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Editing the Sickle Cell Disease Mutation in Human Hematopoietic Stem Cells: Comparison of Endonucleases and Homologous Donor Templates

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Site-specific correction of a point mutation causing a monogenic disease in autologous hematopoietic stem and progenitor cells (HSPCs) can be used as a treatment of inherited disorders of the blood cells. Sickle cell disease (SCD) is an ideal model to investigate the potential use of gene editing to transvert a single point mutation at the β-globin locus (*HBB*). We compared the activity of zinc-finger nucleases (ZFNs) and CRISPR/Cas9 for editing, and homologous donor templates delivered as single-stranded oligodeoxynucleotides (ssODNs), adeno-associated virus serotype 6 (AAV6), integrase-deficient lentiviral vectors (IDLVs), and adenovirus 5/35 serotype (Ad5/35) to transvert the base pair responsible for SCD in *HBB* in primary human CD34+ HSPCs. We found that the ZFNs and Cas9 directed similar frequencies of nuclease activity. *In vitro*, AAV6 led to the highest frequencies of homology-directed repair (HDR), but levels of base pair transversions were significantly reduced when analyzing cells *in vivo* in immunodeficient mouse xenografts, with similar frequencies achieved with either AAV6 or ssODNs. AAV6 also caused significant impairment of colony-forming progenitors and human cell engraftment. Gene correction in engrafting hematopoietic stem cells may be limited by the capacity of the cells to mediate HDR, suggesting additional manipulations may be needed for high-efficiency gene correction in HSPCs.

INTRODUCTION

Site-specific correction of the mutated gene causing a monogenic disease in autologous hematopoietic stem and progenitor cells (HSPCs) has the potential to be used as a treatment for inherited disorders of blood cells, such as hemoglobinopathies and primary immunodeficiencies. Targeted endonucleases may be used to induce a double-strand break (DSB) close to the mutation, and an exogenous homologous DNA donor template possessing the corrective sequence can be utilized by the targeted cells to repair the DSB by homology-directed repair (HDR); if the DSB is fixed by the error-prone non-homologous end joining (NHEJ) pathway, insertions or deletions (indels) may be introduced to disrupt the gene.[1]

Even though the effectiveness of this technology has been broadly demonstrated as proof of principle,[2–6] direct comparisons of the different types of endonucleases and homologous donor templates that can be used for HDR remain necessary. These comparative studies can provide a better understanding of (1) the effects of the endonuclease and homologous donor template type on the DNA repair pathway chosen in the hematopoietic stem cells (HSCs) to correct the DSB and, hence, editing outcomes; and (2) the elements playing a key role in cytotoxicity that will determine the number and engraftment capacity of the edited HSCs. Therefore, we performed a comparative study of two commonly used endonucleases and several homologous donor templates in the context of sickle cell disease (SCD), a monogenic disorder caused by a single point mutation at the *HBB* gene, which represents a suitable model for site-specific gene correction.[3–5]

Zinc-finger nucleases (ZFNs) and CRISPR/Cas9 nucleases have been used to target the *HBB* locus for site-specific correction of the sickle mutation in HSPCs.[3–5] Although transcription activator-like effector nucleases (TALENs) have been utilized to edit the sickle mutation in cell lines[7,8] or in induced pluripotent stem cells (iPSCs) (using

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selection methods),[9] no evidence of their efficacy in HSPCs has been exhibited targeting the *HBB* locus.[10] Thus, ZFNs and CRISPR/Cas9 are the two main systems to be compared in the context of gene editing of HSPCs for SCD. While the most efficient method to deliver ZFNs into the HSPCs is as mRNA,[2,3,11] the Cas9 can be delivered as mRNA[5,6,12,13] or as recombinant protein complexed with the single-guide RNA (sgRNA) forming ribonucleoproteins (RNPs).[4,5,12]

To introduce the homologous donor template, both viral and non-viral systems have been used. As viral vectors, integrase-deficient lentiviral vectors (IDLVs) have been utilized for gene targeting of the *IL2RG* cDNA (exons 5–8) along with the GFP cassette into the *IL2RG* locus in murine and human HSPCs[2] and for site-specific correction of the sickle mutation in *HBB*.[3] Recombinant adeno-associated virus serotype 6 (AAV6) have been shown to be one of the most effective serotypes to transduce human HSPCs,[14–16] and they have been used as a vehicle for site-specific integration of reporter genes in HSPCs derived from mobilized peripheral blood or fetal liver[5,11] or for gene editing of specific diseases, such as SCD[5,12] and X-linked hyper-IgM syndrome.[17] As a third option, adenoviral vectors (AdV), commonly of the Ad35 serotype, have been employed to deliver the DNA template for site-specific insertion of various genes, and they have large enough capacity to also carry the genes encoding the nuclease (ZFN, TALEN, and CRISPR).[18–20]

As an alternative to viral vectors, single-stranded oligodeoxynucleotides (ssODNs) have been broadly utilized to deliver homologous donor templates in HSPCs, either in the setting of using ZFNs[3] or CRISPR/Cas9.[4,6] A recent report described the use of long double-stranded DNA and single-stranded DNA (ssDNA) as donor templates for efficient gene editing in human T cells.[21] In comparison with viral vectors, benefits of using ssODNs include their simple design, short production time, relatively low cost, and reproducibility.[22] We have previously observed higher acute cytotoxicity from the inclusion of ssODN along with the nuclease in the electroporation (EP) than from use of IDLV.[3] Toxicity from the ssODN would reduce the numbers of transplantable HSCs, and this may be due to the ssODN itself, with an additive toxicity from modified bases that may be incorporated to prevent nuclease degradation and increase targeting efficiency.[23,24]

In the context of SCD, previous publications have shown that ZFNs along with IDLV or ssODN donor templates exhibited efficient correction of HSPCs derived from cord blood (CB) or mobilized peripheral blood stem cells (PBSCs) *in vitro*, with significantly lower frequencies of corrected HSPCs *in vivo*.[3] Using CRISPR/Cas9 in PBSCs, either in combination with an AAV6[5] or ssODN[4] donor template, resulted in comparable frequencies of gene correction *in vitro*. However, there was again a substantial decrease of correction by HDR in the engrafting cell of the xenografted mice, partially rescued by utilizing selective methods, which may not be feasible in the clinical setting.[5] The reduced levels of gene correction in the long-term engrafting HSCs could result from the difficulty of editing by HDR in the non-dividing HSCs[25] and/or the higher sensitivity of these HSCs to the toxicity derived from the EP process and reagents.

To assess the efficacy and cytotoxicity derived from the different editing reagents, we performed a direct comparison of two commonly used types of endonucleases, ZFNs and CRISPR/Cas9, in combination with viral (AAV6, IDLV, and adenovirus 5/35 serotype [Ad5/35]) and non-viral methods to deliver the homologous donor template, using SCD as the disease model and targeting the sickle mutation at the *HBB* locus. For this aim, we evaluated the following *in vitro*: (1) viability (by trypan blue exclusion and Annexin V), (2) editing outcomes at the sickle mutation site by high-throughput sequencing (HTS) to determine frequencies of HDR sequence transversion and NHEJ-mediated indels, (3) hematopoietic progenitor potential (by colony-forming unit [CFU] assay), (4) effects on cell cycle status, and (5) differential gene expression (by RNA sequencing [RNA-seq]). We compared the *in vitro* short-term results with the *in vivo* assays to engraft edited HSPCs in immunodeficient mice, where we determined the effects of the different editing reagents on HSPCs survival and function and on editing outcomes (HDR and NHEJ).

RESULTS

Design of ZFNs and CRISPR/Cas9 Targeting the *HBB* Locus and Four Different DNA Homologous Sequence Donor Templates

Identifying the optimal endonuclease, as well as determining the most effective and safest way to deliver the molecule to be used as the donor sequence template to repair the endonuclease-created DSB by HDR, is a critical factor for the application of gene editing. We performed an extensive comparison of two commonly used endonucleases, ZFNs and CRISPR/Cas9, targeting the sickle mutation at the *HBB* locus,[3,4] when co-delivered along with different types of homologous donor templates to correct the single mutation causing this monogenic disease. As homologous donor template, three different non-integrating viral vectors were chosen: IDLV, AAV6, and adenoviruses (hybrid of serotypes 5 and 35, Ad5/35). As non-viral donor templates, ssODNs were designed and modified according to the endonuclease used.

The *HBB* donor sequence carried by the non-integrating viral vectors corresponds to a 1.1-kb segment homologous to the *HBB* locus extending from the 5′ UTR to the beginning of intron II, with the sickle mutation located in the sixth codon of exon I (Figure S1). Due to the limited availability of HSPCs from sickle patients, the DNA donor templates were designed to carry the sickle mutation to be utilized as reverse models in CD34+ cells from healthy donors, causing a *gene transversion* (A→T) rather than a gene correction that would restore the normal sequence. Along with the sickle mutation, a silent base pair change introduces an *HhaI* restriction fragment-length polymorphism (RFLP) site as a surrogate marker of the HDR event.[3] When these donors were tested in the context of the ZFNs, two more silent base pair changes were incorporated at the ZFN-binding site to avoid re-cleavage of the corrected genome;[3] when the CRISPR/Cas9 system was used, a silent base pair change was introduced to the donor to abrogate the protospacer adjacent motif (PAM) sequence for the same purpose.[4]

The ssODN donors used had homology to 100–168 nt of the *HBB* sequences flanking the sickle mutation site, and they carried the same

