

Review Article

The Ongoing Challenge of Hematopoietic Stem Cell-Based Gene Therapy for β -Thalassemia

Ekati Drakopoulou,^{1,2} Eleni Papanikolaou,^{1,2} and Nicholas P. Anagnou^{1,2}

¹Laboratory of Cell and Gene Therapy, Centre for Basic Research, Biomedical Research Foundation of the Academy of Athens (BRFAA), 115 27 Athens, Greece

²Laboratory of Biology, University of Athens School of Medicine, 115 27 Athens, Greece

Correspondence should be addressed to Nicholas P. Anagnou, anagnou@med.uoa.gr

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β -thalassemia is characterized by reduced or absence of β -globin production, resulting in anemia. Current therapies include blood transfusion combined with iron chelation. BM transplantation, although curative, is restricted by the matched donor limitation. Gene therapy, on the other hand, is promising, and its success lies primarily on designing efficient globin vectors that can effectively and stably transduce HSCs. The major breakthrough in β -thalassemia gene therapy occurred a decade ago with the development of globin LVs. Since then, researchers focused on designing efficient and safe vectors, which can successfully deliver the therapeutic transgene, demonstrating no insertional mutagenesis. Furthermore, as human HSCs have intrinsic barriers to HIV-1 infection, attention is drawn towards their *ex vivo* manipulation, aiming to achieve higher yield of genetically modified HSCs. This paper presents the current status of gene therapy for β -thalassemia, its success and limitations, and the novel promising strategies available involving the therapeutic role of HSCs.

1. Introduction

The β -thalassemias represent inherited, monogenic anemias, arising from autosomal recessive mutations, affecting the synthesis of the β -chain of hemoglobin [1]. They are characterized by reduction or absence of β -chain synthesis, resulting in excess of α -chain molecules, which precipitate in red blood precursors, leading to impaired erythrocyte maturation, mechanical damage, and ultimately to apoptosis [2]. Thalassemias are caused by more than 200 mutations affecting the human β -globin gene and are most prevalent in the Mediterranean region, the Middle East, India, and South East Asia, representing a serious health problem. Lately, due to population migration, β -thalassemia presents a clinical problem also in UK, US, and Australia. Globally, it is estimated that there are 80 million carriers [3].

β -thalassemic phenotype is very heterogeneous and directly linked to the genotype. In heterozygotic state, the outcome is consistent with clinically normal individuals, who are largely unaware of their genetic condition. Inheritance

of two copies of β -thalassemia genes causes thalassemia major and usually results in life-threatening anemia and transfusion-dependence treatment for survival. Intermediate clinical forms of the disease exist as thalassemia intermedia, which is characterised by moderately severe anemia with occasional need for blood transfusion.

Current therapies for β -thalassemia include blood transfusions together with life-long iron chelation and hydroxyurea treatment for fetal hemoglobin (HbF) induction. Although these strategies have improved patients' mortality and have significantly delayed the onset of iron-related organ failure, treatment noncompliance is common, leading to cardiac, hepatic, or endocrine failure [4]. Allogeneic hematopoietic stem cell (HSC) transplantation of human leukocyte antigen- (HLA-) matched sibling donors can be curative, reaching cure rates up to 90% in patients younger than 17 years of age [5]. However, it is associated with a number of drawbacks, such as the limited matched related donors and the need for long-term immunosuppression to prevent, treat or delay graft-versus-host disease (GVHD), often associated

with allogeneic HSC transplantation. Therefore, an alternative molecular strategy based on gene therapy is undoubtedly a radical approach that overcomes all the above limitations.

2. Designing Effective Vectors for β -Thalassemia Gene Therapy

Most research efforts towards gene therapy for β -thalassemia have focused on employing retroviral vectors as a means of gene delivery, since these are capable to integrate into the target cell genome, resulting in stable and long-term expression. However, the integration has to be targeted and should occur in a specific manner in order to avoid poor gene expression or even silencing. Therefore, for gene therapy for β -thalassemia to become an effective and realistic therapeutic approach, the following very important criteria need to be met:

- (I) the therapeutic vector should exhibit stability, high titer and erythroid-lineage specificity, via the utilization of respective regulatory elements,
- (II) the transgene must be expressed in therapeutic and sustained levels,
- (III) the therapy itself should be safe and efficient in terms of viral transduction.

