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CRISPR/Cas9-Mediated Correction of the Sickle Mutation in Human CD34+ cells

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Targeted genome editing technology can correct the sickle cell disease mutation of the β-globin gene in hematopoietic stem cells. This correction supports production of red blood cells that synthesize normal hemoglobin proteins. Here, we demonstrate that Transcription Activator-Like Effector Nucleases (TALENs) and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 nuclease system can target DNA sequences around the sickle-cell mutation in the β-globin gene for site-specific cleavage and facilitate precise correction when a homologous donor template is codelivered. Several pairs of TALENs and multiple CRISPR guide RNAs were evaluated for both on-target and off-target cleavage rates. Delivery of the CRISPR/Cas9 components to CD34+ cells led to over 18% gene modification *in vitro*. Additionally, we demonstrate the correction of the sickle cell disease mutation in bone marrow derived CD34+ hematopoietic stem and progenitor cells from sickle cell disease patients, leading to the production of wild-type hemoglobin. These results demonstrate correction of the sickle mutation in patient-derived CD34+ cells using CRISPR/Cas9 technology.

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INTRODUCTION

Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 are genome editing systems that can be used for effective genome modification. ZFNs and TALENs consist of a DNA-binding protein component fused to the FokI endonuclease. Transcription-Activator Like Effectors (TALEs) in TALENs are characterized by 33–35 amino acid repeats in which DNA binding specificity is determined by amino acids in positions 12 and 13, called repeat-variable diresidues.[1] TALENs are composed of multiple repeat-variable diresidue domains that define DNA binding sequences and act as paired TALE binding sites. TALEs are separated by a spacer region of 12 to 20 base pairs that induces cleavage upon FokI endonuclease dimerization.[2] Typically, activation of the FokI nuclease requires correct DNA binding by the ZFN or TALEN pair as a heterodimer. However, off-target cleavage due to homodimerization of the FokI nuclease has been shown to occur. Recently developed FokI domains, known as the ELD/KKR FokI backbone, function as obligate heterodimers and have been shown to increase specificity, but possibly decrease enzymatic activity.[3]

The CRISPR/Cas9 nuclease system is composed of the DNA-binding CRISPR RNA (crRNA) array that encodes for the guide RNA (gRNA), the auxiliary trans-activating crRNA, and the Cas9 nuclease. The crRNA contains a 20-nucleotide (nt) guide sequence which binds to the specific 20 base pair DNA target in the genome through Watson-Crick base pairing.[4] Target recognition by the Cas9 nuclease depends on the protospacer adjacent motif (PAM) sequence associated with the DNA binding site.[2] The Cas9 nuclease induces a blunt, double-stranded break three base pairs upstream of the PAM sequence. Though the CRISPR/Cas9 nuclease system is the most recently developed genome-editing tool, it has become a breakthrough technology due to the ease and efficiency of custom CRISPR gRNA design (a single RNA species created by synthetic fusion of crRNA and trans-activating crRNA).[4]

Each of the three technologies have been tested to target β-globin.[5–7] By relying on the natural repair pathways that take place following the introduction of a double strand break, precise gene correction can be achieved. This stands as an enticing approach for correcting the mutation in β-globin responsible for sickle cell disease(SCD). Due to extensive work previously done in our group using ZFNs,[5] we sought to thoroughly explore the potential for TALENs and CRISPR/Cas9 to lead to gene correction at the SCD mutation in human hematopoietic stem cells. To

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this end, several pairs of TALENs and CRISPR gRNAs have been designed to target in or around exon 1 of the human β-globin gene, the location of the canonical E6V mutation underlying SCD. The work presented here evaluates the ability of these reagents to efficiently cleave the β-globin locus, to elicit gene modification in the presence of a homologous donor template, and their specificity for the target site.

RESULTS

Targeted disruption of β-globin by TALEN and CRISPR/Cas9

To evaluate the efficacy of using TALENs and CRISPR/Cas9 gRNAs for gene correction at the human β-globin locus, multiple pairs of TALENs and several gRNAs (simply referred to as CRISPR 1–6 here) were designed to cleave in exon 1 of human β-globin (**Figure 1a**,**b** and **Supplementary Figure S1**). While multiple Cas9 orthologues are available, in these studies the original *Streptococcus. pyogenes* Cas9 specific to an NGG PAM was utilized. To test nuclease cleavage efficiency, K562 3.21 cells (which harbor the sickle mutation) were electroporated with the plasmids expressing TALENs 2–5, CRISPRs 1–6, truncated CRISPR 3, truncated CRISPR 4, or green fluorescent protein (GFP). Truncated gRNAs, in which 2-3nt in the PAM-distal portion of the gRNA are removed, were evaluated based on reports that these gRNAs provide equivalent cleavage rates with decreased off-target cleavage events.[8] β-globin cleavage ranged from 10–15% in TALEN-treated samples, though 2 TALEN pairs (TALEN 3 and TALEN 4) targeting the same sequence but with different repeat-variable diresidues targeting the nucleotide guanine did not show cleavage (**Figure 2a**,**c**). β-globin cleavage from CRISPRs 1–6 and truncated CRISPRs 3 and 4 was also detected and ranged from 17–39% with all gRNAs showing high cleavage rates at β-globin (**Figure 2b**,**c**). Of note, CRISPR 1 and TALEN 5 are specific to the wild-type base at the SCD mutation location and the cells used here contain this mutation. Average β-globin disruption compared among nucleases was 10–15% for TALENs, and 14–39% for all CRISPRs and truncated CRISPRs (**Figure 2c**). As TALEN 5 and CRISPR 2 (C2) demonstrated the highest cleavage rates at the initial concentrations tested, the plasmids were titrated to determine 1.0 μg for TALENs and 0.5 μg for CRISPRs as the optimal amount of each nuclease to achieve the highest cleavage rates (**Figure 2d**). At all plasmid amounts used, the CRISPR/Cas9 showed higher rates of targeted nuclease activity than the TALEN pair. Thus, several pairs of TALENs and multiple CRISPR/Cas9 gRNAs were successfully developed and resulted in high rates of cleavage at the β-globin locus.

