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Review article

Gene Therapy in Thalassemia and Hemoglobinopathies

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Abstract: Sickle cell disease (SCD) and ß-thalassemia represent the most common hemoglobinopathies caused, respectively, by the alteration of structural features or deficient production of the ß-chain of the Hb molecule. Other hemoglobinopathies are characterized by different mutations in the α- or ß-globin genes and are associated with anemia and might require periodic or chronic blood transfusions. Therefore, ß-thalassemia, SCD and other hemoglobinopathies are excellent candidates for genetic approaches since they are monogenic disorders and, potentially, could be cured by introducing or correcting a single gene into the hematopoietic compartment or a single stem cell. Initial attempts at gene transfer of these hemoglobinopathies have proved unsuccessful due to limitations of available gene transfer vectors. With the advent of lentiviral vectors many of the initial limitations have been overcame. New approaches have also focused on targeting the specific mutation in the ß-globin genes, correcting the DNA sequence or manipulating the fate of RNA translation and splicing to restore ß-globin chain synthesis. These techniques have the potential to correct the defect into hematopoietic stem cells or be utilized to modify stem cells generated from patients affected by these disorders. This review discusses gene therapy strategies for the hemoglobinopathies, including the use of lentiviral vectors, generation of induced pluripotent stem cells (iPS) cells, gene targeting, splice-switching and stop codon readthrough.

ß-thalassemia, sickle cell anemia and other hemoglobinopathies: The thalassemias are a group of disorders due to a large number of heterogeneous mutations causing abnormal globin gene expression resulting in the total absence or quantitative reduction of globin chain synthesis[1]. Mutations in the α- or ß-globin gene lead to α- and ß-thalassemia, respectively[1]. α-Thalassemia is usually due to deletions within the α-globin gene cluster, leading to loss of function of one or both α-globin genes in each locus[2]. However, non-deletion mutations have been described, although they are much less frequent[1]. Depending on the number of genes that are unable to synthesize the α-globin protein, different clinical manifestations can be observed. If one or two α-globin genes are mutated (in *cis* or

trans), normally no or minimal hematological effects are seen, and individuals are normally silent thalassemia carriers or show α-thalassemia trait[1]. If three out of four genes are mutated, the condition is called hemoglobin H (HbH) disease, resulting in a hemolytic anemia that can worsen with febrile illness or exposure to certain drugs, chemicals, or infectious agents. Hemoglobin H disease is characterized by moderate to severe anemia, hepatosplenomegaly, and jaundice. Transfusion may occasionally be required and, if provided frequently, can lead to iron overload. If all four α-globin genes are deleted, the resulting condition is called α-thalassemia major, which is so severe that death occurs in utero. Children rescued through intrauterine transfusions remain dependent on red blood cell transfusions for survival[3].

The thalassemias are characterized by their clinical severity and genetic mutations. Patients with Cooley's anemia, also known as ß-thalassemia major, which is the most severe form of this disease, require many blood transfusions per year and is characterized by ineffective erythropoiesis and extra medullary hematopoiesis (EMH)[1]. If untreated, ß-thalassemia major is fatal in the first few years of life[1]. In ß-thalassemia intermedia, where a greater number of ß-globin chains are synthesized, the clinical picture is milder, and the patients require only infrequent or no transfusions[4,1]. In both thalassemias, with time the spleen is enlarged, the hemoglobin level decreases, and progressive iron overload occurs from increased GI iron absorption in addition to transfusions[1]. The vast majority of ß-thalassemias are caused by point mutations within the gene or its immediate flanking sequences and are classified according to the mechanism by which they affect gene regulation: transcription, RNA processing and mRNA translation[1]. These mutations are also classified as ß0 and ß+ according to the quantity of ß-globin chains synthesized. Mutations that lead to alternative splicing are associated with reduced synthesis of normal ß-globin mRNA and protein and are defined ß+. In contrast, mutations that completely impair ß-globin synthesis (for instance premature termination codons or PTCs) are defined ß0. Depending on the association of these different mutations, patients are classified into three principal groups with none, very low or low ß-globin production (ß0/0, 0/+, +/+ respectively). The levels of fetal hemoglobin (HbF) account for a large part of the clinical heterogeneity observed in patients

with ß-thalassemia. Variation in HbF expression among individuals is an inheritable disease modifier and high HbF (composed from 2 α- and 2 γ-chains) levels generally correlate with reduced morbidity and mortality in this disorder, since the γ-globin chains combine with the excess α-chains.

