2 mL (Order no.: 130-046-702, Miltenyi Biotec, Germany). Both fresh and frozen enriched CD34+ samples were stained with nuclear dye (7AAD) and CD34/CD45 antibodies for enumeration and viability assessment.

Results
Screening for sickle cell status showed that 85.71% of the newborns were AA (homozygous hemoglobin A) and 14.29% were AS (heterozygous hemoglobin S, sickle-cell trait). MACS purification yielded fractions with the maximum purity of 66.3% CD34+ cells for fresh samples and 33.7% for the frozen samples. The median percentage of CD34+ cells in non-enriched samples was 1.17% (IQR, 0.775 – 1.245 %) whereas in enriched samples was 66.65% (IQR, 49.8 – 68.5%). Majority of the enriched cells expressed CD34 antigen alone (66.3%), a small proportion expressed both CD34 and CD45 markers (1.51%), and CD45 was expressed by 32.0%.

Conclusion
The methods for isolating, purifying, and quantifying CD34+ cells were successfully validated in a Tanzania setting, enhancing knowledge of CD34+ cells from African population. This validation of the feasibility of using MACS for CD34+ cell enrichment from
cord blood samples, paves the way for further research and potential clinical applications in the region.

3001 Metabolic Reprogramming with ADA1 and CD26 Overexpression Enhances IL15-GPC3-CAR T Cell Therapy for Hepatocellular Carcinoma and Reduces Cytokine Release Syndrome Risk

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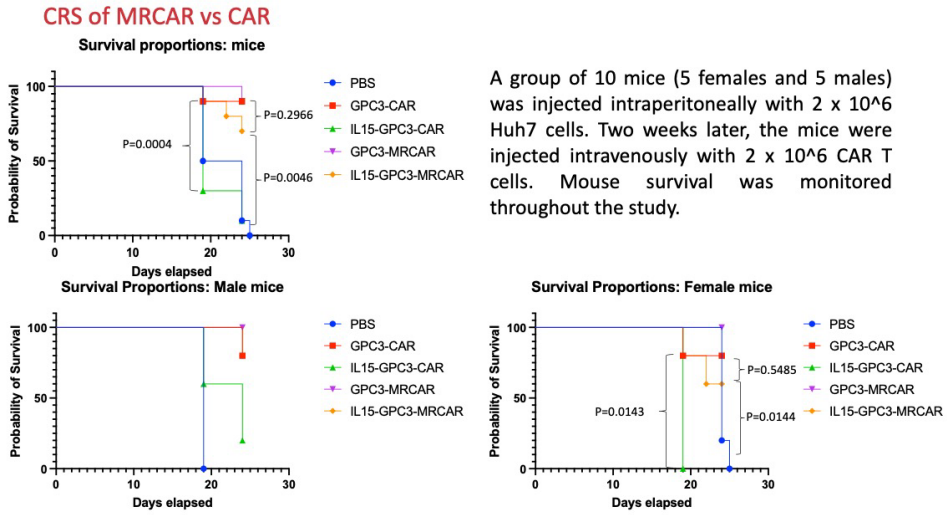
Background:
CAR T cell therapy targeting glypican-3 (GPC3) has shown potential in treating hepatocellular carcinoma (HCC). IL15-GPC3-CAR T cells demonstrated a 33% partial response rate in clinical trials but were associated with severe cytokine release syndrome (CRS) in 75% of patients compared to 8% with GPC3-CAR T cells. Strategies to mitigate CRS while improving therapeutic efficacy are urgently needed, especially for bulky tumors. Previous research established inosine as an alternative energy source for CAR T cells in glucose-deprived tumor microenvironments (TME) (Nat Metab, 2020) and demonstrated that adenosine deaminase 1 (ADA1) enhances T cell survival by converting adenosine to inosine while reducing pro-inflammatory cytokine production (Cell Rep Med, 2024). Additionally, CD26 has been shown to promote T cell trafficking and invasion into solid tumors. This study explores ADA1-driven metabolic reprogramming and CD26 overexpression to optimize IL15-armed GPC3-CAR T cell therapy for HCC by enhancing persistence, invasion capacity, and mitigating inflammatory toxicity.

Methods:
Peripheral blood mononuclear cells (PBMCs) were transduced with retroviral vectors encoding IL15-GPC3-CAR, ADA1, and CD26 (designated IL15-GPC3-MRCAR). Therapeutic efficacy, tumor infiltration, and toxicity were assessed using a Huh7 xenograft mouse model. Cytokine profiles and survival outcomes were evaluated to gauge immune activation, invasion into bulky tumors, and CRS risk.

Results:
IL15-GPC3-MRCAR T cells exhibited enhanced proliferation, reduced exhaustion, and significantly improved invasion into bulky tumors after repeated HCC stimulation in vitro. These cells showed greater cytotoxic activity and deeper tumor penetration compared to IL15-GPC3-CAR or GPC3-MRCAR T cells in vitro and in vivo. While IL15-GPC3-CAR T cells induced severe CRS and high mortality rates, IL15-GPC3-MRCAR T cells significantly prolonged survival with outcomes comparable to GPC3-CAR and GPC3-MRCAR T cells. Tumor infiltration studies confirmed superior invasion of IL15-GPC3-MRCAR T cells due to CD26 overexpression.

Conclusions:
Integrating ADA1 and CD26 overexpression into IL15-GPC3-CAR T cells enhances metabolic fitness, antitumor activity, tumor invasion, and safety by reducing CRS risk. This strategy represents a significant advancement in CAR T cell therapy for HCC, warranting further clinical validation.



3002 Engraftment and HIV Resistance of Gene Modified Peripheral Blood Cells in a Phase I Trial of an HIV Stem Cell Gene Therapy: AIDS Malignancy Consortium 097

Joseph Anderson[1], Julie Beegle[2], Deborah Stewart[1], Christina Dyer[1], Amanda Buck[3], Joanna Donatelli[3], Gunjun Shah[4], David Asmuth[1], Richard Pollard[1], Ariela Noy[4], Mehrdad Abedi[1],

[1]*University of California Medical Center*, [2]*UC Davis*, [3]*University of California San Francisco*; [4]*Memorial Sloan Kettering Cancer Center*

Currently there is no effective cure for HIV infected individuals. Stem cell and gene therapy-based approaches aimed to mimic the result of the Berlin Patient and others, who received a delta-32 CCR5 allograft for leukemia and a functional cure of their HIV, have been developed including ongoing human clinical trials. There is an urgent need, however, for strategies that expand gene therapy beyond CCR5 modification to target multiple stages of HIV replication. Thus, we completed a Phase I clinical trial, AMC097, enrolling HIV-related lymphoma patients requiring an autologous transplant using such a multi-target strategy. Following BEAM conditioning, participants received autologous peripheral blood CD34+ cells transduced with a lentiviral vector containing three HIV-resistance genes including a CCR5 shRNA, a chimeric TRIM5alpha gene, and a TAR decoy. A fourth gene was also included in the vector and used as a selectable marker to purify the gene modified cells prior to transplant. Three cohorts of participants (total N=11) each receiving the gene modified cells in a dose dependent ratio along with unmodified cells: 1:1, 2:1, 1:0 of gene modified to unmodified cells were enrolled. This was to initially evaluate the engraftment of the gene modified while reducing the risk to patients who required immune system reconstitution.

Safety of the vector transduced cells was determined by (1) predetermined engraftment timelines and (2) by average vector copy number in the peripheral blood post-transplant by quantitative PCR using a vector-specific primer and probe set. Flow cytometry of immune cell reconstitution in the peripheral blood was also evaluated. In all participants, including the third cohort which only received the vector transduced cells, successful and steady engraftment was observed over the course of one to two years depending on the continued enrollment of each patient.

One participant underwent an optional analytical treatment interruption (ATI) 42 months post-transplant. Criteria for ATI included CD4 >300 and undetectable plasma viral load. Upon analysis of vector copy number in the peripheral blood, an 8-fold increase in gene marking was observed at seven weeks post-ATI. An in-house single-cell droplet PCR method to evaluate HIV infection in this patient's cells during the ATI estimated that approximately 75% of the gene modified cells were protected from de novo infection compared to cells without detectable lentiviral vector DNA. This protection of infection was similar at both early and late ATI timepoints. ATI was suspended on week 8, after the CD4 and plasma viral load safety thresholds were met. CD4 rose to >300 one week post-treatment resumption and viral load became undetectable by week 8. Our results highlight both the safety and efficacy of our approach and warrants further assessment.

3003 Hematopoietic stem cell gene therapy as a novel therapeutic approach for Angelman syndrome

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[1]*University of California Medical Center,* [2]*UC Davis,* [3]*University of California Davis MIND Institute,* [4]*University of California Davis SOM*

Angelman Syndrome (AS) is a rare genetic neurodevelopmental disorder characterized by developmental delays, impaired communication, impaired motor skills, including coordination, gait and balance. Cognitive dysfunction is illustrated by traits of poor attention, and moderate to profound intellectual disabilities. Novel strategies to treat Angelman Syndrome are critical as there is no proven efficacious cure nor corrective therapy approved by the FDA. A stem cell gene therapy approach has the potential to deliver therapeutic levels of the missing enzyme, ubiquitin ligase E3A (UBE3A) to affected neurons by cross-correction, a successful form of gene replacement therapy, currently utilized with broad successes in various lysosomal storage diseases and leukodystrophies. Transplantation of autologous blood stem cells that are genetically engineered to express

UBE3A/Ube3a could alleviate, restore, and potentially halt the development of AS phenotypes. Our current work illustrates the effectiveness of transplanted human CD34+ hematopoietic stem and progenitor cells that were transduced using a lentiviral vector that expressed a modified mouse isoform 3 of Ube3a using an immunodeficient Ube3a-deficient mouse model. Transplanted mice were assayed using various indices of behavior including motor and cognitive assays, neurophysiology, and power spectral density, in order for us to quantify efficacy of the cell transplants in newborn and adult mice. Assays included open field exploration, beam walking, gait analysis via treadmill walking, novel object recognition, seizure thresholds and numerous indices using EEG. Significant improvements in AS-related clinical phenotypes in all behavioral and neurophysiological domains tested were observed in the mice transplanted with the Ube3a vector transduced cells compared to empty vector control cell transplanted mice. A significant increase in Ube3a protein expression was observed in the brain tissue of these mice. These results highlight this HSCGT strategy as a novel therapeutic approach for the treatment for AS patients.

3004 Significant improvement of clinical phenotypes for Syngap1 and Rett syndrome upon transplantation of lentivector transduced human CD34+ cells into disease-specific mouse models

Joseph Anderson[1], Jill Silverman[2], Camilla Barbaduomo[1], Alyse Lodigiani[1], Julie Beegle[3],
[1]*University of California Medical Center,* [2]*University of California Davis SOM*, [3]*UC Davis*

Neurodevelopmental disorders (NDDs), including autism spectrum disorder (ASD) and intellectual disabilities (IDs) are prevalent and pervasive lifelong disorders for which no symptomatic targeted pharmaceuticals nor precision curative interventions are readily available. Single gene disorders are among the most well understood NDDs given their straightforward inheritance patterns and genetic etiology. With the demonstrated success of cross-correction stem cell gene therapy for a variety of lysosomal storage disorders, leukodystrophies, and in our recent work for Angelman syndrome, we attempted to utilize hematopoietic stem cell gene therapy for other NDDs, including Syngap1, Rett syndrome. Lentiviral vectors were developed to express modified forms of SYNGAP1 and MECP2, respectively. Upon transduction of human CD34+ hematopoietic stem and progenitor cells(HSPC) and transplantation into immunodeficient versions of the Syngap1+/- and Mecp2+/- deficient mouse models, significant improvement in disease-related phenotypes were observed. These included reductions in elevated activity and improved fine motor grasping ability reported previously, in Syngap1+/-- mice. We observed restoration of movement and improved grasping ability in female Mecp2+/- mice. A significant increase in the expression of Syngap1 and Mecp2 were observed in brain tissues of the respective mouse models upon transplantation of vector transduced HSPC. These results highlight the ability of gene modified human CD34+ cells as a novel therapeutic approach for genetic NDDs, broadly, even in cases of highly differing mechanistic etiologies. Future directions include identifying the underlying mechanism of action and comparing and contrasting how a single therapeutic strategy can provide broad efficacy.

