Gene therapy for hemophilia

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Abstract

Hemophilia is an X-linked inherited bleeding disorder consisting of two classifications, hemophilia A and hemophilia B, depending on the underlying mutation. Although the disease is currently treatable with intravenous delivery of replacement recombinant clotting factor, this approach represents a significant cost both monetarily and in terms of quality of life. Gene therapy is an attractive alternative approach to the treatment of hemophilia that would ideally provide life-long correction of clotting activity with a single injection. In this review, we will discuss the multitude of approaches that have been explored for the treatment of both hemophilia A and B, including both *in vivo* and *ex vivo* approaches with viral and nonviral delivery vectors.

Keywords

Gene therapy; Hemophilia; Factor IX; Factor VIII; AAV; Lentivirus; Review

2. INTRODUCTION

Hemophilia is an X-linked inherited disorder resulting in a deficiency in the clotting functionality of blood. Depending on which clotting factor the patient is deficient in, the disease is classified as hemophilia A (deficiency in factor VIII, F.VIII) or hemophilia B (deficiency in factor IX, F.IX). Hemophilia A has a higher prevalence, occurring in about 1:5,000 male births, while hemophilia B occurs in about 1:25,000. The loss of function of either F.VIII or F.IX results in a defect in the intrinsic clotting cascade (Figure 1). In the intrinsic pathway, exposure of circulating F.XII to a damaged surface causes its activation. Active F.XII (F.XIIa) activates F.XI, which then in conjunction with extrinsically activated tissue factor-F.VIIa complex (extrinsic factor Xase) proceeds to cleave the zymogens F.IX and F.X into their active forms, F.IXa and F.Xa. F.IXa is a serine protease whose function depends on the post-translational γ-carboxylation of F.IX by vitamin K. Meanwhile, activation by the extrinsic pathway also results in cleavage of the glycoprotein F.VIII into activated F.VIIIa. F.VIIIa (cofactor) and F.IXa (enzyme) come together to form the intrinsic factor Xase. This complex cleaves F.X into F.Xa at a rate much higher than the extrinsic factor Xase, such that in the end about 90 percent of F.Xa is produced by the intrinsic complex. The activity of the intrinsic factor Xase is dependent on binding to phospholipid

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membranes on endothelial cells or platelets as well as free Ca^{2+}. Activated F.Xa facilitates the conversion of prothrombin into thrombin, which then catalyzes the formation of the fibrin clot. Thus, a genetic defect in F.VIII or F.IX prevents the assembly of the intrinsic factor Xase, significantly impairing the ability to activate F.X and induce formation of the fibrin clot.

While the determination of which factor is missing is important for treatment, the clinical symptoms of hemophilia A and B are essentially comparable. The severity of X-linked hemophilia is dependent on the degree of residual clotting activity. Mild cases (5-40% activity) are typically asymptomatic outside of major trauma or surgery, whereas moderate cases (1-5% activity) are somewhat more vulnerable, and may evidence prolonged bleeding even from minor injuries. However, severe hemophilia (<1% activity) brings additional complications. In addition to the difficulty responding to injury, these patients frequently develop spontaneous bleeds in capillary beds, particularly within joints. Over time, this causes significant chronic deterioration of the joints if not properly managed. Currently, hemophilia is treated by intravenous delivery of replacement clotting factor, either plasma-derived or recombinant. This therapy can be performed on demand, though it has been suggested that prophylactic management (typically 3 injections per week) can reduce joint damage over time (1). Longer-lasting clotting factors that would reduce the required frequency of injections are currently in development (2).

In addition to the inconvenience of these frequent injections, protein replacement therapy also carries the risk of deleterious immune responses against the therapeutic protein. As patients are not naturally producing clotting factor, the immune system can recognize the exogenous protein as a foreign antigen and form antibodies against the protein that prevent its function; these neutralizing antibodies are also known as inhibitors. The frequency of inhibitor formation varies by disease: about 25-30% of hemophilia A, but only about 5% of hemophilia B patients develop inhibitors. The risk for inhibitor formation varies depending on a number of factors, including the severity of the underlying mutation; both preclinical and clinical studies indicate that more residual protein expression reduces inhibitor formation in both hemophilia A and B (3-7). Although the frequency of inhibitors in hemophilia B is reduced, they are typically more severe, with the potential for anaphylaxis as well as nephrotic syndrome due to circulating antigen-antibody complexes (5). Currently, the only treatments for inhibitor formation are immune tolerance induction (ITI) protocols. These procedures are both costly and time-consuming, and during the procedure bypass reagents must be employed which are both less effective and more risky. Additionally, the treatment is typically effective in only around two-thirds of patients (8, 9).

Gene therapy represents an appealing alternative to protein replacement therapy. Instead of repeated injections of protein, it would ideally involve a single injection that would induce long-term production of the defective clotting factor. Expression at only 5% of endogenous levels can improve the disease to a mild phenotype and essentially eliminate the risk of spontaneous bleeding events as well as the need for prophylactic protein therapy. Although a variety of mechanisms to introduce the transgene have been investigated, some of the most popular are recombinant viral vectors. In particular, adeno-associated virus (AAV), a small and non-pathogenic parvovirus with an episomal genome, has been used extensively,

including in multiple clinical trials for the treatment of hemophilia B (10). Additionally, lentiviral vectors (LV) based on HIV-1 which integrate into the host's genome have also been employed in a multitude of preclinical studies both for *in vivo* and *ex vivo* gene transfer (11). Finally, in addition to these gutted viral vectors, research is also being performed on non-viral gene transfer (12). Some examples of sustained correction via liver-directed AAV-mediated gene transfer are demonstrated in figure 2. These include correction of whole blood clotting time in canine studies and activated partial thromboplastin time (aPTT) in mice for hemophilia B, as well as aPTT correction in a murine model of hemophilia A (Figure 2A, C).

However, beyond merely introducing the transgene, it is also important to maintain production of the clotting factor by avoiding the deleterious impact of the immune system on gene transfer, either against the delivery vector or the transgene itself. For instance, preclinical studies with LV vectors have revealed that innate immune responses involving type I interferon (IFN) production can lead to impaired transgene expression and CD8+ T cell responses against the transgene (13, 14). Clinical trials of AAV-mediated gene transfer have also revealed the detrimental impact of pre-existing immunity to the AAV capsid, both in regards to neutralizing antibodies (NAB) preventing transduction as well as a memory CD8+ T cell response to the viral capsid that can eliminate transduced hepatocytes (15). Finally, there is always the risk of an immune response against the clotting factor itself (particularly in the case of hemophilia A), which would inhibit the gene therapy itself as well as obstructing further efforts to treat with recombinant protein (16). Beyond merely avoiding the immune response, though, it is preferable to actually induce immune tolerance to the transgenic protein, ensuring that endogenous production is not eliminated as well as allowing for the administration of supplemental clotting factor (*e.g.* during trauma or surgery) without provoking an inhibitor response (16, 17). Immune tolerance in preclinical studies is typically demonstrated by the intravenous administration of recombinant F.VIII or F.IX. This normally provokes an inhibitor response in hemophilic mice for both diseases; however, following gene transfer, mice that have been tolerized maintain clotting correction and fail to form inhibitory antibodies, as opposed to naïve control animals (Figure 2B, D). A variety of animal models of hemophilia are available for preclinical studies, and clinical trials for both diseases have been attempted as well (Figure 3). In this review, we will provide a comprehensive overview of viral and non-viral gene therapy approaches for both hemophilia B and hemophilia A, with an additional focus on the ability of these approaches to avoid destructive immunity or induce transgene-specific tolerance.

3. GENE THERAPIES FOR HEMOPHLIA B

Of the two diseases, gene therapy for hemophilia B has been more successful, having advanced to multiple recent clinical trials. Primarily, this is due to the simplicity of F.IX compared to F.VIII. The *F9* coding region is only about 1.4 kb, and it encodes a single domain protein of 461 amino acids. This small size allows it to be easily packaged in a recombinant adeno-associated virus, a gene therapy vector that has provided promising results for a variety of genetic disorders (18). Additionally, the posttranslational modification of F.IX can be effectively carried out in skeletal muscle, allowing early studies

to be carried out in a target tissue less risky than a critical organ such as the liver, the natural site of F.IX synthesis (19).

3.1. Adeno-associated virus

Adeno-associated virus (AAV) is a parvovirus with a single-stranded DNA genome of about 4.7 kb. It is a dependovirus that is unable to replicate in the absence of a helper virus such as adenovirus; thus, although it is a common natural infection, AAV is not associated with any known pathogenic infections in humans. Recombinant AAVs are modified by the removal of any DNA encoding for viral protein. Only the inverted terminal repeats (ITRs) required for packaging are retained from the viral genome, giving rAAV vectors a packaging capacity of about 5 kb for the promoter and gene of interest. Several factors make AAV an attractive vector for *in vivo* gene therapy, including its ability to infect non-dividing cells, the maintenance of vector genomes as episomal concatemers (minimizing the risk of insertional mutagenesis), its low immunogenicity, and the wide variety of capsid serotypes that allow gene delivery to numerous target tissues (20-24).

