Re-Creating Hereditary Persistence of Fetal Hemoglobin (HPFH) to treat Sickle Cell Disease and β-thalassemia

POSTER ID: 4708

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Introduction

Overview

Our therapeutic approach for Sickle Cell Disease (SCD) and β-thalassemia (β-thal) uses CRISPR/Cas9 to create genetic edits in patient hematopoietic stem cells (HSCs) that mimic naturally occurring, and protective, hereditary persistence of fetal hemoglobin (HPFH) genotypes. Here we show that we can create the desired genetic edits in human CD34+ hematopoietic stem cells (HSCs) with high on-target and no detectable off-target editing. When performed in patient samples this leads to a meaningful increase in γ-globin expression. Within the CD34+ cells, the HSC population is edited at high efficiency, and these cells retain long-term engraftment potential. This process has been transferred to a GMP-capable manufacturing facility and GLP/toxicology has been initiated in preparation for clinical testing.

Globin Biology, SCD and β-thalassemia

Hemoglobin expression is complex and tightly regulated. In the developing fetus, γ-globin is expressed at high levels and pairs with α-globin to form the $\alpha_2\gamma_2$ fetal hemoglobin (HBF) tetramer. As shown in Figure 1 below, γ-globin expression is repressed and is replaced by β-globin between 0 and 3 months of age. β-globin then pairs with α-globin to form the $\alpha_2\beta_2$ hemoglobin (HBA) tetramer. Patients with SCD or β-thal do not become symptomatic until after HBF has been replaced by HBA, indicating that upregulation of HBF may be a viable therapeutic strategy for these diseases.

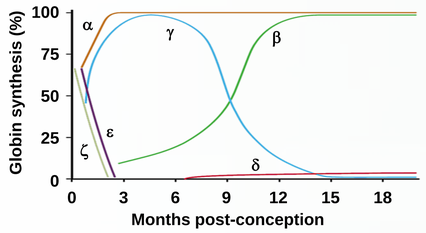


Figure 1. Regulation of hemoglobin subunits during gestation and infancy (modified from Canver and Orkin[1])

Certain individuals have been observed to maintain abnormally high levels of HBF expression into adulthood, a condition termed hereditary persistence of fetal hemoglobin (HPFH). When present in SCD or β-thal patients, HPFH results in a mild or even asymptomatic disease state without evidence of other deleterious effects. As shown in Figure 2 below the severity of disease symptoms correlates inversely with HBF[2,3]. Any amount of HBF is beneficial in both SCD and β-thal, and approximately 25-30% HBF is associated with substantial amelioration of disease in SCD and transfusion-independence in β-thal.

Therapeutic strategy for SCD and β-thalassemia

Our therapeutic strategy for SCD and β-thal uses CRISPR/Cas9 to mimic the genetic mutations that occur naturally in HPFH patients. We plan to isolate patients' hematopoietic stem cells, treat these cells *ex vivo* with CRISPR/Cas9 to create HPFH genetic edits and then reintroduce the edited cells into the patients. We believe that the genetically modified stem cells will give rise to erythrocytes with sufficient HBF expression to significantly reduce disease severity. We have prioritized a number of genetic edits based on the degree of HBF upregulation seen in nature, our ability to re-create theses edits at high efficiency using CRISPR/Cas9, and the absence of off-target editing. We plan to select one of these potential genetic edits as our lead product candidate to advance into clinical trials.

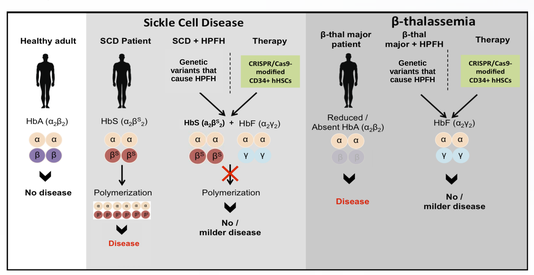


Figure 2. Overview of SCD and β-thal and therapeutic strategy

In vitro proof-of-concept

Lead guides edit with high on-target efficiency

Candidate guide RNAs (gRNAs) were selected computationally and screened for editing efficacy in human CD34+ cells. Figure 3 shows results from one such screen. gRNAs were identified with consistent, high (>70%) on-target editing.

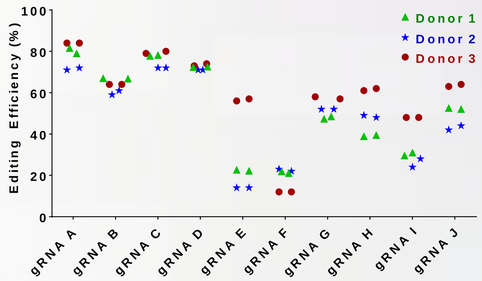
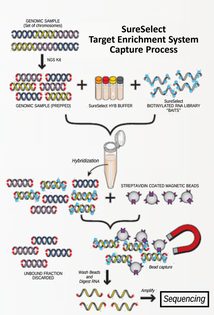


Figure 3. On-target efficacy of gRNAs in human CD34+ cells. Each data point is a single experiment, and symbols represent different healthy CD34+ donors.