Early attempts back in the 1980s and 1990s utilized gammaretroviral vectors to achieve stable and high-level transgene expression, however, with no success. More specifically, Williams et al. [6] managed to introduce a marker gene into murine HSCs, using a vector, derived from murine leukemia virus (MLV) after replacing *gag*, *pol*, and *env* gene with the transgene of interest, while later on, investigators utilized these vectors to drive expression of β -globin genes into murine HSCs [7–9]. The outcome, however, was unsatisfactory, as poor gene-transfer efficiency was obtained, with levels of β -globin reaching 0%–2% of the endogenous RNA levels. In an attempt to increase β -globin expression, Novak et al. [10] incorporated into MLV the newly identified powerful DNA-enhancer elements from the β -globin locus control region (LCR), which is found to be essential for high-level expression of globin genes [11, 12]. Unfortunately, poor vector production and genetic instability of the viral vector genome were observed under these experimental conditions, primarily due to the large size of the fragments incorporated into the vector. Extensive mutagenesis studies of the transduced β -globin gene by Leboulch et al. [13] identified a 372 bp intronic segment and multiple reverse polyadenylation and splicing events, which were responsible for low viral titers and instability of proviral transmission, upon infection. Around the same time, Sadelain et al. [14] managed to generate a high-titer retroviral vector that expressed high levels of β -globin in an erythroid-specific manner, by combining the human β -globin gene and the LCR core hypersensitive sites (HS) 2, 3, and 4; however, the group failed to reduce positional variability of expression. Taken together, the above findings indicated that vector instability might be caused by splicing of the retroviral RNA genome, a consequence of cryptic splice sites within the genomic sequences [13, 14]. A way to circumvent

the above was to turn towards lentiviral vectors (LVs), as the latter, in addition to the common Gag, Pol, and Env proteins, also encode Tat and Rev. A major function of Rev is to mediate nucleoplasmic export of unspliced viral RNA, allowing thus the production of full-length viral RNA genomes [15]. Therefore, Rev expression in a LV packaging line could prevent splicing of a β -globin vector, containing large genomic fragments, leading thus to vector stability.

A major breakthrough in the gene therapy field for hemoglobinopathies took place when May et al. [16] and Pawliuk et al. [17] constructed an HIV-based vector with the β -globin gene along with its LCR and managed to achieve high titers, which in turn allowed high expression of the therapeutic gene and thus disease amelioration. Following this approach, several groups working also on hemoglobinopathies employed β -globin LVs in their studies, obtaining significant results, leading to correction of β -thalassemia [18] in murine models, as will be discussed below. Although the above vectors were able to ameliorate the phenotype of β -thalassemia, the observation that compound thalassaemic patients with the syndrome of hereditary persistence of fetal hemoglobin (HPFH) typically have less anemia, milder clinical symptoms, and are often transfusion-independent, drew the attention towards constructing γ -globin vectors [19].

Persons and colleagues designed such vectors, containing also the extended β -globin LCR, and managed to show significant correction of the thalassaemic phenotype [20]. However, globin expression by these vectors was inconsistent because of chromosomal position effects albeit the curative effect they were capable to demonstrate, and eventually led to the use of chromatin insulators [21].

Insulators represent DNA elements capable to shield the therapeutic gene from the negative and/or positive effects of the surrounding DNA, leading thus to higher and more consistent expression and reducing also vector genotoxicity by preventing the viral regulatory elements to interfere with the expression of flanking genes. A recent study has shown that improved and more consistent globin gene expression can be obtained when a 1.2 kb DNA element from the chicken β -globin locus (cHS4) is incorporated into the globin LV design [21]. Unfortunately, this vector design can lead to a significant reduction in vector production and titer and seriously compromise practical use; the mechanism underlying this decrease has been recently elucidated for the first time [21]. However, when Hanawa et al. [22], included only the 0.25 kb core element of cHS4, it was shown that the specific element could rescue vector titer by alleviating a postentry block to reverse transcription associated with the 1.2 kb element. Also, in an orientation-dependent manner, the 0.25 kb core element significantly increased transgene expression from an internal promoter due to improved transcriptional termination. This element also demonstrated barrier activity, reducing variability of expression due to position effects. Similarly, Lisowski and Sadelain [23] showed that the incorporation of HS1 element enhances the therapeutic efficacy of the globin gene transfer in murine β -thalassemia, compared to HS2-HS3-HS4 alone and, therefore, can lead to even higher globin expression with lower vector copy numbers.