Early studies for gene therapy for hemophilia B with AAV focused on delivery to skeletal muscle, both in animal models (mice and dogs) and humans (25-30). Although more recent clinical trials for gene transfer to skeletal muscle have used AAV serotype 1 (AAV1) vectors due to their superior transduction capacity in myocytes, these early studies used AAV2 vectors (29-34). It was found that expression of functional human factor IX (hF.IX) was possible in skeletal muscle, though potential complications from an immune response against hF.IX could occur in some situations, particularly in cases with more severe mutations (3, 27, 35). The local F.IX expression and anti-F.IX immune response were found to be critically important during muscle-directed gene transfer, and this consequence could be avoided by carefully titering the vector dose per site or with transient immunosuppression (35-39). In a clinical trial, intramuscular delivery of hF.IX to patients with missense mutations met safety requirements and demonstrated that *in vivo* gene therapy with AAV could be a viable treatment strategy; however, although the persistence of transduced fibers was observed for ten years, expression never reached therapeutic levels at any of the doses tested (30, 40). Although some preclinical studies are still studying intramuscular gene therapy, most research for hemophilia B—including clinical trials—has shifted to hepatic gene transfer (41, 42).

The liver represents a superior target for F.IX expression for several reasons. In healthy individuals, F.IX is naturally produced in the liver. Hepatocytes have more efficient secretion machinery than myocytes, allowing them to produce higher transgene levels. Most interesting, though, is the fact that liver-directed gene transfer has been shown to induce transgene-specific tolerance that prevents subsequent antibody and CD8^{+} T cell responses (43, 44). Further studies have shown that this tolerance is mediated by antigen-specific regulatory T cells (Treg) (45-47). Tregs are a subset of CD4^{+} helper T cells that are typically defined as CD4^{+} CD25^{+} FoxP3^{+} lymphocytes, and are regarded as one of the most important regulators of peripheral tolerance. Through a variety of mechanisms, including cytokine release and contact-dependent interactions, they can prevent immune responses in an antigen-specific manner, though they have also been reported to have polyclonal

suppressive effects (reviewed in (48)). Though the mechanism of Treg induction is not entirely clear, it can occur for both secreted and cytoplasmic transgenes expressed in hepatocytes, and it depends on both IL-10 and TGF-β (49, 50). In addition to suppression of *de novo* immune responses against factor IX, hepatic gene transfer can reverse an active high-titer inhibitor response (4). This mechanism depends on induction of Tregs, and the continued presence of these cells is required to maintain tolerance. The high expression of F.IX in mice following hepatic gene transfer (~20-40% of normal) may also be responsible for this rapid clearance of F.IX-specific antibodies due to suppression of memory B cells (4, 51). Though this phenomenon is understandably difficult to verify in human studies, it is encouraging that, to date, none of the patients treated with a liver-directed AAV vector have formed a F.IX-specific immune response (52, 53).

However, clinical trials have revealed an additional complication that was not predicted by preclinical studies: the impact of capsid-specific memory CD8$^+$ T cells (54, 55). The first clinical trial of hepatic gene transfer with AAV vectors for hemophilia B using an AAV2 vector delivered by injection through the hepatic artery revealed a couple of important findings (52). Contrary to previous expectations, it was found that even low-titer anti-capsid neutralizing antibodies could prevent successful transduction. Additionally, in one patient at the highest dose cohort, an initial rise in circulating hF.IX was detected; however, by 4 weeks post-injection, this expression began to decline with a concomitant rise in liver enzymes indicative of hepatic damage (albeit at asymptomatic levels). By 8 weeks, circulating hF.IX was no longer detectable. Further studies revealed that this decline occurred due to a capsid-specific memory CD8$^+$ T cell response (52, 56).

Several advances in AAV vector design were made that were incorporated into the second hepatic gene therapy clinical trial. Rather than using AAV2, this study used an AAV8 vector, a serotype derived from rhesus macaques that has greater liver specificity than AAV2 (31). This allows for elevated transgene expression from an equivalent vector dose, and expression was comparable when the vector was delivered via tail vein or portal vein injection; these greater expression levels also enhanced the F.IX-specific tolerance induced by hepatic gene transfer (57). The prevalence of neutralizing antibodies against AAV8 is lower than AAV2 among the human population, allowing more patients to potentially be treated with AAV8 vectors (58, 59). Additionally, a self-complementary AAV (scAAV) vector rather than a single-stranded AAV (ssAAV) was utilized. This modification, performed by mutating one of the viral ITRs, forces the vector to package double-stranded DNA rather than the single-stranded genome found in the wild-type virus (60). These vectors bypass the need for second strand synthesis, a rate-limiting step during AAV transduction, allowing them to produce higher transgene levels (61, 62). Expression of hF.IX in mice and non-human primates was elevated with scAAV vectors (63-65).

Somewhat worryingly, recent studies have shown that scAAV vectors induce stronger innate immune responses through toll-like receptor 9 (TLR9) than ssAAV that can enhance transgene-specific immune responses during transfer to skeletal muscle (66-68) (reviewed in (69)). Similarly, the removal of CpG motifs sensed by TLR9 from the vector genome can allow expression in skeletal muscle by preventing the formation of a CD8$^+$ T cell response against LacZ (70). In hemophilic mice with a null mutation, we found that scAAV enhances

CD8^{+} T cell but not antibody responses to hF.IX following intramuscular gene transfer (71). However, in transgenic hemophilic mice that are partially tolerant to hF.IX due to expression of truncated, nonfunctional hF.IX, no immune response was observed regardless of the vector genome, suggesting that the enhanced immunogenicity of scAAV vectors may not break tolerance when administration with ssAAV is tolerated. This was also true during hepatic gene transfer, where the enhanced innate immune response to scAAV did not result in transgene-specific immune responses (67). Unlike adenoviral vectors, the innate immune response from scAAV was not sufficient to induce type I interferon-dependent transgene silencing (72). However, the innate immune response may not always be deleterious, as activation of the alternative NF-κB pathway has been shown to enhance transgene expression from AAV vectors (73).

Thus, the second clinical trial of hepatic gene therapy for hemophilia B used a scAAV8 vector delivered via peripheral vein injection (53). As previously observed, a rise in liver enzymes was detected only in subjects treated at the highest dose, though at around 8 weeks rather than 4 weeks post-injection. Administration of prednisolone at this time was able to suppress the CD8^{+} T cell response against the AAV capsid, allowing F.IX activity to persist at around 2% of normal. In a subsequent subject, more careful monitoring around this time point allowed the prednisolone treatment to preserve F.IX expression at ~5% normal. These data represent the first successful clinical gene therapy for hemophilia B. Although this treatment appears to have been successful, there are still problems that limit its applicability.

First, many hemophilia patients, particularly those infected with hepatitis C, are not eligible for general steroidal immune suppression. To this end, several alternatives are being studied in animal models. Targeting of hepatocytes by capsid-specific cytotoxic T lymphocytes (CTLs) requires the degradation of input viral capsid, as the recombinant vectors do not encode for any viral proteins. This process occurs primarily through proteasomal degradation, which is provoked by phosphorylation and subsequent ubiquitination of the AAV capsid (74, 75). The role of the proteasome is supported by a recent study demonstrating that presentation of AAV capsid epitopes in MHC class I (allowing the cell to be targeted by capsid-specific CTLs) requires endosomal escape into the cytoplasm but is independent of nuclear uncoating, suggesting that this degradation occurs in the cytoplasm (76). To avoid this process, mutant AAV2 capsids have been developed in which tyrosine residues have been mutated to phenylalanine (77). Specifically, a combination of three mutations (Y444+500+730F) allows for greatly enhanced transgene expression both *in vitro* and *in vivo* (78). In addition to enhancing transduction, these vectors reduce the ability for capsid-specific CD8^{+} T cells to target transduced hepatocytes (79). Adoptive transfer of *ex vivo* expanded capsid-specific CTLs to immune-deficient mice resulted in more residual hF.IX expression and less elevation in liver enzymes in mice transduced with AAV2(Y-F) vectors than wild-type AAV2. This effect could be further enhanced with a proteasome inhibitor. Interestingly, AAV8 vectors showed prolonged vulnerability to CTL targeting, and additional studies have suggested that, depending on the vector genome, CD8^{+} T cells can detect AAV8 for as long as 6 months in mice (79, 80). This difference in the kinetics of antigen presentation between AAV2 and AAV8 may explain the delay in CTL response observed in the second clinical trial for hepatic gene transfer relative to the first.

