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GENE THERAPY

Development of β-globin gene correction in human hematopoietic stem cells as a potential durable treatment for sickle cell disease

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Sickle cell disease (SCD) is the most common serious monogenic disease with 300,000 births annually worldwide. SCD is an autosomal recessive disease resulting from a single point mutation in codon six of the β-globin gene (*HBB*). Ex vivo β-globin gene correction in autologous patient-derived hematopoietic stem and progenitor cells (HSPCs) may potentially provide a curative treatment for SCD. We previously developed a CRISPR-Cas9 gene targeting strategy that uses high-fidelity Cas9 precomplexed with chemically modified guide RNAs to induce recombinant adeno-associated virus serotype 6 (rAAV6)–mediated *HBB* gene correction of the SCD-causing mutation in HSPCs. Here, we demonstrate the preclinical feasibility, efficacy, and toxicology of *HBB* gene correction in plerixafor-mobilized CD34$^+$ cells from healthy and SCD patient donors (gcHBB-SCD). We achieved up to 60% *HBB* allelic correction in clinical-scale gcHBB-SCD manufacturing. After transplant into immunodeficient NSG mice, 20% gene correction was achieved with multilineage engraftment. The long-term safety, tumorigenicity, and toxicology study demonstrated no evidence of abnormal hematopoiesis, genotoxicity, or tumorigenicity from the engrafted gcHBB-SCD drug product. Together, these preclinical data support the safety, efficacy, and reproducibility of this gene correction strategy for initiation of a phase 1/2 clinical trial in patients with SCD.

INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive monogenic blood disorder, caused by a single point mutation (A>T) in the sixth codon of the β-globin gene. This missense mutation changes the amino acid from glutamic acid (E) to valine (V) and the formation of sickle β chains. The sickle β-globin protein forms abnormal hydrophobic interactions through axial and lateral contacts within sickling hemoglobin (HgbS) in the deoxygenated state leading to hemoglobin polymerization, sickle-shaped red blood cells (RBCs), and hemolytic anemia. SCD affects millions of people worldwide causing both a high medical burden to patients, families, and communities and high financial burden to these same groups and to the broader health care system (*1*). The main disease manifestations of SCD are vaso-capillary ischemia events, causing vaso-occlusive pain events and acute chest crisis. The current standard of care focuses on symptomatic relief involving pain control and blood transfusions, hydroxyurea administration, and recently approved drugs that inhibit HgbS polymerization (*2*) or reduce pain crisis (*3*). The only approved curative treatment to date is allogeneic human leukocyte antigen (HLA)–matched hematopoietic stem cell transplantation (allo-HSCT) (*4*, *5*). In patients undergoing allogenic transplant, long-term persistent mixed-donor chimerism ranging from 10 to 20% corresponded with beneficial clinical results by keeping HgbS concentrations below 50% (*6*–*8*). However, a matched sibling donor is only available in 10 to 15% of cases (*5*, *6*), and the use of haploidentical or unrelated donors is still considered experimental (*9*).

The gene therapy field has recently seen unprecedented efforts to provide alternative and potentially curative treatments for SCD by taking advantage of ex vivo lentiviral transduction of autologous hematopoietic stem and progenitor cells (HSPCs) (*10*–*13*). The reinfusion of autologous genetically modified HSPCs after patient myeloablation obviates the need to find donors for transplantation and decreases the risk of immune-related complications due to HLA mismatch between donor and recipient. Another crucial improvement in HSCT protocols for patients with sickle cell has been the introduction of plerixafor as an alternative HSPC-mobilizing agent to granulocyte colony-stimulating factor (G-CSF), which can cause toxicity and deaths in patients with SCD (*14*–*17*). Now, there are four ongoing clinical trials that use this strategy (clinicaltrials.gov: NCT02186418, NCT02247843, NCT02140554, and NCT03964792), with preliminary reports of therapeutic safety and efficacy emerging (*18*); however, lentiviral vector-related safety concerns and transduction efficiency are challenges (*19*, *20*), warranting further development of gene therapies for SCD.

The CRISPR-Cas9 system (*21*) has proven to be versatile, simple to engineer, and effective for gene editing of human HSPCs ex vivo (*22*–*25*). It can be used to induce precise genetic alterations, by

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exploiting the nonhomologous end joining (NHEJ) DNA repair pathway or by performing "gene correction" and "gene addition" through the homologous recombination (HR) repair pathway, enabled by the supply of a homologous DNA donor template (*26*). Several groups have demonstrated increased HR in ex vivo gene editing with recombinant adeno-associated virus serotype 6 (rAAV6)–mediated delivery of the donor template (*23*, *27*–*34*). Presently, several genome editing approaches in autologous HSPCs using the CRISPR-Cas9 system are being developed for SCD. Some strategies aim to reactivate the expression of the cognate fetal hemoglobin by interfering with the function of the β-globin switch master regulator BCL11A (*35*). Now, there is one open clinical trial (clinicaltrials.gov, NCT03745287) exploring this approach for SCD and two more for β-thalassemia major (clinicaltrials.gov, NCT03432364 and NCT03655678). However, a potentially more definitive approach is to directly correct the pathogenic sickle mutation in the *HBB* gene, thus preserving physiologic regulation of gene expression. Directly correcting the endogenous *HBB* gene will also remove expression of pathologic HgbS, addressing the problem of competition with the highly expressed pathogenic protein. We developed an *HBB* gene correction strategy that combined ribonucleoprotein (RNP) delivery of a high-fidelity (HiFi) Cas9 variant with rAAV6 DNA donor delivery that preserved HSC biological functions and is compatible with a clinical-scale manufacturing process (*23*, *28*).

Here, we describe the preclinical [investigational new drug (IND)–enabling] studies with *HBB* gene-corrected CD34$^+$ HSPCs to treat SCD (gcHBB-SCD), focusing on efficacy, safety, and clinical-scale manufacturing feasibility. In particular, we demonstrate (i) reproducible high-frequency *HBB* gene correction in plerixafor-mobilized HSPCs (plxHSPCs) from healthy donors and patients with SCD, (ii) long-term engraftment of *HBB* gene-corrected HSPCs in mice, (iii) absence of genotoxicity and tumorigenic potential, and (iv) high feasibility of manufacturing a full human clinical dose. Together, these studies provide the foundation supporting an IND submission for a phase 1/2 trial for the treatment of SCD.

RESULTS

HBB gene correction in plerixafor-mobilized, long-term engrafting HSPCs from healthy donors

