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Gene therapy for hemophilia

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Gene therapy offers the potential for a cure for patients with hemophilia by establishing continuous endogenous expression of factor VIII or factor IX (FIX) following transfer of a functional gene to replace the hemophilic patient's own defective gene. The hemophilias are ideally suited for gene therapy because a small increment in blood factor levels (≥5% of normal) is associated with significant amelioration of bleeding phenotype in severely affected patients. In 2011, the St. Jude/UCL phase 1/2 trial was the first to provide clear evidence of a stable dose-dependent increase in FIX levels in patients with severe hemophilia B following a single administration of adeno-associated viral (AAV) vectors. Transgenic FIX expression has remained stable at ~5% of normal in the high-dose cohort over a 7-year follow-up period, resulting in a substantial reduction in spontaneous bleeding and FIX protein usage without toxicity. This study has been followed by unparalleled advances in gene therapy for hemophilia A and B, leading to clotting factor activity approaching normal or near-normal levels associated with a "zero bleed rates" in previously severely affected patients following a single administration of AAV vectors. Thus, AAV gene therapies are likely to alter the treatment paradigm for hemophilia A and B. This review explores recent progress and the remaining limitations that need to be overcome for wider availability of this novel treatment of inherited bleeding disorders.

Learning Objectives

- Understand progress with gene therapy for hemophilia
- Understand the limitations and obstacles

Introduction

Hemophilia A and B are X-linked recessive disorders resulting from mutations in the gene for blood clotting factor VIII (FVIII) and factor IX (FIX), respectively. The incidence of hemophilia A is ~1 in 5000 live male births and that of hemophilia B is 1 in 25 000 live male births. Collectively, they are among the most common inherited bleeding disorders in the world. Despite the genetic and biochemical differences, these disorders are indistinguishable clinically, with the severity of bleeding symptoms varying according to the residual factor activity in a patient's plasma. More than half of patients with hemophilia A or B have factor levels $< 1\%$ of normal.[1] These individuals have a severe bleeding phenotype consisting of frequent spontaneous musculoskeletal and soft tissue bleeding. Repeated episodes of intra-articular bleeding cause severe progressive destructive arthropathy, with deformity leading to complete loss of joint function and attendant disability. Patients with moderate hemophilia may bleed spontaneously and have pronounced bleeding after trauma, whereas in patients with mild hemophilia, bleeding is usually restricted to traumatic events; however, even these individuals have an increased risk for death from intracranial bleeding compared with the normal population.[2]

The current standard of care for severely affected hemophilia patients in countries with developed economies consists of regularly administered prophylaxis with factor concentrates intended to maintain the factor level $>1\%$ of normal. If started in early childhood, arthropathy can be largely prevented by regular prophylaxis.[3] When continued throughout life, prophylaxis leads to near normalization of life expectancy.[4] The relatively short half-life of FVIII and FIX in the circulation necessitates frequent IV administration of factor concentrates (≥2-3 times a week), which is demanding and extremely expensive. Prophylaxis is associated with a "sawtooth" pattern of factor levels in plasma: high immediately after infusion and falling rapidly to near baseline, leading to breakthrough bleeding. Therefore, hemophilia patients have to carefully plan periods of increased physical activities, such as sports, which people living without hemophilia can hardly imagine.

Recent advances in hemophilia treatment and the rationale for gene therapy

New modified synthetic formulations of FVIII and FIX that are pegylated or fused to proteins with a long half-life, such as albumin or Fcγ, have greatly improved the stability profile for FX but have been less impressive for FVIII as a result of the dominant role of von Willebrand factor in determining its half-life.[5] BIVV001, a novel FVIII fusion protein consisting of a single-chain recombinant FVIIIFc fused to the FVIII-binding D′D3 domains of von Willebrand factor, as well as 2 XTEN linkers, has an increased half-life ≥ 38 hours, raising the prospects of extended protection in

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hemophilia A patients with weekly dosage.[6] In patients with hemophilia B, extended half-life products allow reduction of injection frequency to once weekly or even once biweekly while maintaining higher trough levels (FIX > 5%), despite the reduction of injection frequency. Another major development entails the use of nonclotting factor products to secure hemostasis in patients with a bleeding diathesis. For instance, a bispecific antibody (emicizumab) with 1 arm binding to FIXa and the other to FX facilitates the conversion of FX into its active form, leading to restoration of hemostasis to a degree comparable to an FVIII level of ~15% of normal in hemophilia A patients with or without inhibitors.[7] Emicizumab has attractive pharmacokinetic attributes that allow less frequent (weekly to monthly) subcutaneous administration. Unlike FVIII, emicizumab is active in plasma all of the time and is associated with microangiopathy and thrombosis, particularly when used in combination with activated prothrombin complex concentrates.[8] Other novel approaches entail the lowering of endogenous anticoagulants, such as antithrombin or tissue factor pathway inhibitor, with antisense RNA technology (fitusiran)[9] or a monoclonal antibody (eg, concizumab),[10] respectively. These approaches have shown efficacy in reducing the rate of bleeding in hemophilia A and B patients, including those with inhibitors, but their use may be limited by a risk for thrombogenicity.

