Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors

Jianbin Wang1,3, Colin M Exline2,3, Joshua J DeClercq1, G Nicholas Llewellyn2, Samuel B Hayward1, Patrick Wai-Lun Li1, David A Shivak1, Richard T Surosky1, Philip D Gregory1, Michael C Holmes1,4 & Paula M Cannon2,4

Genome editing with targeted nucleases and DNA donor templates homologous to the break site has proven challenging in human hematopoietic stem and progenitor cells (HSPCs), and particularly in the most primitive, long-term repopulating cell population. Here we report that combining electroporation of zinc finger nuclease (ZFN) mRNA with donor template delivery by adeno-associated virus (AAV) serotype 6 vectors directs efficient genome editing in HSPCs, achieving site-specific insertion of a GFP cassette at the CCR5 and AAVS1 loci in mobilized peripheral blood CD34+ HSPCs at mean frequencies of 17% and 26%, respectively, and in fetal liver HSPCs at 19% and 43%, respectively. Notably, this approach modified the CD34+CD133+CD90+ cell population, a minor component of CD34+ cells that contains long-term repopulating hematopoietic stem cells (HSCs). Genome-edited HSPCs also engrafted in immune-deficient mice long-term, confirming that HSCs are targeted by this approach.

Our results provide a strategy for more robust application of genome-editing technologies in HSPCs.

Gene therapy using HSPCs is increasingly being applied to treat severe genetic diseases1–4. A patient’s own HSPCs can be genetically modified following a short ex vivo culture in the presence of hematopoietic cytokines, and integrating viral vectors such as lentiviral vectors are often used to confer long-lasting effects. However the semi-random nature of vector insertion can result in non authentic patterns of gene expression, including silencing over time, or harmful insertional mutagenesis events, such as transactivation of neighboring oncogenes5–7. In contrast, genome editing with targeted nucleases—which include ZFNs, transcription activator-like effector nucleases, and the clustered, regularly interspaced, short palindromic repeats-associated protein 9 (CRISPR-Cas9) system—enables gene correction, correction of a gene mutation or insertion of new DNA sequences in a highly regulated manner at preselected target sites. These nucleases act by catalyzing site-specific DNA double-strand breaks (DSBs)8. Repair of DSBs can proceed by nonhomologous end joining (NHEJ) or homology-directed repair (HDR)9–12, and these pathways are exploited to achieve the desired form of genetic modification13. The therapeutic applications of genome editing that are closest to clinical translation are disruption of the HIV-1 coreceptor CCR5 to treat HIV14 and of the γ-globin repressor BCL11A15 as a therapy for β-globinopathies. Both of these programs involve gene knockout, whereas the ability to correct mutations or add DNA sequences would substantially broaden the impact of gene editing technologies.

HDR-mediated genome editing requires the introduction into a cell of both a targeted nuclease and a matched homologous donor DNA repair template. As both components have to be present only transiently to permanently modify a genome, it is possible to deliver them using nonpermanent delivery vehicles, including nucleic acids (plasmid DNA, mRNA and oligonucleotides) and certain viral vectors (integrate-defective lentivirus (IDLV), adenoassociated virus, and AAV). Application of these methods is now quite straightforward for cell lines and a variety of primary cells16–19, but their use in HSPCs is particularly challenging, especially for insertion of a full transgene expression cassette. Initial attempts at editing human CD34+ HSPCs with IDLVs only achieved efficiencies below 0.1% (ref. 20). More recently, combining the introduction of ZFNs as mRNA with IDLV donor templates resulted in the site-specific insertion of GFP cassettes in ~5% of HSPCs, with a further twofold increase possible when the cells were incubated in the presence of dmPGE2 and SR1 (ref. 21). However, analysis of editing rates in the most primitive HSPCs, identified by expression of CD90 (refs. 22,23) or by studies involving transplantation of cells into immune-deficient mice, have highlighted the difficulty of editing the most primitive, long-term repopulating HSCs compared to more differentiated subpopulations that are also present in the bulk CD34+ HSPC population21,24.

In the present study we evaluated the potential of AAV vectors to function as homologous donor templates. By identifying AAV6 as a capsid variant with high tropism for human HSPCs, and combining this method of donor delivery with mRNA delivery of ZFNs, we demonstrated dose-dependent site-specific insertion of small or large gene cassettes at two different endogenous loci. The high levels of genome editing observed in bulk CD34+ HSPC populations were

1Sangamo BioSciences, Inc., Richmond, California, USA. 2Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, California, USA. 3These authors contributed equally to this work. 4These authors jointly directed this work. Correspondence should be addressed to P.M.C. (pcannon@usc.edu) or M.C.H. (mhomes@sangamo.com).

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also maintained in cells with more primitive characteristics, leading to long-term multilineage production of gene-modified cells following transplantation into immune-deficient mice.

**RESULTS**

**Human HSPCs are efficiently transduced by AAV6 vectors**

We first compared the ability of different AAV serotypes to transduce HSPCs. We used AAV vectors expressing GFP reporter genes, and packaged into capsids from serotypes 1, 2, 5, 6, 8 and 9. These were evaluated at a range of doses on CD34+ HSPCs isolated from either mobilized peripheral blood or fetal liver.

For both types of HSPC, AAV6 gave the highest rates of transduction across a range of vector doses (Fig. 1). The next most-efficient serotype was the closely related variant, AAV1. This agrees with prior reports describing the tropism of AAV6 vectors for human HSPCs.

**HDR-mediated editing in HSPCs by ZFN mRNA and AAV6 donors**

We examined the ability of AAV6 vectors to deliver a homologous donor template to mobilized blood CD34+ HSPCs and thereby direct HDR-mediated genome editing in cells also treated with a targeted nuclease (Fig. 2a). We transduced the cells with varying doses of AAV6 vectors, followed by electroporation 16–24 h later with mRNA expressing a previously characterized CCR5 ZFN pair.

