CRISPR/Cas9- mediated genome editing of Human CD34+ cells upregulate fetal hemoglobin to clinically relevant levels in single cell-derived erythroid colonies

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Introduction

Globin Biology and Sickle Cell Disease (SCD) and β-Thalassemia (β-thal)[1]

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 Patients with SCD or β-thal do not become symptomatic until after HBF has disappeared and been replaced by HBA, indicating that upregulation of HBF in adults may be a viable therapeutic strategy for these diseases.

Certain individuals naturally maintain 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 more mild disease or even asymptomatic state without evidence of any other deleterious effects. As shown in Figure 2 below the severity of disease symptoms correlates inversely with the level of HBF expression. Any amount of HBF is beneficial in both SCD and β-thal, and 25-30% HBF is associated with a near complete amelioration of disease.

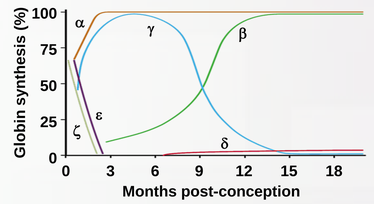
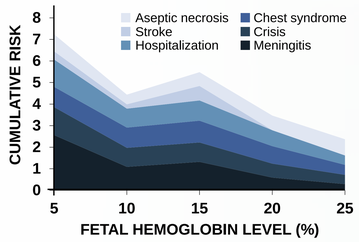


Figure 1. Regulation of hemoglobin subunits during gestation and infancy

Sickle cell disease[2]



β-thalassemia[3]

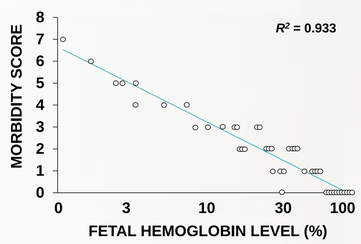


Figure 2. Observed amelioration of symptoms with increasing levels of HBF expression in SCD and β-thal patients who also have HPFH. HPFH arises due to a number of different genetic variants. Several of these are shown in Figure 3 and described below.

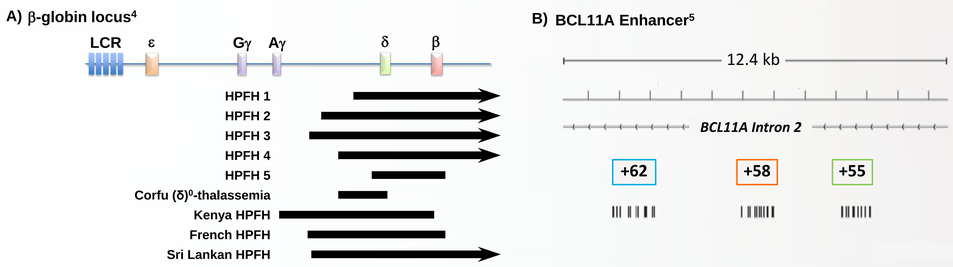


Figure 3. Diagram of several known HPFH variants. A) Deletional HPFH is caused by a variety of deletions overlapping or proximal to the β–globin locus. Non-deletional HPFH is caused by point mutations in the promoter regions of the γ-globin genes. B) Allelic variants within intron 2 of the *BCL11A* gene associated with HPFH.

Non-deletional HPFH[6]

Non-deletional HPFH is caused by point mutations in the promoter regions of the γ-globin genes. These variants have been shown to cause changes in transcription factor binding. Even in heterozygous individuals with the HBF mutation in a single allele, HBF levels can exceed 30% of total hemoglobin .