A single mutation leads to SCD, causing an adenine (A) to thymidine (T) substitution in codon 6 (GAG-GTG), which leads to insertion of valine in place of glutamic acid in the ß-globin chain. The resulting Hb (HbS) has the unique property of polymerizing when deoxygenated[1]. When the polymer becomes abundant, the red cells "sickle", stiff rods form that stretch and distort the red cells. These distorted cells can obstruct blood flow through the small vessels, and the restricted oxygen delivery to the tissues damages cells, injures organs, and produces pain. Similarly to SCD, other hemoglobinopathies can be triggered by the substitution of one amino acid (HbE[5,6,2]), deletion of a portion of the amino acid sequence (Hb Gun Hill[7]), abnormal hybridization between two chains (Hb Lepore[8,9]), or abnormal elongation of the globin chain (Hb Constant Spring[10]). These abnormal Hbs can have a variety of pathophysiologically significant effects, including ineffective erythropoiesis and anemia[1].

SCD and the thalassemias are quite common among Asian, African, African-American and Mediterranean populations[1]. It has been estimated that approximately 7% of the world population are carriers of such disorders, and that 300,000–400,000 children with severe forms of these diseases are born each year[117].

Hematopoietic stem cell transplantation: Current disease management of ß-thalassemia consists of prenatal diagnosis, transfusion therapy, or allogeneic BMT[11-13]. Only the latter is potentially curative[14]. The first successful BMT of ß-thalassemia was reported in 1982[15]. Consequently, several centers have utilized this approach as definitive therapy[16-18]. The most extensive experience in treating ß-thalassemia patients with BMT is that of Lucarelli and coworkers in Italy[18]. Established protocols can lead to a high success of thalassemia-free survival, although the transplant-related mortality is still significant and the chronic graft-versus-host disease is still a potential long-term complication of allogeneic HSCs transplantation[17,19]. In addition, availability of allogeneic bone marrow is limited by finding an identical human leucocyte antigen (HLA) matched bone marrow donor. However, development of new techniques to improve the management of graft-versus-host disease, to perform BMT from

unrelated donors and cord blood stem cells may expand the pool of potential donors in the near future[20].

In addition, patients with severe ß-thalassemia and SCD might benefit from new genetic and cellular approaches. From this prospective, ß-thalassemia and SCD are excellent candidate diseases for genetically based therapies in autologous hematopoietic stem cells (HSCs)[21-23]. Alternatively, somatic cells reprogrammed to induced pluripotent stem cells might also provide a possible new approach to treat ß-thalassemia[24,25].