The other limitation of the current approach is its inability to treat patients with pre-existing neutralizing antibodies (NAB) to AAV8 (reviewed in (81)). Following the first hepatic clinical trial, it was discovered that NAB titers as low as 1:5 could severely impact transgene expression *in vivo* (82-84). Additionally, even if patients initially lack NAB, after the vector injection they will develop an anti-capsid antibody response that will prevent readministration of the vector. Several approaches have been investigated to negate the impact of NAB. Attempts have been made to modify the AAV capsid itself, both by rational design and directed evolution, to negate its susceptibility to NAB binding (85, 86). Plasmapheresis has shown some success at removing NAB from sera, particularly with repeated cycles, though the ability to reach titers sufficiently low to allow transduction may be restricted to individuals with initially low titers (<1:100) (87, 88). Isolation of the liver using balloon catheters and delivering the vector via portal vein injection may also increase the success of gene transfer in the presence of NAB (89). Pharmacological approaches have primarily focused on the use of rituximab, a monoclonal antibody against CD20 that is currently approved for B cell depletion in several autoimmune diseases and B cell cancers. Rituximab alone was able to partially reduce AAV NAB titers in patients with rheumatoid arthritis, though most subjects did not drop below a titer of 1:5 (90). These residual NAB are likely due to an incomplete depletion of B cells by rituximab, as well as the fact that plasma cells do not express CD20, rendering them immune to the cytotoxic activity of rituximab (91). In nonhuman primates, a combination of rituximab and cyclosporine A was more effective at eliminating NAB (92). Additionally, a non-depleting anti-CD4 antibody prevented the development of NAB following AAV gene transfer, though the effects on pre-existing anti-capsid antibodies remain to be seen (93). Finally, the second hepatic clinical trial revealed an interesting phenomenon: although expression levels at the highest dose in both trials were similar, circulating hF.IX expression was observed at low doses in the second trial but not the first. Interestingly, the vector formulation in the second trial contained empty AAV capsids lacking DNA, a byproduct of rAAV production that was removed during the previous trial. Thus, Mingozzi *et al.* have found that the addition of empty capsids can serve as a decoy for pre-existing NAB, with higher titers requiring a greater excess of empties relative to the DNA-containing capsids (94). These empty capsids have been modified to remove the binding site that allows entry into hepatocytes, though it remains to be seen whether this will prevent these decoy capsids from enhancing the CD8^{+} T cell response in human studies (95). As these various approaches each seem to be partially effective, it is possible that some combination of them (or a yet undiscovered therapy) will allow us to bypass NAB and make AAV gene therapies available for patients with pre-existing immunity.

Great strides have already been made in clinical trials for AAV-mediated gene therapy for hemophilia B. Earlier intramuscular studies provided a proof of concept and safety information, while liver-directed gene delivery has seen greater success, though it can still be limited by both NAB and anti-capsid CTL responses (Figure 4). However, there are still problems to solve to improve this strategy, as indicated by the fact that, in addition to the two completed trials, there are currently three active clinical trials using AAV vectors to treat hemophilia B (Table 1).

3.2. Retrovirus and Lentivirus

Although the most clinical success to date has been seen with AAV, numerous studies have also been performed using integrating viral vectors to treat hemophilia B. Unlike AAV, where the maximum duration of expression and stability of episomal concatemers is not yet known, expression from an integrating vector would persist as long as cells from the transfected lineage remain. However, integration also comes with the risk of insertional mutagenesis. Initially, studies focused on the use of γ-retroviral vectors based on Moloney murine leukemia virus (MMLV); however, the inability of these viruses to transduce nondividing cells as well as their propensity for oncogenesis, as seen in a clinical trial for SCID-X1, has caused these vectors to be looked on less favorably (96, 97). Rather, research has shifted focus towards lentiviral vectors (LV). LV is based on HIV-1 (reviewed in (98)). Like γ-retroviral vectors, LV is an integrating ssRNA vector with a packaging capacity of ~10 kb. Unlike the former, though, LV can transduce non-dividing cells (99). Pseudotyping the virus with various envelope proteins allows for significant alterations to tissue tropism and biodistribution. LV is most commonly pseudotyped with the VSV-G protein from vesicular stomatitis virus, though proteins from other viruses—including filovirus, Ebola, LCMV, and rabies virus—have also been employed (100-102). VSV-G pseudotyping allows for transduction of a wide variety of cell types both *in vitro* (*e.g.* CD34$^+$ stem cells) and *in vivo* (*e.g.* liver, brain, and muscle). The integration pattern of LV is somewhat random, though biased towards transcriptional units. Compared to γ-retroviral vectors, which preferentially integrate into transcription start sites, LV prefers to integrate further into the active transcription unit (103, 104). Additionally, some LV incorporate a mutation that eliminates the transcriptional activity of the long terminal repeats; these LV are termed self-inactivating (SIN) (105). Although this modification can also reduce the oncogenicity of γ-retroviral vectors, studies in a cancer-prone mouse model have suggested that integration levels as much as 10-fold higher may be required for LV to reach similar oncogenic potential to γ-retroviral vectors (106-108). Overall, although it is clear that there are risks associated with integrating vectors, the exact degree of risk is currently unclear (109).

One significant advantage of an integrating vector is that it can be applied to cells *ex vivo*, and the transduced cells can then be reintroduced, bypassing any interference from an immune response against the virus (Figure 5). While this strategy is more often applied to gene therapy for hemophilia A, some research has also been done with *ex vivo* gene transfer for hemophilia B. The most common target for this type of therapy is hematopoietic stem cells (HSCs), due to the ease of both harvesting and reintroducing these cells. Following up on earlier studies performed with F.VIII, it was shown that gene transfer to bulk HSCs using LV resulted in sustained F.IX expression that tolerized recipient mice to hF.IX and could be transferred to secondary and tertiary recipients (110, 111). A similar outcome was achieved by targeting expression to cells of the erythroid lineage (112). However, in both studies, mice were lethally irradiated in order to allow engraftment of the transfected HSCs (111, 112). To enhance the clinical relevance of this approach, a dual expression LV expressing both hF.IX and a drug resistance gene (methylguanine methyltransferase mutant P140K, MGMT) was used to achieve correction following engraftment with a moderate busulfan conditioning that was nonmyeloablative (113, 114). In addition to erythroblasts, F.IX expression has also been investigated in platelets following the success seen with that

strategy for F.VIII (115). A transgenic mouse expressing F.IX in platelets demonstrated correction of the bleeding phenotype (116). However, unlike with F.VIII, the activity of F.IX expressed in platelets was still adversely affected by inhibitory antibodies. This effect can also be achieved with gene transfer using LV, and this strategy induces tolerance to hF.IX in treated mice as well (117). More recently, studies have begun to explore expression in alternative types of stem cells. Treatment of murine adipose tissue-derived stem/stromal cells (mADSCs) with LV can induce sustained hF.IX expression *in vitro*, though it remains to be seen whether these cells can engraft into a recipient and provide sustained correction (118). Similarly, menenchymal stem cells (MSCs) derived from human cord blood can be transduced with retroviral or LV vectors to produce F.IX *in vitro* and *in vivo*, though efforts are still underway to optimize the matrix for cell growth and F.IX production (119-122).

In addition to *ex vivo* gene transfer, LV can also be employed for *in vivo* gene therapy. Although some studies have attempted to deliver LV prenatally to induce F.IX expression, these approaches have mostly focused on inducing the expression of F.IX in hepatocytes (123-125). Early studies demonstrated that it is possible to induce sustained hF.IX expression in hepatocytes using LV, and that proliferation is not required for transduction (126-129). F.IX expression has also been induced *in vivo* under the control of tetracycline using LV (130). However, as with AAV, *in vivo* gene transfer revealed barriers to transduction set up by the immune system. Unlike with AAV vectors, hepatic gene therapy with LV did not initially induce tolerance to the transgene. It was discovered that, due to their broad tropism, VSV-G-pseudotyped vectors could transduce antigen-presenting cells (APCs) (131). Since then, a number of steps have been taken to enhance the specificity of expression from LV. The use of a liver-specific promoter allowed for sustained expression of F.IX, but still did not completely eliminate off-target transduction (132). Nonspecific expression in APCs could be further reduced by adding target sequences for a hematopoietic-specific microRNA (miR-142) to the transcript. Mice treated with miR-142-regulated LV showed sustained expression of hF.IX and remained nonresponsive following immunogenic challenge, suggesting that they had been tolerized to hF.IX via a mechanism mediated by regulatory T cells (133, 134). Similar to what was found with AAV vectors, hepatic expression of F.IX via LV was also able to reverse an active inhibitor response, suggesting that hepatic gene transfer can be both therapeutic and tolerogenic even in the presence of an active immune response (135).

As with AAV, eliminating barriers to transduction posed by the adaptive immune system revealed deleterious effects of innate immunity on LV gene transfer. *In vivo* administration of LV triggers type I interferon (IFN) responses that restrict gene transfer and promote vector clearance (13). Consistent with previous *in vitro* findings, this mechanism was shown to be partially dependent on TLR7, which senses the ssRNA genome of LV (14, 136, 137). However, TLR-independent innate immune responses were detected as well (14). Through the use of a reverse-transcriptase inhibitor, it was suggested that these responses were due to the cytoplasmic sensing of viral DNA. Recently, cyclic GMP-AMP synthetase (cGAS) has been implicated as a sensor of cytoplasmic DNA with crucial roles both *in vitro* and *in vivo* (138-142). Interestingly, cGAS has been reported to induce type I IFNs in response to HIV

and other retroviruses, suggesting that it may be responsible for this TLR-independent innate immune response to LV (143).

