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Review

Protein-Engineered Coagulation Factors for Hemophilia Gene Therapy

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Hemophilia A (HA) and hemophilia B (HB) are X-linked bleeding disorders due to inheritable deficiencies in either coagulation factor VIII (FVIII) or factor IX (FIX), respectively. Recently, gene therapy clinical trials with adeno-associated virus (AAV) vectors and protein-engineered transgenes, B-domain deleted (BDD) FVIII and FIX-Padua, have reported near-phenotypic cures in subjects with HA and HB, respectively. Here, we review the biology and the clinical development of FVIII-BDD and FIX-Padua as transgenes. We also examine alternative bioengineering strategies for FVIII and FIX, as well as the immunological challenges of these approaches. Other engineered proteins and their potential use in gene therapy for hemophilia with inhibitors are also discussed. Continued advancement of gene therapy for HA and HB using protein-engineered transgenes has the potential to alleviate the substantial medical and psychosocial burdens of the disease.

Hemophilia A (HA) and hemophilia B (HB) are X-linked bleeding disorders due to inheritable deficiencies in either coagulation factor VIII (FVIII) or factor IX (FIX), respectively.[1–2] The bleeding phenotype is generally related to the residual factor activity: people with severe disease (factor activity <1% normal) have frequent spontaneous bleeds; people with moderate disease (factor activity 1%–5% normal) rarely have spontaneous bleeds, but bleed with minor trauma; and people with mild disease (factor activity 5%–40% normal) bleed during invasive procedures or trauma. Given this well-defined relationship between factor activity and bleeding phenotype, HA and HB are attractive targets for gene therapy as small increases in factor levels are expected to have a meaningful clinical impact. Although a variety of strategies have been investigated over several decades (reviewed in Hough and Lillicrap,[3] Lheriteau et al.,[4] Rogers and Herzog,[5] Arruda and Samelson-Jones,[6] and High[7]), the field has coalesced around the use of adeno-associated virus (AAV) vectors[8–10] delivering transgenes of engineered FVIII or FIX variants with therapeutically advantageous properties not present in the wild-type (WT) protein. Herein, we review the protein engineering strategies that have allowed gene therapy for hemophilia to come to fruition with phase 3 studies announced for both HA and HB. We also review alternative protein engineering approaches being undertaken for FVIII and FIX, as well as other engineered blood proteins that may have potential as transgenes for hemophilia gene therapy. We defer genomic engineering strategies to other reviews.[4]

Overview of Current Therapies for Hemophilia

The armamentarium to treat HA and HB has recently expanded.[11–14] Until a few years ago, the management of HA and HB mostly comprised restoring the FVIII or FIX activity through intravenous infusions of the missing factor protein. Factor replacement was used either "on-demand" to treat acute bleeding or prophylactically to prevent bleeding, which required regular scheduled infusions of factor typically two to four times per week. There are, however, several limitations of factor protein therapy. First, it is estimated that only 20% of people with hemophilia worldwide have regular access to factor replacement because of economic reasons. Second, despite the proven benefits of factor prophylaxis for patients with severe disease,[15,16] ~40% of adult patients with access to factor do not routinely receive prophylaxis.[17,18]

The recent advent of extended half-life (EHL) factor products have allowed for prophylactic regimens with decreased infusion frequency (reviewed in Mannucci et al.,[11] Arruda et al.,[12,14] and Callaghan et al.[13]). These EHL products are altering the standard of care of hemophilia treatment in the developed world and consequently changing the risk-to-benefit discussion of novel approaches such as gene therapy.[19]

The relative safety of recombinant factor protein compared with plasma-derived factor protein was recently questioned in the Survey of Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) clinical trial. In this prospective randomized clinical trial, standard half-life recombinant FVIII products were observed to have increased risk of inhibitor development compared with plasma-derived FVIII products (hazard ratio, 1.87; 95% confidence interval, 1.17–2.96).[20] However, additional independent studies are need to confirm these data. Inhibitors are anti-FVIII or anti-FIX neutralizing alloantibodies that obstruct the hemostatic activity of FVIII or FIX. At high titers, inhibitors render replacement factor ineffective to treat bleeds. Inhibitors are the most substantial complication of factor replacement, and their development is associated with a substantial increase in morbidity and mortality.[21–28] Bleeding in high-titer inhibitor patients

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is managed with bypassing agents, which circumvent the inhibitor to provide hemostasis. Until recently, the only available bypassing agents were recombinant activated factor VII (FVIIa) and activated prothrombin complex concentrate (aPCC). Both recombinant FVIIa and aPCC require intravenous infusions and are dosed for acute bleeds every 2 and 8 h, respectively. Prophylactic regimens with less frequent injections are also established.[29,30]

The first non-factor prophylactic bypassing agent was recently approved for HA patients with and without inhibitors: emicizumab (Hemlibra) is a bispecific antibody that acts as a FVIIIa-mimetic with procoagulant activity.[31–34] Additional non-factor therapies with bypassing activity are also in various stages of clinical development (reviewed in Arruda et al.[12,14] and Callaghan et al.[13]). There is substantial excitement about these non-factor therapies because they can be administered subcutaneously with weekly to monthly dosing. However, questions remain about their safety, especially their thrombotic risk, and how best to integrate them into clinical practice.[12,35,36]

Currently, the only effective therapy for inhibitor eradication is the immune tolerance induction (ITI) protocols, which involve the uninterrupted exposure to factor through regular, typically daily, intravenous administrations for months to years.[37–40] Because of the infusion burden, immune tolerance induction for pediatric patients typically requires central venous catheters, which are associated with thrombotic and infectious complications in ~25% of patients.[39,41] It is also a very challenging therapy for patients, with almost 20% of randomized subjects withdrawing from clinical trials because of compliance issues.[39] Nonetheless, immune tolerance induction has historically been found to be cost-effective compared with a lifetime of bypassing therapy,[42] although the emergence of non-factor therapies requires these assessments to be updated.

Prior to the advent of factor-based treatments, the majority of people with severe hemophilia died before 15 years of age, mostly from spontaneous bleeding or trivial injuries.[43] Viral contamination by hepatitis C virus (HCV) and HIV of plasma-based factor products had a devastating impact on the hemophilia community in the 1980s.[44] New iatrogenic infections are currently exceedingly rare, due to a combination of highly effective virucidal techniques and recombinant factor products. Despite these gains from current therapies, there remains a substantial medical and psychosocial burden from the disease.[45–49]

Success and Challenges of Gene Therapy for HA and HB

The promise of gene therapy for hemophilia is to alleviate these burdens by providing lifelong hemostatic coverage after a single infusion of vector. Recent successful early-phase clinical trials using liver-directed AAV-based gene therapy with bioengineered factor transgene for men with severe HB[50] and HA[51] have reported phenotypic cures and normal or nearly normal factor levels in most subjects. These results, however, are built on several decades of earlier work developing the necessary technologies and protocols to achieve this goal, as detailed in several reviews;[3–7] here, we focus on contextualizing the challenges overcome by engineered FVIII or FIX transgenes.

