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Permalink

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Journal

Science Translational Medicine, 8(360)

ISSN

1946-6234

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Publication Date

2016-10-12

DOI

10.1126/scitranslmed.aaf9336

Peer reviewed

SICKLE CELL DISEASE

Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells

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Genetic diseases of blood cells are prime candidates for treatment through ex vivo gene editing of CD34⁺ hematopoietic stem/progenitor cells (HSPCs), and a variety of technologies have been proposed to treat these disorders. Sickle cell disease (SCD) is a recessive genetic disorder caused by a single-nucleotide polymorphism in the β -globin gene (*HBB*). Sickle hemoglobin damages erythrocytes, causing vasoocclusion, severe pain, progressive organ damage, and premature death. We optimize design and delivery parameters of a ribonucleoprotein (RNP) complex comprising Cas9 protein and unmodified single guide RNA, together with a single-stranded DNA oligonucleotide donor (ssODN), to enable efficient replacement of the SCD mutation in human HSPCs. Corrected HSPCs from SCD patients produced less sickle hemoglobin RNA and protein and correspondingly increased wild-type hemoglobin when differentiated into erythroblasts. When engrafted into immunocompromised mice, ex vivo treated human HSPCs maintain SCD gene edits throughout 16 weeks at a level likely to have clinical benefit. These results demonstrate that an accessible approach combining Cas9 RNP with an ssODN can mediate efficient HSPC genome editing, enables investigator-led exploration of gene editing reagents in primary hematopoietic stem cells, and suggests a path toward the development of new gene editing treatments for SCD and other hematopoietic diseases.

INTRODUCTION

Sickle cell disease (SCD) is a recessive genetic disorder that affects at least 90,000 predominantly African-American individuals in the United States and hundreds of thousands worldwide (1, 2). The genetic and molecular basis of SCD have been understood for nearly 70 years, but curative treatments have lagged (3, 4). SCD is caused by a single-nucleotide polymorphism (SNP) in the seventh codon of the gene for β -globin (*HBB*), one of two globins that make up the major adult form of hemoglobin. The resulting glutamate-to-valine substitution renders hemoglobin prone to polymerization under hypoxic conditions, producing characteristic “sickle”-shaped red blood cells (RBCs). Sickle RBCs have a markedly reduced life span in the bloodstream, damage the vasculature, and cause vaso-occlusion. Major clinical manifestations of SCD are chronic anemia, severe pain episodes, and progressive damage to vital organs such as the brain, lung, and kidney. In the United States, the disease causes a 30-year decrement in life span and a greatly diminished quality of life (2, 5–7).

RBCs are produced from repopulating hematopoietic stem cells (HSCs) in the bone marrow (BM), and allogeneic hematopoietic cell transplantation (HCT) from an unaffected human lymphocyte antigen (HLA)-matched donor is currently the only lasting cure for SCD (8). However, HCT has been used sparingly because of the difficulty in identifying donors, risks associated with the toxicity of the transplant regimen (requiring preparation with chemotherapy and immune suppression),

and potentially fatal graft-versus-host disease (9, 10). Recent transplant advances have reduced these risks in children (11) and have extended treatment to selected adults (12) and individuals for whom only a haplo-identical HLA donor is available (13). Still, the vast majority of individuals with SCD do not pursue allogeneic HCT because of an unfavorable risk-reward profile, especially during early childhood. A curative treatment for SCD that can be safely applied to more people remains an urgent need.

Gene editing has recently emerged as a promising avenue to treat genetic diseases affecting hematopoietic cells (14–16). Ex vivo editing of autologous hematopoietic stem/progenitor cells (HSPCs) could be followed by reimplantation of edited cells, bypassing donor requirements and eliminating the risks of graft-versus-host disease and postgrafting immunosuppression. Because sickle RBCs have a markedly shorter life span in circulation compared to wild-type (WT) RBCs, even low levels of genotypic correction are predicted to generate a clinical benefit (17). Observations in patients after allogeneic HCT suggest that clinical improvement may occur when as few as 2 to 5% of long-term engrafted cells carry a normal *HBB* allele (18–20). An ideal gene editing treatment would exceed this modest target, but to date, even this level of gene editing has not been achieved (14, 21).

During gene editing, a targeted nuclease creates a double-strand break (DSB) that can be repaired by one of two mechanisms: error-prone non-homologous end joining (NHEJ) that results in genomic insertions and deletions (indels), or templated homology-directed repair (HDR) to precisely insert, delete, or replace a genomic sequence (22). The recent development of CRISPR-Cas9, a programmable RNA-guided DNA endonuclease, has ignited an explosion of interest in gene editing to cure many genetic disorders, including SCD (23, 24). Guided by a single guide RNA (sgRNA), the Cas9 nuclease can be programmed to cut a target locus within the genome, allowing rapid iteration and optimization not possible with other gene editing approaches (23, 25).

Optimized methods for efficient ex vivo gene editing of human HSPCs are required to enable a CRISPR/Cas9-based treatment for blood disorders such as SCD. Recent work has demonstrated that Cas9 can be used for

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in vitro reversion of the SCD mutation in laboratory cell lines (26, 27) and induced pluripotent stem cells (iPSCs) (28), as well as efficient knockout of an erythroid enhancer in an immortalized cell line (29). Cas9-mediated in vitro editing at the *HBB* locus in HSPCs has recently been reported as well, using plasmid and mRNA delivery of Cas9 (27, 30). Correction of the SCD mutation in iPSCs has been reported using transcription activator-like effector nucleases (31, 32), and most recently, zinc finger nucleases (ZFNs) have been used to correct the SCD mutation in HSPCs, albeit at levels less than 1% in the long-term repopulating stem cell population (21). To date, gene editing has yet to achieve long-term correction of the SCD mutation in these cells at levels predicted to confer clinical benefit, based on engraftment in immunocompromised mice (21). Previous approaches to HSPC gene editing have furthermore required specialized technologies that are not broadly available, including highly engineered and proprietary ZFNs, chemically protected Cas9 guide RNAs, and viral HDR donors (14, 15, 21, 27). Although these factors are not inherently a barrier to clinical translation, they limit the discovery of efficient gene editing reagents for disease mutations, including SCD.

