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Journal

Molecular Therapy, 26(2)

ISSN

1525-0016

Authors

Long, Joseph
Hoban, Megan D
Cooper, Aaron R
et al.

Publication Date

2018-02-01

DOI

10.1016/j.ymthe.2017.11.001

Peer reviewed

Molecular Therapy
Original Article

Characterization of Gene Alterations following Editing of the β-Globin Gene Locus in Hematopoietic Stem/Progenitor Cells

Joseph Long,[1,2] Megan D. Hoban,[1] Aaron R. Cooper,[3] Michael L. Kaufman,[1] Caroline Y. Kuo,[4] Beatriz Campo-Fernandez,[1] Dianne Lumaquin,[1] Roger P. Hollis,[1] Xiaoyan Wang,[5] Donald B. Kohn,[1,6] and Zulema Romero[1]

[1]Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095, USA; [2]Biology Department, California State University, Northridge, Northridge, CA 91330, USA; [3]Molecular Biology Interdepartmental Ph.D. Program, University of California, Los Angeles, Los Angeles, CA 90095, USA; [4]Division of Allergy and Immunology, Department of Pediatrics, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA; [5]Department of Internal Medicine and Health Services Research, University of California, Los Angeles, Los Angeles, CA 90095, USA; [6]Eli & Edythe Broad Center of Regenerative Medicine & Stem Cell Research, University of California, Los Angeles, Los Angeles, CA 90095, USA

The use of engineered nucleases combined with a homologous DNA donor template can result in targeted gene correction of the sickle cell disease mutation in hematopoietic stem and progenitor cells. However, because of the high homology between the adjacent human β- and δ-globin genes, off-target cleavage is observed at δ-globin when using some endonucleases targeted to the sickle mutation in β-globin. Introduction of multiple double-stranded breaks by endonucleases has the potential to induce intergenic alterations. Using a novel droplet digital PCR assay and high-throughput sequencing, we characterized the frequency of rearrangements between the β- and δ-globin paralogs when delivering these nucleases. Pooled CD34$^+$ cells and colony-forming units from sickle bone marrow were treated with nuclease only or including a donor template and then analyzed for potential gene rearrangements. It was observed that, in pooled CD34$^+$ cells and colony-forming units, the intergenic β-δ-globin deletion was the most frequent rearrangement, followed by inversion of the intergenic fragment, with the inter-chromosomal translocation as the least frequent. No rearrangements were observed when endonuclease activity was restricted to on-target β-globin cleavage. These findings demonstrate the need to develop site-specific endonucleases with high specificity to avoid unwanted gene alterations.

INTRODUCTION

Sickle cell disease (SCD) is one of the most common autosomal recessive disorders worldwide, caused by an adenine-to-thymine transversion in the sixth codon of the β-globin gene (*HBB*).[1] As result of this mutation, SCD red blood cells become adhesive and non-deformable, leading to vaso-occlusive events, organ crises, and chronic pain, compromising the quality of life and lifespan of the patients. Current therapies are based on palliative treatment for painful crises, and allogeneic bone marrow (BM) transplantation is the only available cure.

Autologous stem cell gene therapy has become a promising approach that avoids the immunologic limitations of allogeneic hematopoietic stem/progenitor cell (HSPC) transplantation for the treatment of hemoglobinopathies.[2] Current gene therapy strategies focus on gene addition by integrating viral vectors of an anti-sickling *HBB*,[3–5] a γ-globin gene (*HBG*),[6,7] or short hairpin RNA (shRNA) to the *HBG* repressor BCL11A.[8] However, gene addition by viral vectors has the potential risk of genotoxicity[9–11] and may be limited by gene expression variegation or silencing[12–14] because of semi-random vector integration in the genome.

Precise gene editing of a locus is an attractive alternative to the gene addition approach based on viral vector methods. Zinc-finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that are capable of inducing double-stranded breaks (DSBs) at a specific site in the genome. Different genome editing applications are based on the two main repair pathways used to resolve ZFN-induced DSBs. The homology-based genome editing approach[15] uses homology-directed repair (HDR) and, therefore, involves co-delivery of a homologous DNA donor template along with ZFNs to induce gene correction.[16–18] If the donor template provided carries homology arms flanking a transgene cassette, then a gene-sized heterologous DNA fragment will be inserted into the genome at the target locus after the DSB is induced by the ZFNs,[16] which can be defined as targeted insertion. Conversely, small insertions or deletions produced during non-homologous end joining (NHEJ) result in mutations that can lead to gene disruption when exonic or control regions are targeted.[19,20] More sophisticated gene disruption can be achieved using

Received 8 June 2017; accepted 2 November 2017;
https://doi.org/10.1016/j.ymthe.2017.11.001.

Correspondence: Zulema Romero, Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, 3146 Terasaki Life Science Building, 610 Charles E. Young Drive East, Los Angeles, CA 90095, USA.
E-mail: zulemar@ucla.edu

more than one pair of ZFNs to delete larger gene fragments[21–23] or to study mechanisms of chromosomal rearrangements as duplication and inversion[24] or translocations.[25]

We have previously shown successful gene correction of the SCD mutation in CD34$^+$ HSPCs from donors with SCD by site-specific cleavage using ZFNs designed to flank the sickle mutation at the *HBB* locus in the presence of a homologous DNA donor template, leading to the production of hemoglobin A (HbA). Nevertheless, because of the high degree of homology existing between *HBB* and the paralogous δ-globin gene (*HBD*), off-target nuclease activity has been observed in *HBD* using ZFNs, transcription activator-like effector nucleases (TALENs), or CRISPRs targeted to *HBB*.[17,26] Off-target activity has been observed previously when using ZFNs designed to cleave a gene adjacent to a highly homologous gene in the same chromosome, as is the case with *CCR5* and *CCR2*.[19,21] In that case, the authors observed that the generation of DSBs in two different sites in the genome was sufficient to create undesired genomic deletions, which did not occur in the presence of a single-site DSB.

Given the possibility for on-target cleavage in *HBB* and off-target activity in *HBD* by this specific pair of ZFNs, there is potential for the generation of multiple concurrent DSBs on chromosome 11 (in *cis* or in *trans*). Therefore, we characterized the most likely *HBB-HBD* gene rearrangements as a consequence of multiple-site DSBs by the pair of ZFNs targeting *HBB*. We showed that deletions, inversions, and translocations between the *HBB* and *HBD* loci occurred in cell lines and HSPCs from healthy donors and SCD patients after treatment with the ZFNs. These rearrangements were not seen by the same assays when using a set of ZFNs that did not have off-target activity at *HBD*. Moreover, the effects of co-delivering these nucleases with or without a homologous DNA donor template (as an integrase-defective lentiviral vector [IDLV] or as a single-stranded oligonucleotide) were evaluated. Finally, the relative frequencies of deletions, inversions, and translocations as well as the mono-allelic versus bi-allelic nature of ZFN-based gene modification were assessed in HSPC samples from healthy donors and SCD patients via a novel droplet digital PCR (ddPCR) assay and high-throughput sequencing. The results indicate that creation of multiple DSBs may lead to unwanted gene rearrangement events and emphasize the need for using nucleases with high specificity.

RESULTS

Because of the high sequence homology between the human β-globin-like genes (δ-, ε-, γ A-, γ G-, and β pseudogene 1-globin), extensive work was performed to assess the specificity of the ZFN pair engineered to specifically target the *HBB*.[17] Both the surveyor nuclease assay of *in silico* predicted off-target sites and the less biased IDLV-trapping method showed off-target cleavage of the ZFNs only at the highly homologous *HBD*, located 7 kb away from *HBB*.[17] It is known that introduction of multiple DSBs by site-specific endonucleases has the potential to induce chromosomal alterations such as deletions, inversions, and translocations.[24] Hence, we investigated the possible consequences of off-target cleavage of the ZFNs in *HBD* along with on-target cleavage at *HBB*. Combining the primers previously designed for the surveyor nuclease assay,[17] three sets of PCR primer pairs were developed to detect three potential rearrangements, as shown in Figure 1 and Table 1.