Analysis of nuclease specificity

Due to the high sequence homology between β-globin and other β-globin cluster genes, samples from **Figure 2** treated with TALENs, CRISPR/Cas9 gRNAs, and truncated CRISPR gRNAs were polymerase chain reaction (PCR) amplified in the δ-, ε-, ψ- (pseudobeta), γ1-, and γ2-globin loci to assess levels of off-target globin cleavage. TALEN pairs 2 and 5 showed additional cleavage in δ-globin (**Figure 3a**). Analysis of the CRISPR/Cas9 gRNAs showed no detectable off-target globin cleavage by either the CRISPRs 1–6 or truncated CRISPRs 3 and 4 in this assay (**Figure 3b**). To evaluate nuclease specificity on a genome-wide scale, TALEN 5 and CRISPR 2 were tested using the integrase-defective lentiviral vector (IDLV) trapping method described by Gabriel *et al*.[9] These nucleases were specifically chosen due to their high cleavage rates in cell lines. This assay relies on the nonhomologous end joining -mediated trapping of a GFP-expressing IDLV at the site of both on-target and off-target double-strand breaks. Integration site analysis of cells treated with the IDLV alone (to assess background double strand breaks) or with the nuclease and IDLV was completed. Clustering analysis of the integration sites in each sample revealed that TALEN 5 has off-target activity at the highly-homologous δ-globin locus (see **Supplementary Table S1**). CRISPR 2 on the other hand, did not show any putative off-target sites in this assay (see **Supplementary Table S2**).

Due to the potential for chromosomal rearrangements upon cleavage with multiple nucleases,[10,11] TALEN 5 and CRISPR 2 were evaluated for their ability to induce chromosomal rearrangements at the β-globin and δ-globin loci using a PCR-based analysis. In the event that an on-target cleavage takes place at β-globin and an additional off-target cleavage takes place at the highly-homologous δ-globin on the other chromosome, an interchromosomal translocation could take place (see **Supplementary Figure S2a**). In order to evaluate that the PCR assay did not artificially generate seeming translocation products by template switching from β- to δ-globin sequences that were not contiguous, plasmids containing the β-globin and δ-globin regions were generated and combined at equivalent copy number and subjected to a PCR spanning the β-δ region. While the β-globin and δ-globin plasmids amplified as anticipated, the β-δ translocation event was not detected in these control PCRs (see **Supplementary Figure S2b**). Thus, genomic DNA isolated from both TALEN 5 and CRISPR 2-treated cell lines was subjected to the β-δ translocation PCR. Here, as anticipated based on the Surveyor results in **Figure 3**, a translocation event was detected in the TALEN-treated cells, but not those treated with CRISPR 2 (see **Supplementary Figure S2c,d**).

Successful gene modification of β-globin by TALENs and CRISPR/Cas9 gRNAs

To determine the ability of the nucleases to lead to site-specific gene modification via homology directed repair (HDR), TALENs, and CRISPR/Cas9 gRNAs were co-delivered to K562 3.21 cells alongside a homologous donor template consisting of 1.1 kb of the human β-globin gene spanning the site of nuclease cleavage. TALENs produced average gene modification rates between 8.2–26.6%. In contrast, CRISPR/Cas9 gRNAs and truncated CRISPR/Cas9 gRNAs demonstrated a larger range and overall higher rate of 4.2–64.3% gene modification at the β-globin locus (**Figure 4**). Based on these results, and the high rates of gene modification provided by the CRISPR/Cas9 gRNAs as opposed to the TALEN pairs using the plasmid expression cassettes and cell lines discussed here, the use of CRISPR/Cas9 gRNAs was pursued moving forward to attempt cleavage and correction of HSPCs.

Delivery of CRISPR/Cas9 reagents to CD34+ hematopoietic stem and progenitor cells

As delivery of plasmids to HSPCs resulted in high cell toxicity (data not shown), alternative delivery methods for the gRNA and

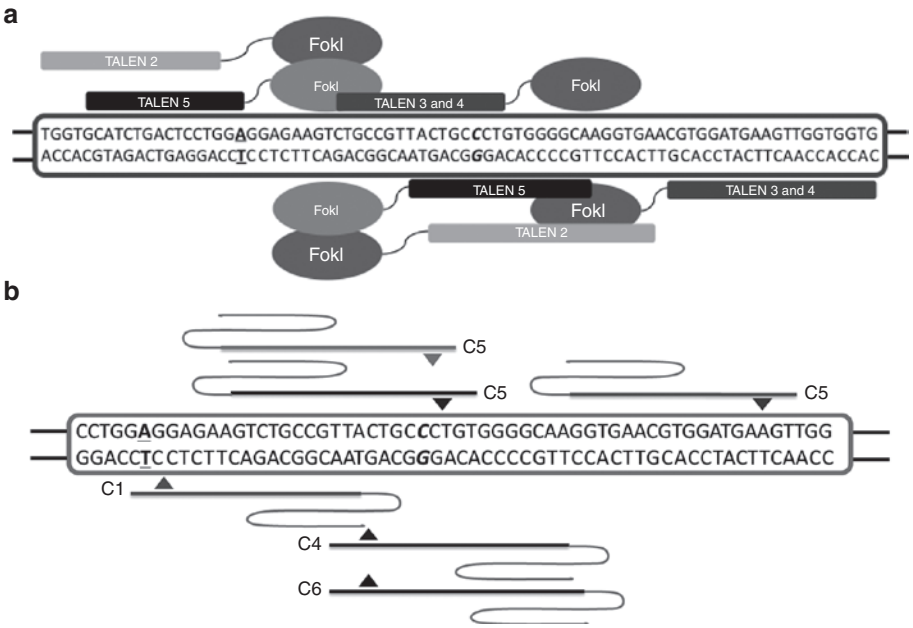

Figure 1 Nuclease binding sites in β-globin: Nuclease binding sites of TALENs 2–5 and CRISPRs 1–6. Sickle mutation location (A/T) in bold and underlined. *Hha*I RFLP (C/G) in bold and italicized. (**a**) Schematic of TALEN binding sites. (**b**) Schematic of sgRNA targets with arrows corresponding to Cas9 cleavage site. TALENs, Transcription Activator-Like Effector Nucleases; CRISPRs, Clustered Regularly Interspaced Short Palindromic Repeats.

