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TREATING HEMOGLOBINOPATHIES USING GENE CORRECTION APPROACHES: PROMISES AND CHALLENGES

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Abstract

Hemoglobinopathies are genetic disorders caused by aberrant hemoglobin expression or structure changes, resulting in severe mortality and health disparities worldwide. Sickle cell disease (SCD) and β-thalassemia, the most common forms of hemoglobinopathies, are typically treated using transfusions and pharmacological agents. Allogeneic hematopoietic stem cell transplantation is the only curative therapy, but has limited clinical applicability. Although gene therapy approaches have been proposed based on the insertion and forced expression of wild-type or anti-sickling β-globin variants, safety concerns may impede their clinical application. A novel curative approach is nuclease-based gene correction, which involves the application of precision genome editing tools to correct the disease-causing mutation. This review describes the development and potential application of gene therapy and precision genome editing approaches for treating SCD and β-thalassemia. The opportunities and challenges in advancing a curative therapy for hemoglobinopathies are also discussed.

Keywords

Hemoglobinopathies; gene therapy; gene correction; nucleases; precision genome editing

Hemoglobinopathies represent major public health burdens with severe mortality and inequalities in their distribution worldwide (Modell and Darlison 2008; Piel et al. 2013b; Weatherall 2010; WHO 2010). According to a recent epidemiological study, sickle cell disease (SCD), a prevalent hemoglobinopathy, occurred in 312,000 births globally in 2010; roughly 75% of these births were in sub-Saharan Africa (Piel et al. 2013b). By 2050, the global annual number of newborns with SCD is projected to increase by 33% as a consequence of population growth and public health conditions (Piel et al. 2013a). In the United States, SCD affects between 72,000 to 98,000 people and is associated with an economic burden exceeding $1.1 billion annually in medical costs (Hassell 2010; Kauf et al. 2009). A related hemoglobinopathy with high global incidence is β-thalassemia occurring in

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40,000 births annually worldwide; 93% of newborns with β-thalassemia are in Asian, Indian, and Eastern Mediterranean regions (Modell and Darlison 2008). As a result of changing demographics, β-thalassemia is becoming increasingly more common in North America and Europe (Vichinsky 2005). The estimates for the burdens of these diseases are limited by a scarcity of accurate data on carrier frequencies and numbers of affected individuals (Piel et al. 2013b; Vichinsky 2005).

Discovered more than 100 years ago, the molecular basis for SCD is an A to T transversion in the β-globin (*HBB*) gene, causing the substitution of a polar glutamic acid by a non-polar valine in the sixth amino acid position of HBB protein (Ingram 1959). The dimerization of these mutant HBB chains with α-globin results in the formation of hemoglobin S (HbS, $\alpha_2\beta^S_2$). This mutation is associated with protection from *Plasmodium falciparum* malaria in people with the sickle cell trait, characterized by heterozygosity for HbS and wild-type adult hemoglobin (HbA, $\alpha_2\beta_2$) without SCD symptoms (Bridges and Pearson 2008; Steinberg and Embury 1994). In patients homozygous for HbS, the most common and severe form of SCD, aberrant polymerization of hemoglobin occurs in deoxygenated red blood cells (RBCs), causing them to deform into a sickle or crescent shape and become rigid and adhesive. Repetitive cycles of sickling as HbS molecules switch from oxygenated to deoxygenated states causes RBC fragility and promotes vaso-occlusion, painful crises, chronic anemia, acute chest syndrome, organ failure, stroke, and death (Bridges and Pearson 2008; Steinberg and Embury 1994). β-thalassemia is caused by point mutations or small deletions in *HBB* resulting in a reduction (β^+) or complete elimination (β^0) of β-globin chains from HbA, excess of α-chains, and ineffective erythropoiesis. Although protecting against malaria in carriers, homozygosity of β-thalassemia mutations results in a disease phenotype with varying severity, from mild forms of anemia referred to as β-thalassemia minor to severe anemia referred to as β-thalassemia major (Bridges and Pearson 2008; Weatherall and Clegg 2001). In addition to anemia, homozygous β-thalassemia major patients have hyperstimulated erythropoiesis and bone marrow cavity expansion by erythroid tissues, resulting in cosmetic deformities (Bridges and Pearson 2008). Co-inheritance of the sickle cell mutation and β-thalassemia results in HbS/β-Thal, a compound heterozygous condition; the severity of which depends on HbS levels and type of β-thalassemia mutation. The most severe form, HbS/β^0-Thal, is nearly phenotypically indistinguishable from homozygotic HbS (Castro et al. 1994; Platt et al. 1991). Detailed reviews on the genotypes of SCD and β-thalassemia could be found in (Ashley-Koch et al. 2000; Cao and Galanello 2010). In this review, we focus on the development of gene therapy approaches for treating β-hemoglobinopathies, including SCD and β-thalassemia major.

The available therapies for SCD and β-thalassemia ameliorate the disease symptoms, but are each associated with undesirable side effects and limitations. Transfusion of normal HbA donor erythrocytes is typically used episodically or long-term, which reduces the proportion of circulating erythrocytes containing HbS, resulting in fewer vaso-occlusion events in SCD patients. However, blood transfusions are limited by risks of blood-borne pathogens, iron overload, hyperviscosity syndrome, alloimmunization, and hemolysis; these transfusion hazards range from mild to fatal. Iron overload complications could be prevented by administration of iron chelation therapy (Bridges and Pearson 2008; Yawn et al. 2014). Alternatively, hydroxyurea therapy is used to induce the expression of γ-globin chains and

elevate the level of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) as well as overall hemoglobin in patients with SCD (Charache et al. 1995; Kinney et al. 1999; Zimmerman et al. 2004) and β-thalassemia (Bradai et al. 2003; Dixit et al. 2005; Fucharoen et al. 1996). In SCD patients, HbF inhibits intracellular HbS polymerization and sickling via the formation of $\alpha_2\beta^S\gamma$ hybrid hemoglobin tetramers (Goldberg et al. 1977; Nagel et al. 1979). Furthermore, hydroxyurea lowers circulating leukocytes and reticulocytes, which contributes to a reduced level of vaso-occlusion events and painful crises (Charache et al. 1995; Hankins et al. 2014). In patients with β-thalassemia, the newly synthesized γ-globin chains induced by hydroxyurea neutralize the excess of α-globin chains thereby improving effectiveness of erythropoiesis and increasing overall hemoglobin levels (Bradai et al. 2003; Dixit et al. 2005; Fucharoen et al. 1996). Clinical studies demonstrated that daily administration of hydroxyurea delayed or discontinued blood transfusions in patients with moderate to severe SCD (Charache et al. 1995) and β-thalassemia (Arruda et al. 1997; Bradai et al. 2003; Dixit et al. 2005). Although hydroxyurea is the only FDA approved drug for treating SCD, it is not effective in treating all patients and is further limited by its myelosuppressive activity in the marrow with unclear risks from long-term treatment (Steinberg et al. 2003; Zimmerman et al. 2004). However, a recent study with a small cohort of patients suggests that, when administrated continuously from infancy, hydroxyurea provides sustained improvement of hematologic features for at least 15 years without major toxicity (Hankins et al. 2014).

Currently, allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative therapy for both disorders (Angelucci et al. 2014). HSCT is a procedure in which a patient receives hematopoietic stem cells (HSCs) from a healthy donor **(Figure 1)**. HSCT studies using HLA-identical sibling donors demonstrate disease-free survival in 80% to 90% of SCD patients without vaso-occlusive crises and anemia, as well as improvement in organ function following stable engraftment (Bernaudin et al. 2007; Walters et al. 1996b; Walters et al. 2000). However, initial transplant studies excluded patients over the age of 16 years old or with extensive end-organ dysfunction, including severe renal and neurologic impairments or stage II/IV sickle cell lung disease (Walters et al. 1996b; Walters et al. 2000). This exclusion was necessary as myeloablative conditioning regiments were used to reduce the risks of graft rejection, which is not well tolerated in many patients, particularly those with severe irreversible complications (Hsieh et al. 2011). A recent Phase 1-2 study showed that nonmyeloablative HSCT could be used safely in adults, including patients with end-organ complications. Nonmyeloablative HSCT resulted in disease phenotype reversal with stable donor engraftment in 87% of patients. There were no cases of acute or chronic graft-verse-host disease reported in these nonmyeloablative transplant studies (Hsieh et al. 2014). One interesting observation reported in HSCT clinical trials is that stable mixed donor-host chimerism is sufficient to reverse the disease phenotype for SCD and β-thalassemia (Hsieh et al. 2014; Walters et al. 2000). Despite the curative effects, HSCT is associated with therapeutic challenges. The major drawback for many HSCT procedures, including nonmyelablative HSCT, is the requirement for an HLA-identical sibling donor (Hsieh et al. 2014). Only 14% of SCD patients have HLA-identical sibling donors for transplantation (Walters et al. 1996a). Additional limitations include the administration of granulocyte colony stimulating factor (GSCF) to mobilize donor stem cells into the peripheral blood, associated with adverse reactions (Hsieh et al. 2014), or harvesting bone marrow to obtain

stem cells (Bernaudin et al. 2007; Locatelli et al. 2013; Walters et al. 2000). Alternatively, umbilical cord blood could be used as a source for stem cells, providing 83% event-free survival in patients with SCD and β-thalassemia at 6 years after HSCT (Locatelli et al. 2013). However, in general, umbilical cord blood would not be sufficient for adult patients. Furthermore, HSCT is limited by risks of graft rejection, graft-verse-host disease, and mortality that must be weighed against the severity of the disease symptoms (Bernaudin et al. 2007; Hsieh et al. 2014; Locatelli et al. 2013; Walters et al. 2000).