Lead guides show no detectable off-target activity

Candidate gRNAs were screened for off-target activity by examining sites computationally identified to be most similar to the target site, and thus have the highest potential for off-target activity. Figure 4 shows the approach and results for the gRNAs tested above. Most guides displayed no detectable off-target activity, even at predicted sites. Only guides C and G show off-target activity, and were thus disqualified from further evaluation.

A)



B)

gRNA	# sites tested	# of sites w/ activity	Max activity
A	365	0	-
B	576	0	-
D	330	0	-
E	696	0	-
F	483	0	-
H	676	0	-
I	677	0	-
J	840	0	-
C	419	8	3.6%
G	530	1	0.6%

Figure 4. A) Schematic of a hybrid-capture assay used to detect editing activity at potential off-target sites. Multiple probes were used for each predicted site to increase assay sensitivity. B) Observed off-target activity via hybrid capture sequencing.

Edits upregulate HBF in patient samples

Candidate gRNAs were used to create specific gene variants, or targets, in erythroid progenitor cells from SCD and β-thal patients, as well as healthy donors. After erythroid differentiation, globin transcript levels were measured. Shown in Figure 5, greater than 30% γ-globin mRNA levels were observed in patient cells edited with gRNAs to create HPFH Targets 5 and 6. SCD and β-thal patient samples exhibited a larger absolute increase in γ–globin than those from healthy donors, consistent with the observation of higher HbF in patients than in heterozygote carriers with HPFH[4].

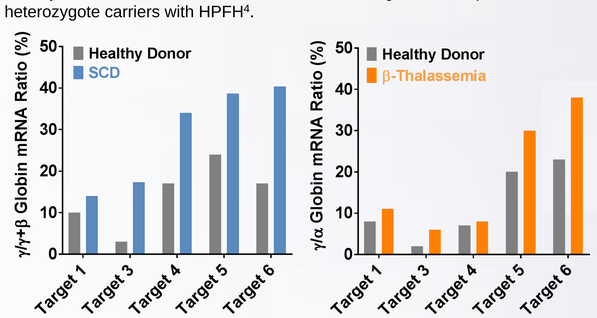


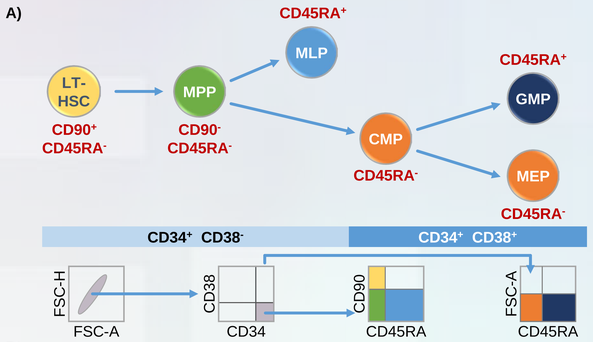
Figure 5. Ratios of globin mRNA levels in cells from SCD and β-thal patients, compared to healthy donors. The level for mock treated cells from each donor was subtracted from the values shown. Data represent a single experiment, except for SCD patient data which represent the mean of 3 different donor samples. Editing efficiency was similar for all experiments.

Engraftment studies

Long-term repopulating cells are edited efficiently

To ensure that editing efficiencies in bulk CD34+ cells are representative of those in long-term repopulating HSCs (LT-HSCs), CD34+ cells were sorted into specific sub-populations and assayed for on-target editing efficiency as shown in Figure 6. Importantly, high editing efficiency was observed in LT-HSCs.

A)



B)

Distribution of cell types remains unchanged after editing

High editing efficiencies in all cell types

Population	Frequency	
	Mock	*Edited*
Bulk	94.83	94.30
LT-HSC	7.82	7.98
MPP	18.97	16.38
MLP	13.23	8.47
CMP/MEP	11.23	11.32
GMP	8.39	13.26

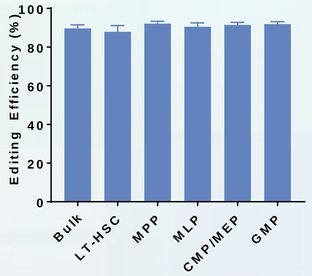


Figure 6. A) Subpopulations of human CD34+ cells, associated surface markers and flow cytometry gating strategy. B) Results indicate a similar distribution of cell types in the mock and edited conditions (Left panel) and similarly high editing efficiencies across the subpopulations compared to bulk (Right panel). Experiments were done using a single gRNA across 4 donors. Bars depict Mean ± SEM. *LT-HSC*, Hematopoietic Stem Cell (highlighted in yellow); *MPP*, Multipotent Progenitor; *MLP*, Multilymphoid Progenitor; *CMP*, Common Myeloid Progenitor; *MEP*, Megakaryocyte Erythrocyte Progenitor; *GMP*, Granulocyte Macrophage Progenitor

Edited cells engraft in mice

In vivo engraftment studies were performed in immunocompromised mice to confirm that gene-edited HSCs retain the potential for long-term repopulation of the hematopoietic system. Human CD34+ cells from healthy donors were gene-edited using different gRNAs and introduced into NSG mice. As shown in Figure 7 for one target, the presence of similar levels of human cells in treated and untreated groups at 16-weeks post-engraftment confirmed that the edited cells retain engraftment potential.