These novel therapies are beginning to change the clinical management of the hemophilias in countries with developed economies by decreasing infusion frequency, thus improving compliance with prophylaxis, offering alternatives to inhibitor patients, and easing the route of administration. To date, none of these advances have impacted the standard of care for 80% of the world's hemophilia patients who live in parts of the world with economies in transition or development.[11] These patients have little or no access to factor concentrates and have a reduced life expectancy, with very few surviving beyond their teenage years.

Despite this widening therapeutic choice for the treatment of the hemophilia, gene therapy has great appeal because it offers the potential for a cure through endogenous production of FVIII or FIX following transfer of a normal copy of the respective gene. The hemophilias have always been considered good candidates for gene therapy, because all of their clinical manifestations are due to the lack of a single protein that circulates in minute amounts in the blood stream. Years of clinical experience and natural history studies show that a small increase in circulating levels of the deficient clotting factor to 5% of normal significantly modifies the bleeding diathesis. Thus, the therapeutic goal for gene therapy of hemophilia is modest in comparison with the majority of monogenetic disorders. Furthermore, tight regulation of transgene expression is not necessary because a wide range of FIX or FVIII is expected to be beneficial and nontoxic.

Early hemophilia gene therapy studies

Early hemophilia gene therapy using viral (eg, oncoretroviral and adenoviral vectors) and nonviral vectors appeared to be safe but did not result in sustained transgene expression at therapeutic levels.[12-15] More recently, the focus has exclusively been on viral vectors, in particular, recombinant adeno-associated viral (AAV) vectors. These vectors have the best safety profile among gene transfer vectors of viral origin, because wild-type AAV has not been associated with human disease. Safety is further enhanced by the dependence of AAV on coinfection with a helper virus (usually adenovirus or herpesvirus) for productive infection. Additionally, recombinant vectors based on AAV are entirely devoid of wild-type viral coding sequences, thus reducing the potential for invoking cell-mediated immune response to foreign viral proteins.

The first study to use AAV vectors in hemophilia patients used ones based on AAV serotype 2, the first serotype to be isolated and fully characterized (Table 1).[16,17] Intramuscular injections of AAV vector encoding the FIX gene in this study were not associated with serious adverse events, but efficacy was not observed in any of the 7 subjects recruited, despite immunohistochemical evidence of FIX expression at the site of injection. Preclinical studies around this time suggested that, for a given dose of AAV, expression was significantly higher following liver-targeted delivery of AAV compared with intramuscular injections, perhaps because the liver is the natural site of FIX synthesis.[18] Therefore, in the second study, AAV2 vectors were infused into the hepatic artery using 3 doses ranging from 0.08 to 2e12 vg/kg in patients with severe hemophilia B. In this study, the low and intermediate vector doses were safe but did not result in a detectable increase in plasma FIX levels, whereas the mixed results were noted in the 2 subjects treated using the high dose (2e12 vg/kg). One subject had higher levels of neutralizing anti-AAV2 antibodies prior to gene transfer, which appeared to block successful transduction and resulted in a lack of transgenic FIX expression. In contrast, FIX levels increased to ~10% of normal levels in the other subject for 4 weeks after vector administration, and then they unexpectedly declined to baseline values. This decline in transgenic protein coincided with a transient tenfold increase in liver transaminases (serum alanine aminotransferase [ALT] > aspartate transaminase [AST]), which spontaneously returned to baseline values over the subsequent weeks, consistent with a self-limiting process. The decline in FIX expression and the liver toxicity were thought to be due to a capsid-specific cytotoxic T-cell response directed against the transduced hepatocytes prompted by the presentation of AAV2 capsid peptide in the context of MHC I molecules.[17] Thus, in this study, humoral and cell-mediated immune responses limited persistent expression of FIX following liver-targeted administration of AAV vectors in humans.

Building on these early studies, a novel approach for hemophilia B gene therapy (St. Jude Children's Research Hospital [St. Jude]/University Collegel London [UCL]; NCT00979238) that addressed some of the limitations of previous trials was developed. First, because the conversion of the single-stranded AAV genome (ssAAV) into a double-stranded form is rate limiting for the onset and levels of transgene expression, we developed a 2.3-kb mini-FIX expression cassette consisting of a codon-optimized version of the human *FIX* gene cloned downstream of a compact synthetic liver-specific promoter (LP1-hFIXco) to enable packaging of 2 self-complementary AAV (scAAV) vector genome within a single virion.[19] These complementary strands come together after uncoating to form a stable double-stranded provirus, eliminating the need for second-strand synthesis, an inefficient process. In preclinical studies in mice and nonhuman primates, scAAV vectors mediated a 10-fold increase in transduction efficiency compared with ssAAV vectors.[19,20] Subsequently, we realized that the covalently closed hairpin structure of scAAV vector inhibited polymerase chain reaction amplification of vector genomes, resulting in an underestimation of vector titer. Thus, the impact of scAAV vectors in animal models may have been overestimated.[21]

Another important aspect of the St. Jude/UCL study was the use of vector pseudotyped with AAV serotype 8 capsid. This had the advantage, over AAV2 vectors, of the remarkable tropism of AAV8 for the liver, leading to efficient transduction of hepatocytes following administration of the vector in the peripheral circulation in animal models.[20,22] Hence, a simple noninvasive route of vector administration that is safer for patients with a bleeding diathesis was used in our study. Additionally, the lower seroprevalence of AAV8