Electroporation of a preassembled ribonucleoprotein (RNP) complex, composed of recombinant Cas9 protein and unmodified in vitro transcribed sgRNA, can be used for gene knockout in a variety of cell types. We have recently extended this approach to enable extremely efficient HDR sequence replacement through rationally designed single-stranded DNA (ssDNA) donors in laboratory cell lines (33–36). Given the efficiency and speed of Cas9 RNP-based editing, we reasoned that a Cas9 RNP-based approach to gene editing could form the basis for an accessible protocol to correct mutations in human HSPCs. We paired the Cas9 RNP with single-stranded DNA oligonucleotide donors (ssODNs), which are generally available, easily designed, and able to mediate efficient sequence replacement in immortalized cell lines (36). Our goal is not only to develop an approach to edit HSPCs as a potential treatment for SCD but also to use reagents that are inexpensive and accessible to a wide variety of researchers, enabling investigator-led studies and rapid optimization to address a multitude of genetic hematopoietic diseases, including polygenic diseases.

We developed a pipeline to enable efficient, RNP/ssODN-based correction of the SCD mutation without introducing a selective marker (Fig. 1A). We first used an erythroleukemia cell line to explore a panel of Cas9 RNPs that cut near the SCD mutation. We then used the most effective RNPs to develop ex vivo editing methods in human HSPCs, achieving up to 33% sequence replacement. We demonstrate efficient correction of the sickle mutation in SCD HSPCs, with corresponding production of WT adult hemoglobin (HbA) RNA and protein in edited, differentiated erythroblasts. After the edited human HSPCs are engrafted in immunocompromised mice, sequence replacement at the SCD locus is retained 4 months after engraftment at levels likely to have clinical benefit.

RESULTS

Prioritizing SCD editing reagents in a model cell line

Pairing Cas9 with various sgRNAs can lead to different activities on the same gene target (37). For HDR-mediated editing, each sgRNA must in turn be paired with an HDR donor template that encodes the desired nucleotide changes. Coupled with subtleties of donor template design and reagent delivery, the complexity of this problem expands rapidly. We chose to use inexpensive, commercially available ssODNs and co-delivered these with Cas9 RNP by electroporation. Using these

components, we were able to quickly iterate combinations of sgRNA, HDR donor, and editing conditions. The rules governing the choice of sgRNA and ssODN are still unclear, although we have developed guidelines based on the mechanism of Cas9's cleavage activity (36).

We searched for maximally active sgRNAs and ssODNs in K562 cells, a human erythroleukemia line that resembles early committed hematopoietic progenitors. Two key restrictions when designing sgRNAs and ssODNs for HDR experiments are the distance between the sgRNA recognition site and the mutation, and the ability to silently ablate the sgRNA protospacer adjacent motif (PAM). This latter constraint ensures that Cas9 cannot recut corrected alleles, thus preventing the introduction of indels into the corrected allele.

To find maximally active sgRNAs compatible with SCD editing, we identified targets in the first exon of the *HBB* gene and tested six of them for which the PAM could be silently mutated (G3, G5, G10, G11, G17, and G18) and one for which it could not (G7) (Fig. 1A). Some of these sgRNAs have been evaluated previously (26, 27), but to our knowledge, they have not been used in a Cas9 RNP format. We also tested truncated versions of three guides (trG3, trG10, and trG11) (table S1) because truncation has been reported to reduce off-target cleavage without compromising on-target efficiency (37). We designed an initial ssODN (T1) that contained silent mutations in the PAMs of all sgRNAs and a WT-to-SCD edit (K562 cells are WT at *HBB*) (fig. S1A). The combination of the WT-to-SCD SNP mutation and removal of the G5 PAM generates a silent Sfc I restriction site, allowing easy tracking of HDR-mediated editing (fig. S1A).

We assembled Cas9 RNPs from each candidate sgRNA and individually delivered them to K562 cells together with the ssODN by electroporation. We used a dose of 100 pmol of each RNP and template per 150,000 K562 cells, based on a previous work (33). A T7 endonuclease I digest of polymerase chain reaction (PCR) amplicons from edited cell pools, which, in this case, detects mismatches arising from both NHEJ- and HDR-mediated repair of Cas9 DSBs, revealed sequence modifications with all but two sgRNAs (G3 and trG3) (Fig. 1B). Sfc I digest showed appreciable HDR-mediated editing of the SCD SNP with three sgRNAs: G5, G10, and trG10 (truncated G10) (Fig. 1C).

We quantified the frequency of HDR and indel formation at the SCD SNP by next-generation sequencing (NGS) of PCR amplicons derived from genomic DNA extracted from pools of edited cells (Fig. 1D) and by droplet digital PCR (ddPCR) (fig. S1B). When the trG10 Cas9 RNP was provided without an ssODN, 90% of NGS reads contained indels, indicating excellent delivery of the RNP and efficacious gene knockout (fig. S1C). We performed experiments using the T1 ssODN and a derivative of T1 bearing only the WT-to-SCD edit and the G5 and G10 PAM mutations (T2) (fig. S1A). Most candidate sgRNAs induced substantial quantities of indels (35 to 40% of reads) (Fig. 1, B and D), and G5, G10, and trG10 also yielded high levels of WT-to-SCD HDR (11% of reads for G5, 41% of reads for G10) from multiple donor designs (Fig. 1 and fig. S1). A phosphorothioate-protected ssODN did not improve HDR frequencies (fig. S1D). The G5 guide targets a sequence very similar to one found in the closely related δ -globin (*HBD*) gene, and we experimentally verified that this guide induces indels in *HBD* (fig. S1E). Hence, we selected the truncated trG10 guide for further testing.

To optimize an ssODN for HDR, we drew guidance from recent work in our laboratory showing that, upon binding its target, Cas9 releases the PAM-distal nontarget strand and that asymmetric homology arms taking advantage of this property can increase HDR efficiency (38). For sgRNAs that target the sense strand, such as G10, the best template to use thus matches the sense strand (annealing to the antisense strand) and