Aside from concerns of immunogenicity, the most important safety concern for LV remains the risk of insertional mutagenesis. Although the degree of risk remains a matter of debate, the potential for tumorigenesis following LV gene therapy has not been eliminated (144). To this end, integrase-defective lentiviral vectors (IDLV) have been developed; expression occurs from these viruses without integration at a rate above random (145-148). The viral genome is stable in episomal form, persisting in nondividing cells but not actively dividing cells (149-151). These findings were confirmed by *in vivo* studies with F.IX. Although the transduction efficiency of IDLV was lower than integrating LV, hepatic expression of F.IX using IDLV is able to induce antigen-specific tolerance and reverse an active inhibitor response (135, 152). However, expression subsided precipitously following partial hepatectomy, indicating a lack of integration. Additionally, in normal mice, F.IX levels declined significantly by 1 year post-injection, suggesting that transgene expression would not be persistent (152). Thus, while F.IX expression can be induced by IDLV, it seems likely that further development of these vectors to achieve persistent expression will be required for them to be a potential therapy for hemophilia B.

3.3. Integrases and non-viral approaches

In addition to the viral gene therapies we have discussed previously, nonviral gene therapy has also been investigated for hemophilia B. At its simplest, this involves the delivery of naked plasmid DNA to target cells. Although modern viral vectors do not produce any viral proteins in target cells, the delivery of naked DNA would also eliminate the immunogenicity of the input viral proteins (such as the CD8$^+$ T cell response to the AAV capsid). However, the problems with this approach are twofold. Firstly, transgene expression from nonviral delivery is typically lower than seen with viral vectors, and without selective pressure or incorporation of the exogenous DNA, expression is short-lived (153). Secondly, it can be challenging to get the DNA into the target cells of interest. *In vitro*, membrane-disrupting procedures such as electroporation and liposome transfection can be employed. For *in vivo* targeting, although a number of targeting approaches are currently under development, hepatic gene transfer is most commonly achieved by hydrodynamic injection of a high volume of DNA solution intravenously (154-157). Efforts are underway to adapt this procedure to larger animal models and eventually humans (158-163).

To bypass the transient nature of plasmid DNA approaches, investigators have recognized the emerging potential of transposons to generate long-lived expression (164). Transposons are naturally occurring DNA elements capable of moving from one chromosomal location to another. They do so by encoding a transposase, a protein which is able to excise the transposon from the donor locus and insert it into a target location. Transposases can act on any DNA sequence flanked by the specific terminal repeat sequences, allowing for integration when DNA encoding for the transposase is added along with the transgene flanked by terminal repeat sequences. Research for gene therapy applications have primarily focused on two transposons: piggyBac (derived from the cabbage looper moth *Trichoplusia ni*) and Sleeping Beauty (SB; a Tc1-like transposon from fish), as well as its hyperactive

mutant, termed SB100X (165-170). The relative activity of these two transposons remains controversial, though it is likely that the rate of integration depends on the cell type being transduced (171, 172). However, it is known that these transposons have distinct integration patterns. SB appears to integrate randomly, whereas piggyBac is biased towards transcriptional start sites, similar to the integration pattern of viral vectors (173-175). By incorporating SB into IDLV, it is possible to replicate this random integration pattern with a viral vector (176). Like LV, the untranslated regions of SB possess transcriptional activity, and incorporation of insulator sequences into this region can reduce the risk of transcriptional activation of host genes proximal to the insertion site (177).

Gene therapy with transposons can be performed directly *in vivo*, or applied *ex vivo* to cells which are then reintroduced into the host. However, likely due to the greater success seen by *in vivo* approaches in gene therapy for hemophilia B, transposon research has focused on that approach. Using hydrodynamic injection, delivery of a SB-containing plasmid along with a plasmid containing F.IX transgene resulted in robust long-term hF.IX expression in mice (178). Similarly, hydrodynamic gene transfer using the ΦC31 integrase (derived from a bacteriophage) induced persistent F.IX expression in hemophilic mice (179-181). However, as previously mentioned, this procedure is currently not applicable to larger animal models. Ironically, in order to achieve targeted delivery of transposon/integrase systems, some investigators have returned to viral vectors. For instance, engineered adenoviral vectors incorporating SB transposons have been used to achieve sustained F.IX expression in mice and in hemophilic dogs (182-184).

Although these approaches have shown some success in pre-clinical models, even the random integration profile of SB transposons is not ideal, as it still carries the potential for oncogenesis. Ideally, targeted integration into 'safe harbor' sites in the genome that are not oncogenic would be employed to eliminate this risk. PiggyBac transposons have been shown to tolerate N-terminal fusion of DNA-binding domains that should constrain its activity to that specific region of the chromosome, whereas a molecular bridge can be employed with SB to fuse it to a DNA-targeting protein (171, 185). Alternatively, targeted gene delivery has been achieved using artificial DNA-recognizing proteins (reviewed in (186)). Zinc-finger nucleases (ZFNs) based on naturally occurring DNA-binding motifs can be engineered to recognize specific DNA sequences by linking together domains that bind specific 3 base pair sequences, allowing for a sequence of 9-18 bp to be identified that should be unique within the human genome (187-189). Similarly, transcription activator-like effector nucleases (TALENs), composed of 33-35 amino acid repeat domains, are able to recognize single base pairs to construct a DNA recognition sequence (190, 191). Finally, the recently discovered clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) systems, which are normally used by bacteria as an adaptive immune system to specifically target phage DNA sequences, use RNA base pairing to DNA to target their cleavage activity (192-197). These targeted DNA integration strategies can approach gene correction in two ways. In addition to the traditional approach of introducing an intact transgene to a safe harbor locus, it is also possible to directly target to mutated gene, replacing it with the corrected sequence (198-200). This second approach may be superior in that it allows the corrected gene to utilize all of the natural regulatory elements of

the gene, such as upstream enhancers that likely would not act on the transgene otherwise. However, it also requires specific design and validation of the targeting nuclease for each disease, whereas insertion into a safe harbor locus could employ the same nuclease while only changing the gene within the delivery vector.

Delivery of these DNA-targeting nucleases remains an issue, as with transposons. Thus, in the treatment of hemophilia B, AAV vectors have been used to deliver a ZFN in conjunction with the hF.IX transgene (201). This genome editing approach cleaved the endogenous F.IX gene after exon 1 (the excised portion encompasses 95% of mutations) and replaced the defective gene with the corrected sequence. Due to the limited packaging capacity of AAV, a dual-vector approach had to be employed in which two AAV vectors were injected simultaneously, one containing the transgene while the other encoded for the ZFN. Although this approach should lead to sustained expression of the ZFN, no adverse consequences (off-target mutations, excision of the transgene, etc.) were observed. This study was initially performed in neonatal mice. Due to their actively growing liver, neonates were anticipated to be capable of homology-directed repair in hepatocytes rather than favoring non-homologous end joining as in quiescent cells (202, 203). However, further investigation determined that this genome editing approach is also effective in adult mice (204).

Finally, a novel approach for nonviral gene therapy involves the oral delivery of plasmid DNA coding for *F9* encased in chitosan nanoparticles (205). In a mouse model of hemophilia B, this approach could induce circulating F.IX and correction of the coagulation defect, particularly when using hyperactive F.IX mutants (206). F.IX expression was restricted to the small intestine. However, despite the tolerogenicity of oral protein delivery, this oral DNA delivery approach was not able to prevent inhibitor formation following protein challenge nor reverse pre-existing inhibitors (206-208).

3.4. Optimization of factor IX

A more recent development in gene therapy for hemophilia B involves alterations to the *F9* transgene itself rather than the delivery vector, a strategy which is applicable regardless of the delivery mechanism chosen. There are a couple potential strategies to achieve this. First, and already in use in the second hepatic clinical trial, is codon optimization (53, 64). This technique is based on the fact that, despite multiple trinucleotide sequences encoding for a single amino acid, certain codons are preferred over others depending on the host organism (mostly due to tRNA frequencies) (209). By making silent mutations within the transgene, it is thus possible to increase translation efficiency by optimizing codon usage for the target cell; this strategy also allows for other changes, such as the removal of negative regulatory *cis*-acting features, to enhance expression (210). Codon optimization of F.IX was reported to increase expression 3-4-fold compared with the unaltered sequence (63). More specific to F.IX, though, is the discovery of mutants with increased clotting activity. Alanine substitution mutation resulted in the artificial generation of F.IX-triple, which has ~10-fold greater specific activity than wild-type F.IX (211, 212). Additionally, a naturally occurring F.IX mutation (R338L), termed F.IX Padua, was discovered that has 5-10-fold higher activity (42, 213-215). Although these hyperactive F.IX variants can cause thrombosis at physiological expression levels, the fact that F.IX circulates in plasma in an inactive form

makes them apparently safe at the expression levels achieved by gene therapy (~5-10% of normal). Interestingly, both variants possessed a mutation at amino acid 338: alanine in F.IX-triple and leucine in F.IX Padua. Adding the leucine mutation to F.IX-triple resulted in an even better clotting factor, with activity ~15-fold greater than normal F.IX (216). A clinical trial is currently underway using an AAV8 vector containing F.IX Padua (NCT01687608).

