Published in final edited form as:

Adv Drug Deliv Rev. 2010 September 30; 62(12): 1204–1212. doi:10.1016/j.addr.2010.09.005.

Delivery of nucleic acid therapeutics by genetically engineered hematopoietic stem cells

Christopher B. Doering, David Archer, and H. Trent Spencer

Aflac Cancer Center and Blood Disorders Service, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia

Abstract

Several populations of adult human stem cells have been identified, but only a few of these are in routine clinical use. The hematopoietic stem cell (HSC) is arguably the most well characterized and the most routinely transplanted adult stem cell. Although details regarding several aspects of this cell's phenotype are not well understood, transplant of HSCs has advanced to become the standard of care for the treatment of a range of monogenic diseases and several types of cancer. It has also proven to be an excellent target for genetic manipulation, and clinical trials have already demonstrated the usefulness of targeting this cell as a means of delivering nucleic acid therapeutics for the treatment of several previously incurable diseases. It is anticipated that additional clinical trials will soon follow, such as genetically engineering HSCs with vectors to treat monogenic diseases such as hemophilia A. In addition to the direct targeting of HSCs, induced pluripotent stem (iPS) cells have the potential to replace virtually any engineered stem cell therapeutic, including HSCs. We now know that for the broad use of genetically-modified HSCs for the treatment of non-lethal diseases, *e.g.* hemophilia A, we must be able to regulate the introduction of nucleic acid sequences into these target cells. We can begin to refine transduction protocols to provide safer approaches to genetically manipulate HSCs and strategies are being developed to improve the overall safety of gene transfer. This review focuses on recent advances in the systemic delivery of nucleic acid therapeutics using genetically-modified stem cells, specifically focusing on i) the use of retroviral vectors to genetically modify HSCs, ii) the expression of fVIII from hematopoietic stem cells for the treatment of hemophilia A, and iii) the use of genetically engineered hematopoietic cells generated from iPS cells as treatment for disorders of hematopoiesis.

Keywords

Hematopoietic stem cell; gene therapy; recombinant lentivirus; hemophilia A; induced pluripotent stem cell

Hematopoietic stem cell gene therapy

Hematopoietic stem cells (HSCs) have been a target of genetic engineering from the earliest gene transfer studies, and they continue to be excellent candidates for systemic delivery of many nucleic acid-based therapeutics. The reason for the continued interest is that these

Correspondence: H. Trent Spencer, 2015 Uppergate Drive, Aflac Cancer Center and Blood Disorders Service, Emory Children's Center, Room 420, Emory University, Atlanta, Georgia.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cells have the ability to regenerate the entire hematopoietic system, which includes all lineages of blood cells such as lymphocytes and monocytes. The HSC is also readily manipulated *ex vivo*, which allows for rapid testing of various parameters relating to gene transfer and the expression of the transferred nucleic acid, *i.e.* the transgene. Importantly, the stable introduction of a transgene into the HSC can result in the expression of the gene product in the progenitor cells derived from the HSC as well as every blood cell derived from the progenitor cells, which can amplify the therapeutic potential of genetically-modified HSCs a thousand fold. Many platforms are now available for introducing nucleic acid sequences into HSCs, some of which are currently in clinical trials [1–7].

Successful initial gene transfer pre-clinical studies used γ -retroviral vectors, such as the Moloney murine leukemia virus (MLV), as nucleic acid-transfer vehicles. The goal of early studies was to develop methods that resulted in 1) stable integration of the transgene into the chromosome of the target cell, 2) efficient nucleic acid transfer, 3) engraftment of significant numbers of genetically-modified HSC, and 4) high-level transgene expression. The mouse proved to be an excellent model system for testing novel gene-transfer methods, and γ -retroviral vectors quickly became the gene-transfer tool of choice. This recombinant γ -retroviral vector system is composed of an expression vector, which contains the transgene of interest, and a packaging cell, which is typically based on cell lines such as NIH3T3 or HEK293T cells. Packaging cells are engineered to express the protein components of the γ -retroviruses, which produces a recombinant viral vector capable of transferring virtually any transgene of interest [1,5,7].

Initial studies targeting mammalian HSCs showed inefficient levels of gene transfer. It quickly was shown that understanding the biology of the target cells was critical to accomplishing efficient gene transfer. HSCs are mainly quiescent and divide relatively infrequently. However, γ -retroviruses require cellular division for successful integration of the transgene into the genome of the HSC. Therefore, various protocols were developed that induced HSC division, most of which focused on identifying effective cytokine cocktails that resulted in efficient murine HSC transduction (>50% transduction of murine HSCs are now readily attained [8–12]). In addition, the introduction of fibronectin fragments was included to the transduction protocol, which further enhanced the efficiency of gene transfer, presumably through co-localization of viral vectors and target cells thereby increasing the effective concentrations of each [13–15].

Safety issues relating to the genetic engineering of HSCs

With efficient packaging cells in hand generating high titers of recombinant virus combined with efficient transduction protocols, HSC genetic engineering quickly moved from preclinical studies to clinical trials. The initial gene therapy trials focused on immunodeficiency disorders, and a clinical trial designed to treat childhood severe combined immune deficiency (SCID) X1 disease provided direction for the entire gene therapy field. In this trial, a serious adverse event was reported in late 2002, which described the development of leukemia in one of the treated patients [16]. Although 9 of 10 children enrolled in the trial showed correction of the immune deficiency for more than five years, an initial patient developed a T cell leukemia-like illness three years after receiving genetically-modified HSCs. Additional children subsequently developed a similar disease, and the FDA put a clinical hold on similar trials. Of the 20 treated children, 5 have developed the T cell leukemia-like disorder. The cause of the leukemia is now well documented, and several outstanding studies have described the molecular events resulting in the leukemia [16,17]. Briefly, the transferred nucleic acid sequence in the SCID X1 trials encoded the common γ chain of the cytokine receptor subunit, which is a component of the IL-2, IL-4, IL-7, IL-9, IL-11, IL-15 and IL-21 signaling receptors. The cDNA was transferred using a recombinant

γ retroviral vector. It is now known that γ –retroviruses integrate into chromosomal sites of active transcription [18–22]. For the children who developed leukemia, the transferred gene integrated near known proto-oncogenes, such as LMO-2. These insertional mutagenic events caused aberrant transcription and expression of the oncogene, and when combined with the enforced expression of the γ chain of the cytokine receptor led to the dysregulation of T-cell expansion and leukemia. Prior to this study, it was predicted that retroviruses integrated randomly. However, it is now well documented that retroviruses can cause severe adverse events through mechanisms associated with insertional mutagenesis. Therefore, investigations focusing on insertion-site analysis have been a major priority in the field of HSC-directed gene therapy. From these studies, it is now clear that under certain circumstances recombinant viruses can alter endogenous gene function at the vicinity of viral nucleic acid integration.

A major issue, therefore, facing the field of HSC-directed gene therapy is that of insertional mutagenesis (Figure 1). Some of these concerns have been alleviated with the introduction of safety-engineered gene-transfer vectors. Recombinant lentiviruses, such as those derived from the human immunodeficiency virus (HIV), are now known to be less genotoxic compared to the γ retroviral vectors used in the initial gene transfer studies. In support of using lentivirus-based vectors, during the past 5 years, VIRxSYS Corp. (Gaithersburg, MD) has transduced and transplanted over a trillion HIV-1-based recombinant lentivirus-transduced CD4⁺ cells in their phase 1 clinical trials for acquired immune deficiency syndrome [23,24]. There is no conclusive evidence showing that patients treated with gene-modified cells have oncogene dysregulation due to recombinant lentiviral infection. It has been proposed that this can be attributed to the tendency of HIV-based vectors to integrate within transcriptionally-active genes as opposed to oncoretroviruses, which tend to integrate near promoter/enhancer regions. Therefore, it is predicted that within genetically-modified HSCs, lentiviral vectors will integrate into known gene loci and hamper cellular gene expression of the gene in which it integrates [25]. However, even if integration occurs within a gene sequence, diploid cells contain a second intact copy of the gene and, therefore, will likely not suffer from the loss of function of one gene copy.