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Table 1. Recent hemophilia B gene therapy trial using AAV

Sponsor	Transgene	No. of CpG motifs in transgene	Serotype	Genome format	Method of vector delivery	Dose range (vg/kg)	Mean stable FIX activity levels	No. of patients with transaminitis	Current status
Avigen/CHOP	Wild-type FIX	19	AAV2	ssAAV	IM	2e11 to 1.8e12	Transient at a maximum level of 1.6%	0	Closed
Avigen/CHOP Coagulin-B	Wild-type FIX	19	AAV2	ssAAV	Intrahepatic artery	2e11 to 2e12	Transient with a maximum of 12%	1 of 2 at highest dose	Closed
St Jude/UCL	Codon-optimized FIX	0	AAV8	scAAV	Systemic	2e11 to 2e12	5.1%	4 of 6 patients at highest dose	Closed
Shire (Baxalta; BAX 335)	Codon-optimized FIX + Padua mutation	99	AAV8	scAAV	Systemic	2e11 to 3e12	Transient except in 1 patient who had expression of 25% at last report	2 of 5 patients treated with ≥1e12 vg/kg	Closed
Spark Therapeutics (SPK-9001)	Codon-optimized FIX containing the Padua mutation	0	AAV- SPK-100	ssAAV	Systemic	6e11	36%	2 of 10 patients	Transitioned to phase 3 with Pfizer
uniQure (AMT-060)	Codon- optimized FIX	0	AAV5	ssAAV	Systemic	5e12 to 2e13	6.9%	2 of 5 at highest dose	In phase 3 trial as AMT-061 using a FIX cDNA containing the Padua mutation
Dimension Therapeutics (DTX101)	Codon optimized FIX	96	AAVrh10	ssAAV	Systemic	1.6e12 to 5e12	6.7%	3 of 3 at highest dose	Closed
Freeline Therapeutics (FLT-180a)	Codon-optimized FIX containing the Padua mutation	5	AAV-S3 synthetic capsid	ssAAV	Systemic	4.5e11	45%	0	Open
Sangamo Bioscience (SB-FIX)	Codon-optimized FIX	Not known	AAV6/zinc-finger–mediated targeted integration into the albumin locus in hepatocytes	ssAAV	Systemic		Unknown	Unknown	Open

cDNA, complementary DNA; IM, intramuscular.

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in humans (~25% compared with >70% for AAV2) enabled exclusion of fewer subjects with preexisting humoral immunity to AAV8.[23]

Ten subjects sequentially received a single IV infusion of scAAV2/8–LP1-hFIXco at a dose of 2e11 vg/kg, 6e11 vg/kg, or 2e12 vg/kg (using a new optimized titration method) over a 2-year period between 2010 and 2012. Two severe hemophilia B patients (FIX < 1%) were enrolled into the first and mid-dose cohorts, with 6 patients treated at the high dose. Stable long-term FIX expression at 1% to 8% of normal was established in all 10 subjects. Asymptomatic transient elevation of ALT, accompanied by a decrease in steady-state FIX, was observed in 4 of the 6 subjects recruited to the 2e12-vg/kg dose level between 6 and 10 weeks after gene transfer. Bilirubin, alkaline phosphatase, and γ-glutamyl-transpeptidase levels remained in the normal range. Each of the 4 patients with increased liver enzymes received a short tapering course of prednisolone, leading to normalization of ALT and AST levels with preservation of FIX transgene expression in the range of 2% to 5% of normal. Cessation of corticosteroids was not associated with an increase in ALT levels or a decrease in FIX activity. Notably, 2 subjects treated at the high-dose level had no evidence of transaminitis and did not require treatment with steroids. They both achieved stable FIX expression of between 5% to 8% of normal. Transgenic FIX activity levels have remained stable in all 10 subjects over an 8-year follow-up, associated with a significant reduction in the annual FIX concentrate usage and frequency of spontaneous bleeding.[24] Importantly, the quality of life of these individuals has improved dramatically because they are now able to undertake activities that previously provoked bleeds without suffering from bleeding episodes. No late toxicity was observed, and neutralizing antibodies to FIX were not detected in any patient. Ongoing monitoring of the liver does not show any evidence of long-lasting damage.[24] Therefore, the key factors that led to the success of the St. Jude/UCL trial were exclusion of patients with preexisting antibodies to AAV serotype and early use of corticosteroids upon detecting an increase in ALT, potentially suppressing an immune response directed against transduced hepatocytes.