Because full-length FVIII cDNA (7 kb) exceeds the packing capacity of AAV vectors (~4.7 kb), most early gene therapy studies focused on HB, because FIX cDNA is ~1.6 kb[52] despite the fact that HB accounts for only 20% of all hemophilia cases. As discussed below in detail, the removal of most of the B-domain of FVIII decreased the cDNA to ~4.4 kb (Figure 1A), and three AAV-based clinical trials for HA have recently reported promising results using this approach (A.C. Nathwani et al., 2018, Am. Soc. Hematology, abstract; K.A. High et al., 2018, Am. Soc. Hematology, abstract).[51] Notably, the first in-human use of liver-directed AAV gene therapy was in HB subjects.[53] This study suggested a critical AAV vector dose-dependent hepatotoxicity that was hypothesized to be due to the destruction of transduced hepatocytes by cellular immunity targeting protein antigens from the AAV capsid; the loss of transduced cells resulted in a loss of circulating FIX protein.[53,54]

The subsequent trial confirmed the AAV vector dose-dependence of this hepatotoxicity and limited the loss of transgene expression by the swift initiation of oral steroids for immunosuppression.[55,56] Although the dose-dependence seems to vary between AAV serotypes (AAV2, AAV5, and AAV8), all AAV gene transfer trials for HA or HB have reported hepatotoxicity in some subjects.[6,51,53,55–57] However, the elapsed time from vector infusion to hepatotoxicity, as well as the lack of identifiable AAV-capsid immune cells in some studies, suggests that distinct biological mechanisms may contribute to the observed hepatotoxicity in different trials.[58] Furthermore, although immunosuppression has reduced the loss of transgene expression in most trials, it has not succeeded in stopping clinically significant declines of FVIII and FIX levels in others (NCT01687608, NCT01620801, NCT02618915, and NCT03003533) (P.E. Monahan et al., 2015, Congr. Int. Soc. Thromb. Haemost., congress; K.A. High et al., 2018, Am. Soc. Hematology, abstract; R. Calcedo et al., 2017, Am. Soc. Hematology, abstract; S. Pipe et al., 2017, Am. Soc. Hematology, abstract),[12,54] although the details of these latter studies have not yet been fully published. The prophylactic use of immune suppression with oral steroids has also been insufficient to prevent hepatotoxicity in at least one study.[51] Lastly, *in vivo* studies of the anti-AAV capsid cellular immune response have required complicated adoptive transfer mouse models[59,60] without proven clinical relevance to date. Combined, these considerations provide a strong rationale for enhancing the potency of the vector, including utilizing bioengineered factor variants with advantageous properties, as a dependable approach to mitigate the risk for hepatotoxicity by reducing the therapeutic vector dose.

Bioengineering FVIII and FIX

Less Is More with B-Domain Deleted FVIII

Full-length FVIII is a large, 280-kDa protein primarily expressed in liver sinusoidal endothelial cells (LSECs),[61,62] as well as extra-hepatic endothelial cells. Its domain structure is shown in Figure 1A. FVIII predominantly circulates as a heterodimer of the heavy chain