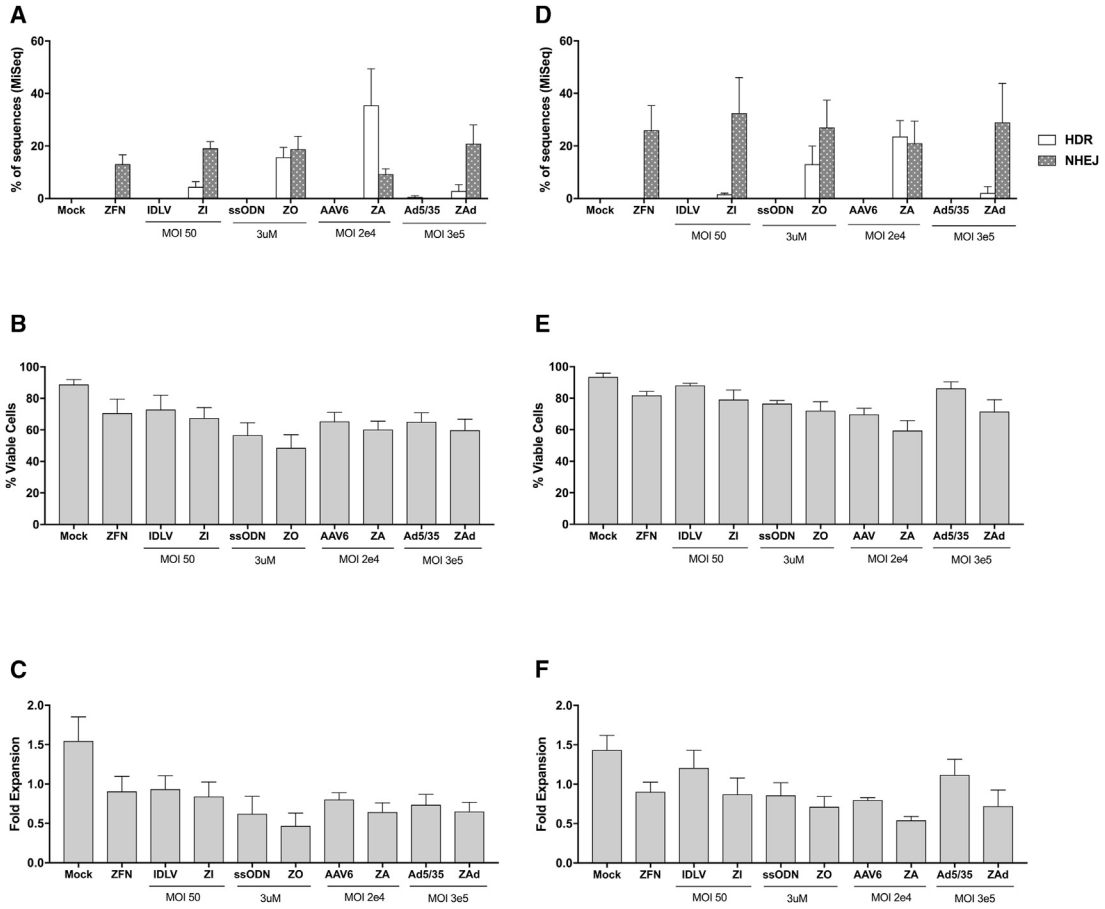

Figure 1. Direct Comparison of Beta-globin Gene Editing with ZFNs Using Four Different DNA Donor Templates (ssODN, IDLV, AAV6, and Ad5/35)

CD34+ cells from pooled CB (A–C) or PBSCs from independent healthy donors (D–F) were pre-stimulated in X-Vivo15 with cytokines (SCF, Flt3-L, and TPO, all at 50 ng/mL) for 48 h before being electroporated with ZFN mRNA (10 μg/mL) and the corresponding donor amount as indicated. All donors contained the sickle mutation as a reverse model for gene transversion. (A and D) The percentages of gene-editing outcomes (HDR and NHEJ) were measured by HTS of *HBB* 4 days post-EP. Samples were counted by trypan blue exclusion 24 h post-EP to evaluate (B and E) viability and (C and F) fold expansion. CB, n = 5–9 biological replicates for controls and experimental samples, respectively, four independent experiments; PBSCs, n = 3–6 biological replicates for controls and experimental samples, respectively, three independent experiments; error bars, mean ± SD. Mock cells were suspended in electroporation buffer for the same time duration that electroporated cells were, but DNA was not added and the mock cells were not electroporated.

features described above when used with ZFNs or CRISPR/Cas9, respectively. The main differences among the design of these ssODN donors were length, symmetry with respect to the nuclease cut site, and strand orientation (see the Materials and Methods for more information).

Editing of the *HBB* Locus Using ZFNs with Four Different DNA Donor Templates

Clinically, three different sources may be used for autologous transplantation of gene-edited CD34+ cells: bone marrow (BM), PBSCs, and CB. BM was initially considered the source of autologous CD34+ cells for gene therapy for SCD patients due to the risks of triggering an acute sickle crisis when granulocyte colony-stimulating factor (G-CSF) was used to mobilize CD34+ cells from peripheral blood.[26,27] However, several recent reports have demonstrated that mobilization of PBSCs from SCD patients using Plerixafor as a single agent can be performed safely and yields sufficient cell numbers for transplant.[28–30] Therefore, PBSCs have become the favored source of CD34+ for these patients. Also, use of autologous CB CD34+ cells may be utilized for gene therapy for infant SCD patients. Therefore, in this study, we performed comparisons of gene-editing efficacy in PBSCs and CB CD34+ cells.

Gene transversion efficacy from editing CB CD34+ with ZFNs was evaluated using IDLV, ssODN, AAV6, or Ad5/35 as DNA donor templates (Figures 1A–1C). The concentrations of ZFN mRNA and ssODN, as well as viral MOIs, were previously established (those providing the best rates of gene transversion but also allowing for the lowest cell death and greatest fold expansion 24 h post-EP) by several titrations of these reagents in CD34+ cells from CB

(ZFN mRNA, ssODN concentrations, and IDLV MOI as in Hoban et al.;[3] AAV6 and Ad5/35 MOI titrations are shown in Figure S2). HDR and NHEJ levels were measured by HTS of *HBB* (or by qPCR for the *HhaI* RFLP, as specified in the figure's legend).

The AAV6 donor (MOI 2e4) provided the highest rates of HDR (~35%) along with the lower levels of NHEJ (~10%). The ssODN donor (3 μM) resulted in levels of HDR and NHEJ that were more equal (~15% and 18%, respectively). The IDLV (MOI 50) and Ad5/35 (MOI 3e5) donors provided the lowest editing rates, with HDR reaching up to 2%–5%, and considerably high levels of NHEJ up to 20%. In terms of toxicity 24 h post-EP, the ssODN donor had the most severe effect on viability, which was reflected in a low fold expansion with respect to the starting numbers of cells. It is worth mentioning that, in this comparison, the AAV6 donor showed a high variability among different CD34+ donors, which has been previously reported in CB CD34+ cells.[16]

The same comparisons of different donor delivery methods were performed with CD34+ from G-CSF-mobilized PBSCs (Figures 1D–1F) (based on prior titrations of the four DNA donor types using mRNA ZFNs in CD34+ PBSCs; Figures S3 and S4). We observed comparable results to those in CB, with the main difference being that, for all the DNA donors used but especially for the AAV6 donor, the HDR:NHEJ ratios were lower with PBSCs than the ones observed for each donor type in CB CD34+ cells. Based on these findings, the IDLV and Ad5/35 DNA donors were excluded from further analyses, due to the low gene transversion levels and the poor HDR:NHEJ ratios.

Editing of the *HBB* Locus: ZFNs versus CRISPR/Cas9 When Delivering the DNA Donor Template as an ssODN or AAV6

Before comparing the activities of the ZFNs and CRISPR/Cas9, the CRISPR/Cas9 reagents were characterized and doses optimized in PBSCs. The sgRNA to *HBB* used here was previously characterized extensively by DeWitt et al.[4] as G10 by both bioinformatic algorithms and genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq), and it was found to have two predominant off-target cleavage sites in two intergenic regions not associated with nearby oncogenes or tumor suppressor genes; the ZFN pair to *HBB* was shown to have only the adjacent and homologous *HBD* locus as a recurrent off-target site.[3,31]

Cutting efficiency and gene transversion rates were assessed when using different amounts of guide RNA (gRNA) and Cas9, delivered either as mRNA (Figure S5) or as recombinant protein in RNP complexes (Figure S6). Comparable rates of gene transversion were observed when using mRNA Cas9 or RNPs, with equivalent viability and fold expansion 1 day post-EP achieved (Figure S7) using the most efficient doses of these reagents (gRNA [5–9 μg]/mRNA Cas9 [5 μg]; and gRNA [5–9 μg]/Cas9 protein [200 pmol]).

Similar optimizations were performed to determine the optimal MOI of the AAV6 donor (Figure S8) or concentration of the ssODN (Figure S9) when using CRISPR/Cas9. Again, an MOI of 2e4 for the AAV6 and a concentration of 3 μM for the ssODN were the most effective amounts of these donors when balancing maximal editing with preservation of viability and fold expansion 24 h post-EP. As no substantial differences in gene transversion were found between RNPs and Cas9 mRNA, the rest of the studies were performed using RNPs. Finally, the optimal time to introduce the AAV6 donor relative to EP was tested. However, adding the AAV6 immediately after the EP, as was done in previous experiments, resulted in the best gene editing and viability rates (Figure S10).

After defining the optimal conditions for use of these reagents, ZFNs and CRISPR/Cas9 nucleases were tested head-to-head in CD34+ cells from CB and PBSCs, comparing ssODN and AAV6 as DNA donor templates with the base pair substitutions corresponding to the modifications of the ZFNs or Cas9 PAM recognition sites. For both sources of CD34+ cells tested with CRISPR/Cas9, the AAV6 donor allowed for the highest frequency of HDR. The HDR:NHEJ ratio was 2:1 when using the AAV6 as DNA donor template and was reversed at 1:2 when using ssODN (Figure 2A). A similar trend was observed with higher HDR with the AAV6 donor when utilizing ZFNs compared to the ssODN donor for both sources of CD34+ cells (HDR:NHEJ 4:1 with AAV6 versus 1:2 with ssODN donor). Therefore, the AAV6 donor template significantly increased the percentage of HDR as well as the HDR:NHEJ ratio compared to the ssODN, regardless of the endonuclease used or the source of the CD34+ cells. Viability was comparable among all the conditions in these specific experiments (Figures 2B and 2C). Note, the higher frequencies of HDR:NHEJ observed previously (Figures 1A and 1D), when CB was used compared to PBSCs (with either the ssODN or the AAV6), were not observed in these experiments, which may reflect CD34+ donor variability.

HDR is known to be restricted to the S/G2 phases of the cell cycle, while NHEJ can occur throughout the cell cycle.[13] Potentially, the higher HDR produced by the AAV6 donor compared to the ssODN donor may be due to effects of either donor on the cell cycle status of the edited cells. Therefore, we analyzed CD34+ cells from CB and PBSCs treated with CRISPR/Cas9 and these two donors using flow cytometry to determine cell cycle status by Hoechst 33342 DNA staining (Figure 2D; Table S1). CB cells and PBSCs edited with either ssODN or AAV6 donors had decreased or a similar percentage of cells in S and G2/M phases with respect to the mock-treated samples and a concordant increase with Sub G1 amounts of DNA, suggestive of apoptosis. These observations do not, however, explain the higher HDR achieved using AAV6.

Since no substantial differences were found among the two CD34+ sources used for the initial *in vitro* studies, PBSCs were chosen for further *in vivo* comparisons of the ssODN and AAV6 in the context of the ZFNs or CRISPRs, as they are the more clinically relevant cell source.