Recent trials of gene transfer in hemophilia B

The gene therapy studies that followed the St Jude/UCL trial differed in their selection of AAV capsid, configuration of the vector genome, design of the expression cassette, and method of vector manufacture (mammalian system vs insect cell–baculovirus method; Table 1). In general, higher vector doses were required for therapeutic transgene expression when the vector preparations were made using the insect cell–baculovirus method. For instance, AAV5 serotype pseudotyped vectors (AMT-060; UniQure Therapeutics, Amsterdam, The Netherlands) made using the insect cell–baculovirus method, but containing the same FIX gene cassette as that used in the St. Jude/UCL trial, resulted in mean FIX activity levels of 6.9%, despite using a log higher vector dose of 2e13 vg/kg.[25] Increased ALT levels were observed in 3 of 10 patients recruited to AMT-060, requiring treatment with corticosteroids. In 3 other unpublished phase 1/2 clinical trials (DTX1010 [NCT02618915], AAV8-hFIX19 [NCT01620801], and BAX 335 [NCT01687608]), transaminitis led to loss of transgenic FIX, despite administration of prednisolone at an AAV dose ≥ 1e12 vg/kg.[26] This suggests that corticosteroids may not work in all circumstances.

The risk of transaminitis appears to be dose dependent, occurring at a high frequency at doses > 1e12 vg/kg. One way to reduce or avoid this complication would be to improve vector performance so that low doses of AAV vectors could be used to mediate plasma FIX activity levels at >5% of normal. With this in mind, the next generation of hemophilia B trials used a FIX complementary DNA (cDNA) containing the Padua mutation, a naturally occurring gain-of-function mutation in humans characterized by leucine (R338L) instead of arginine at position 338 in the catalytic domain. This mutation enhances FIX activity (FIX:C) by fivefold to eightfold for a given amount of FIX antigen. Therefore, a small increase in plasma FIX antigen levels would lead to a substantial increase in plasma FIX clotting activity. There were concerns that the use of a variant transgene would increase the risk of FIX inhibitor development, but preclinical studies in a canine model of hemophilia B showed that FIX-R338L expression after gene transfer did not result in inhibitor formation and, furthermore, was able to induce tolerance.[27]

The first clinical study to evaluate the FIX-R338L transgene in hemophilia B patients (BAX 335 [NCT01687608]) showed that peak FIX activity at 30% to 58% could be achieved at the high-dose level (3e12 vg/kg); unfortunately, expression declined to basal levels in all but 1 patient, who continues to express at the 20% level. The FIX cDNA used in this and the previously mentioned DTX1010 (NCT02618915) study had a higher level of unmethylated cytidine-phosphate-guanosine (CpG) motifs than other studies. Both studies were associated with loss of transgenic FIX despite administration of prednisolone. This led to the hypothesis that an excess of unmethylated CpG motifs, which are common in bacterial, but not mammalian, DNA, triggered a Toll-like receptor 9 response, leading to loss of transduced hepatocytes with an associated transaminitis that is not responsive to corticosteroids.[28]

In the gene therapy trial that followed (SPK-9001), sustained mean FIX activity of 36% was observed in the 10 subjects following a single administration of vector at a dose of 5e11 vg/kg. SPK-9001 consists of bioengineered capsid pseudotyped AAV vector containing the FIX-R338L transgene with a lower number of CpG.[29] Transaminitis occurred in only 2 of 10 subjects who responded well to steroids. All patients were able to stop FIX prophylaxis with a 98% reduction in annualized bleed rates. There was no evidence of inhibitor formation to FIX-Padua. The planned phase 3 trial utilizing this vector is sponsored by Pfizer (New York, NY).

Another study evaluating the FIX-R338L transgene in hemophilia B patients includes AMT-061 from UniQure Therapeutics, which uses the same vector as in AMT-060, with the exception that the FIX cDNA has the R338L (Padua) mutation. The dose under evaluation in AMT-061 is 2e13 vg/kg, the highest used in the AMT-060 Phase 1/2 trial. Mean FIX activity at 36 weeks post–gene transfer in the 3 patients enrolled to date was ~45%, with no reports of transaminitis.[30] Therefore, these results are on par with SPK-9001. Freeline Therapeutics has coupled a synthetic capsid (AAVS3), adapted for more efficient transduction of human hepatocytes, with a modified FIX expression cassette containing the R338L mutation. Preliminary data, reported at the 60th American Society of Hematology Annual Meeting and Exposition (1-4 December 2018, San Diego, CA), from 2 patients treated at a dose of 4.5e12 vg/kg show promise, with steady-state expression at 45% ± 5% and no evidence of transaminitis or neutralizing anti-FIX antibodies.[31]

AAV vectors and gene therapy for hemophilia A

The limited packaging capacity of AAV vectors (4680 nucleotides) and the poor expression profile of FVIII have hindered the use of these vectors for gene therapy of hemophilia A. However, the Nathwani group developed an AAV-based gene-transfer approach

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that addresses the size constraints and inefficient FVIII expression. The FVIII B-domain, which is not required for cofactor activity, was removed to reduce the size of the FVIII expression cassette. Human FVIII expression was improved 10-fold by reorganization of the wild-type cDNA of human FVIII, according to the codon usage of highly expressed human genes.[32] BioMarin Pharmaceutical, who licensed this construct, commenced a phase 1/2 clinical trial using AAV5 pseudotyped vectors made using the baculovirus–insect cell manufacturing method. This vector (AAV5–hFVIII-SQ) was tested in 9 men with severe hemophilia A over a dose range of 6e12 to 6e13 vg/kg in the context of a phase 1/2 dose escalation trial (BMN 270-201).[33] FVIII expression was <3% in the low- and intermediate-dose cohorts. Of the 7 patients treated using 6e13 vg/kg, 6 achieved an FVIII level > 50% (Table 2), using a 1-stage clotting assay, at 1 year. Highly encouraged by these results, a new cohort of 6 patients was treated at a lower dose level of 4e13 vg/kg, leading to sustained FVIII activity of ~30% at 1 year after gene therapy.[33] Across the 13 patients in the 4e13- and 6e13-dose levels, a >90% reduction in annualized bleeding and infusion rates was achieved.