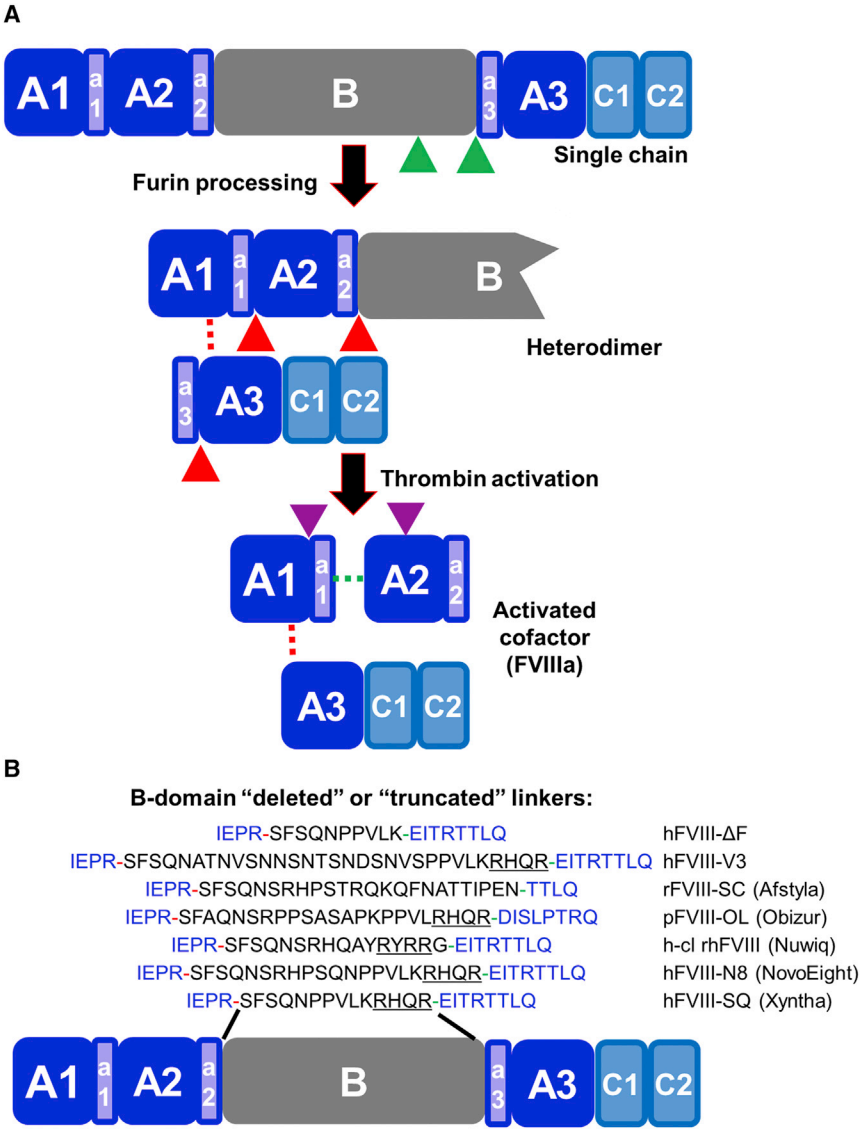

Figure 1. Protein Domains and Life Cycle of FVIII

(A) FVIII is translated as a single-peptide chain (single chain) with the domain structure of A1-a1-A2-a2-B-a3-A3-C1-C2. Proteolytic cleavage of FVIII at R-1313 and/or R-1648 by the *trans*-Golgi protease furin (green triangles) results in *heterodimer* formation. The FVIII heavy chain (A1-a1-A2-a2-B) and light chain (a3-A3-C1-C2) remain associated through non-covalent metal-ion-dependent interactions occurring between the A1 and A3 domains (red dashes). The B-domain undergoes additional non-specific proteolysis in plasma after secretion. During coagulation, FVIII single chain or heterodimer is activated to its heterotrimeric *cofactor* form by cleavage by thrombin at R-372, R-740, and R-1689 (red triangles). A2 remains associated with A1-a1 via non-covalent interactions (green dashes). Inactivation of FVIIIa occurs via spontaneous A2 dissociation and/or proteolytic cleavage, primarily by activated protein C, at R-336 and R-562 (purple triangles). (B) Replacement of the B-domain by linker sequences (black amino acids with blue flanking amino acids from the a2- and a3-domains) has been used for several B-domain "deleted" or "truncated" bioengineered FVIII variants. Most were specifically designed to include furin recognition motifs (underlined). Examples of commercial protein products using each linker are listed in parentheses. FVIII-ΔF and -V3 have demonstrated safety and efficacy as transgenes for AAV-based gene therapy in large-animal models of HA.

(composed of the A1-, A2-, and B-domains) and the light chain (composed of the A3, C1, and C2 domains) bound through non-covalent metal-dependent interactions.[63] The formation of the heterodimer is due to the proteolytic cleavage by the intracellular proprotein convertase furin within the B-domain at either R-1313 and/or R-1648.[64,65] During coagulation, FVIII is activated mostly by thrombin. Once activated (FVIIIa), it acts as a cofactor for activated FIX (FIXa), which is responsible for the activation of factor X (FX) during sustained coagulation.[66]

For reasons that remain incompletely understood, FVIII is poorly transcribed, translated, and secreted in heterologous expression systems, even compared with similarly sized homologous proteins such as coagulation factor V (FV).[63,67–70] Although the plasma concentration of FV is 30-fold higher than FVIII (30 nM versus 1 nM), FV has an analogous biological role as the procofactor for activated FX (FXa) and a similar protein domain structure as FVIII. These properties, as well as the large size of the full-length FVIII cDNA, prevented the incorporation of full-length FVIII into gene therapy vectors. Although the B-domain of FVIII comprises 40% of the total protein, it is not necessary for the clotting activity of FVIII.[71] As such, FVIII variants where the B-domain have been replaced by short peptide linkers (Figure 1B) were developed[65,72,73] and found to have substantially higher expression levels from heterologous systems compared with full-length FVIII. The initial B-domain deleted (BDD; FVIII-SQ in Lind et al.[65]) variant, as well as subsequent modifications, were specifically designed to maintain proteolytic cleavage by furin to ensure comparable heterodimer formation as full-length FVIII.[65,74,75] Cleavage by furin is essential for the biological activity of myriad proteins including a number involved in hemostasis such as FVII, FIX, protein C, protein S, and von Willebrand factor.[76–80] It was also assumed to have a critical role for FVIII; however, our recent findings showed the opposite (as discussed below).

Commercial FVIII-BDD protein has demonstrated equivalent safety, efficacy, and pharmacokinetics as full-length FVIII for the treatment of HA over the last two decades.[74,81–83] In preclinical studies

Table 1. Lessons Learned from Non-human FVIII Orthologs

Species	Insight	hFVIII-Variant	Therapeutic Development	References
Canine	furin evasion is beneficial for secretion and activity	ΔF	preclinical gene therapy studies in large-animal HA models	117–119, 222
Porcine	primary sequence of A1 and A3 impact secretion	ET3	preclinical gene therapy studies in HA mice	108–110
Ancestral primates	reconstructed variants have improved secretion	An-53	preclinical gene therapy studies in HA mice	111

evaluating AAV-based gene therapy in HA models, FVIII-BDD was initially incorporated in a dual-vector system with one vector providing FVIII-BDD heavy chain and a second vector providing FVIII-BDD light chain.[84–87] The identification of small genome regulatory elements[88,89] subsequently allowed FVIII-BDD to be incorporated into a single AAV vector.[87,90] Alternative strategies with other viral vectors have also utilized FVIII-BDD.[91–96] All current gene therapy clinical trials for HA utilize a single AAV vector system and a FVIII transgene with a truncated B-domain (A.C. Nathwani et al., 2018, Am. Soc. Hematology, abstract; K.A. High et al., 2018, Am. Soc. Hematology, abstract).[51]

Although deletion of the B-domain results in ≥15-fold increase in FVIII mRNA and intracellular protein levels, there is only a 2-fold increase in secreted protein compared with full-length FVIII.[97] Several protein interactions along the secretory pathway have been implicated in this ineffective secretion of FVIII-BDD, which has motivated several bioengineering strategies to further enhance secretion. The interaction between FVIII and the endoplasmic reticulum (ER) chaperone BiP (also referred to as 78-kDa glucose-regulated protein [GRP-78] or heat shock 70-kDa protein 5 [HSPA5]) limits FVIII secretion.[98] Disruption of this interaction through specific FVIII amino acid substitutions (e.g., F309S) enhances FVIII secretion 2-fold from mammalian cell culture[99] and several-fold after hydrodynamic injection in HA mice.[100] Elimination of the disulfide bond between C1899 and C1903 (within the A3 domain) through glycine substitutions also increases FVIII secretion by 2-fold from mammalian cell culture;[101] this enhancement is speculated to be due to preventing interactions with free thiols that hinder FVIII secretion.

FVIII and FV secretion also requires LMAN1-facilitated transport from the ER to the Golgi,[102,103] which is dependent on *N*-linked oligosaccharides[104] that occur mostly in the B-domain. FVIII variants with B-domain truncations designed to maintain *N*-linked oligosaccharides demonstrate increased secretion compared with FVIII-BDD with the presence of at least six *N*-linked oligosaccharides conferring an ≥10-fold benefit in secretion from mammalian cell culture[100] and a several-fold benefit after gene transfer in HA mice.[105] This advantage appears to be dependent on the absolute number of *N*-linked oligosaccharides, rather than the native sequence, as a novel 31-amino acid linker designed to incorporate six N-glycosylation sites (named FVIII–V3; Figure 1B) was comparable with the 226-amino acid linker that contained the same number of N-glycosylation sites (226 amino acids/N6).[106] Recently, promising results using this FVIII-V3 transgene in an AAV8 vector to treat HA subjects were reported in abstract form without increased immunogenicity (A.C. Nathwani et al., 2018, Am. Soc. Hematology, abstract).

