



The potential of gene therapy approaches for the treatment of hemoglobinopathies: achievements and challenges

Michael A. Goodman and Punam Malik

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Abstract: Hemoglobinopathies, including β -thalassemia and sickle cell disease (SCD), are a heterogeneous group of commonly inherited disorders affecting the function or levels of hemoglobin. Disease phenotype can be severe with substantial morbidity and mortality. Bone marrow transplantation is curative, but limited to those patients with an appropriately matched donor. Genetic therapy, which utilizes a patient's own cells, is thus an attractive therapeutic option. Numerous therapies are currently in clinical trials or in development, including therapies utilizing gene replacement therapy using lentiviruses and the latest gene editing techniques. In addition, methods are being developed that may be able to expand gene therapies to those with poor access to medical care, potentially significantly decreasing the global burden of disease.

Keywords: gene editing, gene therapy, hemoglobin, hemoglobinopathy, sickle cell anemia, thalassemia

Introduction to hemoglobinopathies

Hemoglobin is a protein found in red blood cells (RBCs) that is critical for the process of oxygen transportation, allowing for oxygen to be transported from the lungs to the tissues. Hemoglobin exists as a heterotetramer, containing two subunits from the α -globin subfamily (ζ , α_2 , and α_1) and two subunits from the β -globin subfamily (ϵ , γ , δ , and β) [Forget and Hardison, 2009]. The expression of the different α - and β -globin family subunits are developmentally-regulated processes. Mutated globin proteins or decreased expression of globin proteins (hemoglobinopathies) can lead to poor oxygen transportation and a variety of other damaging physiological outcomes.

Hemoglobinopathies are a large heterogeneous group of inherited disorders of hemoglobin synthesis that can be further subdivided into thalassemia syndromes and structural hemoglobin variants. Thalassemia syndromes result from diminished or absence of either α - or β -globin, leading to α -thalassemia or β -thalassemia, respectively. Structural hemoglobin variants result from mutations within a globin gene, leading to disruption of the normal peptide structure and function.

Hemoglobinopathies can be found throughout the world, but prevalence is increased among certain ethnicities. In particular, sickle cell disease (SCD) is more common among people with sub-Saharan African or Indian ancestry. As a whole, thalassemias are the most common monogenic disorders in the world [Weatherall, 2001]. According to a World Health Organization (WHO) report, approximately 5% of the world's population carries trait genes for hemoglobin disorders, mainly sickle cell anemia (SCA) and α - or β -thalassemias [WHO, 2011]. Approximately 100,000 Americans have SCA, with 300,000 affected infants born yearly worldwide [Bauer *et al.* 2012; Yawn *et al.* 2014]. The global disease burden of hemoglobinopathies is borne enormously by the African continent, where according to the WHO, 5–16% of under-five mortality in some areas of sub-Saharan Africa is attributed to SCA [WHO, 2006]. Additionally, data suggest that children born in Africa with SCA have an early-life mortality of 50–90% [Grosse *et al.* 2011]. This disproportionate burden on under-developed regions is important to remember when considering application of gene therapies for hemoglobinopathies.

Correspondence to:

Punam Malik, MD

Division of Experimental Hematology and Cancer Biology, Cancer and Blood Diseases Institute Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
punam.malik@cchmc.org

Michael A. Goodman, MD

Division of Experimental Hematology and Cancer Biology, Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

Hemoglobinopathies, especially SCA, are prime targets for gene therapy for a variety of reasons. Their high prevalence, significant morbidity and mortality, and the resulting high cost of medical care portends that a curative therapy can greatly improve patient outcomes and significantly reduce associated medical costs. Due to its genetic etiology, genetically-modified hematopoietic stem and progenitor cells (HSPCs) are able to pass on their modified genome to daughter cells, including RBC precursors. Given the self-renewing capacity of HSPCs, a single treatment is potentially curative. With regards to gene editing strategies, many of the mutations resulting in hemoglobinopathies are single point mutations, which typically allow for greater gene correction efficiencies than more complex mutations. Lastly, the hemoglobinopathies have already been successfully treated with hematopoietic stem cell transplant (HSCT), which similar to many gene therapy techniques; requires engraftment of the long-term repopulating hematopoietic stem cells (HSCs). As will be described, gene therapy for hemoglobinopathies can be divided into four general categories (1) gene addition, (2) gene knock-down to improve the β -globinopathy phenotype, (3) globin gene editing, and (4) gene editing of globin regulatory elements.

Thalassemias

α -thalassemia typically results from functional deletion of two or more of the four α -globin genes. Loss of α -globin chains lead to a reduction in the predominant hemoglobin, hemoglobin A (HbA), composed of a $\alpha_2\beta_2$ heterotetramer. Patients with loss of a single α -globin gene ($-\alpha/\alpha\alpha$) are typically asymptomatic silent carriers. Clinical phenotype of α -thalassemia can range from mild effects on hemoglobin indices to fetal hydrops and intrauterine demise, depending on the number of α -globin genes affected and the specific mutations involved.