We previously demonstrated the proof of concept of successful *HBB* targeting with a reporter donor cassette in long-term engrafting HSCs isolated from mobilized peripheral blood (*23*). We also showed feasibility of *HBB* gene correction for the codon 6 point mutation using an rAAV6 DNA donor sequence bearing six silent mutations (*23*, *36*, *37*). Here, we performed the gene correction process in clinically relevant plxHSPCs from healthy donors (Fig. 1A) (*24*). The *HBB* gene correction process was highly reproducible in five different HSPC donors, with a mean of 80% viability, 92% CD34$^+$ cells by fluorescence-activated cell sorting (FACS), and 22% *HBB* allelic gene correction (gcHBB) assessed by droplet-digital polymerase chain reaction (ddPCR) (fig. S1A). We next tested targeted HSPC engraftment potential and hematopoietic reconstitution in sublethally irradiated nonobese diabetic–scid-gamma (NSG) mice. We compared the systemic intravenous (tail vein) with the intrafemoral (IF) bone routes of delivery by assessing biodistribution and kinetics of HSPCs marked by a luciferase expression cassette targeted to the *HBB* locus (fig. S1B). Gene-targeted HSPCs showed faster engraftment with IF injection, but intravenous injection also resulted in broad biodistribution, with HSPCs homing to multiple bone marrow (BM) sites (fig. S1, C and D). Subsequently, we manufactured plxHSPCs for *HBB* gene correction (gcHBB-SCD) and administered the cells in a dose escalation into NSG mice using both intravenous and IF injection methods. Human engraftment was analyzed in the BM of NSG mice at week 16 after injection and showed comparable engraftment between the two administration routes at the high and intermediate cell doses but a higher engraftment for IF delivery at the low cell dose (fig. S2A). We observed bilineage reconstitution of lymphoid and myeloid cells (fig. S2B), and ~5% of long-term HSPCs retained gcHBB alleles, independent of route of administration (fig. S2C). Molecular analysis in human cells displayed on-site insertion/deletion (INDEL) frequency of 52%, along with an off-target (OT) INDEL frequency of 18% at an identified OT site (OT1) (fig. S2D) (*23*). These results provided the basis to proceed with intravenous injections in subsequent human HSPC engraftment studies using NSG mice. Furthermore, BM–residing HSPCs were also differentiated toward the erythroid lineage ex vivo for 14 to 16 days (Fig. 1A and fig. S3, A and B) and showed 2.5 and 16% gcHBB alleles in two independent experiments (fig. S3C). These results suggest that it is possible to target *HBB* for codon 6 gene correction in long-term engrafting plxHSPCs, albeit the frequencies obtained by these protocol settings might be disease-modifying rather than curative in an autologous transplant setting. This cautious interpretation is based on data from the study of patients having undergone allo-HSCT where claims that curative thresholds range from 5 to 30% for engrafted donor CD34$^+$ HSPCs. There is consensus, however, that the percent of gene-corrected cells needed to cure patients will vary from individual to individual.

Manufacturing protocol optimization to achieve higher frequencies of in vivo *HBB* gene correction with low detectable OT activity

On the basis of the above results, we sought to optimize the genome editing protocol to achieve higher in vivo retention of gene correction alleles with minimal OT activity. We investigated the following parameters: (i) the addition of the UM171 small molecule to the culture medium, which has been shown to promote HSC cycling and self-renewal (*38*), to increase rAAV6 transduction efficiency and HSC recovery (*39*) and to reduce reactive oxidative species (ROS) generation during cell cycle (*40*); (ii) the implementation of the low-density culture condition to promote HSC cycling (*27*, *41*); and (iii) the use of a HiFi Cas9 (R691A) variant that works well in the RNP format (*28*).

Whereas the addition of UM171 to the culture medium did not affect the in vitro gene targeting frequencies (fig. S4A) nor the in vivo engraftment potential of human CD34$^+$ cells (fig. S4B), it increased in vivo retention of gcHBB alleles with respect to the in vitro population [median ratio of 0.75 (in vivo divided by in vitro)] in the BM of transplanted mice (fig. S4C). When calculating the absolute number of human cells with corrected alleles on a mouse-by-mouse basis, we measured a ~3-fold increase in the total number of engrafted gcHBB-SCD cells in the UM171-treated group. Because of the large and known heterogeneity of human engraftment in the NSG model, the result was not statistically significant (unpaired Student's *t* test, $P > 0.05$) (fig. S4D). All gcHBB-SCD cell populations were also able to engraft in secondary transplant experiments (fig. S4E).

As previously demonstrated, low-density cell culture condition primed multilineage repopulating HSPCs for HR (fig. S5, A and B) (*27*, *41*). These findings corresponded with a decrease in proinflammatory cytokines in the cell culture medium (*41*, *42*) (fig. S5C).

