Screening of Chemically Distinct Lipid Nanoparticle *In Vivo* Using DNA Barcoding Technology Towards Effectively Delivering Messenger RNA to Hematopoietic Stem and Progenitor Cells

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Introduction

Using adenine base editors, we aim to treat sickle cell disease by generating single nucleotide polymorphisms in human CD34+ hematopoietic stem and progenitor cells (HSPCs) at specific target sites by mediating A-T to G-C base conversions. While *ex vivo* gene editing approaches show great therapeutic promise, access is limited due to the requirement of an autologous hematopoietic stem cell (HSC) transplant to deliver the *ex vivo* edited cells. To further increase the number of patients eligible for base editing therapy, we are developing an alternative approach to directly deliver base editors to HSCs *in vivo* through non-viral delivery methods. Lipid Nanoparticles (LNPs) are a clinically validated, non-viral approach that enables the delivery of nucleic acid payloads, which may avoid the challenges associated with *ex vivo* approaches including the transplantation of edited CD34+ HSPCs.

Here we describe the development and characterization of LNPs for the delivery of messenger RNA (mRNA) to HSPCs *in vivo* in both mice and cynomolgus macaques. By screening >1,000 chemically distinct LNPs *in vivo* utilizing a DNA barcoding technology, we identified several hit LNPs capable of biodistribution to HSPCs. Upon individual validation of these hit LNPs by delivery of Cre recombinase mRNA in a Cre-reporter mouse model (Ai14), which expresses the fluorescent protein tdTomato under a constitutive CAG promoter following Cre-mediated gene editing, we confirmed that several LNPs efficiently delivered Cre recombinase mRNA to mouse Lin-Sca-1+c-Kit+ (LSK) HSPCs. We next confirmed the most potent hit LNP (LNP-HSC1) identified from the *in vivo* screen to transfect LSK HSPCs in a dose-dependent manner between 0.1 and 1.0 mg/kg Cre recombinase mRNA, transfecting over 40% of LSK HSPCs in Ai14 mice at 1.0mg/kg. In a transfection durability study using Ai14 mice, we observed maintenance of tdTomato+ LSK HSPCs levels in the bone marrow out to 16 weeks post-LNP delivery at two dose levels. Additionally, at 16 weeks post-LNP delivery, tdTomato expression was observed in multiple lineages in blood at levels similar to HSPC percent tdTomato+ in bone marrow.

As LNP-HSC1 had been identified and validated in mice of a C57BL6/j background, we next confirmed its ability to transfect a reporter mRNA into HSPCs in Balb/c mice, humanized mice and in 5 cynomolgus macaques. LNP-HSC1 efficiently transfected LSK HSPCs in Balb/c mice and human CD34+ cells in NSG-CD34+ mice at 1.0 mg/kg. In 5 cynomolgus macaques (n=5 across two experiments), we observed a dose-dependent increase in reporter mRNA delivery with an average of 19% of bone marrow-derived CD34+ HSPCs (n=3) expressing the reporter protein at the highest dose tested.

Taken together, these data demonstrate the value of our *in vivo* high-throughput LNP screening approach to identify novel LNPs capable of delivering to HSPCs, providing a promising delivery platform for an *in vivo* HSC gene editing approach for the treatment of hemoglobinopathies.

Genome Editing with Adenine Base Editors (ABE)

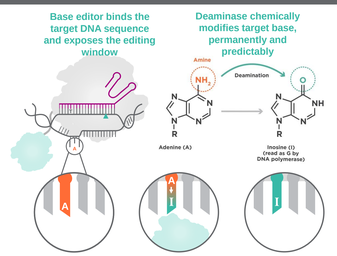

Figure 1. ABE is a fusion protein comprising an evolved TadA* deaminase (teal) connected to a CRISPR-Cas enzyme (grey). The base editor binds to a target sequence that is complementary to the guide-RNA (magenta) and exposes a stretch of single-stranded DNA. The deaminase converts the target adenine into inosine (which is read as guanine by DNA polymerases) and the Cas enzyme nicks the opposite strand. The nicked strand is repaired completing the conversion of an A:T to G:C base pair (1-2).

