CRISPR/Cas9 gene editing for curing sickle cell disease

So Hyun Park, **Gang Bao**[*]

Department of Bioengineering, Rice University, 6500 Main St, Houston, TX, 77030, USA

Abstract

Sickle cell disease (SCD) is the most common monogenic blood disorder marked by severe pain, end-organ damage, and early mortality. Treatment options for SCD remain very limited. There are only four FDA approved drugs to reduce acute complications. The only curative therapy for SCD is hematopoietic stem cell transplantation, typically from a matched, related donor. *Ex vivo* engineering of autologous hematopoietic stem and progenitor cells followed by transplantation of genetically modified cells potentially provides a permanent cure applicable to all patients regardless of the availability of suitable donors and graft-*vs*-host disease. In this review, we focus on the use of CRISPR/Cas9 gene-editing for curing SCD, including the curative correction of SCD mutation in β-globin (HBB) and the induction of fetal hemoglobin to reverse sickling. We summarize the major achievements and challenges, aiming to provide a clearer perspective on the potential of gene-editing based approaches in curing SCD.

1. Introduction

Sickle cell disease (SCD) is the most common monogenic blood disorder affecting ~100,000 Americans and millions more worldwide [1,2]. SCD is caused by a single nucleotide change in the β-globin gene (HBB), replacing a hydrophilic glutamic acid with a hydrophobic valine at the sixth residue. The resulting hemoglobin S (HbS) polymerizes under hypoxic or acidic conditions [3], deforming the red blood cells (RBCs) into a rigid sickle shape with a reduced deformity and a shortened lifespan. Damaged RBCs lead to chronic hemolysis and hemolytic anemia, resulting in severe pain, end-organ damage, and early mortality in SCD patients [4,5].

Despite being the first molecular disease for which the genetic basis was known more than 60 years ago [6], treatment options for SCD remain very limited, and the average lifespan of patients with SCD has not improved over the last few decades [7]. There are only four FDA approved drugs to reduce acute complications; hydroxyurea (approved in 1998), L-

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[*]Corresponding author at: Department of Bioengineering, Rice University, 6500 Main St, BRC 413, Houston, TX, 77030, USA. shp9n@virginia.edu (S.H. Park), gang.bao@rice.edu (G. Bao).

Declaration of Competing Interest

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glutamine (approved in 2018), crizanlizumab-tmca (approved in 2019) and voxelotor (approved in 2020). The only curative therapy for SCD is a hematopoietic stem cell transplant (HSCT), typically from a matched related donor, which is available to only ~15 % of patients [8,9]. Morbidity and mortality from HSCT increase significantly when using matched but unrelated donors [8,9] or haploidentical donors [10]. Furthermore, there are substantial treatment-related risks and complications [11,12], and without modifications to existing regimens, this therapy is not safe for widespread adoption [13].

Autologous gene therapy, whereby in patients' own cells a copy of the "healthy" gene is added, or the mutated gene is corrected, or genes are inactivate, has the advantage of eliminating the need to find a matched donor. *Ex vivo* engineering of autologous hematopoietic stem and progenitor cells (HSPCs) followed by transplantation of genetically modified cells potentially provides a permanent cure applicable to patients regardless of the availability of suitable donors and without the risk of graft-*vs*-host disease [11,12,14]. Sickle RBCs mature inefficiently and have shorter lifespans compared to healthy RBCs, implying selective advantage of gene-corrected HSPCs over SCD HSPCs *in vivo*. As little as 2–5 % of donor chimerism post allogeneic transplantation is adequate to ameliorate SCD-related symptoms in patients with SCD, thus providing the rationale for a gene therapy approach [8,15]. Thus, successful gene addition or correction in relatively few HSCs could translate into a clinically meaningful level of RBC chimerism in the peripheral blood [16].

In the last two decades, gene therapy for SCD using a lentiviral-vector has proven to be curative in preclinical and clinical studies. The first patient with SCD treated with lentiviral vector–mediated addition of an antisickling HBB into autologous HSCs was successful, demonstrating a high level of therapeutic antisickling β-globin 15 months after treatment [14]. A self-inactivating (SIN) lentiviral vector encoding the human anti-sickling HBB, LentiGlobin, is currently being evaluated for safety and efficacy in clinical trials [17]. However, the use of lentiviral vectors poses potential risks such as generation of a replication-competent lentivirus (RCL) capable of infecting non-target cells, and insertional mutagenesis leading to clonal dominance and genotoxicity [18]. Although the recent results from the lentiviral gene therapy clinical trials provide the promise of *ex vivo* engineering of autologous HSPCs, longer follow-up is required to confirm the durability of the safety and efficacy of lentiviral-vector based gene therapy for SCD.

