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# Advancements in gene transfer-based therapy for hemophilia A

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## Abstract

Gene therapy has promised clinical benefit to those suffering with hemophilia A, but this benefit has not yet been realized. However, during the past two decades, basic and applied gene therapy research has progressed and the goal of gene therapy for hemophilia A is once again in our sights. The hemophilia A patient population suffers from a disease that requires invasive, lifelong management, is exorbitantly expensive to treat, has geographically limited treatment access and can become untreatable due to immune reactions to the treatment product. Subsequent to the cloning of the factor VIII gene and cDNA in the early 1980s, academic and commercial research laboratories began to pursue gene transfer-based therapies to supplement or supplant the available protein replacement therapy. However, to date, clinical trials for gene therapy of hemophilia A have been unsuccessful. Three trials have been conducted with each having tested a different gene-transfer strategy and each demonstrating that there is a considerable barrier to achieving sustained expression of therapeutic amounts of factor VIII. Recent progress has been made in gene-transfer technology and, relevant to hemophilia A, towards increasing the biosynthetic efficiency of factor VIII. These advances are now being combined to develop novel strategies to treat and possibly cure hemophilia A.

### Keywords

adeno-associated virus; adenovirus; factor VIII; gene therapy; hematopoietic stem cell transplantation; hemophilia A; lentivirus; oncoretrovirus; transposon; viral vector

## Clinical aspects of hemophilia A

Hemophilia A is a bleeding disorder caused by defects in the gene encoding coagulation factor VIII (fVIII) [1]. fVIII is a prothrombotic, proco-factor that, upon activation, functions to accelerate the blood clotting process. Genetic defects leading to a deficiency in fVIII activity occur in 0.01% of the male population. Primarily males are affected due to the location of the *F8* gene on the X chromosome. The severity of the disease is correlated with the residual fVIII activity level present in the affected individual, and the disease is classified into three groups: severe (≤1% fVIII activity); moderate (1–5%); and mild (5–20%). Patients with severe hemophilia A present with spontaneous bleeding episodes that can be life-threatening. Current treatment involves intravenous infusion of fVIII-containing products, which can either be

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human plasma-derived or manufactured recombinant protein. fVIII circulates at trace levels (approximately 1 nM) in humans and displays expression levels in recombinant systems that are significantly inferior to that of other plasma proteins. Therefore, fVIII products are inefficiently produced commercially and are quite expensive. fVIII product usage for a typical severe hemophilia A patient is US$100,000–300,000 per annum for prophylactic treatment consisting of multi-weekly intravenous injections. For smaller children, a permanent intravenous port is often necessary, which can result in additional adverse complications, such as infection. Despite the various drawbacks, fVIII infusion therapy is effective at controlling ongoing bleeding events and preventing future bleeds if used prophylactically.

Several characteristics of hemophilia A make it amenable to gene transfer-based therapeutic strategies. First, small increases in circulating fVIII level can foster a significant clinical benefit. For example, increasing the baseline fVIII level from below 1% to above 5%, representing an approximate 5–10 ng/ml production boost, eradicates spontaneous bleeding episodes. Second, fVIII can be biosynthesized and secreted into the bloodstream by most cell types with vascular access. Third, the current therapy is expensive and alternative, cost-effective therapies would be beneficial to both patients and insurers. Fourth, inconveniences and inefficiencies remain in intravenous fVIII replacement therapy including invasiveness of treatment, access to treatment (less than a third of the world population is treated) and immune responses to the infused fVIII product that render it ineffective in 20–30% of severe hemophilia A patients. These criteria continue to justify the attention and significant research effort that has been directed towards gene therapy for hemophilia A.

## Early preclinical research

Hopes of using gene therapy in hemophilia A treatment began with the cloning of the *F8* gene and cDNA by a group at Genentech [2,3]. At the time, in 1984, the gene encoding fVIII was the largest ever cloned at 186,000 base pairs in length. The derived mRNA is 9048 nucleotides and encodes a protein of 2351 amino acids (2332 amino acids in the mature form after removal of the signal peptide). Cloning of the *F8* gene, located on the tip of the long arm of the X chromosome at Xq28, showed that the encoded protein has a domain structure designated A1-A2-B-ap-A3-C1-C2, as defined by internal sequence homologies. This domain structure is identical to that of the related coagulation cofactor, factor V. The A domains of fVIII and factor V are homologous to ceruloplasmin and the C domains share homology with discoidin and the milk-fat globule-binding protein, which has implications for their potential roles in metal ion and lipid binding, respectively. The function of the B domain remains poorly understood. The B domain is known not to be necessary for procoagulant function and recent data suggest a role in facilitating secretion from the cell [4]. Owing to the large size and apparent trivial nature of the B domain, it is often deleted in the context of fVIII transgenes, termed B-domain deleted (BDD), that are used in gene therapy delivery vectors. Around the time of *F8* cloning, recombinant viral vector technology emerged and was identified as a potential vehicle for gene therapy applications [5]. In 1990, Israel and Kaufman first demonstrated the retroviral transfer of a human fVIII transgene into cultured cell lines [6]. Soon after, several gene-transfer approaches were tested experimentally and many showed promise in preclinical studies. As no particular gene-transfer strategy was obviously superior to all others, several approaches were pursued. These included retroviral, adenoviral, adeno-associated viral and non-viral gene-transfer methods.