High-throughput barcoding of LNPs enables multiplexed screening of hundreds of LNPs *in vivo* in the same experiment

Figure 2. *In vivo* screening of thousands of chemically-distinct LNPs was conducted in a manner similar to previously described (3). In a single experiment, hundreds of LNPs were formulated with identical molar ratios of cholesterol, PEG-lipid, and phospholipid with various novel ionizable lipid structures to co-encapsulate a mRNA encoding for Cre recombinase and a DNA barcode. Stable and LNPs were pooled together and administered into a single cohort of Ai14 Cre-reporter mice. After 72 hours, bone marrow was isolated and tdTomato-positive cells were FACS isolated. DNA barcodes were recovered from isolated cell populations and sequenced using NGS; after normalization to LNP library administered, LNPs are scored for biodistribution tropism to bone marrow and other tissues.

LNP-HSC1 forms small, stable LNPs and mediated dose-dependent transfection to HSPCs *in vivo*

A

LNP-HSC1 (Cre mRNA)	
Diameter	85nm
Polydispersity	0.08
Encapsulation Efficiency	96.5%

B

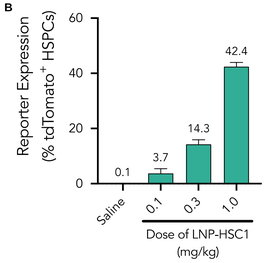

Figure 3. After identification via barcoded screening, LNP-HSC1 was formulated with Cre mRNA. **(A)** Physicochemical properties of LNP-HSC1 were measured including size and polydispersity by dynamic light scattering and encapsulation efficiency. **(B)** LNP-HSC1 transfects LSK HSPCs *in vivo* in a dose-dependent manner between 0.1 and 1.0 mg/kg after IV administration.

LNP-HSC1 leads to durable HSPC transfection *in vivo*

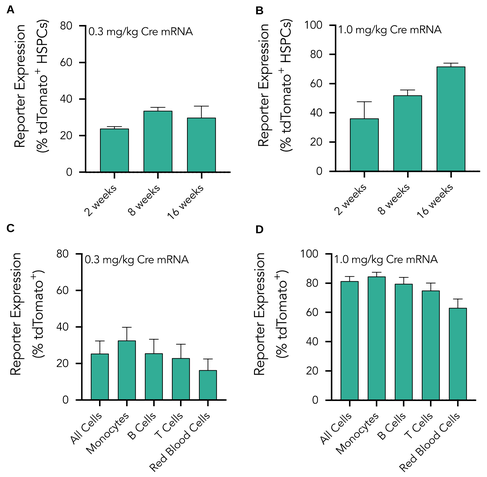

Figure 4. tdTomato+ LSK HSPCs percent were measured in the bone marrow at 2, 8, and 16 weeks post-LNP-HSC1 delivery in Ai14 mice that were administered with doses of **(A)** 0.3 and **(B)** 1.0 mg/kg Cre mRNA. At 16 weeks post-LNP delivery, tdTomato expression was measured in blood following cardiac puncture across multiple different lineages at doses of **(C)** 0.3 and **(D)** 1.0 mg/kg Cre mRNA.

All Cells: Live
Monocytes: LiveCD45$^+$CD11b$^+$,
B Cells: LiveCD45$^+$CD11b$^-$CD11c$^-$CD3$^-$B220$^+$,
T Cells:LiveCD45$^+$CD11b$^-$CD11c$^-$CD3$^+$B220$^-$
Red Blood Cells:LiveCD45$^-$Ter119$^+$

LNP-HSC1 transfects HSPCs in multiple strains of wild-type mice

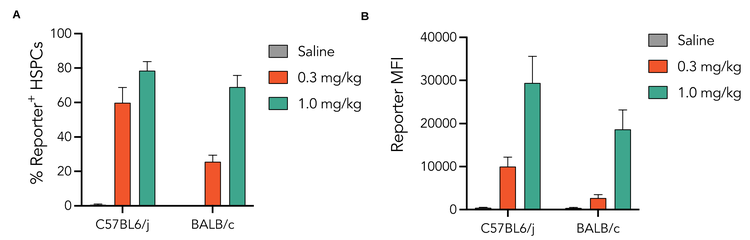

Figure 5. To test LNP-HSC1 in non-transgenic animals, we developed and utilized a mRNA encoding a reporter protein that is detectable via flow cytometry. After validation of this novel reporter protein (dose-response, time-course, signal-to-noise across multiple species), we formulated LNP-HSC1 delivery of mRNA in BALB/c and C57BL6/j mice. We observed dose-dependent increases in **(A)** reporter-positive percent and **(B)** reporter expression (Mean Fluorescence Intensity; MFI) in LSK HSPCs in both mouse strains.