In a separate phase 1/2 clinical trial (GO-8; NCT03001830), the FVIII construct used in the BioMarin study was modified to include a 17-amino-acid peptide comprising 6 *N*-linked glycosylation motifs from the human FVIII B-domain (AAV-HLP-hFVIII-V3) that are highly conserved through evolution.[34] In murine studies, AAV–HLP-hFVIII-V3 mediated expression of FVIII at threefold higher levels compared with AAV–HLP-hFVIII-SQ, encoding the conventional B domain–deleted FVIII. The safety and efficacy of AAV8 serotype pseudotyped HLP-hFVIII-V3, manufactured in mammalian HEK-293 cells, have been assessed in 3 adult men with severe hemophilia A with a short follow-up period of 13 to 47 weeks; FVIII levels of 69% were achieved in 1 of the patients treated at a dose of 2e12 vg/kg. Elevation of ALT was observed in 2 of 3 patients between weeks 4 and 6 after gene transfer, requiring treatment with corticosteroids.[34] No participant has developed a FVIII inhibitor.

In another phase 1/2 hemophilia A gene therapy trial, patients were dosed at between 5e11 and 2e12 vg/kg with SPK-8011, which contains a codon-optimized human FVIII gene under the control of a liver-specific promoter pseudotyped with a bioengineered capsid, LK03.[35] FVIII activity level was between 7% and 13% in the 5 patients treated in the 5e11- and 1e12-vg/kg dose cohorts. Seven patients were treated at the next dose level (2e12 vg/kg), with 5 achieving expression of 30%. All 7 patients received corticosteroids in response to increased ALT levels, but FVIII levels decreased to <5% in 2 patients.

Sangamo Therapeutics (SB-525) announced results of their ongoing phase 1/2 trial in 8 patients with severe hemophilia A treated in 4 dose cohorts (9e11, 2e12, 1e13, and 3e13 vg/kg; n = 2 per cohort.[36] At 6 weeks postinfusion, the 2 patients at the highest tested dose (3e13 vg/kg) reached between 94% and 140% of normal levels. One of the 2 patients treated at the 3e13-vg/kg dose level had ALT elevation requiring a tapering course of oral corticosteroids.

Other hemophilia A gene therapy approaches under evaluation include BAY 2599023 (DTX 201 in partnership with Ultragenyx) and TAK-754 (Takeda).

A key issue with all of these studies will be the durability of transgene expression. In contrast with hemophilia B gene therapy approaches, an oversized transgene is used in most of the hemophilia A studies, which may influence durability. Indeed, longer follow-up of patients in the BMN 270-201 study shows a decline in FVIII activity of 50% between years 1 and 2 post–gene transfer, with a slower decline between years 2 and 3. This decline in transgene expression occurred in the absence of transaminitis, with the greatest decrease in FVIII activity occurring in those patients with the highest level of FVIII expression. For instance, the decrease in expression in the 4e13-vg/kg dose cohort was modest. with a decline in FVIII activity from a mean of 31% at 1 year to 23% at 2 years post–gene transfer. Although the reason for this late decline in FVIII activity remains unclear, potential explanations include loss of the episomally retained oversized FVIII AAV transgene from the transduced hepatocytes and silencing of the FVIII transgene. It is unlikely to be related to the AAV5 capsid, because stable FIX levels for ~3.5 years have been observed in AMT-060, which also used AAV5 pseudotyped vector.

Table 2. Hemophilia A gene therapy trial using AAV

Sponsor	Transgene	Vector	Outcomes	Current status
BioMarin Pharmaceutical (BMN 270)	Codon-optimized BDD-FVIII	AAV5	6e13 vg/kg: FVIII expression in the normal range for 6 of 7 patients. 4e13 vg/kg: FVIII expression in the mild range for 6 of 6 patients. Transient transaminitis at 6-20 wk after gene transfer in 8 of 9 patients	Closed
UCL/St. Jude (GO-8)	Codon-optimized FVIII; B domain replaced with V3 peptide	AAV8	Completed recruitment to low (6e11 vg/kg) and intermediate (2e12 vg/kg) dose levels. FVIII expression = 8-64%. Transient transaminitis in 2 of 3 patients enrolled	Open
Spark Therapeutics (SPK-8011)	BDD-FVIII	AAV-LK03	Completed recruitment to low-dose (5e11 vg/kg), intermediate-dose (1e12 vg/kg), and high-dose (2e12 vg/kg) levels. FVIII expression = 7-30%.	Open
Sangamo Bioscience (SB-525)	BDD-FVIII	AAV6	Enrolled 2 patients in each cohort (9e11, 2e12, 1e13, and 3e13 vg/kg). FVIII expression in the highest-dose cohort = 94-140%.	Open
Ultragenyx Therapeutics (DTX-201	BDD-FVIII	AAVhu37	Trial open	
Takeda (TAK 754)	BDD-FVIII	AAV8	Trial open	

