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Engineered Hematopoietic Stem Cells as Therapeutics for Hemophilia A

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Introduction

Hematopoietic stem cells (HSCs) are the most routinely transplanted adult stem cell. Currently, they are utilized for the treatment of several genetic and acquired diseases including blood cancers, autoimmune disorders, and hematopoietic defects. HSCs are ideal candidates for gene therapy applications because they possess the capacity for self-replication and functionality to propagate the entire hematopoietic lineage, thus facilitating amplification of genetically-modified cells and expression of a transgene product from a multitude of hematopoietic cell types. An additional advantage is the tolerogenic effect HSCs have on host immunity, which in many contexts is a barrier to successful gene therapy. Numerous HSC-targeted gene therapy studies have been conducted in a range of disease settings. Current pre-clinical research for HSC transplantation gene therapy of hemophilia A therapy is focused on i) identification of safe and efficient methods of nucleic acid transfer into HSCs, ii) optimization of the coagulation factor VIII transgene for high expression, iii) minimization of conditioning regimen-related toxicity with HSC engraftment and iv) overcoming complications due to pre-existing factor VIII immunity. Herein, we review the state of the art in HSC transplantation gene therapy of hemophilia A.

Clinical Gene Therapy of Hemophilia A

Loss of circulating factor VIII (fVIII) activity due to mutations within the fVIII gene results in the X-linked, recessive bleeding disorder hemophilia A. The clinical presentation is a mild to severe bleeding phenotype that correlates with the patient's residual plasma fVIII activity level. Hemophilia A has been targeted by numerous academic and commercial entities as a prime candidate for gene transfer-based therapies for several reasons. First, modest increases in fVIII levels (≥ 1% of normal levels) can alleviate spontaneous bleeding episodes. Second, many different cell types are capable of synthesizing functional fVIII protein and virtually any tissue or cell type with access to the bloodstream can be targeted for gene transfer. Third, gene therapy should be more economical and less invasive than protein replacement therapy given that it would consist of limited (possibly only one) treatment events. There have been 3 phase 1 clinical trials of gene therapy for hemophilia A conducted to date and each employed a different gene-transfer strategy (for review see Doering and Spencer, 2010 [1]). The first trial, sponsored by Transkaryotic Therapies, Inc., involved *ex vivo* gene modification of autologous dermal fibroblasts and transplantation into the greater or lesser omentum of twelve male patients [2]. Although no severe adverse events were observed in this trial, designed to assess safety, sustained fVIII levels above 1% of normal were not achieved. In a second study, sponsored by Chiron Corporation, retroviral particles containing a human B-domain deleted (BDD) fVIII transgene were introduced into thirteen male hemophilia A patients via peripheral vein infusion [3]. Again, fVIII levels above 1% of normal were not maintained and the trial was halted. The third trial, sponsored by GenStar Therapeutics, Inc., consisted of a single patient being infused with high-capacity adenoviral particles containing the full-length human fVIII cDNA. Following administration of viral vector, the patient developed transient chills, fever, back pain, and headaches preceding the onset of thrombocytopenia and transaminitis. This patient did achieve fVIII levels >1% of normal that were maintained for several months, but as predicted based on the non-integrating property of adenoviruses, the fVIII activity eventually declined. The trial was halted due to the significant side effects observed. In summary, not only have there been no milestones of success in previous trials, to our knowledge, there are no approved or ongoing clinical trials utilizing gene transfer to treat hemophilia A.

Clinical Hematopoietic Stem Cell (HSC) Therapy

Hematopoietic stem cells first were discovered in the late 1940's as a result of the finding that spleen cells could protect mice from exposure to lethal doses of radiation [4,5]. A comprehensive review of the history of HSC transplantation (HSCT) has been documented by E. D. Thomas, recipient of the Nobel Prize in Physiology or Medicine in 1990 for his pioneering work in this field [6]. Subsequently, HSCs have been implemented in the treatment of several genetic and acquired diseases including leukemia, non-Hodgkin's lymphoma, aplastic anemia, and sickle-cell disease. Annually, more than 20,000 clinical HSCTs are performed. The ability of HSCs to reconstitute all cellular hematopoietic lineages, including myeloid, lymphoid, and erythroid populations through a combination of self-renewal and cellular differentiation endows them with unique clinical utility. Engrafted HSCs are capable of contributing to hematopoiesis for the duration of the patient's life. HSCs are harvested routinely from bone marrow aspirates or peripheral blood since they can be mobilized into the bloodstream using granulocyte-macrophage colony-stimulating factor. For the purpose of gene transfer-based therapies, HSCs can be manipulated successfully *ex vivo*, allowing for the implementation of safety parameters prior to transplantation and subsequent engraftment.