4. GENE THERAPIES FOR HEMOPHLIA A

In contrast to hemophilia B, gene therapy for hemophilia A has seen significantly less recent progress into the clinic, despite it being the more common of the two diseases. Although the vector platforms and advances within them that we have previously discussed for hemophilia B should also be applicable for hemophilia A, there are some additional factors that make endogenous expression of F.VIII more complicated than F.IX. We will subsequently examine some of these issues before discussing the progress that has been made with various vector systems towards a genetic therapy for hemophilia A.

4.1. Additional challenges in hemophilia A

The primary difficulties in gene therapy for hemophilia A stem from the fact that F.VIII is a much more complicated protein than F.IX. As opposed to the single domain of F.IX, F.VIII is produced as a 2351 amino acid protein (encoded by a 9 kb cDNA) that, following secretion, is cleaved into a noncovalent heterodimer of two chains: the heavy chain (A1-A2-B domains) and the light chain (A3-C1-C2 domains) (217). The size and complexity of F.VIII lead to several complications for gene therapy. First, a 9 kb transgene is too large for some vector systems, such as AAV. Additionally, the synthesis and secretion of F.VIII is notoriously inefficient. Using a comparable retroviral vector delivery system, the levels of F.VIII produced *in vitro* were about 2 orders of magnitude lower than F.IX (218). The inefficiency of F.VIII production results from several factors, including inefficient expression of the mRNA, misfolding and degradation of the translated protein, and retention in the endoplasmic reticulum via binding to ER chaperones such as immunoglobulin binding protein (BiP) (218-225).

The synthesis of F.VIII is further complicated by the interaction with von Willebrand factor (vWF), which is required for stabilization of F.VIII (226, 227). Studies *in vitro* have suggested that this stabilizing effect is enhanced when F.VIII and vWF are co-expressed in the same cell, rather than simply adding vWF to the media (219, 228). However, while vWF is believed to be produced in endothelial cells, platelets, and megakaryocytes, the site of F.VIII synthesis is somewhat controversial (229-231). The liver has been implicated as a major site of F.VIII synthesis due to the ability of liver transplantation to cure hemophilia A in canine models as well as human patients (232-234). A number of studies have reported the presence of F.VIII mRNA and protein in hepatocytes *in vivo*, F.VIII production by hepatocytes cultured *in vitro*, and even the ability to restore clotting activity through hepatocyte transplantation (235-239). Conversely, others have reported F.VIII synthesis in liver sinusoidal epithelial cells (LSECs) but not hepatocytes, or even in both cell types (240-246). However, the preponderance of recent evidence implicates LSECs and by extension endothelial cells in other tissues as well, which may explain the observations of

F.VIII production in extrahepatic vascularized tissues such as the kidney, spleen, and lung (247-250). In particular, a pair of recent studies using conditional knockout mice demonstrate a requirement for endothelial cells but not hepatocytes in F.VIII synthesis (251, 252). Thus, the combination of expression in an unnatural cell type and the lack of vWF synthesis in hepatocytes may explain the difficulties that have been encountered in inducing hepatic expression of F.VIII using techniques that have been successful with F.IX.

Although these findings suggest that endothelial cells might be a preferred target for F.VIII expression, moving gene expression away from hepatocytes potentially highlights complications from the immune response to the transgene that are not a factor following hepatic gene transfer. The strength of tolerance induction to F.IX following hepatic gene transfer for hemophilia B is proportional to transgene expression levels (4, 57). Tolerance to F.VIII following gene transfer is likely also mediated by a similar Treg-dependent mechanism (253). Thus, in addition to the difficulty in achieving therapeutic correction, the low expression levels of F.VIII following gene transfer also reveal challenges from the immune system. The endogenous levels of F.IX in plasma (5000 ng/mL) are already significantly higher than F.VIII (200 ng/mL). When this gap is further enhanced by low expression of F.VIII following hepatic gene transfer, hemophilia A mice can generate inhibitors against F.VIII or even be potentiated towards stronger immune responses to i.v. protein challenge than their untreated brethren (254-258). Furthermore, F.VIII seems to be naturally more immunogenic than F.IX, since hemophilia A patients develop inhibitors at about 5-6 times the rate of hemophilia B patients during recombinant protein therapy. Conversely, other studies have suggested that tolerance to F.VIII following hepatic gene transfer with LV may occur or persist in the absence of transgene expression (259). However, these mice were transduced as neonates; the protocol was not tolerogenic in older mice or with non-hepatic delivery routes.

While there are many similarities between hemophilia A and B, these additional difficulties in achieving therapeutic circulating expression and dealing with the immune response to F.VIII have complicated attempts to develop a gene therapy for the former. Keep these challenges in mind as we begin our discussion of the specific approaches used in gene therapy for hemophilia A, as surmounting them has required novel developments and led to some unique approaches that are not effective for hemophilia B.

4.2. Adeno-associated virus

Given the success that AAV has shown as a vector for the treatment of hemophilia B, it is logical that a number of approaches have also attempted to use it for hemophilia A. When considering AAV-mediated delivery of F.VIII, the most pressing concern is the issue of packaging capacity—AAV vectors cannot package the entirety of the *F8* gene. Two strategies to circumvent this limitation have been explored. Firstly, the heavy and light chains of F.VIII can be split into separate vectors, and co-injection of these AAVs can induce the expression of biologically active F.VIII in circulation (260). However, the utility of this approach is limited by the fact that the F.VIII light chain can interact with the heavy chain within the cell, significantly enhancing secretion (261, 262). Thus, only cells that are transduced by both vectors are likely to be involved in F.VIII production. Alternatively, the

use of a shortened promoter sequence allows the packaging of B-domain-deleted (BDD) F.VIII within a single AAV particle (256, 263). BDD F.VIII, as the name implies, is produced by removing the B domain of the *F8* gene. This alteration does not appear to have any adverse effects on procoagulant activity of the protein (264). As the B domain represents over a third of the F.VIII protein, its removal results in a cDNA that can be packaged in an AAV vector and induce sustained F.VIII expression in mice (256, 265). These findings have been replicated in studies in hemophilic dogs, (266, 267). Further studies have indicated that both approaches (BDD and dual-vector F.VIII) can be effective in canine models (268, 269). However, the doses needed to achieve therapeutic correction were significantly higher than the maximum dose of AAV-F.IX administered to humans in clinical trials (52, 53, 270). Given the dose-dependence of the memory CD8^{+} T cell response to capsid that was observed in these trials, these vector doses may not be feasible in human subjects.

By enhancing the expression per AAV particle, it may be possible to reduce this dose. For instance, the use of a full-length rather than a truncated promoter is able to enhance F.VIII expression from AAV vectors (271). Although this required the use of an oversized AAV genome (5.75 kb), some studies have suggested it is possible to produce AAV vectors with an oversized genome, albeit at the cost of reduced packaging efficiency (272, 273). However, further investigation has suggested that these vectors do not actually package oversized genomes; rather, the transgene is fragmented between AAV vectors and complementation in the cell following transduction results in expression of these oversized transgenes (274-277). Thus, this approach would likely be subject to the same limitations as splitting the chains into separate vectors. That expression depends on transduction of a single cell by two separate AAV vectors significantly reduces expression levels below what they would otherwise be. Since proteasome inhibitors can increase transduction by AAV, the use of bortezomib can enhance expression of F.VIII using these oversized AAV vectors (74, 75, 278-280). Additionally, the use of a more strongly liver-specific promoter can enhance transgene expression while simultaneously reducing the propensity for inhibitor formation that can occur with a ubiquitous promoter (281). Similarly, neonatal administration of AAV avoided immune responses against F.VIII (282). Codon optimization of F.VIII significantly enhances transgene expression, as with F.IX (283). In HA mice, a dose used in humans (2×10^{12} vg/kg) of AAV containing codon-optimized F.VIII with a liver-specific promoter induced supraphysiologic F.VIII expression (284). However, even codon-optimized F.VIII is subject to limitations imposed by the immune system. The immunogenicity of codon-optimized F.VIII can vary depending on the genetic background of the treated hemophilic mice (257). In NHP, therapeutic expression was also induced; however, 3 out of 4 macaques developed inhibitors against F.VIII that were resolved with transient immunosuppression (284). Although AAV-mediated gene therapy for hemophilia A is progressing, the immune response to the transgene clearly still represents an additional barrier to transduction that is not fully understood yet.