The creation of self inactivating (SIN) vectors has further increased the safety of lentiviral vectors (Figure 1). SIN vectors take advantage of the fact that the U3 regions of the integrated proviral sequence of both LTRs are generated from the U3 region of the 3' LTR during reverse transcription [26,27]. Therefore, a deletion in this region leads to elimination of transcriptional activity from the viral LTR. SIN vectors decrease the possibility that expression of cellular genes adjacent to the vector integration site will be up-regulated by viral insertion events. However, the viral LTR promoter must be replaced with an internal promoter, such as the elongation factor-1 alpha promoter, which has decreased enhancer activity compared to the viral LTR. Most current HSC-directed gene therapy studies are using SIN lentiviral vectors. Recent studies have shown that SIN lentiviral vectors have a lower oncogenic potential compared to the originally used gamma retroviruses [28,29]. Additionally, lentiviral vectors can transduce HSCs more efficiently compared to γ –retroviral vectors and may require HSCs to be manipulated *ex vivo* for shorter periods of time, which can improve the multipotency and engraftment potential of the transduced cells [30–33]. However, some studies have shown that SIN vectors can have enhanced incidence of transcriptional read-through [34]. Even so, the transforming capacity of SIN vectors is significantly reduced compared to LTR containing vectors [34,35], and SIN-HIV-based vectors have not increased the risk of tumor formation [36]. These recent studies do not end the debate as to the safety issues of retroviral gene transfer, but there is now sufficient evidence to incorporate the use SIN vectors into clinic trials. It should be understood, however, that these modifications may provide some benefit but may also have detrimental

effects on viral titers and gene expression in transduced cells, and clinical trials are being designed to monitor these possibilities.

Based on the results from early clinical trials, the gene therapy field has shifted from a primary focus of determining which diseases are potential candidates for nucleic acid interventions to having a greater interest in safety, which is entirely expected for emerging biotherapeutics. As such, the possible issue of insertional mutagenesis remains a major concern to the field. Routine preclinical HSC-based studies now are designed to include not only analysis of transgene expression but also screening of multiple parameters in animals transplanted with genetically-modified cells. Typical analyses entail: 1) inclusion of cohorts to determine differences in lifespan, 2) flow cytometry studies to monitor the percentages of various hematopoietic lineages over the lifetime of transplanted animals, 3) monthly blood smears to have a Wright-Giemsa staining record over time, 4) analysis to monitor clonal expansion of genetically-modified HSCs, and 5) plans to monitor integration sites in the case of leukemic transformation.

Clinical trials utilizing genetically-engineered HSCs

Even though dramatic improvements have been made with respect to vector design, transduction efficiency of HSCs is still a major concern. For example, in a recent phase 1 HSC gene therapy clinical trial for x-linked adrenoleukodystrophy, CD34⁺ cells were isolated and transduced *ex vivo* with an SIN HIV-based lentiviral vector after the cells were stimulated with a cytokine mixture of IL-3, Flt3-L, megakaryocyte growth and differentiation factor and stem cell factor [37]. Transduction efficiencies approaching 50% were achieved in the CD34⁺ population. However after transplantation, only 9–14% of blood cells were positive for the therapeutic protein. These results clearly show the disconnect between CD34⁺ cell transduction efficiency and engraftment and/or expansion of gene-modified HSCs. One possible reason for the decrease in the percentage of gene-modified cells after transplant is the culture conditions used to transduce HSCs, which can significantly reduce their repopulation potential [38]. Alternatively, HSCs within the CD34⁺ population may be resistant to transduction. Each of these issues is being actively pursued experimentally with the aim of developing better HSC transduction protocols. For example, the CD34⁺ cell population is composed mainly of progenitor cells with an extremely small percentage of HSCs. Better purification techniques to isolate a more pure population of HSCs, such as lineage⁻/CD34⁺/CD38⁻, may provide better transduction efficiency of HSCs and increased engraftment of genetically-modified cells. Although efficient transduction of the HSC can provide treatment options for a wide variety of diseases, some studies have suggested that the HSC may be sensitive to insertional mutagenesis [38]. Therefore, although it would be expected that transduction of highly purified HSCs should be a goal, additional studies are needed to determine the risks and benefits of pursuing this approach.

Thus far, two clinical HSC-based clinical trials have been described in this review, one for x-linked SCID and the other for x-linked adrenoleukodystrophy. The x-linked SCID trials are notable because they highlight the complexity of using HSC-directed nucleic acid therapeutics. Interesting, in contrast to the adverse events observed in the c-chain trials, similar treatment of x-linked SCID disease due to adenosine deaminase (ADA) deficiency has not, to date, resulted in the expansion of leukemic cells [39]. These differences may be due to several factors, including differences in virus preparation, subtleties of the transduction conditions, or differences in the biology of the two diseases. For example, mutations in the c chain results in a pool of lymphocyte progenitor cells that do not progress through the differentiation pathway to become mature lymphocytes. Genetic mutations can build in this pool of undifferentiated cells. After c-chain addition through genetic modification and when combined with the uncontrolled expression of proto-oncogenes due

to insertional mutagenesis as well as constitutive expression of γ c, uncontrolled clonal expansion of leukemic cells can occur. In contrast, cells that lack ADA have a survival disadvantage and the pool of undifferentiated progenitor cells does not occur. Furthermore, the lack of leukemic expansion in the adrenoleukodystrophy study also suggests the biology of the corrected cells may play a role in adverse events resulting from insertional mutagenesis.

Compounding the complexity of understanding how genetic modification effects HSC function, two additional clinical trials have shown puzzling results. A phase I trial for gp91phox deficiency (x-linked chronic granulomatous disease) resulted in the presence of a dominant myeloid-derived hematopoietic clone [40,41]. Although the clone appeared to have a survival or growth advantage, it did not transition to a malignancy. A separate trial for thalassemia [42], the results of which have only been published in abstract form, also showed clonal dominance without leukemic transformation. Although the number of patients enrolled in HSC-based lentiviral clinical trials is low, to date there have not been any reports of pathogenesis due to lentiviral transduction. In addition, no adverse events have been observed in a myriad of lentiviral-transduction studies using genetically-modified T cells as cellular therapeutics, and adverse events have not been observed in rhesus macaques, baboons, dogs, or sheep that have been treated using gene transfer approaches similar to those used clinically [43,44].

HSC transplant (HSCT) gene therapy of hemophilia A

Although there have been some clinical successes using genetically engineered HSCs, additional diseases are candidates for HSC-based gene therapy interventions. For example, hemophilia A is an ideal target for gene therapy, and recent preclinical studies indicate that the major hurdles impeding a successful HSC-based hemophilia A therapy have been overcome. The benefits/risk ratios for treating hemophilia with gene therapy has been discussed extensively, and the consensus is that gene therapy will be the future treatment for hemophilia [45]. Current therapies for hemophilia are hampered by the high cost of treatment, the demanding schedule required to obtain maximal prophylactic benefit, the potential of inhibitor formation, and arthropathy due to joint bleeding, which can occur under ideal treatment conditions. The high cost of hemophilia A treatments result in only approximately 25% of the world hemophilia A population being treated, which can be considered the number one problem with current treatment options. HSC-based gene therapy can provide a one-time treatment resulting in a lifetime cure for this disease.

Hemophilia A is not treatable by hematopoietic stem cell transplantation alone due to the fact that hematopoietic cells are not an endogenous source of coagulation factor VIII (fVIII). However, hemophilia A is amenable to treatment using HSC transplantation (HSCT) gene therapy. Hemophilia A results from deficiency of plasma fVIII activity. Since fVIII is a secreted protein that functions in the bloodstream at sites of vascular injury, it theoretically can be produced by any cell type with access to the bloodstream. Genetic manipulation of a single HSC facilitates the generation of millions of progeny cells, which if an integrating virus was used to perform the gene transfer also will contain the genetic modification of interest, e.g. a functional fVIII transgene. Therefore, HSCs are an ideal drug delivery vehicle for the treatment of hemophilia A. In the current section, we describe advances in HSCT gene therapy for hemophilia A using it as a model disease and example for the use of HSCs as vehicles for therapeutic nucleic acid and protein delivery.