Also, for safety reasons and in order to avoid insertional mutagenesis complications as in the SCID clinical trial [24], all research groups have employed a self inactivating (SIN) configuration in their vectors, by deleting large fragments of the U3 region within the vectors' long terminal repeat (LTR).

3. Hematopoietic Stem Cells (HSCs) and Lentiviral Globin Vectors

A schematic representation of the HSC-based gene therapy for β -thalassemia is shown in Figure 1. HSCs represent a minor population of the adult bone marrow, accounting for 1 in 2,500 to 1 in 10,000 cells in the adult mouse [25, 26]. These cells remain relatively quiescent most of their lives, with murine HSCs entering cell cycle every 1-2 months [27], while in primates and humans, the turnover rate is even slower reaching 1-2 years [28]. Due to the above features, gene therapy for hemoglobinopathies focused on employing such vectors for gene delivery, as they can efficiently infect nondividing cells, and thus manage to deliver the transgene of interest [29]. However, it should be noted that although LVs may be able to infect HSCs in G_0 phase, it has been clearly shown that HSCs exiting G_0 and entering G_1b phase are more readily transduced [30], possibly due to the fact that reverse transcription occurs at this stage [31].

3.1. Correction of Murine β -Thalassemia. As mentioned above, the major breakthrough in the correction of β -thalassemia came from the group of Sadelain in New York, where they managed to correct thalassemia intermedia in a murine model [16] and later in rescuing lethality in a thalassemia major model [32], using TNS9 β -globin vector. In parallel, Pawliuk et al. [17] demonstrated that lentiviral-mediated stem cell transfer of an antisickling variant of the human β -globin chain resulted in hematologic correction and diminished end-organ damage in murine sickle cell disease (SCD). Similarly, Imren et al. [33] using a β -globin vector showed that β -globin expression reached approximately 32% of the total hemoglobin, while in the case of the GLOBE vector, the Ferrari group showed β -globin expression ranging from 14 to 37% [18].

Correction of β -thalassemia was also achieved using γ -globin vectors, as mentioned previously, with the Persons group being the first to construct and test such vector. In their first attempt, using a noninsulated vector, they managed to correct thalassemia intermedia in the murine model. However, the phenotypic correction varied due to chromosomal positioning effects and vector copy number [20]. In an attempt to address these issues, Arumugam et al. [21] incorporated the cHS4 insulator in the D432 β -4 γ vector and succeeded in increasing the transgene's expression, in the expense of viral titers though. Similarly, Hanawa et al. [34] demonstrated that animals receiving transplants of β -thalassemic stem cells transduced with a new LV, containing 3.2 kb of LCR sequences, expressed high levels of fetal hemoglobin, ranging from 17% to 33%, with an average vector copy number of 1.3. The above strategy led to a mean

increase in hemoglobin concentration (26 g/L) and enhanced amelioration of other hematologic parameters [34].

Lastly, Zhao et al. from the Persons group went further and incorporated a drug-resistance gene, methylguanine methyltransferase (MGMT), in the γ -globin vector [35], demonstrating amelioration of murine β -thalassemia and enrichment of the corrected HSC compartment, employing *in vitro* and *in vivo* selection, following drug treatment. However, despite the successful drug-induced enrichment of the HSC pool in the above study, findings from other investigators tend to suggest that MGMT selection approach may not always result in the desired outcome and quite often is accompanied by drug-related toxicity. More specifically, Larochelle et al. [36] failed to induce selection of long-term repopulating HSCs in rhesus macaques and showed that the alkylating agent bis-chloroethyl nitrosourea (BCNU) can result in significant nonhematopoietic toxicity, such as pulmonary congestion/edema and necrohemorrhagic colitis. In addition, recent work by Giordano et al. [37] using a murine serial transplant model demonstrated that MGMT selection approach can also lead to insertional mutagenesis and clonal dominance. Taken together, the above data indicate that although MGMT selection approach can be an effective strategy for enrichment of the corrected HSC compartment, its drug-related toxicity and insertional mutagenesis potential represent considerable risk factors for use in human clinical trials.