Two different homologous donor templates were evaluated as AAV6 vectors, representing both base pair–specific gene editing events (exemplified by the insertion of a restriction site) and insertion of a larger GFP expression cassette. In each case, the AAV6 vectors contained identical homologous CCR5 sequences flanking either an XhoI restriction site (donor CCR5-RFLP) or a PGK-GFP expression cassette (donor CCR5-GFP) (Fig. 2b). These cassettes contain additional sequences between the ZFN binding sites, neither the donor template themselves nor a successfully edited target site would be subject to recutting by the CCR5 ZFNs. The rates of genome editing events were monitored by population deep sequencing and restriction fragment length polymorphism (RFLP) analysis to detect the XhoI insertion, or by flow cytometry and semiquantitative PCR to monitor site-specific GFP addition.

NHEJ and HDR repair are competitive events, and the products of both repair pathways were detected in the treated cell populations by deep sequencing (Fig. 2c and Supplementary Table 1). Increasing the dose of the CCR5-RFLP vector led to an increase in the rate of XhoI insertion at the CCR5 locus, resulting in >20% of alleles being modified (Fig. 2c). This was accompanied by a corresponding decrease in the frequency of the indels characteristic of NHEJ-mediated repair, whereas at the highest AAV6 donor levels, cytotoxicity led to a decrease in both types of genome editing events (Fig. 2c,d). Similarly, increasing levels of stable GFP expression were observed following transduction of the cells with increasing doses of the CCR5-GFP vector, but only in the presence of the CCR5-ZFN mRNA (Fig. 2e–g).

The optimal time for AAV6 transduction relative to ZFN mRNA electroporation was evaluated and found to be between 24 h pre- and 1 h post-electroporation, indicating that this sequential treatment schedule is relatively flexible (Supplementary Fig. 1). The amount of HDR editing at the CCR5 locus achieved under specific conditions also varied between different CD34+ donors (Supplementary Fig. 2).

For example, using AAV6 vector doses of 3,000 vector genomes (vg)/cell, we achieved a mean rate of RFLP insertion in CCR5 of 15.8% (range 8.4–29.2, n = 15 experiments), whereas a dose of 10,000 vg/cell resulted in a mean rate of 20.1% (range 11.4–32.5, n = 11). These levels of HDR-mediated genome editing were also independent of the HSPC cell source because treatment of fetal liver–derived CD34+ cells with the CCR5-GFP vector and CCR5 ZFN mRNA produced similar high levels of stable and site-specific GFP addition (Supplementary Fig. 3).

Programmable site-specific nucleases, including ZFNs, can introduce off-target DNA breaks at sequences with homology to the intended target site, although engineering strategies can minimize such effects. In the absence of extensive homology between an off-target break site and a donor sequence, as is often the case, the most frequent genome editing outcome is NHEJ-mediated repair, which can lead to indels. The off-target profile of the CCR5 ZFNs used in this study has been previously described, and deep sequencing of the top 23 known off-target sites in cells treated with the combination of CCR5 ZFNs and the CCR5-GFP donor gave the expected profile (Supplementary Table 2).

To establish the generality of these results, we performed an analogous set of studies using reagents specific for the AAVS1 ‘safe harbor’ locus. In mobilized blood HSPCs, insertion of a HindIII restriction site occurred, on average, at 28% of the AAVS1 alleles, whereas GFP addition was observed in >30% of the cells (Supplementary Fig. 4). Similar high rates of gene editing at AAVS1 were achieved in fetal liver HSPCs, with stable GFP addition detected in >40% of the cells, without any toxicity (Supplementary Fig. 5). Taken together, these results demonstrate that AAV6 vectors are an effective vehicle for delivering homologous donor DNA templates to CD34+ HSPCs, and that when combined with ZFN mRNA electroporation, the protocol supports both base pair–specific genome editing events and larger gene additions at frequencies in the range of 15–40%.

**AAV6 vectors use HDR pathways to genetically modify HSPCs**

In addition to engaging the cell’s HDR pathways to achieve precise on-target genome editing, AAV genomes can also become inserted at the site of a DSB through NHEJ-mediated end-capture events.
Such events represent on-target gene additions when occurring at the intended nucleasere target site, but are considered off-target when occurring at DSBs generated by either random cellular events or any off-target activity of the nucleasere itself (Supplementary Fig. 6a). To further investigate which mechanism was responsible for the high levels of stable GFP expression observed following the introduction of ZFNs and AAV6 donors into cells, we combined CCR5 ZFN mRNA and AAV6 donor vector electroporation. Cells were collected 3–6 days post-electroporation and analyzed by flow cytometry for % GFP+ results were combined from five experiments using different donors and show mean ± s.d. *P < 0.05, **P < 0.01, unpaired Student’s t-test. (f) Flow cytometry plots from one representative experiment using 3,000 vg/cell CCR5-GFP donor vector, at 6 days post-electroporation. (g) Confirmation of targeted integration of GFP expression cassette at the CCR5 locus by semi-quantitative In-Out PCR, for one representative experiment. Ctrl. is a PCR that serves as a genomic DNA loading control. The % HDR-mediated insertion of GFP was estimated following normalization and by comparison to standards, and numbers are shown for any samples greater than background. Uncropped images of all gels in this figure are available in Supplementary Figure 10.