In 1953, Medawar and colleagues made the fundamental discovery that immune tolerance to allogeneic donor cells could be achieved in mice *in utero* [7]. The result of a successful HSC allotransplant is two genetically-distinct sources of hematopoietic cells, referred to as a state of mixed cellular chimerism, where immunotolerance to the foreign antigens has been established not only for hematopoietic cells, but for any other cell or tissue type derived from the host or donor. For example, acceptance of donor skin grafts often is used to demonstrate this phenomenon. However, with any allogeneic transplantation, there exists significant risk that the donor cells, typically harboring HLA

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mismatches, will identify host tissue as foreign, triggering a cytotoxic immune response clinically described as graft versus host disease (GvHD). GvHD is the major side effect of HSC transplantation and carries with it significant morbidity and mortality risk. Therefore, to avoid the risk of GvHD, the majority of HSCT gene therapy studies have utilized autologous cells. In this setting, the only antigen disparity is that of the transgene product, e.g. factor VIII (fVIII) in hemophilia A gene therapy. As is discussed herein, the immunotolerogenic nature of HSCT is a major benefit to gene therapy of hemophilia A, where the transgene product, fVIII, is a known potent immunogen (for review of fVIII immunogenicity, see [8]).

Strategies for Genetic-Modification of HSCs

Since the emergence of gene-transfer studies in the early 90's, HSCs have been targeted to restore therapeutic levels of certain proteins, the expression of which is affected by deleterious genetic mutations. In addition to the tolerogenic potential of HSCs, targeting this population allows for indefinite expression of a desired protein product from multiple terminally differentiated cell lineages. Several methods have been demonstrated to transfer functional genetic material or to correct existing genetic defects within HSCs, and are reviewed herein (Table 1). Current methods for nucleic acid transfer include viral, chemical, and physical techniques. Viral vectors are the gold standard of genetic transfer into HSCs. Each viral vector system has different integration capabilities, as well as required components for nuclear uptake and expression. Choice of viral vector system is dependent on the transgene, transduction efficiency, and safety requirements. Common viral vector systems are based on retroviruses, lentiviruses, adenoviruses, adeno-associated viruses, and the herpes simplex virus. In addition to viral strategies, cationic lipids and other non-viral synthetic macromolecules have been used widely for cellular introduction of DNA [9]; however, to date, non-viral-based methods targeting HSCs show low potential as a therapeutic approach [10,11]. Physical methods include particle-mediated transfection, electroporation, and hydrodynamics-based transfection. While very little research has been conducted on HSCs using particle-mediated transfections, electroporation has been used successfully to introduce genetic material into hematopoietic progenitors and their progeny [12-14].

The earliest proof-of-concept HSC gene-transfer studies utilized recombinant γ-retroviral vectors derived from murine leukemia viruses (MLV) and produced in recombinant form by transient transfection of NIH3T3 or HEK293T cells with viral packaging and expression plasmids [15,16]. Packaging cells provide the accessory protein components required for the biosynthesis of non-replication competent retroviral vectors. Pre-clinical studies using murine models demonstrated the versatility of this vector system to transfer nearly any transgene into a variety of cell types [17-19]. Soon after, however, it was shown that γ-retroviral vector systems could not transduce HSCs as efficiently as other rapidly dividing cell types. Subsequently, it was shown that γ-retroviral vectors require cellular division within the target cell to facilitate nuclear translocation of the pre-integration complex and integration of the transgene [20,21]. Due to the requirement of cell division and the relatively infrequent replication events of HSCs, protocols incorporating cytokine cocktails were developed and shown to bolster the transduction efficiency of murine HSCs [22-26].