The first hemophilia A preclinical HSCT gene therapy study was conducted by Evans and Morgan in the 1990's [46]. A murine leukemia virus-based γ -retroviral vector was used to deliver a human fVIII transgene to murine bone marrow cells, which subsequently were

transplanted into recipient hemophilia A mice that were conditioned with a lethal dose of total body irradiation (TBI). Correction of the fVIII deficiency was not achieved in this initial study presumably due to low level biosynthesis of fVIII from hematopoietic cells. Subsequently, it was shown by Tonn and colleagues that, using *in vitro* models, genetically-modified erythroid and/or megakaryocytic cell lines secreted higher levels of fVIII than did lymphoblastoid or T-cell leukemia cell lines suggesting a cell lineage-specific biosynthesis differential. This study instilled renewed enthusiasm for HSCT gene therapy and in 2004 and 2005, Hawley and colleagues reported the first preclinical studies successfully utilizing HSCT gene therapy to achieve correction of the fVIII deficiency in hemophilia A mice to a predicted therapeutic level ($\geq 5\%$ of the normal human level or 0.05 units/ml fVIII activity). In their study, they used a bicistronic γ -retroviral vector encoding an EGFP reporter gene in addition to the human fVIII transgene. This modification allowed for the selection of genetically-modified cells by fluorescence activated cell sorting prior to transplantation in order to maximize the engraftment of genetically modified cells and thus increase fVIII production levels [47,48]. Although this technique was successful in the mouse model, it is not clinically feasible and further advancements were needed. To improve upon fVIII expression levels, they incorporated modifications to the human fVIII transgene itself that had been shown by others to increase the secretion rate of human fVIII [49,50]. Incremental improvement also was made by integrating both an enhanced fVIII transgene containing amino acid residues designed for improved expression and decreased immunogenicity as well as a safety optimized retroviral vector gene delivery system [51].

Another successful approach, adopted by our group, was the utilization of the high-level expression property of the orthologous porcine fVIII molecule. The high expression property was identified during the development of a recombinant porcine fVIII product designed, and currently in clinical trials, for the treatment of both congenital and acquired hemophilia A patients with anti-fVIII inhibitory antibodies [52–54]. We have shown in a series of studies that the high expression property of porcine fVIII functions effectively in a HSCT gene therapy setting. In the first study, recombinant murine stem cell virus-based vector encoding a porcine fVIII transgene was used to genetically-modify murine HSCs, which were transplanted into hemophilia A mice preconditioned with TBI [55]. All mice demonstrated complete ($>100\%$ of the normal human plasma fVIII activity level) and sustained correction of circulating fVIII activity levels. Life-long restoration of fVIII activity suggested that genetically-modified HSCs engrafted, proliferated, and expressed fVIII at levels significantly greater than that achieved by Hawley's group using human fVIII variants. This original study demonstrated proof-of-concept that porcine fVIII high-expression elements function effectively *in vivo* in a gene therapy setting. Further progress in the field of HSCT gene therapy for hemophilia A focused on improving the safety profile. For example, in the original studies by Hawley and ourselves, TBI was used as a marrow conditioning regimen. Due to the significant risk of long-term side effects and short-term mortality, TBI is not appropriate for the treatment of patients with hemophilia A especially those that have access to fVIII replacement products. Therefore, investigation into the use of more clinically-relevant HSCT regimens that maintain efficient engraftment and fVIII expression while limiting the development of anti-fVIII inhibitors was necessary. Therefore, we subsequently tested several reduced intensity conditioning regimens and identified several that performed successfully including costimulation blockade (anti-CD40L and CTLA4-Ig) and a combination of busulfan and anti-thymocyte serum (ATS) [56]. Under these conditioning regimens, no mice developed inhibitory anti-fVIII antibodies or obvious leukemic transformations. One conclusion drawn was that T-cell-specific suppression/cytoreduction was a critical component to the successful engraftment of genetically-modified HSCs encoding fVIII. In a related study, we demonstrated that HSCT gene therapy incorporating the high expression porcine fVIII transgene could correct the fVIII deficiency and eradicate pre-existing anti-human-fVIII inhibitors [57]. This was the first report

showing successful treatment of hemophilia A using nucleic acid therapy in the context of fVIII inhibitors and the only report demonstrating inhibitor elimination. As hemophilia A patients with fVIII inhibitors are the most challenging and costly population to treat clinically, they represent an ideal patient population for future gene therapy clinical trials.

The observation that correction of fVIII activity levels can be achieved by a small percentage of genetically-modified hematopoietic cells harboring low proviral copy number supports the feasibility of successfully translating this strategy to human patient care. Clearly, high expression porcine fVIII sequence elements overcome the low-expression barrier observed using standard human fVIII constructs and, more recently, we demonstrated that a high expression hybrid human/porcine (HP)-fVIII construct with as little as 10% porcine amino acid sequence functions indistinguishably in a HSCT gene therapy [58]. In this study, recombinant lentiviral vectors encoding HP-fVIII were used to genetically-modify human hematopoietic cell lines and murine HSCs, the latter of which were transplanted into hemophilia A mice. Similar to the results obtained using the porcine fVIII transgene, HP-fVIII demonstrated significantly greater expression levels than BDD human fVIII supporting its continued development towards clinical application.

One complexity of expressing a heterologous protein in hematopoietic cells is the diversity of this cell population. HSCs give rise to the three committed cell lineages, erithroid, myeloid and lymphoid cell types, each of which contains several distinct subpopulations. For example, myeloid progenitor cells give rise to monocytes, macrophages, neutrophils, eosinophils and basophils. Each differentiated cell type is presumed to possess differential protein synthesis capacity and specificity. These cellular characteristics are important considerations when determining the appropriateness of HSCT gene therapy for a specific disease. For example, it is established that over-expression of fVIII induces endoplasmic reticulum stress in mammalian cells through the unfolded protein response pathway [59]. Prolonged ER stress can lead to apoptosis. Therefore, a relevant concern in HSCT gene therapy for hemophilia A is cellular toxicity in hematopoietic stem, progenitor and terminally differentiated cell lineages due to fVIII expression. Research in this area is ongoing, but in preliminary studies, there have been no reports of toxicity due to fVIII expression following HSCT gene therapy.

If lineage-specific toxicity was observed, one possible solution would be directed expression into lineages that are not sensitive to transgene-related toxicity. In addition, lineage specific expression can limit enhancer-related insertional mutagenesis issues because promoters for lineage expression are only active in a limited subset of cells. Along this line of pursuit, two laboratories have reported the specific targeting of fVIII transgene expression to the megakaryocyte/platelet hematopoietic lineage [60–63]. In concept, the fVIII transgene is transcribed and fVIII biosynthesis occurs in megakaryocytes and the resulting translation product is stored in platelet granules until release at the time of platelet activation. For these studies, DNA constructs encoding a BDD human fVIII transgene under the control of various integrin promoters were used to generate transgenic mice or incorporated into self-inactivating lentiviral vectors and delivered through HSCT gene therapy to recipient hemophilia A mice. These studies provided proof-of-concept that platelet-directed fVIII expression could be used to correct the bleeding phenotype in hemophilia A mice as assessed by tail-clip bleeding assay despite the lack of circulating fVIII activity. Another facet of this strategy is that fVIII stored in platelets is protected from fVIII inhibitors and retains *in vivo* efficacy even in the presence of high titer inhibitors. However, recent results obtained by Poncz and colleagues dispute the interpretation of efficacy using the tail-clip bleeding assay. They conclude that the tail-clip assay is overtly-sensitive to small amounts of fVIII activity due to the nature of the hemostatic challenge and relevant murine physiology [64]. Despite significant progress, further preclinical and clinical testing will be

necessary to determine the translatability of this unique directed expression approach to HSCT gene therapy for hemophilia A. Other hematopoietic cell populations of interest for targeted fVIII expression include plasma B cells, due to their adaptation to the biosynthesis of large quantities of high molecular weight glycoproteins (i.e. immunoglobulins), and erythrocytes, due to their abundant nature and lack of oncogenic disposition.

