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Gene therapy for sickle cell disease: An update

SELAMI DEMIRCI, NAOYA UCHIDA & JOHN F. TISDALE

Molecular and Clinical Hematology Branch, National Heart, Lung, and Blood Institute, Bethesda, Maryland, USA

Abstract

Sickle cell disease (SCD) is one of the most common life-threatening monogenic diseases affecting millions of people worldwide. Allogenic hematopietic stem cell transplantation is the only known cure for the disease with high success rates, but the limited availability of matched sibling donors and the high risk of transplantation-related side effects force the scientific community to envision additional therapies. *Ex vivo* gene therapy through globin gene addition has been investigated extensively and is currently being tested in clinical trials that have begun reporting encouraging data. Recent improvements in our understanding of the molecular pathways controlling mammalian erythropoiesis and globin switching offer new and exciting therapeutic options. Rapid and substantial advances in genome engineering tools, particularly CRISPR/Cas9, have raised the possibility of genetic correction in induced pluripotent stem cells as well as patient-derived hematopoietic stem and progenitor cells. However, these techniques are still in their infancy, and safety/efficacy issues remain that must be addressed before translating these promising techniques into clinical practice.

Key Words: *BCL11A, fetal globin, gene addition, genome engineering, sickle cell anemia*

Introduction

Sickle cell disease (SCD) is a severe hereditary form of anemia that results from a single mutation at the sixth codon of the β-globin chain (from glutamic acid to valine) of the adult hemoglobin (Hb) tetramer ($\alpha_2\beta_2$) [1], which is prone to polymerization at low oxygen levels. It is one of the most prevalent and severe monogenetic disorders, and more than 100 000 individuals in the United States and several million around the world are affected by both acute and chronic manifestations of SCD, such as frequent pain crises, silent cerebral infarct, stroke, end organ damage and early death [2]. Polymerized sickle hemoglobin (HbS, $\alpha_2\beta^S{}_2$) interferes with red blood cell biconcave architecture and flexibility, resulting in crescent-shaped cells with enhanced adherence to the vascular endothelium, and hemolysis, which obstructs blood flow [3]. More detailed reviews on the pathophysiology [4] and genetics [5] of SCD are available in the literature for further reading.

Since its original description more than a century ago [6], treatments that only reduce the symptoms and complications of SCD such as blood transfusions, preventive therapies including penicillin prophylaxis and pneumococcal vaccination, and hydroxyurea therapy have been leveraged in the clinics. Blood transfusion, however, does not correct the phenotype and results in iron overload when not accompanied by aggressive chelation therapy. Hydroxyurea treatment provides clinical benefit through the induction of fetal globin (HbF, $\alpha_2\gamma_2$), which competes with sickle globin; thus, it reduces SCD symptoms, but response to hydroxyurea is not uniform among patients, and concerns for long-term use remain despite abundant evidence for safety [4]. On the other hand, substantial advances in cellular and molecular biology have led to some powerful tools that we have begun to employ. Of those, allogenic hematopoietic stem cell (HSC) transplantation is as of yet the only available curative option for patients with severe disease [7]. Despite the considerably high success rate of HSC transplantation, a significant proportion of the candidates (>80%) do not have a suitable matched sibling donor, and there remains a risk for graft rejection, graft-versus-host disease and transplant-related mortality [8–11]. Improvements have been made with reduced intensity condition; however, this approach also remains limited by donor availability [9].

For those lacking a suitable allogeneic HSC donor, genetic strategies targeting autologous HSCs remains an alternative (Figure 1). In theory, because genetically modified therapeutic cells are of patient origin, the risk for graft-versus-host disease and transplant rejection can be virtually eliminated, abrogating the need for immunosuppression as part of the

Correspondence: **John F. Tisdale**, MD, Molecular and Clinical Hematology Branch, National Heart, Lung, and Blood Institute, Building 10, Room 9N112, 10 Center Drive, Bethesda, Maryland 20814. E-mail: johntis@mail.nih.gov

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conditioning regimen. Primary results obtained from clinical trials with genetically modified autologous HSCs expressing potential therapeutic genes for immunodeficiency disorders [12–21] have encouraged a focus in blood-related diseases. The globin disorders, although long held as a therapeutic target, have proven much more difficult because of the necessity of regulated, lineage-specific, high-level globin expression. In this review, general gene therapy approaches for SCD including gene addition and genome editing technologies for decreasing SCD symptoms by enhancing HbF or correcting the mutation in the β-globin sequence are outlined.