Gene transfer using oncoretroviral vectors: Gene addition mediated by retroviral vectors is an attractive approach for monogenic disorder, However, when applied to hemoglobinopathies, this strategy raises major challenges in terms of controlling transgene expression, which should be erythroid-specific, elevated, position independent and sustained over time. In fact, many studies were performed before positive preclinical data were generated. The first attempts were done using oncoviruses. These viruses belong to the large family of Retroviridae and are characterized by a genome that encodes the genes gag-pol and env[26]. Onco-retroviral vectors, such as those derived from Moloney murine leukemia virus, efficiently transfer therapeutic genes into murine hematopoietic stem cells (HSC) without transferring any viral gene[27]. Recombinant oncoretroviruses were the first viral vectors used to transfer the human ß-globin gene in mouse HSCs[28,29]. These experiments resulted in tissue-specific but low and variable (position-dependent) human ß-globin expression in bone marrow chimeras, usually varying between 0 and 2% of endogenous mouse ß-globin mRNA levels[29,30-33]. Studies aimed at increasing expression levels of transferred ß-globin genes have focused on including locus control region (LCR) elements of the human ß-globin gene locus into oncoretroviral vectors. The LCR contains cis-acting DNase I hypersensitivity sites (HS) that are critical for high-level, long-term, position-independent, and erythroid-specific expression[34,35]. These HS elements contain several DNA-binding motifs for transcriptional and chromatin remodeling factors that facilitates chromatin opening. Also, thes genomic regions allow for binding of other regulatory elements required for high-level expression of the ß-globin gene[36]. Incorporation of the core elements of HS2, HS3, and HS4 of the human ß-globin LCR significantly increased expression levels in murine erythroleukemia (MEL) cells but failed to abolish positional variability of expression[37,35]. Additional efforts aimed to include larger elements resulted in the inability of the vector to incorporate large quantities of genetic material, as shown by the rearrangements of the transferred sequences[38-41]. Since these rearrangements frequently occur because of activation of splicing sites of the LCR sequence contained in the retroviral RNA, additional attempts were done to eliminate these sites. However, even these new vectors failed to include HS elements sufficient large to considerably increase expression of the ß-globin gene[37,35].

Additional erythroid-specific transcriptional elements were investigated within oncoretroviral vectors, including the HS40 regulatory region from the human α-locus[42-44] and alternative promoters. The promoter of ankyrin, a red cell membrane protein, has shown some promise in transgenic mice and in transduced MEL cells[45]. In mice, the ankyrin promoter has been used to drive expression of the human γ-globin gene resulting, at double copy, in an average expression of 8% of that of the endogenous α-globin genes[46]. To overcome transcriptional silencing of the γ-globin promoter in hematopoietic chimeras, mutant γ-globin promoters from patients with hereditary persistence of fetal hemoglobin (HPFH) were also investigated[118,47]. The Greek mutation at position −117 thus appeared to substantially increase γ-globin expression in MEL cells[47]. However, even these vectors failed to increase the level of the ß-globin gene to therapeutic levels.

Although oncoretrovirus vectors integrate into the genome, many integrants undergo transcriptional silencing, posing an additional challenge to the success of gene therapy using these vectors. Kalberer and co-workers attempted to avoid gene silencing by preselecting ex vivo retrovirally transduced hematopoietic stem cells on the basis of expression of the green fluorescent protein (GFP). In this vector the GFP gene was driven by the phosphoglycerate kinase promoter, while the human ß-globin gene by its own promoter and small elements from the LCR[48]. Using this approach, *in vivo* hematopoietic stem cell gene silencing and age-dependent extinction of expression were avoided, although suboptimal expression levels and heterocellular position effects persisted.

Another major limitation is that oncoretroviral vectors need to infect cells before and close to their division, otherwise the viral RNA cannot migrate into the nucleus due to the presence of a nuclear

membrane[49]. Since most hematopoietic stem cells are in a quiescent state, they must be induced with cytokines to divide in order to achieve higher transduction efficiencies and overall expression levels. Stimulation of quiescent hematopoietic stem cells, however, impairs or halts their long-term repopulating capacities[49].

Gene Transfer Using Lentiviral Vectors: With the extensive research on human immunodeficiency virus-1, it has been realized that lentivirus, engineered to be devoid of any pathogenic elements, can become efficient gene transfer vectors. Lentiviruses are characterized by a complex genome that encodes a number of accessory proteins besides the canonical retroviral genes gag-pol and env. They share all the common characteristic of retroviral replication including receptor-mediated entry, capsid uncoating, reverse transcription of the viral RNA, and integration into the host cell genome[26]. In addition, they are able to transduce non-replicating cells, which confers to these viruses a special value for the development of clinically functional gene vectors. Moreover, compared to oncoretroviral vectors, the stabilization of the proviral mRNA genome by the interaction of the accessory protein Rev with its cognate motif Rev-responsive element (RRE), increases their range of application, since larger genomic elements can be introduced in their genome with limited or no sequence rearrangement[50]. Therefore, lentiviral vectors are thus likely to be selected as vectors of choice for the stable delivery of regulated transgenes in stem cell–based gene therapy. The use of lentiviral vectors has allowed the introduction of large genomic elements from the ß-globin locus, different promoters, enhancers, and chromatin structure determinants that led to lineage-specific and elevated of ß-, γ- and α-globin expression *in vivo*. This resulted, in the amelioration or correction of anemia and secondary organ damage in several murine models of hemoglobinopathies, making the recombinant lentiviruses the most effective vector system to date for gene therapy of these disorders.