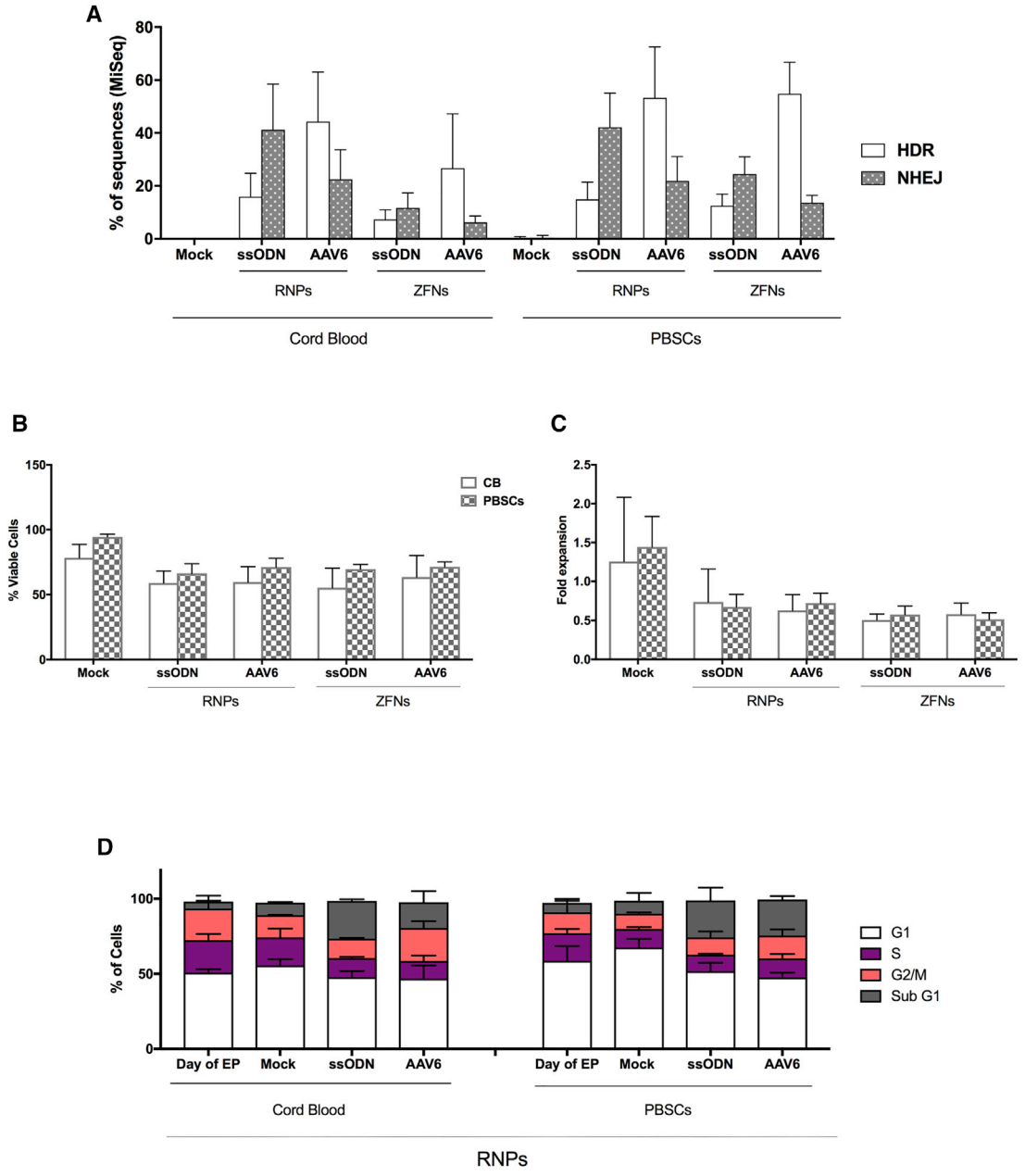

Figure 2. Direct Comparison of Beta-globin Gene Editing with ZFNs and CRISPR/Cas9 Using ssODN or AAV6 as the DNA Donor Template, in CB and PBSC CD34+ Cells

CD34+ cells were pre-stimulated in X-Vivo15 with cytokines (SCF, Flt3-L, and TPO, all at 50 ng/mL) for 48 h before being electroporated with ZFN mRNA (10 μg/mL) or CRISPR/Cas9 RNPs (gRNA 9 μg and Cas9 protein 200 pmol) and the corresponding DNA donor template as indicated. All donors contained the sickle mutation as a reverse model for gene transversion. Mock cells were suspended in electroporation buffer for the same time duration that electroporated cells were, but DNA was not added and the mock cells were not electroporated. (A) The percentages of gene-editing outcomes (HDR and NHEJ) were measured by HTS of *HBB* 4 days post-EP. Samples were counted by trypan blue exclusion 24 h post-EP to evaluate (B) viability and (C) fold expansion (mock, n = 6; CRISPR, n = 9; and ZFN, n = 4 for both CB and PBSCs, from 5 independent experiments). (D) Cell cycle analysis of CB and PBSCs by staining with Hoechst 33342 dye and flow cytometric analysis on the day of the EP (untreated mock samples, n = 2 for both CD34+ sources) and 24 h post-EP for untreated mock samples (n = 2 for both CD34+ sources) and samples treated with CRISPR/Cas9 RNP and the corresponding DNA donor templates as indicated (CB, ssODN n = 2 and AAV6 n = 3; PBSCs, n = 2 for all the treatments). Error bars, mean ± SD.

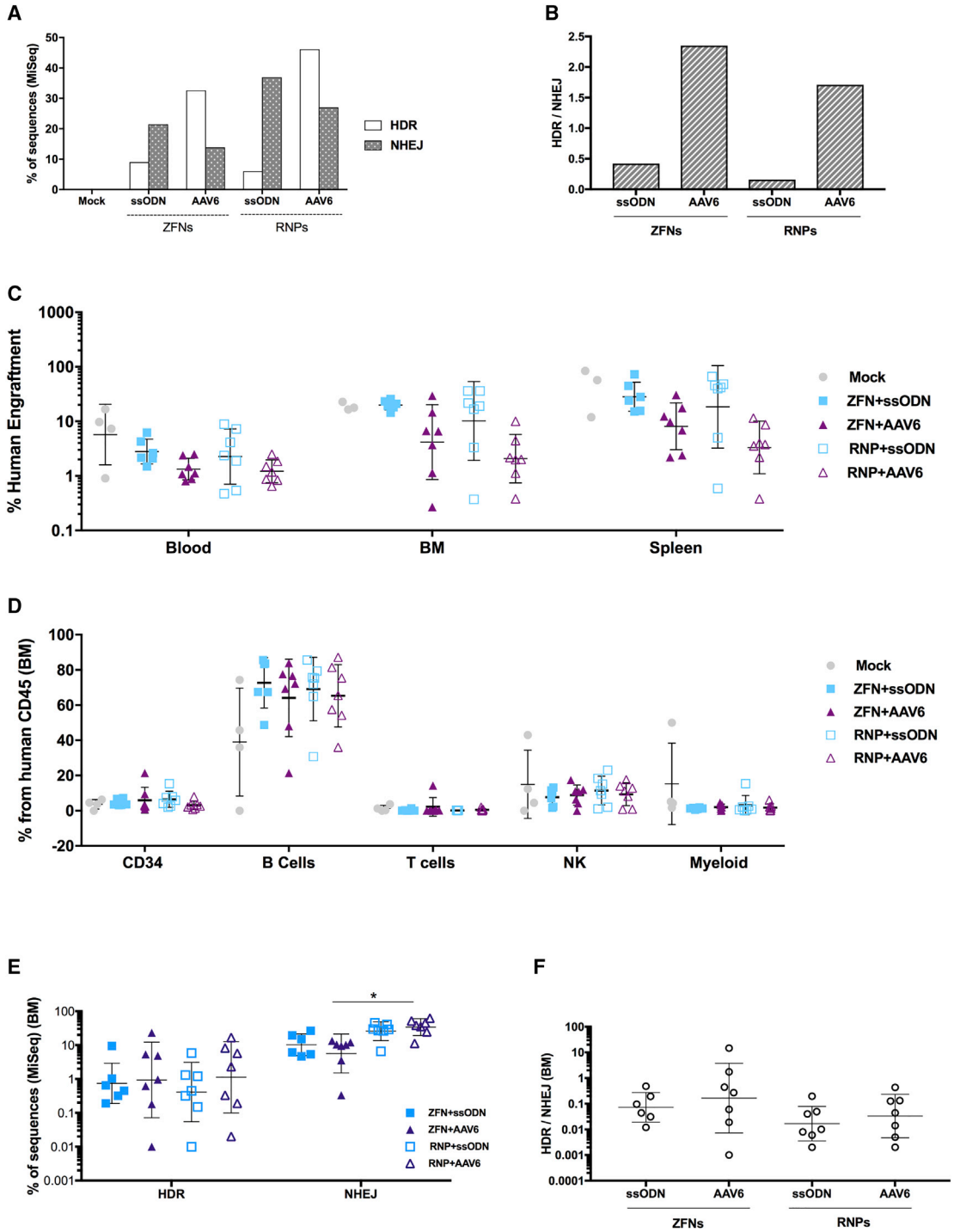

Figure 3. *In Vivo* Comparison of ZFNs and CRISPRs Using ssODN or AAV6 as the DNA Donor Template in PBSC CD34+ cells

(A) Gene-editing rates (HDR and NHEJ) of bulk-transplanted cells kept *in vitro* for 4 days were measured by HTS. (B) HDR:NHEJ ratio from (A). (C) Human engraftment in peripheral blood, bone marrow (BM), and spleen 4 months post-transplant. Human engraftment was assessed by flow cytometry as the percentage of human CD45-positive cells normalized to the percentage of cells positive for human CD45 plus the percentage of cells positive for murine CD45. Error bars, geometric mean ± geometric SD. For this graph, two values (mock BM and mock spleen) were zero or negative and not plotted, since the axis is in logarithmic scale and only values greater than zero can be plotted. Note that the geometric mean is not defined for any value equal to zero or negative. Differences between experimental arms are not significant, based on the Wilcoxon rank-sum test. (D) Immunophenotypic analysis of peripheral blood at 4 months post-transplant by flow cytometry. Percentages of hematopoietic stem cells (CD34), B cells

(legend continued on next page)

In Vivo Assessment of CD34+ PBSCs Treated with ZFNs or CRISPR/Cas9 and the ssODN or AAV6 DNA Donor Template

The prior *in vitro* studies were done at the scale of 2×10^5 cells per electroporated sample for *in vitro* analyses (DNA sequencing of edits, measurements of cell numbers and viability, cell cycle status, etc.). To analyze the edited cells *in vivo* in murine xenografts models that more rigorously assess long-lived stem/progenitor cells, larger numbers of cells were required, on the order of $2–20 \times 10^6$ cells per sample, to divide among the murine recipients. Based on preliminary studies (Figures S11–S14), the EP conditions for editing cells for the xenograft studies were scaled up, maintaining the same concentrations of endonuclease and ssODN and the same AAV6 MOI, adjusted for the increased cell number.