Gene therapy and the emerging precision genome editing based approaches have opened new therapeutic possibilities for SCD and β-thalassemia. This review describes the potential application of gene therapy and genome editing tools for curing SCD and β-thalassemia. The technological and clinical challenges in advancing a novel therapy using each approach are also discussed.

Gene therapy for hemoglobinopathies

The goal of gene therapy is transferring the normal *HBB* into HSCs harvested from the patient for subsequent transplantation as a long-term autologous therapy **(Figure 2)**. HSCs can be isolated from the patient's bone marrow aspirates or peripheral blood by immunolabeling and separating the $CD34^+$ cells. The enriched HSCs are then transduced *ex vivo* using viral vectors containing wild-type *HBB* and transfused back into the patient (Riviere et al. 2012). Alternatively, the patient's whole bone marrow can be transduced and transplanted. Because autologous HSCs have very low potential to activate graft-verse-host-disease in the patient, immunosuppression may not be necessary post-transplantation.

Proof-of-principle studies of the gene therapy approach for treating hemoglobinopathies have been made possible by the availability of transgenic mouse models for SCD (Paszty et al. 1997; Trudel et al. 1991) and β-thalassemia (Skow et al. 1983; Yang et al. 1995). Additional advances leading to seminal studies have been the genomic mapping of the human *HBB* cluster locus control region (β-LCR) (Forrester et al. 1987; Tuan and London 1984; Tuan et al. 1985; Wright et al. 1984) and the identification of regulatory elements within the *HBB* cluster required for high levels of erythroid-specific and vector position independent *HBB* expression (Grosveld et al. 1987; Talbot et al. 1989; van Assendelft et al. 1989). Extensive studies demonstrating correction of *HBB* in murine models applied HIV-1 derived lentiviral vectors containing the *HBB* locus and regulatory elements. Lentiviral vectors efficiently infect non-dividing HSCs, provide long-term expression that depends on the number of copies integrated per cell, and have relatively high RNA processing efficiency (Vigna and Naldini 2000). In a pioneering study by May et al., mouse bone marrow transduced with *HBB* lentiviral vectors and transplanted into lethally irradiated β-thalassemia mice resulted in 13% of the total hemoglobin incorporating the wild-type HBB chain at 24-weeks after transplantation (May et al. 2000). Although the proviral copy number was low, 0.75 per cell, an indication of incomplete transduction, transplanted mice showed correction in phenotypic features of thalassemia indicated by improvements in hematocrit level, RBC count, reticulocyte count, and hemoglobin levels relative to control mice (May et al. 2000). The study by Imren et al. demonstrated the feasibility of transducing all of the hematopoietic cells in the bone marrow through modifications in the lentiviral

vector that enhanced viral potency, as indicated by high-titer and *HBB* expression (Imren et al. 2002). Specifically, infection of bone marrow using the high titer lentiviral preparation containing the wild-type human *HBB* resulted in 3 provirus copies per transduced cell, an indication of transduction in all of the HSCs. Transplantation of the infected cells into severe β-thalassemia mice resulted in 32% of all HBB chains consisting of the wild-type HBB. Further, 95% of RBCs were positive for human HBB. In contrast, lower viral titer preparations provided <40% human HBB positive RBCs, evidence that the viral titer is an important factor for gene transfer efficiency. In thalassemia mice, pan-cellular erythroid expression of human HBB following transplant was sufficient to correct nearly all hematologic indices of anemia and ineffective erythropoiesis (Imren et al. 2002). Additional studies verified that complete reconstitution of the hematopoietic compartment with donor marrow similarly transduced with human *HBB* or γ-globin lentiviral vectors provides permanent human hemoglobin expression and ameliorates the thalassemia phenotype in murine models (Person et al. 2003; Puthenveetil et al. 2004; Rivella et al. 2003).

Transplantation of bone marrow infected with anti-sickling hemoglobin variants and β-LCR resulted in correction of the disease pathology in SCD mouse models. In the study by Pawliuk et al., the $\beta^{A\text{-}T87Q}$ -globin variant, a potent inhibitor of HbS polymerization, having a threonine to glutamine mutation at codon 87, was inserted into a lentiviral vector structurally optimized for erythroid-specific expression and stable gene transfer into HSCs, yielding high viral titers, and multiple chromosomal integration events. Transplantation of $\beta^{A\text{-}T87Q}$ lentivirus-transduced bone marrow from SCD mice into lethally irradiated mouse recipients resulted in up to 52% of total hemoglobin consisting of the $\beta^{A\text{-}T87Q}$ variant. High levels of $\beta^{A\text{-}T87Q}$ hemoglobin containing erythrocytes coincided with corrected RBC and reticulocyte counts and amelioration of SCD-associated splenomegaly and urine concentration defect (Pawliuk et al. 2001). A study by Levasseur et al demonstrated comparable correction of a SCD mouse model using a lentiviral vector containing a different anti-sickling human β-globin variant (Levasseur et al. 2003). In the Levasseur study, minimal amounts of lentiviral vector was used to transduce purified murine HSCs with an β^{AS3}-globin, having substitution of alanine and glutamine at positions 22 and 87 respectively in the HBB polypeptide. Primary and secondary transplant mouse recipients had correction of SCD-associated characteristics, including a significant increase in RBC counts, hematocrit values, hemoglobin levels, and restored urine concentration capacity (Levasseur et al. 2003).

β-globin gene therapy clinical trials

The successful demonstration of gene therapy in mouse models of SCD and β-thalassemia prompted the initiation of human clinical trials in France. The lentiviral vector used in the first trial, HPV569 LentiGlobin, encodes for the therapeutic $\beta^{A\text{-}T87Q}$ gene and was designed with safety features to lower the risks of mutagenic integration observed in the X-linked severe combined immunodeficiency (SCID) gene therapy trial (Cavazzana-Calvo et al. 2000; Hacein-Bey-Abina et al. 2003a; Hacein-Bey-Abina et al. 2003b). Modifications in the lentiviral vector included the removal of functional viral promoter-enhancer activity in the 3’ long terminal repeat (LTR), rendering it a self-inactivating vector. The non-functional 3’ LTR is copied to the 5’ end of the vector during reverse transcription, such that the proviral

promoter and enhancer elements in either LTR have a low probability of activating oncogenes, thus improving safety. Furthermore, insulators containing core elements of the chicken chromatin HS4 was added to prevent activation of neighboring genes. The HPV569 LentiGlobin vector was deemed safe, not showing evidence of activating malignancy in long-term survival studies in mice. The patients selected for the clinical study had severe forms of transfusion-dependent β-thalassemia and SCD and did not have available HLA-matched donors for HSCT. The trial procedure involved isolating $CD34^+$ cells from the patient bone marrow, a portion of which was cryopreserved in the event of graft rejection and the remainder transduced with LentiGlobin. Following *ex vivo* transduction, the treated HSCs were intravenously infused into the myeloablated patient. A sample of transduced HSCs was subjected to replication-competent lentivirus testing to verify safety and quantification of $\beta^{A\text{-}T87Q}$ gene expression. The clinical protocol was designed with an endpoint analysis of transplant success at 2 years post-transplant and requires recipients to be monitored over 15 years for adverse event analysis, replication-competent lentivirus testing, and insertional mutagenesis testing (Bank et al. 2005).