α-Thalassemia could potentially be a target for fetal gene therapy since fetuses with this disorder usually die between the third trimester of pregnancy and soon after birth. The potential use of lentiviral vectors to treat α-thalassemia was investigated a vector containing the HS2, 3, and 4 of the LCR from the human ß-globin locus, and the human α-globin gene promoter directing the human α-globin gene. Using this vector, Han and colleagues performed gene delivery *in utero* during midgestation targeting embryos affected by a lethal form of α-thalassemia. They showed that in newborn mice, the human α-globin gene expression was detected in the liver, spleen, and peripheral blood[51]. The human α-globin gene expression was at the peak at 3–4 months, when it reached 20% in some recipients. However, the expression declined at 7 months. Colony-forming assays in these mice showed low levels of transduction and lack of human α-globin transcript. Thus, lentiviral vectors can be an effective vehicle for delivering the human α-globin gene into erythroid cells *in utero*, but, in the mouse model, delivery at late midgestation could not transduce hematopoietic stem cells adequately to sustain gene expression.

Treatment of ß-thalassemia, SCD and other disorders through lentiviral mediated gene transfer is studied in murine and primate models[52-60]. The original studies in mice showed that lentiviral mediated human ß-globin gene transfer can rescue mice affected by ß-thalassemia intermedia and ß-thalassemia major[61,62,59]. The mouse ß-globin cluster has two adult ß-globin genes, $ß^{minor}$- and $ß^{major}$-globin. Thalassemic mice were generated with deletion of both the $ß^{minor}$- and $ß^{major}$-globin on one allele, designated *th3*/+ mice (63; 64). Also adult *th3*/+ mice have a degree of disease severity (hepatosplenomegaly, anemia, aberrant erythrocyte morphology) comparable to that of patients affected by ß-TI. May and colleagues tested two lentiviral vectors termed RNS1 (carrying minimal core LCR elements) and TNS9 (with large LCR fragments encompassing HS2, HS3 and HS4; approximately 3.2 kb in size) on *th3*/+ mice. Compared to RNS1, mice recipient of the larger TNS9 vector maintained higher human ß-globin transcript levels over time showing amelioration of red cell pathology (anisocytosis and poikilocytosis) and significantly increased hemoglobin levels (from 8-9 g/dL to 11-13 g/dL). The massive splenomegaly found in chimeras engrafted with control *th3*/+ bone marrow was not observed in TNS9-treated animals[61]. This correction was sustained in secondary mice[62].

Mice completely lacking adult ß-globin genes (*th3*/*th3*) die late in gestation, limiting their utilization as a model for Cooley's anemia[64]. For this reason, adult animals affected by Cooley's anemia were generated by transplantation of hematopoietic fetal liver cells harvested from *th3*/*th3* embryos at E14.5 into lethally irradiated syngeneic adult recipients[59]. Hematological analyses of engrafted mice performed 6 to 8 weeks post-transplant revealed severe anemia due not to pancytopenia but rather to low red blood cell and

reticulocyte counts together with massive splenomegaly and extensive EMH[62,59]. These animals could be rescued using TNS9 or by blood transfusions, supporting the notion that their phenotype is due specifically to erythroid impairment[65,59].