In contrast to conventional gene therapy approaches, gene-editing technologies offer the potential to permanently modify disease-causing genes through precise correction, deletion, addition, and disruption of specific sequences [19]. The product for therapy are gene-edited HSPCs from patients with SCD (SCD HSPCs) for autologous transplantation. Several gene editing strategies for curing SCD have shown promise in recent preclinical studies, including: (i) correction of the causative point mutation in HBB [20–24], (ii) induction of fetal hemoglobin (HbF) *via* gene-disruption of γ-globin (HBG) repressors [25–31], and (iii) induction of HbF *via* introducing beneficial hereditary persistence of fetal hemoglobin (HPFH) mutations on the β-globin locus [32–36].

The hemoglobin molecules consist of four subunits, two α polypeptide chains, and two β polypeptide chains. In a healthy adult, the overall hemoglobin composition is 97 % adult

hemoglobin (HbA, $\alpha 2\beta 2$), 3 % or less HbA_2 ($\alpha 2\delta 2$), and up to 1 % HbF (HbF, $\alpha 2\gamma 2$). HPFH is a benign condition caused by mutations within the β-globin gene cluster, which results in elevated HbF levels in adulthood [37]. Patients with SCD who have concomitant HPFH have milder clinical consequences, and elevated levels of HbF are correlated with reduced morbidity and mortality [37]. With an improved understanding of the globin locus regulation, there is considerable interest in developing approaches to induce HbF expression for therapeutic purposes. HbF induction can be achieved by silencing transcription factors such as B-cell lymphoma/leukemia 11A (BCL11A) that mediate silencing of HBG after birth [38] or mimicking beneficial HPFH mutations [39]. In addition, the identification of novel HbF regulators is an active area of research [40].

A gene-editing strategy using engineered nucleases such as TAL-effector nucleases (TALENs), zinc finger nucleases (ZFNs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems, creates a DNA double-strand break (DSB) at a user-defined location. The technology offers the potential to permanently repair disease-causing mutations through correction, deletion, addition, and disruption of the specific sequences mediated by targeted DSB generation followed by non-homologous end joining (NHEJ) or homology-directed repair (HDR) [19]. ZFNs and TALENs have distinct DNA binding domains, and both utilize the FokI endonuclease domain for cleaving the DNA [41]. However, the programming of these nucleases is complicated, time-consuming, and requires significant expertise. The class of programmable nuclease that has proved the most versatile and effective in recent years is the CRISPR/Cas9 system. CRISPR/Cas9 utilizes single guide RNA sequences (gRNA) that bind to a specific target site in the genome and to the Cas9 endonuclease. The Cas9 endonuclease is guided to a specific target site by homology between the gRNA and the target DNA sequences. Although the off-target effect remains a potential issue, it can be significantly reduced by rational gRNA designs or utilizing high-fidelity Cas9 protein [42]. Base editors are created by fusing a nucleotide deaminase with catalytically disabled Cas9 protein. Base editors directly convert one base into another without inducing DSBs and therefore not relying on HDR, enabling the point mutation correction in non-dividing cells. Therefore, base editors are a promising DNA editing tool and considered to be preferable to using Cas9 nuclease which may lead to the generation of unwanted small insertions/deletions (indels), translocations, or chromosomal rearrangements [43]. With the advancement of gene-editing technologies, each of the four technologies (ZFNs, TALENs, CRISPR/Cas9, base editor) have been tested in HSPCs for treating SCD. Studies showed the correction of the SCD mutation by delivering ZFNs [44] or TALENs [45] along with DNA donor template. Other groups developed ZFNs and TALEN targeting the HbF transcriptional repressors or the repressor binding site to induce HbF [26,46]. ZFN targeting the BCL11A locus has been utilized in a Phase-1/2 clinical trial (BIVV003, clinicaltrials.gov).

In this review, we focus on several approaches using CRISPR/Cas9 gene-editing for the treatment of SCD; specifically correcting the sickle mutation in HBB (Fig. 1), producing sufficient levels of HbF to reverse sickling by targeting the HbF transcriptional repressors, and introducing beneficial HPFH mutations. One particular example is the CRISPR/Cas9 gene-editing based Phase-1 clinical trial in a patient with severe SCD (CTX001, clinicaltrials.gov) using autologous CD34^{+} HSPCs in which the erythroid lineage-specific

enhancer of the BCL11A gene was modified to induce HbF expression. We summarize the major achievements and challenges in order to provide a clearer perspective on the potential of gene editing strategies as a cure for SCD. To translate the gene-editing based strategy to the clinic, many challenges exist, including the potential off-target effects, the need to further increase the efficiency of gene correction, and the *in vivo* engraftment of gene-edited HSPCs. Optimization of the genome-editing method, including the CRISPR Cas9/gRNA and donor template as well as the delivery method, is critical in achieving high safety and efficacy. Small improvements in each step is key for clinical translation.