Stable gene addition with lentiviral vectors

HSCs are limited in the human body, and prolonged cultivation of HCSs in *in vitro* conditions changes the potential of stem cells. Therefore, determining efficient gene transfer systems providing stable expression of a target gene at therapeutic levels without leading to any safety concerns after modification, such as an immunogenic response or oncogenesis due to random insertional mutagenesis, is one of the main hurdles to clinical application. After the first gene therapy trial using a γ-retroviral vector to transduce mobilized $CD34^+$ cells for the treatment of severe combined immunodeficiency (SCID) was reported [22], the potential of these viral vectors has been widely investigated for various hematological disorders. However, the use of the γ-retroviral vectors in clinical trials was marred because they are not capable of transducing non-dividing cells, including HCSs in quiescent state; cannot carry large gene sets such as β-globin and its regulatory elements required for high-level expression; and present relatively instable RNA to be reverse transcribed and delivered to the nucleus [23,24]. Most important, treatment with γ-retroviral vector-transduced HSCs in various disorders led to leukemia or myelodysplasia, which was attributed to vector insertion near proto-oncogenes, activated due to enhancer sequences in the retroviral long terminal repeats [25]. The development of human immunodeficiency virus type 1 (HIV-1)-derived vectors, belonging to the lentivirus family, circumvent these safety and efficacy issues. Apart from transducing non-dividing cells and transferring large sequences, they have displayed a safe profile without any sign of insertional oncogenesis or mutagenesis in SCD and *β*-thalassemia patients for 4 to 30 months [26,27]. The self-inactivating design in the U3 region of the 3′ long terminal repeat region, removing accessory genes, and separating packaging components, have made lentiviral vectors a relatively safer option, reducing the possibility of insertional oncogenesis, genotoxicity, and the development of replication competent counterparts [28]. Although lentiviral vectors have the confidence of the scientific community, recent work suggests that foamy viral vectors might also provide a safe option [29,30].

β-globin gene addition

The initial idea of inhibiting HbS polymerization in gene therapy applications emerged with the introduction of a functional β-globin transgene into HSCs. However, efficient and erythroid-specific expression of transgene in reconstituting HSCs remained insufficient in *in vivo* studies [31,32]. Incorporation of the human β-globin locus control region (LCR)