The HPV569 LentiGlobin was used successfully in one patient who received an autologous transplant of $CD34^+$ cells that were transduced with the vector (Payen and Leboulch 2012). The patient, an 18-year-old male at the time of treatment, had severe β-thalassemia and previously required transfusions once per month and did not respond to hydroxyurea therapy. The *ex vivo* transduction efficiency measured at 1 week in culture following transduction was 0.6 vector copies integrated per cell (Cavazzana-Calvo et al. 2010). Transplantation of transduced cells (3.9×10^6 $CD34^+$ cells per kg) into the patient, pre-conditioned by Busulfex treatment, resulted in complete hematopoietic reconstitution. The levels of nucleated blood cells containing the $\beta^{A\text{-}T87Q}$-globin vector stabilized at 11% while 36.2% of HBB chains consisted of $\beta^{A\text{-}T87Q}$ by 30 months after transplantation (Cavazzana-Calvo et al. 2010). The peripheral blood cells containing the $\beta^{A\text{-}T87Q}$ vector gradually increased and stabilized at 3.5 g/dL by 2 years after transplantation (Payen and Leboulch 2012). The patient had corrected mean corpuscular hemoglobin content at 28.4 pg and total hemoglobin between 8.5-10 g/dL, one third of which comprised of the therapeutic hemoglobin $\beta^{A\text{-}T87Q}$ (Cavazzana-Calvo et al. 2010; Payen and Leboulch 2012). Although having subnormal levels of hemoglobin (13.8-17.2 g/dL for normal adult males), the patient was transfusion-independent for 7 years. In a recent press release by BlueBird Bio, this patient recently required two blood transfusions due to anemia (BusinessWire 2015a). DNA pyrosequencing analysis revealed multilineage chromosomal integration of the HPV569 LentiGlobin vector into multiple sites, with the most abundant integration sites found in the *RFX3, ZZEF1, and HMGA2 loci*. HMGA2, a member of the high-mobility group AT-hook family of non-histone chromatin transcriptional factors, is tumorigenic when elevated in adult tissues where it is normally low or undetectable (Lee and Dutta 2007). Interestingly, over time there was a growing dominance of cells with integration within the *HMGA2* locus, specifically found in granulocytes-monocytes and erythroblasts, but not lymphocytes. This increase in myeloid cells positive for disruption in the *HMGA2* suggests the clonal expansion of a transduced HSC having a myeloid bias. The *HMGA2* integration site resulted in a 10,000-fold increase in *HMGA2* expression compared to pre-transplant levels due to combined enhancement from the β-LCR and vector-induced truncation induced insensitivity

to microRNA let-7 regulation (Cavazzana-Calvo et al. 2010). The vector-induced enhancement of *HMGA2* expression is evidence that the core insulators in the vector likely failed to protect against the activation of neighboring genes, thus reinforcing concerns about viral vector safety for therapeutic gene transfer applications.

The disruption of *HMGA2* by a viral vector was implicated in conferring clonal growth advantage in other gene transfer studies (Boztug et al. 2010; Wang et al. 2010). Although the effects of *HMGA2* activation on malignancy are unknown in the patient described in (Cavazzana-Calvo et al. 2010), one can speculate that there is a lingering risk for transformation. In a study by Ikeda et al., transgenic mice with a similar truncation in the 3' untranslated region of *HMGA2* resulted in similar microRNA let-7 insensitivity, *HMGA2* overexpression, increased peripheral blood cells from all blood lineages, splenomegaly, and EPO-independent erythroid colony formation in bone marrow cells (Ikeda et al. 2011). Competitive and serial bone marrow transplant assays in mice revealed that *HMGA2* overexpression conferred clonal growth advantage and self-renewal capacity in HSCs reminiscent of the observations made in the study by Cavazzana-Calvo et al. The results of the Ikeda study also showed upregulation in Jak2 mRNA, pSTAT3, and pAKT proteins with a concomitant decrease in *pSTAT5* expression; implicating the involvement of JAK-STAT5 and P13K-AKT signaling pathways in the proliferation of myeloid cells and hematopoiesis. The transgenic mice did not develop lymphoma, but showed splenomegaly and growth advantage in B- and T-cells as a result of enhanced *HMGA2* expression (Ikeda et al. 2011).

After establishing proof-of-principle application of a lentiviral vector for β-thalassemia, the HPV569 LentiGlobin vector was improved for safety, transduction, and manufacturing efficiency. The insulator domains found to be unstable in the HPV569 vector were removed in the second generation BB305 LentiGlobin to enhance safety. In addition, the promoter-enhancer was changed from the 5' HIV U3-LTR to CMV, resulting in further enhancement in the viral titers and yields. *In vitro* comparison of the two vector designs revealed that the BB305 LentiGlobin provided 3-4 fold higher viral titers and a 2-3 fold higher vector copy number in transduced $CD34^+$ cells relative to the HPV569 vector (Negre et al. 2015). In a murine bone marrow transplant study, peripheral blood from mice engrafted with transduced BB305 LentiGlobin had a 1.1-1.5 fold higher vector copy number relative to the HPV569 vector. Both HPV596 and BB305 LentiGlobin vectors displayed preferred integration into gene coding regions with 51.9% of integrations found in common insertion site regions, which have not previously been associated with adverse events in patients. Preferred insertional integration was not observed in high risk genes LMO2 or MDS1-EVI1, associated with transformation, for either HPV596 or BB305 LentiGlobin vectors in primary and secondary transplanted mouse recipients (Negre et al. 2015), thus providing evidence of vector safety. Phase 1 and 2 clinical trials are currently in progress to establish the safety and efficacy of treating patients with severe β-thalassemia and SCD using the BB305 LentiGlobin vector. Results of this study were recently presented at conferences sponsored by the American Society of Hematology and European Hematology Association respectively, indicating higher transfection efficiencies with vector copy numbers between 0.3-2.1 as well as faster independence from transfusions for the BB305 vector compared to HPV569 (Cavazzana et al. 2015a; Cavazzana et al. 2014; Cavazzana et al. 2015b; Thompson et al. 2014; Walters et al. 2015). In contrast to the HPV569 vector, the BB305 LentiGlobin

provided higher total hemoglobin and therapeutic β^{AT87Q} levels at earlier time points. For example, two patients from the HGB-205 study had 1.5 and 2.1 vector copies resulting in 10.2 g/dL and 11 g/dL of total hemoglobin by Day +180 days after therapy, thereby enabling transfusion independence (Cavazzana et al. 2014). There were no evidence of clonal dominance nor viral vector-related adverse events reported in any of the studies (Cavazzana et al. 2015a; Cavazzana et al. 2014; Kanter et al. 2015; Thompson et al. 2014; Walters et al. 2015). A potential drawback of the BB305 vector is that success at weaning patients off transfusion may be dependent on the β-thalassemia genotype. In the HGB-204 study, all of the patients with a non-β^0 genotype were transfusion independent (Walters et al. 2015). According to the most recent results reported by Bluebird Bio, patients with the β^0 genotype had a 33-100% reduction in transfusion volumes (BusinessWire 2015b).

Despite the initial successes of animal and human gene therapy studies as summarized in **Table 1**, there are challenges that must be addressed to advance its clinical application. The limitations of conventional gene therapy include low gene modification efficiency, insertional mutagenesis risks, toxicity and inflammation due to viral vector exposure in the host, possible immune response, and high costs. Therefore, better approaches are required in order to have gene therapy as a widely applicable treatment strategy for human diseases.

Precision genome editing tools

Precision genome editing using engineered nucleases has the potential to overcome the challenges in conventional gene therapy, and offer gene correction, a new form of gene therapy, for a wide range of therapeutic applications. A major approach in precision genome editing is based on the generation of DNA double stranded breaks (DSBs) in cells at pre-determined loci in the genome, and activating the DNA repair pathway(s) for gene modification. The nuclease induced DSBs are repaired by two main pathways: non-homologous end-joining (NHEJ) and homology directed repair (HDR) (Shrivastav et al. 2008). Repair by the NHEJ pathway results in small deletions and/or insertions at the break site (Shrivastav et al. 2008). The HDR pathway involves high fidelity repair of the broken ends using homologous sequences found in sister chromatids, homologous chromosomes or exogenous donor template DNA containing homologous sequences (Shrivastav et al. 2008). In seminal studies, the homing I-SceI endonuclease (also called meganucleases) from *Saccharomyces cerevisiae*, which generates DSBs at a targeted recognition sequence of 18-bp (Colleaux et al. 1988), was co-transfected along with a homologous donor plasmid DNA into human COS-1 (Rouet et al. 1994) and mouse embryonic stem cells (ESCs) (Smih et al. 1995) to induce HDR mediated knock-in of the donor sequence into the I-SceI target site. Additional studies confirmed that the induction of a DSB by meganucleases can dramatically increase the frequency of HDR by 3-5 orders of magnitude when a homologous exogenous donor DNA is provided (Jasin 1996). Although meganucleases can catalyze DSBs critical for gene modifications, the low targeting specificity of meganucleases such as I-SceI limits their use as a gene-editing tool for site-specific knock-in (Carroll 2011). This challenge inspired the advent of designer nucleases capable of targeting desired sequences in the genome and mediating different types of gene modifications through the activation of NHEJ and/or HDR **(Figure 3)**.