2. Preclinical studies for ex vivo HSPCs gene editing and xenotransplantation

Most preclinical studies utilized *ex vivo* gene-editing of human HSPCs followed by transplantation in an immune-deficient mouse model. This is to assess the long-term engraftment potential of gene-edited HSCs since the durability of an autologous HSCT depends on the ability to modify HSCs permanently.

2.1. Cell culture

Human CD34$^+$ HSPCs can be isolated from several sources, such as cord blood, bone marrow, and mobilized peripheral blood. The majority of genome editing studies utilized peripheral blood CD34$^+$ HSPCs. Isolated CD34$^+$ cells were cultured in pre-stimulation media with cytokines for several days before gene-editing, as post-isolation culture with cytokine exposure has been shown to increase the gene-editing efficiency [47]. Several strategies have been employed to prime HSPCs for efficient gene-editing and stimulate the expansion of gene-edited HSCs.

Low cell density culture conditions and using a hematopoietic stem cell self-renewal agonist such as UM171 and StemRegenin 1 (SR1) have been shown to stimulate the expansion of gene-edited HSPCs as measured by higher engraftment levels in immunodeficient mice [48–50]. Edited CD34$^+$ cells can be cultured in media supporting erythroid differentiation for globin analysis by HPLC and flow cytometry. The functional impact of gene-editing on HSPC lineage commitment is evaluated using the colony-forming unit (CFU) assay by comparing the distribution of CFU between edited and non-edited controls.

2.2. Gene editing reagent delivery

Most studies used the CRISPR/Cas9 system derived from *Streptococcus pyogenes* (Spy Cas9). SpyCas9's gRNAs typically contain a 20-nt guide sequence with a $5^{'}$-NGG-$3^{'}$ PAM requirement. Over the years, the development and optimization of the CRISPR/Cas9 genome editing reagent and delivery method substantially improved the safety and efficiency of gene editing in HSPCs [33]. Early attempts used a plasmid DNA based system for Cas9 and gRNA expression, which resulted in low editing efficiency and high toxicity [51]. Achieving high editing efficiency, however, needs to be balanced with potential safety concern regarding off-target mutations and immunogenicity arising from sustained or excess expression of CRISPR components. Although all editing machinery components elicited immune, stress, and apoptotic responses, delivery of gRNA and Cas9 as a pre-complexed

ribonucleoprotein (RNP) is well-tolerated in CD34+ HSPCs, despite eliciting a DNA damage response (DDR) [52,53]. Electroporation using a nucleofection protocol is often the preferred method for direct delivery of RNPs to HSPCs, as this allows the RNP to enter the cell nucleus quickly, so it can immediately start cutting the genome. The majority of genome editing studies utilized RNP to achieve a high editing efficiency and specificity with lower cytotoxicity in CD34+ HSPCs. Chemical alterations to gRNAs further enhanced genome editing efficiency while reducing toxicity in CD34+ HSPCs [54]. High-fidelity variants of SpyCas9 maintain on-target activities comparable to wild-type SpyCas9 with reduced off-target activities in HSPCs [42]. For SCD mutation correction using the corrective donor template, clinical translation is hindered by a low ratio of HDR to NHEJ in long-term reconstituting HSCs. Cell cycle phase-specific regulation of DNA repair pathways through temporal regulation of Cas9 nuclease activity and transient synchronization of HSPCs in HDR-preferred phases have shown to improve HDR/NHEJ ratio *in vitro* [55]. The chemically modified synthetic gRNAs and high-fidelity Cas9 protein are commercially available, and optimal electroporation conditions for CD34+ cells have been established [21]. Further optimization steps, however, are still needed for each specific application concerning nuclease and donor template amount as *ex vivo* manipulation may negatively impact HSPCs engraftment and long-term repopulation capacity.

2.3. Transplantation study

NOD.Cg-Prkdc^{scid} Il2rg$_{tm1Wjl}$/SzJ (NSG) and NOD,B6.SCID Il2rγ$^{-/-}$ Kit$^{W41/W41}$ (NBSGW) are commonly used xenotransplant hosts for accessing multilineage engraftment of human hematopoietic cells. NSG strain requires sublethal myeloablative irradiation to achieve a high level of human chimerism. Since NSG does not support human erythropoiesis, engrafted HSCs are subjected to *in vitro* erythroid differentiation post-engraftment to assess globin expression and editing frequency in the erythroid lineages. NBSGW is an NSG-derivative strain and does not require preconditioning irradiation to support the engraftment of human HSCs. NBSGW supports not only myeloid and lymphoid but also erythroid engraftment [56]. Despite the widespread use of NSG and NBSGW models, it is difficult to extrapolate from a xenotransplant model of how these cells would behave in a clinical setting. Hans-Peter Kiem and colleagues [35] used a nonhuman primate (NHP) model to closely reproduce human stem cells in terms of kinetics of hematopoietic recovery, immunophenotypic markers, and cross-reactivity between cytokines. The use of a highly clinically relevant large-animal model for *ex vivo* HSPCs gene editing and transplantation offers an opportunity to monitor both long-term engraftment and hemoglobin profile, facilitating the translation of gene editing therapy to the clinic [26,35].