4.3. Retrovirus and Lentivirus

In addition to AAV, significant work has been done exploring the use of retroviral/lentiviral vectors for the treatment of hemophilia A. As previously mentioned, these vectors can be

employed for either *ex vivo* or *in vivo* gene transfer. The first proof of concept for *in vivo* gene therapy used a γ-retroviral vector to express F.VIII in hepatocytes of neonatal hemophilic mice (285). This approach was also successful in a canine model of hemophilia A (286). Though additional studies in mice and rabbits suggested that this approach may also be viable in adult subjects, in human patients, only a sporadic and transient rise in circulating F.VIII was detected (287-289). In addition to γ-retroviral vectors, LV has also been employed in the treatment of hemophilia A via *in vivo* gene transfer. Although F.VIII expression can be achieved with this approach, the therapeutic benefit was hampered by the subsequent development of anti-F.VIII inhibitors in immune competent mice (126, 290-292). Feline immunodeficiency virus (FIV)-based LV have also been employed for hemophilia A gene therapy; these studies have suggested that pseudotyping with the GP64 envelope protein from baculovirus may enhance liver tropism of LV (293-295). By combining this liver-tropic envelope protein with a liver-specific promoter and miR-142 regulation (to prevent transgene expression in APCs), it was finally possible to use LV to express F.VIII in hepatocytes without provoking an inhibitor response (296). Continuing the theme of enhanced immunogenicity of F.VIII relative to F.IX, the GP64 envelope protein was required to avoid inhibitor formation in this case, whereas the F.IX-expressing LV could achieve safe expression with a VSV-G envelope protein (133, 296). Although the miR-142-regulated LV expressing F.IX was tolerogenic, preventing antigen-specific immune responses, it remains to be seen whether these F.VIII-expressing vectors can also prevent inhibitor formation following challenge with recombinant protein (134, 135). Given the ability of AAV-F.VIII vectors to potentiate stronger immune responses in some strains of HA mice, this is a relevant concern if subsequent supplementary doses of F.VIII are required (257). Lastly, although LV vectors have been developed that target gene delivery to endothelial cells *in vivo*, the efficacy of this approach for F.VIII expression remains to be seen (297, 298).

Among the *ex vivo* gene therapies using LV, the most common site of expression is hematopoietic stem cells. Expression of F.VIII in hematopoietic stem cells has been achieved as early as 1992 using γ-retroviral vectors; although this approach did not initially correct the bleeding phenotype *in vivo*, it did appear to partially tolerize mice to F.VIII (299, 300). Further optimizations allowed for therapeutic F.VIII expression *in vivo*, as well as enhanced efficacy of the tolerogenic potential of this treatment (301-304). Similarly, LV can be used to induce F.VIII expression in hematopoietic stem cells as well as human CD34^{+} cord blood cells (305-308). LV-mediated F.VIII expression in B cells also induces therapeutic F.VIII expression and immune hyporesponsiveness (309). An interesting development involves the expression of F.VIII specifically in platelets rather than all hematopoietic cells. Outside of endothelial cells, platelets are the other major source of vWF; platelet-derived vWF is thought to be critical for on-demand coagulant activity, though it may also play a role in clotting hemostasis (310). Given the ability of vWF to enhance F.VIII secretion, producing F.VIII within platelets may allow for efficient on-demand release of F.VIII (311). A transgenic mouse expressing F.VIII under the control of a platelet-specific promoter demonstrated correction of the bleeding phenotype in the absence of circulating F.VIII (312). LV can be used to express F.VIII specifically in platelets, and this strategy also provides therapeutic benefit in the absence of circulating F.VIII (313, 314).

This approach has also proven effective in canine models of hemophilia, and LV-transduced human cord blood cells correct the bleeding phenotype when transplanted into immunodeficient hemophilic mice (315, 316). A surprising consequence of this approach to deliver F.VIII on-demand following a bleeding incident is the ability to bypass concerns of immunogenicity that have plagued other approaches. Mice treated with LV to induce F.VIII expression in platelets show bleeding correction even in the presence of high-titer inhibitory antibodies (317). Transduced mice more rapidly clear inhibitors compared to control animals, though they still had clinically relevant Bethesda titers (BU > 5) six months post-injection. Additionally, the consequences of challenge with recombinant protein remain to be seen. This is in contrast to HSC transduction with retroviral vectors and a less restrictive promoter, which robustly induced tolerance to the transgene (303). The efficacy of tolerance induction following HSC gene transfer appears to be directly correlated with the efficiency of engraftment/expression. The use of safer vectors, more restrictive promoters, or milder conditioning regimens seems to impede the ability to induce transgene-specific tolerance, perhaps suggesting that transgene expression in specific hematopoietic cell types is required for tolerance induction (17, 318). Moreover, other studies have suggested that the ability of platelet-derived F.VIII to bypass inhibitors is improved but limited relative to plasma F.VIII (319). The efficacy of platelet-derived F.VIII can vary depending on the bleeding model used, so further preclinical studies will be required to validate this approach for use in humans (320).

In addition to HSCs, mesenchymal stem cells (MSCs) can be transduced by retroviruses to produce F.VIII (321, 322). In mice, MSCs transduced via LV to express F.VIII were not able to mediate systemic correction; however, following intra-articular injection, they were able to reduce bleeding following joint capsular needle puncture injury (323). When LV-transduced MSCs were injected intraperitoneally into hemophilic sheep, however, they demonstrated widespread engraftment in organs and joints and were able to prevent further spontaneous bleeding as well as resolving pre-existing joint damage (324). In addition to hematopoietic approaches, LV has also been used for *ex vivo* transduction of endothelial cells to produce F.VIII. Although this approach avoids the need for myeloablative conditioning, which can have deleterious side effects, achieving persistent engraftment of endothelial progenitors has been challenging (325). Recent developments in cell sheet transplantation technology have shown promise for achieving sustained F.VIII expression from transduced endothelial cells (326). However, in a canine model, it was recently suggested that the use of an implantation matrix may not always be desirable. Omental implantation of F.VIII-expressing endothelial cells transduced with LV in a fibrin matrix induced an inhibitor response in the presence of sustained F.VIII expression, possibly due to the induction of IL-6 and MCP-1 by the thrombin in the matrix (327). Although anti-F.VIII IgG2 antibodies were detected when cells were implanted in the absence of this matrix, they did not possess inhibitory activity. Finally, LV has recently been used to transduce induced pluripotent stem (iPS) cells. While these iPS cells, which are transcriptionally reprogrammed from adult cells, show some promise, there are still a number of barriers including oncogenicity, genomic instability, epigenetic memory, and the impact of propagation in culture that caution against the use of these cells (328). In nude mice, LV-transduced iPS cells were capable of teratoma formation and the secretion of physiologically

relevant levels of functional F.VIII (329). The impact of the immune system on this approach, however, is still unknown.

4.4. Integrases and non-viral approaches

Research in nonviral gene therapy for hemophilia A has largely mirrored the approaches used for hemophilia B. *In vivo* hydrodynamic injection of F.VIII-expressing plasmid can induce transgene expression; however, unlike with F.IX, this approach is limited by the development of anti-F.VIII inhibitors (254). Subsequent studies with the addition of immunomodulatory therapies have prevented inhibitor formation and induced F.VIII-specific tolerance mediated by Tregs (255, 330, 331). Hydrodynamic injection in conjunction with RNA trans-splicing (splicing therapeutic RNA into abundant albumin mRNA) has also been explored in the treatment of hemophilia A (332, 333). Similarly, sustained gene transfer using the Sleeping Beauty transposon is limited by an immune response to F.VIII, unless tolerance is induced at the neonatal stage (334, 335). Interestingly, in adult mice, using SB to express both F.VIII and the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) induced sustained F.VIII expression and reduced T cell infiltration in the liver (336). In addition to hydrodynamic injection, F.VIII-expressing plasmids have also been targeted to specific cell types using nanocapsules. Targeting SB-mediated transduction of F.VIII to liver sinusoidal endothelial cells (LSECs) of adult HA mice using hyaluronon nanocapsules induced sustained correction of clotting function (337). An alternative approach for *in vivo* gene therapy involves the oral delivery of plasmid DNA encapsulated in chitosan nanoparticles (205). This approach provides phenotypic correction of hemophilic mice, and repeated delivery provides sustained correction in the absence of an immune response to F.VIII (338, 339). In both of these nanoparticle mediated approaches, mice were not challenged with exogenous F.VIII to verify if they were tolerized to F.VIII, or if they merely failed to mount an immune response to the endogenously-produced protein.