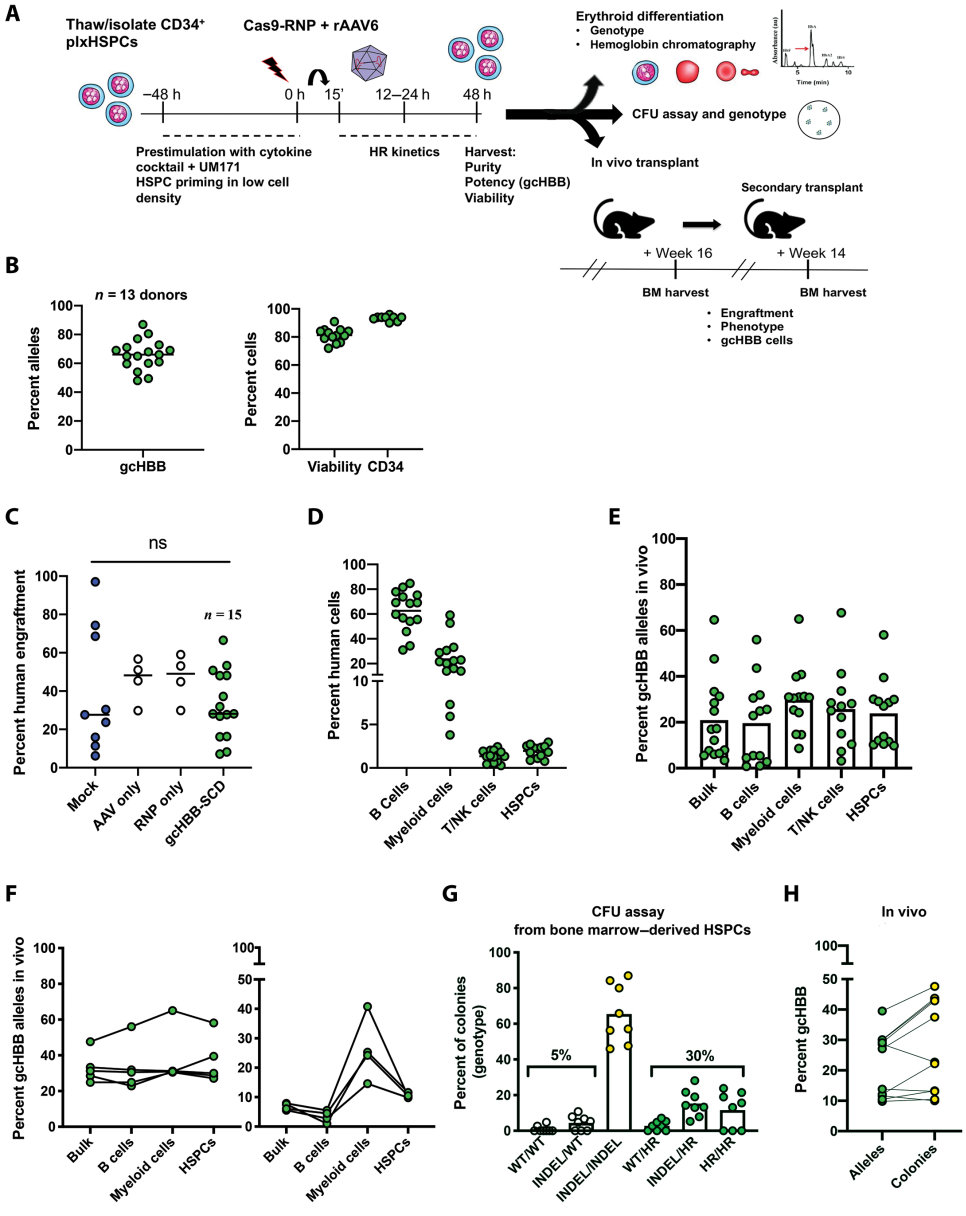

Fig. 1. Gene correction protocol optimization in healthy donor-derived HSPCs. (**A**) Schematics of cell manufacturing protocol, in vitro readouts, and experimental design of in vivo NSG mouse studies. (**B**) Percentage of in vitro gene-corrected alleles (gcHBB) (left), and viability and CD34 expression (right) in up to 13 cell donors; black lines indicate mean values. (**C**) Human chimerism in bone marrow (BM) of NSG mice at week 16 after injection. Control cells were (i) electroporated only (Mock), (ii) coupled with either Cas9 only (RNP only) or (iii) AAV6 only (AAV only). One-way ANOVA Kruskal-Wallis test plus Dunn's multiple comparisons test; ns, not significant. (**D**) Percent distribution of human hematopoietic lineages within the human cell population. NK, natural killer. (**E**) Percent gcHBB alleles in the human cell population (bulk) and in the respective hematopoietic lineages. (**F**) Percent gcHBB alleles in the bulk population and in a mouse-by-mouse analysis of human lineages in BM samples (data points from the same mouse are connected); mice showing good gcHBB allele representation in all lineages (left) or mostly in the myeloid compartment (right). (**G**) Quantification of *HBB* allele distribution in single CFU colonies derived from human HSPCs extracted at terminal analysis from BM of engrafted mice. WT, wild type; INDEL, insertion/deletion; HR, homologous recombination. Data point colors indicate cellular outcome with respect to *HBB* expression: white, "neutral"; yellow, "deficient"; green, "corrective." Brackets group genotypes per cellular outcomes; black bars indicate mean values. (**H**) Percent of gcHBB alleles in vivo (BM) linked to genotype of single sorted HSPC-derived CFUs from the same mouse. Pearson *r* test, $P < 0.001$. Black bars and lines indicate median values if not otherwise specified.

Cytokines such as transforming growth factor–α, interleukin-8 (IL-8), interleukin-1 receptor antagonist, interferon γ-induced protein 10, hepatocyte growth factor, and monocyte chemoattractant protein 1 were at significantly lower concentration in the medium at day 2, indicating a potential decreased effect of inhibitory feedback signals on cell growth [two-way analysis of variance (ANOVA) with Bonferroni posttest; $P < 0.001$) (*41*).

Last, we wanted to improve the editing safety profile by using a HiFi Cas9 protein. This modified Cas9 variant has been shown to retain the high on-target activity of wild-type (WT) Cas9 while reducing OT editing (*28*). When used in the gene-targeting protocol, HiFi Cas9 was equivalent to WT protein in on-target activity at the *HBB* gene, generating an average of 71% HR when coupled with rAAV6 transduction (fig. S5D). HiFi Cas9 markedly decreased INDEL frequency at OT1 below the limit of detection in both the in vitro culture and the in vivo engrafted HSPCs (fig. S5E).

Long-term engrafting HBB-corrected HSPCs in gcHBB-SCD above the predicted therapeutic threshold for SCD

We investigated whether the optimized manufacturing protocol using UM171, low-density culture condition, and HiFi Cas9 (Fig. 1A) would increase the ~5% gcHBB allele frequency in long-term engrafting plxHSPCs. The gcHBB-SCDs were manufactured using plxHSPCs from healthy donors and resulted in an average of 65% gcHBB alleles while consistently showing >80% viability and >90% $CD34^+$ cellular content (Fig. 1B). When studying the kinetics of rAAV6 transduction, we found an 8-hour minimum period of rAAV6 incubation necessary for efficient allele correction frequency, whereas the accumulation of gcHBB alleles was a function of time likely related to the completion of the HR-DNA repair process (fig. S6, A and B). NSG mice were transplanted with cells harvested 48 hours after RNP delivery and AAV transduction. gcHBB-SCD cells showed similar engraftment frequencies (28% median engraftment, $n = 15$) to control

cells, including electroporated only (mock, $n = 9$), RNP only ($n = 4$), and AAV6 only ($n = 4$) (Fig. 1C). Engrafted human cells derived from transplanted gcHBB-SCD were sorted from BM at week 16 using human lineage-specific markers (fig. S7). The human graft was multilineage and consisted mostly of $CD19^+$ B cells and $CD33^+$ myeloid cells (Fig. 1D). Genomic DNA was extracted from the different cell types, and gcHBB allele frequencies were analyzed in specific lineages. Across all the mice transplanted ($n = 15$), there was a median gcHBB allele frequency of 21, 20, 31, 27, and 24% in the bulk human cell population, B cells, myeloid cells, T/natural killer cells, and HSPCs, respectively (Fig. 1E). The rest of the *HBB* alleles analyzed in the bulk human cell population were ~60% INDELs and ~20% WT (fig. S8A). A mouse-by-mouse analysis highlighted that the majority of NSG mice had several human hematopoietic lineages derived from transplanted gcHBB-SCD, with similar frequencies of gcHBB alleles among the different lineages (Fig. 1F, left). A subset of mice had reduced B cell gene correction frequencies but sustained higher correction frequencies in the myeloid lineage (Fig. 1F, right). To estimate the percent of HSPCs that had at least one gcHBB allele in vivo, we single cell–sorted $CD34^+$ HSPCs harvested from BM of NSG mice ($n = 8$) into methylcellulose and genotyped cells derived from single colony-forming units (CFUs). Positive colonies for the gcHBB allele(s) were an average of 30% frequency (up to 50% in some donors), with an average of 12% carrying a biallelic gcHBB (Fig. 1G and fig. S8B). These frequencies of gcHBB positive colonies were ~1.3 times more than the allele frequency in the bulk cell population from NSG BM (Fig. 1H). These results indicate that gcHBB-SCD contains long-term repopulating HSCs with ~30% of cells carrying at least one *HBB*-corrected allele. A 30% cell frequency of at least one corrected allele matches the 30% threshold for donor cell chimerism needed for hematologic cure after allo-HSCT (*8*).

High-frequency *HBB* gene correction in plerixafor-mobilized, long-term engrafting HSPCs from patients with SCD

To understand whether the optimized plxHSPC manufacturing from healthy donors would translate to sickle cell HSPCs, we obtained cryopreserved $CD34^+$ plxHSPCs from two different patients with SCD (NCT03226691) and assessed the gcHBB-SCD manufacturing reproducibility and ability to differentiate into erythrocytes and express HgbA tetramers. In six independent experiments (three technical replicates in each biological donor), we achieved >60% gcHBB alleles (Fig. 2A) with minimal effect on cell viability (>80% viable cells) and expression of CD34 (>90% $CD34^+$ cells) (Fig. 2B), showing feasibility of the gene correction protocol in SCD-derived HSPCs. We plated gcHBB-SCD and mock control (electroporated HSPCs) into an erythroid differentiation medium and obtained >85% $CD45^-/CD34^-CD71^+$/Glycophorin A^+ RBCs (Fig. 2C and fig. S9A). gcHBB-SCD–derived erythroblast in vitro restored output of "corrective" hemoglobin (<10% HgbS, >25% HgbF, and >65% HgbA) in contrast with the mock control–derived erythroblasts (>85% HgbS, >10% HgbF, and 0% HgbA) (Fig. 2D and fig. S9B). As expected, the amount of HgbA restoration increased with the higher gcHBB allele frequencies (Fig. 2A). Of note, a slight increase in HgbF could be observed in both the gcHBB-SCD– and control-derived erythroid cells. This can be attributed to stress erythropoiesis with concomitant up-regulation of γ-globin, and it remains unclear whether this increase will persist in the clinical setting (*43*). We also efficiently manufactured nonmobilized peripheral blood $CD34^+$ cells from patients with SCD (an average of 57% gcHBB alleles) (*23*) and differentiated them toward the erythroid lineage, hence making similar findings that gcHBB alleles produced >90% HgbA (fig. S9C). Collectively, these data indicate that high frequencies of gene correction (for the codon 6 *HBB* gene mutation) can be achieved in SCD patients' HSPCs with restoration of nonpathologic HgbA expression in vitro.