3. HbF induction by BCL11A gene editing

The level of HbF is a key modifier of the clinical severity of SCD, and reactivation of HbF by targeting genes involved in HbF regulation is a promising approach for treating SCD. Genome-wide association studies (GWAS) investigating individuals with HPFH have identified multiple causative genetic loci, and numerous transcription factors have been indirectly implicated in HbF silencing. BCL11A is the chief regulator of HbF level and suppresses fetal hemoglobin expression by association with other DNA-bound factors at

numerous positions within the β-globin locus, including direct HBG promoter repression by BCL11A [57]. Therefore, HbF reactivation through disruption of BCL11A or BCL11A binding motifs represents an attractive and discrete target for therapeutic gene editing for the treatment of SCD.

3.1. BCL11A gene disruption

An earlier study by Humbert et al. [26] validated the HbF repressor function of BCL11A and performed a proof-of-concept transplantation study in the NHP model with TALE nuclease mRNA targeting the BCL11A coding sequence. However, BCL11A plays varied roles in different hematopoietic lineages and coding variation at BCL11A is highly deleterious. Alternatively, naturally occurring genetic variation at the BCL11A enhancer is well-tolerated. Numerous studies have validated BCL11A erythroid enhancer as a target for HbF induction, offering a framework for erythroid-specific therapeutic genome editing by targeting the core sequences of BCL11A enhancer in HSPCs [25]. For example, Canver et al. [25] employed CRISPR lentiviral pooled gRNA screening to perform *in situ* saturating mutagenesis to study the organization and function of the BCL11A erythroid enhancer. A GATA1 motif that forms the core of an enhancer is essential for human erythroid BCL11A expression and HbF repression. Enhancer disruption by individual gRNAs in primary erythroid precursors results in substantial HbF induction while sparing BCL11A expression and function in non-erythroid contexts [25]. Chang et al. [58] and Psatha et al. [28] directly compared the functional consequences of BCL11A exonic *versus* enhancer disruption by using ZFN in HSPCs. BCL11A enhancer disruption showed comparable level of HbF reactivation with the BCL11A coding knockout (KO) while retaining the ability of BCL11A to support HSCs function including differentiation, reconstitution, and long-term engraftment potential [28,58]. Wu et al. [30] demonstrated highly efficient therapeutic gene-editing in HSPCs by CRISPR/Cas9 mediated disruption of GATA1 binding site at the +58 BCL11A erythroid enhancer. This resulted in a erythroid specific reduction of BCL11A expression, and therapeutic induction of fetal γ-globin in engrafting SCD HSCs [30]. The gRNA directly cleaving at the core of the +58 erythroid enhancer of BCL11A gave the highest levels HbF induction in erythroid progeny with the high rate of indels. Based on the clonal analysis of the erythroid progeny of CD34^{+} HSPCs edited at the BCL11A enhancer, biallelic modifications at the cleavage site resulted in robust induction of γ-globin. Edited human SCD CD34^{+} HSPCs were transplanted into immunodeficient NBSGW mice to study the impact of BCL11A enhancer editing on long-term engrafting HSCs. NBSGW supported similar levels of human myeloid, lymphoid, and erythroid engraftment of edited cells compared to unedited cells, validating gene editing of self-renewing HSCs. Indel frequencies at BCL11A enhancer persisted after secondary transplant demonstrating that BCL11A enhancer editing does not have a deleterious impact on stem cell function. Interestingly, the spectrum of indels in bulk HSPCs was different compared to that in the long-term engrafting HSCs, suggesting that engrafting HSCs may favor NHEJ as compared to Microhomology-mediated end joining (MMEJ) repair. Most prior studies reported a reduction of therapeutic allele levels after engraftment which raises questions about the durability of gene-editing in transplantation. The persistence of BCL11A enhancer edited cells suggests that the NHEJ-mediated gene disruption strategy could be more efficient over other gene-editing strategies relying on HDR or MMEJ. This is due to the fact that NHEJ is active in all stages of the cell

cycle and HSCs preferentially undergo NHEJ [30]. This study also tested the specificity and genotoxicity of CRISPR/Cas9 editing and did not observe detectable activities at Circularization for in vitro reporting of cleavage effects by sequencing (CIRCLE-seq) and *in-silico* predicted off-target sites or genotoxicity in terms of TP53 variants and stem cell function. Together, this work demonstrated that editing the BCL11A enhancer by CRISPR/ Cas9 is a practical therapeutic strategy to produce a durable therapeutic level of HbF induction in engrafting HSCs. Vertex Pharmaceuticals and CRISPR Therapeutics have obtained promising results in their Phase-1 clinical trial (CTX001, clinicaltrials.gov) using CRISPR/Cas9 to edit the BCL11A erythroid enhancer to induce HbF expression.