LNP-HSC1 transfects human CD34+ cells *in vitro* in the presence of ApoE3

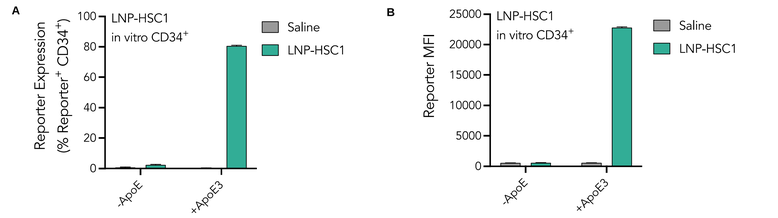

Figure 6. LNP-HSC1 carrying reporter mRNA was co-incubated with human CD34+ cells *in vitro* with or without the addition of ApoE3 into the culture media. **(A)** Percent reporter-positive CD34+ cells and **(B)** reporter expression (Mean Fluorescence Intensity; MFI) was measured after 24 hours by flow cytometry. The presence of ApoE3 significantly increased LNP transfection.

LNP-HSC1 transfects CD34+ HSPCs in humanized NSG-CD34+ mice

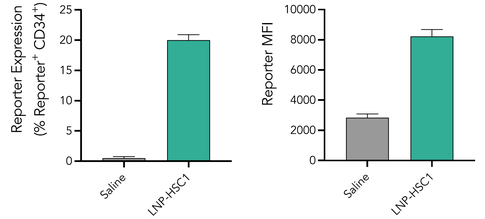

Figure 7. LNP-HSC1 was administered into humanized NSG-CD34+ mice at a dose of 1.0mg/kg reporter mRNA. 24 hours after LNP administration, reporter protein expression was detected with flow cytometry in Lineage$^-$ CD34$^+$ cells as reported by **(A)** percent reporter-positive cells and **(B)** reporter expression (Mean Fluorescence Intensity; MFI)

LNP-HSC1 transfects HSPCs in non-human primates (NHPs)

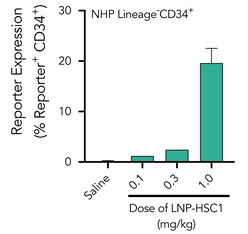

Figure 8. LNP-HSC1 was administered into cynomolgus macaques at dose of 0.1 (n=1), 0.3 (n=1), and 1.0 (n=3) mg/kg reporter mRNA. 24 hours after LNP administration, reporter protein expression was detected in a dose-dependent manner. At 1.0mg/kg, 19% of Lineage$^-$ CD34$^+$ cells were observed to have detectable reporter expression via flow cytometry.

Lineage cocktail included anti-CD3/CD14/CD16/CD20/CD56

Conclusions

We are developing a non-viral approach using LNPs for the delivery of nucleic acids to HSPCs to overcome some of the limitations of current *ex vivo* methodologies. To do so, we are utilizing an *in vivo* LNP screening technology to test >1,000 LNPs for biodistribution to bone marrow and HSPCs. We validated one screening hit, 'LNP-HSC1', to display dose-dependent and durable transfection of Cre-reporter mice. We subsequently demonstrated that LNP-HSC1 could transfect multiple wild-type mice, human CD34+ cells *in vitro,* humanized CD34+ mice, and NHP CD34+ HSPCs. The success of LNP-HSC1 validates the high-throughput LNP screening method for the identification of HSPC-targeted LNPs. Work is ongoing to identify more potent LNPs than LNP-HSC1 for applications in *in vivo* base editing.

References and Disclosures

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2. Gaudelli, N.M., et al. Directed evolution of adenine base editors with increased activity and therapeutic application. Nat Biotechnol (2020).
3. Sago, C., et al. High-throughput in vivo screen of functional mRNA delivery identifies nanoparticles for endothelial cell gene editing. PNAS (2018).

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