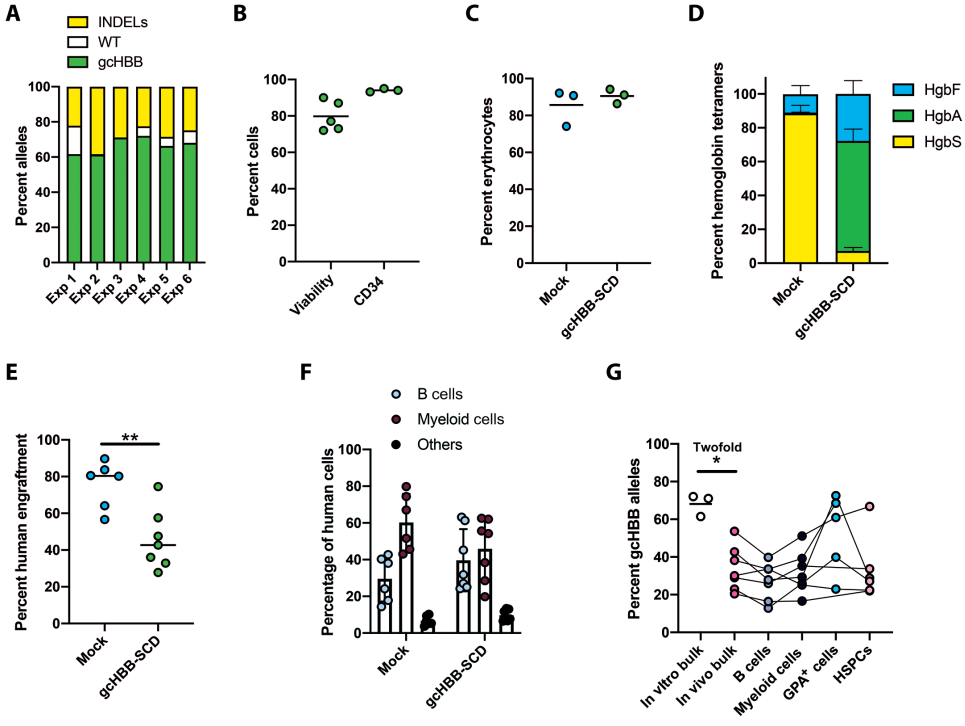

Fig. 2. Effective gene correction, recovery of normal hemoglobin output, and engraftment capability in SCD patient–derived HSPCs. (**A**) Percent allele modification in six gcHBB-SCD biological replicates (Exp 1 to Exp 6). (**B**) Viability ($n = 5$) and CD34 ($n = 3$) expression in vitro. (**C**) Percent of in vitro differentiated erythroid cells displaying $CD71^+$ $CD235a^+$ markers ($n = 3$) (see fig. S9A). (**D**) Quantification of hemoglobin tetramers (by high-performance liquid chromatography) on erythroid differentiated populations ($n = 3$). Two-way ANOVA plus Bonferroni's multiple comparisons test, $P < 0.0001$. (**E**) Human chimerism in the BM of NSG mice analyzed at week-16 after injection ($n = 7$). Mann-Whitney test; $^{**}P < 0.01$. Black bars indicate median values. (**F**) Percent distribution of human hematopoietic lineages in the NSG BM. (**G**) Percent of gcHBB alleles in the human cell population (bulk) in vitro ($n = 3$) and in vivo mouse by mouse ($n = 7$), along with the respective human hematopoietic lineages collected from NSG BM. Mann-Whitney test; $^*P < 0.05$. Black bars and lines indicate mean (± SD) if not differently specified.

We tested the ability of gcHBB-SCD manufactured from patients with SCD to engraft long term and reconstitute hematopoiesis. gcHBB-SCD along with mock controls were injected intravenously into sublethally irradiated NSG mice. BM human chimerism and gcHBB allele frequencies within the human cell compartment were evaluated 16 to 20 weeks after transplantation. Overall, SCD patient–derived plxHSPCs showed robust engraftment capacity with a median human chimerism of 80 and 42% for mock-electroporated HSPCs and gcHBB-SCD, respectively (Fig. 2E). The human graft was multilineage and consisted of mostly $CD19^+$ B cells and $CD33^+$ myeloid cells (Fig. 2F). The bulk human cell population in the BM of mice injected with gcHBB-SCD showed a robust percentage of gcHBB alleles (median of 30%). Furthermore, we isolated B cells, myeloid cells, erythroid cells, and HSPCs from the bulk human population and assessed similar gene correction frequencies in all the human hematopoietic lineages (Fig. 2G). These findings demonstrate long-term reconstitution of *HBB* gene-corrected HSCs with multilineage potential. In the erythroid compartment (GPA^+), gene correction frequencies were higher with a median of 60% gcHBB alleles (Fig. 2G). This frequency suggests that there is an in vivo enrichment of gene-corrected erythroid progenitors, which might have a better fitness compared to the noncorrected sickle cells or cells with biallelic INDELs (biallelic INDEL cells would lead to a β-thalassemia major phenotype because the INDELs are frameshifting). NSG mice engrafted with gcHBB-SCD generated from nonmobilized patient-derived peripheral blood HSPCs showed no decrease in gcHBB alleles in the BM graft with respect to the in vitro cell population (fig. S9, D and E). Engraftment with these cells was almost entirely myeloid, suggesting that the patient-derived nonmobilized $CD34^+$ HSPCs had a substantial myeloid bias (*44*). Nonetheless, even the nonmobilized $CD34^+$ HSPCs retained long-term engraftment potential after transplantation into NSG mice. Overall, these results demonstrate the therapeutic potential of the gene correction platform to treat SCD and directly revert the codon 6 mutation.

No evidence of in vivo oncogenic potential of gcHBB-SCD manufactured at clinical scale

To support the development of a first-in-human clinical trial for a SCD *HBB* gene correction therapy, we performed IND-enabling studies aimed at defining a gcHBB-SCD clinical-scale manufacturing protocol that met specifications for cell product potency (gcHBB alleles), identity and purity ($CD34^+$), and cell viability. To match the planned clinical manufacturing process, we used current good manufacturing practice (cGMP)–compliant cell culture reagents and engaged with key contract development and manufacturing organizations for large-scale provisions of key manufacturing reagents (see the Supplementary Materials). We adapted a protocol for rAAV6 production from previously published two-plasmid system technology (*45*). In particular, we developed the scale-up production process up to 3-liter scale in a suspension culture of human embryonic kidney (HEK) 293T cells. Several mid-scale rAAV6 lots were produced at the Stanford Laboratory of Cell and Gene Medicine (LCGM). Two of these (lot #3 and #4) were used in large-scale manufacturing of human HSPCs for gcHBB-SCD IND-enabling studies (fig. S10). We assessed the following parameters for clinical-scale manufactured gcHBB-SCD: (i) feasibility of $CD34^+$ harvest and manufacturing from plerixafor mobilized healthy donors, (ii) efficacy of gcHBB-SCD cell product manufactured at large scale, and (iii) long-term toxicological and tumorigenic potential in NSG mice.

Apheresis products of plerixafor-mobilized peripheral blood (on average, 50 billion of total nucleated cells) were purchased and freshly enriched for $CD34^+$ HSPCs. Once plated, cells were cultured for 2 days at a concentration of 2.5×10^5 cells/ml in cytokine-supplemented medium. At day 2, cells were collected and underwent the gene correction protocol adapted for large-scale manipulation. The final gcHBB-SCD product was then harvested at day 4 and underwent quality control testing and cryopreservation. We demonstrated the feasibility of manufacturing at clinically relevant cell numbers (2.3×10^7 to 1.3×10^8 $CD34^+$ HSPCs) with a mean of 77% viability, 94% $CD34^+$ cells, and 45% gcHBB alleles (table S1 and Fig. 3A). The CFU ability (CFU assay) of edited cells was twofold lower compared to untreated controls without lineage skewing (Fig. 3B and fig. S11, A and B). Genotype analysis of colonies showed a higher percent of gene-corrected HSPCs than gcHBB alleles (Fig. 3C), with an average 56% of cells having at least one corrected gcHBB allele (Fig. 3D and fig. S11C), of which 32 and 24% were mono- and biallelic events, respectively (Fig. 3D).