Cas9 were explored. Initial attempts at achieving delivery of the gRNA and Cas9 components to HSPCs focused on the use of *in vitro* transcribed RNA for both components. Delivery of these RNAs without a donor template by electroporation to CD34+ cells resulted in allelic disruption levels of <5% on average (see **Supplementary Figure S3**). The low cleavage rates obtained using an RNA/RNA delivery method led to the creation of a lentiviral vector containing the gRNA driven by the U6 promoter, followed by the homologous donor template (see **Supplementary Figure S4**). When packaged as an IDLV, this vector provides the ability to efficiently transduce CD34+ HSPCs while maintaining the desired transience.[12] Electroporation of CD34+ cells from mobilized peripheral blood with Cas9 mRNA along with transduction of the gRNA and donor template-containing IDLV at various doses led to gene modification rates (measured by high-throughput sequencing as the introduction of the single nucleotide base change in the donor template 22 bp downstream of the sickle mutation) of 17.8–18.7% regardless of IDLV dose with minimal associated toxicity (**Figure 5a**,**c**,**d**). In addition, the rates of insertions and deletions (indels) at the target site were quantified by high-throughput sequencing and averaged at 9.0, 12.3, and 9.8% for multiplicity of infection (MOI) 50, 100, and 200, respectively (**Figure 5b**). These results demonstrated the ability to successfully target and modify CD34+ cells using transient delivery of CRISPR/Cas9 reagents and a homologous donor template.

Correction of SCD patient bone marrow CD34+ cells and production of wild-type hemoglobin

To test the ability of CRISPR/Cas9 system to lead to correction of a clinically-relevant cell source, SCD bone marrow (BM) CD34+ cells were electroporated with Cas9 mRNA and transduced with an IDLV carrying the U6-driven gRNA and the wild-type human β-globin gene donor template. 24 hours after treatment, treated CD34+ cells were placed in erythroid-differentiation cultures *in vitro* using an established protocol.[13,14] Gene correction rates by high throughput sequencing averaged 20.6% for CRISPR2 (**Figure 6a**) while the rates of indels averaged 16.3% of all mapped sequence reads (**Figure 6b**). Viability and fold expansion were evaluated 1 day post-electroporation. Minimal toxicity was observed upon codelivery of both the mRNA and IDLV compared with cells treated with either one, though toxicity was evident when compared with nonelectroporated mock controls (**Figure 6c**,**d** and **Supplementary Figure S5d**). To ensure that genome editing does not alter cell differentiation, samples were taken throughout culture and evaluated by flow cytometry for differentiation markers CD34, CD45, CD71, and Glycophorin A (GpA). This analysis showed the expected loss of CD34 expression throughout culture. At day 16, all samples were appropriately differentiating down the erythroid lineage based on the loss of CD45 and high levels of CD71. By the termination of culture, more than 80% of the cells in all samples were GpA+. Treatment with genome editing reagents did not result in any substantial difference throughout culture in any of the makers analyzed compared with mock-treated samples (**Supplementary Figure S5a**). Additionally, hematopoietic colony-forming unit frequency and enucleation rates remained consistent among conditions (**Supplementary Figure S5b,c**).

Importantly, at the end of erythroid differentiation, samples were evaluated by High Performance Liquid Chromatography for globin tetramers. SCD BM CD34+ cells treated with Cas9 mRNA and IDLV showed production of wild-type adult hemoglobin A (HbA) (**Figure 6e**,**f** and **Supplementary Figure S5e**). On average, HbA represented 7.3% of the total hemoglobins with rates as high as 12.6%. These results demonstrate the ability of transiently-delivered genome editing reagents to lead to high rates of correction of the sickle-cell mutation in SCD BM CD34+ cells and the subsequent production of HbA.