Retroviral vectors represent a family of versatile gene-transfer vehicles that display the desirable property of stable integration into the host cell genome. Commonly utilized examples include Moloney murine leukemia virus (MoMLV) and HIV. Each has a relatively large packaging capacity that easily accommodates the BDD fVIII nucleic-acid sequence and can infect a wide range of cell types. MoMLV-based γ-retroviral vectors have been used clinically

in the treatment of X-linked severe combined immunodeficiency (X-SCID) disease [7]. In this setting, gene therapy has successfully cured the disease in the majority of patients. However, severe adverse events have occurred in several patients that presented with subsequent leukemia derived from the genetically modified cells [8,9]. The exact nature of the leukemogenic event remains unclear, but is speculated to have resulted from a combination of factors, including the site of viral integration, vector payload and cell processing protocol. Despite these adverse events, the X-SCID gene therapy story is considered a success owing to the dramatic clinical improvement achieved in the majority of patients with an otherwise dismal prognosis.

Several approaches (*in vivo* and *ex vivo)* for using retroviral vectors have been investigated for the treatment of hemophilia A. *In vivo* approaches involve the direct administration of the vector to the patient, for example, intravenous infusion, while *ex vivo* approaches involve the genetic modification of cells outside of the body, for example, the laboratory, with subsequent introduction to the patient. The first preclinical demonstration of *ex vivo* retroviral gene therapy for hemophilia A was accomplished by transplantation of genetically engineered human dermal fibroblasts seeded onto collagen-coated artificial fibers into the peritoneal cavity of immunodeficient mice [10]. High-level, yet transient, expression of BDD human fVIII was observed. Loss of expression was probably due to cell death within the implanted neo-organ.

Cells obtained from the bone marrow were among the initial cellular targets for retroviral gene transfer because of their accessibility and usefulness in cellular therapy applications, for example, bone marrow transplantation. Bone marrow transplantation protocols have been refined over the past several decades and have become a reliable way to extract, manipulate and re-administer cells with long-term engrafting and expansion potential. Two primary populations of cells exist in the bone marrow and blood compartment. One is of mesenchymal lineage, which has the potential to differentiate into bone, cartilage and adipose cells. The other is hematopoietic in origin, which populates the blood compartment, including myeloid, lymphoid and erythroid lineages. Evans and Morgan reported the initial finding that hematopoietic cells could be genetically modified by retroviral vectors to express human fVIII, albeit at insufficient levels to be detected in plasma [11]. Subsequently, other groups demonstrated *in vitro* that lymphoid cells inefficiently biosynthesize and secrete fVIII compared with other cell types including those of myeloid lineage [12–14]. *In vitro*, genetically modified bone marrow-derived mesenchymal stem/progenitor cells produce high levels of fVIII [15]. Furthermore, they are thought to have long-term engraftment potential and have thus been a target in many preclinical studies incorporating retroviral vectors. However, in these studies, only transient *in vivo* expression was observed, possibly owing to transcriptional silencing and/or transplanted cell death.

In addition to retroviral-based gene transfer systems, adeno-viral-based systems were undergoing extensive testing during the same timeframe. Adenoviral vectors are efficient gene-transfer vehicles composed of nonenveloped, icosahedral virions containing 36-kilobase pair (kb) dsDNA genomes. They infect target cells through interactions with the coxsackie and adenovirus cell surface receptors followed by endosomal internalization and nuclear translocation where the viral genome remains episomal. For gene therapy purposes, part of the viral genome (of varying size) necessary for viral replication is deleted and replaced by a transgene cassette. Adenoviral vectors efficiently infect many cell types, including non-dividing cells such as hepatocytes, and promote high-level transgene expression, which generates enthusiasm for their use in gene therapy applications. However, adenoviral vectors also stimulate the immune system, which has adverse side effects, including hepatotoxicity and acute inflammatory responses, which restrict the dose that can be administered safely.