**Figure 2** Combination of ZFN mRNA and AAV6 vectors promotes high levels of site-specific genome editing at the CCR5 locus in HSPCs. (a) Schematic showing use of AAV vector as a template for HDR for DSBs, as induced by target-specific nucleases. (b) Schematic of AAV vector genomes containing CCR5 homology donors. R and L refer to CCR5 genomic sequences, comprising 1,431 and 473 bp, respectively, and inserted in antisense orientation when compared to the AAV genome20. Vector CCR5-RFLP contains an additional Xho1 restriction site and vector CCR5-GFP contains a promoter GFP cassette with a polyadenylation (pA) sequence. (c) Mobilized blood CD34+ HSPCs were transduced with AAV6 vectors carrying the CCR5-RFLP donor vector at indicated doses for 16 h, then electroporated with CCR5 ZFN mRNA (120 μg/ml). Cells were analyzed 3–5 days post-electroporation by deep sequencing to measure the efficiency of genome modification (% indels and site-specific RFLP insertions). Results from one representative of three experiments using three different HSPC donors are shown. (d) Dose-dependent insertion of Xho1 site at CCR5, confirmed by RFLP analysis. One representative experiment is shown, with % HDR quantitation for any sample greater than background. (e) Mobilized blood HSPCs were treated as described, but using CCR5-GFP donor vectors, with and without CCR5 ZFN mRNA electroporation. Cells were collected 3–6 days post-transduction and analyzed by flow cytometry for % GFP+. Results were combined from five experiments using four different donors and show mean ± s.d. *P < 0.05, **P < 0.01, unpaired Student’s t-test. (f) Flow cytometry plots from one representative experiment using 3,000 vg/cell CCR5-GFP donor vector, at 6 days post-electroporation. (g) Confirmation of targeted integration of GFP expression cassette at the CCR5 locus by semi-quantitative In-Out PCR, for one representative experiment. Ctrl. is a PCR that serves as a genomic DNA loading control. The % HDR-mediated insertion of GFP was estimated following normalization and by comparison to standards, and numbers are shown for any samples greater than background. Uncropped images of all gels in this figure are available in Supplementary Figure 10.

Analysis of HSPCs treated with AAV6 and ZFN mRNA

We examined the ability of mobilized blood HSPCs treated with the CCR5-RFLP vector plus CCR5 ZFN mRNA to proliferate in culture, to differentiate into hematopoietic lineages and to maintain consistent levels of genome-edited cells in the population. When the cells were grown in bulk culture, we observed an initial decrease of 23% in the absolute number of cells present in the population treated with both vector and ZFNs at 1 day post-electroporation, compared to the other
arms of the experiment. However, by 2 days post-electroporation, the rate of growth of these cells had become indistinguishable from a mock-treated population (Fig. 4a). In addition, the frequency of genome-edited events in the population did not vary over 9 days of culturing, with the frequency of site-specific RFLP insertions being 19.56% at day 2 and 19.45% at day 9. Together, these data indicate that although some of the cells in the bulk CD34+ population were sensitive to the AAV6 plus ZFN treatment, the proliferative potential of the surviving cells was not affected, suggesting that any such effects could be compensated for by using higher initial numbers of cells.

We also plated AAV6 and ZFN-treated cells in methylcellulose and analyzed the colonies that formed. Here, we found no difference in the relative percentages of the different colony subtypes that developed in the various treatment arms of the experiment (Fig. 4b). In addition, by picking colonies from the methylcellulose cultures and analyzing their genotype, we confirmed that the levels of genome editing in the myeloid colonies (colony-forming unit granulocyte/macrophage/ granulocyte and macrophage; CFU-G/M/GM) and erythroid colonies (colony-forming unit erythroid and burst-forming unit erythroid; CFU/BFU-E) were indistinguishable from the levels in the bulk liquid

Figure 3 Site-specific genome editing by AAV6 vectors uses HDR. (a) Schematic of AAV vectors used, which all contain a GFP expression cassette. Vector CCR5-GFP additionally contains 1431 and 473 bp sequences with homology to the CCR5 locus, while vector AAVS1-GFP contains 801 and 840 bp of sequences with homology to the AAVS1 (PPP1R12C) genomic locus61. (b,c) Fetal liver HSPCs were mock treated (Mock), or transduced with the indicated AAV vectors for 24 h, then electroporated with CCR5-GFP donors and/or electroporated with GFP+ for representative experiment, and mean post-electroporation. Shown are data from one GFP by flow cytometry at day 1 and day 10 additional colonies. An uncropped image of this gel is available in Supplementary Figure 11. (d) Evaluation of targeted integration of GFP at the CCR5 locus by semiquantitative In-Out PCR, for one representative experiment. The Ctrl. PCR serves as a loading control. Quantitation for the single sample above background control is shown. An uncropped image of this gel is available in Supplementary Figure 11.

(e,f) Mobilized blood HSPCs were transduced without or with 10,000 pg/cell AAVS1-GFP or CCR5-GFP donors and/or electroporated with AAVS1 or CCR5 ZFN mRNA. Cells were analyzed 8 days post-electroporation by flow cytometry. Shown are data from one representative experiment, and mean ± s.d. (f) GFP+ expression from n = 3 samples, except that the no donor, AAVS1 ZFN and no donor, CCR5 ZFN treatments were n = 1. *P < 0.05, one-way ANOVA.