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Obstacles to wider use of AAV vector technology

Safety considerations

Thus far, the risk for liver toxicity accompanied by a loss or reduction in transgene expression appears to be the most worrying toxicity associated with liver-targeted delivery of AAV, as described before. Transaminitis can be readily controlled with a short course of prednisolone in some circumstances, and it appears to be a self-limiting phenomenon with no evidence of late reoccurrence or persistent hepatocellular damage. The precise pathophysiological basis for transaminitis remains unclear, in part because it has not been possible to recapitulate this toxicity in animal models.[27,37] Clinical data show that the increase in ALT post–AAV gene therapy is dependent on vector dose and possibly the number of CpG motifs, as discussed previously, but is independent of AAV capsid, genome configuration, transgene promoter, and method of manufacture.

As expected, all subjects in these trials developed long-lasting AAV capsid–specific humoral immunity. Although the increase in anti-AAV immunoglobulin G does not have direct clinical consequences, its persistence at high titers precludes subsequent successful gene transfer with a vector of the same serotype, in the event that transgene expression should fall below therapeutic levels.

The risk of insertional mutagenesis following AAV-mediated gene transfer has been judged to be low, because proviral DNA is maintained predominantly in an episomal form. This is consistent with the fact that wild-type AAV infection in humans, although common, is not associated with oncogenesis. However, deep-sequencing studies show that integration of the AAV genome can occur in the liver.[38,39] Indeed, a recent publication has found wild-type AAV2 genome fragments integrated in the proximity of known proto-oncogenes in a small percentage of human hepatocellular carcinoma specimens[40]; however, the pathogenic role of AAV2 in this setting is not certain. An increased incidence of hepatocellular carcinoma has been reported in the mucopolysaccharidoses type VII mouse model following perinatal gene transfer of AAV, potentially through integration and disruption of an imprinted region rich in microRNAs and small nucleolar RNAs on mouse chromosome 12.[41] Subsequent studies in other murine models have failed to recapitulate this finding; collectively, the available data in mice, as well as larger animal models, suggest that AAV has a relatively low risk of tumorigenesis.[42] Nevertheless, safety considerations remain paramount and will require careful long-term monitoring of patients, likely beyond the 5 years of follow-up mandated by the U.S. Food and Drug Administration.

Scale-up of vector production

Continued progression toward flexible scalable production and purification methodologies is ongoing to support the commercialization of AAV biotherapeutics. The most widely used method for the generation of AAV entails the transient transfection of HEK-293 cells with plasmids encoding the necessary vector and helper and packaging genes. This method is cumbersome, but progress has been made on improving the productivity to supply phase 3/market-authorization hemophilia gene therapy trials.[43] Another method being used by several biopharmaceutical companies, because of its scalability, is 1 based on baculovirus grown in SF9 insect cells.[44] However, infectivity of AAV made using the baculovirus system is low, due in part to lower levels of VP1 incorporation into the AAV capsid. Attention is currently focused on the downstream purification process so that the purity of clinical-grade AAV preparation can be improved.

Preexisting neutralizing anti-AAV antibody

Between 20% and 70% of patients have preexisting neutralizing anti-AAV antibodies (NAbs) to specific AAV serotypes, which can block efficient gene transfer. These patients are currently excluded from gene therapy trials, but NAbs represent a major limitation to the broad applicability of gene therapy to the hemophilia population. One strategy to overcome NAbs, which works well in animal models, is to switch AAV serotype[20]; however, this may not be applicable in humans because of the cross-reactivity of NAbs. Alternatively, NAbs could be overcome by using immunosuppression or plasmapheresis or by simply increasing vector dose or adding empty capsids.[45]

Affordability of AAV gene therapy

It is likely that gene therapy will command a high price, at least initially, to recoup the development cost. However, successful gene therapy offers the advantage of continuous endogenous expression of clotting factor, which will eliminate breakthrough bleeding and microhemorrhages, thereby reducing comorbidities and the need for frequent medical interventions while improving quality of life. Thus, gene therapy has the potential to yield significant savings for the health care system and society, in general, but it still may prove to be unaffordable for patients living in developing or emerging economies.

Alternative gene therapy approaches that show promise

The AAV genome is maintained in an episomal format, raising the potential for loss of transgene expression with division of the transduced cell. This has engendered interest in targeting approaches.[46] One such approach, "GeneRide," entails the use of AAV vectors to deliver a promoterless FIX gene flanked by a "guide DNA arm" that is several hundred base pairs long and matches a specific locus (eg, albumin locus). Once in the target cell nucleus, the FIX transgene is integrated at a specific site that matches with the "guide DNA arms" within the patient's genome using the host cell's homologous recombination machinery.[47] This approach has been successful in mice but remains to be tested in humans. The same goal can be achieved by using zinc-finger nucleases and CRISPR/Cas9 to mediate targeted integration of the FVIII or FIX gene in a "safe harbor" using nonhomologous end joining.[48] Recently, Sangamo Therapeutics, together with Georgetown University, announced the treatment of the first hemophilia B patient (SB-FIX; NCT02695160) with a gene-editing approach in which the targeted integration of the FIX cDNA into an intron of the host cell albumin gene is promoted by zinc-finger nucleases following systemic administration of 3 separate AAV vectors. A potential concern with this approach is the effect of persistent expression of the endonuclease following AAV gene transfer, given that transgene expression can persist for as long as 8 years.