3.2. BCL11A base editing

A recent study by Zeng et al. [31] demonstrated the feasibility of producing therapeutic levels of base edits in multilineage-repopulating and self-renewing human HSCs. Base editing can potentially offer a high purity gene corrected product compared to nuclease-based editing. Base editors directly introduce base changes without inducing DSBs bypassing the low-efficiency HDR as well as DSB induced unwanted indels and off-target effects [30]. The A3A(N57Q)-BE3 base editor was delivered as an RNP targeting the BCL11A erythroid enhancer in SCD HSPCs. This base editor targets cytosine within the base editing window to disrupt the GATA1 motif. Two cycles of electroporation increased the therapeutic base editing rate but this also resulted in decreased viability and engraftment potential. Biallelic single base edits at the BCL11A enhancer within the GATA1 motif led to potent HbF induction similar to nuclease editing. Following transplantation into NBSGW mice, the base editing frequencies were reduced in engrafted HSCs compared to input HSPCs. Base edited cells showed multilineage reconstitution with similar base editing frequencies in each lineage. There was erythroid lineage specific BCL11A knockdown from erythroid enhancer disruption. For base-editing, both gRNA dependent and independent off-target editing need to be investigated. Although off-target base-editing can be minimized by reducing exposure to RNP and by utilizing the base editor with an attenuated cytosine deaminase domain, comprehensive off-target analysis needs to be performed before clinical implementation of base editing.

4. HbF induction by introducing HPFH mutations

The major fetal hemoglobin gene repressors, BCL11A and Leukemia/lymphoma-related factor (LRF), are directly bound to the HbG promoter at regions residing around 115 bp and 200 bp upstream of the transcription start site, respectively [59]. CRISPR/Cas9 mediated disruption of either the LRF- or the BCL11A-binding site in the HBG promoters induced significant HbF production. Traxler et al. [39] identified a naturally occurring 13 nucleotide (nt) HPFH deletion within the HBG promoter as a DNA target for genome editing. After CRISPR/Cas9 editing, the 13-nt deletion identical to the naturally occurring mutation predominates among other indels because the Cas9 cleavage site is flanked by 8-nt tandem repeats that facilitate MMEJ repair edited progenitors produced RBCs with increased HbF levels that were sufficient to reverse sickling *in vitro* [39]. This strategy has been advanced to demonstrate high-level editing in human HSCs capable of multilineage engraftment after transplantation into immunodeficient mice, and absence of detectable off-target mutations or

deleterious hematopoietic effects [60]. Humbert et al. [35] used an NHP autologous transplantation model to show the curative potential of this approach. The previously validated CRISPR gRNA target sites for human cells were used as the CRISPR target sites as this region of HBG is conserved between human and rhesus macaque. The gRNA target sites are located on the promoter of the homologous HBG1 and HBG2 genes. Considerable levels of large deletions due to simultaneous cleavage have been reported which remove the entire HBG2 gene and part of the HBG1 promoter. Although the frequency of large deletions was significantly reduced post-engraftment in NHP, the underlying mechanism remains unknown and the long-term clinical effect of the large deletion had not been determined [35].

4.1. HBG base editing

A recent study by Wang et al. [61] demonstrated that base editing that induced a single nucleotide substitution at the BCL11A binding site on the HBG promoter was enough to disrupt BCL11A binding and increase HBG expression. Since the base editor mediates base conversion without inducing DSBs, the HBG copy number was not affected, demonstrating that base editing may lead to safer therapeutic applications without creating further DSB induced damage in the genome. The efficiency of this approach has not been tested in engrafting HSCs.

4.2. LRF binding site editing

The transcription factor LRF represses expression of HbF independently from BCL11A [62]. Since knockdown of LRF increases HbF expression but delays erythroid differentiation, targeting of the LRF binding site in the HBG promoter was tested. Disruption of the LRF binding site by CRISPR/Cas9 ameliorated the SCD phenotype. HBG promoter editing is maintained in repopulating HSCs that differentiate into RBCs expressing therapeutically relevant HbF levels [36]. This study by Weber et al. [36] paved the way for simultaneously blocking both LRF and BCL11A which resulted in an additive effect on HbF. Given the independent role of LRF and BCL11A [62] and the efficient multiplex base-editing demonstrated by Zeng et al. [31], base editing to simultaneously disrupt both LRF and BCL11A repressor binding sites in the HBG promoters represent a promising strategy.