Detection of Gene Rearrangements between the β- and δ-Globin Genes Using PCR Amplification

First, the corresponding combination of the primer pairs to detect each event were optimized and tested using genomic DNA (gDNA) from K562 3.21 cells (a variant of K562 cells engineered to have the SCD mutation) (PCR data not shown) and CD34$^+$ cells isolated from umbilical cord blood (CB). As shown in Figure 2, untreated mock- and ZFN-treated CB CD34$^+$ cells were analyzed for each of the rearrangement events. None of the primer combinations used to detect the rearrangements showed amplification in the mock-untreated samples, indicating that products subsequently seen in nuclease-treated cells were not PCR-derived artifacts, such as template skipping. The ZFN-treated samples showed amplification for all of the rearrangement events listed in Figure 1, demonstrating that the rearrangements occurring between *HBD* and *HBB* are the consequence of off-target cleavage of the ZFNs in *HBD* with on-target cleavage in *HBB*. It should be noted that the list of events depicted in Figure 1 and those in Figure 2 represent a subset of possible rearrangements between *HBD* and *HBB*. This analysis was performed using a positive control sample derived from K562 3.21 cells treated with 1 μg of a CRISPR/Cas9 plasmid expressing a gRNA known to possess both on-target *HBB* and off-target *HBD* activity.

After these gene alterations were detected by PCR primer sets in pooled samples from ZFN-treated K562 3.21 and CB CD34$^+$ cells, the same analysis was performed using gDNA isolated from individual colony-forming units (CFUs) derived from SCB BM samples to detect the different rearrangements at a clonal level. Representative gels for each rearrangement event of the CFU analysis are shown in Figure 3. Again, no amplification was observed in the mock untreated samples. However, of the 76 colonies analyzed from the ZFN-treated cells, 33 (44%) were positive for deletions, 10 (13%) for inversions, and 3 (4%) for translocations.

Variations in band sizes across the rearrangement-positive colonies within the same event type could be indicative of different insertion or deletion (indel) sizes, and oscillation in brightness could be due to differences in DNA quality between samples because the same amounts of DNA were used for all PCRs performed. To show that the PCR products represent the proposed rearrangement events, twelve of the 76 PCR product samples amplified from CFU gDNA were analyzed by Sanger sequencing. Of these twelve PCR products, six were from deletion-positive CFUs, four were from inversion-positive CFUs, and two were from translocation-positive CFUs based on the gel analysis of the PCR products. Nine of the CFUs analyzed showed sequences that matched the expected rearrangement. Three of the six deletion CFUs displayed intergenic deletions between *HBD* and *HBB*, with junctional losses from the expected cleavage site ranging between 2–37 bp, and another two had indel-free transitions between *HBB* and *HBD*. Two of the four inversion CFUs showed

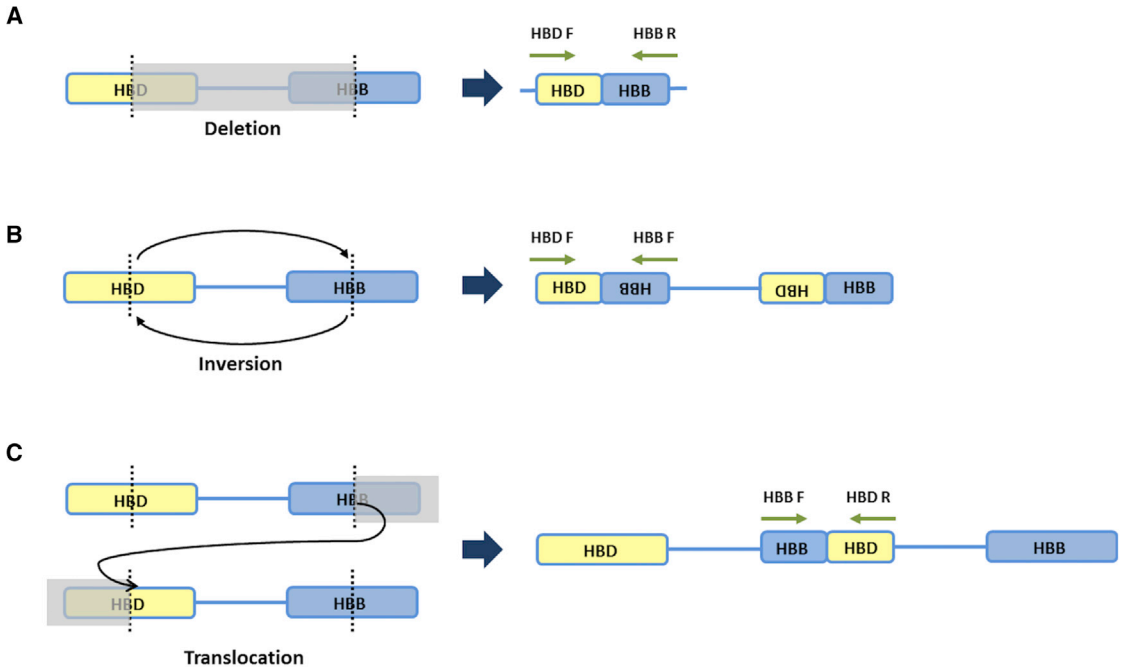


Figure 1. Potential Rearrangement Events Occurring as a Result of On-Target Cleavage in *HBB* and Off-Target Cleavage in *HBD*

The human δ-globin gene (*HBD*) is represented in yellow and the β-globin gene (*HBB*) in blue. The dashed lines indicate off-target cleavage of this ZFN pair in *HBD* and on-target cleavage in *HBB*. (A) Result of a deletion and primer combinations used to detect deletion events: *HBD-Forward* and *HBB-Reverse*. (B) Result of an inversion and primer combinations used to detect inversion events: *HBD-Forward* and *HBB-Forward*. (C) Result of a specific translocation and primer combinations used to detect the β-δ translocation events: *HBB-Forward* and *HBD-Reverse*.

insertions of the intergenic segment in a reversed orientation, and one of the two translocation-positive CFUs showed sequences indicative of this event. Of the four remaining colonies, three had poor sequence quality after the ZFN target site, likely because of the presence of co-mingling CFUs in those samples, and the last sample had a large insertion corresponding to a segment of the *FMO* gene at chromosome 16. This large insertion explains the larger band corresponding to ZFN-treated sample number 20 in Figure 3A. No significant homology exists between the ZFN target site in *HBB* and this portion of the *FMO* gene. This was the only sample detected in the CFU PCR screening analysis with an insertion of this type.

Single examples of Sanger sequences of each event type discussed above are shown in Figure S1. The fact that some colonies were positive for both the deletion and inversion events could be the result of the ZFNs cutting in both chromosomes, with different rearrangements for the two alleles. However, in some cases, the same colonies were positive for all three events, which was not possible based on the proposed rearrangement schematic. This again may indicate that some colonies may not be monoclonal based on the nature of the assay itself or that other types of rearrangements could be taking place.

Gene Rearrangements Dependent on Cleavage at Both *HBB* and *HBD* Rather Than Gene Conversion

To confirm the initial PCR data, a ddPCR assay was designed using the same primer pairs with fluorescently labeled probes, along with primers and a probe for a human gene (*UC378*) used as an internal control for normalization of DNA quantities (Table 2). This ddPCR assay provided an absolute quantification of the percentage of alleles in the populations that were positive for each event. Moreover, ddPCR performed using individual CFUs gave insight into the mono-allelic versus bi-allelic nature of each rearrangement event.

ddPCR was performed on cells treated with ZFNs known to have on-target activity in *HBB* and off-target activity in *HBD* (88/01).[17] Concurrently, for this assay, another pair of ZFNs (55/58), shown to only possess on-target activity in *HBB* and no observable off-target activity in *HBD*, was used. This pair of ZFNs had lower on-target cutting activity than the 88/01 pair used previously. However, the 55/58 ZFN pair was an ideal control to evaluate whether the rearrangements did require the off-target endonuclease activity at *HBD* rather than some type of gene conversion mechanism between the cleaved *HBB* and an intact *HBD* allele. The binding sites of each pair of ZFNs are shown in Figure S2. Off-target activity in *HBD* of both ZFN pairs was analyzed via a surveyor nuclease assay (Figure S3) and confirmed that the 88/01 pair did cleave at *HBD* and that the 55/58 pair did not.

