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Single-Cell RNA Sequencing of Sickle Cell Reticulocytes to Identify Beta-Globin Genotypes and Associated Gene Expression Differences

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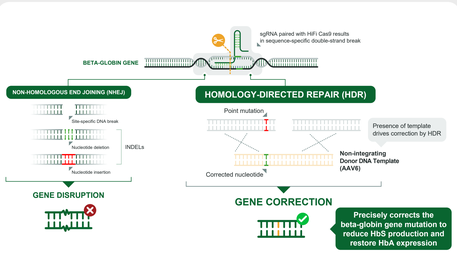
INTRODUCTION

Gene editing as a therapeutic strategy for sickle cell disease

- Sickle cell disease (SCD) is a genetic condition caused by a single point mutation in both copies of the beta-globin (*HBB*) gene, resulting in sickle (S) hemoglobin (HbS) production instead of adult (A) hemoglobin (HbA)
- Previous allogeneic hematopoietic stem cell transplant data have demonstrated a competitive advantage for homozygous (AA) or heterozygous (AS) hemoglobin erythroid progenitors over sickle disease (SS) erythroid progenitors[1]
- GPH101 (nulabeglogene autogedtemcel) is an investigational, gene-edited, autologous hematopoietic stem cell-based therapy in clinical development for SCD that is designed to directly correct the underlying mutation, thereby decreasing HbS production and restoring HbA expression (**Figure 1**)

Figure 1. High-efficiency HDR via CRISPR/HiFi Cas9 precisely corrects the SCD mutation

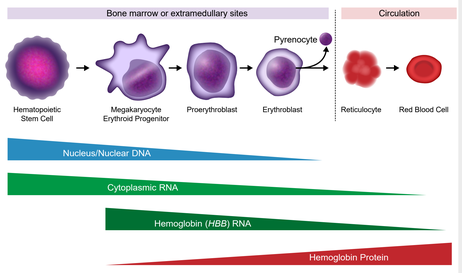


AAV6, adeno-associated virus type 6; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; HbA, adult hemoglobin; HbS, sickle hemoglobin; HDR, homology-directed repair; HiFi, high fidelity; INDEL, insertion and/or deletion; NHEJ, non-homologous end joining; SCD, sickle cell disease; sgRNA, single-strand guide RNA.

The power of reticulocytes in assessing allelic correction

- At early timepoints following infusion of nulabeglogene autogedtemcel, red blood cells (RBCs) expressing corrected HbA cannot be distinguished from HbA in transfused blood
- Because RBCs are enucleated, tracking of gene editing at the genomic level is not possible
- Reticulocytes are immature RBCs that, although also enucleated, retain RNA allowing for allelic correction to be assessed (**Figure 2**)

Figure 2. Reticulocytes are immature RBCs that still contain RNA



RBC, Red Blood Cell.

OBJECTIVE

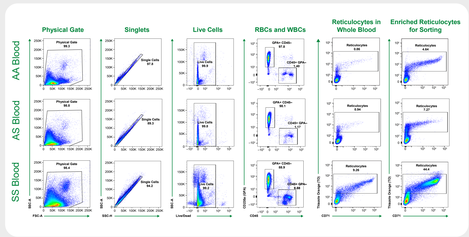
- To develop a single-cell RNA sequencing (scRNAseq) method that could enumerate gene-editing outcomes in peripheral reticulocytes
- To conduct a proof-of-concept study using reticulocytes from different donors to demonstrate the method's ability to call *HBB* genotypes
- To examine single-cell gene expression differences associated with the single-cell *HBB* genotypes

METHODS

Reticulocyte isolation and preparation

- Reticulocytes were isolated from the peripheral blood of healthy (AA), sickle cell trait (AS), and homozygous sickle cell disease (SS) donors using a combination of density-based enrichment and fluorescence-activated cell sorting (FACS) of a Live/CD235a+/CD45-/TO+ population. Phenotyping of reticulocytes also included CD71 surface expression (**Figure 3**)

Figure 3. Reticulocyte enrichment and sorting



Isolated reticulocytes from AA blood were distributed across all stages of maturation. The majority of AS reticulocytes were in the late stage of development. Reticulocytes from SS donor samples had the highest purity and were mostly early stage. SS blood contained approximately 10 times more reticulocytes than AA blood.
CD, Cluster of Differentiation; FSC-A, Forward Scatter Area; GPA, Glycophorin-A; RBC, Red Blood Cell; SSC-A, Side Scatter Area; SSC-H, Side Scatter Height; TO, Thiazole Orange; WBC, White Blood Cell.