4.3. Large deletional HPFH

Several investigators have reported proof-of-concept CRISPR/Cas9 mediated gene editing to recapitulate large deletional HPFH mutation in the β-globin gene cluster as a new approach to treat SCD [32–34]. These approaches rely on NHEJ of paired DSBs to yield precise large deletions to mimic the naturally occurring Sicilian HPFH encompassing the δ- and β-globin genes or the corfu deletion of the γ-δ intergenic region. The efficiency of these maneuvers in engrafting HSCs has not been reported. Introducing a large deletional HPFH mutation requires the simultaneous processing of two DSBs and the re-joining of their distant ends, which led to low rates of large deletions and higher risks of off-target effects. In addition, competing genome editing outcomes, such as small indels and inversions accompanying these deletions, may limit clinical application.

5. SCD mutation correction using DNA donor template

Correction of the disease causing sickle mutation using gene-editing represents the most straightforward therapeutic approach. As shown in Fig. 1, in this approach, the CRISPR gRNA/Cas9 RNP complex targeting HBB together with DNA donor template are delivered into HSPCs isolated from patients with SCD, resulting in the HDR mediated correction of the causative mutation. Many viral based vector options have been evaluated in HSPCs for donor template delivery including integrase-deficient lentiviral systems (IDLVs), adenovirus 5/35 serotype (Ad5/35) and adeno-associated viruses (AAVs) [44]. Compared to other viral vectors, one main advantage of AAV is the low frequency of vector integration into the host genomic DNA and the low risk of related insertional mutagenesis and genotoxicity. Several studies demonstrated efficient targeted integration at the HBB locus in CD34^{+} HSPCs by using RNP combined with single-stranded oligodeoxynucleotides (ssODNs) [20–23,63]. rAAV6 [24,64] and ssODNs [20–23,63] donors have been used by most studies due to safety considerations and efficient HDR mediated by these donors. Differentiated erythroblasts from gene edited cells had an increase in mean levels of HbA and reduced the sickle cell phenotype [21]. Gene edited cells from patients with SCD were able to engraft in NSG [21] or NBSGW mouse transplant models [20] with gene correction observed at standard times post-transplant.

5.1. ssODN vs. rAAV6 donors

Recent reports directly compared the efficiencies of co-delivery of RNP in association with ssODN template or rAAV6-packaged template and demonstrated that the methodology for donor template delivery impacts long-term persistence of HBB gene-modified HSPCs [44,65]. *In vitro*, rAAV6 outperformed ssODN by causing less acute toxicity and inducing greater HDR. The RNP and rAAV6-edited cells showed lower engraftment in the NSBGW [65] or NSG [44] mice, suggesting that rAAV6 caused a decrease in the hematopoietic capacity. These results suggest that the ssODN template is likely to be more amenable for clinical translation than the viral based approach. The use of the ssODN template for gene correction also has the advantages of being easy to produce and having a low manufacturing cost, which will facilitate the application of a gene-editing based cure for SCD.

5.2. Potential risk of inducing β-thalassemia

Clinical translation of SCD mutation correction using the corrective donor template is currently hindered by a low ratio of HDR to NHEJ in long-term reconstituting HSCs. The possibility of inducing β-thalassemia major, intermediate or minor due to Cas9 cutting of *HBB* has not been carefully evaluated. In addition, the *in vivo* effects of Cas9 cleavage of HBB and reduction in functional β-globin levels in a patient with SCD remain unclear and will need to be addressed in a clinical trial.

5.3. HbF induction

Several studies reported upregulation of HbF as a result of HBB disruption in CD34^{+} HSPCs [20–22]. It is possible that the increase in HbF percentage is due to Cas9 cleavage induced HBB KO or due to the increase in hemoglobin formation between α-globin and γ-globin given that β-globin chains are unavailable. However, the molecular mechanism underlying

the HbF induction observed here remains elusive and warrants further investigation. Recently, Boontanrart et al. [66] reported that cellular erythroid stress caused by β-globin knockout can induce robust re-expression of γ-globin, providing mechanistic insight to the poorly understood phenomenon of stress-induced globin compensation. It is also still unclear if Cas9 *HBB*-cleavage induced increase in HbF percentage would have a long-lasting effect at a therapeutically relevant level, and if the resulting benefits to patients with SCD would outpace the potential harm of *HBB* disruption, including the possibility of inducing β-thalassemia major or minor. Addressing these issues will facilitate the safe and effective clinical translation of a gene-editing based treatment strategy for SCD.