3005 Optimization of *CNGA3* Gene Therapy For Achromatopsia Using Stochastic Modeling and Sensitivity Analysis

Niranjana Sendil Kumar
The Charter School of Wilmington

Achromatopsia is a bilateral inherited retinal degeneration disorder caused by a mutation in the CNGA3 (Cyclic Nucleotide Gated Channel Subunit Alpha 3) protein-coding gene, affecting every 1 in 30,000 people. Mutations in the CNGA3 cause myopia, nystagmus, and loss of photopic vision. Achromatopsia gene therapy treats patients by delivering a functional copy of the CNGA3 gene to the affected cone cells. The treatment uses adeno-associated viruses (AAVs) or other viral vectors. A statistical analysis of stochastic models can be used to analyze the randomness in achromatopsia gene therapy, ensuring the ability to configure efficacy by modeling the sensitivities of the delivery efficiency and immune rejection. The variability in the therapeutic outcome requires an understanding of the factors influencing the efficacy.

A stochastic model was developed to assess the variability of the CNGA3 gene therapy in photopic and scotopic vision. Pycharm, a Python programming software was used to study the stochastic models and sensitivity models from the data provided by the NIH's clinical trials. The analysis and visualizations were conducted using Python and Matplotlib. Based on this variability, a sensitivity analysis was used to evaluate the impact of delivery efficiency and immune rejection probabilities on the sensitivity of scotopic and photopic vision.

From the stochastic modeling, it was concluded that photopic vision has higher variability than scotopic vision. The data revealed that poor delivery efficiency was caused by the immune rejection of the delivery vectors rather than the biological component. Delivery efficiency and immune rejection presented an inverse relationship; for example, delivery efficiency increased only when immune rejection decreased.

The efficiency of CNGA3 gene therapy can be enhanced by utilizing immunosuppressive delivery vectors to mitigate vector-related immune responses. Stochastic modeling is valuable in pinpointing critical variables responsible for CNGA3 therapy's success. Stochastic modeling in medicine can help more than 200,000 individuals worldwide benefit from therapeutic CNGA3 gene therapy, enlisting them for absolute freedom.

Figure 1: The Effect of Amplitude (Strength of the Retinal Response to Visual Stimulation) on Frequency

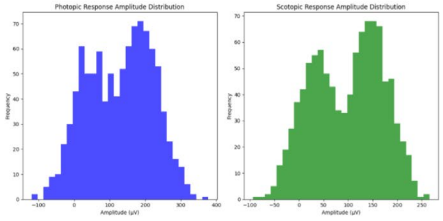


Figure 2: The Effect of Amplitude (Strength of the Retinal Response to Visual Stimulation) on Frequency - Trial 2

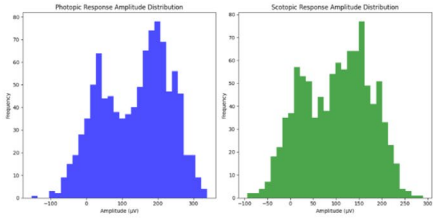


Figure 3: The Effect of Amplitude (Strength of the Retinal Response to Visual Stimulation) on Frequency - Trial 3

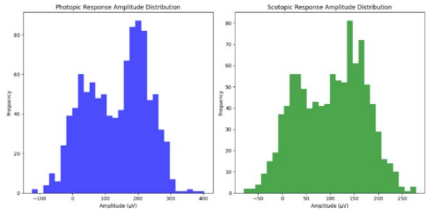


Figure 4: The Effect of Amplitude (Strength of the Retinal Response to Visual Stimulation) on Frequency - Trial 4

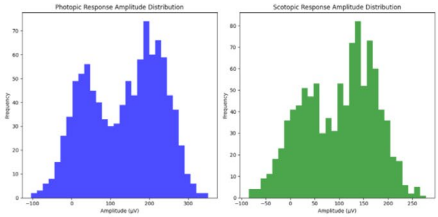
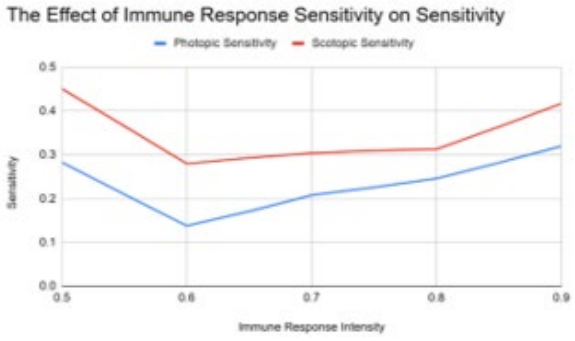


Figure 7: The Effect of Delivery Efficiency on the Scotopic and Photopic Sensitivity



Figure 8: The Effect of Immune Response Intensity on Scotopic and Photopic Sensitivity



3006 Impact of Source Material Refrigerated Shipping and Storage on Isolated B-cell Gene Expression — An Artificial Intelligence and Machine Learning Enhanced Study

Ethan Deitcher[1], Kirk Trisler[1], Hannah Adams[1,2], Steven Deitcher[1]
[1]*Bespoke Biotherapeutics,* [2]*Williams College*

INTRODUCTION:
Apheresis (AP) collection of peripheral blood mononuclear cell (PBMC) products typically requires one- or two-day refrigerated shipping to a central facility for target cell isolation and cellular immunotherapy manufacture. Some have assumed that immune cell populations isolated from shipped PBMC or whole blood (WB) products are unaffected by the delayed processing. We used bioinformatics, artificial intelligence (AI), and machine learning (ML) to compare gene expression profiles of B-cells isolated at 3, 24, and 48 hours after matched healthy PBMC and WB source material collection.

METHODS: WB was collected via venipuncture prior to AP in 3 healthy adult donors. Source materials were transported and stored at 4°C. B-cells were positively isolated using anti-CD19 immunomagnetic MACS® MicroBeads, without prior density gradient centrifugation or red cell lysis, at 3, 24, and 48 hours post-collection. After RNA extraction from the B-cell isolates, individual samples underwent bulk RNA sequencing. Relative proportions of immune cell subtypes were quantified using the iSort™ Fractions v1.4 (CIBERSORT) AI/ML-based digital cytometry deconvolution algorithm applied to bulk RNAseq gene expression data, and by flow cytometry. iSort HiRes v1.1 (CIBERSORT) was run and sequencing reads mapped and quantified by Salmon v1.9 with Gencode v31 annotation. Significant inferred, differentially expressed genes were defined as having log2foldchange >0.5 or <−0.5 and false discovery rate adjusted p-value <0.05. Significance vs. magnitude of expression change was visualized via volcano plots.

RESULTS: Digital cytometry results (mean±SE) for WB and AP-derived PBMC are presented in uploaded Table 1. Immune cell ratios were confirmed by flow cytometry. The number of significantly, differentially expressed genes in B-cells from PBMC and WB are presented in uploaded Table 2. Of 30 evaluated B-cell-specific genes, 24 (80%) were expressed at higher levels in WB- than PBMC-derived B-cells after both 24 and 48 hours simulated shipping. Specifically, CXCR4 expression (i.e., chemotaxis, motility, and survival) decreased and PMAIP1 expression (i.e., pro-apoptosis) increased linearly over time in B-cells from both PBMC and WB.

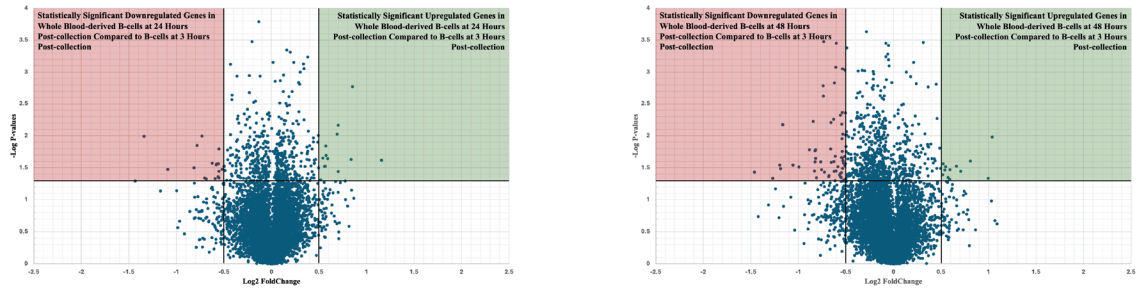
DISCUSSION: The gene expression profiles of human B-cells isolated from AP-derived PBMC products and WB following 24 hours and 48 hours of simulated shipping at 4°C are not identical to those isolated within 3 hours of collection. We used AI/ML-based digital cytometry and inferred B-cell specific gene expression to eliminate misleading gene expression noise from non-B-cells, mostly monocytes and NK cells, found primarily in the B-cell isolates from PBMC. The differential gene expression profiles suggest that, regardless of source material, but more so in AP-derived PBMC, the longer the period of refrigerated “shipping” the more mitochondrially stressed and likely dysfunctional the isolated B-cells. The interval between source material collection and isolation of B-cells, and probably other immune cells, for the manufacture of adoptive cell therapies should be minimized to optimize infused product performance.

Table 1

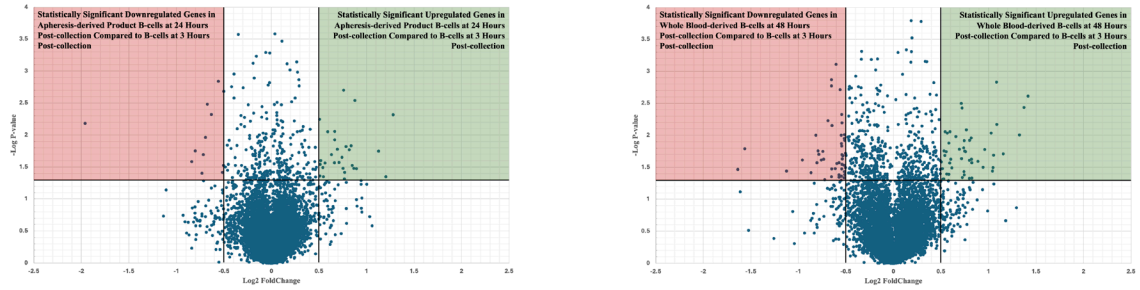
	AP-DERIVED PBMC (%)			VENOUS WB (%)		
	3 Hour	*24 Hours*	*48 Hours*	*3 Hour*	*24 Hours*	*48 Hours*
B-cells	69.7±9.8	63.6±7.1	73.6±2.2	94.5±0.9	92.4±1.5	92.5±0.5
T/NK-cells	5.9±0.1	10.0±1.3	9.4±1.0	5.3±0.8	7.5±1.4	7.3±0.4
Monocytes	24.4±9.8	26.4±7.9	17.0±1.7	0.2±0.1	0.1±0.1	0.2±0.1

Table 2

	AP-DERIVED PBMC		VENOUS WB	
	24 Hrs vs. 3 Hrs	*48 Hrs vs. 3 Hrs*	*24Hrs vs. 3 Hrs*	*48 Hrs vs. 3 Hrs*
Upregulated	29	49	13	12
Downregulated	12	46	18	61



Volcano plots of the inferred gene expression for B-cells isolated from venous whole blood 24 hours post-collection compared to that for B-cells isolated from venous whole blood 3 hours post-collection (left figure above) and for B-cells isolated from venous whole blood 48 hours post-collection compared to that for B-cells isolated from whole blood 3 hours post-collection (right figure above). The red and green shaded areas represent statistically significant and relatively downregulated and relatively upregulated genes, respectively.