Pawliuk and colleagues investigated the efficacy of a lentiviral vector harboring the ß-globin promoter, LCR elements and a mutated human ß-globin gene with enhanced anti-sickling activity (ß87) in two different transgenic mouse models for SCD: SAD and BERK[66,67]. Mice transplanted with BERK and SAD bone marrow cells transduced with this modified ß-globin gene exhibited corrected reticulocyte counts and amelioration of Hemoglobin concentration, anisocytosis, and poikilocytosis. Moreover, the proportion of irreversibly sickled cells, SCD-associated splenomegaly, and characteristic urine concentration defect in SAD and BERK mice were vastly improved or corrected by ß87. Using a similar vector, Levasseur and colleagues obtained equivalent results. They transduced Sca1+c-Kit+Lin− cells rather than unselected bone marrow cells and achieved durable therapeutic results (5–7 months) following transplantation of 100 cells in lethally irradiated C57BL/6 mice[113,114].

Samakoglu and coworkers applied the principle of RNA interference (RNAi) to down-regulate the ß-globin mRNA in CD34(+) cells from patients affected by SCD[116]. They utilized a lentiviral vector harboring a promoterless small-hairpin RNA (shRNA) within the intron of a recombinant γ-globin gene. Expression of both γ-globin and the lariat-embedded small interfering RNA (siRNA) was induced upon erythroid differentiation, specifically downregulating the targeted gene in tissue and differentiation stage-specific fashion. The position of the shRNA within the intron was critical to concurrently achieve high transgene expression, effective siRNA generation and minimal interferon induction.

Miccio and colleagues also utilized an erythroid-specific lentiviral vector driving the expression of the human ß-globin gene from a minimal promoter/enhancer element containing two hypersensitive sites from the ß-globin locus control region in mouse models of ß-thalassemia (68). They showed that genetically corrected erythroblasts underwent *in vivo* selection. The selected erythroblast that derived from progenitors harboring proviral integrations in genome sites and were more favorable to high levels of vector expression. These data suggested that a regimen of partially myeloablative transplantation might be sufficient to achieve a chimerism that would therapeutic in ß-thalassemic patients.

While correction of murine models of ß-thalassemia has been achieved through lentiviral-mediated high levels of globin gene transfer into mouse HSCs, transduction of human HSCs is less robust and may be inadequate to achieve therapeutic levels of genetically modified erythroid cells. Zhao and coworkers therefore developed a double gene lentiviral vector encoding both human γ-globin under the transcriptional control of erythroid regulatory elements and methylguanine methyltransferase (MGMT), driven by a constitutive cellular promoter[60]. MGMT is an alkyltransferase that normally functions to repair cellular DNA damage at the O^6 position of guanine[69,70]. The cytotoxic effects of alkylating agents, such as temozolomide and 1,3-bis-chloroethyl-1-nitrosourea (BCNU), can be prevented if there is adequate expression of MGMT, which removes the O^6 adduct from the modified DNA. Variant MGMT proteins with specific amino acid changes retain significant activity while possessing the useful property of resistance to inactivation by O^6-benzylguanine (BG)[71]. BG can be used to inactivate endogenous MGMT to enhance the specificity of alkylator-mediated cell death to cells not expressing the variant form. Therefore, expression of these variant forms of MGMT provides cellular resistance to alkylator drugs, which can be administered to kill residual untransduced HSCs, whereas transduced cells are protected. To test this hypothesis, mice transplanted with ß-thalassemic HSCs cells transduced with a lentiviral γ-globin/MGMT vector were treated with BCNU[60]. This led to significant increas in the number of γ-globin–expressing red cells, the amount of fetal hemoglobin and resolution of anemia. One important advantage of using the γ-globin gene, normally expressed exclusively during fetal life, is that high level γ-globin expression would be therapeutic not only for ß-thalassemia, but also SCD. Interestingly, selection of transduced HSCs was also obtained when cells were drug-treated before transplantation. These data suggest that coexpression of MGMT allowed autologous, γ-globin vector-transduced ß-thalassemic HSCs to be enriched to therapeutic levels through either pre or post-transplantation selection.