Using the ddPCR assay, it was observed that only the ZFNs possessing both on- and off-target activity (ZFNs 88/01) produced rearrangement events in K562 3.21 cells, CB $CD34^+$ cells, and BM $CD34^+$ cells (Figure 4; Figure S4). The percentage of alleles positive for the deletion event in pooled K562 3.21 cells treated with ZFNs 88/01 ranged

Table 1. PCR Primers to Amplify *HBB* and *HBD* Nuclease Cleavage Sites

Primer Name	Sequence (5′–3′)
HBB-Forward	GACAGGTACGGCTGTCATCA
HBB-Reverse	CAGCCTAAGGGTGGGAAAAT
HBD-Forward	GGTTCATTTTTCATTCTCACA
HBD-Reverse	GTAATCTGAGGGTAGGAAAAC
Event to Be Detected	**Primer Combinations Used**
Deletion	HBD-Forward - HBB-Reverse
Inversion	HBD-Forward - HBB-Forward
Translocation	HBB-Forward - HBD-Reverse

from 24%–34.6% in multiple replicate experiments, whereas the percentage of alleles positive for the inversion or translocation events ranged from 3.8%–4.3% and 4.9%–8.1%, respectively. In the samples treated with the 55/58 ZFN pair, which does not have detectable cleavage activity at *HBD*, no allelic disruption was observed in *HBD*, and no rearrangements between *HBB* and *HBD* were detected (Figures S3A and S4A).

The cleavage activities of the two ZFN pairs described above were not equivalent when using the same amount of ZFN mRNA in $CD34^+$ cells. Therefore, different dosages of ZFN mRNA were titrated to achieve similar levels of cutting to correlate the off-target activity in *HBD* with the *HBB* to *HBD* rearrangement events. For this, CB $CD34^+$ cells were electroporated with 1 μg, 1.5 μg, or 3 μg of either ZFN pair as mRNA and then analyzed by surveyor nuclease assay to compare the percentage of allelic disruption under each condition (Figure S3B). The 88/01 ZFN pair showed higher maximal activity at the *HBB* locus, with 1 μg of ZFNs producing the highest level of allelic disruption at 31% versus 22% for ZFNs 55/58. The 1.5 μg condition produced similar levels of allelic disruption (26%–29%) for both ZFN pairs. Finally, 3 μg of mRNA produced 19% allelic disruption for the 88/01 ZFNs versus 28%–30% for the 55/58 ZFNs. The decrease in allelic disruption of the 88/01 ZFNs at 3 μg suggested that this condition might be toxic for the cells. Therefore, 1.5 μg was selected to compare the levels of rearrangements induced by the two pairs for ZFNs by ddPCR. 3 μg samples were also analyzed to determine whether lower levels of cutting in *HBB* would correlate with less off-target activity in *HBD* and, therefore, lower the rates of rearrangements.

In CB $CD34^+$ cells, at comparable rates of cleavage in *HBB* by both pairs of ZFNs, only the 88/01 ZFNs with off-target activity in *HBD* produced rearrangement events detected by ddPCR. 25%–31% of alleles were positive for the deletion, whereas 5.2% and 1.5%–2.3% of alleles were positive for the inversion or translocation events, respectively (Figure 4). Additionally, the level of rearrangements detected correlated to the level of ZFN activity at *HBB* and *HBD*, assessed by surveyor nuclease assay. 1.5 μg of 88/01 ZFN mRNA, which showed higher allelic disruption in *HBB* compared with 3 μg of ZFN mRNA (which had no detectable cleavage in *HBD* by surveyor nuclease assay), also showed higher levels of deletion- and inversion-positive alleles (Figure 4). However, the levels of translocation events detected remained similar under the 1.5 μg and 3 μg conditions.

BM $CD34^+$ cells electroporated with 1 μg of ZFN mRNA were also analyzed. Again, it was observed that only ZFNs with activity at both the *HBB* and *HBD* loci produced rearrangement events. The percentage of deletion-positive alleles ranged from 7%–10.5%, whereas the inversion- and translocation-positive events ranged between 2%–2.9% and 0.2%–1.2%, respectively (Figure S4B). No rearrangement events were detected with the 55/58 ZFNs.

The results indicate that the production of *HBB-HBD* rearrangements is not driven by gene conversion (utilizing *HBD* as a homologous template for cleaved *HBB*) but, rather, is dependent on having both on-target *HBB* and off-target *HBD* ZFN cleavage. Therefore, these rearrangements can be avoided by using endonucleases lacking off-target cleavage activity in *HBD*, as demonstrated with the 55/58 ZFN pair.

Gene Rearrangements Are Observed Using $CD34^+$ Cells from Bone Marrow from Donors with SCD

Because of their high clinical relevance to correct the sickle point mutation by gene therapy, gene rearrangements occurring after treatment with the 88/01 ZFNs were assessed in $CD34^+$ cells derived from SCD BM. Given that ZFNs 55/58 did not produce any *HBB-HBD* rearrangements, all ZFNs referenced from this point onward will be the 88/01 ZFNs.

SCD BM $CD34^+$ cells from two independent donors were electroporated with 1 μg of ZFN mRNA only or with ZFNs and a HDR DNA donor template (delivered as an IDLV named IHS or a single-stranded oligonucleotide named OHS, and the corresponding frequencies of gene correction were assessed by qPCR as shown in Table S2).[17] Off-target activity in *HBD* was analyzed via surveyor nuclease assay (Figure S5) and again confirmed that the 88/01 pair did cleave at *HBD*. The same analyses by ddPCR were performed to quantify the percentage of alleles containing each rearrangement event. In SCD BM treated with ZFNs only, 33.15% ± 9.77%, 8.21% ± 1.88%, and 0.6% ± 0.33% of alleles were positive for the deletion, inversion, and translocation events, respectively (Figure 5A). With the addition of sickle-correcting donor molecules to SCD BM $CD34^+$ cells treated with ZFNs, the percentage of rearrangement-positive alleles changed slightly without significant differences (Wilcoxon rank-sum test), but the trends were maintained. For the ZFN plus OHS (ZOHS) treatment, 43.69% ± 3.48%, 6.95% ± 0.4%, and 0.81% ± 0.58% of alleles were positive for the deletion, inversion, and translocation events, respectively, whereas, with the ZFN plus IHS (ZIHS) treatment, 49.58% ± 7.41%, 8.84% ± 1.46%, and 0.42% ± 0.0.34% of alleles were positive for deletion, inversion, and translocation events, respectively (Figure 5A). Thus, the presence of an HDR donor template did not cause meaningful changes in the frequencies of rearrangements produced by the ZFNs with off-target activity.

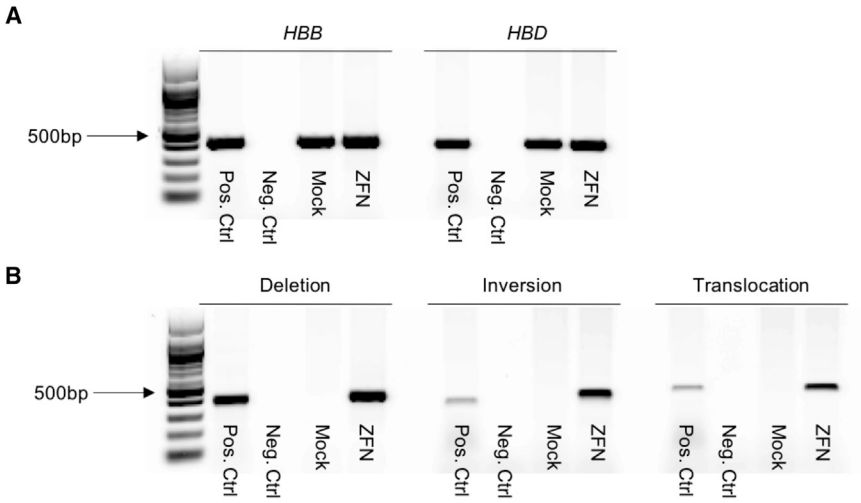


Figure 2. Detection of Rearrangement Events by PCR in CB CD34⁺ Cells Treated with 1 μg of ZFN mRNA

(A) Representative gel showing intact *HBB* and *HBD* by PCR using the primers shown in Figure 1 (HBB-F and HBB-R for *HBB* and HBD-F and HBD-R for *HBD*). (B) Representative gel showing the deletion, inversion, and translocation events using the primer pairs shown in Figures 1A–1C, respectively, present in cells treated with the ZFNs but not in mock-treated cells. Mock: untreated CD34+ samples; ZFN, 1 μg of ZFN 88/01 mRNA-treated CD34⁺ cells; Pos. Ctrl, positive control, CRISPR/Cas9 plasmid-treated K562 3.21 previously detected as positive for the corresponding event; Neg. Ctrl, negative control, as no-template control.