Early studies involved intravenous administration of E1/E3-deleted or E1/E2a/E3-deleted adenoviral vectors encoding human or canine fVIII [16–20]. In these studies, infected mice expressed fVIII in the therapeutic range, yet expression levels gradually returned to baseline levels over time; likely due to immune-mediated destruction of genetically modified cells. Likewise, treated hemophilia A dogs displayed similar transient fVIII expression, but in contrast to the murine studies, loss of fVIII activity corresponded with the development of anti-fVIII inhibitory antibodies [21]. In non-human primates, therapeutic levels of fVIII were achieved in two separate studies using E1/E2/E3-deleted adenoviral vectors [22,23]. Expression persisted for up to 28 days in one study, while the other study was concluded after 1 week. In both studies, transient elevations in liver enzymes, liver inflammation and thrombocytopenia were observed. In the former study, an inhibitory immune response to fVIII was observed in two out of four animals. Due to the immune-stimulating properties of early-generation adenoviral vectors, later-generation vectors were engineered to contain the fewest necessary *cis*-acting elements. These new vectors, termed 'high-capacity' (HC) or 'gutted' adeno-viral vectors, can package above 30 kb of insert DNA, but they are dependent on the use of helper viruses to provide all of the missing functions *in trans* [24]. Helper viruses are not packaged due to mutation of the packaging signal. HC vectors display significantly reduced hepatotoxicity and inflammatory response profiles in both small and large animals compared with early-generation adeno-viral vectors [25–27]. HC vectors encoding human fVIII resulted in significantly higher peak levels of sustained fVIII expression (>1 unit/ml) in hemophilia A mice. fVIII levels subsequently, but gradually, declined over a period of 9 months [28]. No anti-fVIII inhibitory antibodies were detected in these animals. In contrasting studies, although high fVIII levels were also achieved, five out of eight mice developed fVIII inhibitors within 2 months of treatment [29,30]. No vector-related toxicity was observed in either study using vector doses up to $3 \times 10^{11}$ vector particles (vp)/mouse. However, in both canine hemophilia A and non-human primate models, transient hepatic and hematologic toxicities were observed using doses above $3 \times 10^{12}$ vp/kg, which were the minimal doses required to obtain detectable transgene product in the circulation [31].

## Commercial development & human clinical trials

Preclinical results stimulated commercial enthusiasm for clinical gene therapy in hemophilia A treatment and fostered the opening of three Phase I clinical trials. Each clinical trial was sponsored by a separate corporate entity and utilized completely different gene-transfer strategies. The first trial was conducted by Transkaryotic Therapy Inc., (MA, USA) [32]. This study built upon early work targeting human dermal fibroblasts. Unlike the aformentioned published preclinical studies, this gene-transfer strategy involved the electroporation of plasmid DNA into autologous patient fibroblasts. The transgene cassette consisted of a BDD human fVIII cDNA driven by a human fibronectin promoter. Dermal fibroblasts from 12 male hemophilia A patients were expanded *ex vivo* and electroporated in the presence of plasmid encoding the fVIII expression cassette. Clonal cell lines that expressed the highest levels of fVIII *in vitro* and displayed normal cell growth properties were then selected. In addition, the transgene integration pattern was characterized for each clone. Characterized clones were expanded and graded cell doses of 1, 4 or $8 \times 10^8$ cells were surgically implanted into the greater or lesser omentum of the patients under general anesthesia. Follow-up was conducted over a 2-year period. A preliminary report on the first six patients documented no serious adverse events and no detection of anti-fVIII antibodies. However, fVIII levels remained at or near baseline levels (<0.01 units/ml or 1% of normal) in all patients, complicating the interpretation of the lack of immune response. Despite the lack of significant fVIII production, it was reported that several of the patients displayed decreased bleeding frequency following the procedure. This observation must be considered with some degree of skepticism due to the design of the study, which was open-label with no placebo group. For reasons that are not clear,

but likely due to the lack of perceived efficacy of the procedure, this trial was not continued following completion of Phase I.