Imren and colleagues engrafted immunodeficient mice with human cord blood cells infected with a lentiviral vector encoding an anti-sickling ß-globin transgene[35,72]. After 6-months, half of the human erythroid and myeloid progenitors regenerated in

the mice containing the transgene, and erythroid cells derived *in vitro* from these cells produced high levels of the ß-globin protein. In addition, these authors investigated the integrated proviral copies showing that 86% of the proviral inserts had occurred within genes, including several genes implicated in human leukemia. These findings indicate effective transduction of very primitive human cord blood cells achieving robust and erythroid-specific production of therapeutically relevant levels of ß-globin protein. The frequency of proviral integration within genes observed in this study and the data from Miccio and coworkers that indicate that selected erythroblasts were derived from progenitors harboring proviral integrations more favorable to high levels of vector expression, indicate that regulated hematopoiesis might require additional safety modifications to prevent potential genotoxic effects[35,72,68]. This risk is inherent to the integration of foreign genetic material and the risk of insertional oncogenesis has been established both in mice and humans[73-78].

In light of these results, genetic elements with enhancer-blocking properties, such as insulators, could increase the safety of the clinical trails. These elements have been investigated to shelter the vector from the repressive influence of flanking chromatin by blocking interactions between regulatory elements within the vector and chromosomal elements at the site of integration[79-81]. This property of insulators can also be harnessed to diminish the risk that the vector will activate a neighboring oncogene[82,83]. The initial studies indicated that inclusion of the cHS4 insulator element into the 3′ LTR of recombinant murine leukemia virus increases the probability that randomly integrated proviruses will express the transgene[46,84-86]. Puthenveetil and coworkers tested a lentiviral vector carrying the human ß-globin expression cassette flanked by a chromatin insulator in transfusion-dependent human ß-thalassemia major cells[87]. Using this vector, they demonstrated normal expression of human ß-globin in erythroid cells produced *in vitro*. They also observed restoration of effective erythropoiesis and reversal of the abnormally elevated apoptosis that characterizes ß-thalassemia. The gene-corrected human ß-thalassemia progenitor cells were also transplanted into immune-deficient mice, where they underwent normal erythroid differentiation, expressed normal levels of human ß-globin, and displayed normal effective erythropoiesis 3 to 4 months after xenotransplantation. Based on all these preclinical studies on mouse models of ß-thalassemia and SCD, clinical trials have been proposed or are underway[53]. **Figure 1A** depicts this approach.

Alternatively, the homologous recom-bination pathway can be harnessed to avoid random integration. Zinc-finger nucleases (ZFNs) can been used to enhance the frequency of gene correction[88,89]. However, achieving the full potential of ZFNs for genome engineering in human cells requires their efficient delivery to the relevant cell types. Lombardo and colleagues exploited the infectivity of integrase-defective lentiviral vectors (IDLV) to express ZFNs and provide the template DNA for gene correction in different cell types[90]. IDLV-mediated delivery supported high rates (13–39%) of editing at the IL-2 receptor common γ-chain gene (*IL2RG*) across different cell types as well as human embryonic stem cells (5%), allowing selection-free isolation of clonogenic cells with the desired genetic modification. Therefore, this technique opens new and exciting possibilities. By modifying the ZFN binding specificity and selecting an appropriate donor sequence, one could target the IDLV-ZFN system to any individual site in the human genome avoiding random integration (**Figure 1B**) and, potentially, genome toxicity[88-91].