As a model disease for HSCT gene therapy, hemophilia A possesses both desired and disruptive characteristics. However, much research has been invested in this therapeutic arena and significant progress has been made. Although there has not yet been a clinical trial for HSCT gene therapy of hemophilia A, it likely is a not too distant reality based on recent advancements in the field of HSCT gene therapy for other monogenic disorders and the desire for a cure to this physically and economically debilitating disease. Overall, when the potential benefits and risks of gene therapy of hemophilia are weighed with those associated with conventional therapy, the consensus among key opinion leaders in hemophilia is that gene therapy represents the future of hemophilia care [45,65–72].

Alternate sources of HSCs

The propagation of hematopoietic stem cells in vitro has been one of the pivotal challenges for hematologists since the discovery of our ability to transfer hematopoiesis with bone marrow cells. The isolation, phenotypic and molecular identification of the stem cell and its niche has provided considerable insight into the biology of stem cells however, a consistent method of expanding HSC in vitro in the quantities required for transplant, while maintaining their engraftment potential, has remained elusive. Once factors were identified in the late 1980's that could be used to propagate murine embryonic stem cells (mES) [73] more easily, investigators were enthused with the prospect of investigating mechanisms controlling the ontogeny of HSC and of having a potentially unlimited supply of pluripotent cells from which to generate HSC. Subsequent generation of human embryonic stem cells (hES) a decade later reinvigorated research in this area [74] but the technical complexity of hES propagation and politics surrounding hES generation and use provided a challenging working environment. The generation of induced pluripotent stem cells (iPS) in 2006 [75], has provided a readily available mechanism for the generation of pluripotent cell lines from multiple cell types and species. Briefly, the virally mediated ectopic expression of four factors Oct3/4, Sox2, Klf4 and c-Myc in fibroblasts was able to generate cells with similar morphology and differentiation potential to ES cells that could be propagated under ES culture. While iPS cells have been generated from many cell types using a variety of techniques expression of the four genes is usually sufficient to induce pluripotency [76,77]. All four reprogramming genes are now expressed from a single cassette using 2A peptide sequences providing successful iPS generation of mouse and human fibroblasts [78]. Currently, these techniques are very inefficient but as our knowledge of the precise mechanisms involved in reprogramming increases it is more likely that the field will move towards a completely protein or small molecule based approach. Indeed Ichida et al. found that sox2 could be replaced from the standard four gene reprogramming combination by treating with a small molecule based inhibitor of TGF- β that induced the transcription factor nanog [79].

Choosing the optimal source of cells for potential reprogramming is of interest both from a mechanistic perspective and from the practical approach to producing patient specific or banks of iPS lines for clinical use. From the patient perspective, hematopoietic cells are readily available but purifying progenitors can be an expensive and time consuming task. When considering cells banks, there is a practically unlimited supply of pre-existing cord blood units that are already stored and potentially HLA-typed in cord blood banks around the world. Interestingly, mature B cells did not reprogram successfully without addition of

the transcription factor C/EBP α or specific knockdown of the transcription factor Pax5. To further address the idea that terminally differentiated cells may be less amenable to reprogramming Eminli et al selected murine hematopoietic cells at different stages of differentiation and compared the reprogramming efficiency of each lineage [80]. Hematopoietic progenitors of both myeloid and lymphoid lineages produced iPS cells significantly more efficiently than terminally differentiated T or B cells or indeed fibroblasts or keratinocytes, confirming hematopoietic progenitors as a potentially attractive source for iPS induction.

Recently, frozen commercial mononuclear cell preparations from either bone marrow or peripheral blood were successfully reprogrammed using the four transcription factors and a series of cytokine cocktails although, the frequency of iPS generation was low and the phenotype of the cells that were reprogrammed unknown the study confirms that a small blood sample can be sufficient for iPS generation [81]. Human CD34⁺ cells from mobilized peripheral blood, [82] bone marrow, [83] and cord blood were successfully reprogrammed using viruses encoding Oct4, Sox2, klf4 and c-Myc confirming the ability of human hematopoietic progenitors to be reprogrammed. iPS lines were also generated when CD133⁺ cells isolated from cord blood were reprogrammed with constructs encoding all four pluripotency genes but interestingly, reprogramming was also successful when Klf4 or Klf4 and c-Myc were omitted from the protocol[84]. Similarly, the frequency of iPS generation from CD34⁺ cord blood cells was increased about 100 fold when P53 was inhibited during the reprogramming process [85]. These and other studies strongly imply that the efficiency of reprogramming will significantly improve as we gain further insight into the interaction between and specific roles of the genes involved.

Disease Candidates

As discussed in the previous sections, hemophilia A is a natural candidate for gene therapy. To demonstrate the feasibility of an iPS strategy for therapy Xu et al generated murine iPS cells from tail tip fibroblasts of normal mice, differentiated the cells towards an endothelial phenotype, and transplanted them into the livers of irradiated hemophilia A mice leading to the expression of functional levels of factor VIII [86]. Similar studies can be envisioned using human hematopoietic cells to generate the iPS cells.

Another rare genetic disorder affecting hematopoiesis that is a potential target for gene therapy is Fanconi anemia (FA), which is caused by mutations in one of thirteen genes associated with the FA pathway. This syndrome presents with bone marrow failure and an elevated risk of both hematopoietic, typically acute myelogenous leukemia, and solid tumors, the marrow failure usually presenting within the first decade of life. The preferred treatment is hematopoietic stem cell transplantation which has considerable success for those treated before the onset of disease with matched sibling unaffected donors. Unfortunately, most patients do not have donors in this category and despite improving transplant protocols there is still morbidity and mortality associated with transplant (reviewed in [87]). Thus patient specific gene corrected HSC would be particularly beneficial in this disorder, and, depending upon the severity of the disease, the corrected HSC may even have an in vivo proliferation advantage following the transplant [88]. Raya et al generated iPS cells from skin fibroblasts and keratinocytes of patients with FA but the process was only successful when reprogramming was performed in cells that had previously undergone virally mediated gene addition of fanc proteins [89]. This is an important concept indicating that the FA pathway needs to be intact for the successful generation of iPS cells. Furthermore, these corrected iPS cells showed hematopoietic differentiation in vitro that was equivalent to that from control iPS cells and ES cells

indicating that the gene addition was successfully maintained and functional in the differentiated cells.

The hemoglobinopathies as a group are the most common single gene defects in the world. The only curative therapy for beta-thalassemia is hematopoietic stem cell transplantation (reviewed in [90]). While transplantation protocols are improving, the patients who lack a related donor or who have advanced stage disease still have a considerable risk of transplant-related mortality. Therefore, gene therapy of autologous cells to induce beta globin expression would be an attractive approach to treatment. Following considerable work to generate lentiviral vectors that could induce reliable and persistent globin expression in murine cells, transplantation of HSC from β -thalassemic mice transduced to express either β - or γ -globin was shown to correct the β -thalassemic phenotype [91–95].

These experiments were followed by the lentiviral expression of β -globin in human CD34+ cells from a patient with β -thalassemia that corrected the RBC phenotype when engrafted into immune deficient mice [96] and have culminated in a recent clinical trial. In order to validate an iPS approach for thalassemia, fibroblasts from a patient with β^0 -thalassemia were recently successfully reprogrammed to iPS [97]. More interestingly, cells from amniotic fluid and chorionic villus samples were also successfully reprogrammed, suggesting that iPS cells could be generated for use in perinatal transplants. The thalassemias can present with over 200 different mutations [98] so are not the optimal candidates for therapy by gene correction of iPS cells.