To circumvent the need for cellular division, in 1996, Naldini and colleagues reported successful gene transfer in cell cycle arrested fibroblasts using a recombinant HIV-based lentiviral vector system [27]. Fibroblasts in G_1-S, G_2, and to a lesser degree, G_0 cell cycle stages showed increased transduction of firefly luciferase and β-galactosidase reporter genes compared to MLV-based vectors. Furthermore, this vector system was shown to transduce terminally differentiated neuronal cells *in vivo* following direct injection into the rat corpus striatum and hippocampus. This was the first study to demonstrate successful gene transfer into non-replicating cells using a retroviral vector. Although data support the ability of lentiviruses to transduce non-dividing cells, it generally is recognized that dividing cells are transduced more efficiently. A comparative study of transduction efficiencies between MLV-based retroviral vectors and HIV-1 based lentiviral vectors in $CD34^+$ cells revealed the superiority of HIV vector integration into quiescent hematopoietic progenitors [28]. Subsequently, lentiviral vectors have been shown to transduce numerous non-dividing cell types including lung epithelial cells [29], neuronal cells [30,31], and primary hepatocytes [32]. The ability of lentiviral vectors to infect quiescent cells is reliant upon two proteins: *gag* (matrix protein) and Vpr, although Vpr has been deleted in third and fourth generation lentiviral vectors without severe loss of infectivity. These proteins activate nuclear import machinery facilitating transport of the pre-integration complex through the nucleopore, decreasing the need for mitosis-induced nuclear permeability [33,34]. With respect to the targeting of HSCs, recombinant retroviral vectors, and most recently lentiviral vectors, have been the system of choice for proof-of-concept studies in a variety of animal models.

Table 1:

Technologies for HSCT Gene Therapy	Advantages	Disadvantages
γ-Retroviral Vectors	• Ability to target many cell types • Long-term expression due to integration • Increased safety due to SIN development	• Requirement of cellular division • Necessity of cytokine cocktails to stimulate HSC cycling • Insertional mutagenesis potential • Complex manufacturing
Lentiviral Vectors	• Wide range of cell targets • Long-term expression due to integration • Increased safety due to SIN development	• Require multiple plasmids/elements provided *in trans* for production • Risk of insertional mutagenesis • Complex manufacturing
***Sleeping Beauty* Transposon Systems (SBTS)**	• Low complexity • Simple manufacturing (plasmid DNA only) • Potentially reduced immunogenic response	• Lower-level expression of the transgene product • Random insertion pattern • Potential for secondary or tertiary transposition events
Zinc Finger Nucleases (ZFNs)	• Targeted gene correction or addition • Potential to utilize endogenous genetic control elements • Long-term expression through chromosomal integration	• Safety remains undetermined • Risk of off-target mutagenesis • Require additional means of cellular entry • Limited sequence targeting potential
Peptide Nucleic Acids (PNAs)	• Site-specific modification • Useful in gene silencing • In vivo delivery and functionality possible	• Limited research to date • Risk of off-target sites of genetic modification • Low efficiency

In addition to recombinant retroviral vectors, other non-viral technologies are being investigated for genetic modification of HSCs. In eukaryotes, transposon-transposase systems create duplications or transpositions of certain genetic sequences throughout the genome. In humans, transposons have been evolutionarily silenced, but one has been reconstructed from the salmonoid fish genome as a potential gene transfer and therapy tool [35]. Sleeping Beauty transposons systems, named for their evolutionarily dormant phase, employ an enzyme, termed transposase, that catalyzes transposition events. For gene therapy applications, a transgene cassette contained within a donor plasmid serves as the substrate for transposase-mediated transposition into a host cell chromosome. Thus, the only two requirements for the Sleeping Beauty transposon system (SBTS) are i) a transposon containing the gene of interest, and ii) a source of transposase produced either *in cis* or *in trans*. The advantages lie in the simplicity of this design, as well as the reduced risk of immunogenic response [36]. SBTS have been validated in mouse models for the treatment of several genetic disorders, including hemophilia A where they have been shown to drive fVIII expression and phenotypic recovery for over 30 weeks [37-39]. However, as is the case for most SBTS applications, the target cells were not HSCs and expression levels were lower than is routinely achieved using γ-retro- and lentiviral vectors.

Modification of the transposons, and specifically the transposase itself, has led to increased transposition and transgene expression [40]. Recently, hyperactive transposases have been identified through directed evolution-based screening and have been shown to improve the *ex vivo* modification of human cord blood $CD34^+$ cells following electroporation. In that study, genetically-modified cells retained the capacity for differentiation into all hematopoietic cell lineages. However, the efficiency of genetic modification did not exceed 27% [41,42]. Despite significant progress, remaining hurdles for SBTS include i) low gene-transfer efficiency, which requires a method to introduce the transposon and transposase into the target cell, and ii) safety concerns surrounding insertional mutagenesis. Of note, SBTS do demonstrate a more random integration profile than retroviral vectors, which predominantly target promoter regions within gene loci [43,44]. Furthermore, the long-term stability of transposed sequences is not known and it is possible that secondary, tertiary, etc. transposition events could further increase the risk of insertional mutagenesis.