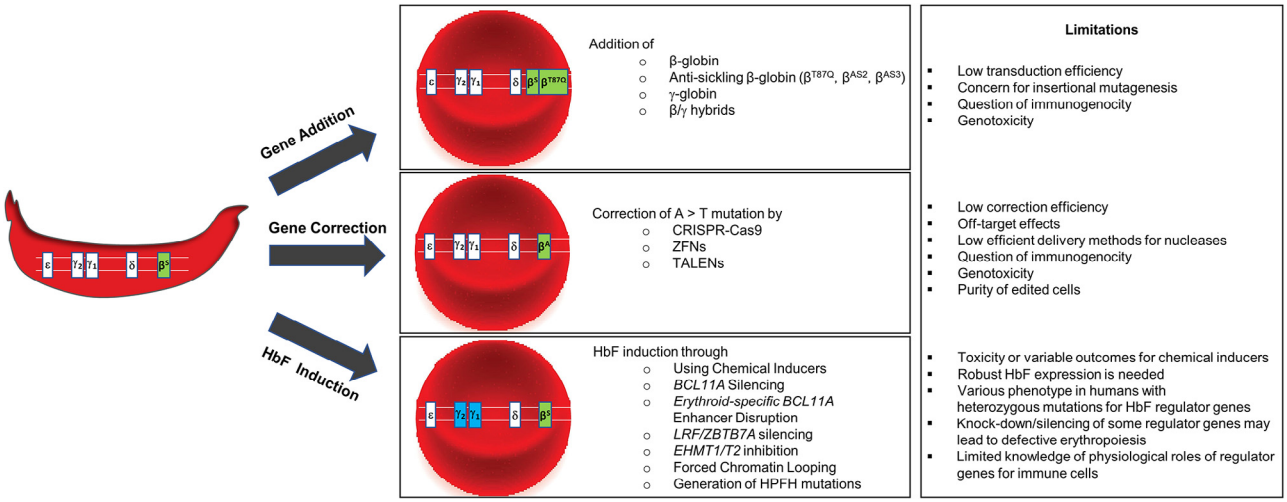

Figure 1. Genetic strategies for sickle cell disease. Anti-sickling protein coding gene addition, fetal globin induction via knocking-down/silencing of repressors of γ-globin gene, and sickle mutation correction with genome engineering tools, particularly CRISPR/Cas9, are the main genetic approaches for sickle cell disease. However, low efficient gene transfer methods, editing rates and safety issues are critical issues that needs to be addressed before starting clinical trials.

became feasible with the development of the lentiviral vector systems based on HIV-1, and the model was first demonstrated in a murine model of β-thalassemia [33]. These results were repeated and extended in both thalassemia and SCD mouse models [34–40], we subsequently established a preclinical, rhesus macaque model for lentiviral globin gene transfer. Mobilized peripheral blood progenitor cells, transduced with a vesicular stomatitis virus-G pseudo-typed, modified HIV-1–based vector [41], expressed significant levels of human β-globin (>50%) among erythroid progeny generated *in vitro*. *In vivo* studies showed more modest levels of human β-globin expression at around 5% early post-transplantation and stabilized at lower levels at 2 years [42], due to species-specific transduction restriction of rhesus progenitor cells by HIV-1 vector. Our newly developed chimeric vector containing the simian immunodeficiency virus capsid to circumvent the species-specific block to HIV in old world monkeys has allowed us to obtain high-level engraftment of genetically modified cells carrying erythroid specific cassettes at levels now exceeding 20% [43] and predicting ultimate clinical success [44].

Anti-sickling β-globin gene addition

Sickle hemoglobin polymerization is the main reason for structural deformation in red blood cells. Initial studies proved that fetal globin or its mixed hybrid tetramer ($\alpha 2\beta^S\gamma$) do not contribute (or to a much lesser extent) to the deoxyHbS polymerization [45]. The amino acid threonine (T87), at position 87, is replaced by a glutamine (Q87) in β-globin, and thus has less tendency to contact the sickle beta subunit valine at position 6 [46].

In a series of studies, the β-globin sequence has been modified to increase the anti-sickling activity based on the observation of less polymerization for $\alpha 2\beta^S\gamma$ with respect to $\alpha 2\beta^S\beta$. Kinetic studies have proved that conversion from T87 to Q87 is the main parameter responsible for most of the sickle inhibiting activity among other 10 amino acid differences between β and γ-globin sequences [47]. Mutation in the sequence of β-globin ($\beta^{A(T87Q)}$) provided strong anti-sickling properties as efficient as γ-globin in reversing the phenotype in two relevant SCD mouse models, BERK and SAD [36]. In addition to its anti-sickling activity, the modified β-globin can be separated from other globins in reverse-phase high-performance liquid chromatography analysis, which makes it more convenient in clinical trials [48]. Results for a multicenter phase 1–2 clinical study for adults with severe SCD have been reported and updated recently [26,49]. A pretreatment transfusion regimen along with improved $CD34^+$ cell collection and manufacturing conditions, along with improvement in myeloablation have resulted in higher peripheral blood vector copy numbers (VCNs) in SCD patients (HGB-206, ClinicalTrials.gov Identifier: NCT02140554) with levels reaching the therapeutic range. In addition, the use of plerixafor, a CXCR4 receptor antagonist, to drive $CD34^+$ mobilization has been adopted in the same clinical trial [50], providing less invasive cell collection with respect to bone marrow harvest and safer mobilization compared with granulocyte colony-stimulating factor treatment, which has historically led to life-threatening complications when used in individuals with SCD [51]. Research-scale levels of transduction with the drug products remained similar for peripheral blood or bone marrow–derived cells from the same patient, indicating plerixafor-based HSC mobilization would be a safer and feasible method for clinical gene therapy applications.