However, sickle cell anemia presents with a single mutation involving the single nucleotide A to T transversion at codon 6 of the β globin gene resulting in the substitution of glutamine by valine in the β globin protein that forms hemoglobin [99]. Tetramers of alpha and the mutant beta globin, form hemoglobin S (HbS, $\alpha_2\beta S_2$) which is predisposed to form polymers when deoxygenated that cause red blood cells to become rigid and deform into the characteristic sickle shape. Direct gene therapy of HSC for the hemoglobinopathies has received considerable attention and lentiviral based vectors containing anti-sickling variants of the human β -globin chain were used to transduce murine bone marrow cells prior to transplant into murine models of sickle cell disease [100,101]. In both studies, transplanted mice showed improvements in hematology and associated sickle pathophysiology. The recognition that persistent expression of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) whether due to the presence of a particular hemoglobin haplotype [102,103] or through induction via treatment with hydroxyurea [104] correlated to a milder clinical course, drove an alternative gene addition strategy which focused on expression of the γ -globin gene.

Pestina et al used a lentiviral vector to transduce bone marrow cells from the Berkeley model of sickle cell disease prior to transplant in to normal mice. Those expressing high levels of gamma globin were spared from the development of sickle cell pathology in multiple organ systems [105]. While these gene addition strategies provide proof of principle data in mice there is still concern over the possibility of insertional mutagenesis, as discussed above. The goal of site directed gene correction for sickle cell disease was achieved in embryonic stem cells derived from a sickle cell mouse created by Drs. Ryan and Townes [106]. They successfully replaced the human γ - β S gene construct that was used to create the sickle cell model by homologous recombination using an allele containing the normal human beta globin gene. Further, mice generated from the corrected ES cells displayed normal hematology. This group has continued to develop the theme by growing skin fibroblasts from the γ - β^S sickle mice and generating iPS cells using retroviruses for Oct 4, Sox2, Klf4 and c-Myc [107]. The iPS cells were again transduced with a virus encoding GFP tagged HoxB4 protein and differentiated towards hematopoiesis on the OP9 stromal cell line. Selected hematopoietic precursors were transplanted into lethally irradiated sickle

cell mice and showed multi-lineage engraftment with correction of sickle hematology and improved organ function. [107] Similarly this group has now generated fibroblasts from a patient with homozygous sickle cell disease, corrected the gene defect using homologous recombination, and then generated iPS cells from the corrected fibroblasts (Tim Townes, personal communication). This work is the closest to realizing the potential of patient specific therapy using iPS technology. However, the major problem that researchers continue to face using either ES or iPS cells for hematopoiesis still remains the generation of human HSCs in sufficient numbers to be clinically relevant.

Summary

The use of stem cells as therapeutics is in the developmental stages with a vast number of preclinical studies advancing to clinical trials. Using stem cells to deliver nucleic acid therapeutics is an attractive treatment approach that can potentially cure diseases that are currently managed through a lifetime of treatment. HSCs are used routinely as therapy for cancer and various inherited diseases. Clinical trials have already shown the usefulness of these cells as vehicles to deliver nucleic acid therapeutics. However, these same trials have shown some of the limitations of introducing nucleic acids into target cells, particularly the HSC. Novel gene transfer vectors have been, and continue to be, developed to more efficiently deliver nucleic acid sequences into cells while decreasing the adverse effects resulting from the introduction of new DNA sequences. The treatment of hemophilia A is excellent example of the potential use of genetically engineered HSCs. Factor VIII is not normally expressed from hematopoietic cells. However, the introduction of the cDNA sequence encoding this protein into HSCs has been used to cure this disease in animal models, and it is predicted that clinical trials will soon test this treatment in humans. Future use of genetically-engineered HSCs may take advantage of alternative sources of this cell. For example, iPS cells can be generated from a variety of differentiated cells, and the conversion of these cells to hematopoietic lineages has already been shown. Introducing therapeutic genes into these cells will not be a major hurdle. The use of HSCs as targets of genetic engineering has unquestionably advanced the field of gene therapy and will likely continue to play important roles in the development of future nucleic acid therapeutics.

References

1. Brenner S, Malech HL. Current developments in the design of onco-retrovirus and lentivirus vector systems for hematopoietic cell gene therapy. *Biochim Biophys Acta*. 2003; 1640:1–24. [PubMed: 12676350]
2. Izsvák Z, Chuah MK, Vandendriessche T, Ivics Z. Efficient stable gene transfer into human cells by the Sleeping Beauty transposon vectors. *Methods*. 2009; 49:287–97. [PubMed: 19615447]
3. Hart SL. Multifunctional nanocomplexes for gene transfer and gene therapy. *Cell Biol Toxicol*. 2010; 26:69–81. [PubMed: 20127400]
4. Shao L, Wu WS. Gene-delivery systems for iPS cell generation. *Expert Opin Biol Ther*. 2010; 10:231–42. [PubMed: 20088717]
5. Miller AD. Retrovirus packaging cells. *Hum Gene Ther*. 1990; 1:5–14. [PubMed: 2081186]
6. Baum C, Schambach A, Bohne J, Galla M. Retrovirus vectors: toward the plentivirus? *Mol Ther*. 2006; 13:1050–63. [PubMed: 16632409]
7. Lundstrom K. Latest development in viral vectors for gene therapy. *Trends Biotechnol*. 2003; 21:117–22. [PubMed: 12628368]
8. Luskey BD, Rosenblatt M, Zsebo K, Williams DA. Stem cell factor, interleukin-3, and interleukin-6 promote retroviral-mediated gene transfer into murine hematopoietic stem cells. *Blood*. 1992; 80:396–402. [PubMed: 1378319]

9. Orlic D, Girard LJ, Anderson SM, Bodine DM. Retrovirus receptor mRNA expression correlates with gene transfer efficiency in pluripotent hematopoietic stem cells. *Leukemia*. 1999; 13(Suppl 1):S52–4. [PubMed: 10232366]
10. Wotherspoon S, Dolnikov A, Symonds G, Nordon R. Susceptibility of cell populations to transduction by retroviral vectors. *J Virol*. 2004; 78:5097–102. [PubMed: 15113891]
11. Ide LM, Javazon E, Spencer HT. Transduction of murine hematopoietic stem cells and in vivo selection of gene-modified cells. *Methods Mol Biol*. 2008; 433:213–28. [PubMed: 18679626]
12. McMillin DW, Landázuri N, Gangadharan B, Hewes B, Archer DR, Spencer HT, Le Doux JM. Highly efficient transduction of repopulating bone marrow cells using rapidly concentrated polymer-complexed retrovirus. *Biochem Biophys Res Commun*. 2005; 330:768–75. [PubMed: 15809063]
13. Pollok KE, Williams DA. Facilitation of retrovirus-mediated gene transfer into hematopoietic stem and progenitor cells and peripheral blood T-lymphocytes utilizing recombinant fibronectin fragments. *Curr Opin Mol Ther*. 1999; 1:595–604. [PubMed: 11249666]
14. Kiem HP, Andrews RG, Morris J, Peterson L, Heyward S, Allen JM, Rasko JE, Potter J, Miller AD. Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. *Blood*. 1998; 92:1878–86. [PubMed: 9731044]
15. Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat Med*. 1996; 2:876–82. [PubMed: 8705856]
16. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003; 302:415–419. [PubMed: 14564000]
17. Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K, Asnafi V, MacIntyre E, Dal Cortivo L, Radford I, Brousse N, Sigaux F, Moshous D, Hauer J, Borkhardt A, Belohradsky BH, Wintergerst U, Velez MC, Leiva L, Sorensen R, Wulffraat N, Blanche S, Bushman FD, Fischer A, Cavazzana-Calvo M. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*. 2008; 118:3132–3142. [PubMed: 18688285]
18. Hematti P, Hong BK, Ferguson C, Adler R, Hanawa H, Sellers S, Holt IE, Eckfeldt CE, Sharma Y, Schmidt M, von Kalle C, Persons DA, Billings EM, Verfaillie CM, Nienhuis AW, Wolfsberg TG, Dunbar CE, Calmels B. Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol*. 2004; 2:e423. [PubMed: 15550989]
19. Bushman F, Lewinski M, Ciuffi A, Barr S, Leipzig J, Hannenhalli S, Hoffmann C. Genome-wide analysis of retroviral DNA integration. *Nat Rev Microbiol*. 2005; 3:3848–58.
20. Schmidt M, Glimm H, Wissler M, Hoffmann G, Olsson K, Sellers S, Carbonaro D, Tisdale JF, Leurs C, Hanenberg H, Dunbar CE, Kiem HP, Karlsson S, Kohn DB, Williams D, Von Kalle C. Efficient characterization of retro-, lenti- and foamy vector-transduced cell populations by high-accuracy insertion site sequencing. *Ann N Y Acad Sci*. 2003; 996:112–21. [PubMed: 12799289]
21. Wu X, Li Y, Crise B, Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. *Science*. 2003; 300:1749–51. [PubMed: 12805549]
22. Cattoglio C, Facchini G, Sartori D, Antonelli A, Miccio A, Cassani B, Schmidt M, von Kalle C, Howe S, Thrasher AJ, Aiuti A, Ferrari G, Recchia A, Mavilio F. Hot spots of retroviral integration in human CD34+ hematopoietic cells. *Blood*. 2007; 110:1770–8. [PubMed: 17507662]
23. Levine BL, Humeau LM, Boyer J, MacGregor RR, Rebello T, Lu X, Binder GK, Slepishkin V, Lemiale F, Mascola JR, Bushman FD, Dropulic B, June CH. Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc Natl Acad Sci U S A*. 2006; 103:17372–7. [PubMed: 17090675]