The long-term toxicology and tumorigenicity study was conducted using the gcHBB-SCD product generated from five of the six preclinical process qualification runs. Manufactured gcHBB-SCD cell products were thawed and injected into sublethally irradiated adult NSG mice. Vehicle control [phosphate-buffered saline (PBS)–injected] and untreated HSPCs were also injected to account for radiation toxicity and influence of treatment (fig. S11D). Mice were observed for the development of any adverse clinical signs and in life parameters (Supplementary Materials and Methods), with no differences seen between controls, mice injected with untreated HSPCs, and mice injected with gcHBB-SCD. Peripheral blood was collected at 8 and 16 weeks after injection and at termination (20 weeks after injection or at early euthanasia for clinical criteria), and samples were submitted to the Comparative Pathology Laboratory for complete blood count and leukocyte differential. Comparing vehicle control and untreated HSPC groups to the gcHBB-SCD group, there were no effects of the gcHBB-SCD treatment on hematological endpoints of the mice transplanted (fig. S12A). Histopathology observations performed in >40 different body tissues showed no gross lesions or adverse effects attributable to cellular infusion in all animals analyzed. All transplanted NSG mice were assessed for engraftment of human cells in the peripheral blood (weeks 8, 16, and 20), BM (week 20), and spleen (week 20). In the mice transplanted with gcHBB-SCD, there was a mean engraftment in the peripheral blood of 3.3, 0.5, and 0.8% at weeks 8, 16, and 20, respectively, whereas mice injected with untreated HSPCs displayed means of 19, 3.3, and 7.3%, respectively (Fig. 3E). Human chimerism in BM was a mean of 3.3 and 20.0% human cells in mice transplanted with gcHBB-SCD (n = 38 mice analyzed) and untreated HSPCs (n = 23 mice analyzed), respectively. There was increased human chimerism in the spleen for both mice groups, mean of 25.1% for gcHBB-SCD group and 66.0% for untreated HSPCs group. We further analyzed engraftment data separating female and male mice. Differences seen between mice transplanted with untreated HSPCs and gcHBB-SCD were statistically significant in most of the grouped analysis [one-way ANOVA Kruskal-Wallis test and Dunn's multiple comparisons test; not significant (ns); $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$; (Fig. 3E)]. Engraftment data in spleens were more similar to those of BM chimerism presented before (in Figs. 1 and 2 and figs. S1, S4, and S9) and indicated preferential homing of HSPCs to the spleen in these cohorts of mice

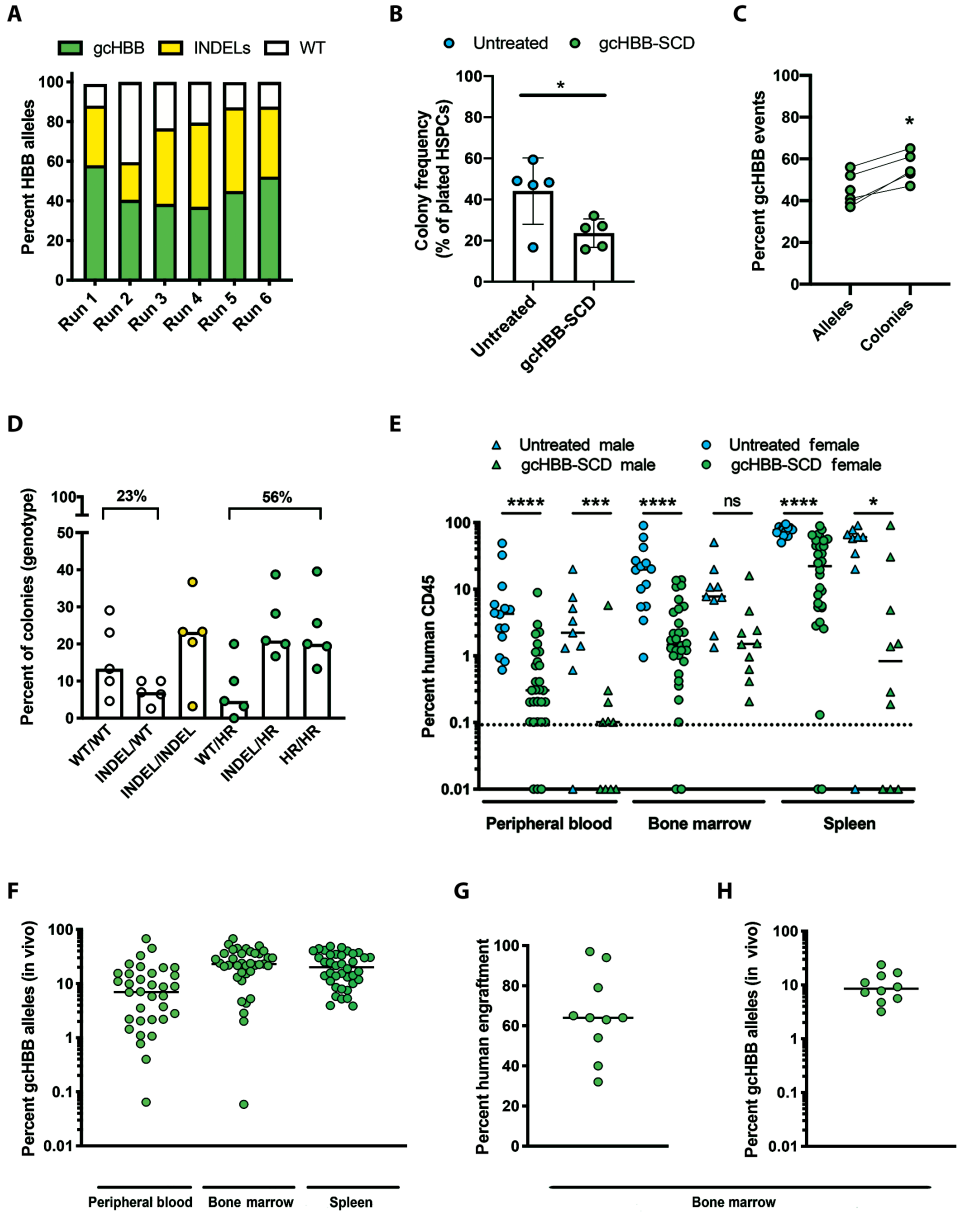

Fig. 3. Efficient scale-up of cell manufacturing in clinically relevant CD34$^+$ cells with lack of tumorigenicity in a long-term toxicology study. (**A**) Percent of allele modification in six medium-scale cell manufacturing runs (Run 1 to Run 6). (**B**) CFU frequency for gcHBB-SCD cell products and untreated cell counterpart. Paired *t* test, $^*P < 0.05$. (**C**) Scatter plot linking gene correction of *HBB* alleles in the bulk cell population to single genotyped colonies. Pearson *r* test, $^*P < 0.05$. (**D**) Quantification of *HBB* allele distribution in single CFU colonies (genotype). Data point colors indicate cellular outcome with respect to *HBB* expression: white, neutral; yellow, deficient; green, corrective. Brackets group genotypes per cellular outcome. (**E**) Human chimerism in hematopoietic tissues of NSG mice in long-term toxicology study. Untreated HSPCs (in blue) and gcHBB-SCD (in green) groups were segregated by sex. One-way ANOVA Kruskal-Wallis test plus Dunn's multiple comparisons test; $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$. (**F**) Percent gcHBB alleles in hematopoietic tissues collected at study end from the gcHBB-SCD NSG cohort ($n = 39$). (**G**) Percent human engraftment and (**H**) percent gcHBB alleles in BM of NSG mice injected with cell manufacturing run 5 at Stanford laboratory ($n = 10$). Black bars and lines indicate median values.