Recombinant human (h) FVIII-BDD is also poorly secreted from heterologous expression systems compared with recombinant porcine (p) FVIII-BDD, which demonstrates 5-fold higher protein yields than hFVIII-BDD.[107] However, this advantage of pFVIII-BDD can be recapitulated by substituting "high-expression" sequences within the A1 and A3 domains of pFVIII into hFVIII-BDD (FVIII-ET3), which appears to improve posttranslational processing, although the molecular details of this enhancement are lacking.[108–110] Interestingly, computationally determined FVIII proteins of primate evolutionary ancestors are also better expressed in such systems.[111] This result suggests, if observations from heterologous expression can inform on endogenous expression, that hFVIII may have evolved to have limited secretory efficiency. These are two examples of hFVIII bioengineering guided by studies of other mammalian FVIII orthologs (Table 1). Recombinant pFVIII-BDD is a US Food and Drug Administration (FDA)-approved hemostatic therapy for acquired hemophilia (a distinct autoimmune disease from HA that typically responds well to immunosuppressive therapy), because pFVIII-BDD can sufficiently evade autoantibodies against hFVIII. However, to date, chimeric FVIII variants have not been evaluated in large-animal models of HA, and the safety concerns related to immunogenicity remain undefined. Indeed, the administration of FVIII orthologs from other species typically results in an anti-FVIII immune response in mice,[112] large-animal models,[106,113] and patients.[114,115] Thus, translation of chimeric FVIII variants is likely to be challenging due to the risk of immunogenicity.

Although FVIII-BDD was designed to maintain furin processing under the untested assumption that heterodimer formation was important for biological activity, attempts to enhance furin cleavage in fact modestly decreased FVIII-BDD secretion.[116] In a comprehensive study of the role of furin in FVIII biology, FVIII-BDD bioengineered to avoid furin processing by deleting the cleavage sequence (FVIII-ΔF; Figure 1B) actually enhanced FVIII secretion 3-fold from both mammalian cell culture and after AAV-based gene therapy in small- and large-animal HA models.[117] The insight that furin processing may hinder FVIII-BDD secretion is another example of protein engineering strategies informed by comparative biology (Table 1). Here, the observation that canine (c) FVIII has a compromised furin recognition motif suggested that furin processing was not necessary for FVIII biological function.[118,119] Both hFVIII-ΔF and cFVIII-ΔF display increased *in vitro* and *in vivo* hemostatic potency compared with their respective FVIII-BDD orthologs.[117]

Because multiple areas of FVIII appear to hinder expression through known and unknown mechanisms, it is possible that combining strategies might have a synergistic biological effect, which is supported by limited examples of combined approaches.[100,101] Moreover, the breadth of strategies that can increase secretion suggest that hFVIII may not be optimized for secretion efficiency, although why this has evolved remains unclear. It may be informative that several other mammalian FVIII orthologs (Table 1) are superior to hFVIII in this regard. Emerging data also suggest that ectopic FVIII expression induces the unfolded protein response (UPR) and markers for ER-stress[120] both in mammalian cell culture[121–123] and after liver-directed gene transfer using a variety of vector systems in mice.[123–126] FVIII secretion may be inversely related to induction of the UPR and ER-stress because FVIII variants with improved secretion appear to have lower levels of UPR and ER-stress biomarkers compared with controls.[121,123] However, only limited physiological sequelae of FVIII transgene-stimulated UPR and ER-stress have been observed. Two studies found no correlation between FVIII inhibitor formation and UPR and ER-stress markers in mice after AAV liver-directed gene therapy,[124,125] although expression of hFVIII in megakaryocytes of transgenic mice was associated with increased apoptosis.[127] It will be interesting to see whether FVIII transgene expression in LSECs,[128] the endogenous site of production, is also associated with increased UPR and ER-stress markers. The clinical consequences of FVIII gene therapy triggering the UPR are unknown. Nonetheless, minimizing induction of the UPR may be an additional motivation for using FVIII variants with enhanced secretion.

At least two current gene therapy trials for HA have reported phenotypic amelioration using FVIII-BDD delivered with AAV vectors (K.A. High et al., 2018, Am. Soc. Hematology, abstract).[51] FVIII activity levels ranged from 19% to 164% 1 year after AAV5-FVIII-BDD gene therapy in the highest dose cohort (6×10^{13} vector genome [vg]/kg, n = 7).[51] However, all of these subjects in the highest dose cohort received steroids for concern for hepatotoxicity, although there was no evidence of an anti-AAV capsid-directed cellular immune response. Speculated causes of this hepatotoxicity include an innate immune response triggered by the vector genome, direct hepatocyte injury due to vector processing, and ER-stress.[51] Although the UPR or ER-stress was not seen in murine studies after administration of the clinical AAV5-FVIII-BDD at doses up to 6×10^{13} vg/kg.[129] Encouraging initial clinical results have also been reported in abstract form using an alternative AAV vector with a transgene encoding for a similar FVIII-BDD protein (K.A. High et al., 2018, Am. Soc. Hematology, abstract) or FVIII-V3 (A.C. Nathwani et al., 2018, Am. Soc. Hematology, abstract). Although promising, ongoing concerns about hepatotoxicity might motivate the use of FVIII variants with further improvements of biological properties to decrease the vector dose and potentially avoid unwanted responses.

FIX-Padua and the Robin Hood Approach to Gene Therapy

The use of the hyperactive FIX variant, Padua (R338L), for HB gene therapy has been termed a "game-changer."[130] We initially identified FIX-Padua as the etiology of a rare X-linked thrombophilia due to a missense mutation in the gene for FIX.[131] The proband of this disorder presented with a spontaneous venous thrombosis at a young age.[131] Both the proband and another affected brother demonstrated an FIX-specific activity 8-fold higher than FIX-WT, whereas their mother, who was heterozygous for FIX-Padua, had a 4-fold increased specific activity, consistent with her being a female carrier.[131] Two earlier unrelated studies had previously identified the position R338 in FIX as being noteworthy, but for different reasons. First, an analysis of the frequencies of HB causing mutations had noted that despite R338 codon containing a CpG mutational hotspot, missense substitutions at this position were underrepresented in the HB mutation database. This insight led to the speculation, over 15 years before FIX-Padua was identified, that missense variants at position 338 might cause a very mild hemophilia or even thrombophilia.[132] Second, in an alanine-scanning study, FIX-R338A was observed to have about 3-fold increased specific activity compared with FIX-WT.[133] Our laboratory demonstrated that the hyperactivity of FIX-R338A could enhance the efficacy of AAV-based gene therapy in murine HB models.[134] Thus, the field was primed to rapidly take advantage of the high specific activity of FIX-Padua with the rationale that lowering the vector dose could mitigate the dose-dependent AAV-capsid-directed hepatotoxicity, which had stymied earlier clinical trials using FIX-WT.[55,56] We refer to this strategy as the Robin Hood approach because it takes from those who have too much FIX activity (the FIX-Padua proband) and gives to those who have too little (HB patients). However, safety concerns needed to be addressed.