One class of engineered nucleases is zinc finger nucleases (ZFNs) **(Figure 4a)**. A ZFN consists of a zinc finger protein (ZFP) DNA-binding domain (zinc finger motifs) (Choo and Klug 1994) fused to the nuclease domain of the *FokI* restriction enzyme, conferring the hybrid protein with DNA binding capability and robust cleavage activity (Kim et al. 1996). The ZFPs contain a tandem array of Cys_2-His_2 units, each roughly 30 amino acids in length, bound to a zinc atom, and recognizing 3 bases of DNA (Pavletich and Pabo 1991). DNA cleavage activity requires dimerization of the *FokI* domains in a pair of ZFNs, each targeting neighboring sequences, arranged in an inverted orientation on the genome, and separated by a spacer region (Smith et al. 2000). The dimerization requirement can be exploited to prevent cleavage events at single binding sites, increasing the overall specificity (Miller et al. 2007). When delivered into cells, ZFNs generate DSBs that are repaired by NHEJ or HDR (Bibikova et al. 2003; Bibikova et al. 2002). In an early study, delivery of a ZFN pair, each having three zinc fingers targeting 9-bp of DNA, along with homologous donor template DNA was used to stimulate gene targeting in the X-chromosome of the *Drosophila melanogaster* germ line (Bibikova et al. 2003). Since then, additional studies demonstrated ZFN mediated gene modification in many different organisms (Meng et al. 2008; Meyer et al. 2010; Morton et al. 2006) and human cell lines (Porteus and Baltimore 2003; Urnov et al. 2005). ZFNs were also designed to disrupt the HIV co-receptor CCR5 gene (Holt et al. 2010) as an HIV therapy currently in Phase 2 clinical trials (Tebas et al. 2014). The co-delivery of *HBB*-targeting ZFNs and a donor template into $CD34^+$ cells provides high levels of gene modification; demonstrating the potential for ZFN-driven gene correction in treating hemoglobinopathies (Hoban et al. 2015). The major drawbacks of ZFN-based approaches include the limited targeting sites available, and relatively low rates of site-specific cleavage (Sebastiano et al. 2011).

Another class of designer nucleases for genome editing is transcription activator-like effector nucleases (TALENs) **(Figure 4b)**. TALENs consist of the DNA binding domain from TALE proteins isolated from bacteria of the *Xanthomonas* genus (Bogdanove et al. 2010), fused to the DNA cleavage domain from the *FokI* restriction enzyme (Kim et al. 1996), similar to ZFNs. A DSB is generated at the target locus by a TALEN pair, each binding adjacent elements on separate strands of the DNA in a tail-to-tail orientation, separated by optimized DNA spacing required for *FokI* dimer formation (Mahfouz et al. 2011). The TALE DNA binding domain is modular, consisting of 15.5-19.5 repeats roughly 34 residues in length that each recognizes a specific nucleotide determined by the repeat-variable di-residues (RVDs) at positions 12 and 13 within the module (Boch and Bonas 2010). The Golden Gate cloning strategy is used to assemble libraries of unique arrangements of RVD repeats for targeting any genomic sequence (Cermak et al. 2011). An attractive feature making TALENs more desirable as a tool for genome editing compared to ZFNs is the availability of the RVD-DNA recognition code enabling design of customized binding domains targeted to desired nucleotide sequences (Boch et al. 2009; Moscou and Bogdanove 2009). In contrast to ZFNs, TALENs have comparable or higher cleavage activities, facile design, and provide a broader target range (Joung and Sander 2013). TALENs have been applied for targeted genome editing in various animal species (Huang et al. 2011; Wood et al. 2011) and human pluripotent cells (Hockemeyer et al. 2011).

The third and newest class of genome editing tools are the recently developed clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) systems **(Figure 4c)** (Jinek et al. 2012). CRISPR/Cas9 systems play a role in the adaptive immune systems for bacteria, providing protection from invading nucleic acids (Barrangou et al. 2007). The Cas9 endonuclease is directed to a target DNA site by a single guide RNA (sgRNA) through Watson-Crick base-pairing (Sander and Joung 2014). In the type II CRISPR system of *Streptococcus pyogenes*, Cas9 endonuclease cleaves a DNA sequence specified by the 20-nucleotide targeting domain of the sgRNA immediately 5' of the proto-spacer adjacent motif (PAM) sequence NGG (Sander and Joung 2014). The commonly used CRISPR/Cas9 system for genome editing involves the co-delivery of the Cas9 endonuclease and sgRNA often encoded on a single plasmid DNA. Redirecting the CRISPR/Cas9 nuclease to desired targets in the genome requires modification of the targeting sequence (crRNA) in sgRNA while the other components remain unchanged, making the CRISPR/Cas9 systems simpler compared to ZFNs and TALENs in genome editing. Furthermore, the Cas9 endonuclease can be co-delivered with two or more sgRNAs targeting multiple sites simultaneously, enabling multiplexed disruption within the same genome (Cong et al. 2013). The potential disadvantage of the CRISPR/Cas9 system is its off-target effects, which may result in gross chromosomal deletions and other types of chromosomal rearrangements (Cradick et al. 2013; Fu et al. 2013; Hsu et al. 2013). Tolerated mismatches and DNA/RNA bulges between the sgRNA and gene sequences were implicated for non-specific cleavage by the CRISPR/Cas9 system (Lin et al. 2014a), providing evidence for the need to carefully identify potential off-target sites and screen for sgRNA designs with high specificity. The CRISPR/Cas9 systems have been applied for efficient generation of a wide range of transgenic animal models (Cho et al. 2013; Kimura et al. 2014; Li et al. 2013a; Li et al. 2013c; Wang et al. 2013) and engineered plants (Li et al. 2013b; Nekrasov et al. 2013; Shan et al. 2013). Therapeutic applications using precision genome editing tools are discussed below and summarized in **Table 2**.

Gene correction using precision gene editing tools

Therapeutic gene correction for hemoglobinopathies can potentially be achieved through the generation of site-specific DSBs and activation of the HDR repair pathway, resulting in the replacement of mutant sequences within the *HBB* locus with a wild-type, homologous donor sequence **(Figure 5)** (Chang et al. 2006; Wu et al. 2006). As discussed earlier, the frequency of HDR can be dramatically increased by inducing a DSB using site-specific nucleases (Choulika et al. 1995; Elliot et al. 1998). Early work by Urnov et al. demonstrated gene correction of a disease mutation in human cell lines and primary cells treated with ZFNs and donor vector. In this study up to 20% gene modification rate was observed when K562 cells, a human leukemia cell line, were treated with *IL2RG*-aiming ZFNs and a donor vector without selection. Comparable levels of HDR were observed in human $CD4^+$ T cells treated. Interestingly, ZFN induced HDR of the target site occurred at the highest frequency in G2 arrested cells, suggesting that gene targeting is favored in the S/G2 stage of the cell cycle (Urnov et al. 2005). The work by Lombardo et al. confirmed that gene targeting in a disease-related gene downstream of its own promoter is feasible using ZFNs and donor templates co-delivered into a variety of human cell types, including ESCs and HSCs (Lombardo et al.

2007). Recent studies have shown that TALENs (Sun et al. 2012; Voit et al. 2014) and CRISPR/Cas9 systems (Cottle et al. 2015; Cradick et al. 2013; Hendel et al. 2014) provide efficient gene targeting of the *HBB* locus near the SCD mutation site in K562 cells.