6.   *In vivo* gene editing

Although *ex vivo* gene-editing has many advantages, including a high editing efficiency and the ability to ablate the unedited HSPCs in the patient, the high cost will prevent the applicability of this therapy to patients with SCD in resource-poor regions. Attempts have been made to develop *in vivo* HSC transduction/selection technology using non-integrating adenovirus. *In vivo* HBG-promoter editing by CRISPR/Cas9 in β-YAC/CD46-transgenic mice has been performed [67]. The human CD46-targeting adenovirus vector (HDAd-HBG-CRISPR/mgmt) expresses CRISPR/Cas9 which targets the HBG promoter for γ-globin reactivation. The vector also contains a O-6-methylguanine-DNA methyltransferase (MGMT^{P140K}) cassette for *in vivo* selection of transduced cells using chemotherapeutic drugs [67]. CD46 is uniformly expressed on HSPCs for hematopoietic tissue targeted viral transduction. The β-YAC/CD46 mice carrying the human β-globin gene locus express human CD46 at a level and in a pattern similar to humans which allows for direct *in vivo* analysis of γ-globin reactivation using the human CD46-targeting adenovirus vector. Because direct transduction of HSPCs localized in the BM is inefficient, the *in vivo* HSPC transduction approach involves HSPCs mobilized from the bone marrow into the peripheral blood followed by intravenous injections of the adenovirus vector (HDAd-HBG-CRISPR/mgmt). This resulted in the reactivation of human γ-globin in erythrocytes of adult animals that was maintained after secondary transplantation of HSPCs [67]. Since mobilized HSCs transduced in the peripheral blood could home to the bone marrow and renew themselves [68], this approach could generate a long-lasting effect.

While promising, *in vivo* gene editing for curing SCD has a lot challenges. Both high *in vivo* delivery efficiency and high editing efficiency in SCD HSCs are required, and off-target cell/tissue editing is a potential concern. Although viral vector based *in vivo* delivery of gene-editing machinery can be highly efficient, it may lead to uncontrollable expression of Cas9/gRNA, causing genotoxicity and activating an immune response [69–71]. On the other hand, non-viral based *in vivo* delivery vehicles may have low efficiencies and broad biodistribution, and repeated injections are often needed for a high delivery efficiency [72]. It is also necessary to compare systemic delivery and local injection to determine the best delivery strategy. The percentage of *in vivo* gene-edited HSCs required for a cure is currently unknown since the unedited HSCs would still produce sickle cells.

7. Potential risks of off-target mutations caused by CRISPR/Cas9

Genome editing poses new challenges since its mechanism of action is different from the conventional gene therapy. In contrast to the knock-in or knockdown gene therapy, which generally requires the continuous and long-term expression of therapeutic genes for treatment, permanent gene modification can be achieved with a single delivery of CRISPR/Cas9. Due to the tolerance for nucleotide mismatches between target DNA and gRNA, the utility of CRISPR-Cas9 systems for genome editing may be compromised by their off-target activity [73–75]. The off-target activity of Cas9 nuclease can cause disruption of normal gene function and genome instability *via* large chromosomal rearrangements [76], which is of serious concern in human gene therapies, potentially leading to difficult-to-predict side effects. Importantly, the long-term expression of Cas9 nuclease *via* plasmid and viral vectors in treated cells means there is a potential for off-target cleavages to accumulate over time [75]. While delivery of gRNA and Cas9 as RNP and utilization of high-fidelity Cas9 have shown to significantly reduce off-target editing, off-target effects are not eliminated. Therefore, better systems for detecting and quantifying these aberrant events are needed to validate potential off-target sites and to aid in optimizing strategies to minimize off-target mutations without sacrificing gene correction efficiency. In addition, a robust, rapid, high-throughput method for monitoring off-target events over time is necessary to assess the long-term toxicity or off-target effects of the system especially for clinical applications of CRISPR-Cas9. Recent advances in off-target site identification using genome-wide unbiased methods such as Chip-seq [77], GUIDE-seq [78], BLESS [79], and END-seq [80] has given rise to a new problem in off-target site validation. The current gold standard for quantifying Cas9 off-target activity is PCR amplification, followed by next-generation sequencing. This method allows for multiple sites to be assessed simultaneously with a high degree of sensitivity. However, recent advances in CRISPR/Cas9 off-target site identification has revealed many sites that cannot be identified by deep sequencing due to a detection limit of 0.1 % by deep sequencing for accurate indel identification [81,82]. Recent publications have reported frequent large deletions and insertions after CRISPR-Cas9 cleavage in mouse embryonic stem cells, mouse hematopoietic progenitors and a human differentiated cell line [83]. Although the large deletions/insertions at the on- and off-target sites and the large chromosomal rearrangements between on- and off-target sites typically have low occurrence, they pose a significant safety concern since even a very small number of HSCs harboring these detrimental events could cause hematological malignancies after HSCT. Next generation sequencing also has significant costs, long turnaround times, and requires the development of robust bioinformatics pipelines, all of which prevent quick sample screening. In addition, any gross chromosomal rearrangements between an on- and off-target DNA break or two off-target DNA breaks cannot be identified by most methods. There are currently no standard methods for quantification of large chromosomal rearrangements induced by CRISPR/Cas9. For therapeutic genome editing, potential off-target effects need to be carefully analyzed because significant challenges exist in both accurately predicting potential off-target sites and in performing genome-wide unbiased searches. As CRISPR/Cas9 moves towards clinical application, there is a need for robust patient follow up and monitoring methods. Just as drug treatments have side-effects, CRISPR/Cas9 treatments will likely have some degree of off-target edits (side effects) that will require careful monitoring over time to ensure that these events do not have a proliferative effect.