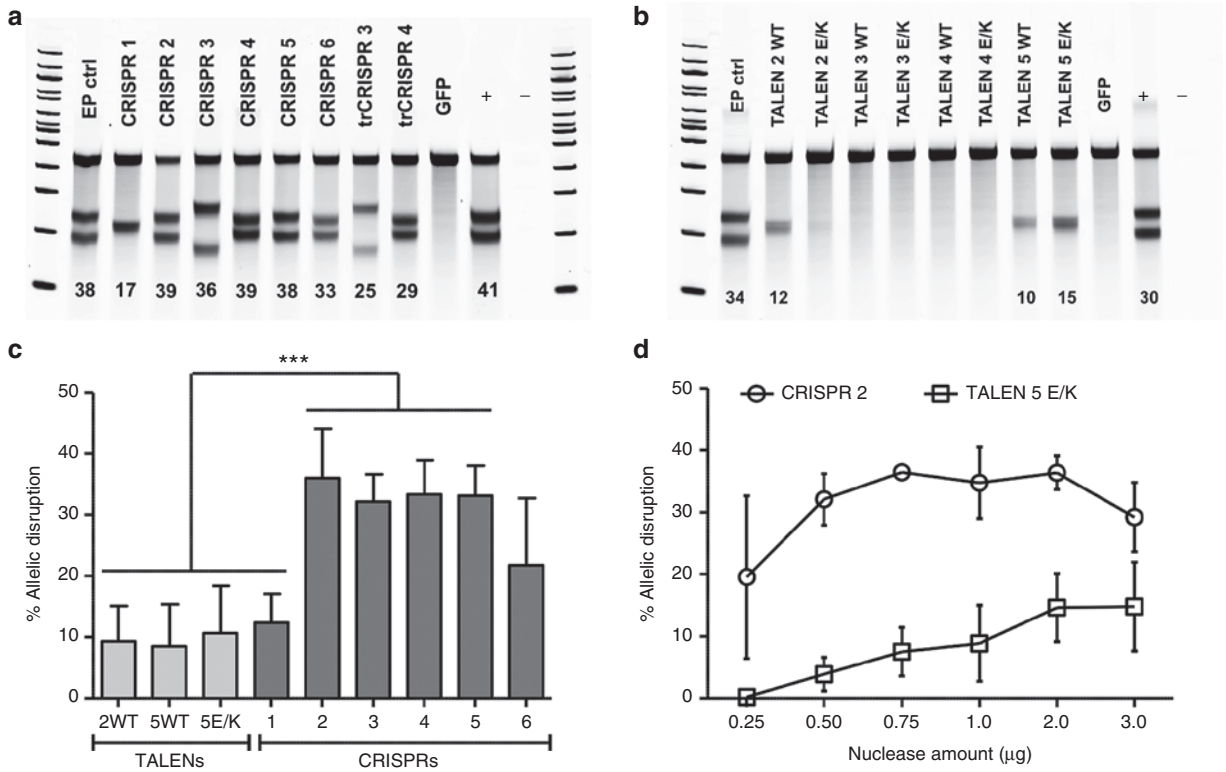

Figure 2 Nuclease cleavage rates: Electroporation of K562 3.21 cells with plasmids expressing TALENs 2–5, CRISPRs 1–6, truncated CRISPR 3, truncated CRISPR 4, and green fluorescent protein (GFP). Genomic DNA was extracted from cells and percent allelic disruption at β-globin was quantified using the Surveyor Nuclease Assay at 3 days postelectroporation. The (EP Ctrl) lane was electroporated (EP) with nucleases known to cleave at β-globin, (–) lane is a no-template PCR control and the (+) lane is genomic DNA from a sample previously shown to successfully cleave the β-globin gene. Numbers below each lane denote percent of cleavage of β-globin based on densitometry analysis. (**a**) Representative gel showing β-globin allelic disruption by CRISPRs 1–6, truncated CRISPR 3, and truncated CRISPR 4. (**b**) Gel demonstrates β-globin cleavage by TALENs 2–5 in either the wild-type or ELD/KKR obligate heterodimer FokI backbones. (**c**) Graph represents percent average β-globin allelic disruption by all nucleases from multiple experiments. Error bars, mean ± SD (three independent experiments, $n = 6$ biological replicates). (**d**) Titration of total plasmid amount by most active nucleases of each type in K562 3.21 cells. Values shown from Surveyor Nuclease Assay and quantification by densitometry. Error bars, mean ± SD. (two independent experiments, $n = 4$ biological replicates). ***$P < 0.005$. TALENs, Transcription Activator-Like Effector Nucleases; CRISPRs, Clustered Regularly Interspaced Short Palindromic Repeats.

DISCUSSION

ZFNs, TALENs, and CRISPR/Cas9 are powerful genome editing tools with the therapeutic potential for SCD and other genetic disorders.[1] While each of these technologies has promise for clinical translation and, in fact, some are already being used in the clinic,[15] a thorough exploration of their ability to drive gene correction for SCD must be completed. In addition to determining which reagent designed for SCD is able to elicit high levels of gene modification, factors such as toxicity and off-target cleavage rates must be analyzed as well. In this study, the on- and off-target cleavage rates and gene modification rates for TALENs and CRISPR/Cas9 targeting β-globin were compared.

The majority of the CRISPR gRNAs tested consistently provided high rates of allelic disruption. On the other hand, only TALEN pairs 2 and 5 showed detectable cleavage rates in these cells and at much lower levels than their CRISPR/Cas9 gRNA counterparts. Of note, TALEN 5 was designed based on a previously described target sequence[7] and, like CRISPR 1, is specific to the wild-type base at the sickle-cell mutation location. While the cleavage rates in wild-type cells were not tested here, Voit *et al* claimed that the nuclease led to similar rates in both wild-type and SCD cells.[7] In addition, the two nucleases were in different expression vectors which could contribute to the differences in their relative cleavage efficiencies.

CRISPR/Cas9 has been praised as a landmark development and offers ease of design and use. Despite this, concerns about off-target effects of CRISPRs remain and many groups are working to understand and improve specificity.[16–25] TALENs typically have a longer target site as they require the binding of two monomers to induce a double-strand break.[1] CRISPRs on the other hand rely on a 20 nucleotide RNA for binding and recognition of the target site.[2] It has been observed that the 10 bases closest to the PAM sequence are crucial to the specificity of the guide.[16] Of the six CRISPR gRNAs tested here, none showed detectable levels of off-target cleavage in the highly-homologous δ-globin gene using the Surveyor Nuclease assay while both TALEN 2 and TALEN 5 showed off-target cleavage at this locus. This could be explained by the lack of sequence divergences in the TALEN target sites from that of δ-globin while each of the CRISPR gRNA target sites had one or more bases in their guide sequences in the 10 bases proximal to the PAM that differed from δ-globin. Genome-wide analysis, while not completely unbiased, provides a wider look at potential off-target sites.[9,26,27] The IDLV trapping-based strategies employed here did not reveal off-target clustered integrations with