In vivo experiments in NOD *scid* gamma (NSG) mice were carried out to compare engraftment capacity and gene transversion frequency of CD34+ PBSCs treated with ZFNs (mRNA) or CRISPR/Cas9 (RNPs) along with a DNA donor template delivered as an ssODN or as AAV6. At 24 h post-EP, small fractions of the edited cells for the transplants were retained for *in vitro* analyses. The remainders of these edited cell samples were used for transplantation of NSG mice, typically at 1×10^6 cells/mouse.

As previously observed, the *in vitro* viability was higher in the samples treated with the AAV6 (60%–65%) compared to the ones treated with ssODN (~45%) (Figures S15A and S15B). Sequencing of the *HBB* gene showed the same trends as previously observed: the AAV6-treated samples resulted in 3- (ZFNs) to 7- (RNPs) fold greater frequencies of gene transversion compared to the ssODN donor (Figure 3A). Moreover, the HDR:NHEJ ratio was approximately 2.0 in both AAV6-treated groups, while it was below 0.5 for the ssODN-treated samples, independent of the endonuclease used (Figure 3B).

At 2 months post-transplant, peripheral blood samples from the mice showed comparable levels of engrafted human CD45+ cells among the mice receiving mock-treated cells or ssODN and endonuclease-treated cells, while the mice receiving AAV6 and endonuclease-treated cells exhibited at least 3-fold lower engraftment of human CD45+ cells, independent of the endonuclease used (Figure S15C; Table S2A). Similar trends were observed in peripheral blood 4 months after transplant, with overall lower engraftment in recipients of AAV6-treated cells (Figure 3C; Table S2A). At necropsy 4 months after transplant, the average engraftment in BM for the mock- and ssODN-treated cells was comparable, 2-fold greater than the engraftment of the AAV6-treated cells when using ZFNs, and 6-fold greater than when using RNPs (Figure 3C; Table S2A). While the overall engraftment percentage in spleen samples was higher than in BM, the same pattern was observed: for the mock- and ssODN-treated samples, engraftment was ~3-fold higher than the engraftment of the AAV6-treated cells when using ZFNs and 7-fold greater than when using RNPs (Figure 3C; Table S2A). Despite these differences in engraftment between the ssODN and AAV6 treatments, the differentiation capacities of the edited cells measured by immunophenotypic markers of the human cells in peripheral blood (Figure S15D) and in BM (Figure 3D) were similar among the five treatment groups measured at 4 months post-transplant. The main component of the human CD45+ cells present in all recipients was CD19+ B cells, as expected for this mouse model. Thus, this *in vivo* experiment demonstrated detrimental effects of the AAV6 donor on engraftment compared to the ssODN-treated group or mock-treated samples.

HBB gene sequence analysis was performed with the BM samples from the NSG mice after 4 months. Unexpectedly, no significant differences in gene transversion frequencies were shown among the four treatment groups, despite the differences observed in the input cells tested *in vitro*. Marked decreases of the HDR levels were detected in the engrafted human cells across all of the treatment groups with respect to the editing frequencies of the input cells, approximately 5-fold when using ssODN and 7- to 10-fold when using AAV6 (Figures 3A and 3E; Table S2B).

Although the frequency of NHEJ also decreased *in vivo* for all the treatments, the magnitudes of the decreases in NHEJ were less than those for HDR. The decrease in NHEJ measured *in vivo* was greater in the ZFN group (~1.7 times lower than *in vitro* for both DNA donors) than in the RNP-treated samples (1.3 to 0.7 lower than *in vitro* for the ssODN and AAV6, respectively) (Table S2B). Therefore, differences in allelic disruption frequencies were observed between the two endonucleases when the same DNA donor template was used, with a greater degree of allelic disruption maintained in the RNP-treated samples compared to ZFNs, although these differences were significant only when the AAV6 DNA donor template was used ($p = 0.02$) (Figure 3E; Table S2B).

Spleens showed a greater loss of HDR *in vivo* than BM for both DNA donors when using ZFNs, while the decrease of HDR for the RNP-treated samples had a pattern more similar to that in BM, independently of the DNA donor template. As for allelic disruption, both tissues displayed a comparable pattern, with spleens showing a significantly greater degree of allelic disruption maintained in the RNP-treated samples for both donor templates (Figure S15E; Table S2B). Overall, the absolute frequencies of HDR measured *in vivo* were 3- to 10-fold lower than those measured *in vitro*, and HDR:NHEJ ratios

(CD19), T cells (CD3), natural killer (NK) cells (CD56), and myeloid cells (CD33) from the total human CD45+ cells are shown. Error bars, mean ± SD. (E) Gene correction rates (HDR and NHEJ) of the human engrafted cells in BM at 4 months post-transplant measured by HTS. Error bars, geometric mean ± geometric SD. Differences are not significant if not specified; *$p < 0.05$ based on the Wilcoxon rank-sum test. (F) HDR:NHEJ ratio from (E). Error bars, geometric mean ± geometric SD. Differences between experimental arms are not significant, based on the Wilcoxon rank-sum test. All female mice: mock, n = 4; ZFN + ssODN, n = 6; ZFN + AAV6, n = 7; RNP + ssODN, n = 7; and RNP + AAV6, n = 7. Mock cells were suspended in electroporation buffer for the same time duration that electroporated cells were, but DNA was not added and the mock cells were not electroporated.

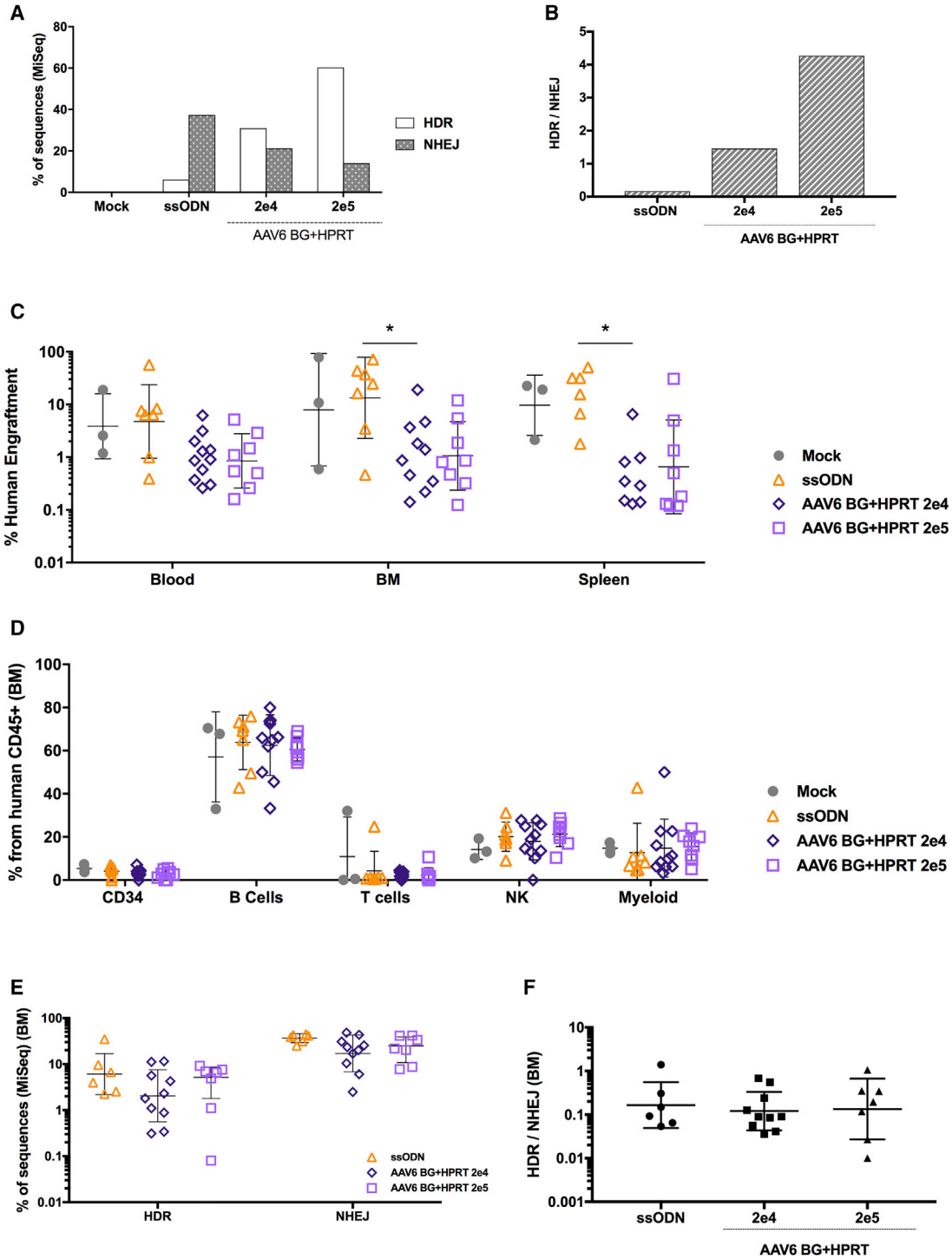

Figure 4. *In Vivo* Comparison of ssODN and AAV6 as the DNA Donor Template Using CRISPR/Cas9 in PBSC CD34+ Cells

(A) Gene-editing rates (HDR and NHEJ) of bulk-transplanted cells kept *in vitro* for 4 days were measured by HTS. (B) HDR:NHEJ ratio from (A). (C) Human engraftment in peripheral blood, bone marrow (BM), and spleen 5 months post-transplant. Human engraftment was assessed by flow cytometry as the percentage of human CD45-positive cells normalized to the percentage of cells positive for human CD45, plus the percentage of cells positive for murine CD45. Error bars, geometric mean ± geometric SD. For this graph, 8 values (ssODN, one for spleen; AAV6_2e4, one for blood, two for BM, and four for spleen) were zero or negative and not plotted, since the axis is in logarithmic scale and only values greater than zero can be plotted. Note that the geometric mean is not defined for any value equal to zero or negative. Differences are not significant if not

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were decreased at least to 3-fold for all the treatments compared to the *in vitro* samples (Figures 3F and S15F compared to Figure 3B).

This first *in vivo* study showed no differences in engraftment, lineage distribution, and gene editing based on which of the two endonucleases was used. In addition, the pair of ZFNs used for these comparisons has been shown to have off-target cleavage at the *HBD* gene,[3] resulting in deletions, inversions, and translocations among *HBB* and *HBD*.[31] Therefore, due to the difficulty of engineering ZFNs and the proprietary nature of the technology, investigations were continued to assess the most effective and safe way to deliver the DNA donor template in the context of CRISPR/Cas9, instead of seeking a more specific pair of ZFNs.