Currently, the majority of gene therapy research consists of exploring and developing gene addition techniques where a functional transgene is introduced into a cell to restore expression. However, several groups now have identified methods for the correction of defective genes *in situ*. There currently are several mechanisms by which gene correction is being pursued. By virtue of its nature, gene correction allows for usage of the endogenous promoter and other regulatory elements and obviates the risk of insertional mutagenesis. Two prominent technologies for gene correction in HSCs include zinc-finger nucleases (ZFNs) and peptide nucleic acid (PNA) conjugates. Currently, both ZFNs and PNA-conjugates are in clinical trials for HIV and cancer treatment, respectively.

Zinc-finger nucleases combine a zinc-finger domain capable of site-specific DNA binding with a non-specific restriction nuclease domain. Upon dimerization at specified nuclear DNA recognition sequences, double-stranded DNA breaks are created that serve as substrates for homology-directed DNA repair (HDR). During HDR, the cleaved 3' ends invade the sister chromatid and replicate using the intact strand as a template. In the case of X-linked diseases such as hemophilia A, HDR is not possible unless a homologous DNA template is provided *in trans*. Without a template, the cleaved DNA is subject to random mutagenic events, i.e. insertions and deletions in an attempt to ligate the cleavage and avoid apoptosis. However, cleavage in the presence of a DNA template containing homologous sequences surrounding the target gene sequence results in HDR directed replacement of the mutation with the corrected sequence now under endogenous promoter and enhancer control. As ZFN-mediated gene correction has been subjected to limited testing in humans, the safety concerns are relatively unknown. Identification of off-target ZFN activity has, however, been demonstrated in pre-clinical studies including a recent study demonstrating the use of ZFNs for *in vivo* treatment of a murine model of hemophilia B [45]. This study utilized adeno-associated viral (AAV) vector to deliver episomally expressed ZFNs and a homologous template encoding the factor IX (fIX) cDNA. The AAV vector transduced primary hepatocytes and the genetically-modified cells expressed fIX at 3-7% normal levels. However, the limited ability of AAV to target HSCs *in vivo* obviates the practicality of this strategy for HSC-directed gene therapy. Similar systems are being developed to target gene addition to specific sequences within the human genome. For example, it was recently shown that the CXCR5 and AAVS1 sites can be targeted for safe harbor gene addition using ZFNs that bind to and specifically cleave sites within these regions [46]. Introduction of donor DNA by an integrase defective lentiviral vector resulted in the introduction of genetic material at these specific and pre-determined sites.

Peptide nucleic acids (PNAs) are similar to ZFNs in that they also stimulate site-directed recombination using co-transfected donor DNA templates. PNAs are polymers containing purine and pyrimidine bases covalently attached to a repeating N-(2-aminoethyl)-glycine backbone. The uncharged backbone diminishes electrostatic repulsion from DNA, allowing for higher affinity binding to recognized sequences. As a result, PNAs can efficiently interrupt normal transcription processes and effectively knock out a gene product. There is ongoing research in exploring the use of PNAs as pharmacological transcription factor decoys to down regulate signaling and expression of oncogenic products [47]. In the case of genetic modification of HSCs, PNAs have only entered into investigation recently. Rogers and colleagues reported *in vivo* genomic modification of HSCs using PNA conjugates fused with a nuclear-localizing protein [48]. Current limitations to this method include the specificity of these PNA conjugates given that genomic modification of somatic tissues also was observed, as well as the low-level percentage of genetically-modified cells, which currently is less than 5%.

Clinical HSCT Gene Therapy

The first clinical gene therapy trials using HSCs revealed many of the safety concerns inherent with somatic cell genetic modification using recombinant retroviral vectors. Despite the pre-clinical and early clinical successes of retroviral based therapy for childhood X-linked severe combined immune deficiency (SCID), a 2002 report revealed the generation of T cell leukemia as a result of genetic-modification in one of the initial patients [49]. This trial was eventually suspended when 5 of 20 patients developed T cell acute lymphoblastic leukemia (ALL). As a result, a large effort was placed on understanding not only the mechanism of integration, but also the location and downstream effects of retroviral transduction. γ-retroviral vectors integrate into chromosomal sites that are actively transcribed, including potential proto-oncogenes, resulting in aberrant transcription patterns and expression [50-54]. To date, insertion-site and clonality analysis remains a top safety parameter for HSC targeted gene therapy.