(one-way ANOVA and Friedman test). Several human hematopoietic lineages were represented within the human CD45 population in BM and spleen, with a prevalence of myeloid cells (fig. S12, B and C). Human cells engrafted in NSG mice in the toxicology study (n = 39 mice analyzed) contained a percentage of gcHBB alleles within ranges previously shown, with a median of 7.0, 23, and 20% in peripheral blood, BM, and spleen, respectively (Fig. 3F). We further confirmed robust engraftment potential of gcHBB-SCD when injecting in parallel cells from toxicology manufacturing run 5 (see table S1) in NSG mice at a Stanford laboratory (Fig. 3, G and H, and fig. S12D). In conclusion, gcHBB-SCD manufactured at clinical scale with cGMP-compatible reagents are capable of long-term engraftment into multiple sites of NSG mice with appropriate hematopoiesis and retention of gcHBB alleles at frequencies similar to our small-scale manufacturing protocols. There was no evidence of tumorigenicity or abnormal hematopoiesis from the manufactured products. There was continued evidence that the genetically modified cells did not engraft as well as control cells, a finding consistent with all genetic engineering approaches, including lentivirus (*46*, *47*).

Low molecular genotoxicity in gcHBB-SCD investigational cell products

Whereas the in vivo mouse study described above did not reveal functional tumorigenicity or abnormal hematopoiesis, a theoretical concern for genome editing using engineered nucleases is the possibility of generating double-strand breaks at OT sites in the genome that can create INDELs and/or translocations at genes regulating critical cell functions. The gcHBB-SCD lots manufactured for the long-term toxicology study were analyzed for genomic alterations. First, cells were karyotyped, and chromosomal analyses were performed. From 150 cell metaphases, there were no detectable clonal gross genomic aberrations (fig. S13, A and B). One gcHBB-SCD lot (LCGM-10819) had three metaphase anomalies reported; however, the cytogenetic report concluded that in the absence of additional like cells, none were considered clonal in nature and were likely technical artifacts. Nonetheless, to determine whether these findings were of clonal nature, we screened 15 additional metaphase spreads from the same gcHBB-SCD lot, and all showed normal karyotypes (fig. S13B). Of note, this gcHBB-SCD lot (LCGM-10819) showed long-term engraftment with no signs of tumorigenicity or abnormal hematopoiesis when injected in NSG mice in the toxicology study.

We performed an extensive OT analysis in the same gcHBB-SCD lots through NGS measurement of INDELs of a master list of putative OTs. The list was developed by cross-referencing three state-of-the-art methods for identifying potential OT sites of single-guide RNAs (sgRNAs) (GUIDE-seq, CIRCLE-seq, and COSMID) (table S2) (*48*). We created a master list of 67 OT sites to analyze by amplicon-based sequencing in gcHBB-SCD cells generated from the toxicology lots (table S2). The analysis showed that only a single OT site, the OT1 site previously mentioned, had measurable INDELs in all samples (0.5 to 2.9%) (Fig. 4A). This site is located in Chr9: 101,833,584 to 101,833,606 that is in an intergenic region (site is ~270,000 nt 3′ to closest gene—*GRIN3A*, Chr9:101,569,353) and was identified by all three methodologies for OT identification and has been previously confirmed as an OT (*23*, *49*). The HiFi Cas9 used in these studies markedly reduced the frequency of INDELs at OT1 (~2 and ~ 30% for HiFi and WT Cas9 nuclease, respectively) while maintaining high on-target activity as previously described (*28*).

We also used high-throughput, genome-wide, translocation sequencing (HTGTS) analysis, which is based on linear amplification–mediated PCR, to look for gross chromosomal rearrangements in cell products manufactured using the WT and HiFi Cas9 (Fig. 4B and fig. S14, A and B) (*50*, *51*). HTGTS confirmed OT1 on chromosome 9 as the main OT site (Fig. 4B) and demonstrated that HiFi Cas9 generated a consistent reduction in translocation frequency between the *HBB* locus and OT1 (Fig. 4, C and D). Specifically, whereas in WT Cas9-treated HSPCs, there was an average of 1.35% translocation frequency (156 *HBB*-OT1 junctions of 11,517 total reads), use of HiFi Cas9 allowed a frequency reduction of *HBB*-OT1 translocations to 0.04% (5 *HBB*-OT1 junctions of 11,517 total reads). These data suggest that the use of HiFi Cas9 to generate the gcHBB-SCD product reduces the chances of translocations by as much as 30-fold compared to WT Cas9.

A portion of junctional reads displayed a frequency that was independent of Cas9 specificity. These events included deletions of various size spanning 4-kb downstream of the break site (fig. S14, D and E). A small number of events included junctions from *HBB* to *HBD* gene and to a breakpoint cluster region. Overall, the frequency of gross chromosomal changes when using HiFi Cas9 was reduced to nearly undetectable levels.

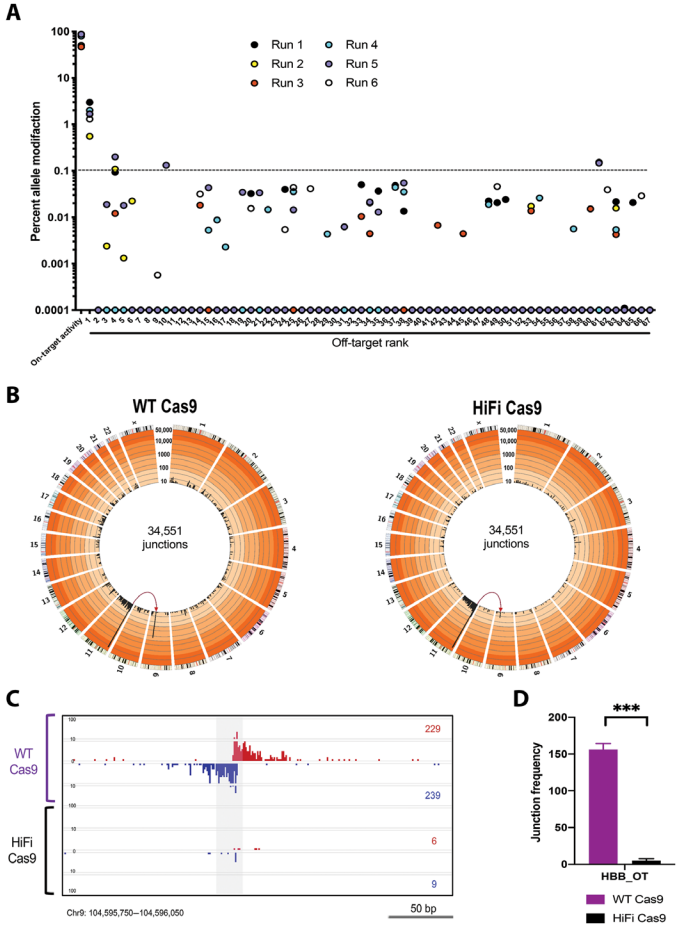

Fig. 4. Next-generation sequencing–based techniques reveal minimal genotoxicity in gcHBB-SCD cell products. (**A**) NGS results of 67 off-targets (OTs) from a master list of putative OTs. NGS was performed in six medium-scale manufacturing runs for toxicology study (Run 1 to Run 6). Dotted line indicates the assay detection threshold. (**B**) Circos plots of genome-wide prey junctions binned into 5-Mb regions (black bars) are plotted on a log scale with indicated ticks; frequency ranges are colored from light orange (10 to 100) to increasingly darker orange colors by factors of 10. Red arrow connects the *HBB* bait site on chromosome 11 to the OT hotspot on chromosome 9 (OT1). (**C**) Integrative Genomics Viewer plot of OT1 (coordinates bottom left); junctions are shown on a logarithmic scale. Shaded area represents the sgRNA footprint. Red and blue numbers indicate the number of junctions from the specified region that translocated in the plus and minus orientation, respectively. The bar (bottom right) indicates 50–base pair (bp) length. (**D**) Junction frequency at OT1 for WT and HiFi Cas9 (means ± SD). Paired *t* test; ***$P < 0.001$.