Several factors may play a role in cell toxicity after AAV6 transduction, such as sub-optimal titers, viral preparation quality (based on differing purification methods by the core or company producing the viral preparations), and intrinsic toxicity of the virus itself. Titers of the viral preparations used for these studies were high enough that viral amounts used were not more than 0.2% of the total culture volume. However, the size of the DNA donor insert (1.4 kb, including the inverted terminal repeats [ITRs]) was below the optimal AAV-packing capacity (~4.7 kb), which could result in higher rates of empty particles, leading to a greater toxicity for the CD34+ cells. To avoid the insert size problem, a 2.7-kb fragment of intron I of the human *HPRT* gene was included as stuffer in the AAV vector backbone, upstream of the 1.1-kb *HBB* insert. The new AAV6 donor (AAV6 BG + HPRT) was packaged and titrated at Virovek and tested in CD34+ cells (Figure S16) before subsequent comparison to the previously used AAV6 donor (hereafter referred to as AAV6 BG), which was packaged and titrated at the UNC Vector Core.

In Vivo Comparisons of ssODN or AAV6 BG + HPRT as the DNA Donor Template Using CRISPR/Cas9 in CD34+ PBSCs

Following the same conditions described in Figure 3, *in vivo* experiments in NSG mice were carried out to compare engraftment capacity and gene transversion rates of CD34+ PBSCs treated with CRISPR/Cas9 (RNPs) along with the previously tested ssODN or two different doses of the AAV6 BG + HPRT donor. As observed in the previous transplants, the viability of the cells to be transplanted 24 h post-EP was lower for the ssODN-treated group (~55%) than for the AAV6 BG + HPRT donor-treated group (~80% at MOI 2e4 and ~75% at MOI 2e5), compared to 95% in mock-treated cells (Figures S17A and S17B). Analysis of gene editing using Cas9 RNPs in the input cells showed higher frequencies of HDR using the AAV6 donors (31% HDR at MOI 2e4 and 60.3% at MOI 2e5) than with the ssODN donor (6.2%), as observed in previous experiments. Frequencies of NHEJ-mediated edits were inversely related to HDR-mediated edits (Figures 4A and 4B).

Engraftment of human cells at 2 months post-transplant assessed by flow cytometry in peripheral blood was comparable between mock- and RNP + ssODN-treated cells, but it was significantly reduced for RNP + AAV6 BG-HPRT-treated cells at the two MOIs used when compared to the RNP + ssODN-treated group (Figure S17C; Table S3A). Similar engraftment patterns were observed in all the tissues analyzed (peripheral blood, BM, and spleen) when the mice were euthanized 5 months post-transplant (Figure 4C; Table S3A). Engraftment of the ssODN-treated cells in blood and BM was similar to the engraftment by mock-treated cells and 10-fold higher compared to the AAV6-treated cells, at both MOIs (but only statistically significant when compared to the AAV6 at MOI 2e4 in BM). Average engraftment in the spleens of the ssODN-treated mice was ~5- to 25-fold higher than in the AAV6-treated mice (significant at a MOI 2e4 [*$p < 0.05$]). Despite the disparity in engraftment based on the DNA donor template used (AAV6 versus ssODN), no differences in lineage distribution were observed in blood (Figure S17D) or BM (Figure 4D) at 5 months post-transplant among all of the treatments and the mock group.

In vivo gene transversion and allelic disruption of the RNP + ssODN-treated mice, analyzed in BM and spleen samples (Figure 4E; Figure S17E; Table S3B) were comparable to the *in vitro* editing rates of ~10% (Figure 4A). In contrast, for the RNP + AAV6 BG + HPRT-treated mice, gene transversion in BM decreased at least one log with respect to the *in vitro* values (from ~30% and 60% to 4% and 5% for MOIs 2e4 and 2e5, respectively) (Figure 4E; Table S3B). Following a similar trend, gene transversion in the spleens decreased 13- to 20-fold in the AAV6-treated groups compared to the *in vitro* cells (Figure S17E; Table S3B). On the contrary, NHEJ frequencies were similar *in vivo* as *in vitro* in BM (Figure 4F; Table S3B) and spleens (Figure S17F; Table S3B).

Characterization of the Hematopoietic Potential of CD34+ PBSCs Treated with CRISPR/Cas9 and ssODN or AAV6 as the DNA Donor Template

A surrogate way to assess the clonogenic and engraftment potential of the treated human CD34+ cells is to perform CFU assays for myeloid-erythroid progenitors. To compare the effect on the hematopoietic potential of cells from the different reagents involved in editing, CFU

specified; *$p < 0.05$, based on the Wilcoxon rank-sum test. (D) Immunophenotypic analysis of peripheral blood at 5 months post-transplant by flow cytometry. Percentages of hematopoietic stem cells (CD34), B cells (CD19), T cells (CD3), NK cells (CD56), and myeloid cells (CD33) from the total human CD45+ cells are shown. Error bars, mean ± SD. (E) Gene-editing rates (HDR and NHEJ) of the human engrafted cells in BM at 5 months post-transplant. Error bars, geometric mean ± geometric SD. Differences between experimental arms are not significant, based on the Wilcoxon rank-sum test. (F) HDR:NHEJ ratio from (E). Error bars, geometric mean ± geometric SD. Differences between experimental arms are not significant, based on the Wilcoxon rank-sum test. Mice transplanted: mock, n = 3 (1 male mouse); RNP + ssODN, n = 7 (1 male mouse); RNP + AAV6 2e4, n = 12 (5 male mice); and RNP + AAV6 2e5, n = 8 (2 male mice). Mice having low numbers of reads in the HTS or with no detectable engraftment but showing background reads in the HTS were excluded from the analysis for (E) and (F) (ssODN, 1 mouse; AAV6_2e4, 2 mice; and AAV6_2e5, 1 mouse). Mock cells were suspended in electroporation buffer for the same time duration that electroporated cells were, but DNA was not added and the mock cells were not electroporated.

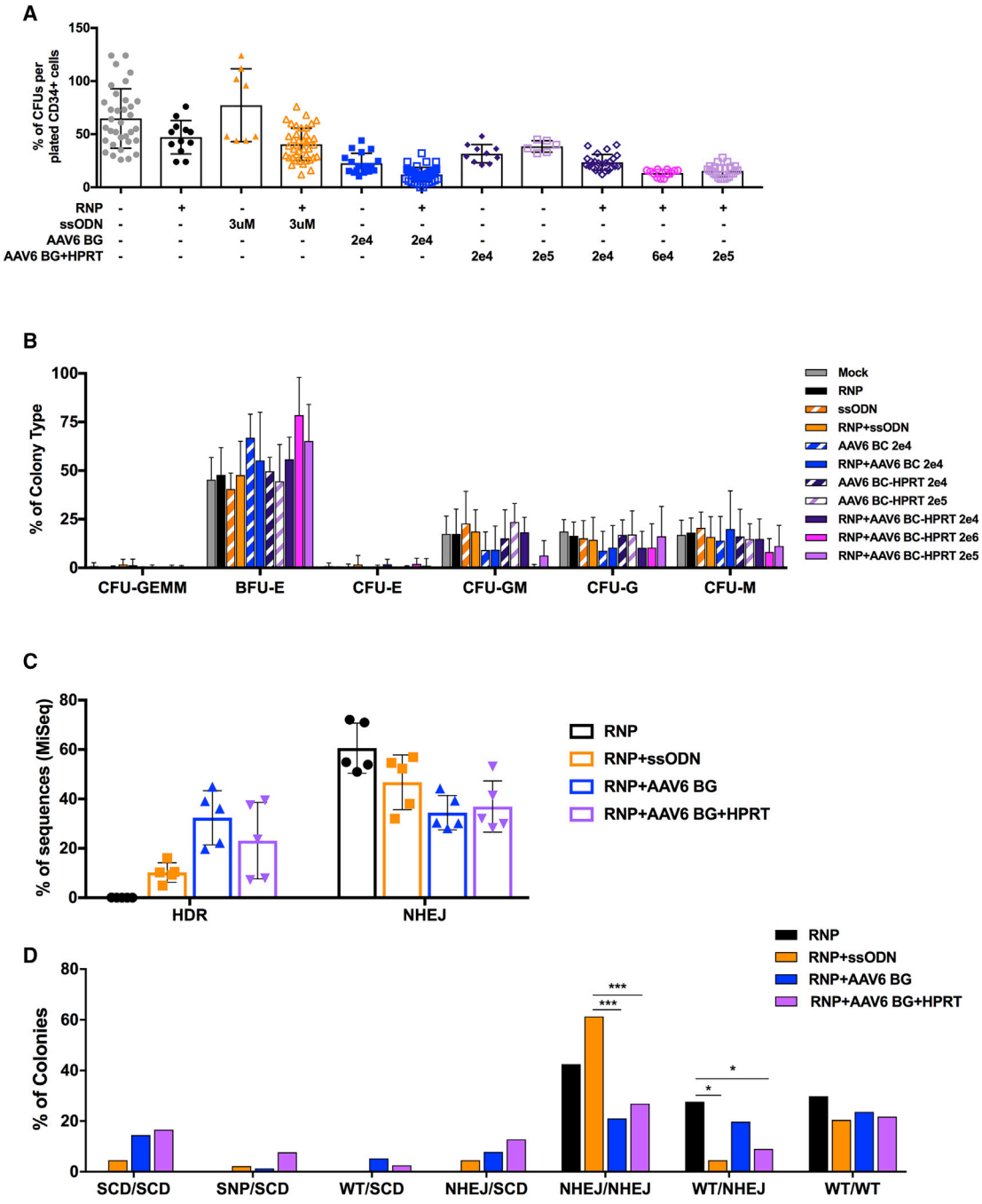

Figure 5. Hematopoietic Potential of PBSC CD34+ Cells after Treatment with CRISPR/Cas9 and ssODN or AAV6 as the DNA Donor Template

(A) At 24 h post-EP, PBSCs were harvested, counted by trypan blue exclusion, and plated in methylcellulose to perform the colony-forming unit (CFU) assay. At 12–14 days after plating the cells in methylcellulose, colonies were enumerated. For each plate, the percentage of cells growing was obtained as the total number of colonies counted normalized to the number of cells plated per dish. Each dot in the graph represents the percentage of cells growing per dish analyzed, for each condition. (B) At 12–14 days after plating the cells in methylcellulose, the different colony types were identified. For (A) and (B), n = 6 independent experiments from five different PBSC donors. Error bars, mean ± SD. (C) Percentage of gene editing (HDR and NHEJ) measured by HTS 4 days post-EP in the bulk population from samples from two of the 6 independent experiments from (A) and (B) (two different PBSC donors). Error bars, mean ± SD. (D) Genomic DNA was extracted from colonies from two (same ones as in C) of the 6 independent experiments from (A) and (B) (two different PBSC donors), and it was sequenced by HTS to determine the frequency of the different possible genotypes shown in the y axes. Differences are not significant if not specified; *$p < 0.05$, ***$p \leq 0.001$, Fisher's exact test; p value adjustment for multiple comparisons. Number of colonies analyzed: RNP, n = 47; RNP + ssODN, n = 44; RNP + AAV6 BC, MOI 2e4 and n = 76; and RNP + AAV6 BC + HPRT, MOIs 2e4 and 2e5 and n = 78. SCD refers to the presence

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assays were performed on CD34+ PBSCs 24 h post-editing with the RNPs only, donor templates only, or RNPs + different donor template combinations. The results from multiple experiments using PBSCs from different healthy donors were compiled. Notably, all treatment groups using only AAV6 vector had a 2- to 3-fold reduction in numbers of colonies formed with respect to the mock, which was worsened with the inclusion of RNPs (***$p < 0.001$, Wilcoxon rank-sum test) (Figure 5A). EP of the CD34+ cells with only the ssODN did not have an effect on the colony formation capacity, whereas EP with only the RNPs or the RNP + ssODN resulted in an ~1.5-fold decreased colony formation capacity with respect to the mock-treated cells (mock versus RNP + ssODN, *$p < 0.05$, Wilcoxon rank-sum test). Regardless of the differences in the colony formation capacity based on different EP reagents, the colony types grown were not affected by any of the treatments (Figure 5B). These results recapitulated the detrimental effect of the AAV6 donor on engraftment capacity of the HSPCs observed in the *in vivo* experiments.