Volcano plots of the inferred gene expression for B-cells isolated from apheresis-derived products 24 hours post-collection compared to that for B-cells isolated from apheresis-derived products 3 hours post-collection (left figure immediately above) and for B-cells isolated from apheresis-derived products 48 hours post-collection compared to that for B-cells isolated from apheresis-derived products 3 hours post-collection (right figure immediately above). The red and green shaded areas represent statistically significant and relatively downregulated and relatively upregulated genes, respectively

3007 Enhancer AAVs for the Treatment of Motor Deficits in Parkinson's Disease.

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Impairment of the nigrostriatal system and decreased levels of dopamine release in the striatum has been recognized as the principal neuropathology underlying Parkinson's disease (PD). However, there is still no cure or therapeutics to slow disease progression. The most widely used treatment is levodopa (L-DOPA), and while effective in the short-term, prolonged usage of this drug has been known to cause dyskinesias (i.e., impairments in regular movements), collectively called LIDs (L-DOPA-induced-dyskinesias). Gene therapy has the potential to provide long-term clinical benefits for debilitating human disorders, including Parkinson's disease. One of these trials showed promising results using AAV2 to drive the expression of human aromatic l-amino acid decarboxylase (hAADC)—a rate-limiting enzyme in dopamine synthesis—under the control of a ubiquitous promoter (CMV). This required a bilateral, intraoperative MRI-guided putaminal infusion, which is expensive, highly invasive, and prone to technical issues. The ability to deliver the therapeutic cargo non-invasively while retaining selectivity would be a huge advancement in the field of gene therapy.

To determine if cell type specificity in delivering hAADC may improve the efficacy of L-Dopa treatment and reduced LIDs, we developed an enhancer-based AAV toolkit to systemically target relevant cell populations (striatal medium spiny neurons (pan-MSNs), striatal cholinergic neurons, and midbrain dopamine neurons) with low off-target expression. We tested the efficacy of our lead pan-MSN vector in a 6-OHDA toxin model and MCI genetic PD model in mice, using multiple behavioral paradigms. Our work showed that when directly injected into the

dorsal striatum of mice, the cell-type specific intervention had more robust rescue than ubiquitous expression, which is currently the benchmark in clinical trials. Furthermore, intravenous delivery of pan-MSN-specific hAADC ameliorated specific motor-related deficits in these models while exhibiting fewer adverse side effects than ubiquitous CMV-promoter AAV. Our enhancer-based strategy needed a lower dose of L-DOPA to restore their motor behaviors, which could improve the quality of life by delaying the onset and possibly diminishing the detrimental side effects of L-Dopa in the patients. A higher dose of L-Dopa over two weeks showed a significantly lower frequency of dyskinesia (LIDs) with MSN-specific enhancer AAV compared to no virus and ubiquitous groups. Intravenous delivery of striatal cholinergic- and midbrain dopaminergic-specific hAADC also showed strong behavioral rescue in the unilateral toxin model at the lower L-Dopa dose but not in the genetic model where the loss of dopaminergic projections to the striatum is more pronounced and developmental. The lead pan-MSN vector was further evaluated in non-human primates (NHP) following intraparenchymal and intracerebroventricular delivery, demonstrating cross-species expression and translational capability.

In summary, our work provides strong proof-of-concept data supporting enhancer-AAVs as potential circuit-selective therapies for PD. Cell-type-specific expression of hAADC via a noninvasive route of administration can improve motor functions in PD patients with a lower dose of L-DOPA. This enables us to bypass invasive brain surgery to achieve a similar or better therapeutic effect while simultaneously reducing the disruptive side effects of the drug treatment.

3008 Establishing Chemistry, Manufacturing, and Controls (CMC) and Its Impact on Critical Components of Clinical Trials

Mo Heidaran
CHCF Brain Foundation, Potomac, MD

There are two broad categories of assays used in cell and gene therapy products from my perspective: **Category 1:** These assays are used to characterize and/or release the drug product. Given the higher risk to the product if a release assay is not qualified and validated, it is important for sponsors to demonstrate that these assays are appropriately qualified and validated for each phase. For instance, prior to the initiation of pivotal studies, assays for safety, identity, purity, and potency should be fully qualified and, in some cases, validated. **Category 2:** These assays are used to test patient materials in clinical trials. Examples include genetic tests for determining patient inclusion or exclusion, assays to measure anti-drug antibodies, and assays used to measure potential surrogate endpoints. Because these assays pose a higher risk to the patient if not controlled, there have been recent changes in the formal expectations for how sponsors should demonstrate their fit-for-purpose and robustness during the clinical trial. Assays in the clinical space may be regulated as companion diagnostics (IVD), laboratory-developed tests (LDTs), or may be performed by CLIA-certified labs. I will discuss different requirements for different assays used to support safety and effectiveness of cell and gene therapy products. Is it LDT, IVD and/or companion diagnostics in view of recent changes to the FDA's final rule on LDTs?

3009 VTX-PID Mediated Optimal Depletion of Anti-AAV3B Neutralizing Antibodies in Human Subjects allows to Expand Patient Eligibility for future AAV-Based Gene Therapies

Jean-Philippe Combal[1], Vernadal Elodie[1], Blanche Tamarit[1], Sonia Valero[1], Veronica Ferrer[1], Anne Douar[1], Céline Bouquet[1], Laia Trigueros Motos[2], Gloria Gonzalez-Aseguinolaza[2], Bernard Benichou[1]
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Preexisting circulating anti-AAV neutralizing antibodies (NAb) prevent AAVs from effectively delivering the therapeutic gene to target cells, reducing the efficacy of gene therapy in a significant proportion of patients ranging from 20 to 65% depending on patient's age and AAV serotype. VTX-PID (imlifidase) is a streptococcal protease that specifically and rapidly cleaves IgG antibodies into F(ab')2 and Fc fragments. Here we report results from a first in man, double blinded and randomized study with single ascending doses (0.075 to 0.6 mg/kg) of VTX-PID in healthy, male subjects, stratified according to their anti-AAV3B NAb levels (intermediate or high) . As of today, twenty-nine subjects have been dosed with VTX-PID or placebo. Within hours, all undigested IgG had disappeared

from plasma at all 4 doses tested and then progressively recovered; similarly, anti-AAV3B NAb levels dropped quickly, with a complete and durable reduction observed at all doses in subjects with intermediate NAb levels. However the effect was more prolonged at the highest dose of 0.6 mg/kg VTX-PID. Three out of 29 currently treated subjects developed transient and self resolving mild or moderate IARs (infusion associated reactions), consisting of hot flushes, facial discomfort, exanthema, and/or abdominal discomfort at the highest dose; 13 experienced mild to moderate reversible AEs consisting of musculoskeletal complaints (arthralgia and/or myalgia) accompanied with hs-CRP elevations and leukocytosis, with or without fever. 2 of these subjects also experienced a transient LFT elevation, which triggered the addition of prophylactic prednisone in the 6 next subjects treated at the highest dose. Based on these data, a PK/PD model has been built including variables from patient-specific factors (e.g. ADA, PK, NAbs level…) to define the most suitable dosing schedule to achieve the optimal window for AAV3B-based treatment. Therefore, VTX-PID-mediated anti AAV NAb removal provides an opportunity to expand the patient population that may benefit from AAV-based systemic gene therapies.

3010 Generation of High Purity Allogeneic CAR-T Cells from CD34+ Cord Blood HSPCs Through the Efficient Delivery of Large Genetic Cargos

Sean Allen[1], Samantha Bremner[1], Nanda Sasi[1], Lanny Gov[1], Jiaji Yu[1], Maha Qubain[1], Jeff Wiezorek[1], JJ Kang[1], Jason Damiano[1]
[1]*Appia Bio*

The development of next-generation allogeneic cell therapies from primary cells requires the efficient delivery of numerous transgenes to maximize product purity and functionality in vivo, necessitating the development of techniques to increase the capacity of genetic cargo delivery. Here we describe an approach that utilizes dual LVV with a “Split TCR” architecture to deliver 5+ transgenes and create stem cell-derived γδT or NKT cells with optimal purity and functional properties. Off-the-shelf chimeric antigen receptor (CAR) expressing T cells derived from hematopoietic stem and progenitor cells (HSPCs) require the stable integration of the CAR transgene (typically >1 kb), a TCR (~1.8 kb), as well as other payloads to provide armoring of the product to improve its potency or persistence. As all viral delivery methods have limitations on the size of DNA that can be packaged and delivered per virion, we have developed a method of transducing cells with two lentiviral constructs simultaneously, where each construct contains one TCR chain. Appia’s ACUA (Appia Cells Utilized for Allogeneic) platform applies selective pressure during cell differentiation and expansion throughout CAR-T manufacturing for cells expressing a complete TCR complex. This allows for cells with the stable integration of both TCR chains to outperform cells with only one chain, resulting in >80% of end-product cells expressing all transgenes. Beyond the CAR and TCR, this "Split TCR" LVV design approach allows for the inclusion of armoring payloads, shRNA expression cassettes, and multiple promoters to provide desirable pharmacological features to drug candidates. “Split TCR” CD19-CAR γδT and NKT cells potently killed target cells in vitro in both short- and long-term cytotoxicity assays, demonstrated robust target-specific expansion in vitro, and displayed high levels of in vivo efficacy in a tumor model. This work demonstrates the ability to efficiently deliver >10 kb of integrated DNA to HSPCs, resulting in robust expression of 5+ transgenes to generate highly efficacious allogeneic CAR-T cells. Furthermore, this process has been found to be compatible with large-scale manufacturing of development candidates to support clinical trials in autoimmune diseases and cancer.

3011 Case study of Single use centrifuges for processing HEK lenti virus in both intensification and viral collection in bench and manufacturing scales

David Richardson
Carrbiosystems

CARR Biosystems™ manufactures three sizes of single-use centrifuges: UFMini®, UniFuge® Pilot, and U2k®. All three centrifuges provide versatility with capabilities to collect concentrate and wash HEK – 293 and Vero to collect both AAV, and Lentivirus. These systems achieve >90% recovery, are scalable, and they reduce filtration. CARR Biosystems single-use centrifuges are in licensed facilities for human therapeutics and have been in the manufacturing environment since 2010. This presentation will examine case study from a viral

vector clinical manufacturing facility. These HEK cell lines are very sticky and tend to clog filters, with our single-use centrifuge- we increase viral titer while reducing filtration.