Figure 4 Rates of bulk culture cell growth and genome modification in erythroid and myeloid lineages. (a) Mobilized blood CD34+ HSPCs were transduced with CCR5-RFLP donor and/or electroporated with 40 µg/ml of CCR5 ZFN mRNA. Mock-treated HSPCs were cultured as a control. Cells were counted at day 1–9 days post-electroporation. Results are shown from one representative experiment from a total of three independent experiments using three different HSPC donors. (b) Cells were also subjected to a colony formation assay at day 24 h post-electroporation, with CFUs evaluated 14 days later. Error bars indicate mean ± s.d. from duplicated samples. No significant differences were detected among the four treatment conditions (P > 0.05, one-way ANOVA). (c) CFUs were also genotyped by deep sequencing to detect rates of insertion of the XhoI site. Between Z3 and 88 validated individual colonies were picked for each colony type. Error bars show mean ± s.d. from two combined experiments using different HSPC donors. No significant differences were detected (P > 0.05, one-way ANOVA). (d) Mobilized blood HSPCs treated with CCR5-GFP donor and CCR5 ZFN mRNA were used for colony formation assays. Representative GFP+ erythroid and myeloid colonies are shown. Scale bars, 100 µm.
Efficient genome editing in primitive subsets of CD34+ HSPCs

CD34+ cells comprise a mixed population of primitive and more differentiated cells that can be further distinguished based on the expression of additional markers. The CD90 subset in particular has been associated with long-term repopulating activity, and CD34+CD90+ cells can provide long-term multilineage engraftment in patients undergoing cancer treatment. However, the most primitive cells, including those defined as CD34+CD133+CD90+, or capable of persisting in transplanted immune-deficient mice, have proven to be the most difficult to edit when ZFNs have been combined with donor templates delivered by IDL.Vs.

To evaluate the ability of AAV6 donors to promote HDR-mediated gene editing in primitive cells, we treated bulk CD34+ populations isolated from fetal liver with CCR5-GFP AAV6 vectors and CCR5 ZFN mRNA and then sorted them into different subsets. We defined subsets within the bulk (B) CD34+ population as primitive (P), early (E), and committed (C) progenitors, based on expression of CD133 and CD90.

Cultured fetal liver HSPCs are quite proliferative, which may increase their permissiveness to HDR-mediated repair. We therefore repeated these experiments using the more clinically relevant mobilized blood CD34+ HSPCs, and using reagents targeting both the CCR5 and AAVS1 loci. These analyses also demonstrated equivalent levels of HDR-mediated gene addition in the CD34+CD133+CD90+ population as in the bulk CD34+ population or the more differentiated progenitors.
template delivery provides the capability of editing even the most primitive compartment of CD34+ HSPCs.

**Genome-edited HSPCs engraft and differentiate in NSG mice**

The long-term engraftment potential of human HSCs can be evaluated by their ability to engraft and differentiate in immune-deficient mice. Such severe combined immunodeficient (SCID)-repopulating cells are considered surrogates for long-term repopulating HSCs. In mouse HSC studies, long-term-repopulating HSCs are also defined by their ability to further persist during secondary transplantations. However, it is especially difficult to demonstrate secondary transplantations when using mobilized blood CD34+ cells, as the transplantation of these cells is usually followed by the presence of unmodified cells in the peripheral blood and bone marrow, as well as in the spleen and tissues. This is true for both the human CD45+ cell populations (Fig. 6b), as well as in individually sorted lineages (Supplementary Fig. 8). Evidence of site-specific GFP insertion at the CCR5 locus in NSG mice was also confirmed in the blood and tissues of individual mice from the CCR5-GFP plus ZFN cohort by In-Out PCR (Fig. 6c). Together, these results demonstrate that modified human HSPCs are capable of engrafting mice and differentiating into multiple different lineages that retain the genomic edits.

Finally, we evaluated the ability of the genome-edited human HSPCs to persist during secondary transplantations. Bone marrow was harvested from two mice each of the separate CCR5-GFP or CCR5-RFLP cohorts and was pooled and used to transplant one additional adult NSG mouse for each group. The bone marrow of these secondary transplant recipients was then analyzed 20 weeks later, revealing that these cells had frequencies of genome editing that were at similar or higher levels when compared to the input primary bone marrow samples (Fig. 6d). As these cells had persisted for a total of 36 weeks in the mice and survived during secondary transplantation, the data support the conclusion that long-term SCID-repopulating cells in the initial population of treated CD34+ cells had been modified.

We also evaluated the extent of genome editing that could be detected in the human cells that persisted long-term (20 weeks) in NSG mice transplanted with mobilized blood HSPCs (Fig. 6e). These cells do not transplant in NSG mice as robustly as fetal liver HSPCs, and the graft declines over time. (Supplementary Fig. 9), making it more difficult to obtain molecular data from human cells at later time points. NSG mice were engrafted with mobilized blood HSPCs that had been treated with AAVS1-ZFN vectors, in the presence or absence of the matched AAVS1 ZFNs, and maintained for 20 weeks. Using an In-Out PCR assay, we were able to demonstrate site-specific GFP insertion at the AAVS1 locus in bone marrow from 6/10 of the mice receiving both the AAV6 vectors and ZFNs, whereas none of the mice receiving just the AAV6 vectors were positive in the same assay (Fig. 6f). The observation of long-term persistence of the GFP cassette at the targeted AAVS1 locus is also consistent with the in vitro CFU analyses (Fig. 4).

Fetal liver CD34+ cells were transduced with CCR5-GFP or CCR5-RFLP vectors followed by electroporation with CCR5 ZFN mRNA, then engrafted into neonatal nonobese diabetic (NOD)-SCID-gamma (NSG) mice and monitored over 16 weeks. Analysis of peripheral blood at weeks 8, 12, and 16 post-transplantation, and the bone marrow and spleen at 16 weeks, revealed robust development of human CD45+ leukocytes at levels that were indistinguishable from mice receiving untreated control HSPCs (Fig. 6a). At each time point, the human cells were further stained for lineage-specific markers and analyzed for the presence of B cells (CD19+), monocytes (CD14+), CD4 T cells (CD4+), and CD8 T cells (CD8+). (Supplementary Fig. 7). This revealed that the treated HSPCs were capable of differentiating into each lineage at rates similar to those of untreated cells, confirming no difference in hematopoietic potential for the treated cells, and agreeing with the observations from the in vitro CFU analyses (Fig. 4).