However, there are current obstacles to successfully apply this therapeutic approach to humans. Some of them include the need for improved efficiency of gene delivery, insertion of the gene into non-oncogenic sites and the potential negative or positive contributions of the ß-thalassemic genotype and potential modifiers to the effectiveness of the gene transfer[1]. Original studies in animal models utilized mice with deletions of the ß-globin genes. These mutations do not reflect the phenotypic variability observed in ß-thalassemic patients. Thus, there is a gap in knowledge between our understanding of the primary mutation, the corresponding phenotype, and the approach to cure an individual patient based on his/her genotype (*i.e.* understanding of the disease and its treatment by genetic modalities). To date this variability has not been addressed and no studies have focused on the efficacy of gene therapy in relation to the different genotypes of the patients. Although gene therapy is an area of active clinical investigation, the aforementioned obstacles limit its use in the management of thalassemia. Nonetheless, as we showed in our review the successful transfer of globin genes into hematopoietic cells of humans has been demonstrated and is encouraging.

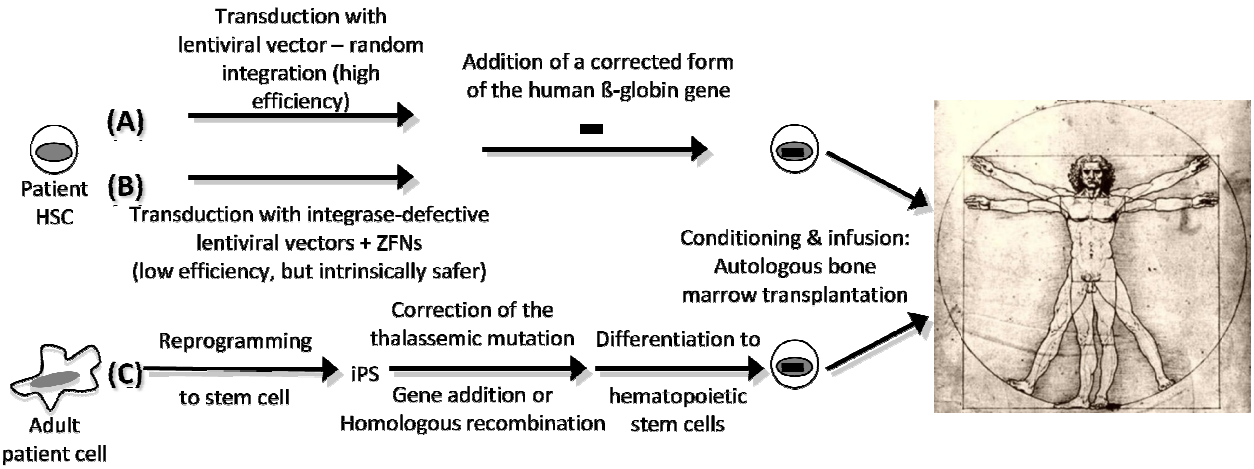

Figure1. Schematic representation of the gene therapy approach mediated, respectively, by (A) gene transfer into hematopoietic stem cells (HSC) using integration competent lentiviral vector (B) gene transfer into HSC by integrase defective lentiviral vectors. ZFN: zinc finger protein. (C) Stem cell therapy by reprogramming of adult cells to stem cells. iPS: Induced Pluripotent Stem Cell.

Gene Correction and Ips Cells: Triplex-forming oligonucleotides and triplex-forming peptide nucleic acids (PNAs) have been shown to stimulate recombination in mammalian cells via site-specific binding and creation of altered helical structures that provoke DNA repair[92,93]. Cotransfection of PNAs and recombinatory donor DNA fragments, Chin and co-workers demonstrated that these complexes can promote single base-pair modification at the start of the second intron of the beta-globin gene, the site of a common thalassemia-associated mutation[94]. This single base pair change was detected by the restoration of proper splicing of transcripts produced from a green fluorescent protein-beta-globin fusion gene. The ability of these PNAs to induce recombination was dependent on dose, sequence, cell-cycle stage, and the presence of a homologous donor DNA molecule. They also showed that these PNAs were effective in stimulating the modification of the endogenous beta-globin locus in human cells, including primary hematopoietic progenitor cells. Enhanced recombination, however, did not exhibit frequencies superior to 0.4%[94]. However, this technology could be a powerful tool in combination with the generation of stem cells. In particular, introduction of the genes Oct3/4, Sox2 with either Klf4 and c-Myc or Nanog and Lin28 genes can induced pluripotent stem (iPS) cells[95,115,24,96]. Ye and coworkers shown that iPS cells can be generated from cells derived from skin fibroblasts, amniotic fluid or chorionic villus sampling of patients with ß-thalassemia[97]. Subsequently, the iPS cells were differentiated into hematopoietic cells that synthesized hemoglobin. Therefore, in the future the mutation in the ß-globin gene of these iPS cells could be corrected by gene targeting and the cells differentiated into HSCs to be returned to the patient[94]. **Figure 1C** depicts this approach. In fact, mice affected by SCD were cured using this strategy[98]. However, there are some obstacles that need to be overcome before iPS treatment of ß-thalassemia will be utilized. One of the most pressing problems is elimination of the transcription factors when they are no longer needed. Second, it is necessary to reestablish the correct re-programming so that the iPS cells do not develop into tumors.