Genetic mutations leading to β -thalassemia may be found within the β -globin genes itself, or external to the genes, within the globin locus. Most commonly, the mutations resulting in β -thalassemia are point mutations. Hundreds of different mutations have been described affecting β -globin levels *via* effects on a wide range of processes, including: transcription, mRNA splicing/processing, RNA stability, translation, and globin peptide stability. The low β -globin content allows the excess α -globin chains to precipitate in

erythroid precursors. The α -globin aggregates cause cell membrane damage and lead to early erythroid precursor death. The resultant ineffective erythropoiesis found in patients, if severe, may necessitate frequent blood transfusions. β -globinopathies typically present 6–12 months after birth when γ -globin expression, which is the predominate globin expressed from the β -globin family during fetal life, begins to diminish to residual amounts typically found throughout the remainder of life. Disease severity may range from asymptomatic to severe.

Structural hemoglobinopathies

The most common structural hemoglobinopathy, SCD, is due to a single point mutation in β -globin, an A to T mutation resulting in a Glu6Val substitution. This mutation leads to a mutated β -globin chain, β^S , and results in production of hemoglobin S (HbS), composed of two α -globin peptides and two mutated β -globin peptides ($\alpha_2\beta^S_2$), instead of the normal HbA ($\alpha_2\beta_2$). Individuals with one copy of β^S are typically asymptomatic and referred to as having a sickle cell trait. Any additional pathological mutation in the other β -globin gene results in SCD. An individual exclusively producing HbS (i.e. with two β^S genes [β^S/β^S] or sickle β^0 thalassemia [β^S/β^0]), is referred to as having SCA. Besides SCA, several other structural hemoglobin variants exist (e.g. hemoglobin C, D, and E). All of the structural hemoglobin variants are inherited in an autosomal recessive manner, and compound heterozygotes for any of these variants along with a sickle allele (HbSC, HbSD or HbSE) phenotypically result in SCD. Similarly, inheritance of a β^+ thalassemia allele along with a β^S allele (HbS- β^+ thalassemia) also results in a SCD phenotype, albeit with milder symptomatology.

HbS, is prone to polymerization under reduced oxygen conditions, leading to the cascade of sickling of RBC, RBC hemolysis and aggregation of RBC and adhesion of white blood cells (WBCs) in the microvasculature, and finally vaso-occlusion. This process can lead to a variety of significant adverse sequelae of varying severity (Table 1). The manifestations of SCD are chronic and progressive with significant associated morbidity and mortality. Similar to severe thalassemia, patients with severe SCD often require frequent blood transfusions, and are at risk of transfusion-induced iron overload. Not infrequently, multiple hemoglobinopathies will occur concurrently (e.g. β^S and

Table 1. Common sequelae of sickle cell disease.

Vaso-occlusive crisis
Acute chest syndrome
Acute stroke
Priapism
Hepatobiliary complications
Splenic sequestration
Acute renal failure
Anemia
Severe infections (e.g. pneumonia)
Chronic obstructive pulmonary disease
Asthma
Pulmonary hypertension
Congestive heart failure
Cardiomegaly
Arrhythmia
Cardiomyopathy
Leg ulcers
Avascular necrosis
Other chronic manifestations
Death

β -thalassemia are often seen together). Concurrent hemoglobinopathies generally lead to a less severe phenotype (i.e. phenotypes of a sickle allele and β^+ thalassemia allele or a sickle allele and an HbC allele are typically milder than two sickle alleles, although exceptions exist, such as HbSD).

Both structural hemoglobinopathies and the thalassemias can be cured by HSCT [Bernaudin *et al.* 2007; Lucarelli *et al.* 2012; Hsieh *et al.* 2014]. While potentially curative, HSCT is only available to a minority of patients due to the lack of appropriately-matched donors for most patients. For instance, only 15–20% of patients with SCD are able to identify an appropriately-matched donor for possible HSCT. Additionally, HSCT is a rigorous treatment, often with its own morbidity and mortality. Graft *versus* host disease, toxic effects of conditioning regimens, prolonged and severe cytopenias, susceptibility to infections, and other adverse effects are commonly observed during HSCT. Some of these effects have been mitigated somewhat with the increasing use of nonmyeloablative conditioning regimens.

Principles of gene therapy

Due to the above considerations, gene therapy utilizing a patient's autologous stem cells hold

great promise, as they would obviate the need to identify matched donors and further mitigate the morbidity and mortality of allogeneic HSCT. A general outline of the steps involved in both current and gene therapies in development is shown in Figure 1. Most commonly, gene therapy is accomplished by first harvesting a patient's HSPCs from either bone marrow, peripheral blood, or umbilical cord blood. Next the cells are exposed to a therapy that introduces designed modifications into the cells genome. Traditional gene therapies currently in clinical trials consist of inserting an additional globin gene (β or γ globin or an anti-sickling β globin) *via* a lentiviral vector that integrates into the host cell's genome. The cells are then transplanted back into the patient where the modified cells proliferate and repopulate the hematopoietic compartment. While engraftment of only a small population of corrected HSPCs can result in amelioration of a hemoglobinopathy, highly efficient gene transfer must occur in order to modify a sufficient number of cells able to achieve long-term engraftment. Current gene therapies in clinical trials achieve this by using a lentiviral vector to insert an additional globin gene.