3.2. In Vitro Studies with Human HSCs. Both β -globin and γ -globin vectors have been used in correcting human erythropoiesis in erythroid cultures and immunodeficient mice. In a detailed study, Puthenveetil et al. [38] tested a lentiviral vector carrying the human β -globin expression cassette, flanked by a chromatin insulator in transfusion-dependent human thalassemia major, and demonstrated that normal amounts of human β -globin were expressed in erythroid cells produced *in vitro*; erythropoiesis was restored and apoptosis significantly reduced. These gene-corrected human β -thalassemia progenitor cells were then transplanted into immunodeficient mice, where they were capable of establishing normal erythropoiesis. In addition, Roselli et al. [39], using the GLOBE vector, reported successful correction of thalassemia major in human cells by achieving high transduction frequency, restoration of hemoglobin A synthesis, rescue from apoptosis, and correction of ineffective erythropoiesis.

Similarly, Persons and colleagues, using different γ -globin vectors and an *in vitro* model of human erythropoiesis, showed recently that both lentiviral-mediated γ -globin gene addition and genetic reactivation of endogenous γ -globin genes have the potential to provide therapeutic HbF levels to patients with β -globin deficiency [40, 41]. Finally, the group of Anagnou, to address the issues of low titer, variable expression, and gene silencing, affecting the gene therapy vectors for hemoglobinopathies, has successfully used the HPFH-2 enhancer in a series of oncoretroviral vectors [42]. Based on these data, the same group [43] generated a novel insulated SIN LV designated as GGHI,

containing the γ -globin gene with the -117 HPFH point mutation and the HPFH-2 enhancer. This vector managed to produce efficient amounts of HbF resulting in phenotypic correction in erythroid cultures of CD34⁺ HSCs isolated from peripheral blood (PB) or bone marrow (BM) [43]. In this specific vector design, the incorporation of the full-length 1.2 kb cHS4 in the U3 region and the SIN configuration had no apparent effect on viral titer, since it reached 2×10^8 TU/mL. Efforts have also been made in the ability of γ -globin vectors pseudotyped with different envelope glycoproteins to transduce human HSCs [44] that resulted in the notion that the vesicular stomatitis virus glycoprotein (VSVG) is more effective in transducing engrafting cells than other gammaretroviral glycoproteins, supporting thus its use in clinical-grade vectors [44].

3.3. The First Clinical Trial. The first human clinical trial using a β -globin LV commenced in France on June 7, 2007, where Leboulch and colleagues selected two β -thalassemia patients who underwent transplantation of LV-transduced HSCs [45, 46]. The first one, a 28-year-old patient experienced a period of prolonged aplasia likely due to the technical handling of the cells, without relation to the gene therapy vector, and despite the absence of any adverse effects, required the administration of untransduced cells kept as a backup, in order to avoid putative infections. As a result, the lentiviral-modified cells did not reach a significant level in PB, neither did the therapeutic hemoglobin, leading to no conclusions regarding the specific patient.

The second patient in the study was a 18-year-old male suffering from HbE/ β^0 thalassemia, a form of the disorder in which hemoglobin production is severely compromised. He was transfusion-dependent since the age of three, requiring 160 mL of packed erythrocytes/kg/year. He received 4×10^6 CD34⁺ cells/kg [46]. The levels of genetically modified cells increased from 2% in the first few months to 11% at 33 months posttransplant. The above rise was also observed in the levels of normal β -globin protein, with 10%-20% of HSCs being genetically modified, leading thus to improved production and quality of red blood cells. Remarkably, a year after the treatment, the patient no longer required blood transfusions. He was last transfused on June 6, 2008, and four years after transplantation, despite being slightly anemic and undergoing repeated phlebotomies for the decrease of iron overload, the patient does not require blood transfusions, which means that this single case can be viewed as a clinical success.

However, despite the successful outcome of the second patient, Cavazzana-Calvo et al. reported that one hematopoietic clone, harboring a vector insertion into the HMGA2 gene showed clonal dominance [46]. At 20 months post transplantation, the specific clone accounted for 50% of the genetically modified HSCs; its contribution, however, to the circulating red blood cells' pool remains at around 3%. The potential clinical relevance of the alteration of HMGA2 expression by the vector integration is highlighted by the fact that this gene may function as a potential oncogene in various types of cancer. However, it remains unclear whether the vector insertion into this gene has actually resulted in the

relatively high contribution of this clone to hematopoiesis, since it is conceivable that the above observation may simply reflect the consequences of engraftment from a small number of transduced HSCs.