We also examined the levels of genome editing in the human cells that developed in the mice over time. Using flow cytometry for cells from the CCR5-GFP or CCR5-RFLP mice, we readily observed edited cells in both the circulation and tissues. This was detected in both the bulk human CD45+ cell populations (Fig. 6b), as well as in individually sorted lineages (Supplementary Fig. 8). Evidence of site-specific GFP insertion at the CCR5 locus was also confirmed in the blood and tissues of individual mice from the CCR5-GFP plus ZFN cohort by In-Out PCR (Fig. 6c). Together, these results demonstrate that modified human HSPCs are capable of engrafting mice and differentiating into multiple different lineages that retain the genomic edits.

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**Figure 6** Engraftment of NSG mice with gene-edited HSPCs. (a) Percentage of human CD45+ cells in peripheral blood of neonatal NSG mice engrafted with fetal liver HSPCs, and treated with AAV6 donors (CCR5-GFP or CCR5-RFLP) plus CCR5 ZFN mRNA. BM, bone marrow. Shown are the combined data from the two separate cohorts of mice. No significant differences were found in the levels of human cells in the blood or tissues between mock (n = 3) and treated samples (CCR5-RFLP n = 5, CCR5-GFP n = 6; two-way ANOVA). (b) Rates of genome modification in human cells were measured in blood and tissue samples from individual mice by flow cytometry for GFP insertions, or deep sequencing for RFLP insertions. na, not available, due to high background autofluorescence of cells. Actual numbers are available in Supplementary Table 3. Cells from mock-treated mice gave only background levels in all assays (not shown). (c) Representative examples of In-Out PCR showing GFP addition at the CCR5 locus in peripheral blood, bone marrow and spleen from individual mice at 16 weeks post-engraftment. Numbers for any samples above the levels of the background controls are shown. (d) Percentage of human CD45+ cell engraftment and gene modification (GFP+ by flow cytometry, RFLP insertion by deep sequencing) in bone marrow from primary HSPC recipient mice and secondary transplant recipients from CCR5-GFP or CCR5-RFLP cohorts. (e) Percentage of human CD45+ leukocytes in bone marrow of mice transplanted with mobilized blood HSPCs, treated with AAVS1-GFP vectors, alone (n = 3) or combined with AAVS1 ZFN (n = 6 for each dose of AAVS1-GFP). (f) Detection of GFP insertion at the AAVS1 locus in bone marrow samples from individual mice, measured by In-Out PCR. Numbers for any samples above the levels of the background controls are shown. Uncropped images of all gels in this figure are available in Supplementary Figure 13.
with an ability to edit the most primitive cells in the mobilized blood CD34+ cell population.

**DISCUSSION**

Precision genome engineering through the use of targeted nucleases is likely to be especially important when the cellular target is a long-lived cell such as an HSC. Achieving HDR-mediated gene editing at efficient levels requires optimization of several steps. First, the targeted nuclease must be introduced into the cell at sufficiently high levels and without toxicity. Next, a homologous donor template must also be introduced. This template can be single-stranded or double-stranded DNA, and presented as a viral vector, bacterial plasmid or synthesized oligonucleotides, although the size limitations of oligonucleotides makes them more suitable for gene correction purposes than transgene addition. A major limitation here for HSPCs can be cytotoxicity, and we have found that plasmid DNA and adenoviral vectors are less well tolerated in HSPCs than in transformed cells. Further, the template must engage the HDR machinery, which is most active in the S/G2 phase of the cell cycle. In the case of CD34+ HSPCs, a further requirement for many applications is that all of these events occur in the most primitive long-term repopulating stem cells, which represent only a minority of the CD34+ population.

We found mRNA electroporation to be a highly effective method to deliver ZFNs, as it resulted in high levels of DNA cleavage at the targeted CCR5 or AAVS1 loci without overt cytotoxicity. We further found that mRNA delivery of ZFNs could be effectively combined with donor template delivery by AAV vectors to achieve HDR-mediated genome editing, with a certain amount of flexibility in the sequence and timing of the two events. A potentially rate-limiting step of transduction of HSPCs by the AAV vectors was overcome by using the HSPC-tropic serotype AAV6. The combination of ZFN mRNA and AAV6 vectors allowed site-specific insertion of a promoter-GFP cassette at the CCR5 and AAVS1 loci at mean frequencies of 17% and 26%, respectively, in mobilized blood HSPCs, and at 19% and 43%, in fetal liver HSPCs.

AAV vectors, in the absence of nuclease, have previously been used to promote HDR, and have found application in transgenics. The AAV genome exists in single-stranded and double-stranded forms, both of which could potentially serve as substrates for HDR, and it has been suggested that AAV inverted terminal repeats may be particularly recombining. The capsid packaging limitation of 4.7 kb can easily accommodate the homology arms necessary to introduce a small gene correction. Furthermore, for gene insertion applications, the specific homology arms we used to target the AAVS1 locus could allow an additional gene cassette of ~3.0 kb. AAV vectors also have the advantage that they are relatively easily manufactured at high titers for clinical applications.

An important consideration when working with CD34+ HSPCs is the ability to modify the most primitive cells in the population, which contribute to ongoing multilineage hematopoiesis. Previous work using IDLV donors has shown that although rates of HDR in the bulk CD34+ population reach ~12% (GFP+ cells) when used in combination with dmPGE2 and SR1 stimulation, the vectors support much lower levels of editing in long-term-repopulating HSCs, as seen in both in vitro cultures and humanized mice experiments. In contrast, we found that donor templates provided as AAV6 vectors were able to direct high levels of genome editing even in the primitive CD34+CD133-CD90+ subset of cells in vitro without additional manipulation and at rates that were indistinguishable from those of the bulk CD34+ population. In addition, the treated HSPCs supported long-term multilineage engraftment of humanized mice, including secondary transplants, consistent with modification of long-term-repopulating HSCs. Furthermore, our data suggest that there is not an inherent defect in the HDR machinery in long-term-repopulating HSCs, at least when the cells are cultured ex vivo for short periods of time.