3012 AAV-Mediated DUX4 Knockdown in Facioscapulohumeral Muscular Dystrophy: Therapeutic Benefits and Challenges for Long-Term Efficacy

Virginie Mariot[1], Solene Sohn[2], Sophie Reid[2], Maximilien Bowen[3], Canille Hudon[2], Emilio Corbex[2], Baptiste Morel-Prieur[3], Christophe Hourde[3], Julie Dumonceaux[1]
[1]*University College London*; [2]*UCL GOSH Institute of Child health*; [3]*Universite Savoie Mont Blanc*

Background:
Facioscapulohumeral muscular dystrophy (FSHD) is characterised by the aberrant expression of the toxic transcription factor DUX4 in skeletal muscle, leading to progressive muscle degeneration. Therapeutic strategies targeting DUX4 inhibition are promising, with AAV-mediated delivery emerging as a key approach. However, the regenerative nature of skeletal muscle poses a challenge to AAV-based therapies, as muscle fibre turnover can diminish AAV transduction efficiency and the persistence of therapeutic transgenes over time. Aims: This study aimed to evaluate the efficacy and durability of AAV-mediated DUX4 inhibition using an AAV vector carrying a short hairpin RNA targeting DUX4 (shDUX4) in a mouse model of FSHD. The goal was to assess this approach's short- and long-term therapeutic impact and identify potential limitations.

Methods:
The ACTA1-MCM; FLExDUX4/+ mouse model, which replicates key features of FSHD, was used. Mice received a single administration of AAV-shDUX4 (MyoAAV 2A capsid), and therapeutic efficacy was assessed one month and 10 months post-treatment. Outcomes included molecular analyses, histopathological evaluation, and functional assessments using force-velocity-endurance (FOvE) tests. The persistence of AAV genomes and DUX4 knockdown efficacy were also evaluated over time.

Results:
One month after AAV-shDUX4 administration, significant improvements were observed in DUX4-associated pathological features, including reduced molecular markers of toxicity, improved muscle histology, and enhanced functional FOvE parameters. However, by 10 months post-treatment, these therapeutic benefits had largely diminished. Most pathological markers remained uncorrected, and muscle force showed no sustained improvement. The decline in therapeutic effect is correlated with reduced DUX4 knockdown and a loss of AAV genomes, which is likely due to muscle fiber turnover and AAV genome dilution.

Conclusion:
AAV-mediated shDUX4 therapy offers significant short-term benefits for FSHD by effectively suppressing DUX4 expression. However, the long-term therapeutic efficacy is limited by muscle fibre regeneration and associated AAV genome loss. These findings underscore the need for strategies to enhance the durability of AAV-based therapies to achieve sustained benefits in FSHD treatment.

3013 Myostatin is a Marker of Disease Severity in Facioscapulohumeral Dystrophy

Virginie Mariot[1], Solene Sohn[2], Sandrine Arbogast[3], Dalila Laoudj-Chenivesse[3], **Julie Dumonceaux**[1]
[1]*University College London*, [2]*UCL GOSH Institute of Child health*,[3]*Universite of Montpellier*

Background:
Facioscapulohumeral dystrophy (FSHD) is a rare genetic disease with a prevalence of 4.5/100000 in Europe. The disease is characterised by weakness and atrophy of specific muscle groups. The likely cause of the disease is the aberrant expression of the double homeobox DUX4 transcription factor in skeletal muscles. Over the past five years, DUX4 has become a favourite target for pharmaceutical companies developing treatments for FSHD, and several approaches to mitigate DUX4's pathological role have been developed, including small molecules, antisense oligonucleotides, and gene therapy vectors, with 2 clinical trials currently underway. However, the clinical trials are complicated by the high variability in disease onset and progression in patients. Moreover, a significant challenge in

the FSHD field is the difficulty in measuring DUX4 expression in patient biopsies, which complicates disease monitoring and therapy development. While circulating biomarkers could provide a quantitative and robust measure of disease severity and therapeutic efficacy, none are currently available.

Aims
This study aimed to evaluate myostatin (MSTN) as a potential biomarker for FSHD disease severity.

Methods
We analysed serum MSTN levels in FSHD patients and healthy controls, followed by a larger analysis of serum from 51 FSHD patients using ELISA. The correlations between MSTN levels and clinical severity scores were evaluated. We then used a DUX4-expressing mouse model, which exhibits chronic and mosaic DUX4 expression and develops an FSHD-like pathology over time to measure Mstn levels at both the mRNA and protein levels in muscle biopsies and serum.

Results
In mice expressing DUX4, Mstn expression was significantly lower at both the mRNA and circulating protein levels than controls, with further decreases observed as the disease progressed. In FSHD patients, MSTN levels were approximately two-fold lower than in healthy controls. In the larger patient cohort, MSTN levels strongly inversely correlated with clinical score (pearson r=-0.74; $p = 9 \times 10^{-10}$).

Conclusion
Our findings suggest that MSTN could be a reliable biomarker of the severity of FSHD disease.

3014 Drug Discovery of Next-Generation Circular mRna Vaccine Platform Targeting Tumor Immune Escape Mechanism

Peng Huang, Tianyun Zhou, Kazuyo Igawa, Hiroyuki Michiue, Motoo Araki
Okayama University

Introduction & Objectives
Immunotherapy represents a novel therapeutic approach to cancer treatment in which the immune system's potential is harnessed to recognize and eliminate tumor cells. mRNA vaccines are regarded as a highly promising avenue for next-generation cancer immunotherapy. The purpose of this study is to create an innovative cancer treatment method that is new mRNA vaccines can cure even advanced prostate cancers that have invaded or metastasized.

Materials & Methods
Using benign prostatic hyperplasia, hormone-sensitive prostate cancer and Castration-Resistant Prostate Cancer tissues from the same patient, multiple tumor-specific mutant mRNAs were identified using a next-generation sequencing approach, candidate circRNA expression was validated by quantitative real- time PCR (RT- qPCR) using divergent primers. Furthermore, mutant mRNAs are delivered by existing nanocarriers, as a novel platform of mRNA vaccines.

Results and Conclusions
We identified multiple neoepitopes from mutant mRNAs that are upregulated in prostate cancer samples from clinical samples and verified with the MiOncoCirc database. Human leukocyte antigen binding affinity of open reading frames from potentially translatable circRNA was assessed using pVACtools. low-dose circRNA nanoparticles elicited potent immune responses in both young adult mice and aged mice, protected mice from target cell challenge, and mediated robust tumor immunotherapy.

In conclusion, this study will lay the foundation to develop circRNA as a novel platform of mRNA vaccines for wide application in the prophylaxis and treatment of cancer.

3015 First-in-human study of cord blood-derived NK cells generated by mRNA-LNP- for relapsed/refractory acute leukemia

Antonio Carrasco[1], **Sonia Macia Escalante**[2], Hassan Bennani[3]
[1]*Clinica Anglo Americana,* [2]*SMED Clinical Research*, [3]*Immunovacell*

Acute myeloid leukemia (AML) is an aggressive cancer in which an excess of myeloblasts is found in bone marrow and blood. AML is the most common type of acute leukemia in adults, accounting for 80% of acute leukemia cases in Western countries. The main cause of therapeutic failure is resistance to chemotherapy by abnormal pathways that allow blasts survive and relapsing even after attaining complete response. Although prognosis for relapsed/refractory (R/R) patients is particularly poor, a proportion can be efficaciously salvaged using chemotherapy-based therapies followed by allogeneic hematopoietic stem cell transplant. However, many AML patients are not considered candidates for front-line or salvage intensive chemotherapy-based strategies. Given the dismal prognosis and the absence of standard/satisfactory treatment options, patients with R/R AML continue to represent a challenging unmet medical need. Cellular immunotherapy is gathering increasing interest for cancer treatment, which can be deployed in different ways: lymphokine-activated killer (LAK) therapy, tumor-infiltrating lymphocyte (TIL) and, for better recognition and killing of tumor cells, modified immune cells expressing antigen-specific T cell receptors (TCRs) or chimeric antigen receptors (CARs).

CAR-NK cells have a potential for generating “off-the-shelf” products that could be readily available and safe for clinical use. CAR-NK cells can be generated from multiple sources, including NK92 cell lines, peripheral blood mononuclear cells, umbilical cord blood, and induced pluripotent stem cells. Their unique biological features include lower risk of on-target/off-tumor toxicity to normal tissues due to their limited lifespan in blood; potentially eliminate cancer cells in a CAR-independent manner, via both CAR-dependent and NK cell receptor-dependent mechanisms.

One of the major barriers for CAR-NK has been the lack of an efficient gene transfer method in the cultured NK cells. This has been overcome by using a lipid nano particle (LNP) for mRNA delivery into cocultured NK cells at very last state and expanded with IL-2 and IL-15for long lasting presence as circulated CAR-NK after infused.

Manufacture “off-the-shelf” CAR-NK products can be ready for clinical use, with less manufacturing time and lower cost, as it eliminates the manufacturing time and allows true point of care administration and can scale to much larger numbers.

This is an open label, Phase 1 study in adult patients with AML to determine the recommended Phase 2 dose (RP2D) of CAR-NK19. Secondary objectives include PK and preliminary anti-tumor activity. Dose escalation will follow an adaptive design, which would allow to optimize different treatment schedules and increase specific cohorts based on response rate.

3016 Unique Surface Anchoring of Cytokines Mediates Improved T-cell Subpopulation Expansion for Immunotherapy

Colleen O’Connor, Andres Campos, Jinchun Wang, Vanessa Garapola, Andries Zijlstra

StimulusBio

Existing cellular expansion technologies are greatly limited because they are unable to specifically expand target cells, require excessive material resources or synthetics, and lead to unintended or undesired phenotypes. This failure is in large part due to the inability of existing methods to recapitulate the full complement of cell signaling required to mediate target cell expansion. The rapid expansion of cell-based therapies and associated research requires effective expansion with reduced costs and improved scalability. A customizable and programmable cellular interface, sbPCI, was designed to promote selective cellular expansion by purposely creating an individualized T-cell cytokine dosing system to decrease cost and technician time while improving the final immunotherapeutic product. To demonstrate the utility of sbPCI, human RBCs were modified to mediate

stimulation of autologous and/or allogeneic T-cells; specifically in this project, to present activating co-stimulatory molecules, as well as cytokines, Interleukin-2 (IL-2) and Interleukin-21 (IL-21). The sbPCI platform presents biomolecules at a density and configuration optimized previously for T-cell specific activation, while the sbPCI is modified to display tethered cytokines. sbPCI bioactivity was demonstrated through a 21-day expansion assay benchmarking performance metrics against industry standards using exogenous human recombinant IL-2 and IL-21. Expanded T cells were enumerated, cryopreserved, and phenotyped weekly. Additional samples were collected daily and twice per week (24hrs post and at 7-day post stimulation) for gene expression analysis to determine immune-specific mRNA changes. Single cell analysis was also conducted to determine individual T-cell cytokine "feeding" patterns with respect to sbPCI co-culture at a 1:1 ratio. Data analyzed included expansion and genotypic changes.