Early studies demonstrating efficient gene targeting by ZFNs (Hockemeyer et al. 2009) and TALENs (Hockemeyer et al. 2011) in human ESCs and induced pluripotent stem cells (iPSCs) led to the idea for a therapeutic strategy involving gene correction in patient-specific pluripotent stem cells. Pioneering work by Yusa et al. demonstrated gene correction of the point mutation in *A1AT* causing α1-antitrypsin deficiency in patient-derived iPSCs using ZFNs and donor vector (Yusa et al. 2011). With drug selection, there was a targeting efficiency of 54% and 4% in one allele and two alleles respectively in iPSC clones and without altering the cell pluripotency (Yusa et al. 2011). A similar approach was used for correcting the SCD mutation where human iPSCs derived from individuals with SCD were transfected with *HBB*-aiming ZFNs and donor vector containing the wild-type *HBB* and a drug selection cassette flanked by loxP sites (Sebastiano et al. 2011). HDR mediated gene correction was achieved in up to 37% of drug resistant clones, each having retained their pluripotency and normal karyotype. A limited investigation of the ZFN specificity showed that there were no mutations generated in cognate off-target sites. The floxed drug resistant gene and reprogramming cassette were excised by transient expression of Cre recombinase in the corrected iPSCs, demonstrating efficient generation of transgene-free corrected iPSC cell lines from SCD patients (Sebastiano et al. 2011). A similar study confirmed the feasibility of generating gene corrected iPSCs derived from SCD patients using *HBB* ZFNs and donor vector (Zou et al. 2011). However, even after excision of the drug selection cassette using the Cre-LoxP system, the endogenous gene expression of the corrected allele was only partially restored in erythroid-differentiated cells, likely because of interfering “scar” sequences remaining after excision (Zou et al. 2011). Challenges in excising the drug selection cassette and differentiating iPSCs after nuclease-based gene correction may limit the clinical applicability of this approach.

HSCs are more clinically relevant compared to iPSCs for targeted genome editing strategies for treating hemoglobinopathies because they can differentiate into normal RBCs *in vivo* following engraftment. Precision genome editing tools were shown to provide efficient NHEJ mediated-gene disruption of *CCR5* in human $CD34^+$ cells (Holt et al. 2010). The work by Genovese et al. was the first to demonstrate successful HDR-mediated gene modification in human HSCs using site-specific nucleases (Genovese et al. 2014). Specifically, delivery of ZFN mRNAs and a donor vector designed to transfer GFP into the mutational hotspot of *IL2RG*, associated with X-linked SCID, and the AAVS1 ‘safe harbor’ site resulted in ~5% HDR in bulk cultured $CD34^+$ cells. Transplantation of these $CD34^+$ cells into non-obese diabetic (NOD)/SCID mice resulted in 3% of GFP positive blood cells due to gene insertion. $CD34^+$ cells were also treated with *IL2RG* ZFNs and a targeting vector spanning exons 5-8 of *IL2RG* to stimulate gene correction of X-linked SCID deficiency. Transplantation of treated $CD34^+$ cells into NOD/SCID mice resulted in the functional reconstitution of *IL2RG*, as evident by T-cells that were physiologically similar to healthy controls. Evaluation of off-target activity by the *IL2RG* ZFNs revealed negligible off-target mutation at previously identified cognate sites (Genovese et al. 2014).

The ZFN based genome editing strategy was applied for correcting the SCD mutation in human HSCs (Hoban et al. 2015). In this study, $CD34^+$ cells treated with *HBB*-aiming ZFNs along with donor templates containing a silent restriction fragment length polymorphism (RFLP) site showed 18.5% average gene insertion. A similar level of gene conversion was measured in healthy $CD34^+$ cells treated with ZFNs and donor containing the SCD mutation, and in $CD34^+$ cells isolated from SCD patients treated with ZFNs and donor encoding wild-type *HBB*. The corresponding HbA protein level was 5.3% in erythrocytes derived from SCD-patient isolated $CD34^+$ cells treated with ZFNs and wild-type *HBB* donor templates, demonstrating functional correction of SCD mutation using genome-editing tools. Although bulk $CD34^+$ cells treated with *HBB*-aiming ZFNs and donor templates engrafted as efficiently as control non-treated $CD34^+$ cells and differentiated into the correct lineages, there was a ~50-fold reduction in gene modified cells in the bone marrow after engraftment in the NOD/SCID/IL2rγ^{null} mice compared to the initial bulk population. High throughput sequencing revealed only 0.85% targeted gene-modification in the bone marrow of mice transplanted with ZFN- and oligonucleotide-treated cells, whereas the bulk population prior to transplantation had 17.3% gene modification. These results suggest that gene modified cells may have a competitive disadvantage for engraftment compared to unmodified $CD34^+$ cells. An assessment of off-target activity revealed that the ZFNs had high levels of specificity with off-target modification occurring only in the homologous δ-globin gene (*HBD*) (Hoban et al. 2015). It is unclear whether gene modification within the *HBD* locus will have adverse consequences in a clinical setting. Although these seminal studies demonstrate the feasibility of efficient gene correction by ZFNs in human $CD34^+$ cells, further investigations that address low levels of engraftment by gene modified cells in the xenograft model, particularly within the context of hemoglobinopathies, will be critical for advancing the nuclease-based gene correction strategy.

Transcriptional silencers of γ-globin expression offer an alternative yet promising therapeutic target for treating hemoglobinopathies. Genome wide association studies revealed small nucleotide polymorphisms in *BCL11A*, encoding for a multi-zinc finger transcription factor, were correlated with variations in HbF levels and the severity of SCD and β-thalassemia (Lettre et al. 2008; Sankaran et al. 2008; Sankaran et al. 2009; Uda et al. 2008). Previously validated as a risk factor for SCD, HbF levels in the 75^{th} percentile (> 8.6%) are associated with reduced severity of anemia and enhanced patient survival (Platt et al. 1994). The BCL11A transcription factor represses HbF in adult erythroid cells and not in fetal erythroid cells. High HbF levels are associated with low BCL11A levels and vice versa, providing evidence for the role of BCL11A in the transcriptional regulation of HbF (Sankaran et al. 2008). Bauer et al demonstrated erythroid-specific gene targeting of BCL11A using designer nucleases (Bauer et al. 2013). In this study, the erythroid-specific enhancer in intron-2 of the *BCL11A* locus was identified using genome wide association studies and was the target for a pair of TALENs designed to cleave the enhancer region, resulting in BCL11A knockdown with concomitant elevated HbF in erythroid cells (Bauer et al. 2013). Recently, knockdown of *BCL11A* expression leading to HbF induction was demonstrated using the CRISPR/Cas9 system (Canver et al. 2015). The level of HbF necessary to ameliorate the morbidity of SCD and β-thalassemia was estimated to be ~20% in an early study that correlated HbF levels with the disease severity as determined by

clinical incidence rates, including the risk for crisis, chest syndrome, hospitalization, and meningitis septicemia (Powars et al. 1984). However, the authors cautioned that the relationship between continuous risk for clinical incidences and the HbF levels may not be linear (Powars et al. 1984). Sangamo Biosciences and Biogen have co-sponsored pre-clinical studies on the efficacy and safety of the nuclease-based strategy in targeting *BCL11A* for treating SCD and β-thalassemia. In recent conference proceedings, the Sangamo and Biogen team reported that erythrocytes differentiated *ex vivo* from human patient $CD34^+$ cells transfected with ZFNs targeting *BCL11A* coding or enhancer regions had clinically relevant elevated levels of γ-globin, up to 79%, as well as long-term engraftment in immunodeficient mice (Ando et al. 2015; Chang et al. 2015; Urnov et al. 2015). In addition to establishing a conclusive threshold value of therapeutic HbF for a therapy, it will be necessary to examine whether *BCL11A* knockdown in erythrocytes may lead to adverse effects. An additional silencer of HbF of similar potency as BCL11A is the lymphoma/leukemia-related factor (LRF) encoded by *ZBTB7A*. Knockout of *LRF* using CRISPR/Cas9 resulted in increased levels of HbF from 3% to >60% in human erythroid knockout clones independent of *BCL11A* expression. Joint knockout of both LRF and BCL11A resulted in >90% HbF expression (Masuda et al. 2016; Masuda et al. 2015). These studies suggest that LRF is a promising new therapeutic target for treating hemoglobinopathies.

Challenges and future directions

Nuclease mediated gene correction is a potentially powerful new autologous therapeutic strategy for hemoglobinopathies. The monogenic mutations responsible for the pathology of SCD and β-thalassemia could be corrected using patient derived HSCs *ex vivo* **(Figures 1, 2, and 5)**. The nuclease-based strategy involves the isolation of HSCs from the patient, modifying specific *HBB* sequences in them *ex vivo*, and infusing them back into the patient. The advantage of the nuclease-based approach over conventional gene therapy is the elimination of risks of insertional mutagenesis or viral-induced immune response (Cavazzana-Calvo et al. 2000; Hacein-Bey-Abina et al. 2003b). However, there are several barriers with the precision genome editing based strategy that must be addressed to advance its clinical application. Firstly, the design of nucleases and donor template needs to be optimized to enable higher levels of HDR mediated insertion of the wild-type donor template into the *HBB* mutation site. Although the design of donor templates is simplified by single-stranded oligodeoxynucleotides, the length and nucleotide sequences need to be optimized. Design and construction of TALENs and ZFNs is difficult to perform, even for experienced researchers, and involves complicated, low throughput cloning steps for assembling multiple plasmids encoding a single nuclease pair (Joung and Sander 2013; Urnov et al. 2010). Although with relatively high specificity, TALEN RVDs have a low specificity for nucleotide G, leading to cleavage activity at potential off-target sites (Joung and Sander 2013). Likewise, ZFNs may have high off-target cleavage activity (Urnov et al. 2010). The specificity for both ZFNs and TALENs can be improved by increasing the lengths of the DNA binding domains or through the use of heterodimeric *FokI* nuclease domains (Miller et al. 2007; Szczepek et al. 2007). There are several online design tools available to help guide the design of TALENs (Lin et al. 2014b; Neff et al. 2013) and ZFNs (Sander et al. 2010; Sander et al. 2007).