In summary, we have demonstrated that homologous donor template delivery by AAV6 vectors can be combined with ZFN mRNA electroporation to achieve high levels of precise genome editing in human HSPCs, including in the most primitive population. As HDR acts downstream of DSB formation, it is likely that AAV6 vectors will have similar utility as partners for all classes of targeted nucleases for this cell population. In this way they provide a means of broadening the application of genome engineering for the treatment of human diseases of the blood and immune systems.

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

J.W., C.M.E. and J.I.D. performed most of the experiments; S.B.H., P.W.-L.L., D.A.S., R.T.S. and G.N.L. developed assays and analyzed samples; J.W., C.M.E., P.D.G., M.C.H. and P.M.C. designed the experiments and analyzed data; J.W., C.M.E., M.C.H. and P.M.C. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Isolation of human CD34+ HSPCs. Leukopaks containing G-CSF mobilized peripheral blood CD34+ HSPCs were purchased (Apheresis Care Group, Inc., San Francisco, CA) and CD34+ HSPCs purified by magnetic bead selection using a Clinimacs cell selection device (Miltenyi Biotec, Auburn, CA). The enriched CD34+ HSPCs were resuspended in mobilized blood CD34 maintenance media: X-Vivo 10 (Lonza, Basel, Switzerland) supplemented with 2 mM l-glutamine, 1% penicillin/streptomycin/amphotericin B (PSA) (Sigma-Aldrich, St. Louis, MO), and 100 ng/ml each of stem cell factor (SCF), fms-like tyrosine kinase 3 (Flt3) ligand and thrombopoietin (TPO) (Peprotech, Rocky Hill, NJ).

Fetal liver samples were obtained from Advanced Bioscience Resources (Alameda, CA) or Novogenix Laboratories (Los Angeles, CA), as anonymous waste samples, with approval of the University of Southern California's Institutional Review Board. Human CD34+ HSPCs were isolated from the tissues following physical disruption and incubation in collagenase to give single-cell suspensions, followed by magnetic-activated cell sorting (MACS) (Miltenyi Biotec), as previously described67. Fetal liver-derived HSPCs were cultured in fetal liver CD34 maintenance media consisting of X-Vivo 15 supplemented with 50 ng/ml each of SCF, Flt3 ligand and TPO (R&D Systems, Minneapolis, MN), plus 1% PSA.

ZFN reagents. ZFNs targeting the CCR5 and AAVS1 loci have been described previously20,21. The following FokI variants were used to construct obligate heterodimeric versions of ZFN46-62: EL:KK (CCR5, experiments with mobilized blood CD34+ cells), ELD:KKR (CCR5, experiments with fetal liver CD34+ cells, AAVS1). An optimized pair of the AAVS1-targeting ZFNs was used in this study (Supplementary Fig. 14). The ZFN coding sequences were cloned into a modified version of plasmid pGEMZ (Promega, Madison, WI) containing a sequence of 64 alanines 3′ of the inserted gene sequence63, which was linearized by Spel digestion to generate templates for mRNA synthesis. mRNA was prepared using the mMESSAGE mMACHINE T7 ULTRA Kit (Life Technologies, Carlsbad, CA) or by Trilink Biotechnologies (San Diego, CA).

AAV vectors. All AAV vectors were produced at Sangamo Biosciences as described below, except for CMV-GFP reporter vectors of different serotypes, used to transduce fetal liver CD34+ cells, which were purchased from the University of Pennsylvania Vector Core (Philadelphia, PA). CCR5 and AAVS1 homologous donor templates 23,25,61 were cloned into a customized plasmid pRS165 derived from pAAV-MCS (Agilent Technologies, Santa Clara, CA), containing AAV2 inverted terminal repeats, to enable packaging as AAV vectors using the triple-transfection method65. Briefly, HEK 293 cells (Agilent Technologies) were plated in ten-layer CellStack chambers (Corning, Acton, MA), grown for 3 days to a density of 80%, then transfected using the calcium phosphate method with an AAV helper plasmid expressing AAV2 Rep and serotype-specific Cap genes, an adenovirus helper plasmid, and an AAV vector genome plasmid containing inverted terminal repeats. After 3 days the cells were lysed by three rounds of freeze/thaw, and cell debris removed by centrifugation. AAV vectors were precipitated from the lysates using polyethylene glycol, and purified by ultracentrifugation overnight on a cesium chloride gradient. Vectors were formulated by dialysis and filter sterilized.

Genome editing of HSPCs. CD34+ HSPCs were stimulated for 16–24 h in cell source–appropriate maintenance media, then transduced with AAV vectors at 1,000 or the indicated vector genome (vg) copy per cell in maintenance media, at a concentration of 1–2 × 10^5/ml, for 16–24 h or the indicated time. The HSPCs were washed 2–3 times with PBS then diluted in BTXpress high performance electroporation solution (Harvard Apparatus, Holliston, MA) to a final density of 2–10 × 10^5 cells/ml for mobilized blood CD34+ HSPCs or 10^6 cells/ml for fetal liver CD34+ HSPCs. This cell suspension was mixed with 40 µg/ml of the indicated amount, of in vitro transcribed ZFN mRNA and electroporated in a BTX ECM830 Square electroporator (Harvard Apparatus) in a 2-mm cuvette using a single pulse of 250 V for 5 ms. Post-electroporation, fetal liver–derived cells were cultured in fetal liver CD34 maintenance media, whereas mobilized blood HSPCs were cultured in mobilized blood CD34 maintenance media plus 20 ng/ml IL-6 (Peprotech), unless indicated.