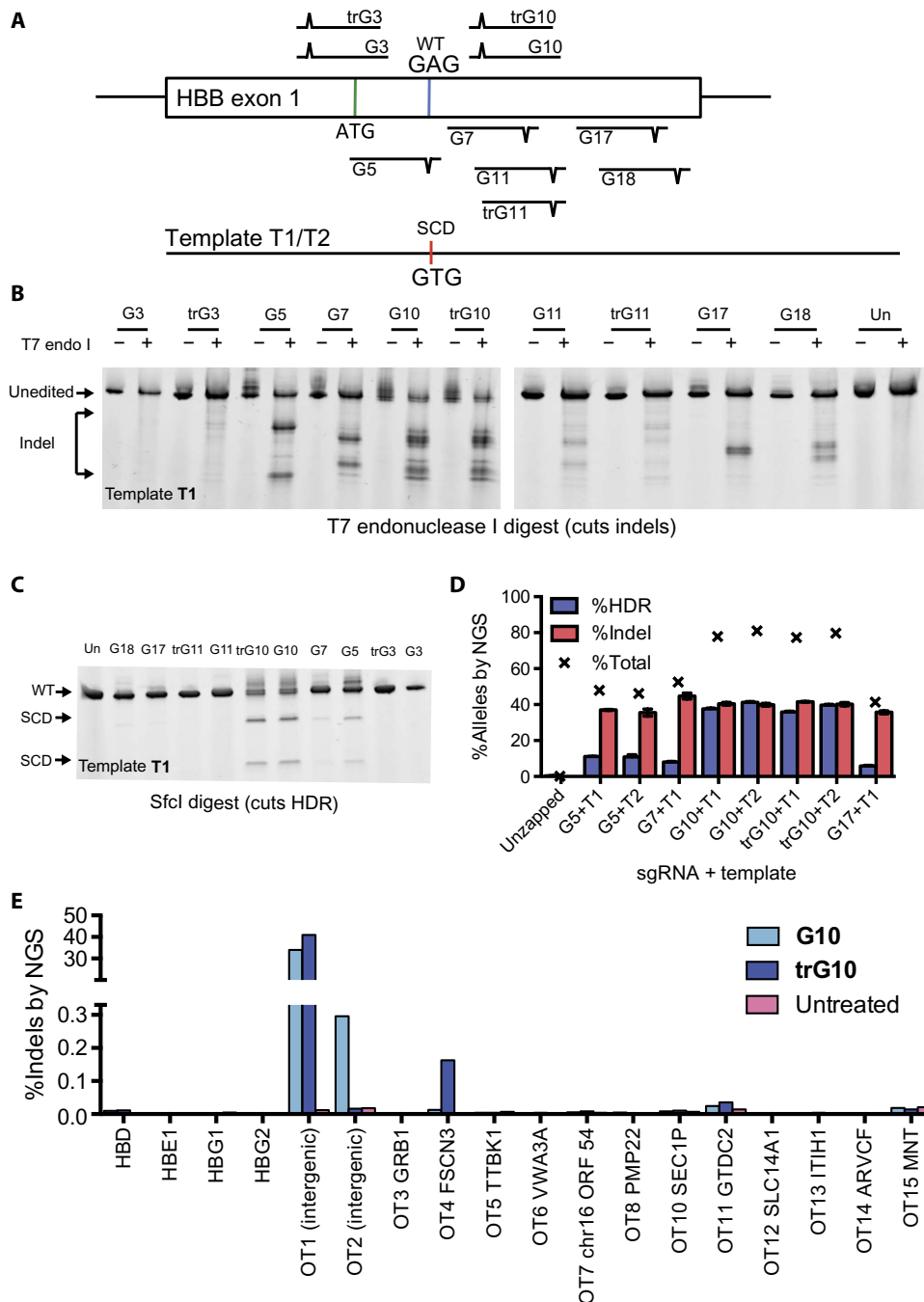


Fig. 1. Editing the SCD SNP in K562 cells. (A) Schematic depicting the experimental approach to editing in K562 cells. A panel of 10 sgRNAs that cut within 100 base pairs (bp) of the SCD SNP was selected. A WT-to-SCD edit was programmed by an ssDNA template (T1) bearing silent PAM mutations for the sgRNAs, which also introduces an Sfc I restriction site. (B) T7 endonuclease I assay depicting indel formation in pools of cells edited by candidate RNPs. (C) Editing of candidate sgRNAs detected by Sfc I digestion. G5, G10, and a truncated variant, trG10, efficiently edit in K562 cells. (D) Gene modification of select sgRNAs and templates at the SCD SNP, assessed by NGS. See fig. S1 for definitions of donors T1 and T2. Data are the average of three biological replicates, and error bars indicate SD. (E) Analysis of off-target cutting by the G10 RNP at sites predicted by the online CRISPR Design Tool, in K562 cells, determined by NGS, of a single replicate.

has a long 5' homology arm and a shorter 3' annealing arm (fig. S1F). On the basis of these principles, we henceforth identify templates by the lengths of their 5' and 3' homology arms relative to the G10 cut site. For example, the initial template T2 is termed T88-107. Unless noted,

all templates bear only the G5 and G10 PAM mutations. We tested the effects of asymmetric templates containing the SCD-to-WT edit on HDR-mediated editing in K562 cells (fig. S1C). We found that a template with a 111-nt 5' arm and a 57-bp 3' arm (T111-57) yielded an HDR frequency modestly higher than the original template T88-107 (33% versus 28.5% in the same experiment). We also found low but measurable rates of conversion (0.5 to 1%) between the HBB coding sequence and the homologous region in HBD, but this was only observed in samples treated with G7, G10, and trG10 (fig. S1G).

The possibility that Cas9 may cut at off-target sites is a concern for the development of Cas9-based therapies (39, 40). This tendency may be reduced by the use of Cas9 RNP delivery, truncated sgRNAs, and other emerging techniques (33, 41, 42). We selected off-target candidates by sequence similarity using a popular off-target prediction tool (40) and used NGS of PCR amplicons to analyze the off-target activity of G10 and trG10 Cas9 RNPs in K562 cells (Fig. 1E). We assessed editing at the two top-scoring off-targets (which are both intergenic), the top 13 exonic off-targets, and predicted off-target sites within the four globin genes (*HBD*, *HBE1*, *HBG1*, and *HBG2*) (fig. S2) (43).

We observed very little exonic off-target activity, with most off-target sites showing no Cas9-dependent indel formation within the limit of detection (~0.001%) (Fig. 1E). Substantial off-target activity was observed for both G10 and trG10 at a top-scoring intergenic site (OT1, chr9:104,595,865, 45% indel formation), which lies ~3 kb from the nearest annotated gene sequence and is nearly identical to the G10 target site. High off-target activity by G10 at this site has been observed previously (26). Off-target activity was detected with G10 at the other intergenic site (OT2, chr17:66,624,238, 0.30%), but this was absent with the truncated guide trG10. Low but detectable off-target activity was observed with both the G10 and trG10 RNPs at two exonic off-targets, FSCN3 and GTDC2. Indel formation was lower for the full-length G10 RNP (FSCN3: 0.013 and 0.16% indel for G10 and trG10, respectively; GTDC2: 0.025 and 0.035% indel for G10 and trG10, respectively). Very few indels were observed at HBD (0.010 and 0.011% indel for G10 and trG10, respectively), and indels at other β -globin genes (*HBE1*, *HBG1*, and *HBG2*) were not detected over background (Fig. 1E).