An updated report for the HGB-205 trial recently published demonstrated that one patient with severe SCD showed no SCD complications for up to 30 months after the LentiGlobin treatment, had stable Hb (12.4 g/dL), $\beta^{A(T87Q)}$ (6.1 g/dL) and peripheral blood VCN (2.3) levels [52]. Consistently, expression of $\beta^{A(T87Q)}$ in the patient that was transplanted with autologous $CD34^+$ cells transduced with LentiGlobin BB305 was sufficient to reverse markers of hemolysis and provide stable hemoglobin levels [53], similar to that seen in sickle cell trait for 15 months of follow-up [54]. Although the initial results are encouraging, clinical trials testing these approaches are ongoing (Table I, as of February 2018), and larger clinical trials, along with extended follow-up, will be required to establish the safety and efficacy of these vector-based approaches for broad application in SCD patients.

Because modification at β16, glycine to aspartic acid, serves a competitive advantage over sickle globin (β^S, HbS) for binding to the α chain, and modification at β22, glutamic acid to alanine, partially enhances axial interaction with α20 histidine, double mutant (β^{AS2}; T87Q and E22A) [55] and triple-mutant β-globin variants (β^{AS3};T87Q, E22A and G16D) [56] have also been developed. These modifications provide anti-sickling properties greater than the T87Q-alone modified variant, and comparable to fetal globin. In an SCD murine model, transplantation of bone marrow stem cells transduced with self-inactivating lentivirus carrying β^{AS3} reversed red blood cell physiology and SCD clinical symptoms [57]. On the basis of these encouraging *in vitro* and *in vivo* data, this variant is currently being tested in a clinical trial (ClinicalTrials.gov Identifier: NCT02247843).

γ-globin gene addition

Globin synthesis in humans is control by developmentally regulated gene expression, known as globin

Table I. Open gene therapy clinical trials for sickle cell disease.

ClinicalTrials.gov identifier	Official title	Therapeutic gene	Age eligibility/phase	Status	Sponsor
NCT02186418	Gene Transfer for Patients With Sickle Cell Disease Using a Gamma Globin Lentivirus Vector: An Open-Label Phase I/II Pilot Study	γ-globin	18–35/Phase I/II	Recruiting	Children's Hospital Medical Center, Cincinnati
NCT02247843	Clinical Research Study of Autologous Bone Marrow Transplantation for Sickle Cell Disease (SCD) Using Bone Marrow CD34$^+$ Cells Modified With the Lenti/βAS3-FB Lentiviral Vector	βAS3-FB (Antisickling β-globin)	≥18/Phase I	Recruiting	Donald B. Kohn, MD (University of California, Los Angeles)
NCT02193191	Safety and Efficacy Trial of Escalation of Plerixafor for Mobilization of CD34+ Hematopoietic Progenitor Cells and Evaluation of Globin Gene Transfer in Patients With Sickle Cell Disease	β-globin	18–65/Phase I	Recruiting	Memorial Sloan Kettering Cancer Center
NCT02140554	A Phase 1 Study Evaluating Gene Therapy by Transplantation of Autologous CD34$^+$ Stem Cells Transduced *Ex Vivo* With the LentiGlobin BB305 Lentiviral Vector in Subjects With Severe Sickle Cell Disease	T87Q (Antisickling β-globin)	≥18/Phase I	Recruiting	Bluebird Bio
NCT02633943	Longterm Follow-up of Subjects With Hemoglobinopathies Treated With *Ex Vivo* Gene Therapy Using Autologous Hematopoietic Stem Cells Transduced With a Lentiviral Vector	T87Q (Antisickling β-globin)	5–50/NA	Enrolling by invitation	Bluebird Bio
NCT02151526	Phase 1/2 Open Label Study Evaluating the Safety and Efficacy of Gene Therapy of the Beta-Hemoglobinopathies (Sickle Cell Disease and Beta-Thalassemia Major) by Transplantation of Autologous CD34$^+$ Stem Cells Transduced Ex Vivo With a Lentiviral Beta-A-T87Q Globin Vector (LentiGlobin BB305 Drug Product)	T87Q (Antisickling β-globin)	5–35/Phase I/II	Active, not recruiting	Bluebird Bio