Preclinical studies in small and large HB animal models supported the use of FIX-Padua to increase the potency of HB gene therapeutics and addressed the salient safety issue of immunogenicity and thrombogenicity.[135–141] The use of FIX-Padua raised several potential immunogenicity concerns. Foremost was the worry that the R338L amino acid substitution would alter the immunogenicity of FIX, such that expressing FIX-Padua after gene therapy could break the previously established immune tolerance in HB subjects to FIX-WT protein. The second potential immunogenicity concern was that the lower antigen levels associated with FIX-Padua could increase the risk of an anti-FIX immune response; this concern was based on earlier studies in HB mice where the ability of AAV liver gene therapy with FIX-WT to induce immune tolerance required surmounting a threshold level of FIX expression.[142] However, the specific threshold for immune tolerance was dependent on both the type of promoter used as well as the background strain of the HB mouse. Addressing these immunogenicity concerns was necessary for the development of FIX-Padua gene therapy because, although inhibitors are rare in HB (<3% of patients), they are very difficult to treat and are associated with a substantial increase in morbidity and mortality.[37]

In HB mice, we observed that AAV liver-directed gene therapy with hFIX-Padua or hFIX-WT induced immune tolerance that was maintained despite a provocative antigen challenge consisting of subcutaneous administration of hFIX-WT or hFIX-Padua protein, respectively, mixed with adjuvant.[135] Lentivirus (LV) liver-directed

gene therapy with hFIX-Padua similarly tolerized HB mice to FIX-WT challenges.[137] In outbred HB dog models, we and others have observed that cFIX-Padua and cFIX-WT have similar immunogenicity.[135,136,138,140] Notably, both AAV liver-directed[135] and muscle-directed[140] gene therapy with cFIX-Padua induces immune tolerance in an inhibitor-prone dog model. In this inhibitor-prone HB model, which is due to an early stop codon in the cFIX gene resulting in no cFIX transcript, a single infusion of cFIX protein concentrate results in inhibitor development.[143] After gene therapy with cFIX-Padua, these animals are tolerant to even widely spaced infusions of cFIX-WT protein.[135,140] Moreover, AAV liver gene therapy with cFIX-Padua induced immune tolerance even in a dog from this colony with a pre-existing anti-FIX-WT immune response.[135]

Four HB dogs from a different colony that we treated with AAV-cFIX-Padua have had sustained FIX antigen levels ranging from undetectable (~0.3% normal) to 1.5% normal.[136,140] Despite these minimal antigen levels, none of these animals have developed an immune response to cFIX even after multiple exposures with recombinant cFIX-WT protein. The concern raised from HB mouse studies that low antigen levels may promote an anti-FIX immune response[142] is not supported by our results in outbred canine models.[136,140] In summary, extensive *in vivo* studies in small- and large-animal HB models suggest that FIX-Padua has a similar immunogenicity as FIX-WT, even at minimal levels. These extensive preclinical studies established the immunogenic safety for the clinical use of FIX-Padua in gene therapy. Moreover, these data suggest that it is the continuous uninterrupted expression of antigen provided by gene therapy, rather than exceeding an antigen threshold level, that is critical for successful immune tolerance induction.

Despite FIX-Padua being initially identified as a cause of a thrombophilia, the accumulated experimental data indicate that FIX-Padua and FIX-WT have comparable thrombogenicity. We observed that mice expressing extremely high levels of FIX-WT or FIX-Padua after AAV gene therapy (up to 2,000% normal activity) had increased mortality and elevated markers of coagulation (thrombin-antithrombin complex and D-dimer levels), but there was no difference between the variants.[135] Furthermore, normal levels of markers of coagulation were observed in mice expressing up to 340% normal FIX activity[137,139] and dogs expressing up to 240% normal FIX activity[135,140] after gene therapy with FIX-Padua. Combined, these results implicate the high levels of FIX activity as the prothrombotic etiology in the Padua proband, rather than the specific R338L substitution. The proband likely developed thrombosis because of his very elevated FIX activity ($\geq$700% normal levels) rather than the specific R338L substitution itself.[131] Indeed, his heterozygous mother with FIX activity levels of about 300% normal never had thrombotic complications, including during three successful pregnancies,[131] a highly prothrombotic condition.

Preclinical studies also supported the efficacy of using FIX-Padua to enhance gene therapy for HB. Experimental injuries in HB mice after gene therapy demonstrate the *in vivo* hemostatic effectiveness of FIX-Padua,[137,139,144] although direct comparison against FIX-WT at equivalent activity levels has not been reported. However, FIX activity $\geq$1% normal after AAV (n = 10) or LV (n = 1) gene therapy with cFIX-Padua in HB dogs was sufficient to completely abrogate the severe bleeding phenotype with zero bleeds observed with 529 months of cumulative follow-up[135,136,138,140] (B. Samelson-Jones et al., 2018, Mol. Ther., abstract; unpublished data). Notably, this includes two dogs (O20[140] and O59[138]) that expressed between 1% and 2% normal FIX activity and $\leq$0.16% normal FIX antigen. The lack of bleeding and immune response to the transgene, even with minimally therapeutic FIX activity and antigen levels, demonstrates the *in vivo* hemostatic efficacy of FIX-Padua.

These comprehensive preclinical studies bolstered the use of FIX-Padua to test the rationale of the Robin Hood approach for HB gene therapy. We recently reported the successful utilization of FIX-Padua in an AAV liver gene therapy clinical trial for HB.[50] The use of FIX-Padua allowed for a lowering of the therapeutic vector dose by 4-fold, but an increase in the sustained FIX activity levels (mean ~30% normal) and a decrease in the number of subjects requiring immunosuppression to 2 out of 10 compared with 4 out of 6 observed in earlier trials using FIX-WT.[14,50] FIX-Padua antigen levels ranged from 1.2% to 7% normal.[50] As was expected, the annualized bleeding rate decreased from 11 to 0.4 after gene therapy.[50] There were no immunogenic or thrombogenic concerns in this trial. Similarly promising results were recently reported using FIX-Padua for AAV-based gene therapy in another early-phase trial for HB subjects (P. Chowdary et al., 2018, Am. Soc. Hematology, abstract).