Optimizing candidate Cas9 RNPs and ssODNs to edit CD34⁺ HSPCs

The results of RNP delivery to K562 cells encouraged us to investigate using this approach with HSPCs, recognizing that editing efficiency can differ between cell types. We used the trG10 sgRNA, which showed robust HDR and few off-target effects in K562, and reoptimized HDR in adult mobilized peripheral blood HSPCs, which are the cells most relevant for therapeutic gene editing to address SCD. Because these cells were obtained from healthy WT donors, our initial experiments used ssODNs bearing a WT-to-SCD mutation.

We first explored optimization of electroporation conditions, template design, and RNP/ssODN dose in HSPCs (fig. S3, A and B). To selectively interrogate editing in viable cells, we used NGS to assay HSPCs cultured under erythroid expansion conditions for 7 days after editing (erythroid-expanded), along with edited HSPCs cultured for only 2 days (unexpanded). During initial treatments, we found that treatment with 100 pmol of RNP led to a decline in viability (fig. S3C), and used 75 pmol of RNP as a lower dose in subsequent in vitro experiments. We also tested the effects of the small molecule SCR7 on editing in HSPCs. This NHEJ inhibitor has been reported to increase HDR-mediated editing in some cell types, but we did not observe an improvement in HDR in HSPCs (fig. S3A) (44, 45).

We observed appreciable initial levels of editing at the sickle SNP in HSPCs, with HDR rates between 6 and 11% (Fig. 2, A and B). After 5 days of erythroid expansion, HDR rates increased, with up to 33% editing at both high and low doses of RNP (150 and 75 pmol, respectively). Total editing (HDR + %NHEJ) in expanded HSPCs was between 66 and 72%, indicating good delivery of the trG10 RNP to HSPCs. In general, higher editing was accompanied by some reduction in viability, as measured by fewer cells remaining after treatment, particularly at a high, 150 pmol dose of RNP (fig. S3C).

The asymmetric ssODN was most effective in K562 cells (T111-57) drove HDR more efficiently at a lower Cas9 dose of 75 pmol RNP per 150,000 HSPCs, whereas a shorter template (T111-27) was more efficient at a higher dose of Cas9 (150 pmol RNP per 150,000 HSPCs) (Fig. 2A). In a separate experiment, we confirmed high rates of HDR by ddPCR (fig. S3D). These experiments demonstrate efficient in vitro editing of CD34⁺ HSPCs using the Cas9 RNP, including HDR-mediated

sequence replacement using ssODNs without a selection marker. As was the case with K562 cells, in HSPCs we found low but measurable conversion of HBB coding sequence to HBD (0.2 to 2%), and rates of conversion increased after expansion of edited cells (fig. S3E).

To analyze how allele frequencies in the HSPC population translate to the editing of alleles in individual HSPCs, we repeated our best editing condition in CD34⁺ HSPCs (75 pmol trG10 RNP and 100 pmol T111-57),

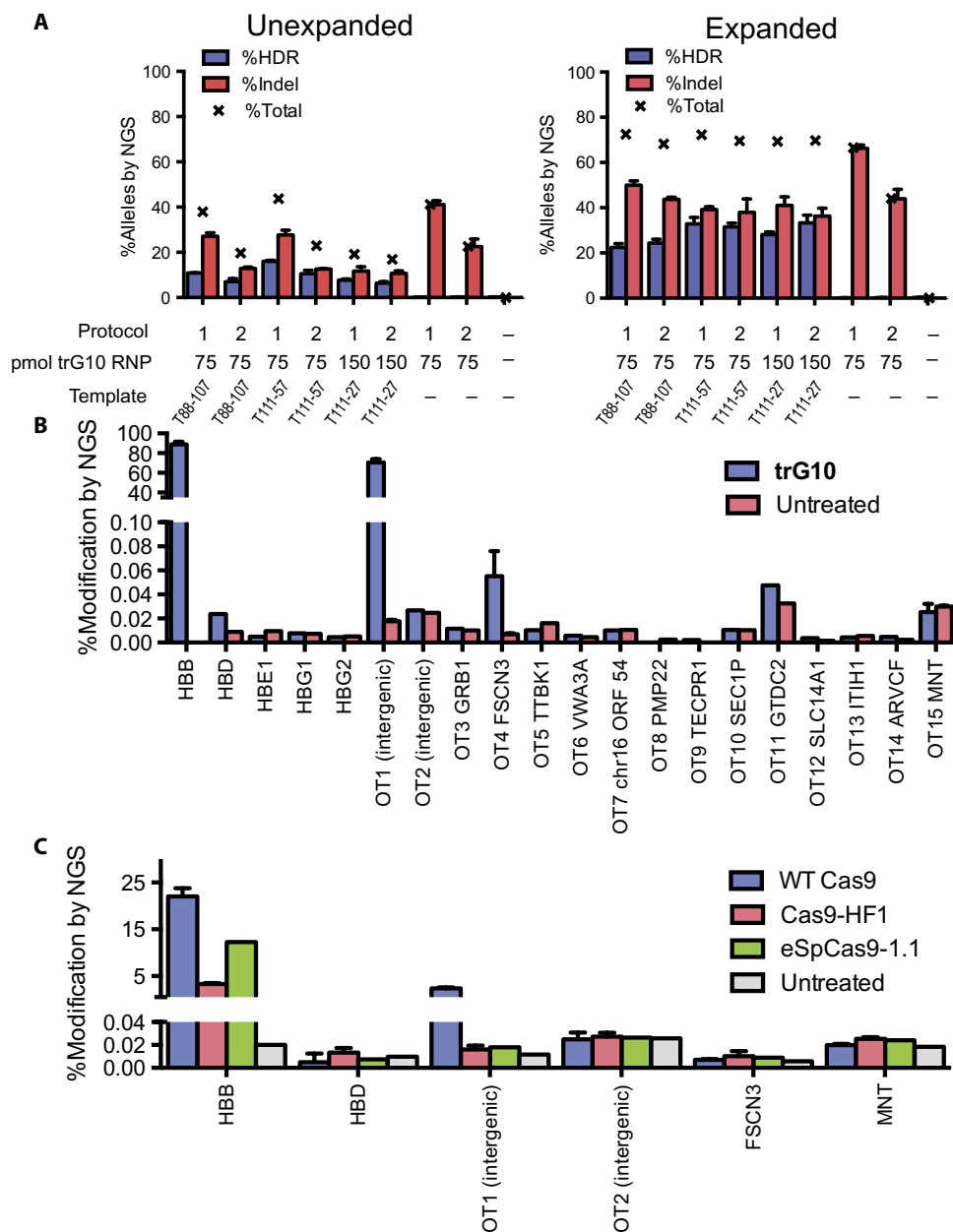


Fig. 2. Editing of WT human CD34⁺ HSPCs by the Cas9 RNP. (A) Analysis of editing in unexpanded HSPCs (left) and erythroid-expanded HSPCs (right), using trG10 RNP and under conditions as indicated. Templates, which are asymmetric about the G10 cut site, were designed as described in the text. All graphs show three biological replicates, and error bars are ±SD. (B) Modification (HDR+Indel) at off-target sites in HSPCs edited with the trG10 RNP and template T88-107, as compared to untreated cells. The bars for HBB, OT1, FSCN3, and MNT represent three biological replicates, with error bars (±SD); the others are each a single replicate. Targets were selected using the online CRISPR Design Tool. (C) Indel formation at on- and off-target sites by Cas9 mutants with increased specificity (HF1 and eSpCas9-1.1), in HSPCs, as compared to WT Cas9, all complexed to the G10 sgRNA and no ssODN. The absence of an ssODN reduced indel formation overall. All data are shown as n = 3 biological replicates, with error bars (±SD).