In addition to the absolute quantification of rearrangement events in pooled SCD BM, ddPCR analyses were performed on individual CFUs derived from the same SCD BM CD34⁺ cells treated with ZFNs and OHS or IHS donors. These analyses allowed assessment of the mono-allelic versus bi-allelic nature of the different rearrangement events. The results were detected as a ratio of the corresponding rearrangement-containing alleles to total alleles present in each CFU sample, which was determined using the *UC378* reference gene. A mono-allelic rearrangement event was observed as a ratio of ~0.5 and a bi-allelic event as ~1.0. Representative ddPCR plots for CFU sample rearrangement detection can be seen in Figure S6.

In the ZFN-only treated samples, 67 colonies were analyzed for the deletion rearrangement, and 25 were positive for the deletion event. Of the deletion-containing CFUs, 55.23% ± 16.23% (13/25) were mono-allelic, 32.28% ± 1.45% (8/25) were bi-allelic, and 12.5% ± 17.68% (4/25) were positive for both the deletion event as well as the inversion event in that analysis (referred to as "bi-allelic modification"; Figures 5B and 5C). Of the 107 CFUs analyzed for the ZOHS samples, 57 were positive for the deletion. Of the deletion-containing CFUs, 62.18% ± 9.3% (38/57) were mono-allelic events, 28.11% ± 7.34% (14/57) were bi-allelic events, and 9.72% ± 1.96% (5/57) were positive for both the deletion as well as an inversion. Finally, for the ZIHS treatment, 82 CFUs were analyzed, with 54 of them positive for the deletion event. Of the deletion-containing CFUs, 50.6% ± 10.89% (25/54) were mono-allelic, 35.7% ± 3.39% (20/54) were bi-allelic, and 13.67% ± 7.54% (9/54) were positive for a deletion and inversion event (Figure 5B).

In the case of the inversion rearrangement, for the ZFN-only-treated samples, of the 67 colonies analyzed, 13 were found to be positive. Of the inversion-containing CFUs, 72.2% ± 39.32% (8/13) were mono-allelic events, 5.55% ± 7.85% (1/13) were bi-allelic events, and 22.2% ± 31.4% (4/13) were positive for both an inversion and a deletion. For the ZOHS samples, of the 107 total CFUs analyzed, 17 colonies were positive for inversions. Of the inversion-containing CFUs, 72.85% ± 18.17% (12/17) were mono-allelic, and 27.15% ± 18.17% (5/17) were from inversion- and deletion-positive CFUs, with no bi-allelic inversion events detected for this condition. In the ZIHS samples, of the 82 total CFUs analyzed, 18 colonies were positive for the inversion. Of the inversion-containing CFUs, 51.5% ± 40.31% (7/18) were mono-allelic, 7.7% ± 10.89% (2/18) were bi-allelic, and 40.75% ± 29.34% (9/18) were positive for an inversion and deletion (Figure 5C). With respect to the bi-allelic modified samples that contained both the deletion and inversion events, it is important to note that the same CFUs were counted for the deletion and inversion datasets. For the translocation event, only one sample (from the ZOHS condition) was positive from among all of the CFUs analyzed, which is consistent with its relative rarity among bulk samples.

Overall, there were no significant differences in frequency (unpaired t tests) between the mono-allelic and bi-allelic events, except in the ZOHS treatment for the inversion event, in which the mono-allelic events were significantly more frequent than the bi-allelic ones. However, there was a trend for the mono-allelic events to be more frequent than the bi-allelic events. This was true regardless of the type of rearrangement, the source of HSCs, or the type of HDR donor template provided. Furthermore, the bi-allelic events were at least half as frequent as the mono-allelic events in the deletion analysis and lower or absent in the inversion rearrangement analysis.

High-Throughput Sequencing to Further Characterize Rearrangement Events

To further confirm the frequency of each rearrangement event in a pooled population and to detect other rearrangement events not explored previously, high-throughput sequencing was performed on peripheral blood stem cells (PBSCs) treated with 1 μg of ZFN mRNA and an untreated mock sample (see Table S1 for high-throughput sequencing primers). To detect all possible chromosomal

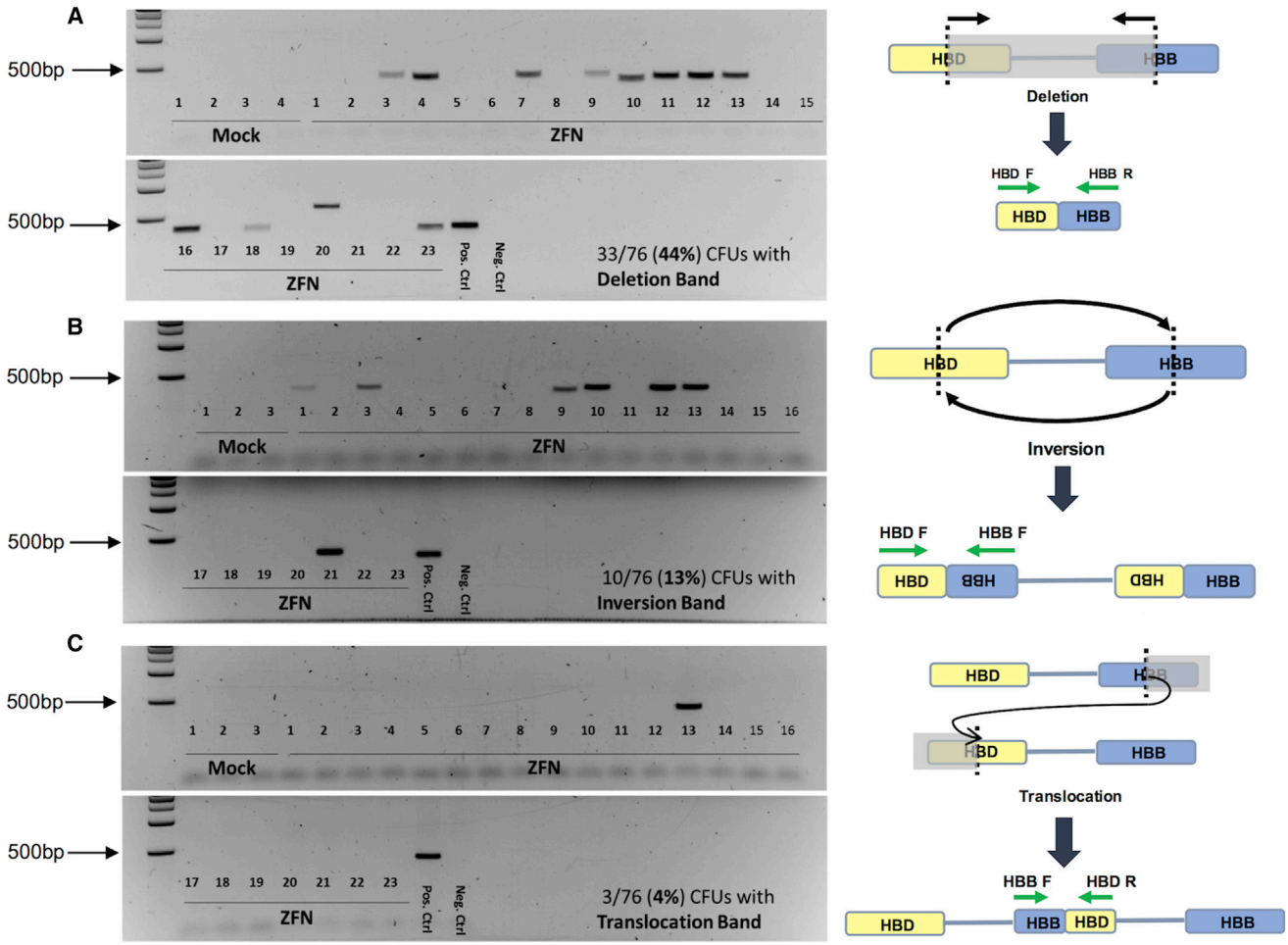


Figure 3. Detection of Rearrangement Events by PCR in CFUs from CD34$^+$ Cells Treated with 1 μg of ZFN mRNA