Deletional HPFH[6]

Other forms of HPFH are caused by large deletions 3' of the γ-globin genes, which remove regulatory elements while also bringing the γ-globin genes into proximity with enhancer elements. These variants result in increased γ-globin expression, and can also result in loss of the δ and/or β genes. When in a compound heterozygous state with β^0-thalassemia, these deletions can lead to HBF levels up to 75-85% total hemoglobin. Homozygous deletions can lead to HBF levels up to 90% of total hemoglobin

BCL11A[7,8]

Allelic variants in *BCL11A*, a well characterized β-locus regulatory gene, also lead to increased HBF levels. Naturally occurring heterozygous disruptions of *BCL11A* upregulate HBF up to 30%. However, BCL11A gene disruption is also associated with other neurologic or immunologic defects. Allelic variants within an erythroid-specific enhancer in intron 2 of BCL11A have also been linked to HPFH, and disruption of BCL11A expression through this enhancer appears to be well-tolerated due to its restriction to the erythroid lineage.

References

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Approach and experimental setup

Therapeutic strategy for SCD and β-thal

Our therapeutic strategy uses CRISPR/Cas9 to mimic natural HPFH variants. We plan to isolate patients' hematopoietic stem cells, treat these cells *ex vivo* with CRISPR/Cas9 to create the desired genetic edits, and reintroduce the edited cells back into patients. The edited stem cells are intended to give rise to erythrocytes that contain HBF with the potential to significantly reduce the severity of disease symptoms. We have prioritized a number of different HPFH variants based on the degree of HBF upregulation seen in nature, ability to recreate the variants at high efficiency using CRISPR/Cas9, as well as other considerations. From this set a single lead product candidate will be prioritized for advancement into clinical trials.

Experimental approach for bulk and clonal analysis

Shown are results from recreation of six different HPFH variants, or editing "targets", in human mPB CD34+ cells. In Figure 4 at right, the CD34+ cells were treated with CRISPR/Cas9, differentiated into erythrocytes, and then assayed for HBF mRNA and protein expression in bulk and colonies. Bulk analysis confirmed HBF upregulation and allowed for the prioritization of targets that demonstrated the highest levels of HBF. Clonal analysis allowed confirmation that genetic edits caused by CRISPR/Cas9 were indeed the cause of the increase in HBF at the individual cell level.

Experimental setup

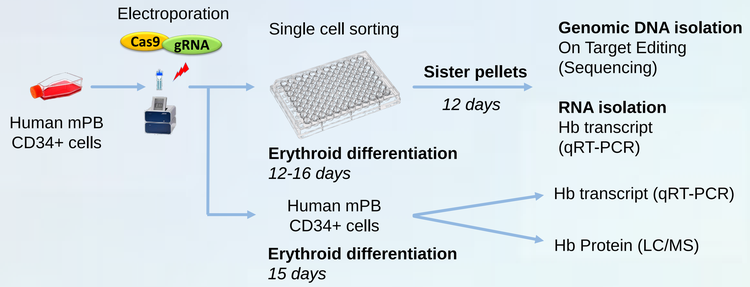

Figure 4. Overview of experimental approach for bulk and clonal analysis of hemoglobin mRNA and protein levels in erythroid cell populations derived from CRISPR/Cas9 gene edited human mPB CD34+ cells

Edits upregulate HBF in bulk culture

Figure 5. HBF upregulation in bulk differentiated human mPB CD34+ cells modified with different targeted edits. Results are from 3 different donors for targets 1-3, and 7 different donors for targets 4-6. The background level for mock treated cells has been subtracted. Data is mean ± SEM. A) mRNA transcript levels by qRT-PCR. B) Protein levels by LC/MS.