DISCUSSION

We described the preclinical development of an investigational autologous cell therapy that uses ex vivo Cas9-RNP and rAAV6 for *HBB* gene correction in human HSPCs to correct the SCD causing point mutation. The gene correction protocol was optimized in clinically relevant plxHSPCs and preserved the viability and HSC repopulation capacity in a xenotransplantation model. The overall gene correction frequencies and preservation of gene-corrected cells after transplantation into NSG mice exceeded what has been previously published using single-stranded oligonucleotide delivery (*44*, *52*, *53*).

A common challenge using genome editing strategies by many groups that exploit HR has been low numbers of gene-edited cells and their poor persistence after transplantation into NSG mice (*23*, *52*, *54*, *55*). We introduced protocol modifications that led to high frequencies of gcHBB alleles in vitro and increased the long-term engraftment of human cells with gcHBB alleles in vivo. In particular, the human engrafted population in NSG mice increased ~4-fold (from ~8 to ~30%) as did the frequency of gene-corrected alleles (from ~5 to ~20% gcHBB alleles) using healthy donor HSPCs. The percent human engraftment and gcHBB alleles were even higher in HSPCs derived from patients with SCD, with a median of 40 and 30%, respectively. In the majority of NSG mice analyzed at study termination, we further observed an enrichment of gene correction in GPA^+ erythroid cells (median of 50% gcHBB alleles), indicating a selective advantage of gene-corrected RBCs in vivo. The in vivo findings, combined with detecting >90% HgbA production in vitro derived from gene-corrected sickle cell HSPCs, provide strong evidence that erythroid lineage correction can be achieved in vivo in humans. In patients undergoing allogenic transplant, long-term persistent mixed-donor chimerism ranging from 10 to 20% corresponded with beneficial

clinical results (*6*–*8*, *56*). Likewise, even 1 to 5% normal donor HSPCs can result in the production of >50% nonsickling RBCs in the periphery due to the 10- to 30-fold survival advantage of nonsickling RBCs over sickling RBCs (*57*–*59*). These findings support the hypothesis that long-term engraftment with 10 to 20% "corrected" $CD34^+$ HSPC may be beneficial in reducing SCD symptoms. However, estimates of long-term engraftment from allo-HSCT may not completely reflect the biological process of engraftment of autologous gene-edited cells.

We believe that there are three key features of this *HBB* gene correction approach that might make it a more suitable therapeutic strategy than the recently reported investigational treatments based on HgbF up-regulation (*60*, *61*): (i) Each corrected *HBB* allele, in contrast to interfering with BCL11A expression, has a predetermined editing outcome; (ii) *HBB* gene correction directly converts HbS to HbA alleles, providing therapeutic function through β-globin expression, simultaneously reducing pathological HgbS, and increasing HgbA; and (iii) HgbA demonstrates a normal oxygen-binding affinity, whereas HgbF, conversely, has a higher oxygen affinity. This innate characteristic of HgbF is necessary for the survival of the fetus within the placenta but is ultimately shut off as it could impair oxygen delivery to tissues as development progresses. This can become problematic for future patients, especially in women as it has been reported that persistent elevated HgbF can complicate their pregnancies by intrauterine fetal growth restriction as compared to women with low to normal amounts of HgbF (*62*).

The NCT03282656 study uses lentiviral-mediated gene transfer of an shRNA for the erythroid-specific down-regulation of the BCL11A expression (*60*), and the NCT03745287 study performs targeted disruption of the erythroid specific BCL11A enhancer (*61*). These two ex vivo therapies, albeit representing differences in technology to modify HSPCs, reported similar therapeutic outcomes at early time points of follow-up (3 to 15 months), showing stabilization for some SCD clinical indices with ~30 to 40% HbF expression of total β-globin chains and with a clear selective advantage of the HgbF-expressing cell counterpart. The authors concluded that additional follow-up will determine the long-term effects of HgbF reactivation, although patients with hereditary persistence of fetal hemoglobin are far better clinically than patients with SCD. However, some questions remain for these approaches, specifically the use of LV as a method to express shRNA, associated risk of insertional mutagenesis for the former, and the unpredictable editing outcomes and genotoxicity when inducing a high number of NHEJ events for the latter. The long-term hematopoietic effects in humans of attempting to disrupt the *BCL11A* gene in the erythroid lineage are also unknown. Whereas targeting the BCL11A pathway shows great promise, one specific advantage of the direct gene correction approach described here is that it simultaneously increases HgbA and decreases pathologic HgbS.

In the scale-up of manufacturing of gcHBB-SCD, we encountered challenges, especially in product manufacturing runs 2 to 3, because of high RBC contamination negatively affecting purification of HSPCs from healthy donors after a single dose of plerixafor. This was an artifact of using healthy donors and would not be expected on the basis of the published results of plerixafor mobilization in patients with SCD who mobilize with faster kinetics and higher numbers than healthy donors.

The in vivo toxicology study demonstrated no evidence of tumors or abnormal hematopoiesis but did show reduced engraftment of edited cells compared to control cell populations. The reduced engraftment underscores the importance of initially infusing an adequate and safe cell dose to patients in a phase 1 trial to ensure hematopoietic reconstitution in a timely fashion.

The molecular analyses of genotoxicity demonstrated measurable OT changes, including OT INDELs at a single intergenic region and rare translocations between the on- and single OT site. The risk of tumorigenicity from these detectable changes is predicted to be low as the OT1 lies in an intergenic region of no known function, and there is no predictable oncogenic potential of the translocation. In human leukemias, for example, translocations into the *HBB* gene have never been described.

The limitation of this work is the consistent fall in percentage of gene-corrected cells after engraftment. The exact mechanism for this decrease is not known and is likely multifactorial resulting from, among others, the limitations of the highly oligoclonal model, some decrease in engraftment potential in cells having undergone gene correction, and a lower frequency of gene correction in human HSPCs that preferentially engraft after transplantation into immunodeficient mice. The results from the first human patients will give important information as to the relative importance of these different potential mechanisms. Nonetheless, the level of engraftment of gene-corrected cells still exceeded what is believed to be needed for patient benefit. In summary, given the tremendous unmet medical need and the potentially definitive therapeutic benefit of this investigational product, these preclinical data demonstrate safety and efficacy profiles that support the initiation of a phase 1/2 clinical trial in patients with severe SCD.

MATERIALS AND METHODS

Study design

The overall goal of this study was to assess the efficacy of an autologous cell therapy product of which uses ex vivo Cas9-RNP and rAAV6 to correct the SCD point mutation in human HSPCs. These preclinical (IND-enabling) studies, deemed sufficient for the proposed trial, focused on efficacy, safety, and clinical-scale manufacturing feasibility to warrant the initiation of a phase 1/2 clinical trial. Six- to 8-week-old NSG mice were enrolled in this study to assess the efficacy of the small-, medium-, and clinical-scale manufacturing protocol of *HBB* gene-corrected $CD34^+$ HSPCs. Small-scale manufacturing process studies followed an experimental protocol approved by the Stanford University's Administrative Panel on Laboratory Animal Care. Medium-scale manufacturing process studies took place in process development laboratories according to the standard operating procedures (SOPs) at the Stanford LCGM. Clinical-scale manufacturing process studies were conducted by In Vivo Services at the Jackson laboratory, Sacramento facility, an Office of Laboratory Animal Welfare–assured and American Association for Accreditation of Laboratory Animal Care–accredited organization. This study was performed according to an Institutional Animal Care and Use Committee–approved protocol and in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011). Each experiment derives results from at least three biological (or technical where warranted), unless indicated otherwise. Animal caretakers and investigators involved in specimen processing and analysis were blinded to the conditions of each respective study group until results were processed.