Analysis of genome modification. For experiments using GFP donors, cells were collected at different time points post-treatment and analyzed for GFP expression by flow cytometry, using either a BD FACS Canto II (BD Biosciences, San Jose, CA), or Guava EasyCyte 6-2L or EasyCyte SHT (EMD Millipore, Billerica, MA). Data acquired were analyzed using FlowJo software version 9.5.3 or version X (Treestar, Ashland, OR), or InCyte version 2.5 (EMD Millipore). In each independent analysis, GFP+ populations of cells were defined using gates assigned using untreated or mock-treated populations cultured in parallel, with the criteria that 0.1% or less of the cells from the untreated or mock-treated populations would be included in the GFP+ gate.

In addition, a semiquantitative In-Out PCR was used to measure the rates of GFP integration at the CCR5 locus. The assay uses two simultaneous PCR reactions. One primer pair amplifies HDR-mediated insertion events because one primer recognizes a sequence contained in the AAV vector, whereas the other binds only to a genomic sequence outside of this donor. Specifically, one primer binds within the poly A region of the GFP cassette and the other binds to a sequence 3′ (beyond) the end of the right CCR5 homology arm (Supplementary Table 4, CCR5 In-Out primers). This PCR product, designated CCR5-HDR, was normalized by comparison to the product resulting from a control primer set (Ctrl.), which recognizes sequences in the CCR5 locus that are not included in the homology donor (Supplementary Table 4, CCR5 control primers). The concentration of the In-Out primer set was two times that of the control primer set, to increase detection sensitivity. The relative intensities of each CCR5-HDR PCR product were normalized for DNA input and quantitated by comparison to a set of standards, generated from genomic DNA isolated from a K562 cell line with a constant level of GFP integration at the CCR5 locus, as quantitated by Southern blot analysis. The parental K562 cells were purchased from ATCC (Manassas, VA). A similar PCR reaction was used to detect site-specific integration of the GFP cassette at AAVS1, using specific primer sets (Supplementary Table 4, AAVS1 In-Out and control primers), and standardization against a K562 cell clone with a single GFP integrant at one of the AAVS1 loci.

For experiments using RFLP donors, RFLP assays and Illumina deep sequencing were used to quantify the frequency of genome modification. The RFLP assay was performed as described45, with some modifications. Briefly, a pair of CCR5 or AAVS1 out-out primers (Supplementary Table 2, out-out1), located outside the region of homology contained within the CCR5 or AAVS1 donor molecules, was used for PCR amplification. The PCR products were then digested with XhoI (CCR5) or HindIII (AAVS1) and resolved on 1% agarose or 10% polyacrylamide gels. Alternatively, for CCR5, the gel-purified out-out PCR product was re-amplified using CCR5 in-in primers (Supplementary Table 4) before XhoI digestion. For Illumina deep sequencing, gel-purified PCR products were amplified with a target-specific MiSeq adapter primer pair (Supplementary Table 4, adapter primers) and sequence barcodes were added in the subsequent PCR reaction using the barcode primer pairs. Alternatively, 1/5,000 of the PCR products amplified using primer pair out-out were re-amplified with primer pair out-out2 (Supplementary Table 4), then 1/5,000 of the second PCR products were amplified using the MiSeq adapter primers. The final PCR products were cleaned and sequenced in an Illumina MiSeq sequencer, essentially as described by the manufacturer (Illumina, San Diego, CA). For analysis of genome modification levels, a custom-written computer script was used to merge paired-end 150-base sequences, and adapter trimmed via SeqPrep (John St. John, https://github.com/jstjohn/SeqPrep, unpublished).

Reads were aligned to the wild-type template sequence. Merged reads were filtered using the following criteria: the 5′ and 3′ ends (23 bp) must match the expected amplicon exactly, the read must not map to a different locus in the target genome as determined by Bowtie2 (ref. 66) with default settings, and deletions must be <70% of the amplicon size or <70 bp long. Indel events in aligned sequences were defined as described previously25, with the exceptions that indels of 1 bp in length were also considered true indels to avoid undercounting real events, and true indels must include deletions occurring within the sequence spanning the penultimate bases (adjacent to the gap) of the binding site for each partner ZFN. Events with expected RFLP modification were defined based on perfect alignment with the DNA sequence containing the novel restriction site (CCR5 RFLP: AGTGGTGTCTCGAGGTGATGA; AAVS1 RFLP: AAGTGGCGGAAACTTACTAGGG) of the expected sequences.
Colony forming unit and cell growth assays. Cells were cultured in X-Vivo 10 media with 100 ng/ml each of SCF, Flt3 ligand, and TPO, and 10 ng/ml interleukin (IL)-6 (PeproTech). To monitor cell growth, cells were collected at indicated time points for cell counting by flow cytometry (Guava EasyCyte HT) after addition of 5 µg/ml propidium iodide (PI), to exclude dead cells, and flow count beads (Beckman Coulter) for reference.