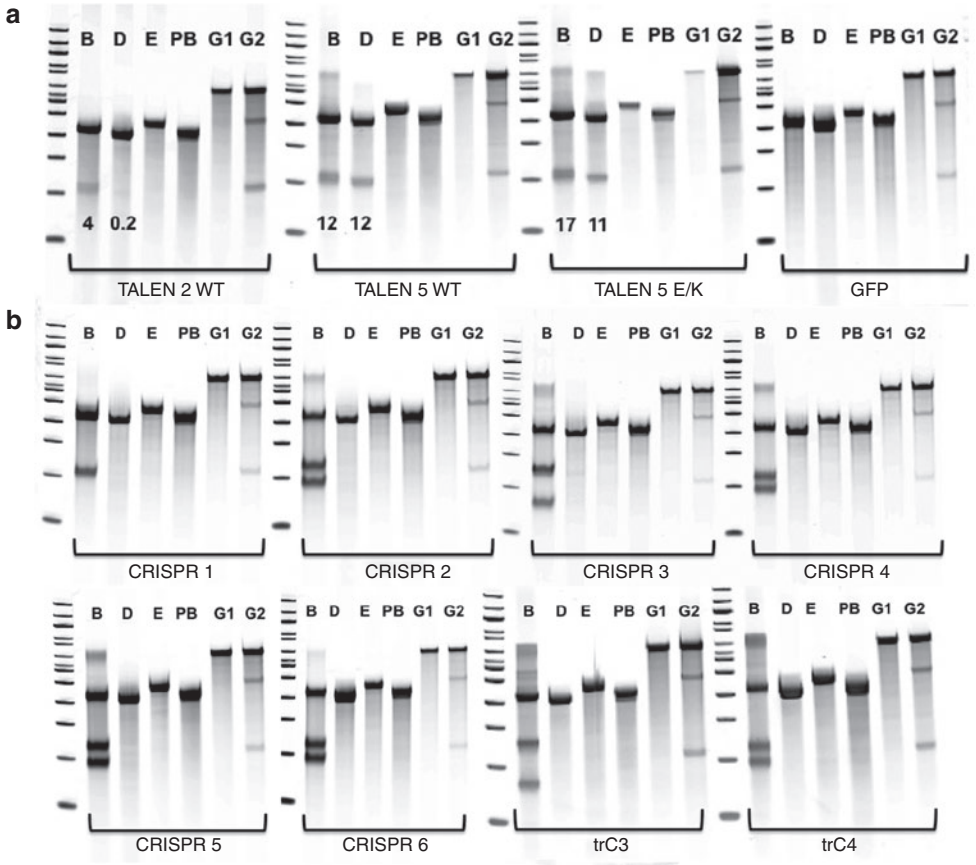

Figure 3 Nuclease Specificity Analysis: Off-target cleavage of globin genes from K562 3.21 cells electroporated with plasmids expressing TALEN 2 wild-type, TALEN 5 wild-type, TALEN 5 ELD/KKR, CRISPRs 1–6 and GFP. Genomic DNA was PCR amplified in the β- (B), δ- (D), ε- (E), ψ- (PB), γ1- (G1) and γ2- (G2) globin regions and analyzed through the Surveyor Nuclease Assay. (**a**) Numbers below lanes B and D denote percent of allelic disruption of β-globin and δ-globin for TALEN-treated samples. Although additional bands appear in the γ2 region, the same band pattern is seen in the GFP treated samples which suggests that additional bands were due to a heterozygous silent nucleotide polymorphism in the γ2 locus in these cells. (**b**) CRISPR 1–6 and truncated CRISPR-treated samples as in (**a**). TALENs, Transcription Activator-Like Effector Nucleases; CRISPRs, Clustered Regularly Interspaced Short Palindromic Repeats; PCR, polymerase chain reaction.

significant homology to the target site for the CRISPR-treated cells.

Identification of off-target cleavage sites continues to be a developing arena, but it is important to also characterize the result of such cleavage events. Here we show the potential for genomic rearrangements following cleavage at both the on- and off-target sites. This follows from work utilizing targeted nucleases to model cancer-causing events by inducing chromosomal rearrangements.[11,28] As translation of genome editing therapies in HSPCs will likely involve the bulk treatment of CD34+ cells, thorough analysis of the genomic integrity of every cell is not feasible before reinfusion of the final cell product. Therefore, understanding which rearrangements are most likely to take place, how to easily assay for them, and how to avoid or decrease them will be imperative.

Efficient, transient delivery of CRISPR/Cas9 HDR reagents to CD34+ cells without need for selection remains an area of development. In the experiments presented here, electroporation of *in vitro* transcribed Cas9 mRNA along with transduction of an IDLV carrying the gRNA and donor template led to high rates of gene correction in both mobilized peripheral blood and BM CD34+ cells. Other delivery methods including the use of modified RNAs and ribonucleotide proteins could also be explored further in combination with a donor template for HDR.[29]

Recent work by our group and others using ZFNs has highlighted the challenges to moving forward clinically with nuclease-mediated HDR-based strategies in hematopoietic stem cells (HSCs).[5,30,31] In these studies, despite high rates of HDR in the bulk CD34+ population, following transplantation into an immunocompromised mouse model which interrogates primitive HSCs more stringently, rates of HDR-modified cells were significantly lower. In spite of this, cells containing nonhomologous end joining-mediated indels were better maintained. While the exact cause for decreased rates of HDR in primitive HSCs has not been fully elucidated, it stands to reason that the preference for nonhomologous end joining as a natural repair mechanism in these quiescent cells is a key factor.[32] Further investigation is needed to ensure that this observation holds true for TALEN and CRISPR/Cas9-based strategies, but if the repair pathway of choice is indeed the predominant hurdle, similar results are likely. However, recently, high rates of HDR were achieved in primitive HSCs using ZFNs (delivered by electroporation of *in vitro* transcribed mRNA) and a donor template delivered by adeno-associated virus serotype 6 (AAV6).[33] The use of AAV6 as a donor template could be applied