Future gene therapies may make use of induced pluripotent stem cells (iPSCs) to circumvent the need for high-modification efficiencies. This route involves first isolating somatic cells (e.g. fibroblasts) from a patient. Somatic cells are then reprogrammed into a pluripotent state by expressing certain factors (originally Oct3/4, Sox2, c-Myc, and Klf4) [Takahashi and Yamanaka, 2006]. The iPSCs are then treated to achieve the desired genetic modification. A correctly modified clone is then expanded *in vitro*. After sufficient expansion, the iPSCs are differentiated into HSPCs. These cells are then transplanted back into the patient. While this strategy would overcome many of the limitations in using HSPCs harvested directly from a patient, it is not yet feasible to use iPSC-derived HSPCs for HSCT, as they currently have only limited repopulation potential.

Gene delivery mechanisms

A variety of gene delivery mechanisms have been devised, but we will focus only on those applicable for treatment of hemoglobinopathies. The applicable delivery mechanisms can be divided into viral and nonviral methods. As mentioned above, traditional gene therapies utilize viral vector delivery systems. Having evolved the ability to

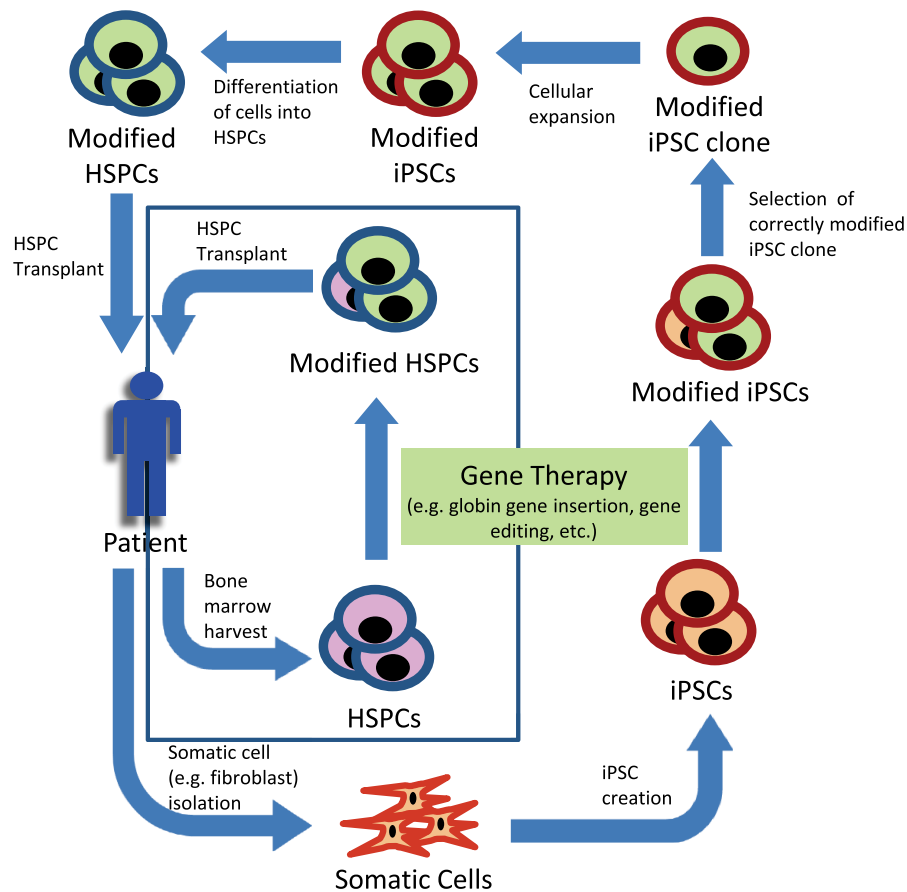


Figure 1. Typical process of gene therapy for hematopoietic disorders. Hematopoietic stem and progenitor cells (HSPCs), induced pluripotent stem cells (iPSCs). HSPCs can be modified directly, as is the case for currently used therapies. Alternatively, somatic cells, e.g. fibroblasts, can be first reprogrammed into iPSCs before being modified. A correctly modified iPSC clone is then selected and expanded before being differentiated into HSPCs. In either case, the genetically modified HSPCs are then transplanted back into the patient. When HSPCs are modified directly, modification may not occur in every cell.

transduce mammalian cells over 100 million years ago [Katzourakis *et al.* 2009] viral vectors are able to deliver engineered genetic material with very high efficiency. Numerous viral systems have been harnessed by researchers for genetic transfer, including gammaretrovirus, lentivirus, foamy virus, adenovirus, and adeno-associated virus (AAV). The most common viral vector used currently is lentivirus. Depending on the nature of the therapy, viral vectors can be chosen based on their ability, or lack of ability, to integrate into the host cells genome. For example, traditional gene therapy, involving insertion of a globin gene, requires genomic integration. Gammaretrovirus, lentivirus, and foamy virus all efficiently integrate into a host cells genome, and are therefore frequently used for this type of therapy. However, many new gene editing techniques benefit from avoiding genomic integration. Constitutive

expression of the DNA-cutting elements found in gene editing modalities can lead to increased off-target effects. Therefore, it is often best to express these elements only transiently, which is best achieved using nonintegrating viral vectors. In particular, adenovirus, which does not integrate into a host cells genome, and AAV, which has very low rates of integration, are ideal for this purpose. Additionally, integration-deficient gammaretroviruses and lentiviruses have been engineered, also with low rates of integration.