8. Future perspectives and challenges

With the advancement of CRISPR/Cas9 technology, autologous transplant of gene-edited hematopoietic stem cells could potentially provide a cure for most patients with SCD. However, to translate the gene-editing based SCD treatment strategy to the clinic, many challenges exist, including the need for high editing efficiency and low off-target effects. Quantitative understanding of the genotypic and phenotypic consequences of a diverse array of mutations in the CRISPR/Cas9 edited SCD CD34+ cells is essential for safe clinical applications. The development of the editing strategies which allow high yields of long-term repopulating HSCs that have a polyclonal and a high proportion of gene-edited cells after engraftment remains a challenge. Furthermore, there is limited knowledge of the impact of SCD pathology on HSPC viability and engraftment potential, particularly in patients exposed to years of SCD related chronic inflammation. To date, most SCD-related *in vivo* engraftment studies are performed with cells derived from healthy individuals, limiting our understanding of the effects of chronic systemic inflammation and ineffective erythropoiesis associated with HSPCs from patients with SCD. The source of HSPCs and the SCD pathology of the individuals, including differences in patient conditions, could have a significant effect on both the gene-editing outcomes and the engraftment potential. Genetic and environmental factors could likely influence the viability and functions of SCD HSPCs.

Current *ex vivo* gene-editing approaches have some shortcomings throughout the process. Only a small percentage of CD34+ cells from patients with SCD are typically HSCs. Harvesting HSCs from the bone marrow is invasive. Patients undergoing myeloablative chemotherapy also experience chemotherapy related side effects such as low blood counts and infections. *In vitro* culture and gene editing of HSCs lead to loss of HSC pluripotency and engraftment potential. Furthermore, providing an *ex vivo* gene-editing based cure to patients may be prohibitive due to the high cost which is driven by the need for highly specialized facilities and the technical expertise required. *In vivo* gene-editing of HSCs can potentially overcome the limitations of *ex vivo* gene-editing since administration of *in vivo* therapy could be minimally invasive and cost effective; therefore, more readily available in resource-poor regions. However, major challenges exist in developing *in vivo* gene-editing as a clinically viable approach, including achieving both high *in vivo* delivery efficiency and high editing efficiency. Although the development of *in vivo* gene-editing based therapies for SCD is still in its infancy, the collaborative between the NIH and the Bill and Melinda Gates Foundation to support the development of a curative *in vivo* gene therapy approach for SCD will greatly accelerate technological development and innovation.

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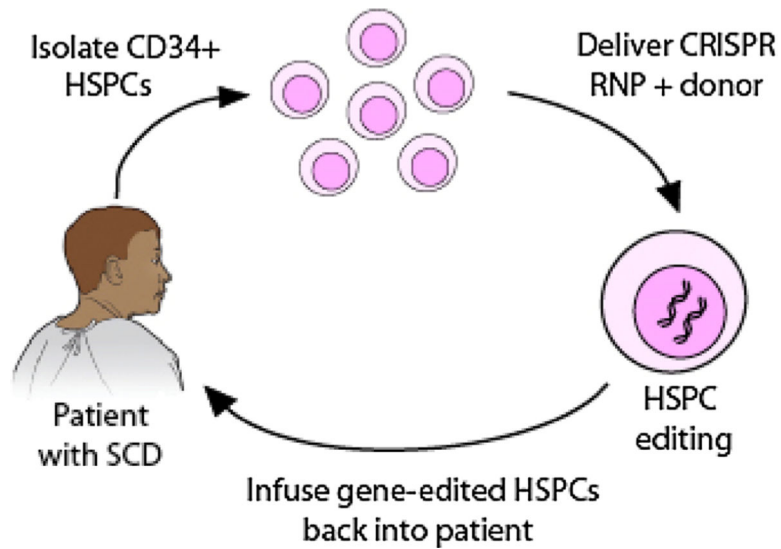
Fig. 1. Genome editing based strategy for treating sickle cell disease.
CD34+ HSPCs are first isolated from a patient with sickle cell disease. The RNP (ribonecleoprotein) complex of CRISPR guide RNA with Cas9 protein and DNA donor template are delivered into the nuclei of HSPCs *via* electroporation for gene correction. The gene-edited HSPCs are then infused back into the patient to reverse the disease phenotype. To make the gene-editing strategy a clinically viable approach, both high efficacy and adequate safety need to be achieved.