24. Wang GP, Levine BL, Binder GK, Berry CC, Malani N, McGarrity G, Tebas P, June CH, Bushman FD. Analysis of lentiviral vector integration in HIV+ study subjects receiving autologous infusions of gene modified CD4+ T cells. *Mol Ther*. 2009; 17:844–50. [PubMed: 19259065]
25. Hayakawa J, Washington K, Uchida N, Phang O, Kang EM, Hsieh MM, Tisdale JF. Long-term vector integration site analysis following retroviral mediated gene transfer to hematopoietic stem cells for the treatment of HIV infection. *PLoS One*. 2009; 4:e4211. [PubMed: 19148292]
26. Iwakuma T, Cui Y, Chang LJ. Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virology*. 1999; 261:120–32. [PubMed: 10441560]
27. Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol*. 1998; 72:8150–7. [PubMed: 9733856]
28. Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C, Naldini L. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol*. 2006; 24:687–96. [PubMed: 16732270]
29. Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, Benedicenti F, Sergi LS, Ambrosi A, Ponzoni M, Doglioni C, Di Serio C, von Kalle C, Naldini L. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J Clin Invest*. 2009; 119:964–75. [PubMed: 19307726]
30. Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science*. 1999; 283:682–686. [PubMed: 9924027]
31. van der Loo JC, Liu BL, Goldman AI, Buckley SM, Chrudimsky KS. Optimization of gene transfer into primitive human hematopoietic cells of granulocyte-colony stimulating factor-mobilized peripheral blood using low-dose cytokines and comparison of a gibbon ape leukemia virus versus an RD114-pseudotyped retroviral vector. *Hum Gene Ther*. 2002; 13:1317–1330. [PubMed: 12162814]
32. McKenzie JL, Gan OI, Doedens M, Dick JE. Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of CD122+ cells. *Blood*. 2005; 106:1259–61. [PubMed: 15878972]
33. Guenechea G, Gan OI, Inamitsu T, Dorrell C, Pereira DS, Kelly M, Naldini L, Dick JE. Transduction of human CD34+ CD38- bone marrow and cord blood-derived SCID-repopulating cells with third-generation lentiviral vectors. *Mol Ther*. 2000; 1:566–73. [PubMed: 10933981]
34. Modlich U, Bohne J, Schmidt M, von Kalle C, Knöss S, Schambach A, Baum C. Cell-culture assays reveal the importance of retroviral vector design for insertional genotoxicity. *Blood*. 2006; 108:2545–53. [PubMed: 16825499]
35. Modlich U, Navarro S, Zychlinski D, Maetzig T, Knoess S, Brugman MH, Schambach A, Charrier S, Galy A, Thrasher AJ, Bueren J, Baum C. Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. *Mol Ther*. 2009; 17:1919–28. [PubMed: 19672245]
36. Bauer G, Dao MA, Case SS, Meyerrose T, Wirthlin L, Zhou P, Wang X, Herrbrich P, Arevalo J, Csik S, Skelton DC, Walker J, Pepper K, Kohn DB, Nolta JA. In vivo biosafety model to assess the risk of adverse events from retroviral and lentiviral vectors. *Mol Ther*. 2008; 16:1308–15. [PubMed: 18461052]
37. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, Vidaud M, Abel U, Dal-Cortivo L, Caccavelli L, Mahlaoui N, Kiermer V, Mittelstaedt D, Bellesme C, Lahlou N, Lefrère F, Blanche S, Audit M, Payen E, Leboulch P, l'Homme B, Bougnères P, Von Kalle C, Fischer A, Cavazzana-Calvo M, Aubourg P. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009; 326:818–23. [PubMed: 19892975]
38. Kustikova OS, Schiedlmeier B, Brugman MH, Stahlhut M, Bartels S, Li Z, Baum C. Cell-intrinsic and vector-related properties cooperate to determine the incidence and consequences of insertional mutagenesis. *Mol Ther*. 2009; 17:1537–47. [PubMed: 19532134]
39. Cassani B, Montini E, Maruggi G, Ambrosi A, Miolo M, Selleri S, Biral E, Frugnoli I, Hernandez-Trujillo V, Di Serio C, Roncarolo MG, Naldini L, Mavilio F, Aiuti A. Integration of