Traditional gene therapy was first developed in replication-deficient gammaretroviruses. These studies demonstrated the ability to insert globin transgenes into HSPCs, but expression of the globin chain was low and unstable [Dzierzak *et al.* 1988; Bender *et al.* 1989; Bodine *et al.* 1989; Lung *et al.* 2000]. Subsequently, a variety

of modifications were made to the gammaretrovirus vectors, resulting in improved gene expression. Alterations included: inserting portions either the α -locus HS-40 regulatory region or β -globin locus control regions, the main gene regulators of the α - and β -globin gene clusters, respectively [Chang *et al.* 1992; Plavec *et al.* 1993; Ren *et al.* 1996; Nishino *et al.* 2006]; using the ankyrin gene promoter, promoter of another gene expressed in RBCs, to drive expression of a globin transgene [Sabatino *et al.* 2000]; or utilizing promoters associated with hereditary persistence of fetal hemoglobin [Katsantoni *et al.* 2003; Fragkos *et al.* 2005]. A major problem with early gammaretrovirus vectors that carried the human β -globin gene and β -locus control region (LCR) was that they were significantly unstable with frequent rearrangements [Orkin, 1990]. This instability was overcome through careful optimization of the viral genome, LCR elements, and the β -globin gene (including elimination of an intronic segment and multiple reverse polyadenylation and splicing signals) [Leboulch *et al.* 1994; Sadelain *et al.* 1995]. However, despite the above modifications, gene expression levels remained subtherapeutic.

A breakthrough in gene therapy occurred with the advent of lentiviral vector-based vectors [Naldini *et al.* 1996]. Lentiviral vectors, based on human immunodeficiency virus (HIV)-1, offer several unique advantages compared with those vectors preceding it, including (1) the ability to infect quiescent cells, (2) the ability to package large transgene cassettes, and (3) reduced genotoxicity. As opposed to gammaretroviruses, which integrate preferentially near transcriptional start sites, lentiviruses preferentially integrate within transcribed genes, resulting in lower genotoxicity and insertional oncogenesis. Additionally, safety was increased by the ability to generate vectors efficiently with a self-inactivating (SIN) vector design that deletes the promoters and enhancers from the viral long terminal repeat (LTR) in the provirus. SIN vectors are produced by deleting the viral enhancer/promoter sequences in the U3 region of the 3' LTR. This deletion is then transferred to the 5' LTR after reverse transcription and integration into a host cells genome. Without the deleted sequences the LTR is transcriptionally inactive and no viral genes are able to be transcribed, thereby significantly improving the safety profile of the vector, and allowing the vector to be solely driven by the promoter of choice. While first described using gammaretroviral vectors,

similar systems were later adapted for use in lentiviral vectors [Yu *et al.* 1986; Miyoshi *et al.* 1998; Zufferey *et al.* 1998]. A total of two seminal studies demonstrated the ability of novel lentiviral vectors to correct relevant mouse models of β -thalassemia and SCD. In the first, a lentivirus carrying DNase I hypersensitive site 2 (HS2), HS3, and HS4 of the LCR and the human β -globin gene was able to ameliorate disease in a β -thalassemia mouse model [May *et al.* 2000]. In the second, a lentivirus containing a mutant β -globin chain ($\beta^A\text{-T}^{87}\text{Q}$) known to have anti-sickling properties and the HS2, 3 and 4 fragments of the LCR was able to ameliorate disease in two mouse models of SCD [Pawliuk *et al.* 2001].

In addition to viral delivery methods, transposases have been developed that also allow for integration of a transgene into a target cell's genome. This system consists of a transposase enzyme that is able to cut and paste DNA transposons, such that a transposon supplied on a donor DNA molecule can be pasted into genomic DNA. Transposons consist of a given DNA sequence, flanked by inverted terminal repeats (ITRs). One such transposase is the sleeping beauty transposase system [Geurts *et al.* 2003; Aronovich *et al.* 2011; Ivics and Izsvak, 2011]. In this system, a transposon (transgene flanked by ITRs) is inserted into the host cell's genome at random TA-dinucleotides. An advantage of this system over viral delivery systems is the increased randomness of the integration compared with lentivirus or gammaretrovirus vectors, which have a bias towards integrating into gene rich regions. A version of this system has been used to effectively deliver an anti-sickling globin gene into HSPCs derived from a patient with SCD. The cells derived from the modified HSPCs demonstrated reduced cellular sickling metrics [Sjeklocha *et al.* 2013].

Significant developments in gene editing/targeting and the advantages of having DNA cutting elements only transiently present has led to an increase in the use of nonviral delivery methods. Common methods include nucleofection, electroporation, and lipofection [Toneguzzo and Keating, 1986; Harrison *et al.* 1995; Floch *et al.* 1997; Levetzow *et al.* 2006; Manzini *et al.* 2015]. In addition to the ability to deliver DNA, these methods are also capable of delivering RNA and proteins. The ability to deliver proteins is particularly useful for gene editing, as it allows for the delivery of the designer nucleases (discussed

below). Nucleofection and electroporation utilize electrical charges to permeabilize cell membranes and deliver the desired agent into the cell. Lipofection utilizes lipid reagents to form liposomes around the agent being delivered. Fusion of the liposomes with the cell membrane results in introduction of the agent into the cellular cytoplasm.