A second clinical trial using gene transfer to treat hemophilia A was sponsored by Chiron Inc. (San Diego, CA, USA) [33]. This trial developed through extensive preclinical testing of an amphotropic enveloped, oncoretroviral, MoMLV-based vector encoding BDD human fVIII driven by the MoMLV long terminal repeat (LTR). This candidate vector was tested extensively in several animal models and the data reported by Jolly and colleagues [34]. In both juvenile rabbits and hemophilia A dogs, circulating human fVIII was observed following intravenous infusion of the recombinant virus. In rabbits, fVIII production persisted for more than 6 months, while in dogs, fVIII expression was transient disappearing after 2 weeks and coinciding with the appearance of neutralizing antibodies, which confounded the experimental interpretation. Despite this shortcoming, a Phase I clinical trial was initiated with 13 adult male hemophilia A patients. The trial was an open-labeled, single agent, dose-escalation ($2.8 \times 10^7$–$8.8 \times 10^8$ transducing units [TU]/kg), multicentered design. The vector was delivered intravenously, via peripheral vein, on 3 consecutive days at equal split dose. No serious adverse events associated with vector delivery were observed. Although viral DNA could be detected by PCR in peripheral blood mononuclear cells 1 year post-administration, none of the patients displayed sustained, therapeutic fVIII activity levels. However, some patients on occasion did have fVIII activity levels above baseline, fewer bleeding episodes and required fewer protein infusion treatments than prior to treatment. One serious adverse event was observed in a single patient who tested positive for vector DNA in semen 9 weeks postvector infusion. All subsequent tests on the same patient were negative, supporting the conclusion that the transient signal observed was a false-positive effect. Again, since it was a Phase I safety study, no placebo control group was included. Therefore, conclusions regarding efficacy could not be drawn and the study did not proceed beyond Phase I.

The third clinical trial utilized a HC adenoviral vector and was sponsored by GenStar Therapeutics Inc. (CA, USA). The reduced toxicity and increased transgene expression levels generated from recombinant HC adenoviral vectors compared with the early-generation vectors created hope that they may be used in the treatment of numerous genetic diseases in addition to various cancers. The candidate vector chosen for clinical development included the full-length human fVIII cDNA under the control of a human albumin promoter. As described above, fVIII expression was observed following vector administration in several pre-clinical models including mice, dogs and non-human primates. However, there was a progressive decrease in fVIII expression and corresponding increase in toxicity observed in each model system moving from murine to canine to non-human primate. The results of the truncated Phase I trial followed this trend. A single patient was treated by intravenous infusion with a dose of $4.3 \times 10^{10}$ vector genomes (vg)/kg. The patient expressed at least 1% normal fVIII activity for several months, but the trial was halted following the observation of transient infusion-related adverse events, including fever, chills, achiness and headache as well as subsequent elevations in liver enzymes and thrombocytopenia. Although all clinical parameters returned to baseline within 3 weeks, the trial was put on hold and never recommenced. The cause of toxicity was not determined in the clinical trial, but is generally believed to have resulted from an immune response to the vector particles themselves and been independent of the transgene product. The toxicity results of this study, combined with similar results obtained from other studies targeting different diseases have led to the general opinion that unless vector-related toxicity can be averted, adenoviral vectors will not be utilized in future clinical gene therapy trials to treat genetic diseases where alternative therapies are available. However, adenoviral vectors likely will play an important clinical role in acute cancer gene therapy.

Collectively, the results of the three clinical gene therapy trials for hemophilia A are disappointing in terms of signs of efficacy and lack of progression beyond Phase I. However,

it is clear that fVIII expression remains a major barrier to gene therapy for hemophilia A. Also disappointing is the lack of recent indications of novel clinical trial development beyond publications describing preclinical murine (and some canine) studies. Although it is not clear when another clinical trial for gene therapy of hemophilia A will be conducted, there are promising new developments in both the fields of gene therapy and blood coagulation factor research that will spawn the next clinical trial. The remainder of this review focuses on these promising new developments and speculation towards future progress.

## Recent preclinical advances

### Factor VIII bioengineering

One obstacle possibly not fully appreciated by those conducting previous gene-therapy trials and preclinical studies is the poor expression property of human fVIII. Human fVIII is produced at levels 100–1000-fold lower than other recombinant proteins (for a review see [35]). Extensive research efforts have been directed towards deciphering the bottlenecks in fVIII biosynthesis, with the goal of increasing expression for both recombinant protein and gene transfer-based treatments. fVIII biosynthesis begins with transcription of the *F8* gene into a primary RNA transcript followed by processing of the transcript and exportation from the nucleus. Previous studies have identified sequences within the human fVIII cDNA that inhibit transcription and/or accumulation of fVIII mRNA within heterologous cells [36,37]. FVIII mRNA encodes a signal peptide that targets the mRNA-ribosome–peptide complex to the rough endoplasmic reticulum (ER). Following co-translational translocation into the ER lumen, N-linked glycans are added to the nascent protein and trimmed by glycosidases I and II. Interaction of newly translated fVIII with the ER resident chaperones BiP, calnexin and calreticulin has been demonstrated experimentally [35,38–40]. These proteins play a direct role in the maintenance of quality control within the ER by retention of unfolded proteins (for review of ER quality control, see [41]). For many secreted proteins, including fVIII, transit from the ER to the Golgi is rate limiting [39,42]. Genetic mapping in patients with combined factor V/fVIII deficiency identified two proteins, designated LMAN1 and MCFD2, located within the ER–Golgi intermediate compartment that facilitate transport of fVIII from the ER [43,44]. This interaction requires oligosaccharide structures attached to the B domain [45, 46]. Following exit from the ER, fVIII is released from the cell within 4 h indicating efficient transport through the Golgi and secretory vesicles [47].