Variations in the intentional mechanical stressors on the RBC as well as cytokine tethers triggered membrane alterations that resulted in cyclical sbPCI presentation over 5 to 7 days. Continuous cytokine cycling within the co-culture triggered nuanced phenotypic and genotypic T-cell differentiation. The sbPCI drove exponential T-cell expansion with a mean viability ≥ 90%. A 48-hour sbPCI/PBMC co-culture resulted in ≥ 60% of activated T-cell expansion as defined by elevated expression of Granzyme B and IFN-y. By day 7, the cultures were primarily composed of the desired $CD3^{+}CD8^{+}$ T cells. Gene expression was consistent with activation in response to biostimulatory molecule presentation, revealing exploitable T-cell variations accessible through selective programming. Irreversible cellular exhaustion was not observed with the use of sbPCI.

The current implementation of sbPCI emphasizes polyclonal expansion of cytotoxic T-cells. Furthermore, specific tuning of sbPCI expression will be leveraged in future programming to selectively expand variations that emulate the expansion of therapeutically active populations *in vivo*. In doing so, sbPCI can produce subtypes tailored to safely support and improve CAGT efficacy leading to a narrowed production window.

3017 Access to Curative Gene Therapies in Africa: A Syatematic Analysis of Barriers and Enablers

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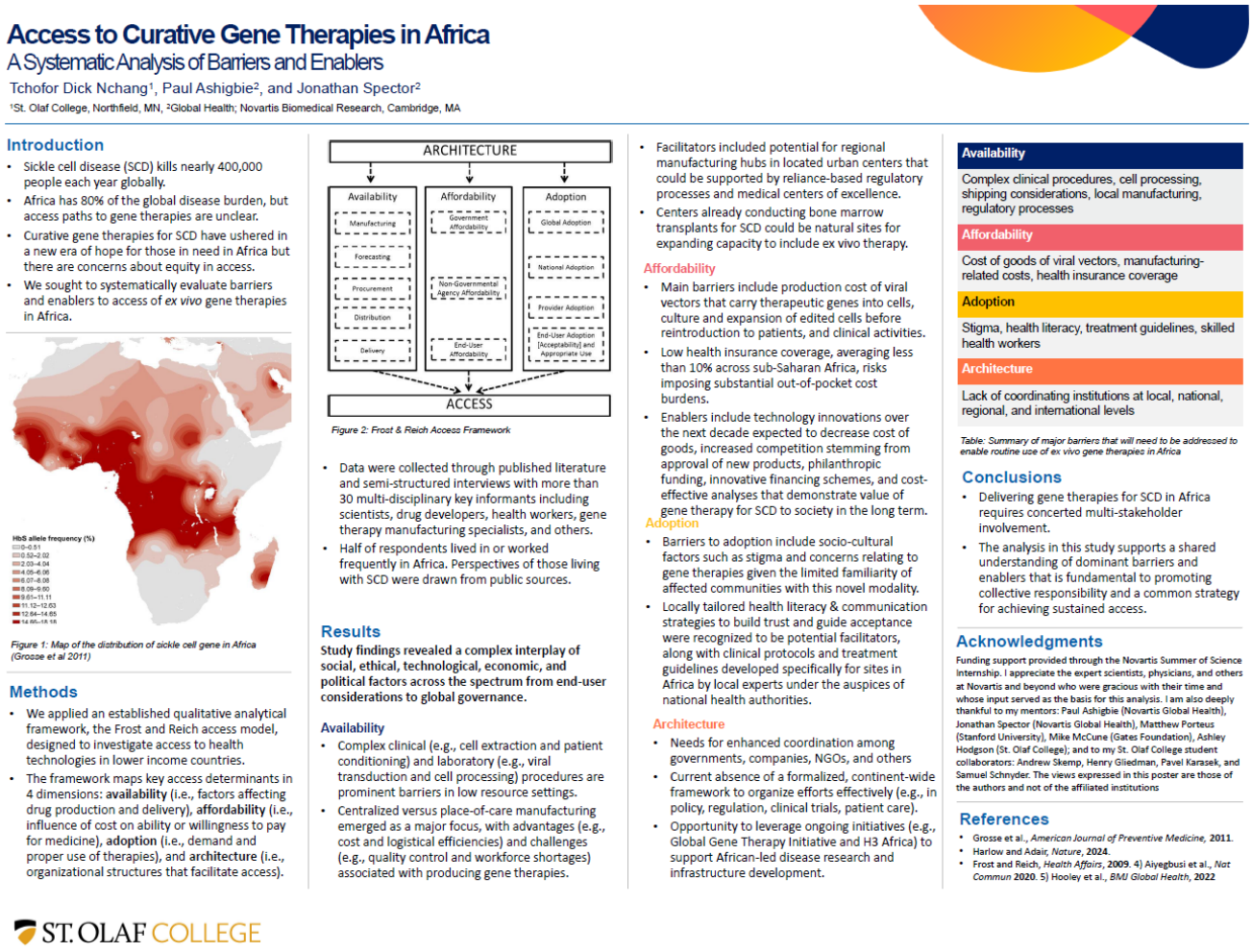
Background
The recent approval of curative gene therapies for sickle cell disease (SCD) is to be celebrated even as concerns have been voiced about equity in access to medicines in Africa, home to 80% of births affected by SCD. This study systematically evaluated barriers and enablers to ex vivo gene therapy distribution in Africa using an established qualitative analytical framework designed to investigate access to health technologies in lower-income countries.

Methods
The model used in this study maps key access determinants across four dimensions: availability (factors affecting drug production and delivery), affordability (cost influence on ability or willingness to pay), adoption (demand and proper use of therapies), and architecture (organizational structures facilitating access). Data were gathered through published literature and semi-structured interviews with over 30 multi-disciplinary experts including scientists, clinicians, and gene therapy specialists. More than half of respondents worked in Africa. Publicly available perspectives of individuals living with SCD were also included.

Findings
The study revealed a complex interplay of social, technological, economic, and political factors ranging from end-user aspects to global governance. Regarding availability, complex clinical (e.g., cell extraction) and laboratory (e.g., viral transduction) procedures were prominent recognized barriers, while the potential for regional manufacturing hubs in urban centers, supported by reliance-based regulatory processes, emerged as a facilitator. Affordability concerns are driven by high production costs and low health insurance coverage, which risks imposing substantial out-of-pocket expenses. Enablers include future technological innovations expected to lower costs, increased market competition, and innovative financing schemes. Adoption is hindered by socio-cultural factors including stigma and unfamiliarity with gene therapies and enabled by locally tailored health literacy programs and clinical protocols developed by African experts. Architecture challenges include the absence of a formalized,

continent-wide framework to coordinate efforts, although leveraging existing initiatives such as the Global Gene Therapy Initiative could help address this gap.

Conclusion
Delivering gene therapies for SCD in Africa will require concerted multi-stakeholder involvement. This analysis promotes a shared understanding of dominant barriers and enablers that is fundamental to encouraging collective responsibility and a common strategy for achieving sustained access.



3018 A "Transcriptional Repressor Substrate Reduction Strategy" for Tay-Sachs and Sandhoff diseases

Alessia Medugno[1], Rosa Maritato[1], Emanuela D'Andretta[1], Claudio Mussolino[2], Enrico Surace[1]
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Tay-Sachs disease (TSD) and Sandhoff disease (SD) are lysosomal storage disorders caused by mutations in the HEXA and HEXB genes that result in the accumulation of GM2 ganglioside and progressive neurodegeneration. Current treatment options are limited and do not effectively target central nervous system involvement. To address this challenge, we are developing a gene therapy approach that uses transcriptional repressors to regulate genes involved in ganglioside biosynthesis, thereby reducing substrate accumulation. We initially mapped B4GALNT1

regulatory elements in cis by assessing the accessibility-occupancy of the proximal regulatory region of B4GALNT1 by bioinformatics analysis. We then generated constructs with deletions and site-specific modifications of B4GALNT1 regulatory elements and assessed their impact on B4GALNT1 expression by gene reporter assay. Once we identified the critical regions that functionally control B4GALNT1 expression, we validated their relevance by editing these cis-regulatory elements in the native B4GALNT1 locus by genome editing (CRISPR/Cas). In parallel, we are perturbing /interfering the expression of B4GALNT1 by targeting the identified cis targets in trans through (i) ectopic expression of endogenous transcription factors (TFs), (ii) synthetic transcription factors (TFs) and (iii) CRISPR inhibition systems (CRISPRi). We are currently implementing this transcriptional repression strategy also in fibroblasts from patients with Tay-Sachs and Sandhoff diseases.

3019 Engineered Polyclonal Anti-tumor Antibodies to Counteract Tumor Escape

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Counteracting tumor adaptation is a long-standing challenge in oncology. Targeted therapies are susceptible to escape following mutation, shedding, or downregulation of the tumor antigen. Polyclonal treatments targeting multiple antigens may improve outcomes by impeding tumor resistance. Tumor-Infiltrating Lymphocytes are polyclonal T cell therapies clinically approved for the treatment of selected solid tumors. However, these autologous medicines are complex to manufacture. To conceptualize a strategy to overcome tumor resistance, we turned to tumor-infiltrating antibody expressing lymphocytes. Tumor-infiltrating B cells are linked to favorable prognosis and were demonstrated to promote response to immune checkpoint blockade.

We harvest tumor-infiltrating B cells to produce a polyclonal, anti-tumor antibody therapy for multiple malignancies. We enrich, culture and differentiate tumor-infiltrating lymphocytes towards antibody-secreting cells. Polyclonal antibodies purified from tumor-infiltrating B cell cultures effectively bind patient-derived tumor cells and cell lines from similar tumor types.

Importantly, to elicit supraphysiological responses, we engineer tumor-infiltrating antibody-secreting cells. Specifically, we employ CRISPR-Cas9 RNP and AAV transductions to target the constant segments of the antibody coding genes to force the secretion of T cell engager molecules, without modifying the polyclonal variable domains. We achieve up to 23% engineering in patient derived samples. Antibodies purified from engineered tumor-infiltrating lymphocyte cultures engage T cells without significant impairment in tumor binding capacity.

Anti-tumor antibodies may also be extracted from tumor-associated fluids. Indeed, ascites-infiltrating antibody expressing cells are more frequent than in peripheral blood, enabling robust cell cultures from very large samples. Polyclonal antibodies derived from ascites fluids or from ascites infiltrating B cells cultures also display anti-tumor activity, but not antibodies derived from the sera of healthy donors or antibodies from the sera of patients with similar tumor types. Polyclonal antibodies derived from these strategies did not bind healthy cells, indicative of tumor specificity and facilitate tumor cell killing in co-cultures with effector cells.

Uniquely, our method produces engineered polyclonal anti-tumor antibodies for a wide variety of solid malignancies. This therapy is non-autologous and combining several sources of antibodies may produce a potent therapeutic with cross-tumor reactivity. We propose that polyclonal anti-tumor antibody therapy should be considered as a novel strategy to counteract tumor escape.

