GMP-compliant Non-viral CRISPR-mediated Process Correcting the Sickle Cell Disease Mutation in SCD Patient CD34+ Cells Achieves 60% Wild Type Adult Hemoglobin Expression in Differentiated Erythrocytes.

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Introduction

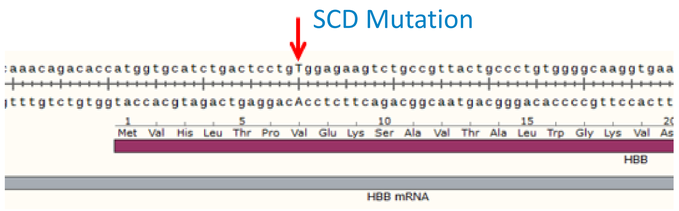
The c.20A>T mutation of β-globin gene causes sickle cell disease (SCD). Allogeneic hematopoietic stem cell (HSC) transplantation can cure SCD, but most lack a suitable donor. *Ex vivo* gene therapy strategies, including lentiviral mediated gene transfer or endonuclease mediated BCL11a knockdown allowing fetal hemoglobin (Hb) induction, are currently under evaluation. Correction of the SCD mutation by non-viral gene editing of autologous HSCs would add an alternative strategy and permit endogenous gene expression at its natural regulatory locus and the beneficial reduction of the pathogenic sickle Hb production. Allogeneic transplantation has established that the therapeutic threshold for clinical benefit is ≥20% donor chimerism. We previously reported efficient correction of a monogenic "hotspot" mutation in the CYBB gene in X-linked chronic granulomatous disease (CGD) patient HSCs with a robust, scalable, cGMP, and regulatory compliant process (*Sci Transl Med* 2017) that we now apply to SCD.

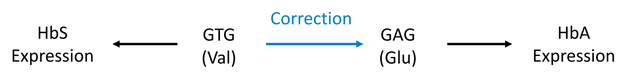
In initial studies using a B cell line (B-LCL) created from SCD patient and healthy volunteers' CD34+ HSCs, we developed a SCD mutation specific guide RNA, and a normal β-globin specific guide RNA (converse). The converse guide differed by only one nucleotide from the SCD mutation specific guide, where each guide could be used together with a single stranded DNA donor to effectively alter the wild type to SCD and the SCD to wild type, respectively (ASGCT 2017).

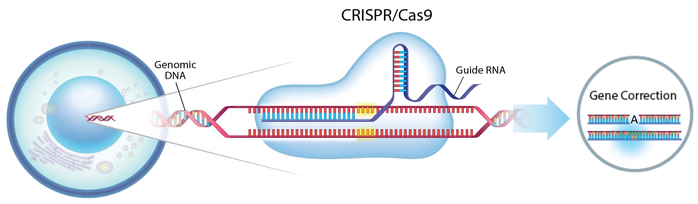
At first, we optimized homology directed repair (HDR) at the SCD locus by integrating a HindIII enzyme site. We observed efficient site-specific insertion of the HindIII-marker in the B-LCL as evidenced by HindIII digestion of the PCR products (~50%), and targeted sequencing (~35% HDR and ~50% Indel). The optimized process was applied to correct SCD CD34+ HSCs to achieve similar biallelic HDR rates for HindIII site insertion as well as gene correction from the SCD mutation to the normal β-globin sequence (up to ~35% correction and ~50% Indel). Interestingly, this correction was maintained during erythroid differentiation in culture. Among erythrocytes differentiated from corrected SCD CD34+ cells in vitro, wild type adult Hb protein levels were above 60% as assayed by both reverse phase HPLC and Hb electrophoresis, and sickle Hb production decreased from 100% to 20% after correction.