Gene therapy approaches

As mentioned above, current and proposed methods of gene therapy for hemoglobinopathies can be divided into four general categories (1) gene addition, (2) gene knockdown to improve the β -globinopathy phenotype, (3) globin gene editing, and (4) gene editing of globin regulatory elements.

Gene addition

As noted above, traditional gene therapy for hemoglobinopathies involves inserting a globin transgene into a patient's HSPCs. The globin transgene introduces either an additional copy of an endogenous globin gene, or a globin gene engineered to have disease-ameliorating effects. Engineered globin genes frequently make use of natural polymorphisms that have been shown to reduce the severity of SCD or thalassemia, such as polymorphisms that impair the normal developmental switch from γ -globin to β -globin that occurs during terminal hemoglobin switching [Akinsheye *et al.* 2011]. Normally the β -globin family members γ - and δ -globin are expressed only at very low levels (<2%) after terminal hemoglobin switching to HbA, and are unable to compensate for hemoglobinopathies [Thein *et al.* 2009]. However, polymorphisms that impair this switch result in hereditary persistence of fetal hemoglobin (HPFH). In this condition, increased amounts of γ -globin are produced, which is able to form heterotetramers with α -globin ($\alpha_2\gamma_2$) referred to as fetal hemoglobin, or HbF. When HPFH occurs concurrently with β -thalassemia, it reduces the amount of free α -globin chains and its resulting toxicity. RBCs containing HbF are also less prone to β^S -induced cell damage and RBC aggregation, resulting in diminished vaso-occlusion [Akinsheye *et al.* 2011]. Incorporating these polymorphisms into designed globin transgenes thus offers a potential therapeutic advantage. Previous clinical trials using additive globin techniques have proven successful in treating β -thalassemia [Cavazzana-Calvo *et al.* 2010].

Currently, there are several ongoing clinical trials for SCD and β -thalassemia sponsored by St. Jude Children's Research Hospital; Cincinnati Children's Hospital Medical Center; bluebird bio; University of California, Los Angeles; Memorial Sloan Kettering Cancer Center; and IRCCS, San Raffaele, Italy. There are presently no clinical trials for α -thalassemia. The lentiviral vectors used in the above trials have been extensively reviewed previously and summarized briefly below; readers are directed to the following reviews for a more indepth discussion [Villamizar *et al.* 2001; Dong *et al.* 2013; Finotti *et al.* 2015].

Briefly, successful correction of β^0/β^E thalassemia major has been shown with a β -globin vector carrying a T87Q mutation by Leboulch and coworkers [Bank *et al.* 2005; Cavazzana-Calvo *et al.* 2010] (and subsequently licensed by the commercial company, bluebird bio), where all patients received myeloablative chemotherapy conditioning, and following gene therapy achieved a median increase in hemoglobin (contributed by the β^{T87Q} transgene) of 5 g/dl at 6 months, and all patients became transfusion-independent. For β^0 thalassemia patients, partial correction occurred in most of the patients with significantly reduced transfusion requirement; one β^0/β^0 patient attained transfusion independence. Overall, the vector produced approximately 5 g Hb/vector copy. The same vector resulted in correction of the sickle phenotype in at least one of four patients, where high vector copies were achieved (results presented at the American Society of Hematology Meeting, 2015 [Cavazzana-Calvo, 2015]); in the other three patients, modest increases in β^{T87Q} globin was modest. A β -globin lentivirus vector generated by Sadelain and colleagues was used in a β -thalassemia gene therapy trial by Memorial Sloan Kettering with a reduced intensity busulfan conditioning [Sadelain *et al.* 2010]. The gene marking efficiency was low and therefore, subtherapeutic expression was achieved, with a significant reduction in transfusion in one patient, and a good partial response in a second patient. However, two patients did not show a clinically significant response, of which one was given myeloablative doses of busulfan; and was attributed to low gene-modified HSC. In another gene therapy trial for β -thalassemia being conducted in IRCCS, San Raffaele, Italy by Ferrari and colleagues under the auspices of Glaxo-Smith-Kline using a β -globin lentivirus vector and myeloablative conditioning, one patient has received gene modified cells. Although it is short-term follow up, this patient

has shown promising transfusion independence within a month (results presented at the [Cooley's Anemia Foundation Meeting, 2015]). We have recently opened a gene therapy trial for SCA using a fetal hemoglobin expressing vector and reduced intensity transplant at Cincinnati Children's Hospital Medical Center and results of this trial are awaited. Another trial for SCA has been opened by Kohn and colleagues [Urbani *et al.* 2015; Romero *et al.* 2013] using an anti-sickling β -globin lentivirus vector designed by Townes and colleagues [Levasseur *et al.* 2003] and results are awaited. In summary, lentivirus vectors are successfully treating patients with hemoglobinopathies, although optimal hemoglobin levels and reduced chemotherapy conditioning may still be barriers that need to be overcome. Importantly, long-term follow up of patients treated with lentivirus vectors for hemoglobinopathies and other diseases has not shown evidence of genotoxicity thus far.