The creation and implementation of self-inactivating (SIN)

viral vectors is predicted to alleviate some of the concern regarding integrating viral vectors. Deletions in the 3' LTR of retroviral vectors are transferred to the 5' proviral LTR resulting in transcriptionally inactive viral particles [55]. This is important because in lentiviruses the LTRs are promoters with strong enhancer capabilities. As has been demonstrated, the strong enhancers can induce the transcription of genes near the integrated proviral sequence [56]. This safeguard, however, requires the insertion of a new promoter into the vector sequence, such as the elongation factor-1 alpha or cytomegalovirus (CMV) promoters. Promoter selection enables control over which cells express the transgene product and at what level. Current pre-clinical research and clinical trials are exploring the use of SIN lentiviral vectors because of their lower genotoxic profile and increased predicted ability to modify HSCs *ex vivo* compared to the original γ-retroviral vectors [57-61]. Although proof-of-concept studies clearly have shown the benefits of using SIN-lentiviral vectors, it is possible that these vectors may have adverse effects on viral titer, engraftment potential, and/or transgene expression. Current clinical trials are designed to answer these specific questions, and the gene therapy field has made safety of viral integration a primary concern.

Recent advancements in the safety profile of γ-retroviral vectors have led to two successful clinical trials of HSCT gene therapy for adenosine deaminase (ADA) deficiency and X-linked SCID. In August 2011, Gaspar et al. inserted the ADA cDNA into a γ-retroviral vector pseudotyped with the gibbon-ape-leukemia-virus envelope [62,63]. Six children ceased enzyme replacement therapy prior to treatment and were conditioned with either 140mg/m^2 melphalan or 4mg/kg busulfan intravenously prior to autologous HSCT gene therapy. Four of six subjects recovered immune function and three subjects no longer required ADA or immunoglobulin replacement therapy. All patients survived and no leukemia or other adverse events were observed within the 24 to 84 month follow-up. ADA expression was sustained in all hematopoietic lineages resulting in restored metabolic function, and functional T cell levels were elevated and sustained over 5 years post gene therapy. This trial is now one of two studies to present long-term, safe therapy using genetically-modified HSCs for the treatment of a monogenic disease [64]. In the treatment of X-linked SCID, Gasper et al. also used a similar γ–retroviral vector encoding the common γc subunit of the interleukin 2 receptor (IL2RG) cDNA to transduce CD34$^+$ bone marrow cells stimulated with stem cell factor, thrombopoietin, interleukin-3, and Flt-3 ligand prior to transplantation [65]. Nonmyelosuppresive conditioning was used in this study and 10 of 10 patients showed elevated, functional polyclonal T cell populations over a 54-107 month follow-up. While most side effects were minimal and overcome with standardized course of action, one patient did develop T cell ALL due to up-regulation of the LMO-2 proto-oncogene. However, this patient maintained a polyclonal T cell population and currently is in remission. While the usage of γ-retroviral vectors has proven to be an effective and predominantly safe treatment option against ADA-deficiency SCID and X-linked SCID, the risk of oncogenic up-regulation is still a concern. ADA-deficiency SCID treatment has not, to date, resulted in the expansion of leukemic cells [66]. The emergence of T-ALL in X-linked SCID treatment but not ADA-SCID despite similar viral vector preparation methods suggests that the discrepancies in safety are the result of transduction methods, biology of the corrected cells, and/or differences in the two disease states.

Immunological Aspects of HSCT Gene Therapy

A major risk of allogeneic transplantation and even autologous transplants containing neo-antigens is that of immunological response and rejection. This issue is relevant to hemophilia A gene therapies because transplantation of HSCs expressing fVIII can induce an immune response to fVIII. Non-specific immunosupressants have shown success in reducing transplant rejection, however, they pose significant risk of subsequent infection. As a result, efforts to prevent specific inhibitor formation in gene therapy strategies have become high priority. Several studies have combined gene therapy approaches with immune tolerance strategies to prevent this inactivation response. HSCs remain ideal targets because the immune system is derived from these cells, and expression of transgenes within hematopoietic cells can induce immune tolerance.