switching. HbF is the dominant hemoglobin type after the first trimester of gestation, which is gradually replaced by adult hemoglobin after birth. The contribution of HbF in adults is generally lower than 1% and is not evenly distributed in red cells but, rather, concentrated in particular cells, referred to as F cells [58]. The appreciation of the ameliorative role of HbF in the blood of patients with SCD began 70 years ago, when Janet Watson and colleagues demonstrated deoxygenated erythrocytes derived from infants with SCD showed delayed sickling compared with their sickle mother's erythrocytes in *in vitro* conditions [59]. This low rate of sickling was attributed to high levels of HbF in the infants' blood, with increased sickling noted at later time points after a decline of HbF. This initial observation was confirmed by others, with the demonstration that SCD and β-thalassemia patients with hereditary persistence of fetal hemoglobin (HPFH) syndrome, having red blood cells with pan-cellular distribution of HbF with a typical level of 30%, have a marked reduction of their clinical and hematologic severity [60–62].

After discovering the ameliorative effect of HbF for SCD patients, efforts to increase γ-globin expression by direct or indirect approaches were vigorously followed. As observed for β-globin encoding vectors, γ-globin encoding vectors were similarly insufficient [63]. The issue of tissue specificity have been partially overcome using different vector designs, such as linking the ankyrin promoter to the γ-globin cassette to provide erythroid-specific expression [64] or using β-globin regulatory sequences and promoter elements to provide cell specificity and high-level expression [65]. As a different approach, Samakoglu and her colleagues used a γ-globin transgene expression and concomitant β^S RNA interference under the control of LCR and γ-globin promoters in sickle cell patient–derived $CD34^+$ cells [66]. With both a reduction in β^S and an increase in γ-globin, an enhancement of anti-sickling activity would be expected [67]. To provide stable and therapeutic level of gene expression for clinical studies, γ/β hybrids have been constructed to benefit from LCR regulatory elements of β-globin and anti-sickling properties of γ-globin. One of those γ/β hybrids gene carrying construct, driven by ankyrin-1 promoter and controlled by two erythroid-specific enhancers (HS-40 plus GATA-1 or HS-40 plus 5-aminolevulinate synthase intron 8 [I8] enhancers) provided 43%–113% human γ-globin/copy of murine α-globin [38].

Targeted γ-globin induction

Having the knowledge of chromosomal orientation, various transcription factors regulate the expression of the globins; controlling these parameters to induce HbF has always been an attractive research area for SCD. γ-to-β globin switching in neonatal period is driven by β-globin LCR, modulating β-like globin expressions by direct contact with their promoters. The shift of interaction between promoters and the LCR region is driven by transcription factors including *Gata1*, *Tal1*, *E2A*, *Lmo2*, and *Ldb1* [68]. Recently, an innovative strategy based on artificially changing the binding site of LCR has emerged to mediate globin switching. Deng and colleagues reported that fusion protein of artificial zinc finger (ZF) and *Ldb1*, or its self-association domain, can rescue the interaction between β-globin promoter and LCR in Gata-*null* erythroblasts [69]. The ZF-Ldb1 can be effectively targeted to the γ-globin promoter, leading to reactivation of γ-globin expression (85% of total β-like globins) and a concomitant reduction in β-globin expression in primary adult human erythroblasts [70]. The approach was tested in hematopoietic progenitor cells derived from sickle patients as well [71]. Transducing SCD $CD34^+$ cells with a lentiviral vector encoding ZF-Ldb1 upregulated HbF production and reduced erythrocyte sickling *in vitro* with greater efficiency than chemical HbF inducers including butyrate and hydroxyurea. From an SCD perspective, decreasing sickle globin along with augmenting anti-sickling γ-globin expressions would increase the clinical benefits.

ZF designs were also fused with transcription factors regulating γ-globin expressions. ZF directed to the 117-position of γ-globin promoter, which is open to DNA-binding proteins and close enough to known transcriptional regulators of the gene, were fused with the potential transcriptional factors [72]. Transducing K562 cells with retrovirus expressing gg-VP64, a transcriptional activator domain, induced HbF expression 7- to 16-fold. Wilber and colleagues brought this technology to human $CD34^+$ cells [73]. Significant induction of HbF (up to 20%) was achieved by overexpressing gg-VP64 domain driven by an erythrocyte-specific promoter, ankyrin-1.