(A) Representative gel showing the deletion event in CFUs from SCD BM CD34 cells. (B) As in (A) for the inversion events. (C) As in (A) and (B) for the translocation events. Mock, untreated CD34$^+$ samples; ZFN, 1 μg of ZFN 88/01 mRNA-treated CD34$^+$ cells, Pos. Ctrl, K562 3.21 cells treated with a different endonuclease and previously detected as positive for the corresponding event. (A total of 76 CFUs were analyzed for each event.)

rearrangement events, the samples were prepared as two separate sequencing libraries designed to detect any sequence that had translocated to *HBB* after ZFN treatment. One library focused on detecting sequences bound to the portion of *HBB* upstream of the ZFNs cut site (HBB_Upstream), and the other library focused on detecting sequences bound to the portion of *HBB* downstream of the ZFNs cut site (HBB_Downstream) (Figure 6). With respect to the deletion, inversion, and translocation events explored here, the deletion event was expected to be detected only by the HBB_Downstream library; the inversion event may be detected by both the HBB_Upstream and HBB_Downstream libraries, and the translocation event by the HBB_Upstream library only.

The percentages of each rearrangement were reported as the number of sequencing templates containing each event with respect to the total number of templates for that sample. In the PBSCs CD34$^+$ sample treated with ZFNs, it was observed that 18.92% ± 1.92% of templates contained the deletion event, 5.3% ± 1.2% contained the inversion event (average of the upstream and downstream libraries), and 1.18% ± 0.45% contained the expected translocation event (Figures 6 and 7). These data further confirm the trend shown by previous methods that the deletion event is the most frequent, followed by the inversion, and the translocation as the least frequent.

Beyond deletions, inversions, and the specific inter-chromosomal translocation, other rearrangement events were detected as well. For example, a *HBB*-to *HBB*-translocation was observed among several replicates at a very low frequency in the ZFN-treated samples and not in the mock samples (Figure S7). Although many other apparent translocation events connecting *HBB* to other non-*HBB* or *HBD* loci were detected rarely, none of these events were detected in more than one replicate, and many were present in the mock

Table 2. Probes and Primers Used for Droplet Digital PCR

Primer Name/Probe	Sequence (5′–3′)
HBB-Probe	5′ 6-FAM/TAGACCTCA/ZEN/CCCTGTG GAGCCACACC/3′ IABkFQ
HBD-Probe	5′ 6-FAM/AACCCTGCT/ZEN/TATCTTA AACCAACCTGCT/3′ IABkFQ
UC378-Forward	CGCCCCCTCCTCACCATTAT
UC378-Reverse	CATCACAACCATCGCTGCCT
UC378-Probe	5′ HEX-TTACCTTGCTTGTCGGACCAA GGCA/3′ Iowa Black FQ
Event to Be Detected	Primers and Probe Combinations
Deletion	HBD-Forward - HBB-Reverse - HBD-Probe
Inversion	HBD-Forward - HBB-Forward - HBB Probe
Translocation	HBB-Forward - HBD-Reverse - HBB Probe

untreated samples, and were thus determined to likely not be the result of ZFN activity (Figure S8). These findings demonstrate that, within the samples analyzed in this study, the *HBB-HBD* deletion and inversion events represent the vast majority of total ZFN-induced rearrangement events present. In addition, no other rearrangement events produced by this ZFNs pair recurred at any significant frequency, consistent with prior analysis by IDLV-trapping, which did not detect recurrent off-target cutting by this ZFN pair except at *HBD*.[17]

DISCUSSION

Site-specific endonucleases, such as ZFNs, TALENs, and CRISPR/Cas9, are powerful gene editing tools that have the potential to be utilized for the treatment of many genetic disorders.[15,27] Although these designed nucleases can produce high levels of specific on-target DSBs, identification of off-target sites and characterization of the result of their cleavage activities are essential in terms of biosafety. This is particularly important when targeting genes with known adjacent homologous genes, such as *HBB* or *CCR5*, in which concurrent DSBs have the potential to produce unwanted gene alterations.[21] The results shown here demonstrate that high levels of rearrangements between *HBB* and *HBD* are present in human HSPCs treated with a ZFN pair targeted to *HBB* and known to have off-target activity in *HBD* (see Table S2 for a summary).

We have previously[17] characterized the gene correction and allelic disruption levels that can be achieved *in vitro* in $CD34^+$ cells from SCD BM using the 88/01 pair of ZFNs along with the OHS or IHS donor templates. Therefore, in this study, we explored genetic modifications beyond gene correction, on-target cleavage, and off-target cleavage. These additional modifications included *HBB-HBD* intergenic deletions and inversions and an inter-chromosomal translocation. All three rearrangement events were detected in cell lines and HSPCs treated with ZFNs by PCR with primer combinations specific to each of the three events. In all cell types tested, the deletion event was the most frequent rearrangement detected, followed by the inversion, and the translocation as the least frequent.

Because of the surprisingly high levels of rearrangements observed, possible sources of over-estimation of rearrangement detection were addressed. First, the specificity of these primers in detecting each event was established by a lack of amplification seen in mock untreated samples. In addition, through Sanger sequencing, it was confirmed that the rearrangements detected were the result of true NHEJ-driven events rather than gene conversion products. Gene conversion in this case refers to cleavage by ZFNs in *HBB* being repaired via HDR by utilizing a non-cleaved *HBD* allele as a template or vice versa. Gene conversion could thus produce rearrangement-like sequences and result in overestimation of true chromosomal rearrangement events. This concern was further addressed using a second pair of ZFNs (55/58) targeted to *HBB* and known not to have any off-target activity in *HBD*. When $CD34^+$ cells were treated with either pair of ZFNs at equivalent levels of allelic disruption in *HBB*, no rearrangement events were detected in cells treated with the 55/58 ZFN pair. Thus, the rearrangements observed were dependent on the presence of concurrent DSBs in *HBB* and *HBD* and not by gene conversion between the cut *HBB* gene and a non-cleaved *HBD* gene. However, it should be noted that, if gene conversion occurred beyond the binding site of the primers designed for the library generation (which would require an HDR track of at least 200 bp), then those molecules would not be amplified.

These results, showing an absence of *HBB-HBD* junctions when the ZFNs did not cleave *HBD*, are in contrast to a prior report studying CRISPR/Cas9-mediated editing of *HBB*.[28] When they used CRISPR guide RNAs targeting *HBB* without detectable off-target activity in *HBD*, apparent gene conversion was detected in a low percentage of the total editing events. The different results observed between our work and that of Bothmer et al.[28] may be based on the use of different endonucleases and the kinds of DNA lesions and overhangs created.

Although this work focuses on gene and chromosomal rearrangements produced by a specific pair of ZFNs, we previously observed that TALENs and CRISPR/Cas9 nucleases targeted to *HBB* and showing off-target activity in *HBD* also produce rearrangement events between the two genes.[26] Moreover, CRISPR guide RNAs with on-target activity in *HBB* and no off-target activity in *HBD* did not produce any detectable rearrangement events. This prior result is consistent with our findings here that rearrangements between *HBB* and *HBD* are dependent on the combination of on-target activity in *HBB* and off-target activity in *HBD* and are independent of the nuclease type.