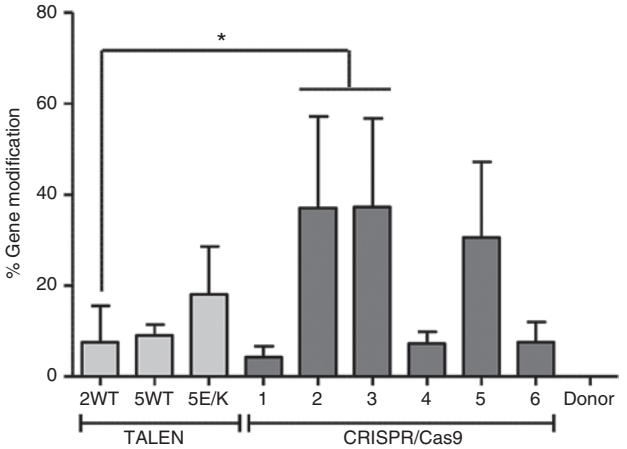

Figure 4 Gene modification by TALENs and CRISPRs. K562 3.21 cells were electroporated with plasmids expressing TALEN 2 wild-type, TALEN 5 wild-type, TALEN 5 ELD/KKR, or CRISPRs 1–6, along with a β-globin gene (1.1 kb) plasmid donor template containing the wild-type base. Percent of gene modification at the RFLP analyzed by qPCR is shown for each nuclease. Error bars, mean ± SD (two independent experiments, $n = 4$ biological replicates). *$P < 0.05$. TALENs, Transcription Activator-Like Effector Nucleases; CRISPRs, Clustered Regularly Interspaced Short Palindromic Repeats; qPCR, quantitative polymerase chain reaction, RFLP, restriction fragment length polymorphism.

for the correction of the SCD mutation described here as well. By delivering both the gRNA and the homologous donor template in a single AAV6, high rates of HDR in primitive HSCs may be possible and is currently under investigation.

Correction rates in BM CD34+ cells from SCD patients using CRISPR 2 averaged 20% and led to the production of corrected HbA at rates of 7%. These were similar to the rates already reported using ZFNs.[5] Importantly, in contrast to the results in SCD BM using ZFNs, here gene correction rates were higher than indel percentages. Whether this difference is due to target site, double strand break characteristics between FokI and Cas9, or the delivery approach remains unknown. In addition, the full implications of generating alleles with indels at the β-globin locus in HSCs also remain unknown. These reagents would need to be compared head-to-head to confirm that this difference is consistent. Data from allogeneic HSC transplants for SCD suggest that donor chimerism of 10–30% could lead to substantial clinical improvement.[34–37] If the correction rates presented here could be maintained in long-term, repopulating HSCs, the potential for clinical benefit is high. Thus, the use of the CRISPR/Cas9 system to correct the sickle-cell mutation in HSCs provides another strategy which could be further pursued for gene therapy for SCD.

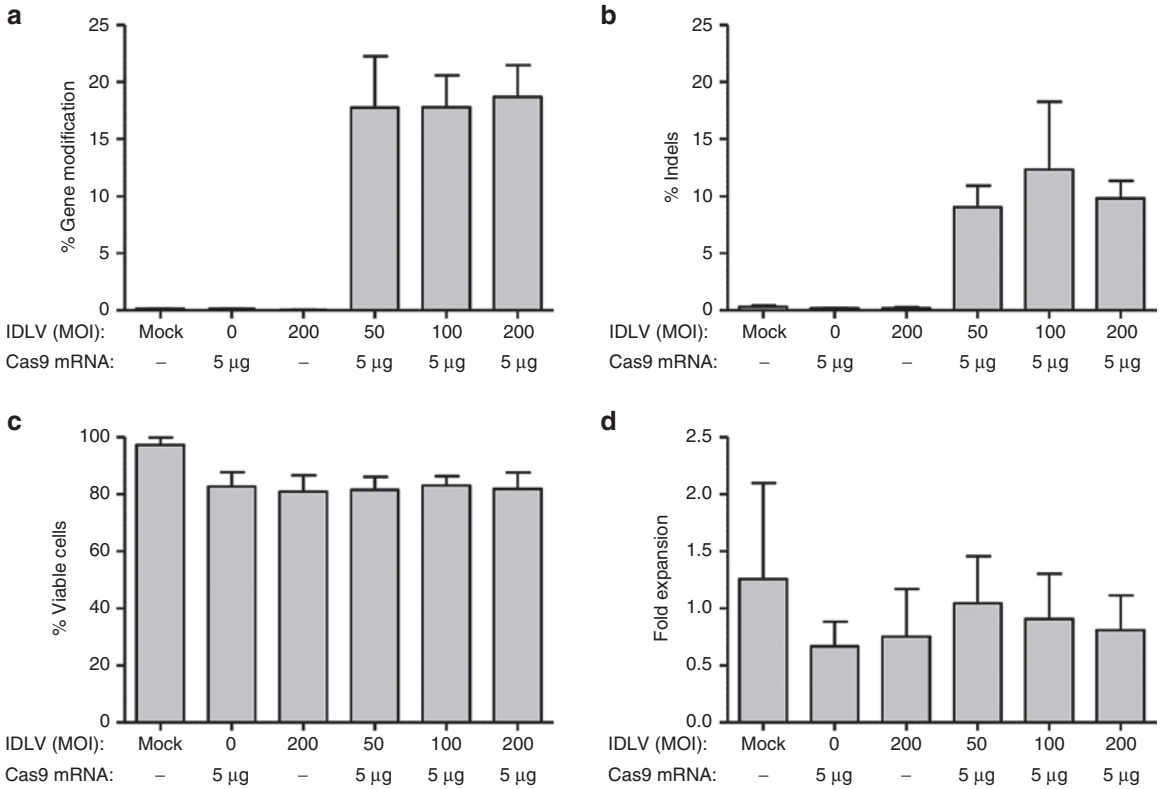

Figure 5 Gene modification of mobilized peripheral blood (mPB) CD34+ cells. (**a**) Gene modification rates of the RFLP and (**b**) Indel rates of mPB CD34+ cells treated with Cas9 mRNA and an IDLV containing the gRNA and β-globin gene (1.1 kb) donor template. Rates were determined by high throughput sequencing of the β-globin locus 4 days following treatment. Cell counts were performed using trypan exclusion dye 1 day postelectroporation to determine (**c**) percentage of viable cells and (**d**) fold expansion from cell numbers on day of electroporation. IDLV, integrase-defective lentiviral vector containing the U6-driven CRISPR 2 guide RNA and the homologous β-globin gene donor template; MOI, multiplicity of infection. Error bars, mean ± SD. (Three independent experiments, Mock $n = 6$, Cas9 $n = 2$, IDLV $n = 6$, MOI 50, 100, and 200 $n = 6$ biological replicates.) TALENs, Transcription Activator-Like Effector Nucleases; CRISPRs, Clustered Regularly Interspaced Short Palindromic Repeats; RFLP, restriction fragment length polymorphism.