Nonviral gene transfer has also been employed *ex vivo* for the treatment of hemophilia A. In human patients, this *ex vivo* approach was used to transduce autologous dermal fibroblasts and select for F.VIII-producing cells (340). Delivery of these genetically modified fibroblasts into hemophilic patients provided a slight decrease in the number of bleeding events, and no inhibitor formation was detected. However, these clinical improvements only lasted for about 10 months, likely due to loss of the transduced cells. Given these promising results, subsequent studies have focused on finding a superior cell type for *ex vivo* transduction, both in terms of secretion capacity as well as persistence. In particular, blood outgrowth endothelial cells (BOECs) have shown promise for this approach. These cells, derived from circulating endothelial cells in peripheral blood, display many of the characteristics of endothelial cells, including vWF expression (341). Additionally, BOECs grow extremely well in culture; after 65 days, they can be expanded from about 20 cells to 10^{19} cells (341). Following gene transfer, selection, and injection into mice, genetically modified BOECs induced therapeutic or even supraphysiologic circulating levels of hF.VIII, depending on the cell dose (342). These cells maintained an endothelial phenotype and accumulated primarily in spleen and bone marrow. Further studies have also achieved persistent seeding of liver and lung in addition to the spleen and bone marrow (343). Other approaches have involved nonviral modification of hepatocytes and embryonic stem cells

(344, 345). F.VIII-expressing endothelial cells derived from iPS cells have also shown promise for the treatment of hemophilia A (346). iPS cells have also been modified with human artificial chromosomes (HACs), constructs that mimic a human chromosome. HACs are maintained separately from the host genome (minimizing the risk of insertional mutagenesis), persist through cell divisions due to their ability to bind centrosomal proteins, and allow for delivery of large constructs that can mimic physiological gene regulation (347-350). Megakaryocytes/platelets derived from iPS cells have been generated that produce F.VIII following transduction with a HAC, though their *in vivo* efficacy has not yet been demonstrated (351).

4.5. Optimization of factor VIII

Finally, the optimization of the F.VIII transgene represents a vector-agnostic approach to improving gene therapy. As previously mentioned, codon optimization of F.VIII has been shown to enhance transgene expression (283). However, the inefficient secretion of F.VIII provides additional routes for transgene optimization. Interestingly, porcine F.VIII is secreted by cells more efficiently than human F.VIII (352-354). HSCs expressing porcine F.VIII are able to correct the bleeding phenotype following implantation into hemophilic mice (303, 304, 355). Further studies have incorporated porcine sequences into hF.VIII to enhance secretion of the clotting factor while still maintaining a largely human protein; this hybrid protein can also mediate clotting activity *in vivo* following implantation of transduced HSCs (356-358). Another interesting aspect of porcine F.VIII is its differential immunogenicity relative to hF.VIII. Although the magnitude of the response to both proteins is largely comparable, antibody responses preferentially target different epitopes in porcine or human F.VIII, and some reports have suggested that porcine F.VIII may be able to bypass inhibitors directed against hF.VIII (359-362). Similarly, canine F.VIII is more stable and exhibits greater specific activity than hF.VIII (363). Incorporation of a point mutation (R1645H) from canine F.VIII into hF.VIII conferred many of these properties to the new transgene, and expression of this altered F.VIII via an AAV vector was more effective and comparably immunogenic to BDD-F.VIII (364). Other strategies for enhancing secretion include the incorporation of a fragment of the light chain into the heavy chain (whose secretion is the rate limiting step) or the use of chemical chaperones (365, 366). Alternatively, one can simply bypass F.VIII altogether. Expression of activated factor VIIa in hepatocytes or platelets has been shown to provide bleeding correction in the absence of thrombotic events (367, 368). F.IX variants that do not require F.VIII, or an antibody that mimics F.VIII's role in the intrinsic factor Xase by bringing F.IX and F.X into close proximity are also able to provide therapeutic benefit (369, 370). By completely avoiding F.VIII, these strategies sidestep complications posed by the immune system. In addition to preventing the risks associated with a *de novo* inhibitor response to the gene therapy, they would also be appropriate for use in patients with preexisting inhibitors without being concerned with tolerance induction. However, there are also potential risks for thrombosis when bypassing the natural regulation of the coagulation cascade, and these mechanisms will likely need thorough safety studies before being applied in the clinic.

5. CONCLUSION

Clearly, the field of gene therapy for hemophilia is being thoroughly explored. Given its recent clinical success, AAV-mediated hepatic gene transfer is likely to be the primary direction going forward for hemophilia B. However, there are still a number of problems that limit the broad applicability of the current approach, particularly the current immunosuppressive regimen and pre-existing neutralizing antibodies. Perhaps these barriers to transduction will be fixed with more specific therapies or a combinatorial approach of several techniques to bypass NAB. Alternatively, a different approach that does not have to deal with these anti-vector immune responses, such as an *ex vivo* or nonviral technique, might become a more effective way to administer gene therapy to the broadest base of patients. For hemophilia A, while AAV-mediated gene therapy has potential, a number of limitations reduce its desirability, including packaging capacity and inefficient expression. While a number of transgene modifications have increased the expression levels, the vector doses required to achieve corrective F.VIII expression remain significantly higher than with F.IX. These expression limitations lead to further concerns about immune responses both to the capsid and, if expression levels are not sufficient, the transgene. As such, *ex vivo* gene transfer may be more effective for hemophilia A due to its ability to enhance expression through cellular division. Specifically, gene transfer to platelets is a promising example of this technique. This approach appears to bypass barriers posed by the immune system, as the local expression and release of F.VIII can correct the bleeding phenotype in the presence of inhibitors. This technique, too, has its drawbacks, particularly in terms of delivery. Current techniques involve the use of integrating viral vectors, for which concerns about oncogenicity have not been fully addressed. Thus, while a number of promising approaches for gene therapy for hemophilia have been elucidated, there are clearly numerous problems that still need to be addressed to develop approved gene therapies for both hemophilia A and B for use in humans.

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Abbreviations

F.VIII	factor VIII
F.IX	factor IX
F.VII	factor VII
F.X	factor X
ITI	immune tolerance induction
AAV	adeno-associated virus
ITR	inverted terminal repeat
Treg	regulatory T cell

scAAV	self-complementary AAV
ssAAV	single-stranded AAV
TLR9	toll-like receptor 9
CTL	cytotoxic T lymphocyte
MHC	major histocompatibility complex
NAB	neutralizing antibody
MMLV	Moloney murine leukemia virus
LV	lentivirus
HIV	human immunodeficiency virus
VSV	vesicular stomatitis virus
LCMV	lymphocytic choriomeningitis virus
SIN-LV	self-inactivating lentivirus
HSC	hemaptopoietic stem cell
mADSC	murine adipose tissue-derived stem/stromal cells
miR-142	microRNA 142
IFN	interferon
TLR7	toll-like receptor 7
cGAS	cyclic GMP-AMP synthetase
IDLV	integrase-defective lentivirus
SB	Sleeping Beauty
ZFN	zinc-finger nuclease
TALEN	transcription activator-like effector nuclease
CRISPR	clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated systems
BiP	immunoglobulin binding protein
vWF	von Willebrand factor
LSEC	liver sinusoidal endothelial cell
i.v.	intravenous
BDD-F.VIII	B-domain-deleted factor VIII
FIV	feline immunodeficiency virus
BU	Bethesda unit
MSC	mesenchymal stem cell

IDO	indoleamine 2,3-dioxygenase
BOEC	blood outgrowth endothelial cell
iPS	induced pluripotent stem cell
HAC	human artificial chromosome