Planned phase 3 AAV gene therapy studies using the FIX-Padua transgene have been announced by Pfizer (using the Spark Therapeutics program) and uniQure. The early-phase clinical study evaluating the uniQure vector utilizing FIX-WT achieved FIX activity levels of only between 3% and 13%,[57] compared with the Spark Therapeutics product that used FIX-Padua and achieved levels between 14% and 81% at a more than 10-fold lower vector dose.[50] uniQure subsequently announced for their pivotal trial that they would also use a FIX-Padua transgene.[130]

Uncertainties in Defining FVIII and FIX Activity

A variety of assays and reagents are used to measure FVIII or FIX activity in order to determine the severity of hemophilia, define the potency of protein factor products, and monitor post-infusion levels after therapeutic administration. One-stage clotting assays (OSAs) are the most widely available clinically. In OSAs, sample plasma is mixed with factor-deficient plasma, and the time until fibrin clot formation is monitored after coagulation is initiated by phospholipids, calcium, and a contact activation reagent such as silica, ellagic acid, or kaolin. Chromogenic substrate assays (CSAs), in contrast, make use of chromogenic FXa substrates to detect FX activation after sample plasma is mixed with phospholipids, calcium, and defined amounts of purified clotting factors necessary for FXa generation.[145] CSAs have lower interlaboratory variability, likely because they are not plasma based, and are the recommended method for potency

Table 2. One-Stage Assay Measurements of FIX-Padua-Specific Activity

FIX Ortholog	FIX-Specific Activity (Fold of FIX-WT)	One-Stage Assay Activator (Reagent)	References
Human	8	ellagic acid (Actin)	131
Human	7	ellagic acid (Actin FSL)	141
Human	5–6	silica (STA-PTT Automate)	139
Human	10–15	silica (TriniCLOT)	50
Canine	8–12	silica (TriniCLOT)	135,136
Canine	7	ellagic acid (Actin FSL)	138

assignments by the European Medicine Agency. However, long-recognized and well-described discrepancies exist between the factor activity measured by OSA and CSA for different FVIII and FIX molecules.[145–149] This issue of assay discrepancy has become more prominent as bioengineered EHL FVIII and FIX molecules have entered clinical use that requires specific assays and reagents to monitor post-infusion levels.[14,149] As perhaps should have been anticipated given the experience of other new therapies for hemophilia, OSA and CSA discrepancies were also recently noted in the FVIII-BDD and FIX-Padua activity in gene therapy subjects.[50,51]

There is a tendency for the measured activity of recombinant FVIII products to be lower with OSAs compared with CSAs, especially with FVIII-BDD variants.[145,150] Surprisingly, in HA subjects expressing FVIII-BDD after AAV5-based gene therapy, the measured FVIII activity with an OSA was ~1.7 times higher than with a CSA,[51] the opposite that has been observed for FVIII-BDD protein. Preclinical studies of AAV liver-directed gene transfer in HA mice and dogs have mostly relied on a CSA[87,96,151,152] with reasonable agreement between the CSA-determined FVIII activity and ELISA-determined FVIII antigen.[117,119] However, in a limited number of HA dogs (n = 6) expressing FVIII after viral vector liver-directed gene therapy, the measured FVIII activity with an OSA was 0.7 times lower (range 0.3–1) than with a CSA.[90,92] The cause of these differences is unknown, but it is a critical issue because sustained factor activity level is a proposed core outcome for gene therapy for hemophilia.[153] Being able to better understand these discrepancies will likely be needed before AAV products can be licensed for HA.

The activity of recombinant FIX (WT) protein products measured by OSA is ~1.4 the activity measured by CSA, which had not been observed for plasma-derived FIX products.[146] Interestingly, a similar assay discrepancy in FIX activity was observed in HB subjects expressing FIX-Padua after AAV gene therapy.[50] We have also observed a similar discrepancy between the measured FIX activity by OSA and CSA in HB dogs expressing cFIX-Padua (unpublished data). The specific activities of hFIX-Padua and cFIX-Padua appear similar whether determined with a silica or an ellagic acid-activated OSA clotting assay (Table 2). Likewise, we recently reported in an abstract a similar pattern of discrepancies for the measured FIX activity of recombinant FIX-Padua or FIX-Padua expressed in subjects that received AAV gene therapy for different OSA reagents, although it does not appear to be related to the specific activator (M. Robinson et al., 2018, Gene Delivery Am. Soc. Hematology, abstract). The etiology of these discrepancies is unknown, but is a salient issue for the clinical development and potential licensing of AAV-FIX-Padua. Recombinant FIX-Padua protein calibration curves may help better define the amount of FIX-Padua expression in gene therapy subjects.

Alternative Protein Engineering Approaches to Enhance FVIII or FIX Gene Transfer

New FVIII or FIX bioengineered variants that have demonstrated advantageous properties as proteins may be amenable to incorporation into gene therapy vectors. However, *in vivo* studies are required to ensure adequate expression and secretion from the gene therapy-targeted tissue, which cannot be assumed.

Designing FVIII to Resist Inactivation. Most bioengineering approaches investigated to enhance the hemostatic effect of FVIII have focused on designing FVIIIa to resist inactivation. Once activated, FVIIIa is inactivated by the dissociation of the A2-domain from the heterotrimer and/or by the proteolytic inactivation by activated protein C (APC) (Figure 1A). Successful strategies to impede A2 dissociation have included: (1) removing the thrombin cleavage sites R740 and R1649 in a B-domain truncated FVIII such that A2-domain remains covalently bonded through the peptide chain to the A3-domain;[154,155] (2) introducing a disulfide bond connecting the A2-domain with the A3-domain through amino acid substitutions (Y664C/T1826C);[156] and (3) eliminating potentially unfavorable electrostatic interactions by the replacement of charged amino acids with hydrophobic amino acids in A2.[157–159] This latter approach has yielded a FVIII-BDD variant, D519V/E665V, that demonstrates about 2-fold increased hemostatic efficacy in *in vitro* and *in vivo* assays.[157] APC inactivates FVIIIa by proteolytic cleavage at R336 (A1-domain) and/or R562 (A2-domain). Amino acid substitutions at these positions have also resulted in FVIII variants with increased hemostatic efficacy in *in vitro* and *in vivo* assays (N.A. Parsons et al., 2017, Am. Soc. Hematology, abstract).[154,160] To date, none of these bioengineered FVIII variants appear to provide enough hemostatic enhancement over FVIII-WT to easily justify the potential immunogenic risk of introducing neo-epitopes; however, synergistic combinations and/or novel approaches may yet prove sufficient.