Molecular analyses of editing outcomes in the bulk cell population (from two of the six independent experiments completed) and single colonies were performed for the following treatments: RNP only, RNP + ssODN, RNP + AAV6 BG, and RNP + AAV6 BG + HPRT. As seen before, the use of the AAV6 donors with RNPs led to higher frequencies of HDR sequences, compared to the ssODN donor with RNPs *in vitro* (Figure 5C). Genotyping of individual colonies showed that transversion of the sickle mutation to homozygosity (SCD/SCD) was higher in the RNP + AAV6-treated samples than in the RNP + ssODN group, but not statistically significantly. Transversion of the sickle mutation to heterozygosity (SNP/SCD, wild-type [WT]/SCD, and NHEJ/SCD) was also more frequent in the RNP + AAV6-treated groups than in the RNP + ssODN group (Figure 5D). On the other hand, disruption of both alleles to homozygosity (NHEJ/NHEJ) was the most frequent event observed for the RNP + ssODN treatment, and it was significantly higher than in the RNP + AAV6 treatments (***$p \leq 0.001$, Fisher's exact test) or the RNP-only-treated colonies (not significant); while allelic disruption to heterozygosity (WT/NHEJ) was significantly low in the RNP + ssODN colonies with respect to the RNP-only treatment (*$p < 0.05$, Fisher's exact test), but not with respect to the RNP plus any of the AAV6 donors. These observations correlate with the HDR and NHEJ frequencies observed in all the treatments in the bulk population *in vitro*.

Identification of the Effects of the Reagents Involved in Editing on Gene Expression by RNA-Seq

Next, we sought to assess the effects of the reagents involved in editing on gene expression profiles of CD34+ PBSCs. We performed RNA-seq of total RNA isolated from cells 24 h post-editing. Data were generated from three independent biological replicates for each condition, and they were used to quantify expression of all annotated genes (see the Materials and Methods).

Principal-component analysis (PCA) revealed substantial changes in overall gene expression profiles as a result of the majority of the treatments, and replicates for each condition clustered together (Figure 6A). Samples treated with the RNPs, either alone or in combination with the different DNA donor templates, were separated from all other samples along PC1. Samples treated with either of the AAV6 vectors (AAV6 BG or AAV6 BG + HPRT) were separated from all other samples along PC2. Samples treated with a combination of RNPs and either of the AAV6 vectors were separated from mock-treated cells along both PC1 and PC2. At the same time, samples treated with ssODN only or the RNP + ssODN were clustered close to mock-treated and RNP-only samples, respectively, indicating minimal effects of ssODN on gene expression. Overall, the results of PCA suggest strong and independent effects of RNP and AAV6 treatments on gene expression by the edited CD34+ cells.

To further characterize changes in gene expression, we performed pairwise comparisons between mock and each of the single and double treatments (Figure 6B). Differentially expressed genes (DEGs) were identified at a false discovery rate (FDR) of 1% and minimum fold change of 2 (Tables S4 and S5). The smallest number of DEGs was detected for the ssODN-only treatment, and the largest number of DEGs was detected for the RNP + AAV6 double treatments. Expression patterns of the top 20 DEGs from each comparison are shown in Figure 6C. Many of the top DEGs were common among different comparisons (i.e., top 20 DEGs from seven comparisons in total comprised 60 genes). Virtually all top DEGs were upregulated in samples treated with the EP reagents compared to mock samples. In agreement with the results of PCA, distinct groups of genes were upregulated in response to RNP and AAV6 treatments (most of the top DEGs were common between AAV6BG and AAV6BG + HPRT treatments). The same groups of genes were upregulated in corresponding double treatments, e.g., most of the top DEGs detected in RNP-only and AAV6 BG-only samples were also upregulated in RNP + AAV6 BG double treatment samples.

Manual inspection of the top DEGs revealed that many of them represented genes involved in cell communication and responses to various stimuli, including responses to stress and immune response (Figure 6D). Notably, many DEGs specifically upregulated in response to RNPs were also associated with responses to viruses; the use of *in vitro*-transcribed gRNAs may have played a role in eliciting the interferon response signature seen. Another prominent category of top DEGs included genes associated with cell proliferation and apoptotic processes, with the number of DEGs associated with these two categories being two times greater in the AAV6-treated

of the introduced thymine causing sickle cell disease; SNP refers to the presence of either or both of the polymorphisms of the donor creating an *HhaI* restriction site or the PAM site mutation; WT refers to the adenine base at the sickle mutation site, indicating no change for the sequence present in the healthy donor cells; NHEJ indicates an indel was detected (see also Figure S1). Mock cells were suspended in electroporation buffer for the same time duration that electroporated cells were, but DNA was not added and the mock cells were not electroporated.

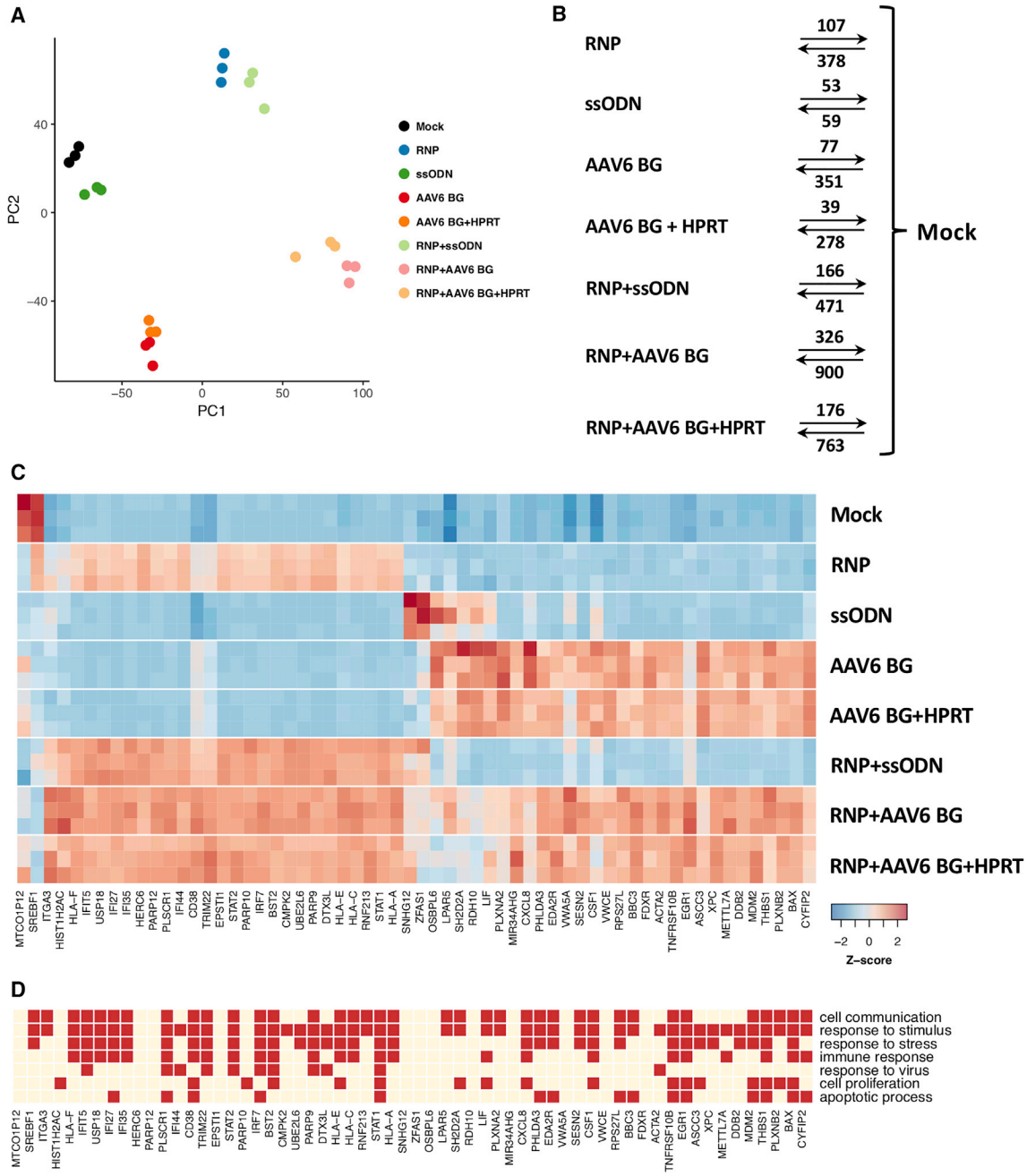

Figure 6. Transcriptional Effects of Different Reagents Involved in Editing

(A) Principal-component analysis (PCA) plot based on expression patterns of all genes. PC1 separates all RNP samples (both in single and double treatments) from other samples, including control samples (mock). Similarly, PC2 separates all AAV6 samples from other samples. (B) Summary of differential expression analysis. Each treatment was compared to mock. Arrows and numbers indicate direction and number of upregulated genes in each comparison, e.g., 107 genes were upregulated in mock compared to RNPs, and 378 genes were upregulated in RNPs compared to mock. (C) Expression patterns of top 20 differentially expressed genes from all pairwise comparisons. Values are shown in scaled expression values (*Z* scores), as in scale. Three biological replicates are shown for each condition in separate rows. (D) Selected gene ontology categories (GO terms) for the same set of genes as in (C). Associations with particular GO terms are indicated in red. Complete lists of identified DEGs and enriched GO terms are provided in Tables S4, S5, and S6.

groups than in the RNP-treated groups. For example, we observed a more than 10-fold increased expression of *CDKN1A* encoding $p21^{CIP1}$ in all samples exposed to AAV, which may be responsible in part for the decreased colony formation and engraftment, as recently observed by Schiroli et al.[32] (To assess the effects of editing on apoptosis by another method of analysis, Annexin V/DAPI staining was performed on the all the reagents involved in the EP as an additional analysis for apoptosis; Figure S18.) In addition, we performed systematic gene ontology (GO) analysis to identify biological processes associated with sets of identified DEGs (Table S6)

DISCUSSION

In the last decade, great improvements have been achieved in gene-editing technology, and site-specific correction of autologous HSPCs to treat monogenic blood cell diseases has been broadly demonstrated *in vitro* and *in vivo*.[2–6,17,33] Common current approaches use CRISPR/Cas9 (or other site-specific endonucleases, e.g., ZFNs, TALENs, etc.) to initiate HDR at a disease-related locus in HSPCs with a homologous sequence donor, either as ssODN or carried by an AAV6 vector, to provide the gene-editing template. Frequencies of HDR-mediated gene editing that have been attained in the studies of *IL2Rγ*, *HBB*, *CYBB*, and *CD154* editing using long-term xenograft HSC assays in NSG mice have ranged from 5%–15%. These activity levels may be sufficient for disease remediation in some gene therapy applications, but they are likely at the lower end of the therapeutic threshold.