Quantification of chromosomal rearrangements by next-generation sequencing or ddPCR has been previously performed in cancer-related studies.[29] With respect to gene therapy, previous studies have used high-throughput sequencing methods for the identification of off-target loci or PCR-based methods and Sanger sequencing of clonal populations to identify and quantify deletions, inversions, and translocations between several gene targets.[21,30] Additionally, a previous study identified a single endonuclease-induced rearrangement, a deletion between *HBD* and *HBB*, and quantified this deletion

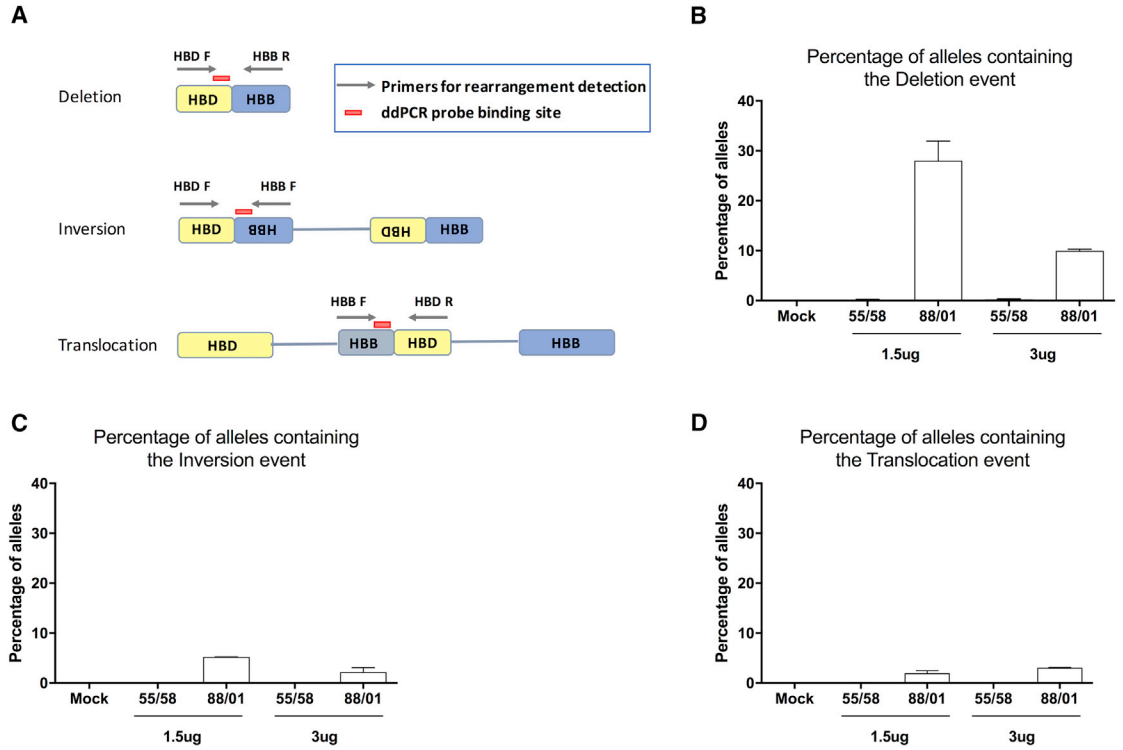


Figure 4. Detection of Rearrangement Events by ddPCR in CD34⁺ Cells from CB Using ZFNs Not Having (55/58) or Having (88/01) Off-Target Cleavage at *HBD*

(A) Schematic showing the binding site for primers and probes for the different rearrangement event detections. (B) Percentage of alleles containing the deletion event in cells treated with 1.5 μg or 3 μg of mRNA from ZFNs 55/58 or ZFNs 88/01. (C) Percentage of alleles containing the inversion event in cells treated with 1.5 μg or 3 μg of mRNA from ZFNs 55/58 or ZFNs 88/01. (D) Percentage of alleles containing the translocation event in cells treated with 1.5 μg or 3 μg of mRNA from ZFNs 55/58 or ZFNs 88/01. Mock, untreated CD34⁺ samples (n = 2 for each treatment). Error bars represent mean ± SD.

via quantitative PCR.[31] The use of ddPCR and next-generation sequencing here has provided valuable information for the absolute quantification of rearrangement events in pooled cell populations in the presence or absence of sickle mutation-corrective donor DNA molecules as well as for determining the mono versus bi-allelic frequencies of these events. By ddPCR, it was determined that the majority of ZFN-induced rearrangement events were deletions, in both cell lines and primary cells, and that, in SCD BM-derived CFUs, the deletions and inversions mostly occurred as mono-allelic events, whereas bi-allelic events were present at much lower frequencies. High-throughput sequencing analysis of the bulk population corroborated the frequency of the deletions and inversions, with lack of a significant presence of any other type of translocation.

With the use of endonucleases for targeted gene therapy, the necessity of finding off-target cleavage sites is critical for subsequent clinical applications. Although off-target sites were confirmed here via surveyor nuclease assay, as can be done when targeting sites with known adjacent homologous sequences such as *HBB* or CCR5, other methods now available can provide an unbiased genome-wide profiling of off-target cleavage sites such as Guide-seq, BLESS and Digenome-seq.[30,32] Future studies utilizing these methods, in combination with the rearrangement detection methods described here or other high-throughput sequencing-based, chromosomal alteration detection methods such as high-throughput genome-wide sequencing (HTGTS),[33] would achieve a more complete off-target analysis of a lead therapeutic endonuclease.

Despite the extensive work to identify and quantify these gene and chromosomal alterations between *HBB* and *HBD*, the full biological effect of these rearrangements remains unexplored. One possibility that could be considered is the production of HBB/HBD fusion proteins. It is likely that any *HBB/HBD* gene fusions produced from rearrangements would not express a functional form of the globin protein because of the presence of indels as a result of endonuclease cleavage. However, Sanger sequencing of deletion-positive CFUs did show several clones possessing indel-free events, and indels produced in multiples of 3 bp would maintain the reading frame, making the idea of a globin fusion protein possible. Nevertheless, given that the deletion event is the most frequent rearrangement produced by this pair of ZFNs, the most common *HBD/HBB* fusion gene would thus only be expressed using the weak (in adults) *HBD* promoter.[34,35] One factor potentially affecting the frequency of rearrangements observed that was not explored here is the size of the intergenic gap between *HBB* and *HBD*. Although the presence and frequency of these rearrangements was determined here

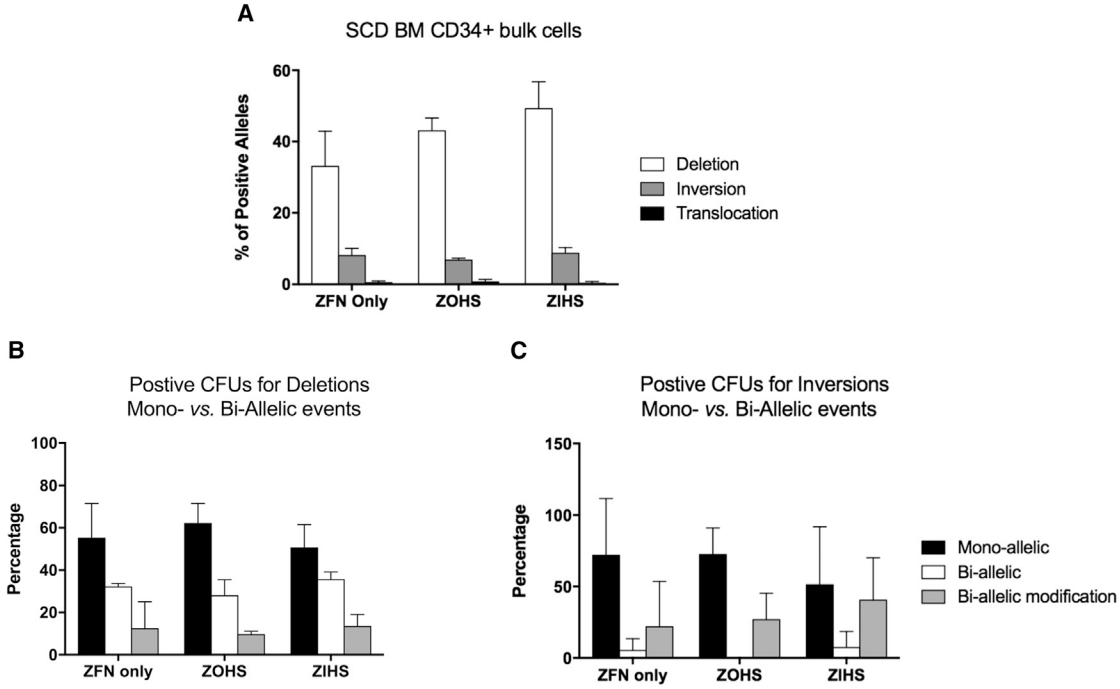


Figure 5. Detection of Rearrangement Events by ddPCR in Bulk Cells and CFUs Derived from SCD BM CD34$^+$ Cells