Increasing FIX-Specific Activity beyond Padua. Several alternative missense variants besides R338L demonstrate increased FIX activity compared with FIX-WT (Table 3). Several combinations of these amino substitutions appear to have a synergistic effect on FIX activity approaching 20-fold higher than FIX-WT activity. However, the biochemical and epistasis mechanisms of these enhancements have not been studied in detail. As the use of FIX-Padua allowed for a lowering of the AAV vector dose to 5×10^{11} vg/kg, which mitigated but did eliminate the hepatotoxicity associated with AAV liver-directed gene therapy,[50] there is a strong rationale for utilizing even more active FIX variants to further enhance the vector potency.

Table 3. Hyperactive FIX Variants

Amino Acid Substitutions[a]	Fold Change in FIX Activity (Relative to FIX-WT)[b]	References
G4Y	1.2	161
V10K	1.6	161,211
V86A	1.1	212
K265T	1.9	213,214
E277A	1.3	212
R338L	8.0	131
N346A	1.2	215
S377W	1.4	161
E410H	4.6	216
R338L + S377W	12	161
V10K + R338L + S337W	19	161
R318Y + R338E + T343R	17	(S.-B. Hong et al., 2016, Am. Soc. Hematology, abstract)
V86A + E277A + R338L	22	141
G4Y + V86A + R338L + S337W	15	161

[a]Numbering is based on mature FIX protein without propeptide sequence; if multiple hyperactive variants occur with substitutions at the same amino acid position, only the reported highest activity variant is included.
[b]Activity based on reported one-stage clotting assay.

Indeed, AAV8 vector doses of 2×10^{11} vg/kg resulted in sustained FIX-WT levels of 2% and were not associated with hepatotoxicity (n = 2).[55,56] The use of bioengineered FIX variants with specific activity greater than FIX-Padua would allow for further decreases in the vector dose while maintaining comparable plateau FIX activity levels. However, only a few studies have evaluated the efficacy of these FIX variants *in vivo*.[141,161] There are also no published reports that delineated the immunogenic risk of multiple amino acid substitutions in FIX, which remains a major safety concern (as discussed below). The preclinical development of FIX-Padua may provide a roadmap for how the efficacy and safety concerns of immunogenicity and thrombogenicity could begin to be addressed for these variants.[135–140]

Extended Half-Life Factors in Gene Therapy. An alternative method to enhance the potency of gene therapy vectors may be to use bioengineered EHL FVIII or FIX fusion proteins as transgenes. These EHL products fuse half-life extending proteins, such as the IgG1-Fc domain or albumin, with FVIII or FIX variants.[11–14] The rationale of employing EHL variants in gene therapy is that higher sustained circulating activity levels may be achievable as long as the EHL variants continue to display prolonged half-lives after gene transfer without compromised expression or secretion compared with the WT factor. Because of the packaging constraints of AAV vectors discussed above, only FIX fusion proteins would be amenable to incorporation into AAV vectors. However, recent work evaluating AAV gene therapy with transgenes of FIX fused with albumin or the Fc domain in HB mice was unsuccessful because of poor transgene expression compared with FIX-WT.[162] Intriguingly, several gene-editing approaches have also investigated inserting the FVIII or FIX gene into the albumin locus as an integrative gene therapy strategy to ensure long-term expression of WT FVIII or FIX despite hepatocyte cell division.[163–165] Whether it is possible to modify these gene integrative approaches to express *in vivo* an FIX-albumin fusion protein similar to the current EHL product FIX-FP (Idelvion, Coagulation Factor IX [Recombinant], Albumin Fusion Protein) remains to be determined.

Engineering FVIIa for Gene Therapy

Recombinant FVIIa (eptacog alfa [NovoSeven]) is a successful acute and prophylactic protein bypassing agent for hemophilia with inhibitors.[30,166] Its major efficacy limitation is its short half-life (~2 h), which often necessitates frequent intravenous infusions. Thrombotic complications remain the major safety concern.[167] Stable continuous protein expression provided by gene therapy could overcome the efficacy limitation imposed by its short half-life while potentially minimizing its thrombotic complications (Figure 2). To this end, a FVII variant was engineered to be secreted as FVIIa rather than zymogen FVII by introducing a furin recognition motif (RKRRKR) at the cleavage site of FVII that yields FVIIa (Figure 2).[168,169] This strategy is the converse employed for the furin-evading FVIII variant FVIII-ΔF.[117] AAV-based liver-directed gene therapy with this FVIIa variant resulted in significantly improved hemostasis in HB mice and an amelioration of the bleeding phenotype in both HA and HB dogs.[168,170] It also improved the bleeding phenotype in HA mice with inhibitors[171] and decreased the clot time in HB mice with inhibitors.[172] Although there are no reports demonstrating enhanced hemostasis after FVIIa gene therapy in large-animal models with inhibitors, it is expected that FVIIa should provide the same improvement of hemostasis regardless of the presence of inhibitors because of its bypassing mechanism.

However, the minimal efficacious and the maximal safe level of FVIIa after gene therapy has yet to be rigorously determined because a cessation of spontaneous bleeding was observed in one HB dog despite no measurable increase in the FVIIa levels,[170] whereas HB mice expressing FVIIa levels greater than 2 μg/mL (mean 3.2 μg/mL) exhibited thrombosis and premature mortality.[173] As such, it remains unclear whether current AAV-based gene transfer technology can provide clinically relevant FVIIa levels in patients with hemophilia with inhibitors. Indeed, the AAV vector doses used for FVIIa gene transfer in the canine hemophilia model[170] were ~4-fold higher than used in analogous FVIII or FIX gene transfer studies in the same model.[87,174,175] As such, there is a strong rationale for developing new FVIIa variants with advantageous properties to potentially lower the vector dose while maintaining hemostasis.

Protein-Engineered Bypassing Agents

Novel hemostatic agents have also been engineered from blood proteins besides FVIII and FIX, including FVIIa variants with enhanced