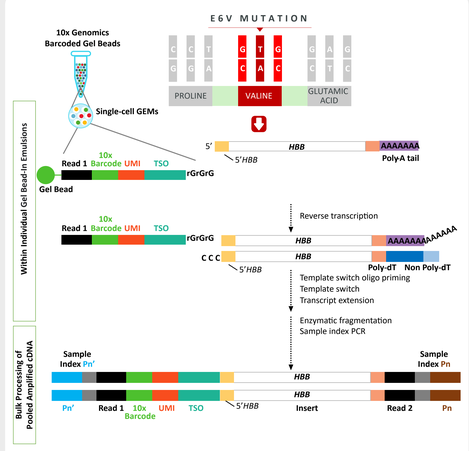
RNA bulk sequencing of individual sorted reticulocyte samples as well as blood and reticulocyte mixes

- RNA was extracted from individual AA, AS, and SS reticulocyte samples, as well as even mixes of blood volumes and sorted reticulocytes
- *HBB* transcripts were sequenced using a targeted cDNA sequencing assay. Briefly, RNA was converted to cDNA using a standard reverse transcription reaction. The region of *HBB* containing the edit site was PCR amplified, indexed, and sequenced

scRNAseq library workflow

- AA, AS, and SS reticulocytes were mixed evenly based on cell counts, re-quantified, and diluted to target loading amounts before starting the scRNAseq workflow
- We utilize the 10x Genomics 5′ scRNAseq kit to ensure adequate sequencing coverage across the 5′ end of *HBB* transcripts. The 10x scRNAseq workflow tags individual mRNA molecules with cell barcodes, as well as unique molecular identifiers (UMIs) that enable transcript quantification. Sequencing is performed with a long Read 1 as outlined in the standard 10x Genomics scRNAseq protocol in order to sequence far enough into the 5′ end of *HBB* transcripts (**Figure 4**)

Figure 4. 10x Genomics scRNAseq workflow captures the 5′ end of the *HBB* transcripts



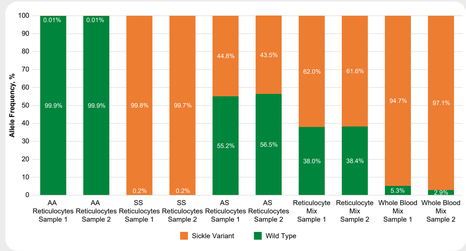
The 10x Genomics scRNAseq workflow captures the 5′ end of *HBB* transcript to ensure high-resolution coverage of the E6V mutation, which resides close to the transcription start site in exon 1.
GEM, Gel Bead-In Emulsions; *HBB*, beta-globin; PCR, polymerase chain reaction; scRNAseq, single-cell RNA sequencing; TSO, template switch oligo; UMI, unique molecular identifier.

RESULTS

Bulk cDNA sequencing reproducibly estimates *HBB* allele content but RNA content differs between AA and SS donors

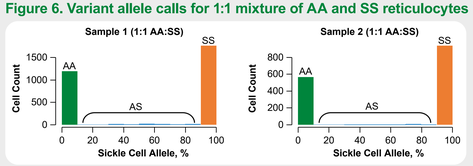
- *HBB* allele frequencies were reproducibly called using cDNA sequencing of reticulocyte pools and whole blood samples (**Figure 5**)
- Bulk cDNA sequencing of reticulocyte and blood mixes could overestimate the number of SS cells
  - cDNA sequencing of pure AA and SS reticulocytes results in very clean and expected allele frequency calls. The AS sequencing results are slightly skewed toward the A allele
  - For the reticulocyte mix, even numbers of AA, AS, and SS reticulocytes were combined. The flow data showed that the SS reticulocytes had brighter thiazole orange staining indicative of a higher RNA content. The higher *HBB* transcript levels in the less mature SS reticulocytes resulted in an elevated S allele frequency for the reticulocyte mix
  - For the whole blood mix, the effect of SS reticulocytes was even more pronounced. The reticulocyte frequency in SS blood was 10 times higher than in AA or AS blood, and as such a mix of even blood volumes resulted in a very high S allele frequency

Figure 5. Bulk cDNA sequencing of reticulocytes and blood mixes could overestimate the number of SS cells



- To determine if the *HBB* genotypes of individual cells could be accurately called from scRNAseq data, we ran a proof-of-concept, single-cell experiment with a mixture containing only wild-type (AA) and sickle disease (SS) reticulocytes to demonstrate that *HBB* genotypes of individual cells can be clearly called using our custom bioinformatics pipeline
- Histograms of the sickle allele frequencies show that most cells either have 0% or 100% S allele frequency. Very few reticulocytes carry A and S alleles, indicative of mixed cells after single-cell sequencing (**Figure 6**)