(A) Percentage of alleles containing each rearrangement event in SCD BM CD34$^+$ cells from two independent SCD BM donors, treated with 1 μg of ZFN 88/01 as mRNA only (4 biological replicates) or with the addition of the oligonucleotide (OHS) or integrase-defective lentiviral vector (IHS) donor templates (6 biological replicates per treatment). (B) Percentage of deletion-positive CFUs with mono-allelic or bi-allelic deletion events (ZFN only, n = 25; ZOHS, n = 57; ZIHS, n = 54). (C) Percentage of inversion-positive CFUs with mono-allelic or bi-allelic deletion events (ZFN only, n = 13; ZOHS, n = 17; ZIHS, n = 18). Bi-allelic modification refers to detection of both a deletion and an inversion event within the same sample. Nonparametric Wilcoxon rank-sum test (A) and unpaired t tests (B and C) were used to determine statistical significance between ZFN only-ZOHS, ZFN only-ZIHS, and ZOHS-ZIHS conditions and to determine statistical differences between mono- versus bi-allelic modifications within the same treatment (ZFN only, ZOHS, and ZIHS). For all comparisons, $p \geq 0.05$. Error bars represent mean ± SD. The legend shown in (C) also applies to (B).

between a single target site in *HBB* and its corresponding off-target site in *HBD,* with a gap size of ~7 kb, these results may not be indicative of what would be seen when considering sites with different gap sizes. A prior study observed a lower frequency of nuclease-induced intergenic deletions with an increase in size between cleavage sites.[24]

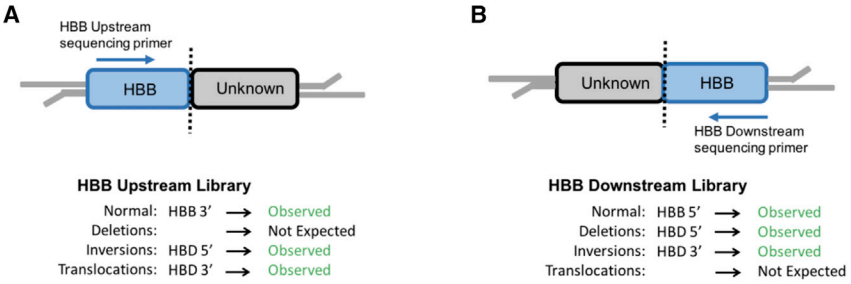
The gene and chromosomal alterations shown here, as the result of known high-level off-target cutting at a nearby sequence with high homology to the on-target gene, are an extreme example of the risks for interactions between multiple simultaneous cut sites using endonucleases. However, they illustrate the potential unwanted results of making two simultaneous double-stranded DNA breaks in cells, either by a combination of on-target and off-target cutting, as with a nuclease of low specificity, or by intentionally making two simultaneous cuts in gene editing strategies targeting multiple loci in a single process. Additionally, they suggest that determining the presence of off-target loci only by the occurrence of indels may underrepresent the full extent of endonuclease off-target activity and that these alternative types of chromosomal alterations need to be considered. Although many inter-chromosomal translocations may either be benign or cause cell death, or as detrimental as the isolated *HBB* NHEJ, some may potentially be oncogenic and could pose rare but serious risks to the use of gene editing for cellular therapies. Use of very specific nucleases that do not cleave highly similar off targets, such as the 55/58 ZFN pair or the CRISPR/Cas9 system with a specific guide RNA, as discussed above, can prevent this potential risk. Thus, nucleases to be used for clinical cell editing should be carefully investigated to understand their safety profile by characterizing their specificity.

MATERIALS AND METHODS

Cell culture, primary human CD34$^+$ cell processing, electroporation, mRNA production, and donor template construction were performed as described previously.[17,26] All human BM samples from volunteers with SCD were used following UCLA IRB protocol 10-001399 with written informed consent. Use of umbilical CB collected at normal births was deemed exempt from IRB review as anonymous medical waste.

Cel-1 Surveyor Nuclease Assay

A surveyor nuclease assay (Cel-1) was used to determine ZFN-induced site-specific allelic disruption at the on-target site in *HBB* and at the off-target site in *HBD*. A 410-bp region surrounding the



C

Sample	Normal Templates	Deletion Templates	Inversion Templates	HBB-HBD Translocation Templates	%Deletion	% Inversion	% HBB-HBD Translocation
mock_up_1	8869	15	60	13	0.17%	0.67%	0.15%
mock_up_2	9135	7	23	9	0.08%	0.25%	0.10%
ZFN_up_1	6006	18	394	107	0.28%	6.05%	1.64%
ZFN_up_2	6397	6	509	51	0.09%	7.34%	0.73%
ZFN_up_3	7242	49	364	91	0.63%	4.70%	1.17%
mock_down_1	9179	61	10	-	0.66%	0.11%	-
mock_down_2	8652	42	38	-	0.48%	0.44%	-
ZFN_down_1	6086	1603	351	-	19.94%	4.37%	-
ZFN_down_2	6552	1474	411	-	17.47%	4.87%	-
ZFN_down_3	6456	1635	361	-	19.34%	4.27%	-

Figure 6. High-Throughput Sequencing of *HBB* Rearrangements in ZFN-Treated PBSCs

(A) Schematic displaying the orientation of *HBB* upstream of the cut site and an unknown connected sequence downstream of the cut site. This represents the potential rearrangement product after ZFN treatment, shearing, and Illumina adaptor ligation (gray bars adjacent to the molecule). The directionality of sequencing is shown, starting upstream of the ZFN cut site (indicated by the dashed lines) in *HBB* and sequences toward the rearrangement junction (designated as HBB upstream). The expected rearrangement events and whether they were observed are specified below the schematic. (B) Schematic displaying the orientation of *HBB* downstream of the cut site and an unknown connected sequence upstream of the cut site. This represents the potential rearrangement product after ZFN treatment, shearing, and Illumina adaptor ligation (gray bars adjacent to the molecule). The expected rearrangement events and whether they were observed is specified below the schematic. (C) A summary table showing the samples sequenced in both the upstream and downstream libraries. The number of normal templates (for *HBB*); templates for the deletion, inversion and translocation; and the percentage of deletion, inversion, and translocation events are shown. The percentages of deletion, inversion, and translocation events were calculated as the number of templates positive for each event over the total number of templates for that sample. Mock_up and ZFN_up refer to samples from the HBB_Upstream library versus the Mock_down and ZFN_down samples, which were from the HBB_Downstream library.

ZFN binding site in *HBB* was PCR-amplified from 200 ng of gDNA using primers HBB-Forward and HBB-Reverse or in *HBD* using primers HBD-Forward and HBD-Reverse using Accuprime Taq Hi-Fi (Life Technologies, Carlsbad, CA). Denaturation, reannealing, digestion, and electrophoretic and densitometry analyses were completed as described previously.[36]

Rearrangement Event Detection by PCR

For gene and chromosomal rearrangement detection, a 400-bp region surrounding the nuclease cleavage site was PCR-amplified from 10–200 ng of gDNA using Accuprime Taq Hi-Fi (Life Technologies, Carlsbad, CA) with the following cycling conditions: initial denaturation at 94°C for 5 min; amplification for 35 cycles at 94°C for 30 s, 54°C for 30 s, and 68°C for 1 min; and final extension at 68°C for 5 min. The PCR products were resolved on a Novex 8% TBE gel (Thermo Fisher Scientific, Waltham, MA) at 120 V for 50 min and imaged on a Typhoon FLA 9000 Biomolecular Imager (GE Healthcare, Chicago, IL). Primer sequences and combinations used to detect each event are summarized in Table 1.