recovered the cells in HSPC expansion medium for 2 days, and plated single edited HSPCs by limiting dilution in erythroid expansion medium. After 14 days of growth, 96 edited clones were individually genotyped by multiplexed NGS (Table 1). In this experiment, 21% of alleles were HDR. However, these alleles were spread among 32% of the cells. This increased prevalence of edited cells relative to edited alleles is predicted by the independent assortment of alleles within a population (although we observed that homozygous genotypes were still overrepresented relative to a prediction based on random assortment) (46).

We analyzed off-target activity of the trG10 RNP in HSPCs using the target selection criteria described above for K562 cells (Fig. 2B). Most predicted genic off-targets showed no detectable indel formation, although cutting at the previously observed intergenic site remained high (OT1, ~80% indel). The rates of off-target cleavage observed in HSPCs generally corresponded to observations in K562 cells, although the rates were often reduced (for example, rate of FSCN3 is ~0.05% in HSPCs versus up to 0.16% in K562 cells).

As an additional test of the effects of off-target activity of the trG10 RNP, we used overamplification PCR to detect the presence or absence of translocations between the on-target site at HBB and selected off-target sites (OT1, HBD, FSCN3, and MNT) in both K562 cells and HSPCs edited with trG10 (fig. S4). Translocations between OT1 and HBD with HBB were observed in K562 cells. Most sites showed no translocations in HSPCs; one translocation may have occurred between OT1 and HBB in HSPCs, but the frequency of this event is not known.

We also tested whether trG10 has off-target activity at cancer-associated genes. Although these sites bear little similarity to the trG10 protospacer, even low levels of activity in such locations would be a concern. We used a capture library (Illumina TruSight Cancer) to sequence 94 genes and 290 cancer-associated SNPs to ~8000-fold coverage each. Relative to unedited cells, we found a small number of indel mutations enriched in K562 cells edited with the trG10 RNP, typically at less than 1% of alleles (table S2). Almost all of these mutations were present in unedited cells.

In contrast, no indels were detected in similarly edited HSPCs. These results highlight the importance of performing rare off-target event detection in the target primary cell type and suggest that the trG10 RNP may not have substantial genotoxic liabilities, although this has not been tested at clinical scale.

Two mutant variants of the Cas9 protein have been reported to reduce off-target effects in cell lines, even at highly similar off-target sites such as OT1 for the G10 RNP (47, 48). We expressed and purified the eSpCas9-1.1 and HF1 variants of Cas9, paired them with the G10 sgRNA to form RNPs, and used them to edit WT CD34⁺ HSPCs in the absence of an ssODN. We used NGS to determine the on- and off-target editing frequencies (Fig. 2C). As expected based on our recent observation of enhancement of indel formation by oligonucleotides, the on-target indel formation was reduced in the absence of an ssODN (Fig. 2B versus Fig. 2C) (38). Compared to WT Cas9, both HF1 and eSpCas9-1.1 showed even further decreased on-target indel formation at the HBB locus, for example, an almost fivefold decrease in indels for HF1. However, both modified enzymes also completely eliminated all previously observed off-target events, including the prevalent OT1 intergenic off-target (Fig. 2C).

Correction of the SCD mutation in SCD HSPCs to produce WT hemoglobin

Our success in WT-to-SCD editing in HSPCs implies that the same method could be used to edit SCD to WT in HSPCs derived from SCD patients. Because human erythropoiesis does not occur when human HSPCs are xenografted into mice, and the availability of SCD HSPCs is limited, we evaluated the effects of correcting the SCD mutation in HSPCs in vitro, by carrying out erythroid differentiation of edited HSPCs. We obtained CD34⁺ HSPCs from whole blood discarded after exchange transfusion of SCD patients. Because the HF1 and eSpCas9-1.1 proteins yielded reduced levels of on-target editing and the predominant off-target from WT Cas9 lies in an intergenic region with no known function, we focused on experiments using more efficacious WT Cas9. We corrected the SCD mutation using the trG10 RNP and ssODNs carrying an SCD-to-WT edit. These SCD-to-WT templates, denoted by the suffix “S,” encode the same number of mutations as the WT-to-SCD templates, with the base identity different only at the SCD SNP. Measuring editing by both NGS and ddPCR, we found that SCD HSPCs were edited at levels similar to those observed in WT HSPCs from mobilized blood, with up to 25% of alleles corrected to WT at high RNP dose and 18% corrected at low RNP dose (Fig. 3A and fig. S5).

To analyze the hemoglobin production potential of corrected HSPCs, we differentiated pools of treated HSPCs into enucleated erythrocytes and late-stage erythroblasts and measured hemoglobin by high-performance liquid chromatography (HPLC) (21, 28, 49). We found that corrected HSPC pools produce substantial amounts of WT HbA, with a concomitant decrease in sickle hemoglobin (HbS) (22.2 to 22.4% HbA, 48.0 to 50.6% HbS at low-dose RNP, 29.3% HbA, and 38.7% HbS at high-dose RNP) (Fig. 3, B and C). We also observed a substantial increase in fetal hemoglobin (HbF) in edited cell pools (16.3 to 17.4% HbF in edited cells versus 2.0% HbF in unedited cells).