Figure 6. Variant allele calls for 1:1 mixture of AA and SS reticulocytes

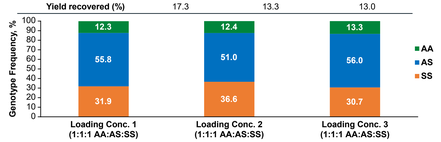


scRNAseq estimates zygosity of AA/AS/SS alleles

- We then applied this approach to a mixture of AA, AS, and SS reticulocytes. The 10x Genomics approach was performed using different loading concentrations to examine the impact on cell yield and genotyping
- The percentage of cells recovered after single-cell sequencing was lower than anticipated based on other experiments with reticulocytes. Reticulocytes express fewer genes than most of the cells typically assessed by single-cell sequencing, but based on 10x Genomics QC metrics, such as percentage of reads assigned to cells, this appears to have little impact on cell identification. Overall, even the lowest input resulted in well over 1000 single cells identified and genotyped
- Despite low recovery, results from these experiments show all 3 *HBB* genotypes can be reproducibly called using the scRNAseq 5′ protocol (**Figure 7**) and are sufficient to assess editing zygosity and the impact editing might be having on a cell's gene profile

Figure 7. Variant allele calls among 1:1:1 mixture of AA, AS, and SS reticulocytes

Target cell recovery	8,000	12,000	16,000
Recovered cells	1,384	1,591	2,072
Yield recovered (%)	17.3	13.3	13.0

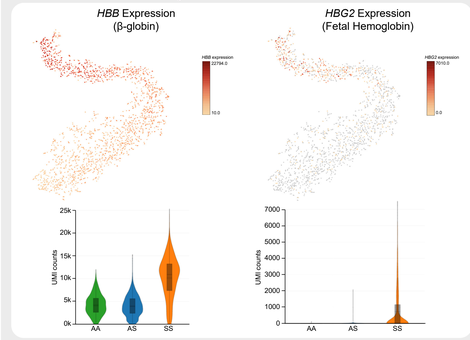


scRNAseq is able to differentiate SS from AA & AS reticulocytes

- Overlaying the genotype calls on single-cell clustering results (t-SNE plot) shows that the SS reticulocytes form a separate population from the AA and AS reticulocytes
- Differential gene expression analysis on the 3 genotypes also highlight the similarities of AA and AS reticulocytes and their marked difference from the SS reticulocyte population (**Figure 8**)
- In comparison to AA and AS reticulocytes, SS reticulocytes had higher *HBB*, *HBG2* (fetal hemoglobin), and overall higher transcript (UMI) counts (**Figure 9**)
- Interestingly, both total and *HBB* transcript counts align with thiazole orange staining results, suggesting total UMI counts could be used to classify reticulocyte age/maturity
- Although SS reticulocytes had higher total expression levels, their ratio of *HBB* to HBA1/HBA2 expression is lower than in AA and AS reticulocytes (**Figure 10**)

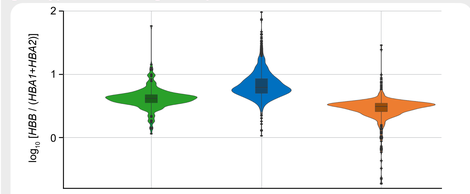
Figure 8. mRNA expression profile and t-SNE plots of AA, AS, and SS reticulocytes

Figure 9. SS reticulocytes had higher *HBB* and *HBG2* expression than AA and AS reticulocytes



UMI, unique molecular identifier

Figure 10. The scRNAseq data can be used to examine genotype-associated gene expression differences



HBA1, alpha-globin subunit 1; *HBA2*, alpha-globin subunit 2; *HBB*, beta-globin

CONCLUSIONS

- We have developed a workflow and bioinformatic pipeline that enables genotype calling and classification of editing outcomes from scRNAseq data
- In this proof-of-concept study, we show that this approach can call the *HBB* genotypes of individual reticulocytes and that it will be amenable to determining *HBB* editing outcomes in these erythroid progenitors
- Additionally, we show that this analysis enables correlation of genotype with gene expression profiles, as demonstrated by the distinct expression profiles of HbAA and HbAS versus HbSS cells
- Our data demonstrate how this scRNAseq approach will be used to evaluate differential *HBB* editing outcomes in erythroid progenitors from patients treated with nulabeglogene autogedtemcel to support clinical development
- In principle, our approach can be applied to any region of an expressed edited gene of interest via single-cell targeted RNA sequencing

Disclosures

All authors are current employees and equity holders in Graphite Bio, Inc. Kristina Krassovsky also reports former employment at UC Berkley within the last 24 months.

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- Figures created with BioRender.com

References

1. Wu CJ, et al. *Blood*. 2005;106(10):3639–3645.

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