Several strategies have been implemented to increase the biosynthetic efficiency of fVIII. Increased mRNA expression has been achieved by removal of the B-domain sequence [48–50]. Importantly, the removal of the B domain from recombinant fVIII does not alter its procoagulant function and recombinant BDD human fVIII products, ReFacto™ and Xyntha™, have been approved for clinical use. In preclinical studies, the insertion of intron 1 from the coagulation factor IX gene into the fVIII cDNA at positions corresponding to the fVIII introns, 1 and 13, increased fVIII mRNA and secreted fVIII levels 13-fold compared with an intron-less fVIII construct [51]. However, increased fVIII secretion also coincided with a 100-fold increase in intracellular fVIII antigen indicating that secretory efficiency remains limiting. Reducing the interaction with protein chaperones in the ER through targeted mutagenesis is another strategy that has been employed to increase fVIII production. Mutagenesis of sequences within the A1 domain involved in BiP-binding proved successful in increasing fVIII expression two- to three-fold [52]. Presumably due to the lower N-glycan content, BDD human fVIII does not interact with LMAN1/MCFD2 resulting in lesser secretory efficiency compared with full-length human fVIII. Pipe and colleagues demonstrated that addition of limited B-domain sequence, encoding multiple predicted glycosylation sites, to BDD human fVIII resulted in enhanced production *in vitro* in mammalian cell culture systems and *in vivo* following hydrodynamic injection of naked plasmid DNA into mice [4]. However, to date, the

enhanced expression properties of B-domain fragments have not been demonstrated using gene-therapy approaches.

Recently, a third strategy for increasing fVIII production utilizing the high-expression properties of porcine fVIII was described [53]. It was demonstrated that BDD porcine fVIII is expressed at ten- to 100-fold greater levels than recombinant BDD human fVIII *in vitro* from COS-7 and baby hamster kidney-derived (BHK-M) cells. Interclonal variation in fVIII protein expression correlated with steady-state mRNA levels. However, porcine fVIII was expressed at higher levels than human fVIII on a per mRNA basis suggesting a translational or post-translational regulatory differential. In a subsequent study, hybrid human/porcine (HP)-fVIII molecules containing single or combinatorial domain substitutions were constructed and it was found that the interspecies expression differential results from porcine-specific sequences within the A1 and ap-A3 domains [54]. This effect appears to be synergistic since a construct containing only porcine A3 sequence was expressed at levels indistinguishable from human fVIII and the construct containing only porcine sequence in the A1 domain was expressed at an intermediate level between human fVIII and porcine fVIII. Metabolic labeling experiments with cells stably expressing either human fVIII or the construct containing the porcine A1 and A3 domain substitutions revealed that the interspecies expression differential results from enhanced secretion. Although the evolutionary significance of this observation is not well understood, porcine and HP-fVIIIs containing porcine A1 and ap-A3 domain sequences remain the single most efficient fVIII production constructs identified to date and could be vital to overcoming the fVIII expression barrier identified in gene-therapy clinical trials.

## Hematopoietic stem cell gene therapy

Bone marrow and, more recently, hematopoietic stem cell transplantation (HSCT) has been used for the treatment of various blood cancers and defects of hematopoiesis. From a theoretical perspective, HSCT gene therapy is very attractive for the treatment of many disorders, especially those due to deficiencies in circulating proteins. The attractiveness comes from the fact that HSCs that have been genetically modified through integration of retroviral vectors have the capacity to engraft in the patient and reside for the patient's lifetime while constantly undergoing division and self-renewal. The genetic material of the HSC is passed on to all sister and daughter cells, which then divide themselves and give rise to all the cells that comprise the hematopoietic lineage. This results in dramatic amplification of the transferred genetic material from as few as one HSC to millions of myeloid, lymphoid and erythroid cells per day. This amplification relieves an important limitation that viral transduction efficiency imposes on clinical gene therapy. To date, several groups have conducted clinical trials incorporating HSCT gene therapy, although many of these were non-therapeutic, gene-marking studies. HSCT is also being tested clinically as a treatment option for a variety of human autoimmune disorders, including multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis and Crohn's disease [55–59]. In animal models, HSCT gene therapy has been shown to facilitate the introduction of and tolerance to neoantigens [60–66]. However, successful HSCT often involves conditioning of the patient with radiation or chemotherapeutic agents that kill resident HSCs thereby making 'space' for the transplanted, genetically modified HSCs. These conditioning regimens are associated with non-trivial side effects and significant risk of adverse events. Therefore, more research effort is required to identify safer yet still effective HSCT protocols.