3020 Challenges and Advancements in GLP-Compliant Preclinical Toxicology Studies for ATMPs

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Aptuit (Verona) Srl, an Evotec Company

Preclinical toxicology studies play a critical role in the drug development t ensuring their safety and effectiveness before entering clinical phases. These studies must comply with Good Laboratory Practice (GLP) principles, following international regulatory guidelines (FDA, OECD, EMA, and ICH). While these regulations are well-defined for traditional small-molecule drugs, their direct application to Advanced Therapy Medicinal Products (ATMPs) presents unique challenges. Unlike conventional pharmaceuticals, ATMPs often feature highly diverse and complex mechanisms of action, combining multiple therapeutic strategies, such as gene editing, cell therapy, and tissue engineering, within a single product, and therefore, their unique nature demands a product-specific preclinical development approach. A major hurdle in ensuring GLP compliance for ATMPs lies in addressing critical aspects such as Test Article characterization, manipulation, formulation analysis, stability testing, and the establishment of well-defined acceptance criteria. These challenges are further compounded by the need for specialized delivery techniques, including surgically based in situ administration, which requires meticulous planning, validation, and execution. Additionally, study protocol endpoints must be carefully defined and managed to ensure that the generated toxicology and biodistribution data are not only scientifically robust but also interpretable for regulatory submissions. Given the variability inherent to ATMPs, traditional GLP study designs often require modifications or refinements to accommodate the specific properties of these advanced therapies.

Over the past five years, Evotec's GLP Test Facility has played a pivotal role in refining processes to navigate these complexities, conducting a range of toxicology and biodistribution studies tailored to different ATMP modalities. These include gene therapies mediated by adeno-associated viruses (AAVs) and oncolytic viruses, as well as cell-based therapies utilizing diverse cell types with various administration routes and delivery platforms. In this work, implemented quality risk assessments and optimized processes adopted by Evotec GLP Testing Facility were described. In particular, proper definition of the Test Article characteristics, test article formulation analysis and stability, acceptance criteria, and various management approaches based on different ATMP products were explored. Thanks to this work, the facility has enhanced the reliability and reproducibility of preclinical data while maintaining full GLP compliance. This approach has strengthened the integrity of toxicology and biodistribution evaluations, ensuring that preclinical studies for ATMPs align with both scientific rigor and evolving regulatory expectations.

These advancements highlight the importance of an adaptive, science-driven approach in the preclinical development of ATMPs. By integrating regulatory compliance with innovative risk assessment strategies, research facilities can generate high-quality preclinical data that support the successful clinical translation of these groundbreaking therapies. The ongoing refinement of GLP methodologies specific to ATMPs will be crucial in overcoming current and future challenges, ultimately accelerating the safe and effective development of next-generation cell and gene therapies.

3021 An AAV GMP Manufacturing Solution for Large Clinical Demand Indications

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Lexeo Therapeutics

The high cost of goods (COGs) and low yields of current AAV manufacturing processes preclude development pipelines from considering indications with large patient and/or dose demands due to the significant capital required to supply the clinic and to commercialize the drug product. The combination of costly manufacturing and small number of patients has driven up the price of AAV therapies to record-setting heights, further limiting patient access and under-serving many patient populations. We have developed a highly reproducible and scalable 200L GMP AAV production process using the Sf9-baculovirus system. The process is relatively simple – there is no need for costly plasmids, time-intensive generation of producer cell lines, intensification, or large-scale production of recombinant baculovirus, and purification is a straightforward 2-column process. The overall process delivers over 1E15 vg/L of purified AAVrh.10 product that contains less than 25% empty capsids. A 200L GMP batch can yield approximately thirty (30) 6E13 vg/kg doses for Lexeo's LX2020 PKP2 program. Produced this way, Lexeo's AAV

drug substance has shown consistent potency from *in vivo* and IND-enabling studies through GMP production of clinical-grade material and is currently supplying Lexeo's clinical-stage programs.

The current state of AAV manufacturing has significantly limited the progression of AAV gene therapy to successful commercial viability. The universal problem of high COGS and low yield has not allowed gene therapy to have the deep impact on healthcare that it has the potential to do. Lexeo's manufacturing process and overall CMC strategy can dramatically expand patient access and transform the gene therapy field.

3022 AI-powered therapeutic aptamer drug discovery: Targeting CTGF CT-domain for Duchenne muscular dystrophy

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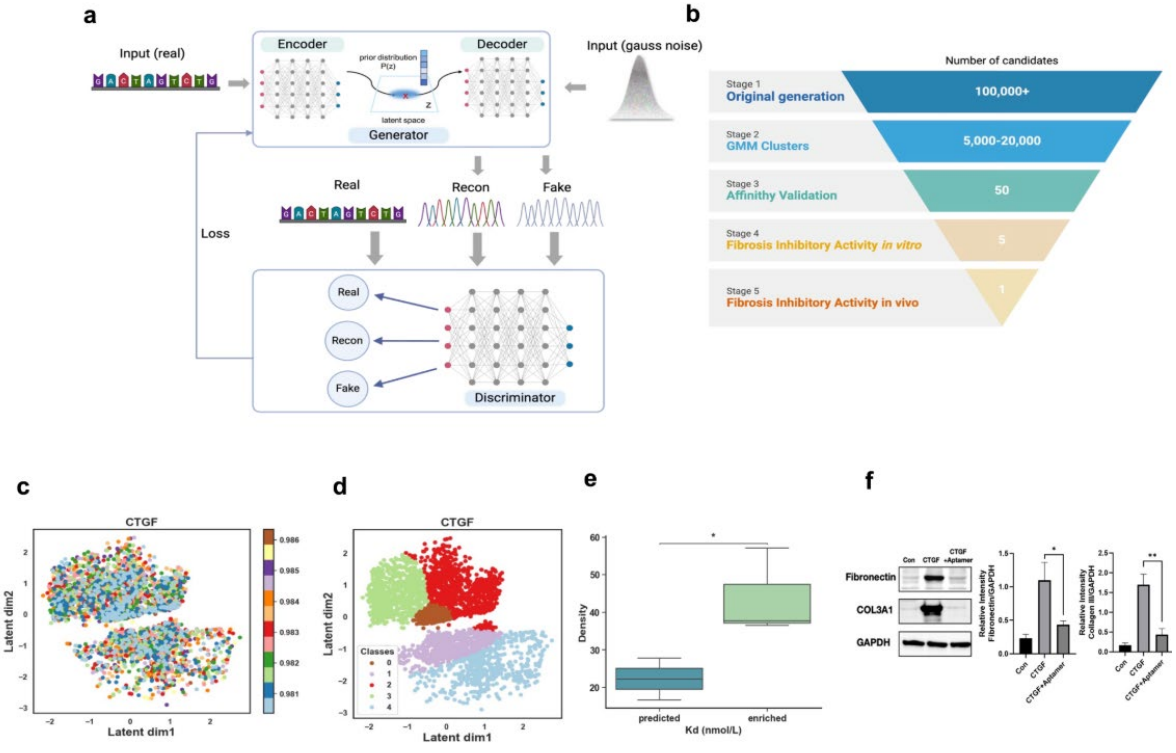
Following the clinical failure of the connective tissue growth factor (CTGF) VWC-domain specific antibody (FG-3019) in Duchenne muscular dystrophy (DMD), we identified the CT-domain as an optimal target than the VWC-domain by experimental validation. The inherent flexibility of the CTGF CT-domain presents a challenge for traditional small molecule approaches due to the absence of stable binding pockets, while monoclonal antibody strategies are primarily challenged by lengthy antigenic immune cycles and poor muscle tissue permeability. To address these limitations, we employed our generative AI method, AptGen, to rapidly discover a potent CTGF CT-domain specific aptamer within 42 days. This chemically modified aptamer could distribute and remain in muscle tissues for an extended period, whereas FG-3019 could not do so. Importantly, this aptamer demonstrated better fibrosis inhibitory activity in vitro and in mdx mice when compared to the FG-3019, with no detected lesions in the major organs. Within 10 months, we went from target domain discovery, aptamer drug discovery, and then obtained the orphan drug (DRU-2024-10138) and pediatric rare disease designation (RPD-2024-838) by US FDA.



3023 Adeno-associated Virus (AAV) Inverted Terminal Repeats (ITRs) Induce Toxicity in Breast Cancer Stem Cells

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The adeno-associated virus (AAV) inverted terminal repeats (ITRs) are the only viral cis elements that remain in the recombinant AAV (rAAV) genomes currently used for gene therapy applications. These 145-nucleotide hairpin-like structures are required for rAAV production and flank both ends of the single-stranded (ss) AAV transgene cassette. Previous reports have suggested that the presence of the AAV-ITR alone, which elicits a cellular DNA damage response (DDR), exhibits significant anti-tumorigenic activity in a subset of cancer cells and cytotoxicity in certain stem and progenitor cells. Because cancer stem cells (CSCs) emerge upon activation of the epithelial to mesenchymal transition (EMT), a transcriptional program involved in the reversion of differentiated epithelial cells to a partially undifferentiated state that mimics the molecular and physiological traits of human embryonic stem cells (hESCs), it was posited that rAAV ITRs could be harnessed to induce selective cell death in CSCs. To investigate this hypothesis, a large and molecularly diverse panel of well-characterized breast cancer cell lines (BCCLs, N=11) were transduced with self-complementary (sc) AAV2 expressing a green fluorescent protein (GFP) reporter under the control of a cytomegalovirus (CMV) promoter (scAAV2-CMV-GFP). Growth rates, cell viability, CSC marker expression, transduction efficiencies, and viral entry were investigated. Cell viability assays completed eight days post-transduction resulted in variable reductions in cell viability across the panel; however, the most sensitive cell lines belonged to the "claudin-low" intrinsic molecular subtype class which is characterized by enrichment of EpCAM-/CD49f+ cell populations (CSCs) and EMT-associated transcriptional profiles. The EpCAM-/CD49f+ CSC population of the two most sensitive BCCLs (MDA-MB-436 and BT549) were further characterized via flow cytometry after treatment with either doxorubicin, a first-line chemotherapy reagent, or scAAV2-CMV-GFP. As expected, both cell lines demonstrated an increase in the percentage of CSCs (>80% of the remaining cells) following treatment with doxorubicin, confirming that the CSC population is chemoresistant. In contrast, three days post-transduction with scAAV2-CMV-GFP, the percentage of CSCs was reduced by up to 20%, suggesting that transduction resulted in CSC death. Data will also be presented to further define the mechanism of AAV-ITR2-associated cell death in CSCs, including the use of small molecule inhibitors of apoptosis and a comparison of ssAAV2-CMV-GFP vectors containing either a wild-type ITR2 (wtITR) or a mutant synthetic ITR (synITR), which has been shown to attenuate p53-dependent apoptosis in hESCs.

3024 In vitro and clinical assessments of muscle specific promoters in driving gene expression and long-term cardiac efficacy in Duchenne muscular dystrophy

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BACKGROUND:
AAV derived gene therapy has been the primary approach for gene therapy in Duchenne muscular dystrophy (DMD). Three of these products include zildistrogene varoparvovec (SGT-001), an AAV9 vector containing a CK8 promoter, fordaditrogene movaparvovec (PF-06939926), an AAV9 vector containing an hCK promoter, and delandistrogene-moxeparvovec (SRP-9001, ElevidysÒ), an AAVrh74 vector containing a MHCK7 promoter. There is ongoing discussion surrounding the efficacy of these treatments to prevent or delay the cardiac manifestations of the disease. Here, we aim to demonstrate the effect of each muscle specific promoter from in-vitro and clinical data.