Moreover, challenging limitations still remain to be solved for the most effective translation to the clinic. Ideal gene-editing strategies would make universal gene correction in 100% of the treated HSCs, with no disruptive indels—on-target or off-target—with absence of cytotoxicity or other deleterious effects on HSC engraftment or function. While relatively high frequencies of gene correction by HDR can be achieved in hematopoietic progenitor cells analyzed *in vitro*, gene correction in long-term engrafting HSCs *in vivo* is often less efficient, disruptive indels may be relatively more abundant, and the editing manipulations may lead to some loss of HSC activity.

To understand factors governing gene-editing efficiency and toxicity in HSCs, we analyzed critical elements of gene-editing protocols to transvert the sickle mutation, including different HSPC sources (CB versus G-CSF-mobilized PBSCs), different site-specific endonucleases (ZFNs versus CRISPR/Cas9), and various methods to deliver homologous donor sequences (ssODN, AAV6, IDLV, and Ad5/35). Similar results were achieved in CD34+ cells from either CB or PBSCs. There were not significant differences in the effectiveness of HDR at the sickle cell mutation by either ZFNs, delivered as *in vitro*-transcribed (IVT) mRNA, or by CRISPR/Cas9, delivered as IVT mRNA or as pre-formed RNP complexes. The IDLV and Ad5/35 vectors that we evaluated supported minimal levels of HDR gene editing (~2%–5% and 2%, respectively), and they were not examined beyond initial studies.

The major differences observed related to the different editing outcomes and effects on cell function from the two donors analyzed extensively: ssODN and AAV6. In edited CD34+ cells analyzed after short-term culture, AAV6-mediated homologous donor delivery strikingly led to the highest frequencies of site-specific gene transversion of the sickle cell mutation in *HBB*. AAV6 donors showed a dose-dependent increase of HDR to frequencies as high as 50%–60%. This high activity of AAV6 donors is in accord with prior reports from other investigators.[5,11] The ssODN donor led to lower frequencies of HDR than with AAV6 donors, consistently in the range of 5%–15%. The use of the ssODN donor generally allowed higher frequencies of indels (20%–40%) than with AAV6 donors (10%–20%), resulting in a significantly worse ratio of favorable HDR-mediated edits to unfavorable NHEJ-mediated edits.

HTS analysis in single clones showed that the NHEJ/NHEJ genotype was the most common event in the RNP + ssODN group (significantly higher than in the RNP + AAV6 treatments), suggesting that, in the bulk population, some fraction of the alleles that indicate NHEJ are bi-allelic disrupted. It remains unknown what the biological consequences of this will be after transplant of a mixed edited population of cells; presumably, bi-allelic disruption of the *HBB* gene will lead to some degree of ineffective erythropoiesis, as in beta-thalassemia.

The editing outcomes differed when analyzed using short-term *in vitro* culture assay compared to using long-term *in vivo* analyses of reconstituting HSPCs in NSG mice xenografts. Surprisingly, when gene-editing assessments were made *in vivo* in the long-term HSPCs engrafted in NSG mice, the frequencies of both HDR and NHEJ were quite similar using either an AAV6 or ssODN donor. The considerably higher levels of HDR from AAV6 donors observed *in vitro* did not carry over to the cells engrafted in the mice. This suggests that the levels of gene editing *in vivo* may be limited by the capacity of long-term reconstituting HSCs to mediate HDR,[13] resulting in similar levels of engrafted edited HSCs in mice independently of the nuclease or DNA donor type used. We have previously shown that editing of the *HBB* gene in human CD34+ cells, using the same sgRNA and AAV-mediated donors, was significantly reduced in immunophenotypic HSCs and multi-potent progenitors sorted from the bulk CD34+ cells after editing, supporting our observations here about the lower *in vivo* frequency of HDR-mediated edits.[13]

In terms of effects on HSPCs survival and function, the AAV6 vectors caused minimal acute cytotoxicity, based on cell counts and viability measurements 24 h post-EP. The ssODN led to larger decrements to viable cell numbers 24 h after EP. (Our prior efforts to ameliorate the acute cytotoxicity from ssODN donors using three phosphorothioate-modified bases at either the 5′ or 3′ or both ends of the ssODN led to higher toxicity at equal ssODN concentrations and similar toxicity if the modified ssODN was titrated down to lower concentrations that led to equivalent frequencies of HDR as the higher amounts of the unmodified ssODN.) Despite the relative non-toxicity of the AAV6 vector when assessed for acute viability, we observed consistent impairment of *in vivo* engraftment of the gene-edited HSPCs when AAV6 vectors were used. Compared to the level of engraftment by

the mock-edited control cells and the endonuclease + ssODN-treated cells, the AAV6-edited cells had 3- to 10-fold lower engraftment in the BM of the NSG mice. This decrease in hematopoietic capacity was observed with multiple lots of AAV6 vector purchased from different commercial sources. We also observed that AAV6 + endonuclease suppressed *in vitro* progenitor-derived colony formation by a similar 4- to 5-fold. None of the editing manipulations led to alterations in the lineage differentiation potential of cells *in vitro* or *in vivo*, with the full expected spectrum of progenitor-derived colony types *in vitro* and blood cell lineages formed in similar ratios to mock-treated cells *in vivo*.

The relative engraftment capacity of edited cells has been rarely compared to non-edited control cells to assess potential impairment, and those studies that did demonstrate losses of engraftment from 2- to 10-fold.[5,17] While impaired engraftment caused by gene-editing manipulations may be overcome in experimental models by increasing the transplanted cell dose, in the clinical situation, the absolute numbers of available HSCs is limited by the efficiencies of mobilization, collection, and enrichment methods. Inclusion of small molecules, such as SR1, UM171, or PGE2,[34–37] may modestly compensate for cell loss during culture, but it does not significantly expand HSC number to overcome engraftment impairment.

RNA-seq analysis performed 24 h post-EP showed that the two main editing reagents triggering upregulation of a larger and distinct sets of genes with respect to the non-treated mock cells were the RNPs (as a single treatment or in combination with either of the donor templates) and the AAV6 (as a single treatment or in combination with the RNPs). In contrast, treatment with ssODN only resulted in only modest changes in gene expression. Many top genes upregulated in response to RNPs and AAV6 were involved in cell responses to various external stimuli and immune response. While the RNPs upregulated the expression of genes involved in response to viruses, the AAV6 upregulated a greater number of genes associated with apoptosis and cell proliferation. Cromer et al.[12] studied the effects of gene editing on HSCs using microarray analysis of gene expression, and they reported minimal perturbations with AAV6 vectors. In contrast, Schiroli et al.[32] used RNA-seq analysis and found significant changes in gene expression of TP53 pathway genes upon exposure of human CD34+ cells to AAV6 vectors, notably, elevations of $p21^{CIP1}$ (encoded by the *CDKN1A* gene) to which they attributed the cell inhibitory effects seen in samples treated with AAV6. The latter results are similar to our findings with AAV6 and with minimal effects on transcript levels for *TP53* but more than 10-fold increases in *CDKN1A* transcripts in all samples exposed to AAV6 (Figure S19; Table S5). The TP53 DNA repair response is post-translational, with modifications (e.g., phosphorylation) of existing p53 protein or changes in upstream or downstream proteins affecting p53 function, without a significant change in its transcription.

From the *in vivo* experiments performed, it was observed that the RNP + ssODN treatment had a less detrimental effect on the human engraftment than any of the RNP + AAV6 treatments. In these *in vivo* experiments, single treatments, as RNP only or donor only, were not performed. However, the CFU assay showed that the AAV6-only treatments resulted in a 2- to 3-fold reduction in the numbers of colonies formed with respect to the mock, while the RNP-only and ssODN-only treatments did not significantly affect the colony formation capacity. Based on this, it could be concluded that the set of genes with differential expression due to the RNP-only treatment might have less severe effects on engraftment; while, in the case of AAV6, it is not clear which of the altered genes may play a role in the impairment of engraftment induced by AAV6 vector exposure.

Another potential difference between the use of oligonucleotides and AAV vectors for homologous donor delivery is their potential for differential frequencies of random integration in the host genome. Double-stranded oligonucleotides are end captured by NHEJ into DSB, the basis for unbiased techniques to detect off-target nuclease cutting such as GUIDE-seq,[38] but the frequency of end capturing ssODN is not known. AAV vectors have some preferred integration sites in the human genome (e.g., the well-known AAVS1 site), but they may also be end captured, as reported.[20] The methods we used to detect HDR start with PCR primers that amplify from outside of the donor sequences for either the oligonucleotide or the AAV, so we should not be falsely detecting randomly integrated donor sequences as an indication of on-target HDR.

Thus, the ideal donor remains elusive. AAV6 vectors caused less acute toxicity than the ssODN donor, but AAV6 diminished the proliferative and/or engraftment capabilities of the treated HSPCs. Use of novel serotypes of AAV that are more efficient at transducing human HPSCs (e.g., AAV-HSC[39]) may support high-frequency HDR with less adverse effects on hematopoiesis. While the IDLV donor showed low donor activity, there was no detectable cytotoxicity. Recent advances in identifying compounds that enhance transduction by lentiviral vectors may allow IDLV donors to reach increased efficiency.[40–43]

Whereas we provided *in vitro* and *in vivo* evaluations of some of the most commonly used endonucleases and homologous donor templates for gene editing, the next steps needed to translate these protocols to the clinic will involve testing reagents more suitable for clinical settings. These may include using chemically synthesized (and base-modified) gRNAs to potentially reduce toxicity[44] and more specific endonucleases, such as the high-fidelity versions of the Cas9 protein,[45] to reduce off-target activity, as well as performing cell processing scale-up involving electroporator systems able to handle larger cell doses.[6] These advances will allow site-specific correction of relevant genes in HSCs causing monogenic disorders to be used as a treatment for inherited disorders of blood cells.