In summary, based on these in vitro correction rates confirmed by targeted sequencing, wild type adult Hb protein expression, and substantially decreased sickle Hb amounts, we are starting to evaluate engraftment of corrected SCD patient HSCs in immunodeficient mice. The high rate of engraftment in immunodeficient mice of similarly corrected HSCs observed in our published CGD study puts these results observed for in vitro correction of SCD within the therapeutic window of reversing SCD.

Scientific Rational

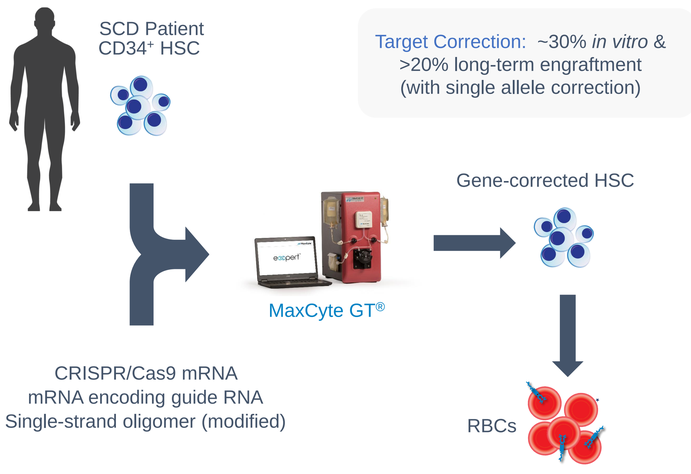






Mutation & CRISPR-mediated Correction in SCD Patient HSCs. SCD is a monogenic mutation in 6th codon of the β^A-globin (HBB) gene from an A to a T, resulting in a Glu to Val amino acid change. CRISPR/Cas9, gRNA and a DNA oligo template are used to mediate gene correction in SCD patient HSCs. Correcting the SCD mutation with ≥ 20% in a single allele provides SCD patients with significant clinical benefits.

SCD Correction Scheme



Assessment of Gene Correction:
1. *In vitro* differentiation into red cells
   - Healthy β-globin expression
2. Sequencing

Product Concept and Manufacture Procedure. MaxCyte's clinically and commercially validated Flow Electroporation™ Technology is used for delivery of CRISPR and Oligo for gene correction in HSC and patient B cells. In this developmental study, HDR of integration of HindIII recognition 6-nucleotide sequence, or correction of SCD mutation into wild type genotype, or converse of wild type genotype into SCD mutation is performed. The detection of the HDR efficiency is performed by HindIII digestion following agarose gel analysis, and sequencing of the PCR-amplified genomic DNA 3d post correction. Based on published studies, the target is set up with single allelic gene correction of ≥30% *in vitro* and ≥20% *in vivo* engraftment.

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Materials and Methods

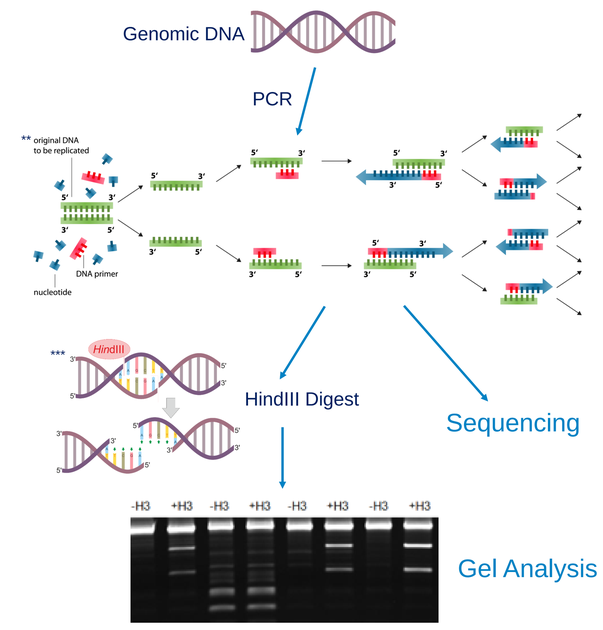
Materials

- gRNA & sequencing service were purchased from Genomic Engineering Center, Washington University at St Louis
- Oligo purchased from IDT
- mRNA by in house IVT (Ambion Kit)