Splice-Switching and Stop Codon Readthrough: Defective ß-globin gene expression and ß-globin deficiency can be attributed to almost 200 thalassemic mutations. However, only 10 mutations are responsible for the majority of cases worldwide and some of the most frequent cause aberrant splicing of intron 1 (IVS1-110, IVS1-6, IVS1-5) or intron 2 (IVS2-654, IVS2-745)[99,112]. These mutations lead to incorrectly spliced mRNAs, even though the correct splice sites remain undamaged and potentially functional. Use of small nuclear RNA (snRNA) and splice-switching oligo-nucleotides represents a promising approach since these molecules can restore the corrected splicing re-establishing the synthesis of the normal protein[94,100-108]. Therefore blocking the aberrant splice sites with antisense oligonucleotides forces the splicing machinery to reselect the existing correct splice sites. Expression of antisense sequences targeted to the aberrant splice sites in thalassemic pre-mRNA has been successful, restoring the correct splicing pattern and ultimately restoring hemoglobin synthesis[102,93]. This was demonstrated in HSCs and erythroid progenitor cells from a patient with IVS2-745/IVS2-1

thalassemia. After transduction of the patient cells with a lentiviral vector that express a snRNA targeting the mutant RNA, the levels of correctly spliced ß-globin mRNA and adult hemoglobin were approximately 25-fold over baseline[108]. Similarly, the correct splicing pattern was restored in a mouse model of IVS2-654 thalassemia. This was achieved by delivery *in vivo* of a splice-switching oligonucleotide, a morpholino oligomer conjugated with an arginine-rich peptide. Repaired ß-globin mRNA restored significant amounts of hemoglobin in the peripheral blood of the IVS2-654 mouse, improving the number and quality of erythroid cells[107].

Another approach showing a great potential for the treatment of genetic disorders characterized by to premature termination codons (PTCs) is the use of drugs to induce stop codon readthrough. These modified RNA would protected against non-sense mediated mRNA decay (NMD) and allow production of a protein[109]. Aminoglycoside antibiotics can decrease the accuracy in the codon-anticodon base pairing, inducing a ribosomal read through of premature termination codon. These findings have led to the development of a pharmacologic approach to treat thalassemic patients carrying the ß0-39 mutation, which introduces a PTC in codon 39 of the ß-globin gene and is one of the most frequent thalassemic mutations in the Mediterranean littoral[1]. Aminoglycosides and analogous molecules were tested in their ability to restore ß-globin protein synthesis on human erythroid cells (K562) carrying a lentiviral construct containing the ß0-39 globin-gene[110]. Treatment of these cells with geneticin (G418) and other aminoglycosides restored the production of ß-globin[110]. Moreover, after FACS and high performance liquid chromatography (HPLC) analyses, G418 was also demonstrated to partially correct the biological function of the ß0-39 globin mRNA in erythroid precursor cells from ß0-39 homozygous thalassemia patients[111]. This study strongly suggests that ribosomal read-through should be considered a novel approach for treatment of ß0 thalassemia caused by premature stop codon mutations and NMD.

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