MATERIALS AND METHODS

Briefly and as previously described by Hoban et al.[3] and DeWitt et al.,[4] ssODN homologous donors were designed to be optimized for each endonuclease. The ssODN designed for the ZFNs was a fragment of 100 nt (ordered from Integrated DNA Technologies) in reverse orientation to the direction of transcription with the SNP at the sickle

mutation site centered with respect to the cut site.[3] The ssODN used for CRISPR/Cas9 was 168 nt in length (Integrated DNA Technologies) in the forward orientation and asymmetric with respect to the cut site. This design was based on the DNA strand bound by the gRNA used to target the sickle mutation.[4,46] Both ssODN donors contained only native deoxynucleotides.

Use of umbilical CB collected at normal births was deemed exempt from Institutional Review Board review because it was anonymous medical waste. Mobilized peripheral blood (mPB) was collected from normal, healthy donors on days 5 and 6 after 5 days of stimulation with G-CSF and purchased from HemaCare (HemaCare BioResearch Products, Van Nuys, CA).

Pre-stimulation

Healthy human CD34+ cells from CB or mPB (PBSCs) were thawed in pre-warmed X-VIVO 15 medium (Lonza, Basel, Switzerland) with penicillin, streptomycin, and glutamine (Pen/Strep/Glu; Gemini BioProducts, Sacramento, CA); pelleted at 500 × *g* for 5 min; and resuspended at 5×10^5 cells/mL in pre-warmed X-VIVO 15 medium with Pen/Strep/Glu and cytokines (50 ng/mL stem cell factor [SCF], 50 ng/mL fms-related tyrosine kinase 3 ligand [Flt3-L], and 50 ng/mL thrombopoietin [TPO]; PeproTech, Rocky Hill, NJ). Cells were pre-stimulated at 37°C in a 5% CO_2 incubator for 48 h.

EP

For EP, 2×10^5 cells (or 1×10^6 cells for mice experiments) per condition were pelleted at 90 × *g* for 15 min at room temperature (RT); resuspended in 100 µL BTXpress Electroporation buffer (Harvard Bioscience, Holliston, MA), combined with the indicated amounts of (1) ZFN mRNA and/or oligonucleotide as applicable; (2) Cas9 mRNA and gRNA and/or oligonucleotide as applicable; or (3) Cas9 protein and gRNA and/or oligonucleotide as applicable; and pulsed once at 250 V for 5 ms in the BTX ECM 830 Square Wave Electroporator (Harvard Apparatus). Following EP, cells were rested for 10 min at room temperature and then recovered with 400 µL (or 2.4 mL for 1×10^6 cells) X-VIVO 15 medium with cytokines and containing the corresponding viral vector (IDLV, AAV6, or Ad5/35) when applicable and at the indicated MOI or concentration. Mock cells were suspended in EP buffer for the same time duration that electroporated cells were, but DNA was not added and the mock cells were not electroporated. (Prior EP controls without added editing reagents revealed minimal cytotoxicity and, thus, were not performed further.) After EP, cells were cultured in a 24-well (or 6-well for 1×10^6 cells) plate at 37°C in a 5% CO_2 incubator.

At 24 h post-EP, the cells were harvested and counted by hemocytometer with trypan blue to determine viability (viable cells normalized by the total of number of cells counted) and fold expansion (total number of cells 24 h post- EP normalized to the starting number of cells the day of the EP). Cells were replated into 1 mL (or 5 mL for 1×10^6 cells) myeloid expansion medium (Iscove's modified Dulbecco's medium [IMDM, Thermo Fisher Scientific, Waltham, MA] + 20% FBS [HI fetal bovine serum, Gibco/Thermo Fisher Scientific, Waltham, MA] + 5 ng/mL interleukin-3 [IL-3], 10 ng/mL interleukin 6 [IL-6], and 25 ng/mL SCF [PeproTech, Rocky Hill, NJ]), and they were cultured for 4–5 days (unless otherwise specified) prior to harvesting for gDNA PureLink Genomic DNA Mini Kit (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA).

mRNA and sgRNA *In Vitro* Transcription

To make the mRNA template, T7 expression plasmids were linearized with *SpeI* (New England Biolabs, Ipswitch, MA) and purified using a PCR purification kit (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA), according to the manufacturer's protocol. *In vitro* transcription was performed using mMessage mMachine T7 Ultra Kit (Thermo Fisher Scientific). mRNA product was purified using the RNeasy MinElute Cleanup Kit (QIAGEN, Valencia, CA), following the manufacturer's protocol. The sgRNA template was prepared as previously described (https://www.protocols.io/view/in-vitro-transcription-of-guide-rnas-and-5-triphos-nghdbt6), and RNA was purified using the RNeasy MinElute Cleanup Kit (QIAGEN), following the manufacturer's protocol.

CFU Assay

CFU assays were performed by the manufacturer's protocol using Methocult H4435 Enriched methylcellulose (STEMCELL Technologies, Vancouver, BC, Canada). 5,400 cells from PBSCs were resuspended in IMDM + 2% FBS, and serial dilutions were performed to obtain 900, 300, and 75 cells in a final volume of 600 µL in IMDM + 2% FBS. To achieve a final number of 300, 100, and 25 cells/35-mm plate, 300 µL of each serial dilution was added to a 3-mL methylcellulose aliquot, briefly vortexed, and 1.1 mL methylcellulose was dispensed per 35-mm dish with grid in duplicate per experimental sample. Dishes were incubated at 37°C and 5% CO_2 for 14 ± 2 days and humidified atmosphere. The different types of hematopoietic colonies were identified and scored. Plucked CFUs were incubated at least for 1 h in PBS and genomic DNA was isolated (NucleoSpin Tissue XS, Clontech Laboratories) for HTS analysis.

HTS, Sequencing Analysis, and Calculations

These were performed as described by Lomova et al.[13]

Gene Modification at HhaI by qPCR

This was performed as described by Hoban et al.[3]

Cell Cycle

2–1 × 10^6 cells were harvested per condition, fixed in 1 mL ice-cold 70% EtOH, and kept at −20°C for at least 2 h and up to 2 weeks. Fixed cells were washed two times with PBS + 2% FBS and spun down at 700 × *g* for 10 min after each washing. Cells were stained during 1 h at 37°C with 5 µg/mL Hoechst 33342 (BD Biosciences, San Jose, CA) in PBS + 2% FBS at a final volume of 250 µL to 1 mL, depending on the number of cells.

Annexin V Staining

7×10^4 to 1×10^5 cells were harvested per condition and washed twice with PBS. Cells were resuspended in 100 µL Annexin V Binding

Buffer (BioLegend, San Diego, CA) plus 5 μL allophycocyanin (APC)-Annexin V antibody (BD Biosciences, San Jose, CA) and 1 μg/mL DAPI (Thermo Fisher Scientific, Waltham, MA) incubated for 15 min at room temperature. 200 μL was added per reaction before flow cytometry analysis was performed within the following hour.

Mouse Transplants

All work with mice was done under protocols approved by the UCLA Institutional Animal Care and Use Committee. Mouse work was performed according to the animal research committee (ARC) protocol number 2008-167.

Transplant

For the assessment of human engraftment of gene-edited cells and the levels of gene editing in a long-term HSC population, PBSCs were transplanted into NSG mice (The Jackson Laboratory, Bar Harbor, ME). PBSCs were pre-stimulated and electroporated as previously described using a 1×10^6 cell EP protocol. At 24 h post-EP, the cells were harvested and counted by trypan blue. 5×10^4 to 1×10^5 cells were kept in myeloid culture to determine gene-editing levels *in vitro* prior to transplant. 1–1.3 $\times$ 10^6 cells were resuspended in 103 μL PBS + 1.5 μL OKT3 antibody (BioLegend, San Diego, CA) to be retro-orbitally transplanted per mouse. Transplants were performed 3–5 h after 250-cGy total body irradiation.

Harvest and Analysis

These were performed as in Lomova et al.[13] Mouse harvest, flow cytometry, and sequencing analyses were performed in a blinded manner.

RNA-Seq

1×10^5 cells/condition were resuspended in RLT buffer + beta-mercaptoethanol (β-ME), following the manufacturer's protocol (RNEasy Micro kit, QIAGEN, Venlo, the Netherlands), and snap frozen in LN2 to be stored at −80°C until RNA extraction (RNEasy Micro kit, QIAGEN, Venlo, the Netherlands). RNA-seq libraries were prepared using the Universal Plus mRNA-Seq kit (NuGEN, Redwood City, CA) and sequenced using Illumina HiSeq3000 platform (single-end 50-bp reads). Total numbers of reads varied from 20 to 40 million per sample.

RNA-seq reads were aligned to the human reference genome (hg38) using spliced transcripts alignment to a reference (STAR).[47] Uniquely mapped reads comprised from 84% to 88% of total reads, and they were used to quantify expression levels of all annotated genes (Ensembl version [v.]92). After filtering out lowly expressed genes, raw expression value of 13,703 genes (with at least 50 reads in at least 2 samples) were normalized to counts per million (CPM) values using EdgeR.[48] Scaled CPM values for all expressed genes were used to perform PCA and to visualize gene expression patterns. DEGs were identified using EdgeR-QLF.[48] DEGs were detected at FDR 1% and a minimum fold change cutoff of 2. The sequences for RNA-seq data were deposited to NCBI Gene Expression Omnibus (GEO): GSE131387.

Statistical Analysis

Descriptive statistics such as number of observations, mean, and SD were reported and presented graphically for quantitative measurements. Normality assumption was checked for outcomes before statistical testing. Nonparametric Wilcoxon rank-sum tests were used to compare two experimental groups and conditions for outcome measures such as cell viability and apoptosis; gene-editing rates (HDR and NHEJ); HDR:NHEJ ratios; human engraftment in peripheral blood, BM, and spleen; and hematopoietic potential of CD34+ PBSCs (% CFU/plated cells). Dwass, Steel, Critchlow-Fligner Method[49] was used for p value adjustment due to multiple comparisons. Comparisons of percentages of colonies between different genotypes were performed via Fisher's exact test with Benjamini-Hochberg[50] multiple testing adjustment. 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).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.05.014.

AUTHOR CONTRIBUTIONS

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

CONFLICTS OF INTEREST

The authors declare no competing interests.

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