Low-level biosynthesis of human fVIII from genetically modified hematopoietic cells has been demonstrated to be the major obstacle preventing expression of therapeutic levels of fVIII. This was identified in the first preclinical HSCT gene therapy study for hemophilia A, conducted by Evans and Morgan [11]. More recently, this finding was confirmed by Hawley and colleagues, who used a vector construct that expressed an EGFP reporter gene in addition

to the fVIII transgene so that genetically modified cells could be cell sorted prior to transplantation in order to maximize the level of genetic modification and thus fVIII production [63,67]. Although useful as proof-of-concept studies, at present, selection of genetically modified cells prior to transplant is not clinically applicable.

The high-level expression property of porcine fVIII has recently been shown to function effectively in gene-transfer studies and overcome the expression barrier. Recombinant retroviruses encoding a BDD porcine fVIII transgene were used to genetically modify HSCs, which were then transplanted into hemophilia A mice preconditioned with a lethal dose of total body irradiation (TBI) [60]. All mice demonstrated sustained plasma fVIII activity levels approaching or exceeding 100% of the normal human level for greater than 1.5 years post-transplantation. Long-term restoration of circulating fVIII activity suggests that genetically modified HSCs engrafted and proliferated, and their progenies expressed fVIII. This study demonstrates proof-of-concept that porcine fVIII high-expression elements function *in vivo* in a gene therapy setting. However, the transplantation protocols' use of a lethal dose of TBI poses an inherent safety concern that does not warrant clinical use in patients with hemophilia A. Therefore, more clinically relevant HSCT regimens that lead to efficient engraftment and expression of high-expression fVIII transgenes while limiting the development of anti-porcine fVIII inhibitors were tested. Towards this end, sustained high-level fVIII expression was observed in hemophilia A mice that received genetically modified HSCs under reduced intensity conditioning regimens, including sublethal TBI (5.5 Gy), costimulation blockade (anti-CD40L and CTLA4-Ig) or a combination of busulfan and anti-thymocyte serum [62]. In these studies, no mice developed inhibitory anti-porcine fVIII antibodies or displayed signs of leukemic transformations. This study demonstrated that T-cell suppression is a critical component to the successful engraftment of genetically modified HSCs encoding BDD porcine fVIII. Due to the observation that high-level fVIII activity can be achieved at low systemic copy number, these results clearly show that high-expression porcine fVIII sequence elements overcome the low-expression barrier observed using standard human fVIII constructs.

Most recently, it was demonstrated that a HP-fVIII construct with as little as 10% porcine amino acid sequence also functions in the HSCT gene therapy setting to overcome the fVIII expression barrier [68]. In this study, recombinant simian immunodeficiency virus-based vectors encoding HP-fVIII were used to genetically modify human hematopoietic cell lines and murine HSCs, the latter of which was also transplanted into hemophilia A mice. Both *in vitro* and *in vivo* HP-fVIII demonstrated significantly greater expression levels than BDD human fVIII, supporting its continued development towards clinical application.

## Gene therapy for patients with fVIII inhibitors

Inhibitory antibody development in hemophilia A patients remains one of the most significant clinical complications and affects 20–30% of patients with severe hemophilia A. Nucleic acid-transfer techniques have provided some insights into the mechanism of inhibitor development. The cytokine and immunoglobulin profiles observed in inhibitor patients and in animal models have shown that the anti-fVIII response is a mixed Th1 and Th2 response, indicating that cell-mediated and humoral immunity are involved in the anti-fVIII immune response. This information has led several groups to test various methods that could be used to decrease antibody development during gene therapy treatments. For example, using human fVIII encoding plasmids, Miao and colleagues have demonstrated that immunomodulation at the time of plasmid administration can eliminate the production of anti-fVIII antibodies [69,70]. In addition, it has recently been shown that Tregs can aid in the establishment of a long-term tolerance to fVIII following injection of plasmid-based [71] or recombinant lentiviral vectors into hemophilia A mice [72].

Historically, a proportion of this patient population has been treated effectively (at least acutely) with plasma-derived porcine fVIII. This clinical experience provided the basis for the preclinical testing of a HSCT gene therapy protocol incorporating the BDD porcine fVIII transgene in the inhibitor setting. In order to induce a humoral immune response in hemophilia A mice, recombinant human fVIII (10 units/ml) was administered intravenously (weekly) for 4 weeks. A significant anti-human fVIII inhibitory response was observed in all animals that displayed 10–14% cross-reactivity to BDD porcine fVIII. Myeloablative HSCT gene therapy resulted in high-level donor cell engraftment and expression of therapeutic levels of fVIII (3.6 ± 1.3 units/ml) [61]. Similar success was achieved using a regimen of busulfan (or sublethal-TBI) and anti-thymocyte serum [62]. These results demonstrate the feasibility of using non-myeloablative conditioning in a HSCT gene therapy approach in hemophilia A inhibitor patient populations that otherwise suffer from inadequate and unreliable treatment options.