gRNA-Oligo Sequence		
gRNA 1	Taret To SCD	5'GTAACGGCAGACTTCTCCTC
gRNA 2	Target To Health	5'GTAACGGCAGACTTCTCCAC
oligo 1	H in SCD	5' tcatccacgttcaccttgccccacagggcagtaacggcagacttctcAAGCTTcAcaggagtcagatgcaccatggtgtctgtttgaggttgctagtgaa
	Correct SCD	5' ttcatccacgttcaccttgccccacagggcagtaacggcagacttctccTcaggagtcagatgcaccatggtgtctgtttgaggttgctagtgaacacag
Oligo 2	H in Health	5' tcatccacgttcaccttgccccacagggcagtaacggcagacttctcAAGCTTcTcaggagtcagatgcaccatggtgtctgtttgaggttgctagtgaa
	Converse To SCD	5' ttcatccacgttcaccttgccccacagggcagtaacggcagacttctccAcaggagtcagatgcaccatggtgtctgtttgaggttgctagtgaacacag

Oligo and gRNA. Two gRNA targeting either SCD mutation (gRNA 1) or wild type (gRNA 2) corresponding to the same site at the SCD mutation. Transfection was performed to to integrate HindIII or correct mutation into wild type in SCD patient cells with oligo 1, and to integrate HindIII or converse wild type into SCD mutation in health cells with oligo 2.

HDR Analysis

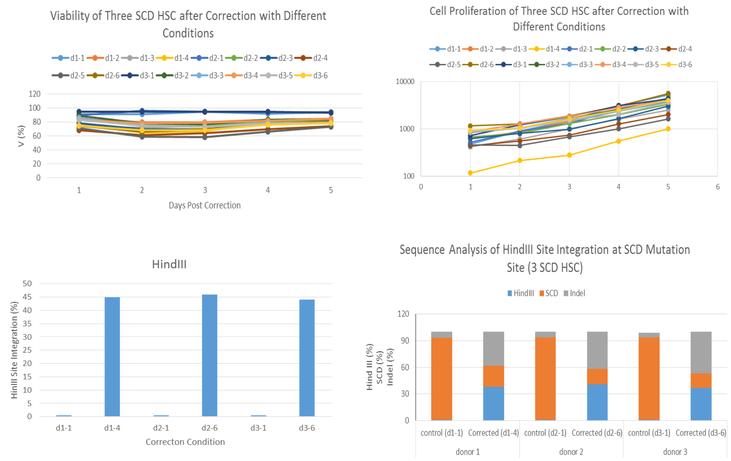


HDR Analysis. Genomic DNA was isolated following cell electroporation. The interested area at the SCD mutation site was amplified by PCR. The amplicon was either digested with HindIII to detect the integration of HindIII recongnition 6-nucleotide sequence by gel, or sequenced to detect the integration of HindIII recognition 6-nucleotide sequence, correction of nucleotide T in SCD to A in wild type, or maintenance of the A to T mutation.