In summary, the above patient represents the proof of principle that gene therapy can be a successful therapeutic approach. This successful clinical trial demonstrated that large amounts of a therapeutic protein can be produced *in vivo* in a lineage-specific manner and validated somatic gene transfer using a lentiviral SIN vector for transducing long-term repopulating HSCs. It also demonstrated that somatic gene transfer *ex vivo* can provide transfusion independence for patients with severe forms of thalassemia.

4. Enhancing Lentiviral Gene Transfer Efficiency in Human and Nonhuman Primate HSCs

As discussed above, studies from many laboratories have shown that murine HSCs can be genetically modified using both β -globin and γ -globin LVs and engraft, resulting in amelioration of β -thalassemia. However, this is not the case for both human and nonhuman primate HSCs. These types are more resistant to lentiviral gene transfer and so far, no more than 10%–15% of genetically modified peripheral blood cells have been achieved. Recent work on rhesus macaques [47] using GFP LV demonstrated an average of 7% LV-bearing peripheral blood cells, a finding that comes into agreement with previous studies on pigtail macaques [48, 49]. Similarly, the 18-year-old patient in the first clinical trial discussed above showed an average of 15% genetically modified HSCs [46], supporting even further the notion that higher percentages of genetically modified HSCs in the case of human and nonhuman primates is not an easy task. The most effective strategies shown to increase HSC yield are described below and are also shown in Figure 1.

4.1. HSCs and Cellular Factors. The resistance of primitive human and nonhuman primate HSCs to infection by HIV-based vectors has drawn a lot of attention in the past decade. Recent work by the Naldini group [50] demonstrated that lentiviral gene transfer is limited by the proteasome through the regulation of one or more of the cellular postentry steps of the vector particle. Experiments involving the proteasome inhibitor MG132 during LV transduction demonstrated a 4-fold increase in gene transfer into human CD34⁺ cells [50]. In this study, the transient and reversible inhibition of proteasome function did not lead to any obvious HSC defects. However, as proteasome is the major cellular proteolytic machinery, inhibition of its function may lead to cellular toxicity, through rapid accumulation of proteins in cells, resulting in impairment of their survival. Thus, the use of proteasome inhibitors as a means of increasing lentiviral HSC transduction needs further investigation, before it is considered safe to use in clinical trials. Lastly, in a different study by Zhang et al. [51], cell-cycle protein p21, which is found at high levels in HSCs, was identified as a unique molecular barrier to HIV infection, through its