### Platelet-targeted gene therapy

Other laboratories have reported the specific targeting of fVIII transgene expression to the megakaryocyte/platelet hematopoietic lineage [73–75]. Studies have been performed using both transgenic animals produced by pronuclear injection of DNA constructs encoding a BDD human fVIII transgene under the control of various integrin promoters and HSCT gene therapy using self-inactivating lentiviral vectors encoding a similar expression cassette [76]. Both strategies are effective at correcting the bleeding phenotype in hemophilia A mice as assessed by tail-clip bleeding assay despite the lack of circulating fVIII activity, which is probably due to sequestration within platelets until release following platelet activation, in these animals. Furthermore, fVIII stored in platelets is protected from fVIII inhibitors and retains *in vivo* efficacy in their presence, suggesting a potential use in inhibitor patients. However, recent results obtained by Poncz and colleagues dispute the interpretation of these results, specifically the tail-clip bleeding assay, and the relevance to correction of the bleeding phenotype in hemophilia A patients [77]. Further preclinical and clinical testing will be necessary to judge the effectiveness of this unique approach.

## Expert commentary

During the last decade there has been tremendous effort directed toward the development of a nucleic acid therapy for hemophilia A. Parallel to the development of optimized fVIII transgenes has been the development of several novel gene-transfer platforms, including viral and non-viral systems, as well as a better understanding of how to deliver gene-based therapies. These efforts are now converging to the point where novel clinical trials for hemophilia A are being discussed. Unfortunately, despite an abundance of recent and ongoing preclinical research, there is uncertainty as to what the next gene-therapy trial(s) for hemophilia A will entail or when it (they) will be initiated. This uncertainty stems primarily from the difficulty in successfully translating preclinical gene transfer successes into clinical realities, as evidenced by the three clinical trials conducted to date. In addition, significant effort has been devoted to issues that are not directly related to the successful transfer of fVIII transgenes, such as developing a better understanding of the molecular events that induce the generation of anti-f VIII antibodies. Although not directly related to the genetic manipulation of target cells, these studies may provide the basis for future therapeutic interventions that accompany the gene-transfer procedures. Furthermore, commercialization of gene transfer-based pharmaceuticals as a whole suffers from the lack of a validated business model and the related difficulties associated with raising research and development capital. These are formidable obstacles for the field to overcome. However, there have been some clinical successes using HSCT gene therapy, and it should be expected that hemophilia A can be treated using similar approaches. More recently, an adeno-associated viruses (AAV)-based gene therapy was demonstrated to improve vision in patients with Leber's congenital amaurosis [78,79]. Hopefully, successes

such as these will generate sufficient momentum to propel gene therapy for hemophilia A back into the clinic. Proven technologies such as lentiviral HSCT and AAV-based gene transfer are at the forefront of this effort.

## Five-year view

Several alternative gene-transfer platforms are under development, which may eventually prove beneficial for gene therapy of hemophilia A. The next 5 years will undoubtedly see progress towards the clinical development of novel gene-transfer systems. For example, nonviral gene-transfer technologies are being steered toward the treatment of hemophilia A. One promising development for gene therapy is the Sleeping Beauty transposon, which has been demonstrated by several groups to be an effective technique for transferring fVIII transgenes [80,81]. However, gene-transfer efficiency into primary mammalian cells has been shown to be limiting. Recently, a hyperactive transposase (SB100X) with approximately a 100-fold enhancement in efficiency was identified [82], and SB100X was shown to support efficient gene transfer (35–50%) into human HSCs. Therefore, SB100X could be adapted to HSCT gene therapy for hemophilia A. Another recent advance involving Sleeping Beauty transposon is cell type-specific gene targeting using hyaluronan- and asialoorosomucoid-coated nanocapsules to direct fVIII expression to liver sinusoidal endothelial cells [83]. Sleeping Beauty transposon DNA encoding BDD canine fVIII was encapsulated in hyaluronan nanocapsules and injected intravenously into hemophilia A mice. Subsequently, plasma fVIII activity was restored to wild-type levels for almost 1 year. No antibody formation was observed suggesting an immune privileged property of liver sinusoidal endothelial cells, which have been suggested as an important site of endogenous fVIII biosynthesis [84]. Although the clinical usefulness of these non-viral transfer systems for diseases, such as hemophilia A, has not been demonstrated, preclinical studies such as those described herein are in progress and may lead to clinical application.