We used RNA sequencing (RNA-seq) to measure globin transcript abundance in pools of edited SCD HSPCs differentiated to erythrocytes (50). Globin transcript levels showed a trend similar to protein levels after editing, with sickle HBB transcripts decreasing from 56.7% of all transcripts to ~9% and WT HBB transcripts increasing from 0.1 to 13% across all three editing conditions (Fig. 3D). Consistent with the increase in HbF protein, we observed about threefold increase in the expression

Table 1. Zygosity of clonal colonies of CD34⁺ HSPCs edited with the trG10 RNP. HSPCs were edited with 75 pmol of the trG10 RNP (similar to Fig. 2) and cloned by limiting dilution. Of the resulting clones, 96 were then genotyped by NGS. The fraction of all three alleles, the frequency of clones with at least one copy of each of the three alleles, and the frequency of all six genotypes are indicated.

n = 96 colonies						
	%WT	%Indel	%HDR			
All alleles	46	33	21			
Clones with one allele %WT/_ %Indel/_ %HDR/_						
Actual	60	48	32			
Predicted	71	55	37			
Clones by genotype						
	%WT/ WT	%WT/ Indel	%WT/ HDR	%Indel/ Indel	%Indel/ HDR	%HDR/ HDR
Actual	32	18	10	18	13	9.4
Predicted	21	30	19	11	14	4.3

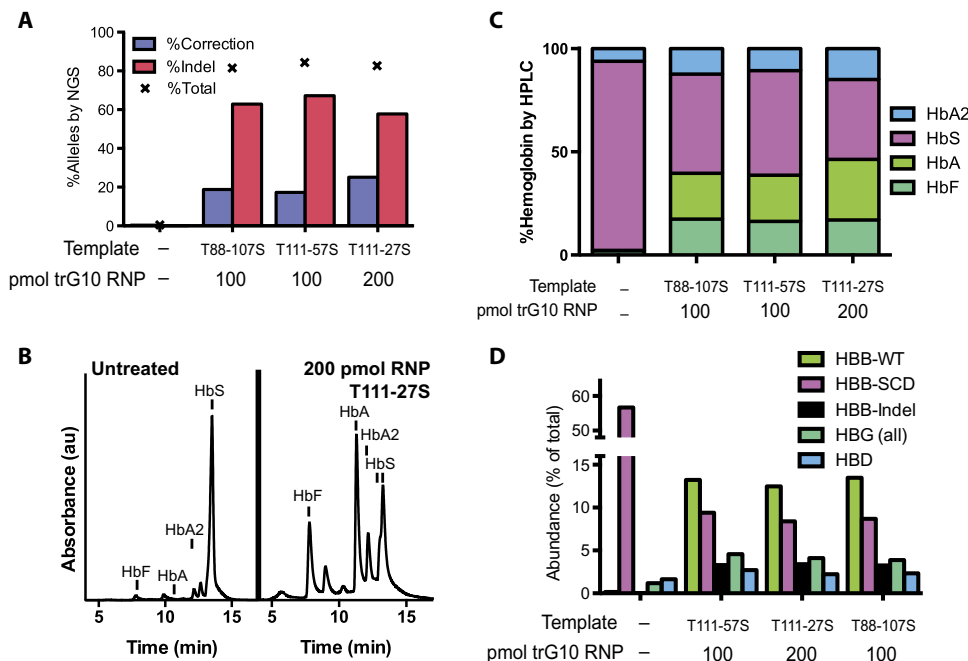


Fig. 3. Correction of the SCD mutation in SCD HSPCs. (A) Editing the SCD mutation in unexpanded CD34⁺ HSPCs from the whole blood of SCD patients, assessed by NGS. (B) HPLC trace depicting hemoglobin production in SCD HSPCs edited with 200 pmol of the trG10 RNP and the T111-27S donor, as compared to untreated HSPCs, after differentiation into late-stage erythroblasts. Increases in HbA, HbF, and HbA2 are apparent. au, arbitrary units. (C) Stacked bars showing HPLC results, with HSPCs edited as indicated before differentiation into erythroblasts. (D) Globin gene expression in SCD HSPCs edited as in (C), determined by RNA-seq. All experiments include a single replicate for each of the four treatment conditions.

of γ -globin (HBG1 and HBG2) mRNA. Thus, corrected pools of cells produce decreased sickle β -globin, increased adult WT β -globin, and increased fetal (γ) globin.

Repopulation by edited HSPCs in vivo

The trG10 Cas9 RNP and an ssODN donor template can efficiently edit the SCD mutation in CD34⁺ HSPCs, and erythrocytes derived from these cells have altered hemoglobin levels consistent with substantial gene correction. However, in order for gene correction to manifest in vivo, edited repopulating stem cells must engraft and repopulate within a recipient (51). A powerful method of assaying repopulating stem cells is through long-term xenografting in an immunodeficient mouse model, such as the NOD/SCID/IL-2 γ^{null} (NSG) mouse (52). In this model, edited human stem cells must engraft in the mouse and persist over several months for them to be observed in subsequent experiments. After 16 weeks, progenitor cells should be lost from the system, and human cells in the BM should be derived from long-term repopulating stem cells within the initial HSPC population (21, 52). Because SCD HSPCs are difficult to obtain at the necessary scale, we tested engraftment by editing WT HSPCs to SCD and implanting them in NSG mice.

We injected NSG mice with pools of WT CD34⁺ HSPCs edited with the trG10 RNP and the T88-107 template (seven mice over three treatments, 1×10^5 cells per mouse), along with two uninjected NSG mice (52). Engraftment was monitored by fluorescence-activated cell sorting (FACS) analysis of blood draws at 5 and 8 weeks after injection. Final engraftment was assessed at 16 weeks after injection, when mice were sacrificed and BM cells were harvested and subjected to FACS-based lineage analysis (six mice) (Fig. 4A and fig. S6). Substantial numbers of hematopoietic (CD45⁺) cells were detectable in BM of all injected mice at 16 weeks ($37 \pm 21\%$

human CD45, mean \pm SD), demonstrating that edited cells maintain long-term repopulating potential. Within the BM, engrafted human CD45⁺ cells were primarily B cells (CD19⁺; $57 \pm 18\%$, mean \pm SD) and myeloid cells (CD33⁺; $25 \pm 18\%$, mean \pm SD) (fig. S6B).

Genotyping of edited HSPCs was performed by NGS immediately after editing and before injection (Fig. 4B) and from mice at weeks 8 (blood) and 16 (BM and spleen) (Fig. 4, C and D). The input populations had $55 \pm 19\%$ indel alleles and $11.8 \pm 3.7\%$ HDR alleles (mean \pm SD) (Fig. 4B). NGS analysis revealed consistently high levels of indel alleles after 16 weeks ($46 \pm 6\%$ in BM, mean \pm SD), along with maintenance of HDR-mediated editing at the SCD SNP throughout the lifetime of the xenograft in both BM and spleen ($2.3 \pm 1.8\%$ in BM, $3.7 \pm 1.4\%$ in spleen; mean \pm SD). Human cells engrafted in one mouse maintained HDR-mediated editing in BM at a markedly high level (6.2%), although the reasons for this difference are unclear. To ensure that editing was present in the progenitor (CD34⁺) cells and not overrepresented in the B lymphocyte (CD19⁺) populations, marrow from two mice was sorted for these markers and

genotyped by NGS. Editing was maintained within both populations, indicating durable editing of the long-term stem cell (HSC) population (fig. S6). Despite the decreased HDR rate relative to input, this level of sequence replacement using Cas9 RNP and ssODNs is more than ninefold greater than the previously reported editing of the SCD mutation in HSCs using ZFN mRNA electroporation and ssODNs (21).