The *S.* pyogenes (*Spy*) CRISPR/Cas9 system is easier to design and construct, and it only requires modifying the 20-nucleotide target sequence within the sgRNA to cleave a new gene locus. However, off-target activity might be an issue for their clinical applications (Cradick et al. 2013; Fu et al. 2013; Hsu et al. 2013). The specificity of CRISPR/Cas9 systems can be improved through the Cas9 nickase system, shown to result in efficient gene editing with >50-fold lower off-target activity (Mali et al. 2013; Ran et al. 2013). It has also been demonstrated that by modifying the Cas9 protein, or using Cas9 orthologs, the specificity of CRISPR/Cas9 systems can be improved (Kleinstiver BP 2016; Lee et al. 2016a; Slaymaker et al. 2016). The recent characterization of the Cpf1-containing class 2 CRISPR systems revealed unique features for genome editing; the Cpf1 nuclease makes staggered DSBs and has a simpler and shorter guide RNA design. The shorter guide RNA could enable fabrication of RNA oligos cheaper and the staggered cuts with 5' overhangs has the potential for the precise insertion of exogenous genes via non-HDR mechanisms (Zetsche et al. 2015). In the last few years many CRISPR design tools have been developed, including tools for identifying target sites and target validation (Hodgkins et al. 2015; Hsu et al. 2013; Montague et al. 2014; Xie et al. 2014; Zhu et al. 2014) and for predicting potential off-target sites using *in silico* methods (Cradick et al. 2014; Fine et al. 2014; Hsu et al. 2013). A recent review of these tools can be found in Lee et al. (Lee et al. 2016b). Given the simplicity of the CRISPR/Cas9 system, further improvements in the safety will likely result in its dominant application over ZFNs and TALENs in future gene therapies.

To increase the HDR rates in $CD34^+$ cells, it is necessary to have a better understanding of DSB repair events in cells after co-delivery of nucleases and donor templates, and develop new methods to alter the pathway choice (HDR vs. NHEJ). The traffic light reporter system has been used for measuring both NHEJ and HDR in single cells treated with gene editing reagents (Certo et al. 2011), but this system is time consuming and difficult to construct. Alternatively, single molecule real-time (SMRT) sequencing or the next-generation deep sequencing (NGS) can be used to provide highly accurate quantification of NHEJ- and HDR-mediated gene modification at on- and off-target sites (Hendel et al. 2014). Recently, Tracking of Indels by Decomposition (TIDE) was developed as a faster and more cost-effective approach for accurate quantification of targeted gene modifications (Brinkman et al. 2014), which may enable more efficient optimization of *HBB* targeting nucleases and donor designs. Although new methods have been developed to increase the HDR rates, their applicability to $CD34^+$ cells needs to be further studied (Maruyama et al. 2015).

Further, efficient gene targeting requires a sufficient amount of nuclease proteins and donor templates in the target cells, such as $CD34^+$ cells. Integrase deficient lentivirus (IDLV) has been used to deliver ZFNs and donor templates into $CD34^+$ cells, resulting in low levels of gene targeting (Lombardo et al. 2007). A limitation of this approach is low permissiveness of human HSCs to multiple IDLV infections (Lombardo et al. 2007). In contrast, delivering the donor template using IDLV and ZFN mRNAs using electroporation resulted in considerably higher levels of gene targeting (Genovese et al. 2014; Hoban et al. 2015). It was found that cell cycle activation through culture stimulation is necessary to obtain optimized levels of lentiviral delivery and gene targeting, particularly in primitive HSCs (Genovese et al. 2014). The addition of aryl hydrocarbon receptor antagonist and 16,16-dimethyl-prostaglandin E2 to the culture media was found to reduce differentiation and may

contribute to enhanced gene targeting (Genovese et al. 2014). For the CRISPR/Cas9 systems, the possible delivery approaches, include plasmid encoding both Cas9 and gRNA, Cas9 mRNA along with gRNAs, or Cas9 protein complexed with gRNA molecules (RNP). It has been shown that the specificity and toxicity can be improved through the delivery of the CRISPR/Cas9 system as RNP complexes (Zuris et al. 2015).

The therapeutic benefit of gene corrected HSCs depends on their capacity to engraft and provide long-term production of healthy RBCs in a patient. Therefore, a critical challenge for genome-editing based treatment of hemoglobinopathies is the low engraftment potential of human $CD34^+$ cells treated with nucleases and donor templates (Hoban et al. 2015). Currently it is unclear how to increase the amount of gene corrected HSCs from a hemoglobinopathy patient that would engraft, particularly within the context of an autologous transplant. Addressing this issue may require the development of xenograft models of hemoglobinopathies that better recapitulate the disease phenotype. It is also necessary to evaluate the consequences of both on-target and off-target cleavages in $CD34^+$ cells induced by nucleases, and non-specific integration of donor templates.

In summary, targeted gene correction of the *HBB* locus or reversal of HbF suppression through the application of precision genome editing strategies may enable the development of a novel curative autologous therapy for hemoglobinopathies. Further research efforts addressing the design, optimization, and delivery of nucleases and donor templates for *HBB* gene correction in HSCs, and the development of better mouse models for accurately quantifying the repopulation efficiency of gene-corrected human HSCs are critical for the clinical translation of the gene correction strategies in treating hemoglobinopathies.

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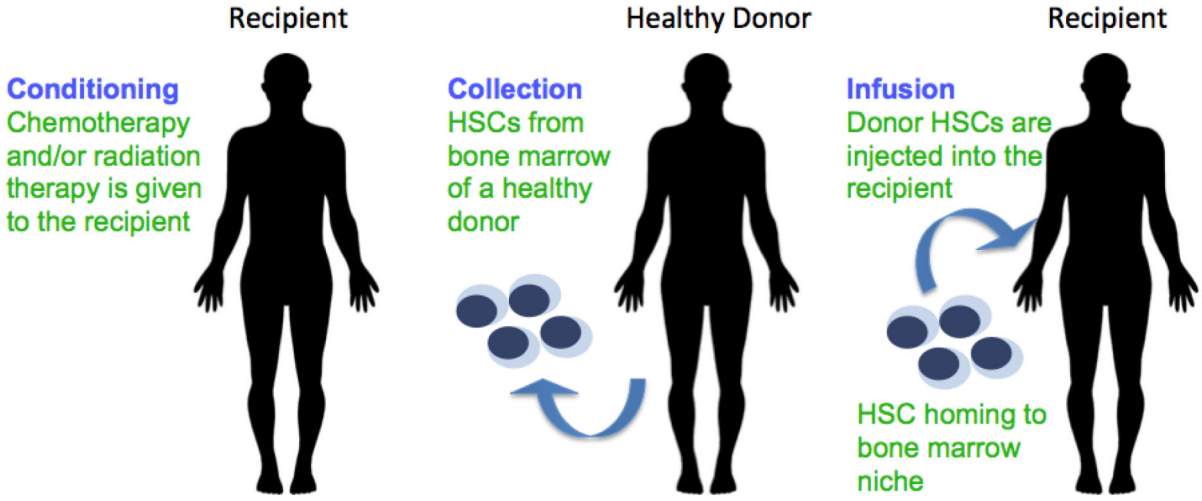

Figure 1. Schematic of allogeneic HSCT

The patient is first treated with radiation therapy and/or chemotherapeutic drugs to ablate the bone marrow. Bone marrow from a healthy donor is harvested and enriched for $CD34^+$ cells using immunolabelling and cell separation techniques. Alternatively, $CD34^+$ immobilized using granulocyte colony stimulating factor (GSCF) could be collected from the peripheral blood of a healthy donor. The donor HSCs are infused into the patient to repopulate the entire blood system, thereby replacing the defective RBCs with healthy RBCs.