For colony formation assays, cells were plated as a single-cell suspension at a density of 200–800 cells/ml in semi-solid methylcellulose-based medium containing 50 ng/ml SCF, 20 ng/ml GM-CSF, 20 ng/ml IL-3, 10 ng/ml IL-6, 20 ng/ml G-CSF and 3 units/ml erythropoietin (EPO) (StemCell Technologies Inc., Vancouver, BC, Canada), at 24 h post-electroporation. After 2 weeks of incubation, CFUs were classified and enumerated by trained operators on the basis of size and morphological characterization under a light microscope. Individual CFUs were then picked into 50 µl QuickExtract DNA extraction solution (Epicentre Biotechnologies, Madison, WI). DNA was extracted from colonies and subjected to deep sequencing analysis as described above. Colonies with two or more sequences comprising >10% of reads were treated as mixed clones and excluded from final genotyping analysis. Unique sequences comprising <10% of total sequence reads for a given sample were considered to be the result of processing errors and/or sample contamination and were also excluded from the analysis. Individual CFUs were identified as wt/wt (CFU count: a), wt/indel (b), wt/RFLP (c), indel/RFLP (d), indel/indel (e), and RFLP/RFLP (f). Frequency of RFLP modification in the population was then calculated using the following: % RFLP = (c+d+2f)/(2a+2b+2c+2d+2e+2f)*100%.

HSPC subset analysis. Mobilized blood or fetal liver–derived CD34+ HSPCs were treated with AAV6 vectors and ZFN mRNA as described above. One or 2 days post-mRNA electroporation, cells were washed, blocked in FCS (Denville), and stained with the following fluorophore-conjugated antibodies: CD34 (581) (BD Biosciences), CD90 (5E10) (BD Biosciences) and CD133/2 (293C3) (Miltenyi Biotec). Cell sorting into subsets based on expression of these markers was performed using a BD FACS Aria II (BD Biosciences), with all compensations performed using Diva software (BD Biosciences). Subsets were defined as primitive (P; CD34+CD133+CD90+), early (E; CD34+CD133+CD90+), and committed (C; CD34+CD133−CD90+) progenitors. The subsets derived from fetal liver HSPCs were cultured in fetal liver maintenance media, and the subsets derived from mobilized blood HSPCs were maintained in SFEM-II media (Stemcell Technologies, Vancouver, Canada) supplemented with SCF, Flt3 ligand, TPO and IL-6, for a further 6–7 days. GFP expression was determined in each population by flow cytometry, and site-specific insertion of GFP at either the CCR5 or AAVS1 loci was analyzed by In-Out PCR, as described above.

Mouse engraftment and human cell analysis. Fetal liver HSPC engraftment of 1- to 2-day-old NOD.Cg-PrkdcscidIL2rgtm1Wjl/SzJ (NSG) neonatal mice was performed as previously described, using 1 × 10^6 CD34+ cells per mouse.47 No preference was given to any animal property at the time of engraftment and mice were randomly assigned to each engraftment group. HSPCs from two different tissue donors were either mock treated, or treated with AAV6 donors (CCR5-GFP or CCR5-RFLP) plus CCR5 ZFN mRNAs. Genome editing levels in the input HSPCs were 9.1% and 12% for CCR5-GFP treated cells, by flow cytometry, and 6.7% and 11.2% for CCR5-RFLP treated cells, by deep sequencing. Two separate litters of mice were engrafted with HSPCs from each fetal liver donor to limit possible litter effects on results. Peripheral blood (70 µl) was sampled every 4 weeks from 8 weeks of age, and spleen and bone marrow were isolated at necropsy, as described. Whole blood and tissue samples were blocked in FCS (Denville) and stained with the following antibody-fluorophore conjugates: CD4-V450 (RPA-T4), CD3-PE (UCHT1), CD19-APC (HIB19), and CD45-PerCP (2D1) (BD Biosciences) for 15 min at room temperature. Red blood cells were lysed after staining by incubation in BD Pharm Lyse buffer (BD Biosciences). Lysis was halted by the addition of PBS, and cells were analyzed by flow cytometry using a BD FACS Canto II (BD Biosciences). Compensation samples were created with BD CompBeads (BD Biosciences). Analysis of flow cytometry data was performed using FlowJo software version 9.5.3 or version X (Treestar, Ashland, OR). Compensation for stain overlap was performed post-acquisition using the tools included in FlowJo software. Initial gating was performed as forward scatter height versus forward scatter area to obtain the single-cell population; the resulting population was plotted on a side-scatter area versus forward-scatter area grid to gate for live lymphocyte populations. Subsequent gates were set using full minus one controls such that <0.1% of cells not receiving a specific stain were considered positive for that stain. There was no operator blinding in the analyses. Secondary transplants were performed using 1×10^7 mouse bone marrow cells harvested from the upper and lower limbs of two separate mice for each condition (CCR5-RFLP/ZFN and CCR5-GFP/ZFN). Each pooled bone marrow sample contained 16% human CD45+CD34+ cells, and was transplanted into 8-week-old female NSG mice, as described.47 For experiments using mobilized blood HSPCs, adult (7 week old) female NSG mice were engrafted by retro-orbital injection of 1×10^6 cells, as described. The input HSPCs were treated with AAVS1-GFP, with and without AAVS1 ZFN. Levels of GFP+ cells, measured by flow cytometry at five days post-transfection in culture, were 0.72%, 20.5% and 30.7% respectively, for cells receiving 10,000 vg/cell AAVS1-GFP alone, 3,000 vg/cell AAVS1-GFP plus ZFNs, and 10,000 vg/cell AAVS1-GFP plus ZFNs. Twenty weeks later, bone marrow was harvested and analyzed for human CD45+ cells, as described above.

Mouse blood and tissue samples were also subjected to genomic DNA purification using NucleoSpin Tissue XS kits (Macherey-Nagel, Bethlehem, PA) and subsequent molecular analysis as described above. All animal studies were performed in compliance with the regulations and with the approval of the University of Southern California Institutional Animal Care and Use Committee.

Statistical analysis. Statistical analyses were performed using the software suite GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) or the Excel Analysis ToolPak. Off-target data analysis used the method described by Guilinger et al.67 with Bonferroni correction.

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