Integrating vectors, based on lentivirus to propagate integration of the FIX or FVIII transgene into target cells, is also under evaluation in hematopoietic stem cells or blood outgrowth endothelial cells following ex vivo manipulations. The development of a new generation of lentiviral vectors designed for efficient delivery of the transgene to the liver following systemic delivery of vectors carrying FVIII and FIX shows great promise and supports the further evaluation of this approach in the clinic.[49]

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Conclusions

The availability of convincing evidence for long-term expression of transgenic FVIII and FIX at therapeutic levels, resulting in amelioration of the bleeding diathesis following AAV-mediated gene transfer, is an important step toward development of curative gene therapy. Several obstacles still remain, but the field is evolving at a rapid pace, raising the prospects of eventual licensure of gene therapy for the hemophilias. Such a product would change the treatment paradigm for patients with severe hemophilia and facilitate the development of gene therapy for other monogenetic disorders, particularly those with limited or nonexistent treatment options.

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References

1. Nathwani AC, Tuddenham EG. Epidemiology of coagulation disorders. *Baillieres Clin Haematol.* 1992;5(2):383-439.
2. Loomans JI, Fijnvandraat K. Mortality caused by intracranial bleeding in non-severe hemophilia A patients: reply. *J Thromb Haemost*. 2017;15(8): 1710-1711.
3. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med.* 2007;357(6):535-544.
4. Darby SC, Kan SW, Spooner RJ, et al. Mortality rates, life expectancy, and causes of death in people with hemophilia A or B in the United Kingdom who were not infected with HIV. *Blood*. 2007;110(3): 815-825.
5. Lambert T, Benson G, Dolan G, et al. Practical aspects of extended half-life products for the treatment of haemophilia. *Ther Adv Hematol*. 2018; 9(9):295-308.
6. Konkle BA, Shapiro A, Quon D, et al. BIVV001: The first investigational factor VIII therapy to break through the VWF ceiling in hemophilia A, with potential for extended protection for one week or longer. *Blood*. 2018;132(suppl 1):636.
7. Shima M, Hanabusa H, Taki M, et al. Factor VIII-mimetic function of humanized bispecific antibody in hemophilia A. *N Engl J Med*. 2016; 374(21):2044-2053.
8. Oldenburg J, Mahlangu JN, Kim B, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med.* 2017;377(9):809-818.
9. Sehgal A, Barros S, Ivanciu L, et al. An RNAi therapeutic targeting antithrombin to rebalance the coagulation system and promote hemostasis in hemophilia. *Nat Med*. 2015;21(5):492-497.
10. Eichler H, Angchaisuksiri P, Kavakli K, et al. A randomized trial of safety, pharmacokinetics and pharmacodynamics of concizumab in people with hemophilia A. *J Thromb Haemost*. 2018;16(11):2184-2195.
11. Ljung RC. Prophylactic infusion regimens in the management of hemophilia. *Thromb Haemost*. 1999;82(2):525-530.
12. Roth DA, Tawa NE Jr, O'Brien JM, Treco DA, Selden RF; Factor VIII Transkaryotic Therapy Study Group. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med.* 2001;344(23):1735-1742.
13. Roth DA, Tawa NE Jr, Proper J, et al. Implantation of non-viral ex vivo genetically modified autologous dermal fibroblasts that express B-domain deleted human factor VIII in 12 severe hemophilia A study subjects. *Blood.* 2002;100:116a.
14. Qiu X, Lu D, Zhou J, et al. Implantation of autologous skin fibroblast genetically modified to secrete clotting factor IX partially corrects the hemorrhagic tendencies in two hemophilia B patients. *Chin Med J (Engl).* 1996;109(11):832-839.
15. Mannucci PM. Ham-Wasserman Lecture: Hemophilia and related bleeding disorders: a story of dismay and success. *Hematology.* 2002; 2002(1):1-9.
16. Manno CS, Chew AJ, Hutchison S, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood.* 2003;101(8):2963-2972.
17. High KA, Manno CS, Sabatino DE, et al. Immune responses to AAV and to factor IX in a phase I study of AAV-mediated liver-directed gene transfer for hemophilia B. *Blood.* 2003;102(11):154a-155a.
18. Nathwani AC, Davidoff A, Hanawa H, Zhou JF, Vanin EF, Nienhuis AW. Factors influencing in vivo transduction by recombinant adeno-associated viral vectors expressing the human factor IX cDNA. *Blood.* 2001;97(5):1258-1265.
19. Nathwani AC, Gray JT, Ng CY, et al. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood*. 2006;107(7):2653-2661.
20. Nathwani AC, Gray JT, McIntosh J, et al. Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood.* 2007;109(4):1414-1421.
21. Fagone P, Wright JF, Nathwani AC, Nienhuis AW, Davidoff AM, Gray JT. Systemic errors in quantitative polymerase chain reaction titration of self-complementary adeno-associated viral vectors and improved alternative methods. *Hum Gene Ther Methods*. 2012;23(1):1-7.
22. Thomas CE, Storm TA, Huang Z, Kay MA. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. *J Virol*. 2004;78(6):3110-3122.
23. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA*. 2002;99(18):11854-11859.
24. Nathwani AC, Reiss U, Tuddenham E, et al. Adeno-associated mediated gene transfer for hemophilia B: 8 year follow up and impact of removing "empty viral particles" on safety and efficacy of gene transfer. *Blood.* 2018;132(suppl 1):491.
25. Miesbach W, Meijer K, Coppens M, et al. Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B. *Blood*. 2018;131(9):1022-1031.
26. Doshi BS, Arruda VR. Gene therapy for hemophilia: what does the future hold? *Ther Adv Hematol*. 2018;9(9):273-293.
27. Finn JD, Nichols TC, Svoronos N, et al. The efficacy and the risk of immunogenicity of FIX Padua (R338L) in hemophilia B dogs treated by AAV muscle gene therapy. *Blood*. 2012;120(23):4521-4523.
28. Vandamme C, Adjali O, Mingozzi F. Unraveling the complex story of immune responses to AAV vectors trial after trial. *Hum Gene Ther*. 2017; 28(11):1061-1074.
29. George LA, Sullivan SK, Giermasz A, et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *N Engl J Med*. 2017; 377(23):2215-2227.
30. Von Drygalski A, Giermasz A, Castaman G, et al. Phase 2b trial of AMT-061 (AAV5-Padua hFIX): translation into humans of an enhanced gene transfer vector for adults with severe or moderate-severe hemophilia B. *Haemophilia*. 2019;25:30.
31. Chowdary P, Shapiro S, Davidoff AM, et al. A single intravenous infusion of FLT180a results in factor IX activity levels of more than 40% and has the potential to provide a functional cure for patients with haemophilia B. *Blood*. 2018;132(suppl 1):631.
32. McIntosh J, Lenting PJ, Rosales C, et al. Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. *Blood*. 2013;121(17):3335-3344.
33. Rangarajan S, Walsh L, Lester W, et al. AAV5-factor VIII gene transfer in severe hemophilia A. *N Engl J Med*. 2017;377(26):2519-2530.
34. Nathwani AC, Tuddenham E, Chowdary P, et al. GO-8: preliminary results of a phase I/II dose escalation trial of gene therapy for haemophilia a using a novel human factor VIII variant. *Blood.* 2018;132(suppl 1):489.
35. George LA, Ragni M, Sullivan SK, et al. SPK-8011: Preliminary results from a phase 1/2 trial of investigational gene therapy for hemophilia A. *Haemophilia*. 2018;24:30-31.
36. Konkle BAKS, Visweshwar N, Harrington T, et al. Initial results of the Alta study, a phase 1/2, open label, adaptive, dose-ranging study to assess