DISCUSSION

The treatment of genetic diseases by gene editing to replace an endogenous sequence is a long-standing goal of regenerative medicine. Unlike gene therapy using integrating viral vectors, wherein regulation of the introduced gene may be compromised and endogenous genes may be disrupted, gene editing corrects the disease mutation at the endogenous locus. In the case of most genetic blood disorders, lasting correction requires HDR-mediated editing of endogenous genes in repopulating HSCs. Ideally, these edits should be efficient enough to operate without the introduction of a nonnative selection marker. This is technically challenging, particularly because the edits do not typically confer a selective advantage in the target cell compartment, as is the case for correction of the HBB sickle mutation in BM stem cells (14, 21).

Here, we used the Cas9 RNP and ssODNs to develop a rapid and extensible gene editing pipeline to introduce SNPs into human adult HSPCs, focusing on the SCD mutation. We used K562 cells to iterate combinations of Cas9 RNPs and ssODNs that edit the HBB gene and then applied these reagents to human HSPCs. These reagents efficiently induce HDR-mediated editing of the SCD mutation in HSPCs with minimal genic off-target activity. Previous reports of gene knockout in HSPCs by Cas9 mRNA delivery required chemical protection of the

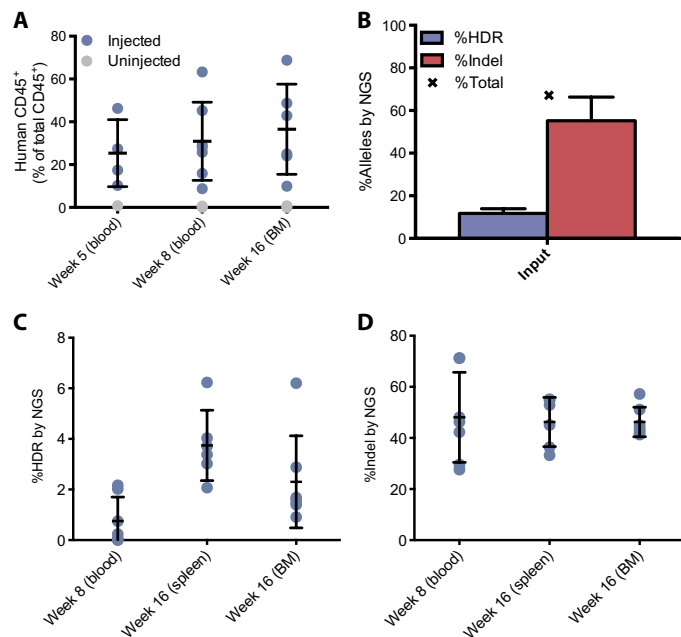


Fig. 4. Engraftment of edited HSPCs into NSG mice. (A) Engraftment of human CD45⁺ cells in NSG mice injected with edited HSPCs, compared to two uninjected mice. (B) Analysis by NGS of editing at the SCD SNP in cells before engraftment. Error bars indicate mean \pm SD of three separate experiments with cells from two healthy donors. (C) HDR-mediated editing and (D) indel formation at the SCD SNP in human cells engrafted in mouse blood, spleen, and BM at 5 and 16 weeks after injection. Error bars indicate mean \pm SD over either six mice (spleen) or seven mice (BM and blood).

sgRNA (27), but we found that RNP delivery yields efficient knockout and HDR even with unmodified sgRNAs and ssODNs. Although we used a truncated guide (trG10) here, we found no apparent decrease in off-target activity compared to the full-length guide, possibly due to the extremely high homology between the on- and off-target sites. RNP delivery has been associated with reduced off-target effects (41), suggesting that this modality could allow efficient editing of disease mutations in HSPCs using full-length unmodified sgRNAs such as G10.

We performed extensive off-target analysis using the trG10 RNP in HSPCs. We found few genic off-target events and one major intergenic event that had been described previously (26, 27). We did not find clear evidence of frequent translocation events between targeted loci in HSPCs. Similarly, we found low-frequency mutations at a few cancer-associated loci in edited K562 cells, typically at sites with measurable mutations in unedited cells, but did not observe any mutations in HSPCs. This suggests that off-target analysis should be performed in the target primary cell type and not cancer cell lines. To reduce off-target activity, we tested several recently characterized high-fidelity Cas9 variants as RNPs, but chose to pursue WT Cas9 due to their reduced on-target activity (47, 48).

Recent gene editing efforts in hematopoietic cells have focused on the use of viral HDR donors that are often delivered at very high multiplicity of infection (15, 16, 21). Advances in viral delivery technology have greatly improved safety, and viral donors are a valid option for gene correction. However, genomic insertion of even nonintegrating vector sequences has been observed during genome editing, and high levels of off-target integration have been observed in HSPCs (53, 54). Furthermore, the intensive engineering associated with viral donor design restricts optimization. By contrast, the co-delivered RNP/ssODN approach described here is nonviral, modular, and readily optimized.

Here, we describe levels of sequence replacement in long-term stem cells that may be clinically relevant (21). However, HDR was still diminished relative to the input CD34⁺ populations. Reductions in HDR frequency during long-term engraftment have been observed previously and remain a major impediment to bringing HDR-based therapies to the clinic (14, 15, 21). It may be that HDR-edited HSCs do not engraft as well as unedited cells, that the HDR donors are themselves toxic, or that HSCs are intrinsically more difficult to edit than other CD34⁺ cells. We found that relatively high doses of ssDNA donor do not affect indel formation even during long-term engraftment, implying that donor delivery is not in itself toxic, but that the stem cells may instead be intrinsically refractory to HDR-based editing or that HDR causes HSCs to lose their long-term regenerative potential.

Corrected SCD HSPCs produced greatly reduced sickle and increased WT hemoglobin protein and β -globin mRNA. Edited HSPCs also produced more γ -globin mRNA and HbF. Although the cause of this increase is unclear, it is possible that indels within HBB cause selective expansion of HbF-expressing cells during differentiation or that indels within HBB stimulate a cell-intrinsic up-regulation of HbF. The molecular basis by which alterations at β -globin cause changes in HbF abundance warrants further investigation.