Several labs have conducted HSCT gene therapy using mouse models to study immune tolerance through molecular chimerism. For example, it has been shown that retroviral gene transfer of the porcine glucosyltransferase UDP galactose gene, αGT, into naïve murine bone marrow cells induced tolerance to porcine xenografts containing the foreign epitope [67]. Furthermore, analysis of the B cell population revealed that specific antibody-producing cells against the xenograft were eliminated during immune reconstitution. In a subsequent study, retroviral gene transfer of the cDNA encoding H-2K^b, a murine major histocompatibility class I antigen, into HSCs resulted in long-term expression of the antigen in hematopoietic lineages. Furthermore, genetically-modified T cells facilitated tolerance to transplanted H-2K^b expressing targets, but retained the capacity to reject third-party grafts, suggesting that T cells are capable of induced tolerance via gene therapy [68]. Additionally, it was confirmed that this gene therapy approach induced negative selection of cells expressing the alloreactive T cell receptor in the thymus, and that thymic re-education is possible through genetic modification [69]. As described below, under certain conditions, expression of fVIII from genetically-modified HSCs induces long term tolerance in transplanted mice.

HSCT for the Treatment of Hemophilia A

Hemophilia meets several criteria for HSCT gene therapy. First, it is a monogenic X-linked disease caused by the deficiency of a single, essential blood coagulation factor. Second, fVIII functions in the circulation. Thus, hematopoietic cells are ideal vehicles for its delivery to the bloodstream. Third, mere picomolar concentrations of fVIII are sufficient to alleviate spontaneous bleeding episodes. Fourth, virtually all cell types tested have the capacity to biosynthesize fVIII, albeit at varying levels. Currently, there is no cure for hemophilia A and state of the art fVIII replacement therapy is cost prohibitive to the majority of people with the disease. Lastly, protein replacement therapy is plagued by complications arising from the necessary intravenous route of administration. The risk/benefit ratio of gene therapy as a treatment for hemophilia A has resulted in the consensus that gene therapy is the most promising therapeutic advance on the horizon [70].

Evans and Morgan conducted the first preclinical study of HSCT-based gene therapy of hemophilia A [71]. Using a murine leukemia virus-based γ-retroviral vector, the human fVIII cDNA was transferred to murine bone marrow cells, which subsequently were transplanted into hemophilia A mice that were pretreated with a lethal dose of total body irradiation (TBI). In this study, correction of the fVIII deficiency was not achieved, but it provided an early indication of the low level biosynthesis of human fVIII as a major barrier to the development of hemophilia A gene therapy applications. In 2002, Tonn et al. showed that there are hematopoietic lineage specific differentials in fVIII biosynthesis [72]. It was observed that erythroid and megakaryocytic cells secreted higher levels of B-domain deleted (BDD) human fVIII compared to lymphoblastoid or T cell leukemia cell lines. The first

demonstration of HSCT gene therapy based induction of therapeutic fVIII levels (generally accepted to be >5% or 0.05 U/ml fVIII activity) came in 2004 and 2005 by Hawley and colleagues [73,74]. Using a bicistronic γ-retroviral vector encoding the BDD human fVIII transgene as well as an EGFP reporter transgene, genetically-modified cells were selected prior to transplantation leading to higher level engraftment of genetically-modified cells. Recognizing the technical expression limitations in these studies, a greater effort was placed in engineering the fVIII transgene for higher level expression from hematopoietic as well as other cell types. Point mutagenesis of endoplasmic reticulum chaperone immunoglobulin-binding protein (BiP) recognition site, specifically at residue 309, increased expression 2 – 3 fold [75]. Further characterization and engineering led to the generation of constructs containing 6 additional N-linked glycans in the B-domain that were shown to further improve secretion [76]. Although it is predicted that these constructs with enhanced fVIII expression will benefit HSC-directed gene therapy, this has not been conclusively demonstrated in a head to head study against standard BDD human fVIII. In another study, Hawley and colleagues explored the use of a simian immunodeficiency vector system containing a B cell specific enhancer/promoter to drive fVIII expression [77]. Therapeutic levels of fVIII were induced and intracellular fVIII was detected in $B220^+$ B cells and $CD138^+$ plasma cells, but not in HSCs. Transplantation under non-myeloablative conditioning induced a minor immunological response after subsequent challenge with fVIII. By targeting B cells exclusively, tolerance to fVIII through molecular chimerism can be achieved. However, future studies are required to elucidate lineage-specific limitations in expression.