Recombinant adeno-associated viruses have been studied in clinical trials for hemophilia B and some preclinical studies have shown that AAV can be used to transfer fVIII cDNA sequences. Recombinant AAV vectors have been used in preclinical studies using small and large animal models [85–90]. Employing various delivery conditions, long-term fVIII expression has been achieved. Studies taking place over the next 5 years will likely focus on safety issues surrounding the use of AAV transfer, such as effects of pre-existing immunity to the AAV vector, the route of gene delivery and the optimal cell type to target. These issues have been reviewed recently by Youjin and Jun [91], and Mingozzi and High [92].

Other gene-transfer technologies have been reported, but are not discussed at length in this review because significant advancements are necessary prior to their potential clinical application. These include the use of blood outgrowth endothelial cells (BOECs) as a fVIII delivery system and the use of mRNA editing technologies, such as spliceosome-mediated RNA trans-splicing (SMART). BOECs are attractive target cells because they can be isolated easily from blood and expanded *ex vivo* and *in vivo* expression using transfected or lentivirally transduced cells have shown high-level fVIII expression [93,94]. However, averting the development of anti-fVIII antibodies following BOEC administration and maintaining fVIII expression over time despite cell death both require further refinement. SMART technologies take advantage of a natural cellular process that splices RNA molecules into pre-existing RNAs. Recently, Wang *et al.* demonstrated that the nucleic acids encoding fVIII can be spliced into the albumin pre-mRNA molecule, which results in sustained, albeit low-level, fVIII expression in hemophilia A mice [95].

Although at present there are no clinical trials approved or publically announced, several technologies or therapies can be viewed as being in the later stages of preclinical development.

Significant progress has been made toward increasing fVIII expression levels following gene transfer, for example, the use of high-expression porcine fVIII elements and inclusion of oligosaccharide modifications present in the B domain. Lentiviral-based gene-transfer systems are undergoing intensive preclinical and now clinical testing. They have become a leading gene-transfer platform for transferring bioengineered high-expression fVIII constructs based on their perceived safety advantage over oncoretroviruses owing to differential integration site preference and affect on nearby gene expression. Furthermore, genetically modified HSCs have been used successfully in several non-hemophilia clinical trials, and this cell population may also prove to be an ideal target cell population for hemophilia A. Therefore, a HSCT gene therapy clinical trial incorporating the use of bioengineered high-expression fVIII variants is possibly a near future reality.

Gene therapy has not delivered on the initial hype of the late 1980s and early 1990s, which was a time when the challenges to the clinical translation of this new technology were not known. During the past two decades, these challenges have become clear, and with respect to developing a gene therapy treatment for hemophilia A, they are substantial, but certainly not insurmountable. If gene therapy as a field is viewed in the same manner as monoclonal antibody-based therapy, then the use of nucleic acids as therapeutics is still on track to deliver significant clinical benefit to patients. Within a mere 10 years of investigation of gene therapy for hemophilia A, many accomplishments have been made and many lessons learned. The next 5 years will see further progress towards a gene transfer-based treatment for this disease. However, with tight budgetary constraints currently in place, future clinical studies will likely require governmental, private institution and philanthropic support.

## Key issues

- Treatment of hemophilia A is complicated by many factors, including access, cost, invasiveness and development of anti-factor VIII inhibitory antibodies.
- Hemophilia A has many characteristics that make it amenable to gene transfer-based therapy, including a large therapeutic window, vast range of cell types capable of factor VIII (fVIII) biosynthesis and an adequate patient population.
- Clinical gene therapy has been unsuccessful at demonstrating sustained correction of the fVIII deficiency.
- Inefficient biosynthesis of human fVIII remains a significant barrier to gene therapy of hemophilia A.
- Progress towards improving the biosynthetic efficiency of fVIII has been made through bioengineering and interspecies fVIII analysis, for example, HP-fVIII.
- Recent preclinical success towards curing hemophilia A has been demonstrated using hematopoietic stem cell transplantation retroviral, adeon-associated virus, and high capacity adenoviral-based gene transfer as well as nonviral gene transfer alternatives.
- Although no hemophilia A gene therapy clinical trials are ongoing, there should be continued optimism that gene therapy will provide benefit to patients with this disease.
- Future progress towards a cure for hemophilia A utilizing gene-transfer technology depends on improved understanding of several factors, including overcoming the fVIII expression barrier, avoidance of anti-fVIII immune responses and maintenance of a favorable risk–benefit ratio. Furthermore, sufficient financial investment to support the translation of these advances will be critical.

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