METHODS:
This is a two-part study looking at in-vitro and clinical data using 7 unique muscle specific promoters. Part 1 (In vitro): C2C12 cells underwent lipofectamine transfection of ITR plasmids encoding *Firefly* luciferase downstream of one of seven muscle specific promoters (Desmin, CK8, TNNT, MHCK7, hCK, TCK, SP-5-12). Co-transfection with an ITR plasmid encoding *Renilla* luciferase downstream of the constitutively active EF1α promoter enabled

normalization of luciferase luminescence. A positive control consisting of the constitutively active CBh promoter was included, as well as several negative controls. *Firefly* luminescence was measured at 590 nm while *Renilla* luciferase luminescence was measured at 460 nm. Part 2 (Clinical): Echocardiogram (ECHO) (N=13) and cardiac MRI (cMRI) (N=10) findings for subjects dosed in four DMD gene therapy clinical trials were analyzed (NCT03368742, NCT03362502, NCT03769116, NCT04626674). ECHO and cMRIs were collected at Baseline, Day 360, Day 730 and Years 3-6. cMRIs were only collected in subjects dosed with SGT-001 or PF-06939926. ECHO and cMRIs from baseline and yearly up to year 6 after dosing were included.

RESULTS: Part 1 (In vitro): All muscle promoter groups showed evidence of positive luminescence at both 590 nm and 460 nm. Negative control showed minimal luminescence at comparable levels (mean: 7.7 rlu at 590 nm; 8.3 rlu at 480 nm). The CBh positive control showed the highest normalized luminescence. Of the muscle specific promoters, Desmin showed the highest overall normalized luminescence (mean: 11.6) which was greater than all other promoters on one-way ANOVA ($p < 0.001$). Part 2 (Clinical): Thirteen subjects met criteria. Seven subjects received SGT-001, three received PF-06939926 and three received SRP-9001. Median age (range) at dosing was 7y (4-14). All were on a daily steroid regimen and under standard cardiac care. Left Ventricular Ejection fraction (LVEF) on ECHO analysis remained within normal range for subjects 2-13, median 66% (53-76) and on cMRI analysis for subjects 2-10, median 58.2% (52.5-64.3). The oldest subject (14y, Subject 1) exhibited a decreased EF via cMRI at Year 5 (39%) when compared to his pre-treatment EF (50.9%) however, had near normal left ventricle (LV) endo Global circumferential strain (GCS)% at Year 5 (–16.8).

CONCLUSION: This two-part study illustrated the Desmin promoter was superior to the others in transfecting skeletal muscle and cardiac function was preserved over time in patients treated with gene therapy. Further studies are needed to test efficacy of each promoter in human cardiomyocytes and additional long term follow up is needed to understand the effects of DMD gene therapy are sustained in cardiac muscle.

Figure 1. Echocardiogram (ECHO) Analysis for Subjects 1-13. Shaded areas denote natural history ranges for Left ventricular internal diameter at end-diastole (LVIDd) and Left ventricular ejection fraction (LVEF) of untreated DMD patients.

SGT-001: Circles indicate patients receiving the low dose (5E13 vg/kg), triangles indicate patients receiving the high dose of (2E14vg/kg). PF-06939926: Circles indicate patients receiving the low dose (1E14 vg/kg), triangles indicate patients receiving the intermediate dose (2E14 vg/kg). SRP-9001: Triangles indicate patients receiving 2E14 vg/kg and circles indicate patients receiving 1.33E14 vg/kg.

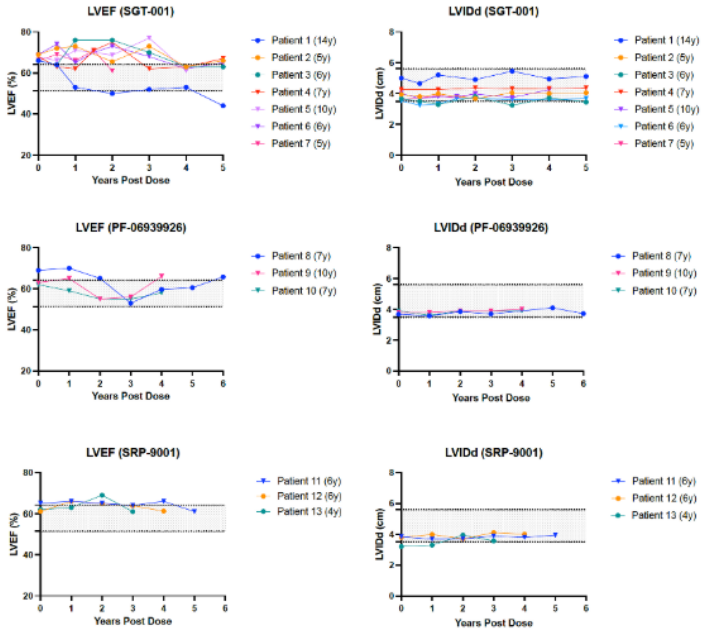
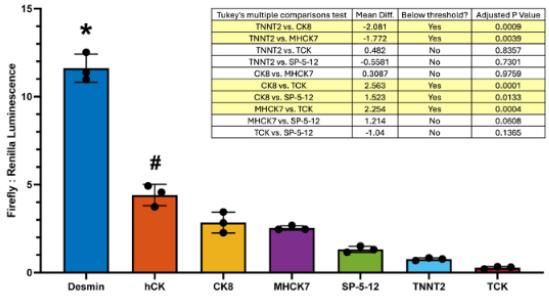


Figure 2. Luciferase luminescence results from transfection experiments using muscle-specific promoters in C2C12 cells. Data are presented as the ratio of *Firefly* to *Renilla* luminescence. One-way ANOVA revealed a significant main effect of promoter type on luminescence ($F(6,14) = 224$, $p < 0.0001$). Tukey's post-hoc test was performed to access pairwise comparisons. The desmin promoter showed significantly higher luminescence compared to all other promoters (* $p < 0.0001$). The hCK promoter showed significantly higher luminescence than all other promoters expect desmin (# $p < 0.05$). The remaining post-hoc comparisons are provided in the table below.



Tukey's multiple comparisons test	Mean Diff.	Below threshold?	Adjusted P Value
TNNT2 vs. CK8	-2.081	Yes	0.0009
TNNT2 vs. MHCK7	-1.772	Yes	0.0039
TNNT2 vs. TCK	0.482	No	0.8357
TNNT2 vs. SP-5-12	-0.5581	No	0.7301
CK8 vs. MHCK7	0.3087	No	0.9759
CK8 vs. TCK	2.563	Yes	0.0001
CK8 vs. SP-5-12	1.523	Yes	0.0133
MHCK7 vs. TCK	2.254	Yes	0.0004
MHCK7 vs. SP-5-12	1.214	No	0.0608
TCK vs. SP-5-12	-1.04	No	0.1365

3025 Leveraging Biobank Scale Data to Uncover Novel Actionable Genetic Variants

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Alzheimer's disease (AD) is the most common form of dementia and has increasing prevalence in global populations. An *APOE-ε4/ε4* genotype is one of the strongest risk factors for AD, though this risk is diminished in individuals with African ancestry. Administration of *APOE* bearing protective variants such as the *APOE* Christchurch missense change represent powerful candidates for AD therapeutics. To uncover novel protective variants for AD and other conditions, we leveraged ~245,400 individuals with short-read whole genome sequencing information of which ~185,000 were unrelated and had matching electronic health record data (EHR) from the NIH *All of Us* dataset. Samples included roughly 32,500 African, 3,200 East Asian, 2,000 South Asian, 98,600 European, 28,200 Admixed American, and 650 individuals of Middle Eastern ancestry. We performed phenome-wide association studies (PheWAS) to identify which of 1,816 potential phenotypes is significantly associated with single nucleotide variants after adjusting for age, sex, and the first three principal components. We confirmed robust association with known variants enriched in African or European populations such as rs334 in sickle cell anemia ($p=1\times10^{-305}$) and rs113993960 in cystic fibrosis ($p=1.91\times10^{-123}$). The ApoE4 defining risk variant rs429358 was associated with AD, but primarily in individuals of European ancestry (OR 2.92; $p=7.31\times10^{-25}$) with less of an effect in individuals of African ancestry (OR 1.59; $p=0.07$). In individuals of African ancestry, we identified several variants that explained this reduced risk, which we confirmed in functional assays in HMC3 and SH-Sy5y cells. Interestingly, we identified several co-morbidities associated with APOE risk or protective variants including hyperlipidemia (OR 1.21; $p=1.1\times10^{-55}$) and hypercholesterolemia (OR 1.24; $p=3.3\times10^{-38}$). Understanding genotype-phenotype correlations is critical for assessing functional effect of therapeutics, especially in a population-specific manner. Addressing phenotypes at scale can help predict and proactively respond to side-effects or co-morbidities of therapy. Collectively, our tools create a powerful roadmap for identifying and acting on protective genetic variants in AD and other human diseases.

3026 Comparison of 8 DNA payloads for universal CAR-T engineering with multi-loci gene editing—cell tolerance, efficiency and accuracy

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Universal CAR-T therapy engineered via non-viral CRISPR gene editing approach could empower CAR-T therapy with more advantages: as targeted precise CAR gene knock-in to ensure its location, copy number per cell and expression level, which make the safety and quality more under the control of engineering process; besides CAR gene knock-in, multiple gene knock-out can be managed simultaneously to eliminate the trigger of host immune

response. This one-step non-viral manufacture process could even further shorten the turn-around time and total cost for CAR-T therapy. Large fragment knock-in by CRISPR DSB (double-stranded break) and HDR (homology-directed repairing) based precise knock-in is widely used for cell therapy development. Managing the balance between editing efficiency and cell viability, the format and quality of DNA payloads is critical to achieve high proportion of CAR expressing cell and good cell fitness after gene editing and manufacture. To provide a thorough comparison of different DNA payloads, we adopted the GFP insertion at TRAC exon 1 in primary T cell via CRISPR gene knock-in, plus B2M and PDCD1 gene knock-out to abrogate surface expression of MHC-I and PD-1 as a multiple-genes editing allogenic universal CAR-T engineering model. Here we collected 8 types of different DNA payloads encoding the same GFP and 300bp homology arms flaking the DSB site of TRAC, to test their performance in the scenario of only TRAC gene knock-out and TRAC+B2M+PDCD1 triple gene knock-outs. For HDR template, we prepared 1) linear ssDNA, 2) classic 5'-ssCTS with annealing oligo, 3) 5'-ssCTS modified as hairpin structure, 4) circular single-stranded DNA, 5) open-ends linear dsDNA prepared by PCR amplification, 6) closed-ends linear dsDNA as GenWand dsDNA, 7) super-coiled circular dsDNA with minimized backbone as GenCircle, and 8) the classic DNA vector as pUC57 plasmid to compare.

For editing efficiency, overall, double-stranded DNA templates (~30%) have lower editing efficiency than single-stranded DNA payloads (40-60%), due to their cytotoxicity caused by trigger cGas-Sting pathway during electroporation. Linear dsDNA has slightly higher editing efficiency than circular dsDNA, but not significant. Single-stranded DNA either in linear or circular format show similar editing efficiency as well. Linear single-stranded DNA with CTS sequence modification at 5'-end has high editing efficiency (60%), due its low cytotoxicity, increase nucleus delivery and better stability with Cas9 RNP protects the 5'-end of linear ssDNA. To our surprise, when we replace the partial dsDNA structure of CTS as self-annealed hairpin structure than the annealing complex of ssDNA and oligo, the editing efficiency decreases, although we can prove the binding of RNP in vitro.

We also measured the editing accuracy by amplifying the insertion flanking region and long-reads nanopore sequencing. Among the three loci editing samples, GFP fragment insertion at other loci than TRAC, especially partial insertion at B2M and PD-1 gene were observed on sequencing reads. Among the 8 DNA payloads, circular ssDNA gives the highest 90% on-target editing accuracy, and ssCTS 88% with improved editing accuracy by RNP binding and guiding, while blunt-ends dsDNA shows multi-loci off-target insertion.