γ-globin repressor silencing

Early works and genome-wide association studies in blood donors revealed DNA polymorphisms in *BCL11A* and *HBS1L-MYB* genes that were associated with elevated HbF expression [62,74,75]. The newly discovered fetal globin regulating candidate, *BCL11A*, was confirmed by its γ-globin repressing roles in primary erythroid cells by re-configuration of the locus region by binding to transcription factors such as *GATA-1* and *SOX-6* [76]. Moreover, conditional deletion of *Bcl11a* in erythroid cells using an SCD mouse model suggested that targeting this repressor was sufficient to reverse the SCD manifestations

in vivo [77], showing the importance of this gene as a potential target for further clinical studies. The question of to what extent *BCL11A* controls HbF has been partly answered by two recent clinical studies reporting on patients with autism spectrum disorder with rare microdeletions of 2p15-p16.1 who had haploinsufficient *BCL11A*, expressing ~20% HbF levels, indicating the central role of *BCL11A* on γ-globin silencing [78,79]. In addition to *BCL11A*, other key trans-acting factors such as *Klf1* and *Myb* have been studied in genetic and functional models [80,81]. Continued investigation of these and other factors involved in fetal hemoglobin repression will help inform clinical approaches leveraging genome editing technologies.

Genome editing technology

Along with the development of human induced pluripotent stem (iPS) cells by Yamanaka and colleagues [82] through the introduction of specific reprogramming factors, circumventing the ethical issues surrounding embryonic stem cells, solid advances in genome editing with methods including zinc finger nucleases (ZFNs), transcription-activator–like effector nucleases (TALENs) and clustered regularly interspaced palindromic repeats (CRISPR)-associated protein-9 nuclease (Cas9) have brought the potential of introducing promising ideas to the clinic. These nucleases are proven to efficiently edit genomes of various animal models and human cells, and great efforts to bring these tools to the clinic have been pursued by academia and industry. These editing tools efficiently disrupt genes at defined loci and can also be used to promote homology directed repair (HDR) as a means of gene correction, although HDR is less efficient. Because β-globin disorders such as SCD are monogenic diseases, genome editing aiming to correct the mutated β-globin gene would be applicable to all affected patients, making this disorder an ideal target. Additionally, the higher efficiency of cutting (over-correcting) genes at specific loci along with the ameliorative effect of HPFH on the SCD phenotype make genome editing at HPFH loci an attractive initial approach. The induction of HbF synthesis by editing regulatory sequences such as promoters or other regulatory sequences such as *BCL11A* are straightforward approaches for a radical cure of the beta-hemoglobinopathies. Genome editing tools are mainly based on the creation of a double-strand break using an endonuclease at a specific site in the genome, leading to activation of an auto-repair machinery system using either non-homologous end joining or the error-free HDR pathways [83]. The CRISPR/Cas9 system for genome editing contains an endonuclease that cuts DNA, a guide RNA to direct where the DNA cut occurs, and template DNA that serves as the correct copy of the targeted gene. There are a number of delivery systems for transporting these tools to cells that depend on the application, but efficiency is increasing such that double-strand breaks at specific loci can be achieved at 90% or higher rates, with lower rates of HDR achieved thus far. As HCSs are mostly quiescent (G_0/G_1), gene disruption will prove easier than gene correction, which is more likely among committed progenitor cells (S/G_2) [84]. Regardless of such drawbacks, understanding the mechanism of action for each editing system, introduction of new chimeric technologies and continuous interest of scientists would help meet these kinds of technical challenges.

Gene correction through genome editing

Although gene addition strategies have recently enjoyed outstanding progress, with translation into clinical research now ongoing, genome editing methods with programmable nucleases targeting precise sites of disease-associated mutations have been introduced to tackle fundamental concerns of gene addition, such as insertional mutagenesis and low transduction efficiency of the viral systems transferring large cargos. The holy grail of genome editing for the β-hemoglobinopathies is the correction of the β-globin mutation in either patient-derived HSCs or patient-derived iPSCs, which, although easier, would then require conversion to engraftable HSCs. Indeed, iPSCs are infinitely more amenable to genome editing than primary cells [85], but they remain encumbered because HSC derivation from pluripotent stem cells with engraftment potential remains insufficient at present, and safety concerns remain to be addressed before iPSCs can be used to treat β-globin disorders. Patient-derived HSCs, however, remain a suitable option for corrective applications given that iPSCs are not currently applied in the clinical setting due to safety concerns. However, experimental data obtained from animal models conducted with HSC-like cells derived from iPSCs are encouraging. In a humanized SCD mouse model, transplantation of hematopoietic progenitors obtained from autologous skin fibroblast-derived iPSCs, corrected with normal β-globin, rescued sickle cell manifestations [86]. As a starting point to transfer the method to humans, earlier attempts to correct β-globin mutation in human SCD patient-derived blood progenitor cells or iPSCs by ZFNs [85,87,88] and TALENs [89–91] have shown the potential of genome engineering to cure the disease. Even though these nucleases can be designed to be quite specific and off-target concerns are generally not an issue, they are expensive, labor-intensive, time-consuming and require expertise. The CRISPR/Cas9