- retroviral vectors induces minor changes in the transcriptional activity of T cells from ADA-SCID patients treated with gene therapy. *Blood*. 2009; 114:3546–56. [PubMed: 19652199]
40. Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, Glimm H, Kühlcke K, Schilz A, Kunkel H, Naundorf S, Deichmann A, Fischer M, Ball C, Pilz I, Dunbar C, Du Y, Jenkins NA, Copeland NG, Lüthi U, Hassan M, Thrasher AJ, Hoelzer D, von Kalle C, Seger R, Grez M. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med*. 2006; 12:401–9. [PubMed: 16582916]
 41. Kang EM, Choi U, Theobald N, Linton G, Long Priel DA, Kuhns D, Malech HL. Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long-term correction of oxidase activity in peripheral blood neutrophils. *Blood*. 2010; 115:783–91. [PubMed: 19965657]
 42. Bank A, Dorazio R, Leboulch P. A phase I/II clinical trial of beta-globin gene therapy for beta-thalassemia. *Ann N Y Acad Sci*. 2005; 1054:308–16. [PubMed: 16339679]
 43. Kohn DB, Sadelain M, Dunbar C, Bodine D, Kiem HP, Candotti F, et al. American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells. *Mol Ther*. 2003; 8:180–187. [PubMed: 12907140]
 44. Porada CD, Park PJ, Almeida-Porada G, Liu W, Ozturk F, Glimp HA, Zanjani ED. Gestational age of recipient determines pattern and level of transgene expression following in utero retroviral gene transfer. *Mol Ther*. 2005; 11:284–93. [PubMed: 15668140]
 45. Tuddenham EG. Gene therapy for hemophilia? Gene therapy for hemophilia is both desirable and achievable in the near future. *J Thromb Haemost*. 2005; 3:1314. [PubMed: 15946224]
 46. Evans GL, Morgan RA. Genetic induction of immune tolerance to human clotting factor VIII in a mouse model for hemophilia A. *Proc Natl Acad Sci USA*. 1998; 95:5734–39. [PubMed: 9576953]
 47. Moayeri M, Hawley TS, Hawley RG. Correction of murine hemophilia A by hematopoietic stem cell gene therapy. *Mol Ther*. 2005; 12:1034–42. [PubMed: 16226058]
 48. Moayeri M, Ramezani A, Morgan RA, Hawley TS, Hawley RG. Sustained phenotypic correction of hemophilia A mice following oncoretroviral-mediated expression of a bioengineered human factor VIII gene in long-term hematopoietic repopulating cells. *Mol Ther*. 2004; 10:892–902. [PubMed: 15509507]
 49. Miao HZ, Sirachainan N, Palmer L, Kucab P, Cunningham MA, Kaufman RJ, Pipe SW. Bioengineering of coagulation factor VIII for improved secretion. *Blood*. 2004; 103:3412–19. [PubMed: 14726380]
 50. Swaroop M, Moussalli M, Pipe SW, Kaufman RJ. Mutagenesis of a potential immunoglobulin-binding protein-binding site enhances secretion of coagulation factor VIII. *J Biol Chem*. 1997; 272:24121–24. [PubMed: 9305856]
 51. Ramezani A, Hawley RG. Correction of murine hemophilia A following nonmyeloablative transplantation of hematopoietic stem cells engineered to encode an enhanced human factor VIII variant using a safety-augmented retroviral vector. *Blood*. 2009; 114:526–34. [PubMed: 19470695]
 52. Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. High level expression of recombinant porcine coagulation factor VIII. *J Biol Chem*. 2002; 277:38345–49. [PubMed: 12138172]
 53. Doering CB, Healey JH, Parker ET, Barrow RT, Lollar P. Identification of porcine coagulation factor VIII domains responsible for high level expression via enhanced secretion. *J Biol Chem*. 2004; 279:6546–52. [PubMed: 14660593]
 54. Dooriss KL, Denning G, Gangadharan B, Javazon EH, McCarty DA, Spencer HT, Doering CB. Comparison of Factor VIII Transgenes Bioengineered for Improved Expression in Gene Therapy of Hemophilia A. *Hum Gene Ther*. 2009; 20:465–78. [PubMed: 19222367]
 55. Gangadharan B, Parker ET, Ide LM, Spencer HT, Doering CB. High-level expression of porcine factor VIII from genetically modified bone marrow-derived stem cells. *Blood*. 2006; 107:3859–64. [PubMed: 16449528]
 56. Ide LM, Gangadharan B, Chiang KY, Doering CB, Spencer HT. Hematopoietic stem-cell gene therapy of hemophilia A incorporating a porcine factor VIII transgene and nonmyeloablative conditioning regimens. *Blood*. 2007; 110:2855–63. [PubMed: 17569821]

57. Doering CB, Gangadharan B, Dukart H, Spencer HT. Hematopoietic Stem Cells Encoding Porcine Factor VIII Induce Pro-coagulant Activity in Hemophilia A Mice with Pre-existing Factor VIII Immunity. *Mol Ther.* 2007; 15:1093–9. [PubMed: 17387335]
58. Doering CB, Denning G, Dooriss K, Gangadharan B, Johnston JM, Kerstann KW, McCarty DA, Spencer HT. Directed Engineering of a High-expression Chimeric Transgene as a Strategy for Gene Therapy of Hemophilia A. *Mol Ther.* 2009; 17:1145–54. [PubMed: 19259064]
59. Malhotra JD, Miao H, Zhang K, Wolfson A, Pennathur S, Pipe SW, Kaufman RJ. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc Natl Acad Sci U S A.* 2008; 105:18525–30. [PubMed: 19011102]
60. Ohmori T, Mimuro J, Takano K, Madoiwa S, Kashiwakura Y, Ishiwata A, Niimura M, Mitomo K, Tabata T, Hasegawa M, Ozawa K, Sakata Y. Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein I α promoter: in vivo model for platelet-targeting gene therapy. *Faseb J.* 2006; 20:1522–4. [PubMed: 16723382]
61. Yarovoi HV, Kufrin D, Eskin DE, Thornton M, Haberichter S, Shi Q, Zhu H, Camire R, Fakharzadeh S, Kowalska M, Wilcox D, Sachais B, Montgomery R, Poncz M. Factor VIII ectopically expressed in platelets: efficacy in hemophilia A treatment. *Blood.* 2003; 102:4006–13. [PubMed: 12881300]
62. Yarovoi H, Nurden AT, Montgomery R, Nurden P, Poncz M. Intracellular interaction of von Willebrand factor and factor VIII depends on cellular context: lessons from platelet-expressed factor VIII. *Blood.* 2005; 105:4674–76. [PubMed: 15731176]
63. Shi Q, Wilcox DA, Fahs SA, Weiler H, Wells C, Cooley B, Desai D, Morateck P, Gorski, Montgomery R. Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J Clin Invest.* 2006; 116:1974–82. [PubMed: 16823491]
64. Gewirtz J, Thornton MA, Rauova L, Poncz M. Platelet-delivered factor VIII provides limited resistance to anti-factor VIII inhibitors. *J Thromb Haemost.* 2008; 6:1160–1166. [PubMed: 18433455]
65. Laurian Y. Future perspective for the treatment of hemophilia A. *J Thromb Haemost.* 2005; 3:1320–1321. [PubMed: 15946228]
66. Brettler DB. Gene therapy for hemophilia? *J Thromb Haemost.* 2005; 3:1317–19. [PubMed: 15946227]
67. Hoots WK. Gene therapy for hemophilia? *J Thromb Haemost.* 2005; 3:1316–17. [PubMed: 15946226]
68. DiMichele D. Gene therapy for hemophilia? The debate reframed. *J Thromb Haemost.* 2005; 3:1315–16. [PubMed: 15946225]
69. Doering CB, Spencer HT. Advancements in Gene Transfer-based Therapy for Hemophilia A. *Expert Rev Hematol.* 2009; 2:673–683. [PubMed: 20577574]
70. Srivastava A. Gene therapy for hemophilia? *J Thromb Haemost.* 2005; 3:1313. [PubMed: 15946223]
71. Hough C, Lillicrap D. Gene therapy for hemophilia: an imperative to succeed. *J Thromb Haemost.* 2005; 3:1195–1205. [PubMed: 15946210]
72. Negrier C. Gene therapy for hemophilia? Yes. *J Thromb Haemost.* 2004; 2:1234–5. [PubMed: 15304020]
73. Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature.* 1988; 336:688–690. [PubMed: 3143917]
74. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998; 282:1145–1147. [PubMed: 9804556]
75. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006; 126:663–676. [PubMed: 16904174]
76. Okita K, Yamanaka S. Induction of pluripotency by defined factors. *Exp Cell Res.* 2010
77. Kiskinis E, Eggan K. Progress toward the clinical application of patient-specific pluripotent stem cells. *J Clin Invest.* 2010; 120:51–59. [PubMed: 20051636]

78. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature*. 2009; 458:771–775. [PubMed: 19252477]
79. Ichida JK, Blanchard J, Lam K, Son EY, Chung JE, Egli D, Loh KM, Carter AC, Di Giorgio FP, Koszka K, Huangfu D, Akutsu H, Liu DR, Rubin LL, Eggan K. A small-molecule inhibitor of tgfbeta signaling replaces sox2 in reprogramming by inducing nanog. *Cell Stem Cell*. 2009; 5:491–503. [PubMed: 19818703]
80. Eminli S, Foudi A, Stadtfeld M, Maherali N, Ahfeldt T, Mostoslavsky G, Hock H, Hochedlinger K. Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. *Nat Genet*. 2009; 41:968–976. [PubMed: 19668214]
81. Kunisato A, Wakatsuki M, Shinba H, Ota T, Ishida I, Nagao K. Direct generation of induced pluripotent stem cells from human non-mobilized blood. *Stem Cells Dev*. 2010
82. Loh YH, Agarwal S, Park IH, Urbach A, Huo H, Heffner GC, Kim K, Miller JD, Ng K, Daley GQ. Generation of induced pluripotent stem cells from human blood. *Blood*. 2009; 113:5476–5479. [PubMed: 19299331]
83. Ye Z, Zhan H, Mali P, Dowey S, Williams DM, Jang YY, Dang CV, Spivak JL, Moliterno AR, Cheng L. Human-induced pluripotent stem cells from blood cells of healthy donors and patients with acquired blood disorders. *Blood*. 2009; 114:5473–5480. [PubMed: 19797525]
84. Giorgetti A, Montserrat N, Aasen T, Gonzalez F, Rodriguez-Piza I, Vassena R, Raya A, Boue S, Barrero MJ, Corbella BA, Torrabadella M, Veiga A, Izpisua Belmonte JC. Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. *Cell Stem Cell*. 2009; 5:353–357. [PubMed: 19796614]
85. Takenaka C, Nishishita N, Takada N, Jakt LM, Kawamata S. Effective generation of iPS cells from CD34+ cord blood cells by inhibition of p53. *Exp Hematol*. 2010; 38:154–162. [PubMed: 19922768]
86. Xu D, Alipio Z, Fink LM, Adcock DM, Yang J, Ward DC, Ma Y. Phenotypic correction of murine hemophilia A using an iPS cell-based therapy. *Proc Natl Acad Sci U S A*. 2009; 106:808–813. [PubMed: 19139414]
87. MacMillan ML, Wagner JE. Haematopoietic cell transplantation for Fanconi anaemia - when and how? *Br J Haematol*. 2010; 149:14–21. [PubMed: 20136826]
88. Rio P, Meza NW, Gonzalez-Murillo A, Navarro S, Alvarez L, Surrallés J, Castella M, Guenechea G, Segovia JC, Hanenberg H, Bueren JA. In vivo proliferation advantage of genetically corrected hematopoietic stem cells in a mouse model of Fanconi anemia FA-D1. *Blood*. 2008; 112:4853–4861. [PubMed: 18812474]
89. Raya A, Rodriguez-Piza I, Guenechea G, Vassena R, Navarro S, Barrero MJ, Consiglio A, Castella M, Rio P, Sleep E, Gonzalez F, Tiscornia G, Garreta E, Aasen T, Veiga A, Verma IM, Surrallés J, Bueren J, Izpisua Belmonte JC. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature*. 2009; 460:53–59. [PubMed: 19483674]
90. Smiers FJ, Krishnamurti L, Lucarelli G. Hematopoietic stem cell transplantation for hemoglobinopathies: current practice and emerging trends. *Pediatr Clin North Am*. 2010; 57:181–205. [PubMed: 20307718]
91. May C, Rivella S, Callegari J, Heller G, Gaensler KM, Luzzatto L, Sadelain M. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature*. 2000; 406:82–86. [PubMed: 10894546]
92. Imren S, Payen E, Westerman KA, Pawliuk R, Fabry ME, Eaves CJ, Cavilla B, Wadsworth LD, Beuzard Y, Bouhassira EE, Russell R, London IM, Nagel RL, Leboulch P, Humphries RK. Permanent and panerythroid correction of murine beta thalassemia by multiple lentiviral integration in hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 2002; 99:14380–14385. [PubMed: 12391330]
93. May C, Rivella S, Chadburn A, Sadelain M. Successful treatment of murine beta-thalassemia intermedia by transfer of the human beta-globin gene. *Blood*. 2002; 99:1902–1908. [PubMed: 11877258]

94. Rivella S, May C, Chadburn A, Riviere I, Sadelain M. A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human beta-globin gene transfer. *Blood*. 2003; 101:2932–2939. [PubMed: 12480689]
95. Hanawa H, Hargrove PW, Kepes S, Srivastava DK, Nienhuis AW, Persons DA. Extended beta-globin locus control region elements promote consistent therapeutic expression of a gamma-globin lentiviral vector in murine beta-thalassemia. *Blood*. 2004; 104:2281–2290. [PubMed: 15198957]
96. Puthenveetil G, Scholes J, Carbonell D, Qureshi N, Xia P, Zeng L, Li S, Yu Y, Hiti AL, Yee JK, Malik P. Successful correction of the human beta-thalassemia major phenotype using a lentiviral vector. *Blood*. 2004; 104:3445–3453. [PubMed: 15292064]
97. Ye L, Chang JC, Lin C, Sun X, Yu J, Kan YW. Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases. *Proc Natl Acad Sci U S A*. 2009; 106:9826–9830. [PubMed: 19482945]
98. Giardine B, van Baal S, Kaimakis P, Riemer C, Miller W, Samara M, Kollia P, Anagnou NP, Chui DH, Wajcman H, Hardison RC, Patrinos GP. HbVar database of human hemoglobin variants and thalassemia mutations: 2007 update. *Hum Mutat*. 2007; 28:206. [PubMed: 17221864]
99. Ingram VM. Gene mutations in human haemoglobin: the chemical difference between normal and sickle cell haemoglobin. *Nature*. 1957; 180:326–328. [PubMed: 13464827]
100. Levasseur DN, Ryan TM, Pawlik KM, Townes TM. Correction of a mouse model of sickle cell disease: lentiviral/antisickling beta-globin gene transduction of unmobilized, purified hematopoietic stem cells. *Blood*. 2003; 102:4312–4319. [PubMed: 12933581]
101. Pawliuk R, Westerman KA, Fabry ME, Payen E, Tighe R, Bouhassira EE, Acharya SA, Ellis J, London IM, Eaves CJ, Humphries RK, Beuzard Y, Nagel RL, Leboulch P. Correction of sickle cell disease in transgenic mouse models by gene therapy. *Science*. 2001; 294:2368–2371. [PubMed: 11743206]
102. Perrine RP, Pembrey ME, John P, Perrine S, Shoup F. Natural history of sickle cell anemia in Saudi Arabs. A study of 270 subjects. *Ann Intern Med*. 1978; 88:1–6. [PubMed: 619731]
103. Powars DR, Chan L, Schroeder WA. The influence of fetal hemoglobin on the clinical expression of sickle cell anemia. *Ann N Y Acad Sci*. 1989; 565:262–278. [PubMed: 2476064]
104. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, McMahon RP, Bonds DR. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N Engl J Med*. 1995; 332:1317–1322. [PubMed: 7715639]
105. Pestina TI, Hargrove PW, Jay D, Gray JT, Boyd KM, Persons DA. Correction of murine sickle cell disease using gamma-globin lentiviral vectors to mediate high-level expression of fetal hemoglobin. *Mol Ther*. 2009; 17:245–252. [PubMed: 19050697]
106. Wu LC, Sun CW, Ryan TM, Pawlik KM, Ren J, Townes TM. Correction of sickle cell disease by homologous recombination in embryonic stem cells. *Blood*. 2006; 108:1183–1188. [PubMed: 16638928]
107. Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science*. 2007; 318:1920–1923. [PubMed: 18063756]

Figure 1. Schematic showing the integration of three viral vectors into the genomic DNA of a target cell

The first vector, a MLV-based vector, integrates near the promoter of gene 1. Potential results of integrating near or within promoter sequences is the dysregulation of the downstream gene. In addition, the enhancer activity of the MLV promoter can activate nearby genes (within ~300 kb), such as gene 2 in the Figure. The second vector, an HIV-based vector, also integrates into gene 1, but instead of targeting the promoter region, recombinant HIV vectors integrate within the gene, typically downstream of the promoter. This integration pattern can disrupt expression of the gene in which the vector integrates, eg gene 1, and similar to the MLV vector nearby genes can also be disrupted, such as gene 2 within the Figure. The third vector, an HIV-SIN-based vector, integrates within a gene sequence, similar to HIV vectors, and will potentially disrupt expression of the targeted gene, which is gene 3 in the Figure. Because the viral LTR promoter sequences are inactivated expression of nearby genes, such as gene 4 in the Figure, are not affected.