AAV6 vector design, production, and purification

AAV6 vector plasmids were cloned into the pAAV-MCS plasmid (Agilent Technologies, Santa Clara, CA, USA), with inverted terminal

repeats derived from AAV2. Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA, USA) was used for the creation of the gcSCD-AAV6 vector as per the manufacturer's instructions. The gcSCD-AAV6 vector includes 2.4 kb of homologous sequence to *HBB* with aforementioned single-nucleotide polymorphism correction and silent mutations (*23*). In addition, an AAV6 vector, designed for tracking of targeted HSPCs after transplantation, included a bicistronic Nluc complementary DNA (cDNA)–T2A-TurboGFP cDNA cassette driven by spleen focus-forming virus with 400-bp homology arms to *HBB*. AAV6 vectors were either produced as described previously (*27*, *63*) or purchased from Vigene Biosciences.

The mid-scale AAV6 lots were produced at the Stanford LCGM. A bank of HEK293T cells adapted to suspension growth was developed for AAV6 vector production. Cells were thawed at 37°C and expanded in FreeStyle F17 medium supplemented with 5 mM GlutaMAX and 0.2% (w/v) Pluronic F68. The cells were expanded up to a final volume of 3 liters in spinner flasks and cotransfected with the two plasmids described above, using a polyethylenimine-based transfection method. Two days after transfection, the transfected cells were harvested by centrifugation, the supernatant removed, the cell pellets resuspended in Dulbecco's PBS (DPBS), and then stored at −80° ± 20°C.

The cell suspension underwent chemical lysis using Triton X-100 [0.8% (v/v)] and clarification by depth filtration. The gcSCD-AAV6 was then purified from the clarified cell lysate by affinity column chromatography (AVB sepharose resin, Cytiva; formerly GE Healthcare) followed by ultrafiltration on a centrifugal filter unit, to concentrate and buffer exchange into the final formulation buffer, DPBS with 0.001% Pluronic F68. The product was filtered with an EKV sterilizing grade filter (0.2 μm; Pall Corporation) (fig. S10). In-process testing was performed on the eluate fractions from the purification, including silver stain SDS–polyacrylamide gel electrophoresis analysis and viral genomic titer determination by quantitative PCR. The final gcSCD-AAV6 products were an average titer of 8×10^{11} vg/ml.

All gcSCD-AAV6 lots were stored at −80° ± 20°C until use. All AAV6 vectors were further titered using ddPCR to measure the number of vector genomes as previously described (*64*).

In vitro culture of CD34$^+$ HSPCs

Human CD34$^+$ HSPCs were cultured in small-scale conditions as previously described (*23*, *24*, *27*, *65*, *66*). Frozen-purified CD34$^+$ HSPCs were sourced from plerixafor- and/or G-CSF–mobilized peripheral blood (AllCells, Alameda, CA, USA and STEMCELL Technologies, Vancouver, Canada). Frozen-purified CD34$^+$ HSPCs from plerixafor-mobilized peripheral blood of patients with SCD were provided by J.T., National Institutes of Health, Bethesda, MD. CD34$^+$ HSPCs were cultured at 1.0×10^5 to 1×10^6 cells/ml (as indicated in results) in StemSpan Serum-Free Expansion Medium II (STEMCELL Technologies, Vancouver, Canada) or Good Manufacturing Practice Stem Cell Growth Medium (SCGM, CellGenix, Freiburg, Germany) supplemented with four human cytokine (PeproTech, Rocky Hill, NJ, USA) cocktail, stem cell factor (100 ng/ml), thrombopoietin (100 ng/ml), Fms-like tyrosine kinase 3 ligand (100 ng/ml), Interleukin 6 (100 ng/ml), streptomycin (20 mg/ml), and penicillin (20 U/ml). StemRegenin1 (0.75 μM; STEMCell Technologies, Vancouver, Canada) and/or UM171 (35 nM; STEMCell Technologies, Vancouver, Canada) were added to the culture medium depending on experimental requirements (as indicated in Results).

The medium-scale cell manufacture for use in the nonclinical tumorigenicity/toxicology study took place in the unclassified process development laboratories according to the SOPs at the Stanford LCGM. Briefly, the manufacturing process for the gcHBB-SCD drug substance started with the immunomagnetic selection of CD34$^+$ HSPCs from G-CSF or plerixafor-mobilized apheresis products from healthy donors (AllCells and Hemacare). Before CD34$^+$ selection, the apheresis product was washed with CliniMACS PBS/EDTA buffer containing 0.5% (v/v) human serum albumin to remove platelets. This wash step was performed on the LOVO Cell Processing System. CD34$^+$ HSPCs were then isolated by positive selection on the CliniMACS Plus instrument (Miltenyi Biotec, Bergisch Gladbach, Germany) and were either freshly plated in culture (for manufacturing runs 1 to 4) or frozen before manufacturing (for manufacturing runs 5 and 6).

Gene correction procedure

The *HBB* synthetic chemically modified sgRNA was purchased from Synthego (Menlo Park, CA, USA). A large-scale production of sgRNA was provided by Agilent according to the manufacturer's proprietary process development (Agilent, Santa Clara, CA, USA). The synthetic modifications compose of the 2′-*O*-methyl-3′-phosphorothioate at the three terminal nucleotides of the 5′ and 3′ ends described previously (*22*). The target sequence for the *HBB* sgRNA (R-02) is as follows: 5′-CTTGCCCCACAGGGCAGTAACGG-3′ (*67*, *68*). The WT and HiFi Cas9 protein variants (Alt-R S.p. Cas9 nuclease V1, V2, and V3) used was purchased from Integrated DNA Technologies (Coralville, IA, USA) and Aldevron (Fargo, ND, USA). RNP electroporation (*24*–*26*, *30*) was performed using the Lonza 4D-Nucleofector in (micro)Nucleocuvette and 4D-Nucleofector LV Unit in 1-ml Nucleocuvette Cartridge (medium-scale) (program DZ-100). Electroporated cells were then plated at 2.5×10^5 cells/ml in the cytokine-supplemented medium. Immediately after, rAAV6 was dispensed onto cells at multiplicities of infection of 2.5×10^3 to 1.0×10^4 vector genomes per cell on the basis of titers determined by ddPCR and incubated for 24 hours. After, a medium addition or medium exchange was performed to dilute or remove any residual gcSCD-AAV6, and the CD34$^+$ HSPCs were cultured for an additional 12 to 24 hours.

Statistical analysis

All statistical tests on experimental groups were done using Prism7 GraphPad Software. The exact statistical tests used for each comparison are noted in the figure legends. For making multiple comparisons, we used one-way ANOVA Kruskal-Wallis multiple comparisons test with one variable and two-way ANOVA with Bonferroni posttest in case of two variables. For comparing the average mean of two sample groups, we used the unpaired Student's *t* test to reject the null hypothesis ($P < 0.05$). Further details and descriptions of Materials and Methods are included in the Supplementary Materials.

SUPPLEMENTARY MATERIALS

stm.sciencemag.org/cgi/content/full/13/598/eabf2444/DC1
Materials and Methods
Figs. S1 to S14
Tables S1 to S3
Data file S1
References (*69*–*77*)

View/request a protocol for this paper from *Bio-protocol*.

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