3027 Dynamic of DNA payloads for CRISPR-HDR editing on primary T cell via electroporation

Lumeng Ye, *GenScript USA Inc.*

During the protocol optimization of non-viral CRISPR gene editing for CAR-T engineering, the fact that different editing efficiency by different HDR DNA payload formats on the same editing design draw our attention. We assume this may due to the cytoplasm delivery efficiency of DNA payload via electroporation, and the consequence delivery efficiency into nucleus and cytoplasm stability. To solve this puzzle, we adopted the GFP insertion at TRAC exon 1 in primary T cell via CRISPR gene knock-in as testing model, and prepare 8 different DNA formats, to be delivered with eSpCas9 protein and sgRNA either targeting TRAC to mimic the condition of CRISPR-based knock-in (HDR group), or with sgRNA that targeting RAB11a locus, to measure the stability of DNA payloads under the condition of double-stranded breaking, but no intend gene knock-in via homology-directed repairing (no HDR group). T cell electroporation was done on Neon^{TM} platform 10 µL setting, 0.5 million 48h activated T cell for each run. All the single-stranded DNA payloads were delivered as 2 µg, theoretical calculation is about 4.4 million copy per cell; both of the linear dsDNA payloads were delivered as 0.5 µg due to its toxicity, theoretical calculation is about 0.5 million copy per cell, GenCircle and pUC57 vector were delivered as 1 µg, calculated as 0.88 and 0.43 million copy per cell, respectively. DNA payload stability was measured by harvesting the T cell after electroporation immediately (day 0), and day 3, day 5, day 7, and day 10. Then use qPCR to quantify the copy number of DNA payloads from the washed T cell lysate. Editing efficiency were measured at day 3, 5, 7, and 10 as well.

From the DNA payload copy number measurement on day 0 sample, circular ssDNA was detected as the highest with 0.2 million copy per cell, about 4.7% delivery by electroporation. The second is ssCTS with 0.18 million copy

per cell, about 4.0% delivery efficiency. By comparing the copy number detected at different days, circular ssDNA is observed with the slowest degrading rate, which may explain the toxicity and editing efficiency drop of the 2 μg group on editing results. The stability of ssCTS is higher than the linear ssDNA without CTS design, indicated the 5'-end protection and stability improvement by RNP binding, and its stability data correlates to the high editing efficiency.

In the no HDR group, all 8 DNA payloads continuously degrading. While in the HDR group, elevation of DNA copy number at day 5 compares day 3 observed for most of the DNA payloads with >30% editing efficiency. Another major difference is on the cells with two linear dsDNA payloads, although they have the same copy number at day 10. However, closed-ends dsDNA was detected with higher copy number from day 3 to day 7 in no HDR sample indicates the improved stability by closed-ends, but open-ends dsDNA sample has higher detected copy number from day 5 to day 7, but lower detected editing efficiency. Overall, circular ssDNA and ssCTS degrades the slowest, while GenCircle and pUC57 vector degrades the fastest. To our surprise, the residue of 2 μg cssDNA sample was detected lower than 0.5 μg sample, given the fact of decreased editing efficiency, we hypothesis that an extra DNA degradation event happened in the T cell electroporated with 2 μg cssDNA due to overdosing.

3028 Anti-HIV Function of duo CAR-T Cells is Enhanced by Treatment with an Immunotherapeutic Combining IL-15 Stimulation with TGF-β Inhibition

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Transforming growth factor-β (TGF-β) is a critical immunosuppressive cytokine that suppresses T cell cytotoxic function. Elevated levels of TGF-β are common in people with HIV (PWH) even if treated with antiretroviral therapy (ART) and is associated with systemic inflammation and immune suppression. We previously described a duoCAR-T cell therapy which targets two gp120 sites, the CD4 binding site with the CD4 D1 domain and the coreceptor binding site with a human VH based single domain antibody (sdAb) to target and eliminate HIV-infected cells while protecting the duoCAR-T cells from HIV infection. Despite its potent anti-HIV activity, the suppressive function of duoCAR-T cells may be inhibited by systemic inflammation and elevated TGF-β in PWH. We hypothesized that inhibiting TGF-β activity while stimulating IL-15 signaling using a bifunctional fusion molecule, HCW9218, may unleash a more potent anti-HIV immune response mediated by our duoCAR-T cells. HCW Biologics developed bifunctional immunomodulatory proteins including HCW9218, composed of TGF-βRII and IL-15/IL-15Rα. TGF-βRII acts as a TGF-β sink, effectively neutralizing its activity and the linked IL-15/IL-15Rα activates NK and CD8+ T cells. As controls, we used HCW9228, composed of TGF-βRII with IL-15/IL-15Rα mutated to reduce activity and HCW9238 with a functional IL-15/IL-15Rα but inactivated TGF-βRII. TGF-β neutralizing activity of HCW proteins were evaluated by treating duoCAR-Ts with HCW proteins and TGF-β, followed by quantifying TGF-β induced-phosphorylation of SMAD2/3 (Fig. 1A, B). IL-15 function was assessed by staining for IL-15 induced STAT5 phosphorylation in HCW protein treated T cells and measuring cell growth of duoCAR-Ts stimulated with HCWs and TGF-β after 5 days (Fig. 1C). Anti-HIV activity of duoCAR-Ts in the presence of TGF-β and the HCW proteins was evaluated by HIV viral suppression assays using autologous HIV-LucR-infected CD4+ T cells and cytotoxicity assays targeting 293T-gp120-Luc cells (Fig. 1D, E).

TGF-β-induced SMAD2/3 phosphorylation was inhibited by HCW9218 but not HCW9238 which lacks functional TGF-βRII. HCW9218 displayed robust IL-15 activity by phosphorylating STAT5 in isolated T cells and stimulating proliferation of an IL-15 dependent cell line and HCW9218 IL-15 activity stimulated significantly greater cell growth of duoCAR-Ts compared to HCW9228 treatment (Fig. 1C). duoCAR-Ts treated with TGF-β maintained cytotoxicity and HIV suppression (n=3 donors) in the presence of HCW9218 but not HCW9238 (Fig. 1D, E).

In summary, our findings demonstrate that HCW9218 neutralizes TGF-β activity and thereby prevents TGF-β-mediated inhibition of duoCAR-T cell proliferation, cytotoxicity, and HIV suppression. HCW9218 may serve as an adjunct therapeutic to contribute to a functional cure for HIV by increasing the anti-HIV activity of duoCAR-Ts in PLWH with elevated levels of TGF-β, thereby sustaining their anti-HIV immune responses to suppress the HIV-1 reservoir.

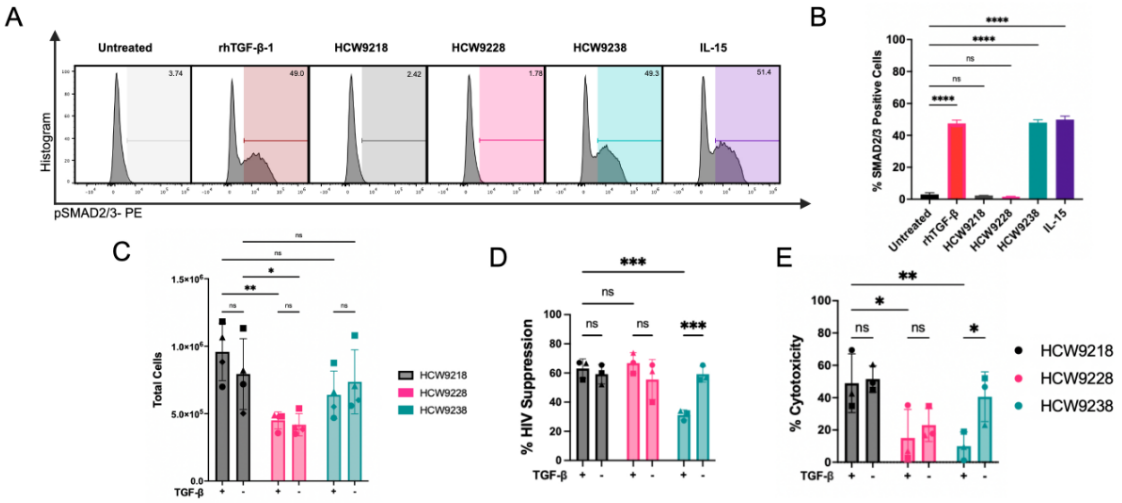

Figure 1. HCW9218 inhibits TGF-β induced suppression of duoCAR-Ts. (A) Representative histograms of flow cytometry analysis for pSMAD2/3 staining of duoCAR-T cells. **(B)** Bar graph showing % SMAD2/3 positive cells in duoCAR-T cells based off the flow cytometry plots. **(C)** CAR-T cells from 4 independent donors were cultured with 50 nM of HCW proteins, with or without 10 ng/mL TGF-β, for 5 days. **(D)** HIV suppression assay in healthy donors. CD8 CAR-T cells were generated and pre-treated with indicated HCW proteins and with and without TGF-β for 3 days. CAR-T cells were then cultured at an [E:T] of 1:60 with autologous HIV infected CD4 T cells with indicated HCW proteins or cytokines with and without TGF-β for 24 hours, n=3. **(E)** Luciferase cytotoxicity assay against 293T-gp120-Luc cells. CD4/CD8 duoCAR-T cells were generated from healthy donors and cultured at an E:T of 2:1 with 293T-gp120-Luc for 48 hours with and without 10ng/mL TGF-β supplemented in the media. Cytotoxicity was measured based on luciferase released in the supernatant upon lysis of cells, n=3. Data was analyzed by two-way ANOVA using multiple comparison. All statistical analysis was done in GraphPad Prism 9.5.0. For all significance values, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

3029 AAV-Based Homeostatic Modulation for RHO-ADRP Gene Therapy

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Transcriptional modulation is becoming increasingly important in various fields of gene therapy. In the context of transcriptional silencing, this can be achieved by cis-sequence mutagenesis using CRISPR/Cas9 or base editors (BE), or by trans-acting DNA-binding proteins tethered to repressor domains such as epigenetic modifiers (CRISPR inhibition, CRISPRi). In our quest for an effective treatment for rhodopsin autosomal dominant retinitis pigmentosa (RHO-ADRP) - a severe and currently untreatable retinal degeneration - we established a robust proof of principle using an unconventional approach. Specifically, we used a zinc finger (ZF) DNA-binding protein (ZF-RHO) to silence endogenous RHO expression independently of tethered repressor domains. This silencing strategy is combined with gene replacement by introducing a functional copy of RHO within the same AAV vector containing two expression cassettes. Here to ensure optimal transgene expression of both cassettes we exploited the intrinsic regulatory syntax of RHO expression itself. We recently identified a compact RHO regulatory unit (RRU) that controls RHO expression via a novel gene regulatory code (Rosa Maritato et al., 2024). The use of the regulatory lexicon of RHO enables to drive balanced expression of both the silencing (ZF6) and replacement (exogenous RHO) components. We achieved “homeostatic”, stable and therapeutically relevant gene expression levels with a well-distributed transgene expression profile across the retina, while maintaining low AAV vector doses. Our findings represent a significant advance in gene therapy for RHO-ADRP, paving the way for safer and more effective interventions for this and potentially other blinding diseases.