The benefits of using fVIII orthologs, however, have been well characterized. Characterization of porcine fVIII, has contributed greatly to overcoming the fVIII expression barrier. BDD porcine fVIII expresses at 10-100 fold greater levels than BDD human fVIII *in vitro* and *in vivo* [78-80]. The use of the porcine fVIII cDNA in gene therapy strategies was founded on the clinical history of plasma derived porcine fVIII in the treatment of acute bleeding episodes in persons with anti-human fVIII inhibitory antibodies. Of note, no species-based incompatibilities have been observed between porcine fVIII and the human blood coagulation components, including binding to von Willebrand factor. Currently, a recombinant BDD porcine fVIII product is undergoing late-stage clinical testing in inhibitor patients. Therefore, there is no technical obstacle to the utilization of the high expression property of porcine fVIII in gene therapy applications to overcome the fVIII expression barrier. Investigation into the mechanism responsible for the expression differential revealed that the dominant characteristic in high level expression is improved post-translational secretory efficiency [79]. In support of this mechanism, it was demonstrated that recombinant porcine fVIII induces the unfolded protein response (UPR) to a lesser extent than human fVIII, thus explaining the previously observed differential in post-translational secretory transport [81]. Additionally, it was shown that pharmacogenetic knockdown of GRP78/BiP, a master regulator of UPR, using shRNA technology increased human fVIII production, and overexpression of X-box-binding protein 1 (XBP1), another UPR regulator, resulted in increased production of both human and porcine fVIII. Therefore, we are beginning to understand the basic mechanisms governing human and orthologous fVIII biosynthesis.

Several studies have demonstrated the utility of high expression porcine fVIII sequences in gene transfer-based applications for hemophilia A. For example, the BDD porcine fVIII transgene was transferred into HSCs using recombinant murine stem cell viral vector (a γ-retrovirus-derived vector) and the transduced cells were transplanted into hemophilia A mice subjected to a lethal dose of TBI [82]. All experimental mice expressed circulating fVIII activity levels near or exceeding 100% normal human levels that were sustained for over 18 months after transplantation. Subsequently, reduced-intensity conditioning regimens were explored including sub lethal TBI (5.5 Gy), costimulation blockade (anti-CD40L and CTLA4-Ig), and a combination of busulfan and anti-thymocyte serum. Each of these regimens combined with HSCT gene therapy incorporating the BDD porcine fVIII transgene resulted in successful engraftment and sustained therapeutic fVIII expression in all hemophilia A mice [83]. Additionally, it was shown that mice engrafted under the reduced-intensity conditioning regimens did not elicit an immune response following challenges with human fVIII. Furthermore, T cells from these animals were not activated upon stimulation with porcine fVIII suggesting that immunologic tolerance to fVIII was induced as a result of the HSCT gene therapy [84]. From this, it can be concluded that T cell suppression is critical to successful engraftment of genetically-modified HSCs encoding f VIII.

Investigations into the mechanisms underlying fVIII production and secretion differentials within orthologous and bioengineered transgenes have been pursued concomitantly with their application in gene therapy strategies. The use of porcine and high-expression human/porcine (HP) hybrid constructs have reduced the gene transfer requirements in terms of the genetically-modified cell dose and proviral copy number, thus increasing the safety profiles of proposed gene therapy protocols. Additionally, through the use of HP constructs, it is possible to conserve the high expression characteristics of porcine fVIII within a predominantly human fVIII transgene. In one study, a HP construct containing a 9:1 ratio of human to porcine amino acid composition respectively, showed identical therapeutic performance to BDD porcine fVIII in the hemophilia A mouse HSCT gene therapy model [85]. These results are relevant and potentially critical to the design of future clinical gene therapy applications in light of the previous failures to achieve therapeutic fVIII expression levels in clinical trials.

Prior to approval for clinical testing in humans, a proposed gene therapy product must demonstrate pre-clinical safety and efficacy using multiple *in vivo* experimental systems. For hemophilia A, several model systems exist including the hemophilia A mouse model [86], canine model [87], and ovine model [88]. While characterization and experimentation with the ovine model is in the relatively early phases [89], the murine and canine models have been studied and utilized extensively in the pre-clinical development of novel hemophilia A therapeutics including gene-transfer based therapies (for review of the canine studies, see [90]). In the context of the broader application of HSCT gene therapy, most large animal studies have been performed using canine and non-human primate models (for review, see [91]). These models have been instrumental in the development of methods for stem cell harvest, *ex vivo* manipulation, and gene transfer as well as studying the biology of transplanted genetically-modified cells. Recently, Wilcox and colleagues demonstrated correction of canine Glanzmann thrombasthenia (GT), a rare platelet adhesion disorder, using HSCT gene therapy [92]. Affected dogs underwent autologous HSCT gene therapy incorporating a HIV-1-based lentiviral vector encoding a functional integrin αIIbβ3 gene. Post-transplantation analysis revealed approximately 5,000 αIIbβ3 receptors on 10% of platelets, resulting in improved bleeding times and reduced blood loss up to 5 years after treatment. However, as is the case for any model