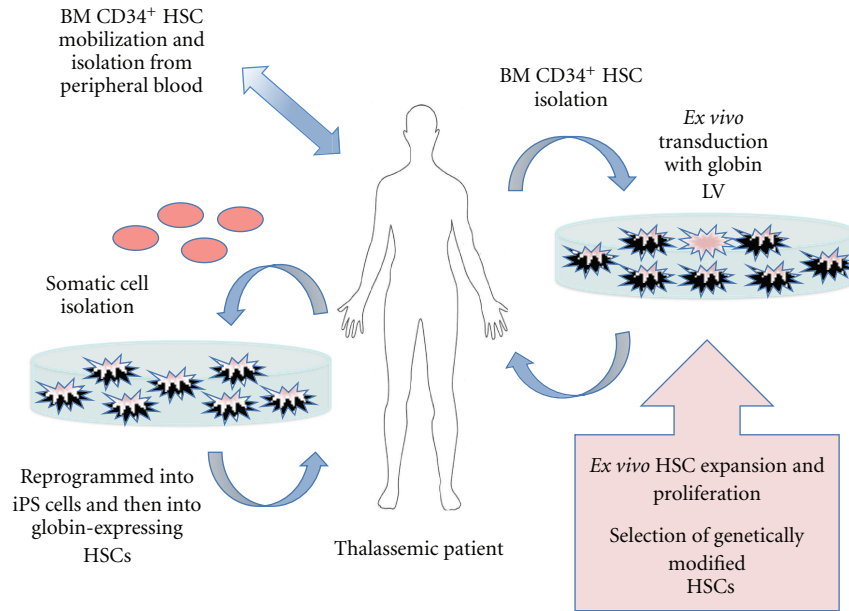


FIGURE 1: Schematic representation of HSC-based gene therapy for β -thalassemia. BM CD34⁺ cells are removed from the patient (right panel), transduced *in vitro* with the therapeutic globin LV, carrying the preferred envelope glycoproteins, and returned to the patient intravenously. At this stage, and primarily in the case of human HSCs, *ex vivo* expansion and proliferation can be induced; alternatively, selection of genetically modified HSCs may take place. Both strategies can lead to increased yield of corrected HSCs, which will contribute to the HSC compartment, when returned to the patient. Alternative strategies for obtaining higher yield of CD34⁺ cells are continuously emerging (left panel). These include mobilization of BM CD34⁺ HSCs with mainly G-CSF and then isolation of these cells from peripheral blood. Moreover, iPS cell strategy suggests that somatic cells from the patient can be reprogrammed to iPS cells and after genetic manipulation, involving genetic correction for the mutations by homologous recombination, these cells can give rise to globin-producing HSCs, following directed hematopoietic differentiation.

interactions with the viral integrase, leading to inhibition of chromosomal integration [51].

4.2. HSC Cycling and Expansion Ex Vivo. Apart from cellular factors being important for lentiviral gene transfer into human HSCs, another crucial characteristic that impairs the efficiency of the gene transfer is the low levels of *ex vivo* HSC cycling. As mentioned earlier, human HSCs remain quiescent longer than murine ones, with the former entering G₁ every 30–40 weeks [28], compared to just 4–8 weeks [27] in the murine case. These findings, together with the fact that HSCs are more readily transduced when they enter cell cycle, point towards the need for *in vitro* proliferation and expansion of HSCs destined for lentiviral gene transfer. Major efforts have focused on research into novel culture conditions, which can lead to HSC expansion and proliferation. Promising candidates for these features are angiopoietin-like proteins, which are shown to induce *ex vivo* expansion of human cells that repopulate immunodeficient mice [52, 53]. Also, transcription factors such as HOXB4 [54] and NUP98-HOX fusion protein [55] have also been shown to induce HSC expansion. Furthermore, a purine derivative called stem regenin1 (SR1) was also shown to promote *ex vivo* expansion of CD34⁺ cells, by acting as an aryl hydrocarbon receptor (AHR) antagonist, leading to a 50-fold increase in CD34⁺ cells and a 17-fold increase in cells that retained their ability to engraft immunodeficient mice [56].

SR1 was shown to directly bind AHR and inhibit AHR signaling, which is implicated in hematopoiesis regulation. Recent work by Wang et al. [57] showed that inhibition of mitogen-activated protein kinase (MAPK) p38 could also result in *ex vivo* expansion of HSCs. This increase in HSC expansion is likely attributable to the p38 inhibitor-mediated inhibition of HSC apoptosis and senescence and to the upregulation of HOXB4 and CXCR4. Although the above study used murine lineage negative cells, the fact that resulted in HOXB4 upregulation, already shown to be essential for human HSC expansion, suggests that it is very likely to be effective in human HSCs case as well.

4.3. Selection of Genetically Modified HSCs. As mentioned above, increasing the levels of genetically modified human HSCs following lentiviral gene transfer is not an easy task. Various approaches have been employed in order to achieve this goal. Zhao et al. [35] incorporated the drug-resistance gene MGMT, which confers resistance to several potent hematopoietic toxins such as BCNU, in their γ -globin LV and managed to show significant amelioration of the disease in the murine model [35]. The globin-expressing HSCs were selected *in vivo* by cytotoxic drug administration and reached high levels, sufficient to ameliorate the disease. Moreover, this system allowed also an *in vitro* selection of the transduced cells, prior to transplantation, which led to enrichment of the γ -globin-expressing cells compartment.

However, despite the successful application of the above approach in murine models, there are major caveats for clinical applications, primarily due to the alkylating agents-related genotoxicity and the insertional mutagenesis potential, as discussed previously in this paper. Lastly, it is feasible in a system where cell numbers are not a limiting step, which usually is not the case for human HSCs.