system has emerged as another option that has proven easy to design and can deliver high-efficiency genome editing. As with all such systems, minimization of off-target effects remains crucial. Improving DNA specificity by modified nucleases such as Cas9 nickases (Cas9n), dimerization dependent dead Cas9-FokI chimeric enzyme, or high-fidelity versions of Cas9 mutated in DNA binding groove to increase the nuclease specificity, have reduced off-target activity (reviewed in Genovese *et al.*) [84]. Also, the correction rates in sickle-patient-derived progenitor cells are encouraging. Hoban *et al.* presented 20% sickle correction rate in bone marrow $CD34^+$ cells electroporated with Cas9 mRNA and transduced with integrase-free lentiviral vector carrying gRNA and β-globin gene donor template [92]. This level of correction might be clinically relevant because 20% donor chimerism provides significant improvements and reverse the disease manifestations in allogenic HSC transplanted SCD patients [44]. Almost 30% correction of red blood cells derived from edited sickle $CD34^+$ cells was recently reported [93]. Transplantation of these corrected cells to NSG mouse revealed long-term maintenance and repopulation of HDR-edited HSCs in both bone marrow (2.3%) and spleen (3.7%). Although the results are quite encouraging, correction of cells with long-term repopulating activity requires further optimization. To overcome this limitation, the truncated nerve growth factor receptor (tNGFR) was recently used to enrich HBB-targeted HSCs using magnetic bead separation [94]. These strategies are beginning to show some promise for SCD patients, and a focus on limiting off-target activity is currently being vigorously pursued.

Targeted γ-globin induction through genome editing

Elevated HbF has a long-known beneficial role in SCD, and experiments of nature have suggested increasing HbF as a treatment for SCD, such as in HPFH. Increasing the HbF levels in sickle erythrocytes before severe organ damage might be beneficial in the preventing the relentless complications of this disease. Elevating HbF levels through specific genome editing tools has emerged as a promising strategy for SCD treatment. *BCL11A* was one of the first targets for gene editing after the introduction of these robust and relatively simple genome engineering approaches. However, complete knockout of *Bcl11a* is lethal in mice due to the deficiencies in neural and lymphoid development [95]. Therefore, rather than creating full knockout of *BCL11A* activity in HSCs, a search for the erythroid-specific enhancer could provide a target free of these complications. In a recent study, CRISPR/Cas9-mediated saturating mutations have been introduced to erythroid cells to evaluate effects on HbF levels in red blood cells. This tour de force led to the identification of a particular site in the *BCL11A* erythroid-specific enhancer region, enabling the production of significant HbF without effecting HSC behavior or lymphoid development [96,97]. In a similar approach, Tan *et al.* used specific ZFNs for targeting two regions of *Bcl11a* to increase HbF production in bone marrow–derived $CD34^+$ cells. Ablation of *Bcl11a* resulted in reactivation of HbF in erythroid cells and maintained the engraftment capacity, indicating the clinical relevance of current gene editing strategies [98]. More recently, disruption of exon-2 and GATAA motif in the intronic erythroid-specific enhancer of *BCL11A* in bone marrow–derived $CD34^+$ using targeted zinc finger nucleases was reported to elevate HbF expression, whereas exon-2 deletion negatively affected *ex vivo* enucleation and engraftment in immunodeficient mice [99].