system, there are limitations to its ability to mimic human testing. For example, many reagents used in HSCT and HSCT gene therapy, including cytokines, stem cell markers, gene transfer vectors and conditioning agents, display species specificity. Therefore, reagents and dosing schemes may not be translatable from preclinical to clinical studies. Currently, the most relevant preclinical test of a HSCT gene therapy product is the ability to genetically-modify human $CD34^+$ cells without significantly diminishing their engraftment and hematopoiesis potential. To examine this, murine (NOD.C*g*-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ, referred to as NSG mice) xeno transplantation models are the gold standard. Therefore, there is no consensus on the best path toward regulatory approval for HSCT gene therapy and what role, if any, large animal models of hemophilia A will play in this process.

HSCT Gene Therapy for Patients with Inhibitors

The development of neutralizing antibodies against fVIII represents the most challenging and costly complication in the treatment of hemophilia A. Inhibitors emerge in 20-30% of patients with severe hemophilia and typically prevent future treatment with human fVIII replacement products. Instead, patients are routinely treated using fVIII bypassing agents such as recombinant activated factor VII or activated coagulation factor concentrates or, historically, porcine fVIII product. Currently, a recombinant BDD porcine fVIII product is in phase 3 clinical testing. Unfortunately, each of these treatments has significant limitations, e.g. subsequent immunity to porcine fVIII, and are best utilized on an acute basis and not for lifelong prophylaxis. Therefore, inhibitor patients represent the most at risk hemophilia A population in the developed world and, therefore, can be considered prime candidates for novel experimental therapies such as HSCT gene therapy. However, due to the complexities of fVIII gene transfer and expression, historically, little attention has been devoted to this patient population.

Recently, it was demonstrated that the porcine fVIII transgene can be used to induce fVIII expression in mice with preexisting antibodies to human fVIII. To study the use of HSCT gene therapy for patients with inhibitors, humoral immunity to human fVIII is induced in mouse hemophilia A models by weekly intravenous administration of human fVIII over 4-6 weeks. Typically, significant anti-human fVIII titers are observed in all animals, and in one study it was shown that 10 – 14% of these antibodies have cross-reactivity to BDD porcine fVIII. Even in this unfavorable environment, using a gene transfer strategy incorporating porcine fVIII and myeloablative conditioning, complete correction of the fVIII deficiency (3.6 ± 1.3 U/ml) and eradication of the fVIII inhibitors ($t_{1/2}$ of 16 days) was observed (93). This was the first successful report of HSCT gene therapy in an anti-fVIII inhibitor model where fVIII activity was restored and the inhibitors were eliminated. Despite the inherent difficulty of treating hemophilia A patients with pre-existing inhibitors, these results demonstrate the feasibility of HSCT gene therapy in this high-risk disease setting.

Summary

The field of HSCT gene therapy has advanced from proof-of-concept studies to the treatment of humans with acquired and genetic diseases. Several clinical trials have resulted in life-saving successes, and the curative potential of genetically-modified HSCs is now a reality with the number of disease applications growing rapidly, including chronic granulomatous disease, Wiscott-Aldrich disease, Fanconi anemia, β-thalassemia, and sickle cell disease. Hemophilia A remains a prime candidate for HSCT gene therapy. Advancements in fVIII transgene design, viral vector engineering, and immunological conditioning have cured this disease in animal models and show promise for upcoming clinical trials. Additional studies continue to elucidate methods and technologies to identify and isolate HSCs and improve transduction of this important gene therapy target. In addition, overcoming the current limitations of fVIII expression, as well as reducing the risks of the gene transfer procedure, such as insertional mutagenesis, is possible. Further advancements in the field of HSCT gene therapy have included novel mechanisms to target site-specific gene insertions or corrections using engineered nucleases or PNA-complexes. Through ongoing intensive research, the field of HSCT gene therapy is progressing towards safer, more efficient, and cost effective treatment options.

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