4.4. Packaging Envelope Glycoproteins. Another promising strategy for increasing human HSC lentiviral transduction is by designing vectors that carry ligands on their envelope, which match the receptors on target cell. In a recent pioneering work by Verhoeven et al. [58], it was shown that HSC gene transfer is significantly increased when lentiviral particles are engineered to display early acting cytokines on their surface. The rationale behind the above strategy is that these modified LVs would selectively and minimally stimulate HSCs within the CD34⁺ cell population, leading to increased transduction and thus gene transfer. Finally, the use of the alternative envelope protein RD114 [59, 60] represents another good candidate for enhancing viral transduction and might be used as an alternative to the more cytotoxic VSVG protein.

5. Alternative Strategies for Obtaining More HSCs with Less Effort

Apart from manipulating the *ex vivo* culture conditions and the HSC state, researchers have demonstrated additional means of increasing the HSC yield, such as HSC mobilization and the generation of induced pluripotent stem (iPS) cells (Figure 1) as described below.

5.1. HSC Mobilization. The term HSC mobilization refers to the forced migration of HSCs from the BM to the bloodstream. Mobilized PB can then be used as an alternative source for CD34⁺ cells for lentiviral transduction, gene transfer, and eventual transplantation for the treatment of β -thalassemia, yielding a 3 to 4-fold enrichment [61]. Lately, this strategy has drawn the attention of many research groups, with granulocyte colony-stimulating factor (G-CSF) being the major inducer of peripheral blood stem cell (PBSC) mobilization, alone or together with chemotherapy [61, 62], and has specifically gained ground in the field of gene therapy for β -thalassemia. Particularly, Li et al. [63] assessed the administration of G-CSF in mobilizing stem and progenitor cells in thalassemic major pediatric patients and compared the kinetics of CD34⁺ cells and lymphocyte subsets with those of healthy PBSC donors. Results showed that CD34⁺ cells in 20 thalassemic patients and 11 healthy donors were effectively mobilized by G-CSF in concentrations of 10–16 $\mu\text{g}/\text{day}/\text{kg}$ of weight. No significant difference was observed in the levels of daily stem cell counts between the two groups of subjects, demonstrating that under close monitoring of CD34⁺ cell levels in PB, the mobilization by G-CSF and collection of PBSCs in β -thalassemia patients are feasible [63]. Recently, Yannaki et al. [64] have mobilized murine HSCs using G-CSF and

showed that thalassemic mice mobilized less efficiently than their control counterparts due to increased splenic trapping of HSCs and progenitor cells. The reduced mobilization efficiency was restored when splenectomy was performed in HBB^{h-3} mice, suggesting that for human gene therapy, HSC mobilization may require more than one cycle or alternative protocols, so as to yield sufficient HSCs for genetic manipulation and transplantation.

Regarding the French ongoing clinical trial, following authorization from the regulatory agency may already use either BM-derived or peripheral blood G-CSF mobilized CD34⁺ cells [45].

5.2. Induced Pluripotent Stem (iPS) Cells. iPS cells are generated by reprogramming a differentiated somatic cell into a pluripotent embryonic stem cell (ESC) [65]. These iPS cells, which are identical to human ESCs, have the potential to give rise to every cell type in the human body. The genes and surface proteins expressed in these cells are almost identical to those expressed in ESCs and, therefore, can be eventually used to correct mutant cells or tissues by homologous recombination. Thus, somatic cells from a patient may be isolated and reprogrammed to iPS cells, employing genes such as Oct3/4, Sox2 with either Klf4 and c-Myc or Nanog and Lin28, and after genetic manipulation and differentiation to the desired cell lineage, they could be administered back to the patient, maintaining, at least theoretically, the same properties and characteristics [65]. There are different techniques for pluripotency induction, and these are extensively reviewed by Patel and Yang [65] and have been presented in detail in a recent special issue of this journal [66]. Briefly, they include somatic cell transfer, cell fusion, reprogramming through cell extracts, and direct reprogramming using mainly viral vectors, proteins, RNAs, microRNAs (miRNAs), and small molecules.