Gene knockdown to improve the β -globinopathy phenotype

In addition to HPFH, other concurrent genetic variations have inspired potential gene therapies. It has been found that loss of one of the four α -globin genes concurrent with certain β -thalassemia mutations results in a less severe phenotype than β -thalassemia by itself [Kan and Nathan, 1970]. The loss of an α -globin gene leads to less free α -globin, and therefore less toxicity. One way to reduce gene expression is to use RNA interference, whereby defined RNA sequences are able to facilitate mRNA degradation or block protein translation of a specific RNA transcript. Overall, two groups have used RNA interference to decrease α -globin expression in murine primary erythroid cells and a β -thalassemia mouse model [Xie *et al.* 2007; Voon *et al.* 2008; Xie *et al.* 2011]. Knockdown of α -globin resulted in a reduction in reactive oxygen species (ROS) production, improvement in red cell morphology, and RBC counts. While the referenced studies did not perform complete gene knockout, the findings, combined with the patient findings noted above, suggest that deletion of an α -globin may be a plausible approach for treatment of β -thalassemia.

Globin gene editing

Recent advances in the area of gene editing have revolutionized the field of gene therapy. Using designer nucleases, it is now possible to develop

therapies that achieve precise genetic modifications (e.g. gene correction). Depending on the delivery system, they are able to avoid nonspecific integration and significantly reduce or completely avoid insertional oncogenesis. Gene editing techniques are not devoid of genotoxicity, as off-target DNA damage can occur at varying rates and nonspecific integration of DNA can occur if a donor sequence is required, the latter at typically low frequencies. The three designer nucleases that are predominantly used are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR associated protein (CRISPR/Cas).

The basic steps involved in the use of designer nucleases to perform site-specific gene editing are depicted in Figure 2. All of the designer nucleases create double strand breaks (DSBs) at precise locations in the genome. Each of the three designer nuclease systems function in a slightly different manner. ZFNs are user-defined zinc finger domains for recognition of specific DNA sequences and a nonspecific FokI catalytic domain for DNA cleavage upon dimerization. By targeting ZFNs to either side of the desired cut site, FokI dimerization occurs, activating its nuclease activity and creating the DSB. TALENs contain multiple DNA-binding domains arranged in tandem (TALE repeats), which are linked to a FokI catalytic domain. Each binding domain contains two defined amino acids that are able to recognize a specific DNA base pair [Moscou and Bogdanove, 2009]. Similar to ZFNs, a pair of TALENs are targeted to either side of the desired cut site, allowing their FokI domains to dimerize and create the DSB with high efficiency [Boch *et al.* 2009; Miller *et al.* 2011; Bedell *et al.* 2012]. The CRISPR/Cas system is composed of a guide-RNA (gRNA) and a Cas protein. The gRNA contains a defined 17–20 nucleotide portion that allows for targeting specific complementary genomic sequences. gRNA first forms a ribonucleoprotein (RNP) complex with a Cas protein and then directs the protein to the correct genomic location. Complementary genomic sequences that are followed by a specific DNA sequence, referred to as a protospacer adjacent motif, are able to be cut by the Cas protein. Several types of both natural and engineered Cas proteins, and a similarly functioning protein, CpfI, have now been described [Ran *et al.* 2015; Zetsche *et al.* 2015; Kleinstiver *et al.* 2016; Slaymaker *et al.* 2016].

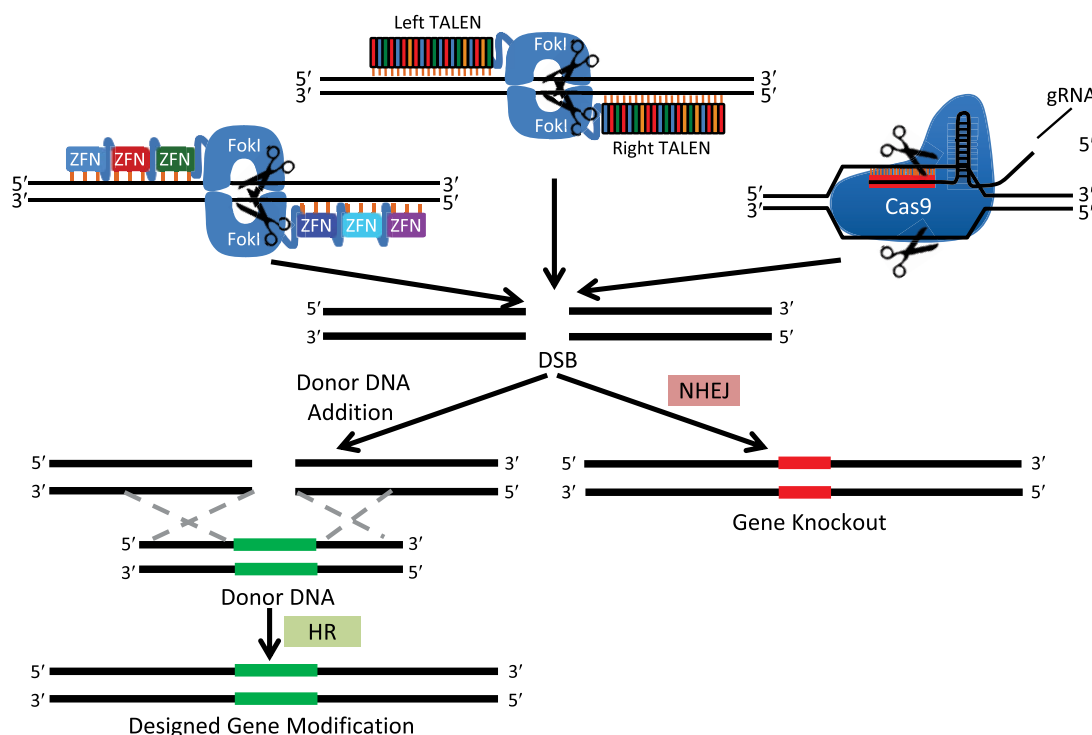


Figure 2. Three most common gene modification strategies using designer nucleases (ZFN, TALEN, and CRISPR/Cas).