Sanger Sequencing of PCR-Amplified Rearrangement Events in CFUs

For sequence analyses, PCR products corresponding to deletion, inversion, or translocation events in the CFUs were purified using the PureLink Quick PCR Purification Kit (Invitrogen, Carlsbad, CA) and then Sanger-sequenced using the corresponding PCR amplification primers.

Rearrangement Event Detection by ddPCR

For quantification of each rearrangement event using ddPCR, the same combinations of primers as described previously (Table 1) were used, and additional fluorescently labeled probes were designed for ddPCR analysis of the *HBD* and for *HBB* as detailed in Table 2. For absolute quantification of each rearrangement event, ddPCRC analysis of the human gene (UC378) was used as an internal control for normalization.

Reaction mixtures of 20 μL volume were prepared containing 1 × ddPCR Master Mix (Bio-Rad, Hercules, CA), relevant primers and probe (400 nM primers and 100 nM probe), and 1–2 μL of gDNA (3–20 ng for CFUs samples or 10–75 ng for bulk cells). Droplet generation was performed as described by Hindson et al.[37] The droplet emulsion was transferred to a 96-well propylene plate (Eppendorf, Hamburg, Germany) and amplified in a conventional thermal cycler (T100 thermal cycler, Bio-Rad, Hercules, CA). Thermal cycling conditions consisted of 95°C for 10 min; 94°C for 30 s, 54°C for 1 min, and 72°C for 2 min (54 cycles); 98°C for 10 min (1 cycle); and 12°C hold. After PCR, the 96-well plate was transferred to a droplet reader (Bio-Rad, Hercules, CA). Acquisition and analysis of the ddPCR data were performed with the QuantaSoft

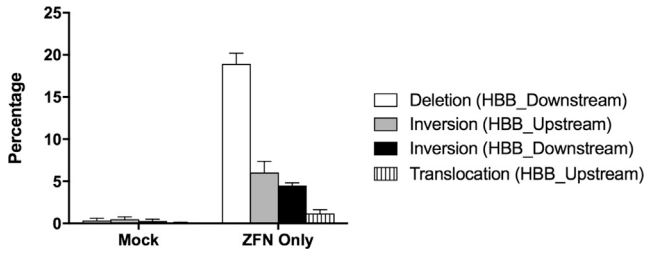


Figure 7. Detection of Rearrangement Events by High-Throughput Sequencing in CD34⁺ Cells Treated with 1 μg of ZFN mRNA

Percentage of total templates of each sample possessing either the deletion (only detected by the HBB_Downstream library), inversion events (detected by both the HBB_Upstream and HBB_Downstream libraries), or translocation events (detected by the HBB_Upstream library) in PBSCs treated with 1 μg of ZFN mRNA. Inversion (HBB_Upstream) identifies one junction of this event, whereas inversion (HBB_Downstream) identifies the other junction. Mock, untreated BM CD34⁺ sample. Unpaired t test was used to determine statistical significance in ZFN-only treatment between deletion and inversion (HBB_Upstream) ($p < 0.001$), deletion and inversion (HBB_Downstream) ($p < 0.001$), inversion (HBB_Upstream) and inversion (HBB_Downstream) ($p = 0.12$), deletion and translocation ($p < 0.001$), inversion (HBB_Downstream) and translocation ($p < 0.001$), and inversion (HBB_Upstream) and translocation ($p = 0.004$). Error bars represent mean ± SD.

software (Bio-Rad, Hercules, CA), provided with the droplet reader. The relative frequencies of each rearrangement event were calculated by dividing the concentration (copies per microliter) of the corresponding event by the concentration of the *UC378* gene, used to normalize per human genome.

High-Throughput Sequencing

Genomic DNA from ZFN-treated PBSC CD34⁺ cells was sheared with a Covaris S200 sonicator to an average length of 500 bp and then end-repaired, A-tailed, and ligated to Illumina adapters containing an 8-nt random molecular index. The samples were prepared as two separate libraries designated as HBB_Upstream or HBB_Downstream. An initial round of PCR was performed using primers specific to HBB upstream or downstream of the expected ZFN cut site and designated (HBB_Up-1) or (HBB_Down-1), respectively. A second nested PCR was performed to amplify the target region and for the incorporation of a 6-nt sample index sequence. Samples were then quantified by densitometry, mixed, and purified. The final library was quantified by ddPCR and then submitted for sequencing as a MiSeq paired-end 2 × 150 run. It should be noted that the samples analyzed by this high-throughput sequencing method were replicates of a single ZFN-treated sample or mock sample and, thus, not true biological replicates.

Sequence reads were first demultiplexed using index sequences installed by unique PCR primer for each sample during the first PCR step in library preparation. After demultiplexing, paired-end reads were joined using the flash2 utility. Only reads that overlapped sufficiently to be combined were kept for further analysis because non-overlapping paired-end reads likely came from long sheared DNA fragments that were not completely sequenced in the paired-end 150 MiSeq run. For deduplication, identical sequence reads with similar unique molecular indexes (UMIs) installed in a ligated linker during library preparation were collapsed to reduce read duplications. The resulting reads were aligned to the hg19 assembly of the human genome using BLAT (BLAST-like alignment tool). Alignments were processed by a custom Python script, and split alignments to *HBB* and other non-*HBB* sites were further examined.

Statistical Analysis

Descriptive statistics, such as number of observations and mean and SD, were reported and presented graphically for quantitative measurements. Normality assumption was checked for outcomes before statistical testing. Nonparametric Wilcoxon rank-sum test was used to compare the percentage of positive events in SCD BM CD34⁺ bulk cells between experimental arms (Figure 5A). Unpaired t test was used to compare the percentage of mono- versus bi-allelic events in deletion- and inversion-positive CFUs between experimental arms (Figures 5B and 5C). To evaluate the difference between the deletion (HBB downstream), the inversion (HBB upstream), the inversion (HBB downstream), and the translocation (HHB upstream) in the ZFN-only group, unpaired t tests were performed (Figure 7). For all statistical investigations, tests for significance were two-tailed. A p value of less than the 0.05 significance level was considered to be statistically significant. All statistical analyses were carried out using statistical software (SAS version 9.4, SAS Institute, 2013).

ACCESSION NUMBERS

Sequence data are available in the Sequence Read Archive: SUB2691555 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA387385).

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and two tables and can be found with this article online at https://doi.org/10.1016/j.ymthe.2017.11.001.

AUTHOR CONTRIBUTIONS

Conceptualization, J.L., M.D.H., and Z.R.; Methodology, J.L. and Z.R.; Software, A.R.C. and M.L.K.; Validation, C.Y.K., B.C-F., and R.P.H.; Investigation, J.L., Z.R., and D.L.; Formal Analysis, X.W.; Writing – Original Draft, J.L.; Writing – Review & Editing, Z. R. and D.B.K.; Supervision, D.B.K. and Z.R.; Funding Acquisition, D.B.K.

ACKNOWLEDGMENTS

These studies were supported by research grants from the California Institute for Regenerative Medicine (CIRM TR4-06823, CL1-00505-1.2, and FA1-00613-1), the Doris Duke Charitable Foundation (Innovations in Clinical Research Award 2013158), an Interdisciplinary Training In Virology and Gene Therapy award (grant 5 T32 AI060567 to M.D.H.), the Whitcome Predoctoral Training Program, the UCLA Molecular Biology Institute (to M.D.H.), the Ruth L. Kirschstein National Research Service Award (grant GM007185 to A.R.C.), and the Initiative to Maximize Student Diversity (NIH grant GM55052 to D.L.). J.L. is the recipient of the CSUN-UCLA Bridges to Regenerative Medicine Program (CIRM TB1-01183) to California State University, Northridge and University of California, Los Angeles.

The DNA Sequencing Core of the Eli & Edythe Broad Center of Regenerative Medicine & Stem Cell Research provided essential support.

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