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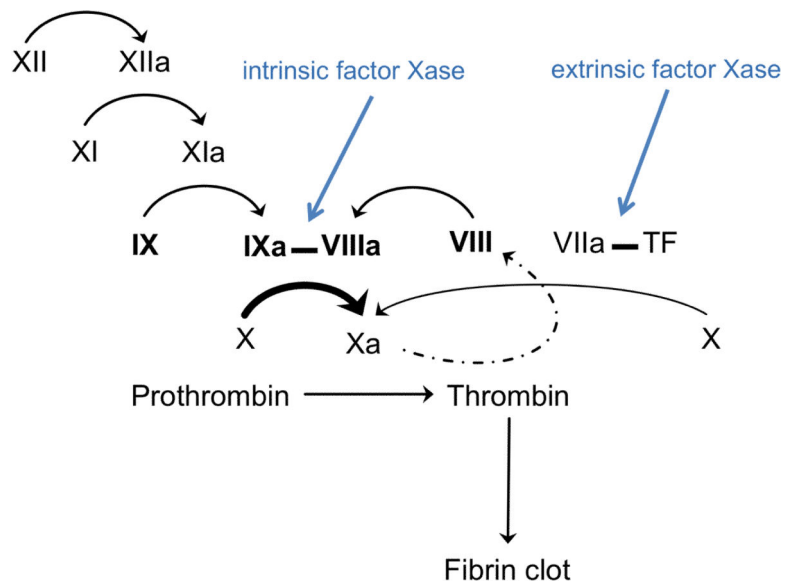
Figure 1.
Diagram of the coagulation cascade. Activation of F.XII by exposure to a damaged surface causes sequential activation of F.XI and F.IX. Similarly, activation of the extrinsic factor Xase (F.VIIa and tissue factor) leads to limited activation of F.X. This limited activation induces a feedback loop by activating F.VIII, which combines with F.IXa to form the intrinsic factor Xase. The intrinsic factor Xase then cleaves high levels of F.X, which induces the activation of prothrombin to thrombin, leading to formation of the cross-linked fibrin clot.
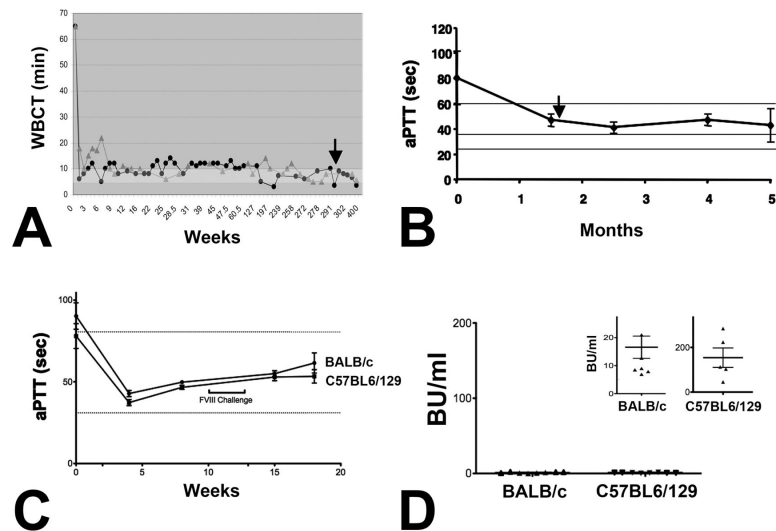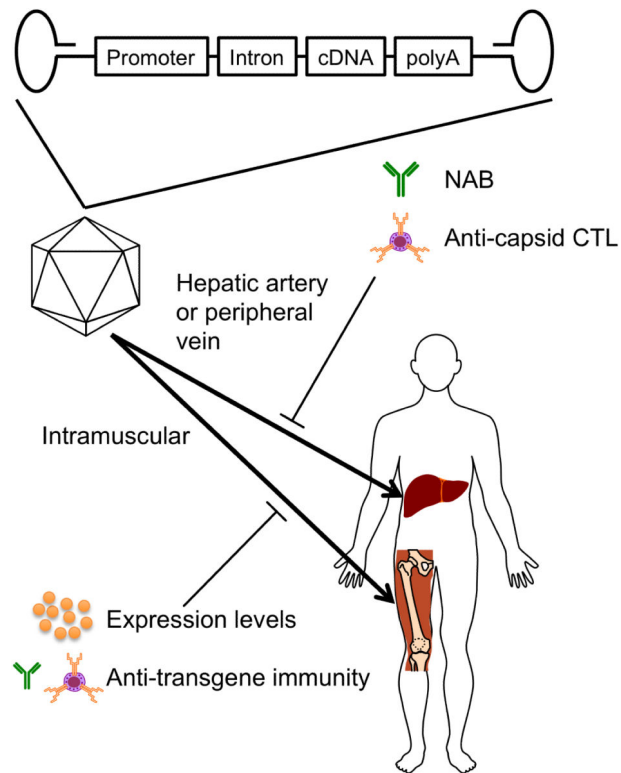
Figure 2.
Examples of sustained correction of hemophilia in animal models by hepatic AAV gene
transfer. **A.** Sustained correction of the whole blood clotting time after hepatic AAV2-canine
F.IX gene transfer in 2 hemophilia B dogs with F9 null mutation (Niemeyer *et al., Blood*
2009). **B.** Sustained correction of the activated partial thromboplastin time (aPTT) after
hepatic AAV2-human F.IX gene transfer in hemophilia B mice (n=4) with F9 gene deletion
(Dobrzysnki *et al., Proc Natl Acad Sci* 2006). Arrows in A and B indicate challenge with/
immunization against FIX protein. **C.** Sustained correction of the aPTT after hepatic AAV8-
human FVIII gene transfer in hemophilia A mice (of 2 different strain backgrounds, n=8 per
strain) with F8 exon 16 gene deletion (Sack *et al., PLoS One* 2012). Mice were challenged
with F.VIII protein injections at the indicated time interval. **D.** Lack of inhibitor formation in
the hemophilia A mice treated with gene therapy and challenged with F.VIII protein (insert
shows inhibitor titers in BU/ml in response to FVIII in control mice that had not received
gene transfer).

| Models	Mouse	NHP	Dog	Sheep	Human
Hemophilia A	+	-	+	+	+
Hemophilia B	+	-	+	-	+
Weight	25 g	6-8 kg	10-40 kg	45-160 kg	60-90 kg
AAV dose (2×10^{12} vg/kg)	5×10^{10}	1.4×10^{13}	6×10^{13}	1.2×10^{14}	1.5×10^{14}

Figure 3.
Animal models of hemophilia. Preclinical studies of gene therapy for hemophilia have access to a variety of animal models. Models of both hemophilia A and B are available in mice, whereas dogs typically serve as the large animal model for both diseases. Although studies are performed in nonhuman primates, there are not hemophilic models of these animals available. Though not used very often, there is a model for hemophilia A in sheep. Finally, humans obviously suffer from both hemophilia A and B, and studies for both have been performed in clinical trials. An average range of weights for each animal is given below, and the average AAV vector dose that would be required for delivering 2×10^{12} vg/kg to each animal indicates how rapidly the vector titers required can increase with larger animals.

Figure 4.
AAV-mediated gene therapy for hemophilia B. A recombinant AAV vector is produced, whose genome includes the viral ITRs, as well as a promoter, intron, F.IX cDNA, and polyA tail. AAV has been used for intramuscular gene transfer, which was limited by nonphysiological expression levels in human trials, as well as anti-transgene immunity during some preclinical studies. Circulatory delivery of AAV for hepatic gene transfer, either via the hepatic artery or peripheral vein, has seen more success. However, this route can still be limited by anti-capsid NAB as well as reactivation of a memory CD8+ T cell response to the input viral capsid.
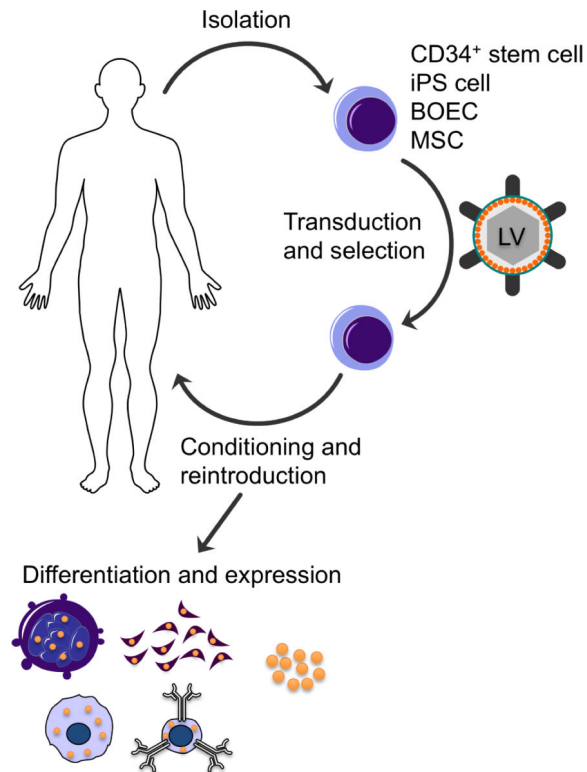
Figure 5.
Ex vivo gene transfer. Gene transfer to cells *ex vivo* is typically initiated by isolation of the relevant cell population (such as CD34^{+} HSCs, iPS precursors, BOECs, MSCs, etc.) from the patient. Modification with LV or other integrating gene transfer system is performed, followed by a selection for transduced cells. If necessary, a conditioning regimen is given to the patient prior to reintroduction of the transduced autologous cells to ensure efficient engraftment. Finally, in the patient, these precursors are distributed and differentiated into the cells desired for expression, whether these are megakaryocytes, platelets, B cells, endothelial cells, MSCs, or some other cell type of interest.

Table 1

Clinical trials of AAV-mediated gene therapy for hemophilia B

Vector	Route of administration	Institute	Status	Reference/Identifier
ssAAV2-CMV-hF.IX	Intramuscular	Children's Hospital of Philadelphia/ Stanford/Avigen	Complete	(30)
ssAAV2-hAAT-hF.IX	Hepatic artery	Children's Hospital of Philadelphia / Stanford/Avigen	Complete	(52)
scAAV8-LSP-hF.IXco	Peripheral vein	St. Jude Children's Hospital/University College London	Ongoing	(53)
ssAAV8-hAAT-hF.IXco	Peripheral vein	Children's Hospital of Philadelphia	Ongoing	NCT01620801
scAAV-TTR-hF.IXco-Padua	Peripheral vein	University of North Carolina	Ongoing	NCT01687608