The given nuclease system identifies a specified region of the genome through base pair recognition (orange hash marks) and creates a DSB (scissor). In the absence of a repair donor, NHEJ DNA repair creates an 'indel' (red DNA region) resulting in a gene knockout. If a repair donor is supplied, HR may occur, resulting in incorporation of a designed modification (e.g. insertion, deletion, substitution).

Cas, CRISPR associated protein, CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double strand break; gRNA, guide-RNA; HR, homologous recombination; NHEJ, nonhomologous end-joining; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.

All three systems outlined above result in formation of DSBs that are typically repaired by an innate DNA repair process, nonhomologous end joining (NHEJ). The NHEJ repair process is error prone, such that a significant proportion of repair events will result in the addition or subtraction of base pairs at the location of the DSB. This insertion/deletion is referred to as an 'indel'. Indels can be used to disrupt DNA binding sites and splice sites. Additionally, if an indel occurs within a coding region, a frameshift mutation can result, resulting in formation of a truncated protein, or degradation of the mRNA transcript *via* nonsense-mediated decay. To achieve a variety of more complicated gene modifications (e.g. gene correction, precise deletions, or insertions) a donor DNA sequence is provided. This allows a cell to make use of another innate DNA repair process, homologous recombination (HR). The donor sequence contains the desired sequence to be introduced into the cell, flanked by homology arms that align with DNA sequences on either

side of the region being modified. HR results in seamless and precise repair of the desired locus, replacing the cellular sequence with that in the donor template.

When devising a gene editing modality, multiple factors must be considered, such as choice of endonuclease, donor design, delivery modality, and genomic target of nuclease. Given the variety of considerations, comparisons between different modalities is difficult. Huang and colleagues have reported a side-by-side comparison of ZFNs, TALENs, and CRISPR/Cas and their ability to modify the β -globin locus [Huang *et al.* 2015]. ZFNs proved to be the least efficient while CRISPR/Cas was the most efficient. Successful use of ZFNs to correct the β^S mutation have been reported [Sebastiano *et al.* 2011; Zou *et al.* 2011; Hoban *et al.* 2015]. Most notably, using a ZFN method Hoban and colleagues demonstrated initial significant correction of CD34⁺ HSPCs isolated from both human umbilical cord blood as

well as mobilized peripheral blood, but ultimately poor correction of long-term repopulating HSCs was observed in a xenograft mouse model [Hoban *et al.* 2015]. Multiple studies have reported on the use of TALENs to correct either the β^S or β -thalassemia mutations in iPSCs [Ma *et al.* 2013; Ramalingam *et al.* 2014; Sun and Zhao, 2014; Xu *et al.* 2015]. Instead of correcting β -globin mutations, Voit and colleagues reported the TALEN-facilitated insertion of the entire β -globin cDNA immediately in front of the native β -globin gene [Voit *et al.* 2014]. In this strategy, the inserted β -globin cDNA is immediately followed by a polyadenylation signal, preventing read-through of the native β -globin gene. Potential advantages of this strategy are its applicability for treatment of any mutation found within the β -globin gene and its ability to express engineered globin genes. Lastly, several studies have demonstrated correction of both β -thalassemia and β^S mutations in iPSCs utilizing CRISPR/Cas editing techniques [Xie *et al.* 2014; Huang *et al.* 2015; Song *et al.* 2015].

While the above studies are promising, several obstacles must be overcome for these therapies to become clinically relevant. Most of the above studies necessitate utilizing a selectable marker to select for modified cells. Without the selectable marker, it would be unable to obtain a population of cells with a sufficient proportion of modified cells. This type of selection is feasible when working with iPSCs, which can be maintained in culture without the loss of the self-renewing potential. However, the culture time needed to select cells, and to later remove the marker, would not allow for retention of long-term repopulating HSCs. Editing efficiency of long-term repopulating HSPCs without the use of a selectable marker must be improved in order to achieve therapeutic efficacy. Another obstacle is the need to increase the desired precise HR outcomes over NHEJ. HR and NHEJ are competitive processes, with NHEJ typically being the dominant process with most gene editing systems.