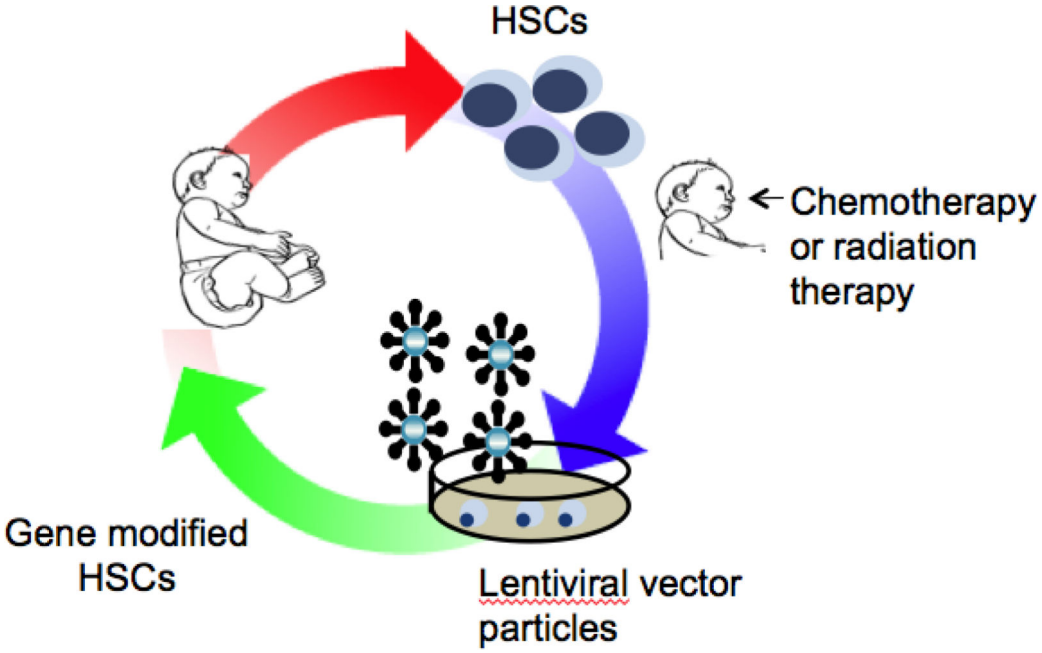

Figure 2. Schematic of gene therapy for hemoglobinopathies

$CD34^+$ cells are collected from the patient with β-thalassemia or SCD and treated with lentiviral vector particles containing the normal *HBB* and β-LCR elements. The patient is given chemotherapy and/ or radiation therapy to ablate the bone marrow and make room for engraftment by gene modified HSCs. Following viral transduction, the gene modified HSCs are infused into the patient.

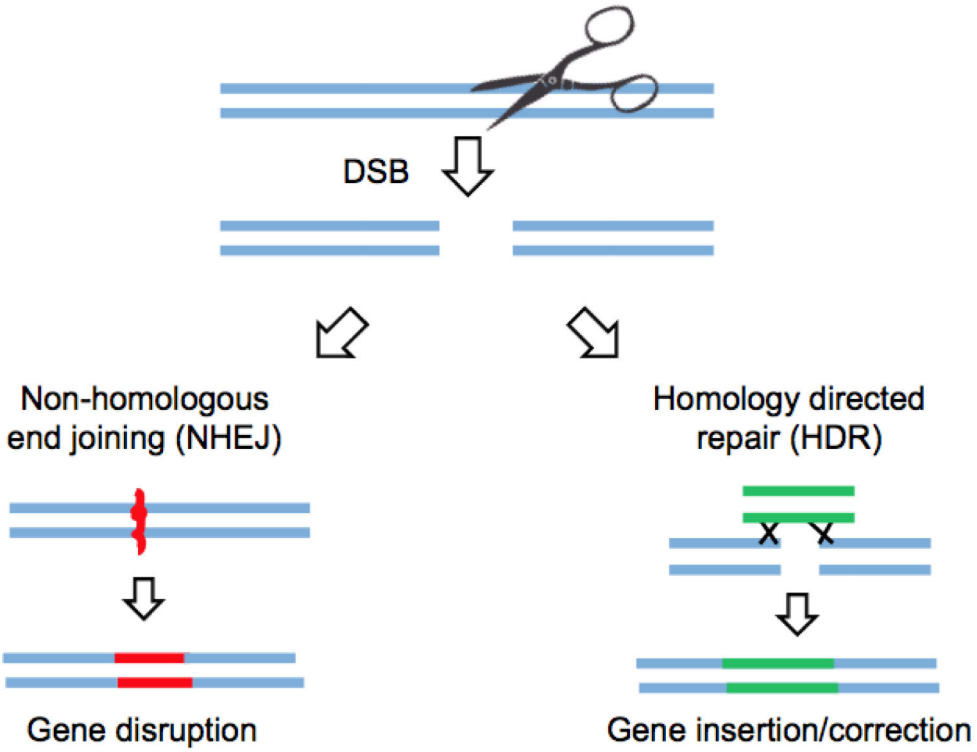

Figure 3. Gene modification outcomes following the induction of a DSB by precision gene editing tools

The DSB is resolved by endogenous repair machinery involved in the NHEJ and HDR pathways. Activation of the NHEJ repair pathway leads to insertions and/or deletion of sequences at the DSB site to enable resecting of both strands for repair, resulting in gene disruption. The simultaneous delivery of nucleases and donor repair template DNA enables repair using the HDR pathway, whereby the information encoded on the donor template is transferred to the target site for gene insertion or gene correction.

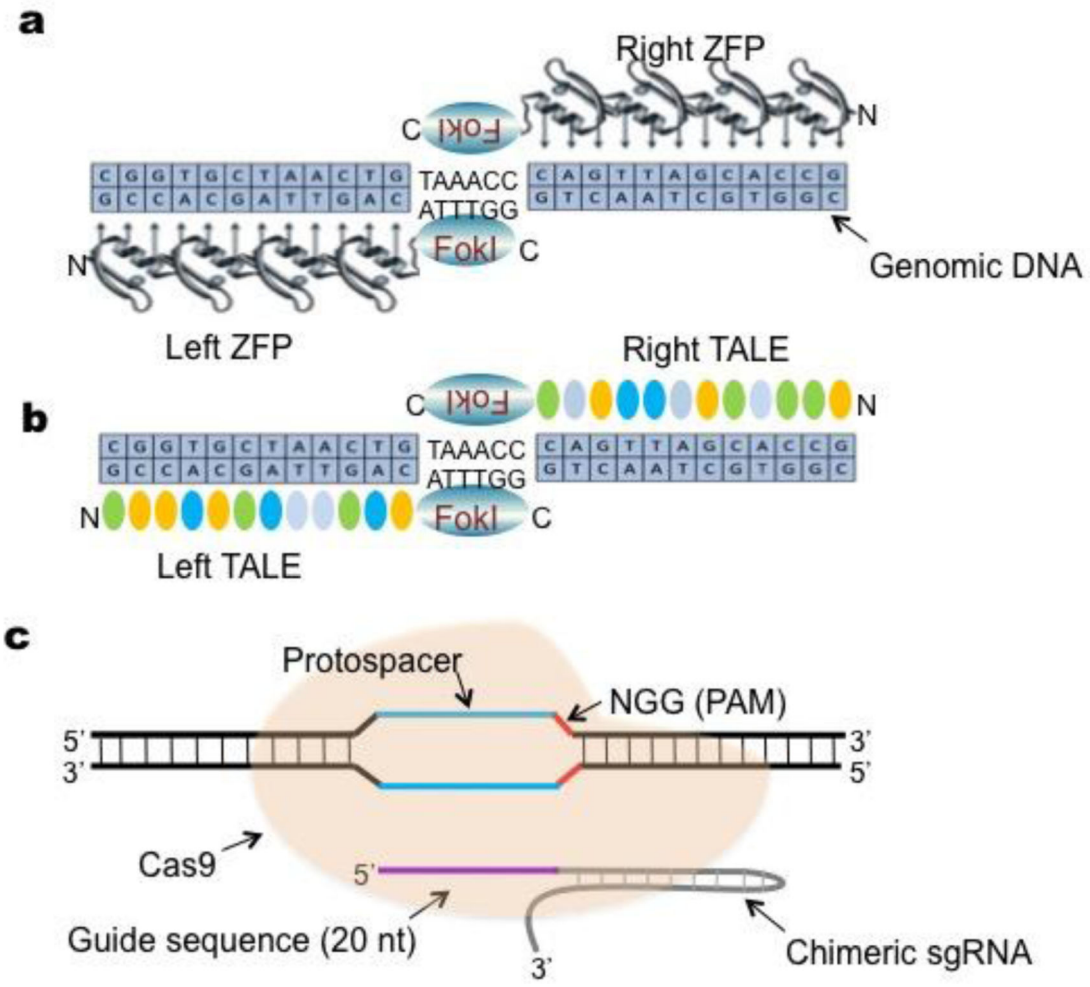

Figure 4. Schematic of precision gene-editing tools

(a) ZFNs consist of a DNA binding ZFP fused to a *FokI* restriction enzyme. Each of the ZFP units recognizes 3 nucleotides and are linked together to mediate binding to a desired target site. A pair of ZNFs binds to the DNA target sites in a head to tail orientation. **(b)** TALENs consist of TALE repeat domains, each recognizing a single nucleotide, fused to *FokI*. The effector domains from a TALEN pair bind to adjacent effector elements in a tail to tail orientation with optimized spacing. The generation of a DSB within the spacing region between TALE or ZFP pairs requires the dimerization of the *FokI* domains. **(c)** In the CRISPR/Cas9 systems, a Cas9 endonuclease is guided to a target site by a chimeric sgRNA containing the complementary guide sequence (purple) to the target site immediately 5' of the NGG PAM sequence (red).