Other than *BCL11A*, known HPFH mutations in the β-globin locus were also created in $CD34^+$ cells using CRISPR/Cas9 that increased γ-globin expression in RBC generated in *ex vivo* culture [100,101]. HbF upregulation in modified progenitor cells is promising, and efforts to achieve reliable engraftment should be pursued because the technologies require electroporation of HSCs, a process that initially led to the development of viral vectors more than a quarter of a century ago due to the deleterious effects of electroporation on viability.

Although there is a great focus on genome editing research, especially for monogenic disorders such as SCD, there are still serious limitations and considerations. Several delivery methods for genome editing tools in the forms of DNA, RNA or protein have been presented in the literature, including electroporation, cationic lipids, cell-penetrating peptides, nanoparticles and viral vectors, offering variable pros and cons (reviewed in LaFountaine *et al.* and Shim *et al.*) [102,103]. The use of integrating viral vectors with persistent expression of Cas9 and guide RNAs allows study of the system's activity, but persistent Cas9 expression is not desirable for most clinical applications [104]; hence, transient nuclease activity would be preferable and convenient from a clinical perspective. Nucleases can be applied both *ex vivo* and *in vivo* to target the HSCs, but *ex vivo* editing would be advantageous because HSCs can be cultured and manipulated *ex vivo* allowing controlled delivery of enzymes especially where off-target modifications are problematic, and higher levels of editing rates are needed. However, HSCs can lose their stem cell properties and engraftment ability in culture conditions due to epigenetic instability in laboratory conditions [105]. A reduction in engraftment ability can be augmented by delivering ablative conditioning, but ablative conditioning carries significant risks [104].

Improved conditioning for better tolerability

Better conditioning regimens offering less overall toxicity and higher specificity would circumvent the concerns over current chemoradiotherapy-based regimens and increase the success rate of HSC-based genetic therapies. Targeting HSCs in bone marrow while sparing non-hematopoietic cells using specific antibodies has emerged as an attractive alternative to genotoxic chemoradiotherapy. These nontoxic approaches are especially appropriate for blood disorders such as SCD where end accumulated organ damage compounds toxicity of existing conditioning regimens. A monoclonal antibody, ACK2, targeting c-kit was recently demonstrated with or without low-dose irradiation to eliminate host blood progenitor cells before donor cell transplantation [106,107]. Considerable level of endogenous HSC clearance and 90% donor chimerism after subsequent transplantation were achieved. As a different strategy, others have targeted CD45 with antibody conjugated to ribosome-inactivating protein, Saporin (CD45-SAP) to target HSCs in an immunocompetent mouse model [108]. Efficient donor cell engraftment (>90%) was obtained with a single dose of CD45-SAP administration without neutropenia and anemia, and, most interestingly, rapid recovery of T and B cells and sustained antifungal immunity were reported, along with correction of sickle manifestations *in vivo*. These promising strategies could transform transplantation approaches for genetic diseases affecting the blood if these results can be replicated in large animals and humans.

Conclusion

Recent advances in gene transfer and editing technologies, better clarification of erythropoiesis regulation and improved transplantation methods have driven optimism in developing a one-time definitive cure for SCD. Gene addition trials have already shown that high-level expression of anti-sickling β-globin can reverse the complications of the disease [53]. With improvements in cell processing, viral vectors and transduction methods, we anticipate validation of these encouraging results over the short term, allowing broader application of potentially curative strategies to patients with SCD. In addition, nature has already shown us that persistent, high HbF expression through coinheritance of HPFH mutations can ameliorate the clinical severity of SCD, opening another potentially curative strategy. If genetic approaches can be adapted and standardized at high efficiency, increasing HbF protein levels in the blood of patients with SCD by gene addition or controlling gene repressors and activators of γ-globin through gene editing would provide additional options, and promising preclinical results indicate the possibility of clinical trials in the coming year. Finally, a dream therapy for SCD has recently become imaginable through correction of the disease-causing mutation in patient-derived iPSCs and blood progenitor/stem cells with the introduction of relatively easy and feasible genome editing tools, particularly CRISPR/Cas9. Several issues remain to be addressed, including delivery of these tools to HSCs without compromising their engrafting ability, achieving high-efficiency correction and minimizing or eliminating undesirable off-target editing. Overcoming the current technical challenges of the newer candidate approaches appears surmountable, and continued rapid progress should lead to widely available curative approaches for the hemoglobinopathies.

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