iPS cell technology seems quite promising in the context of human gene therapy for β -thalassemia, as it provides an alternative and patient-friendly strategy for obtaining higher number of HSCs for genetic manipulation and transplantation. The first reported gene correction in the context of hemoglobinopathies, using iPS cells, was performed in an SCD mouse model. Hanna et al. [67] harvested cells from the skin of the SCD mouse and reprogrammed them to ESCs, by retrovirally delivering Oct4, Sox2, Lf4, and c-Myc genes. After removing *c-Myc* to decrease or eliminate putative tumorigenesis in treated mice, ESCs were cultured to produce BM stem cell precursors, and following replacement of the defected gene with a normal one, via homologous recombination, they were transplanted back to SCD mice. The outcome was disease amelioration in these mice, with blood and kidney function returning to normal levels. Following the murine study, Ye et al. [68] were the first to show that this issue is also feasible in humans, as they managed to reprogram skin fibroblasts from a thalassemic patient with β^0 thalassemia into iPS cells, and demonstrated that the latter, following gene targeting, could differentiate into hemoglobin-producing HSCs.

Such gene targeting, however, needs to be highly controlled, as randomly integrated transgenes may result in

oncogenicity, and therefore, a general need for a strategy to introduce transgenes into “safe” regions in iPS cells is imperative. A good approach to overcome this obstacle came recently from the Sadelain group [69], where they managed to induce β -globin transgene expression in iPS cell clones, where the LV had integrated in “safe harbors” throughout the genome. iPS cells in this study originated from skin fibroblast or BM mesenchymal cells from patients suffering from β -thalassemia major. In order to identify safe harbors for transgene integration in the human genome, they employed bioinformatics and functional analysis. Retrieval of safe harbor sites (i.e., safe integration sites) met the following five criteria: (i) distance of at least 50 kb from the 5' end of any gene, (ii) distance of at least 300 kb from any cancer-related gene, (iii) distance of at least 300 kb from any miRNA coding gene, (iv) location outside a transcription unit, and (v) location outside ultraconserved regions (UCRs) of the human genome. Measurement of β -globin expression in these progenitors reached high levels, suggesting that the above strategy, once improved, can be very efficient for β -thalassemia.

6. Summary

The efficient gene delivery of therapeutic transgenes using LVs has become a milestone in the field of gene therapy for hemoglobinopathies. Improved LV design enabled successful introduction of transgenes into both murine and human HSCs, leading to amelioration of β -thalassemia in murine models, and restored erythropoiesis *in vitro*. Extensive studies in the field led also to the success of the first clinical trial in France, in June 2007, where a 18-year-old HbE/ β^0 thallemic patient was treated with a β -globin vector and a year after BM transplantation managed to become transfusion-independent. As of today, he remains transfusion-independent, for over three years, in spite of repeated phlebotomies aimed to decrease iron overload. Despite the efficacy of LVs to introduce therapeutic transgenes into HSCs, there is also the risk for insertional mutagenesis, and therefore, an extensive work is currently focused on generating safe vectors for gene therapy. The above is usually achieved by incorporating elements, which make the vector tissue-specific and also shield the transgene from neighbouring effects, upon integration. Regardless the need for constantly improving vector design, a lot of attention has been drawn also towards strategies that result in higher numbers of genetically modified HSCs, which will in turn contribute to the HSC pool in the patient. The latter, together with extensive research towards alternative HSC sources, such as iPS cells, will undoubtedly set the ground for more successful clinical trials.

List of Abbreviations

HbF: Fetal hemoglobin
 HSC: Hematopoietic stem cells
 HLA: Human leukocyte antigen
 GVHD: Graft versus host disease
 MLV: Murine leukemia virus

LCR: Locus control region
 HS: Hypersensitive site
 LV: Lentiviral vector
 HPFH: Hereditary persistence of fetal hemoglobin
 cHS4: Chicken hypersensitive site 4
 SCID: Severe combined immunodeficiency
 SIN: Self-inactivating
 LTR: Long terminal repeat
 SCD: Sickle cell disease
 MGMT: Methyl guanine methyl transferase
 BCNU: Bis-chloroethyl nitrosourea
 PB: Peripheral blood
 BM: Bone marrow
 VSVG: Vesicular stomatitis virus glycoprotein
 SR1: Stem-regenin 1
 AHR: Aryl hydrocarbon receptor
 MAPK: Mitogen-activated protein kinase
 iPS: Induced pluripotent stem
 G-CSF: Granulocyte colony-stimulating factor
 PBSC: Peripheral blood stem cell
 ESC: Embryonic stem cell
 miRNA: microRNA
 UCRs: Ultraconserved regions.

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