Observations of transient mixed chimerism (2 to 5%) after allogeneic HCT for SCD suggest that correcting the sickle allele in relatively few BM stem cells could translate into a clinically meaningful increase in the numbers of nonsickling circulating RBCs (see Supplementary Discussion) (18–20). The abrogation of ineffective erythropoiesis intrinsic to sickle cell anemia and the greatly increased life span in circulation of WT RBCs carrying a WT β -globin allele imply that correction of a small fraction of HSCs can substantially increase HbA level and lower the HbS fraction in the blood. Furthermore, we found that alleles assort largely independently within the edited population, such that a given percentage of allele correction of alleles in a population corresponds to correction of nearly twice as many cells. This should be taken into account when comparing the allelic correction data presented here with observations of mixed chimerism (for example, correction of 1 to 3% of alleles may correct 2 to 6% of cells) (table S3 and Supplementary Discussion).

An approach to SCD treatment that integrates a gene editing approach could have several limitations. First, it is possible that the allelic correction frequency we observed in the HSPC population will ameliorate but not eliminate the clinical expression of SCD. Second, although autologous HSCT should be safer than allogeneic HSCT, it is still an intensive procedure, requiring myeloablative conditioning, which can have serious side effects. Finally, clinical translation of an HSCT-based therapy is not feasible in developing countries, where SCD is most prevalent. We anticipate that further developments will address these remaining issues.

The approach described here allows researchers to edit SNPs at endogenous loci in human adult HSPCs using readily available reagents that are conducive to rapid iteration and optimization. Given the low barrier to entry, we anticipate that these democratizing methods will enable investigator-led gene editing studies in a wide variety of disease areas. We predict that the methods outlined can be improved and scaled up for a gene editing treatment for SCD.

MATERIALS AND METHODS

For detailed Materials and Methods, please see the Supplementary Materials.

SUPPLEMENTARY MATERIALS

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Discussion

Materials and Methods

Fig. S1. Additional data showing editing of K562 cells with the Cas9 RNP.

Fig. S2. Genomic context of predicted off-target cut sites for the G10 RNP.

Fig. S3. Additional data showing editing of CD34⁺ HSPCs with the Cas9 RNP.

Fig. S4. Qualitative detection of chromosomal translocations by overamplification PCR.

Fig. S5. Confirmation of efficient correction of SCD HSPCs by ddPCR.

Fig. S6. Additional data on engraftment and editing of HSPCs in NSG mice.

Table S1. Oligonucleotides used in this study.

Table S2. Indel mutations at cancer-associated genes in K562 cells and HSPCs edited with the trG10 RNP, as compared to unedited cells.

Table S3. Estimates of cellular editing in mice engrafted with edited HSPCs.

Table S4. Tabulated data from Figs. 1 to 4 (provided as an Excel file).

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Acknowledgments: We thank C. Jeans of the QB3 MacroLab at University of California (UC), Berkeley, for expression and purification of the Cas9 protein variants used in this study. We thank S. McDevitt and the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley, which is supported by NIH S10 Instrumentation Grants S10RR029668 and S10RR027303. We thank H. Turner and M. J. Kim [Children's Hospital Oakland Research Institute (CHORI)] for advice and assistance with mouse work and L. Parker (CHORI) for advice and assistance with flow cytometry. We thank J. Doudna and M. Botchan (UC Berkeley), and B. Lubin and E. Penhoet (UCSF Benioff Children's Hospital Oakland) for their contributions to the initiation of this project. **Funding:** J.E.C., M.A.D., D.C., N.L.B., T.M., and T.W. were supported by the Li Ka Shing Foundation. M.A.D. was a California Institute for Regenerative Medicine postdoctoral fellow under training program TG2-01164. D.C. is supported by NIH grant R01 GM078571 and was supported by the Siebel Scholars Fund at Berkeley. D.I.K.M., D.B., and S.-J.H. were supported by NIH grants 5R21AA022753 and 1R56ES022377-01; D.I.K.M., W.M., M.C.W., and D.P.M. were supported by the Jordan Family Fund at CHORI. D.B.K. and F.U. were supported by a research grant from the Doris Duke Charitable Foundation to D.B.K. (Innovations in Clinical Research Award #2013158). **Author contributions:** J.E.C., D.C., M.C.W., and D.I.K.M. conceived the project. J.E.C., D.I.K.M., M.A.D., D.C., D.P.M., D.B., W.M., D.B.K., and J.R.B. designed the experiments. W.M., M.A.D., D.C., T.W., S.-J.H., and F.U. performed the experiments. N.L.B., T.M., S.-J.H., M.A.D., W.M., and D.C. analyzed the data. M.A.D., J.E.C., D.I.K.M., M.C.W., and D.C. wrote the manuscript. **Competing interests:** J.R.B. is an employee of Bio-Rad Inc. J.E.C. is an inventor on a patent submitted by UC Berkeley that covers asymmetric HDR donors, which were used during this work. All other authors declare that they have no competing interests. **Data and materials availability:** All NGS data have been deposited at the National Center for Biotechnology Information Sequence Read Archive under accession no. SRP090223. Tabulated data for Figs. 1 to 4, including references to the original NGS data, are presented in table S4.

Submitted 21 April 2016

Accepted 22 September 2016

Published 12 October 2016

10.1126/scitranslmed.aaf9336

Citation: M. A. DeWitt, W. Magis, N. L. Bray, T. Wang, J. R. Berman, F. Urbinati, S.-J. Heo, T. Mitros, D. P. Muñoz, D. Boffelli, D. B. Kohn, M. C. Walters, D. Carroll, D. I. K. Martin, J. E. Corn, Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci. Transl. Med.* **8**, 360ra134 (2016).

Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells

Mark A. DeWitt, Wendy Magis, Nicolas L. Bray, Tianjiao Wang, Jennifer R. Berman, Fabrizia Urbinati, Seok-Jin Heo, Therese Mitros, Denise P. Muñoz, Dario Boffelli, Donald B. Kohn, Mark C. Walters, Dana Carroll, David I. K. Martin and Jacob E. Corn

Sci Transl Med **8**, 360ra134360ra134.
DOI: 10.1126/scitranslmed.aaf9336

Hammering out the sickle cell mutation

Sickle cell disease is a genetic disorder caused by a mutation in one of the hemoglobin genes, which causes deformation of red blood cells and results in occlusion of blood vessels, severe pain crises, and progressive organ injury. To correct the mutation that causes this disease, DeWitt *et al.* modified hematopoietic stem cells from sickle cell disease patients using a CRISPR/Cas9 gene editing approach. The authors showed that the corrected cells successfully engrafted in a mouse model and produced enough normal hemoglobin to have a potential clinical benefit in the setting of sickle cell disease.

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