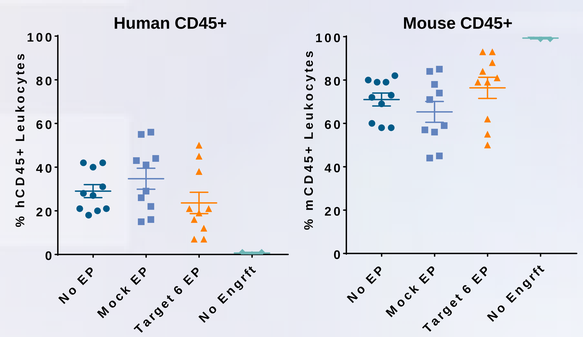


Figure 7. Analysis of CD45+ cell populations in NSG mice 16-weeks post-engraftment. Data points represent the percentage of human CD45+ (left panel) or mouse CD45+ (right panel) relative to total CD45+ cells in individual animal. Bars depict Mean ± SEM. *No EP*, untreated cells; *Mock EP*, cells were electroporated, but not gene-edited; *Target 6 EP*, cells electroporated with Target 6 editing reagents, *No Engrft*; no human cells were injected.

Scale-up and IND/CTA enabling studies

Process has been transitioned to clinical scale

Process development has been initiated at a GMP-capable facility in preparation for clinical studies. As shown in Figure 8, no significant loss of gene editing efficacy was observed at clinical scale in a GMP-compatible process.

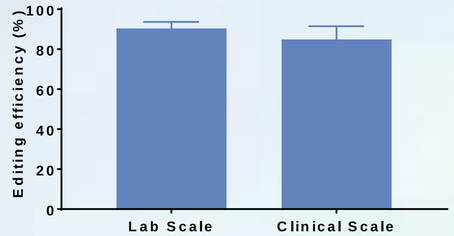
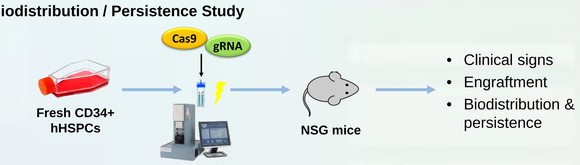


Figure 8. Average editing efficacy of a candidate target in human CD34+ cells at laboratory and clinically relevant scales. Data represent Mean ± SD across 4 or more experiments.

GLP/Toxicology studies have been initiated

In preparation for a regulatory filing planned for late 2017, GLP/toxicology studies have been initiated. Two studies in NSG mice will allow for a comprehensive characterization of biodistribution and toxicology of edited CD34+ cells.

Biodistribution / Persistence Study



Tumorigenicity / General Toxicology Study

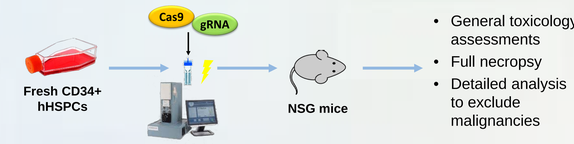


Figure 9. Overview of GLP/Toxicology study design

Conclusions

- CRISPR/Cas9 gene editing has been employed to create genetic modifications in patient hematopoietic stem cells (HSCs) that mimic naturally occurring, and protective, HPFH genotypes.
- HPFH modifications have been re-created with high efficiency and no detectable off-target modification elsewhere in the genome.
- Clinically relevant increases in γ-globin expression, a key component of HBF, were observed, after re-creating these specific HPFH modifications in either healthy donor or patient cells
- The long-term repopulating HSC subset of CD34+ cells is edited at high efficiency and edited CD34+ cells are able to engraft in mice *in vivo* with no significant loss of efficiency.
- The editing process, and high editing efficiency, have been successfully transferred to a GMP-capable manufacturing facility, and GLP/toxicology studies have been initiated.
- These ongoing studies are designed to support the regulatory filings that will enable clinical testing in patients.

References

1. Canver and Orkin, 2016. *Blood* 127:2536
2. Musallam et al., 2012. *Blood* 119:364
3. Powars et al., 1984. *Blood* 63:926
4. Weatherall and Clegg 2008. *Chapter 10: Hereditary Persistence of Fetal Hemoglobin* in The Thalassemia Syndromes

Conflicts of interest disclosure: MIL, EJP, BM, SC, YC, KT, AS, AW, YZ, MJL, MA, TJC, TC, ST, LK – CRISPR Therapeutics, employment; SM, MW, BE – Vertex Pharmaceuticals Incorporated, employment; MP – CRISPR Therapeutics, consultancy, equity ownership, advisory board; CML, GB, AM, AL – CRISPR Therapeutics, research funding; FM – CRISPR Therapeutics, consultancy, research funding, AdVerum Therapeutics – consultancy, advisory board; CAC, ASL – CRISPR Therapeutics, employment and equity ownership; RN – CRISPR Therapeutics, employment, equity ownership, and board membership