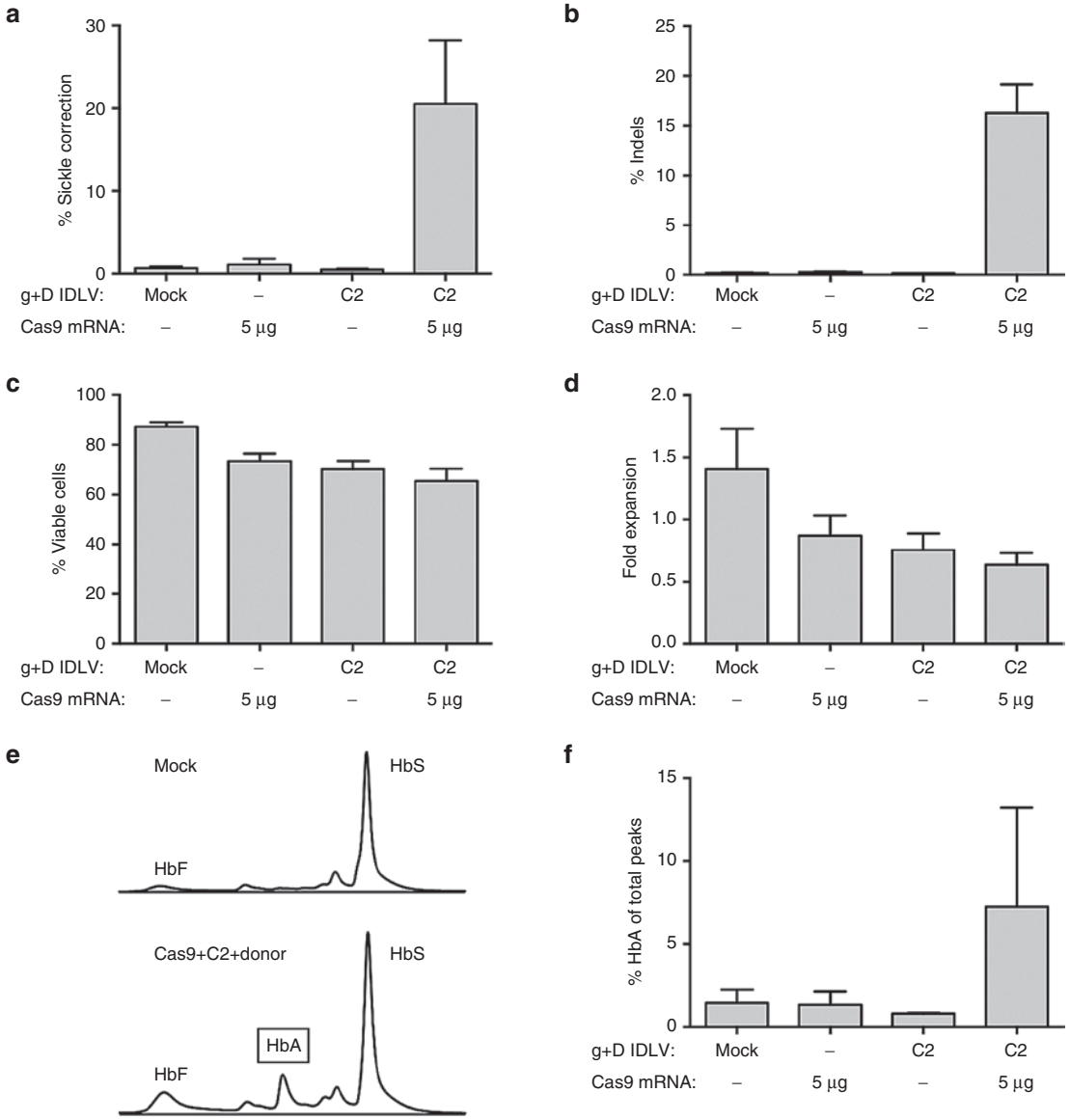

Figure 6 Correction of SCD BM CD34+ cells. SCD BM CD34+ cells were electroporated with Cas9 mRNA (50 µg/ml) and transduced with an IDLV containing the CRISPR 2 (C2) gRNA and donor template (MOI = 50). (**a**) Gene correction rates at the sickle mutation as determined by high-throughput sequencing of the β-globin locus. (**b**) Indels as determined by high-throughput sequencing of the β-globin locus. Cells were counted with trypan exclusion dye 1 day postelectroporation to determine (**c**) viability and (**d**) fold expansion. (**e**) Representative HPLC plots for mock-treated and CRISPR/Cas9-treated cells from the conclusion of erythroid culture. (**f**) HPLC results at the termination of erythroid culture showing the percentage of wild-type adult hemoglobin tetramers (HbA). g+D IDLV, integrase-defective lentiviral vector containing the U6-driven guide RNA and the homologous β-globin gene donor template; MOI, multiplicity of infection. Error bars, mean ± SD. (two independent experiments, Mock $n = 3$, Cas9 $n = 3$, C2 IDLV only $n = 3$, C2 IDLV + Cas9 $n = 4$ biological replicates). SCD, sickle cell disease; TALENs, Transcription Activator-Like Effector Nucleases; CRISPRs, Clustered Regularly Interspaced Short Palindromic Repeats; HPLC, High Performance Liquid Chromatography.

MATERIALS AND METHODS

See **Supplementary Materials and Methods**.

Cell culture. The K562 3.21 subclone was generously provided by Sangamo BioSciences and was generated from K562 cells (ATCC, Manassas, VA) to remove the single nucleotide polymorphism at the β-globin locus and to contain the sickle mutation. K562 3.21 cells were cultured in RPMI1640 Roswell Park Memorial Institute (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (Gemini Bio-products, West Sacramento, CA) and penicillin/streptomycin/L-glutamine (Gemini Bio-products).