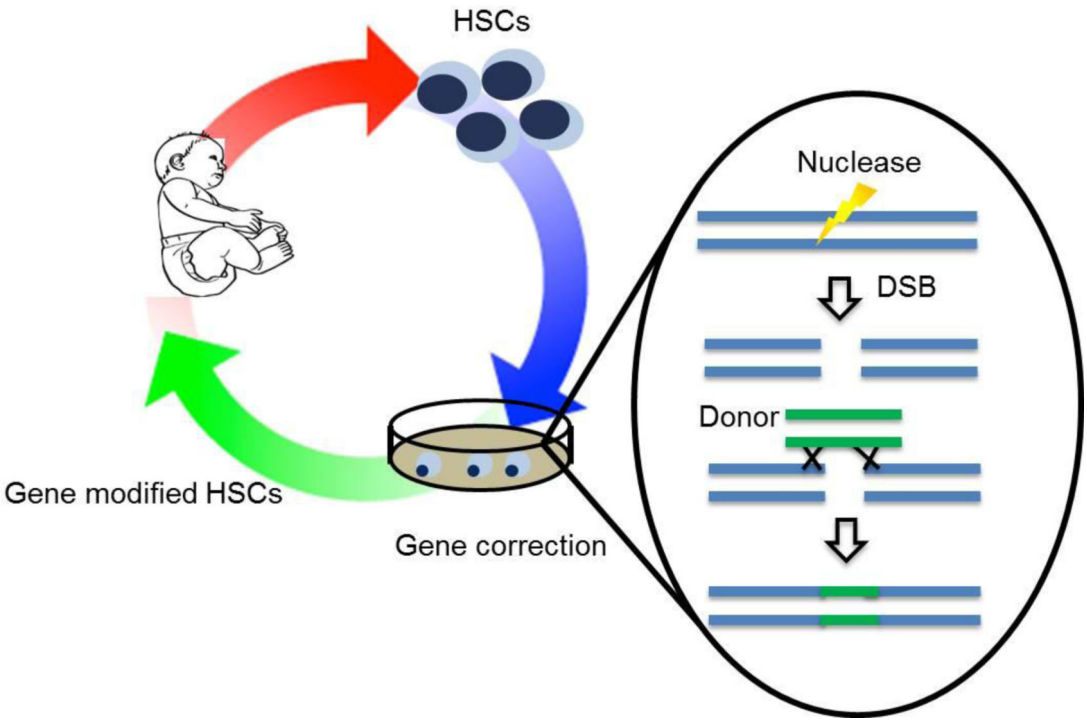

Figure 5. Schematic of gene correction approach for treating hemoglobinopathies

$CD34^+$ cells are collected from the β-thalassemia or SCD patient and introduce with a nuclease targeted to the *HBB* genomic locus and a donor template DNA. HDR-mediated repair of the DSB stimulated by the nucleases will enable replacement of the sickle *HBB* with the wild type *HBB* in the genome. Transfusion of the gene modified HSCs will provide long-term replacement of the sickled RBCs with healthy RBCs for the lifetime of the patient.

Table 1

Summary of gene therapy studies using viral vectors

Disease	Vector	Species	Results/Drawbacks	References
β-thalassemia	Lentiviral vector containing wild-type human *HBB*	Mice	• Hemoglobin levels depends on vector copy number • High copy number in bone marrow cells results in 32% total hemoglobin consisting of human HbA and correction of hematologic indices of disease	(Imren et al. 2002; May et al. 2000)
SCD	Lentiviral vector containing $\beta^{A\text{-}T87Q}$ -globin	Mice	• 52% of total hemoglobin consisting of the $p^{A\text{-}T87Q}$ variant • Corrected RBC and reticulocytes, splenomegaly, and urine concentration defect	(Pawliuk et al. 2001)
SCD	Lentiviral vector containing β^{AS3}-globin	Mice	• Mean proviral copy number of 2.2 • 20% total hemoglobin consisting of HbAS3 • Correction of SCD phenotype	(Levasseur et al. 2003)
β-thalassemia	HPV569 LentiGlobin	Human	• 1 of 2 subjects had transfusion independence for 1-7 years after therapy • Low vector copies (0.6 per cell) • Partial dominance of clone with *HMGA2* integration	(BusinessWire 2015a; Cavazzana-Calvo et al. 2010; Payen and Leboulch 2012)
β-thalassemia and SCD	BB305 LentiGlobin	Human	• High vector copy numbers (0.3-2.1) • Higher total and therapeutic hemoglobin levels at earlier time points in contrast to HPV569 • Patients are transfusion independent earlier compared to HPV569 • No adverse events or clonal dominance • Patient outcome may depend on β-thalassemia genotype	(Cavazzana et al. 2015a; Cavazzana et al. 2014; Cavazzana et al. 2015b; Kanter et al. 2015; Thompson et al. 2014; Walters et al. 2015)

Table 2

Summary of gene therapy studies using genome-editing tools

Disease	Nuclease	Correction Mode	Results/Drawbacks	References
X-linked SCID	IL2RG-aiming ZFNs and targeting vector	• *In vitro* in K562 and human embryonic kidney 293 cells • *Ex vivo* in human T-cells, ESCs and *CD34*$^+$ cells	• 5% HDR in bulk cultured CD34$^+$ cells • Engraftment of gene modified HSCs in NOD/SCID mice with correction of T-cells • HDR mediated gene targeting dependent on cell cycle stage	(Genovese et al. 2014; Lombardo et al. 2007; Urnov et al. 2005)
A1-antitrypsin	A1AT-aiming ZFNs and donor vector	• *Ex vivo* in patient derived iPSCs	• Up to 54% targeting efficiency with drug selection while maintaining pluripotency	(Yusa et al. 2011)
SCD	HBB-aiming ZFNs and donor vector	• *Ex vivo* in patient derived IPSCs • *Ex vivo in CD34*$^+$ cells	• 37% of drug resistant iPSC clones were positive for HDR-mediated gene correction • 5.3% HbA in erythrocytes derived from SCD *CD34*$^+$ cells transfected with ZNFs targeting HBB and donor template vector • Excision of drug resistant cassette in iPSC using Cre recombinase could leave interfering “scar” sequences •Gene modified cells have less engraftment in contrast to unmodified *CD34*$^+$ cells	(Hoban et al. 2015; Sebastiano et al. 2011; Zou et al. 2011)
β-thalassemia and SCD	Double pair of ZFNs, TALENs or CRISPR/Cas9 nucleases targeting *BCL11A*	• *In vitro mouse erythroleukemia cell line* • *Ex vivo* in patient *CD34*$^+$ cells	• Erythroid specific BCL11A knockout and elevated HbF expression in erythroid cells • Long-term engraftment of *CD34*$^+$ cells transfected with BCL11A-aiming ZFNs	(Ando et al. 2015; Bauer et al. 2013; Canver et al. 2015; Chang et al. 2015; Urnov et al. 2015)
β-thalassemia and SCD	CRISPR-Cas9 targeting ZBTB7A	• *Ex vivo* in human immortalized erythroid (HUDEP-2) cells • *Ex vivo* in	• *ZBTB7A* knockout clones had >60% HbF levels in contrast to 3% HbF in unmodified controls • Double knockout of *ZBTB7A* and *BCL11A* resulted in 91-94% HbF levels	(Masuda et al. 2016; Masuda et al. 2015)