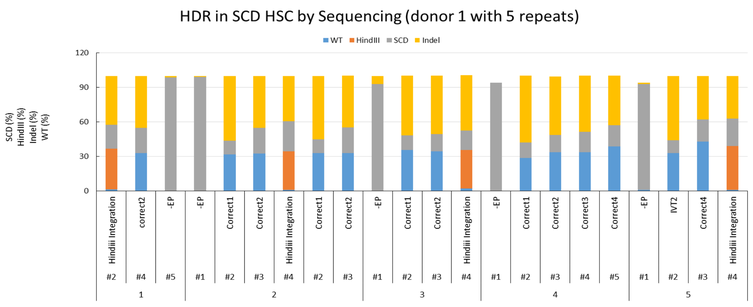
MaxCyte Delivery Platform Overview

- High viability and loading efficiency
- Consistent product quality and function
- Scalable (*5 x10^5 in seconds up to 2 x10^{11} cells in <30 min)*
- ISO9001:2008 certified Quality Systems
  - Rapid, automated platform with single-use disposable technology
  - No human or animal-derived materials employed
- Regulatory Support
  - Master File with US FDA and Health Canada
  - CE-marked
- Clinical / Commercial Validation
  - 15+ licenses for clinical development
  - 2 commercial partners
  - 50+ partnered programs

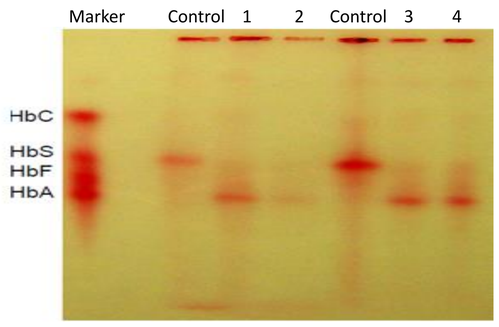
Correction in SCD HSC



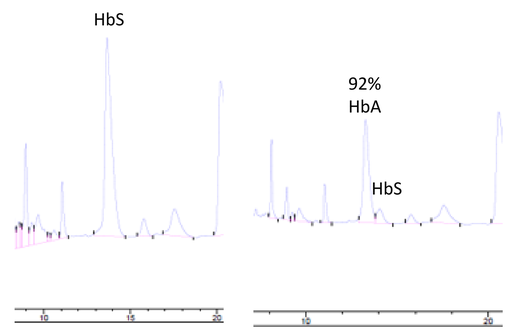
Gene correction of SCD HSC(n=3). Plerixafor-mobilized HSCs from three donors were processed by the MaxCyte GT system. Processed SCD HSC were maintained *in vitro* for up to 5 days for follow-up analysis. The viability is presented in the top left graph; cell proliferation is presented in the top right graph; HindIII digestion is presented in the bottom left graph; and the sequencing results presented in the bottom right graph. Consistent integration of HindIII recognition site among the three donors was observed.



Consistency of Correction. Plerixafor-mobilized HSCs from a SCD patient were corrected using the MaxCyte GT system with multiple correction conditions. Corrected SCD HSC were non-differentiated and/or differentiated into erythroid cells *in vitro* for 17days. Correction rates were maintained through differentiation. Gene editing efficiency by deep sequencing is shown with high consistency.



Efficient WT adult hemoglobin (HbA) expression following gene correction as assessed by globin electrophoresis gel analysis. Plerixafor-mobilized HSCs from a SCD patient were corrected using the MaxCyte GT system. Corrected HSCs were differentiated into erythroid cells *in vitro* for 17 days. Globin electrophoresis was performed to analyze the correction efficiency at the HbA level. Consistent and efficient correction from HbS to HbA is achieved. Some Hbγ is induced during erythroid differentiation for both control and corrected cells. ≥90% HbA is expressed after correction if Hbγ induced by *in vitro* differentiation is disregarded.



Efficient WT adult hemoglobin (HbA) expression following gene correction assessed by HPLC analysis. Plerixafor-mobilized HSCs from a SCD patient were corrected using the MaxCyte GT system. Corrected HSCs were differentiated into erythroid cells *in vitro* for 17 days. Protein was analyzed by HPLC. >90% hemoglobins are the corrected HbA, if Hbγ-induced through *in vitro* differentiation is disregarded.

Summary

- Therapeutically-relevant levels of SCD mutation correction is achieved using a non-viral cell approach.
- The gene correction procedure results in consistent gene correction efficiency.
- The achieved level of gene correction lead to ≥90% HbA expression.
- The correction rate is believed to be therapeutically beneficial for SCD patients.