Gene editing of globin regulatory elements

Transcription of β -globin family members is controlled by a large number of transcription factors and other regulatory mechanisms. Researchers have attempted to exploit many of these mechanisms to increase production of fetal hemoglobin. Kruppel-like factor 1 (KLF1) is an erythroid-restricted transcription factor that binds the CACCC box of the β -globin gene in mice and

humans and is critical for the expression of the β -globin gene. Manchinu and colleagues were able to increase δ -globin expression by inserting a binding site for the KLF1 transcription factor into the promoter region for δ -globin [Manchinu *et al.* 2014]. When combined with a mouse model of β -thalassemia, the inserted KLF1 binding site was able to improve the phenotype. Another important transcription factor is BCL11A, which is an important suppressor of γ -globin expression. Guda and colleagues designed an erythroid-specific RNAi technique to knockdown BCL11A, and demonstrated a significant de-repression of γ -globin expression [Guda *et al.* 2015]. Furthermore, Canver and colleagues were able to knockdown BCL11A in the erythroid lineage by using a CRISPR/Cas technique to delete an erythroid-specific enhancer for BCL11A [Canver *et al.* 2015]. This resulted in reactivation of fetal hemoglobin. Researchers have also been able to create synthetic transcription factors to influence globin expression. Costa and colleagues increased γ -globin expression by creating an artificial transcription factor composed of a ZFN binding domain recognizing the γ -globin promoter fused to a activation domain (VP64) [Costa *et al.* 2012].

Conclusion

Effective gene therapy for hemoglobinopathies requires several requirements to be met [Chandrakasan and Malik, 2014]: (1) Therapy must be efficiently transferred into the target cell population. Currently, this requires high-efficiency transfer into HSPCs. However, lower efficiencies would be required for therapies that may one day utilize iPSCs, as this would allow identifying and propagating only corrected clones. (2) Minimal genotoxicity. Therapies that result in insertion of a viral vector may have genotoxic effects if the vector integrates near a putative oncogene. This occurs when the vector either enhances the activity of nearby gene promoters, or functions as a promoter itself. Genotoxicity may lead to formation of clonal dominance and uncontrolled proliferation, often resulting in leukemia. In the case of gene editing strategies, therapies can have varying degrees of off-target effects caused by creation of DSBs at locations other than the desired genomic location. These DSBs can cause genetic mutations, deletions, translocations, and other mutations. (3) Phenotype correction. Therapies must show correction, or at least significant disease amelioration, to be pursued as viable treatment options. There are additional requirements for therapies involving

insertion of engineered genes. (4) Consistent, integration site-independent, high-level expression of inserted gene. (5) Erythroid lineage-specific and developmental stage-specific expression of the inserted gene.

Access to genetic therapies

Given the successes of therapies both in clinical trials and in development, gene therapy has the potential to have a profound impact on the treatment of hemoglobinopathies. However, these therapies currently require expensive and sophisticated resources for their implementation, including Good Manufacturing Practice (GMP)-certified manufacturing of viral vectors and hematopoietic stem cell graft products, and facilities capable of performing hematopoietic stem cell transplants. This limitation places these therapies out of reach for most of the patients suffering from hemoglobinopathies, as the vast majority of these patients live in underdeveloped countries and have extremely poor access to even basic medical care, and virtually no access to facilities capable of implementing these treatments. Nevertheless, new techniques are emerging that may increase the availability of these new therapies, especially to those with limited access to medical care.

Gene therapy for hematopoietic disorders has traditionally been achieved by first isolating a patient's own HSPCs, and genetically altering the cells *ex vivo*, before transfusing them back to the patient. This method is technically challenging and requires extensive precautions to insure the graft product retains both its sterility and stemness. Direct infusion of a viral vector obviates these considerations. Several studies have demonstrated this methodology, using either gene editing or traditional gene therapy, as a viable alternative to *ex vivo* manipulation [Carbonaro *et al.* 2006; Li *et al.* 2011; Burtner *et al.* 2014]. Given the high transduction/editing efficiencies often required to achieve a therapeutic effect, *ex vivo* manipulation of cells may be unavoidable for some therapies.

A new device, the CliniMACS Prodigy (Miltenyi Biotec, Germany), is now available that has the ability to automatically perform cell preparation, enrichment, activation, transduction, and expansion in a closed GMP-certified system [Apel *et al.* 2013]. This system reduces the technical expertise required by the operator as well as dependency on sophisticated ancillary facilities

required for product preparation. Additionally, investigators are developing increasingly less toxic nonmyeloablative transplantation regimens. Less toxicity leads to less transplant complications, which will increase the number and type of facilities capable of performing transplants. Wang and colleagues have provided evidence that STAT5 modulation may increase nonablative stem cell replacement [Wang *et al.* 2009]. Additionally, Xue and colleagues have demonstrated the potential of a pre-transplantation conditioning using an antibody targeting V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (KIT) to facilitate engraftment of transplanted hematopoietic stem cells [Xue *et al.* 2010]. As new methodologies and technologies advance, there will continue to be a corresponding increase in the access to gene therapy. Hopefully, this will eventually open up these novel therapeutic treatments to populations that have the highest prevalence but poorest healthcare access.

Hemoglobinopathies represent a broad range of inherited diseases leading to reduced hemoglobin levels or improper hemoglobin function. The disorders have significant global prevalence, morbidity, and mortality, but the burden of disease is felt greatest in sub-Saharan Africa, where access to medical care is often limited. For the minority of patients with access to medical care and an available matched donor, HSCT remains a viable curative option. However, gene therapies in clinical trials and earlier in development hold enormous promise for potentially providing a curative therapy to all patients. While promising, significant technical challenges must be overcome for these therapies to reach patients in those remote areas where disease burden is greatest.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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