Edited colonies produce HBF mRNA

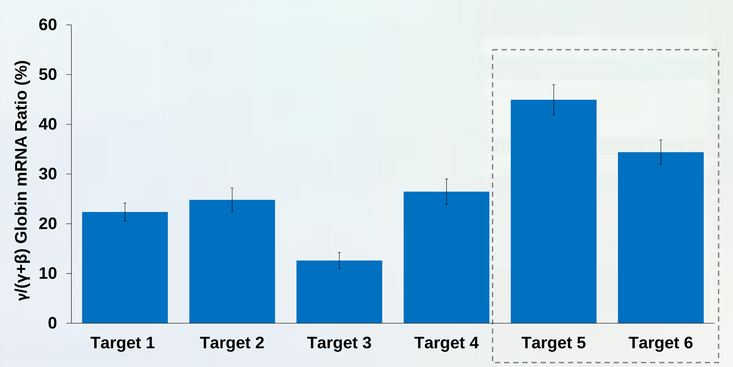

Figure 6. HBF upregulation in individual colonies of differentiated human mPB CD34+ cells modified with different target edits. Results are from a single donor, and 50-80 colonies per target. mRNA transcript levels were measured by qRT-PCR. Data is mean ± SEM. Targets 5 and 6 displayed the highest HBF levels and were analyzed further below. Data is mean ± SEM.

HBF levels correlate strongly with genotype in edited colonies

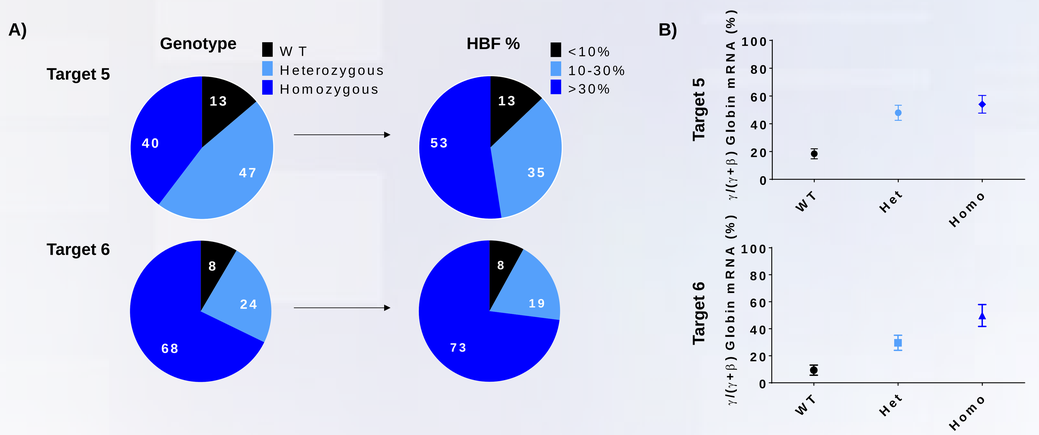

Figure 7. Genotype to phenotype correlation in Target 5 and Target 6 edited colonies of differentiated human mPB CD34+ cells. WT denotes colonies that do not show evidence of gene editing, Heterozygous or Het denotes colonies with one allele edited, and Homozygous or Homo denotes colonies with both alleles edited. A) Left hand charts show % of colonies with each genotype, and right hand charts show percent of colonies with each level of HBF upregulation (expressed as γ/(γ+β) globin mRNA ratio). B) Charts show mRNA transcript levels, for groups of colonies with similar genotypes. Data is mean ± SEM.

Conclusions

- CRISPR/Cas9 has been used to edit mPB CD34 cells at six different targets, each mimicking a different HPFH variant
- HBF upregulation was observed at both the transcript and protein levels, varies across different targets, and was in the range believed necessary for substantial amelioration of symptoms
- Clonal analysis revealed a correlation of the genotypic results of the phenotypic results of editing with upregulation of HBF
- A relationship has been observed between populations of clones with 0, 1 and 2 alleles edited, and the low, medium and high HBF producers
- This evidence supports the causal relationship between the genetic edits produced, and the desired upregulation of HBF, providing further validation for the proposed therapeutic strategy

Conflicts of interest disclosure: BM, SC, MIL, EJP, YZ, RL, KT, MAP, AS, MA, TJC, MJ, LK,TC – CRISPR Therapeutics, employment; CAC, ASL – CRISPR Therapeutics, employment and equity ownership; RN – CRISPR Therapeutics, employment, equity ownership, and board membership