Primary human CD34+ cell processing. Cord blood (CB) samples were acquired according to guidelines approved by the University of California, and exempt from IRB review as they are deemed anonymous medical waste. BM aspirates from SCD volunteer donors were obtained with informed consent under UCLA IRB protocol #10-001399. Mononuclear cells were isolated from BM and cord blood using Ficoll Hypaque (GE Healthcare, Little Chalfont, UK) density centrifugation. The mobilized peripheral blood apheresis products were purchased from the Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center (Cincinnatti, OH) and isolated with the CliniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). CD34+ cells were enriched using anti-CD34 microbeads and immunomagnetic column separation (Miltenyi Biotec) per the manufacturer's instructions. Cells were frozen in 10% Dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO), 90% fetal bovine serum and cryopreserved in liquid nitrogen.

Electroporation. K562 3.21 cells (2.0E+05 per sample) were spun at 90×*g* for 10 minutes and then resuspended in 20 µl SF buffer solution (Lonza, Basel, Switzerland). Equimolar amounts of TALEN (2.0 µg total) or CRISPR (1.0 µg) plasmid DNA were added to each sample and placed in the cuvette. In experiments tested for gene modification, a plasmid DNA donor (containing a 1.1 kb fragment from the 5′ end of the wild-type human β-globin gene surrounding the sickle-cell mutation with a *Hha*I restriction site added as a 1 bp silent polymorphism and cloned into a TOPO backbone) was added to each sample (1.5 µg DNA) (Invitrogen, Waltham, MA). Cells were electroporated using the Amaxa 4D Nucleofector X Unit (Lonza, Basel, Switzerland) and the FF-120 program as optimized by the manufacturer. Cells were rested at room temperature for 10 minutes after electroporation before 80 µl of prewarmed RPMI+10% fetal bovine serum was added to the cells and transferred to a 24 well tissue culture treated plate with 400 µl RPMI+10% fetal bovine serum for a final volume of 500 µl.

CD34+ cells (2.0E+05 per sample) derived from cord blood, mobilized peripheral blood, or BM were electroporated as described.[5,38] Briefly, cells were spun at 90×*g* for 10 minutes, resuspended in 100 µl BTXpress buffer (Harvard Apparatus, Holliston, MA), mixed with indicated amounts of nuclease mRNA and electroporated with the ECM 830 Square Wave Electroporator at 250 Volts for 5 milliseconds with one pulse before resting for 10 minutes at room temperature and the addition of recovery medium (XVivo 15 with penicillin/streptomycin and 50 ng/µl each of SCF, TPO, and Flt3-ligand). As applicable, an IDLV carrying the β-globin gene homologous donor sequences was present in the recovery medium at the indicated amounts and washed out the following day.

High throughput sequencing. For library preparation, an initial PCR was completed outside of the donor template region using primers as described.[5] An inner PCR was completed using primers HBB Fwd 5′ – ACACGACGCTCTTCCGATCTNNNNGGCAGAGCCATCTATTGCTT – 3′ and HBB Rev 5′ – GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTGGTCTCCTTAAACCTGTCTTG – 3′ to amplify the specific 198 bp region of *HBB* surrounding the sickle mutation along with primers to add Illumina adapters and indexes. Libraries were prepared for high-throughput Illumina paired-end 150 sequencing.

Off-target analyses. For β-globin cluster genes analyzed by the Surveyor Nuclease Assay, sites were PCR-amplified with the following primers: HBD: 5′-ggttcatttttcattctcaca-3′ and 5′-gtaatctgagggtaggaaaac-3′; HBBP1: 5′-cacccttgaccaatagattc-3′ and 5′-gagactgtgggatagtcata-3′; HBE1: 5′-cattatc acaaacttagtgtcc-3′ and 5′-agtctatgaaatgacaccatat-3′; HBG1: 5′-gcaaaggctat aaaaaaaattagc-3′ and 5′-gagatcatccaggtgctttg-3′; HBG2: 5′-ggcaaaggctataaaa aaaattaagca-3′ and 5′-gagatcatccaggtgcttta-3′.

GFP IDLV trapping assays for genome-wide off-target analyses were completed as described[5] with the following adjustments: For TALEN samples, 10 µg/ml of each TALEN 2 plasmid was electroporated into K562 3.21 cells and then transduced with an MND-driven GFP IDLV (MOI = 100). CRISPR samples were treated with 5 µg/ml of plasmid and transduced with the MND-GFP IDLV (MOI = 30). GFP IDLV MOIs were adjusted based on pilot experiments optimizing the trapping efficiency. Control samples consisted of electroporated cells that were transduced with the GFP IDLV at the appropriate MOI but did not receive nuclease. Eight biological replicates were completed for each condition. Cells were cultured for 65 days before sorting GFP+ cells. After a 3-day expansion, genomic DNA was extracted and samples were subjected to nonrestrictive Linear Amplification-Mediate PCR. Analysis was completed as described[5] with the homology (Homology (%) column) calculated based on TALEN spacer lengths of 5–35 nt and CRISPR homology dependent on the identification of an NGG PAM sequence on either strand.

SUPPLEMENTARY MATERIAL

Materials and Methods

Figure S1. Nuclease binding sites: Nuclease binding sites of TALENs 2-5, CRISPR 1-6 and truncated CRISPR 3-4.

Figure S2. Characterization of chromosomal rearrangements due to off-target nuclease cleavage.

Figure S3. Allelic disruption by CRISPR/Cas9 RNA in CD34+ cells derived from cord blood.

Figure S4. Schematic of gene modification reagents.

Figure S5. Correction of SCD BM CD34+ cells.

Table S1. Clustered integration site (CLIS) analysis for TALEN5.

Table S2. Clustered integration site (CLIS) analysis for CRISPR 2.

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