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the safety and tolerability of SB-525 gene therapy in adult subjects with severe hemophilia A. In: Proceedings from ISTH 2019: Practice in Thrombosis and Hemostasis, 2019, Melbourne, Australia.
37. Simioni P, Tormene D, Tognin G, et al. X-linked thrombophilia with a mutant factor IX (factor IX Padua). *N Engl J Med*. 2009;361(17): 1671-1675.
38. Nowrouzi A, Penaud-Budloo M, Kaeppel C, et al. Integration frequency and intermolecular recombination of rAAV vectors in non-human primate skeletal muscle and liver. *Mol Ther*. 2012;20(6): 1177-1186.
39. Li H, Malani N, Hamilton SR, et al. Assessing the potential for AAV vector genotoxicity in a murine model. *Blood*. 2011;117(12):3311-3319.
40. Nault JC, Datta S, Imbeaud S, et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat Genet*. 2015; 47(10):1187-1193.
41. Donsante A, Miller DG, Li Y, et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science*. 2007;317(5837):477.
42. Kay MA. AAV vectors and tumorigenicity. *Nat Biotechnol*. 2007;25(10): 1111-1113.
43. Grieger JC, Samulski RJ. Adeno-associated virus vectorology, manufacturing, and clinical applications. *Methods Enzymol*. 2012;507: 229-254.
44. Cecchini S, Negrete A, Kotin RM. Toward exascale production of recombinant adeno-associated virus for gene transfer applications. *Gene Ther*. 2008;15(11):823-830.
45. Mingozzi F, Anguela XM, Pavani G, et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. *Sci Transl Med*. 2013; 5(194):194ra92.
46. Davidoff AM, Nathwani AC. Genetic targeting of the albumin locus to treat hemophilia. *N Engl J Med*. 2016;374(13):1288-1290.
47. Barzel A, Paulk NK, Shi Y, et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature*. 2015;517(7534):360-364.
48. Sharma R, Anguela XM, Doyon Y, et al. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. *Blood*. 2015;126(15):1777-1784.
49. Milani M, Annoni A, Moalli F, et al. Phagocytosis-shielded lentiviral vectors improve liver gene therapy in nonhuman primates. *Sci Transl Med*. 2019;11(493):eaav7325.

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