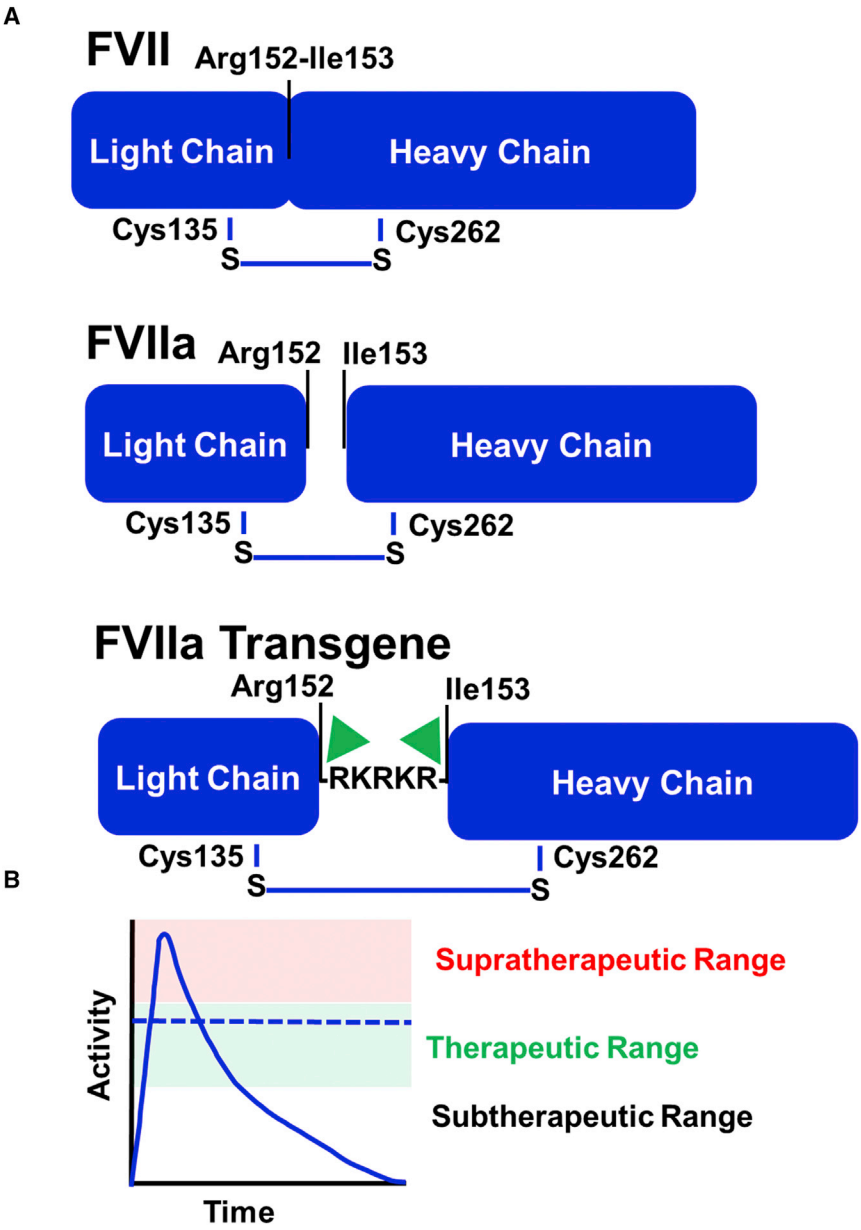

Figure 2. Adopting FVIIa for Gene Therapy

(A) Proteolytic cleavage of zymogen FVII between R152 and I153 results in the two-chain activated protease FVIIa composed of a heavy chain and a light chain held together by a disulfide bond. In the design of FVIIa for gene therapy, the furin recognition motif RKRKR was inserted into the transgene such that cleavage by furin (green triangles) would result in a secreted two-chain FVIIa molecule. (B) Illustration of potential advantages of stable activity level provided by FVIIa gene therapy (dashed line) compared with the peaks and troughs associated with current FVIIa protein therapy (solid line). In the latter, peaks at supratherapeutic levels pose a thrombotic risk, whereas troughs at subtherapeutic levels do not provide sufficient hemostasis.

biological properties (Table 4). Like FVIIa, these proteins are designed to promote hemostasis through mechanisms that bypass both FVIII and FIX activity. They therefore have the potential to treat bleeding in hemophilia patients with inhibitors. To date, most studies have focused on evaluating these variants as protein therapeutics. An exception is the assessment of the hyperactive FVIIa variant, FVIIa-VEAY, as a transgene for AAV-based liver-directed gene therapy in HA mice.[172] The use of this hyperactive variant allowed for an ~100-fold lower vector dose compared with FVIIa-WT, while maintaining a comparable response to hemostatic challenges and a normalization in the clot time.[172] Although several other engineered FVIIa variants progressed to late-stage clinical trials as protein bypassing agents, their clinical development has been discontinued because of immunogenicity concerns (see below). Even if these immunogenicity concerns could be addressed, it remains to be determined whether the use of hyperactive FVIIa transgenes can lower the vector dose sufficiently in large-animal models such that clinical development could be contemplated.

Other protein-engineered bypassing strategies have extended the biological half-life of activated coagulation factors downstream of FVIII and FIX. ^{SUPER}FVa is designed to resist inactivation by limiting the A2-domain dissociation and APC proteolysis.[176,177] Similar strategies have been applied to FVIII to also avoid inactivation, as discussed above.[154,156,160,178] Zymogen-like FXa is engineered to evade antithrombin inhibition by maintaining the zymogen-like conformation until bound to its cofactor FVa.[179] It has demonstrated hemostatic efficacy as a protein therapy in hemophilia models.[180,181] It is currently being developed for acute hemorrhagic conditions and has demonstrated safety in an early-phase clinical trial with hemostatically normal subjects.[182,183] As such, its future use in hemophilia could be envisioned.

Protein engineering has also yielded new non-factor therapies for hemophilia designed to rebalance the hemostatic system by lowering endogenous anticoagulants to compensate for the decrease in procoagulant activity caused by the deficiency in FVIII or FIX.[12,14] An α-1-antitrypsin variant (A1AT-KRK) specifically designed to irreversibly inhibit APC, but not thrombin, demonstrated hemostatic efficacy as a protein therapeutic in murine hemophilia models.[184] This approach is supported by previous studies demonstrating that the FV variant Leiden that is resistant to APC inactivation ameliorates the

Table 4. Non-FVIII or -FIX Bioengineered Blood Proteins for Hemophilia Therapy

Proteins	Variant	Mechanism	Therapeutic Development	References
FVIIa				
VEAY	L305V	10-fold increased FX activation due to optimized active site	efficacy in murine studies	172,217,218
	S314E			
	K337A			
	F374Y			
DVQ, NN1731, vatreptacog alfa	V158D	50-fold increased FX activation due to partial mimicking of TF-bound conformation	11% (n = 8) of subjects in phase 3 study developed ADA	192,217,219
	E296V			
	M298Q			
BAY86-6150	P10Q	increased affinity to activated membranes	10% (n = 1) of subjects in phase 3 study developed ADA	191,220
	K32E	prolonged half-life		
	A34E			
	R36E			
	T106N			
	V253N			
marzeptacog alfa	T128N	7-fold increased FX activation	phase 2 for subcutaneous delivery	H. Levy et al., 2018, Int. Soc. Thromb. Haemost., abstract; 221
	P129A	prolonged half-life		
	Q286R			
	M298Q			
FVa				
^{SUPER}FVa	H609C	engineered disulfide bond between A2 and A3 domain	efficacy in murine studies	176,177
	E1691C	APC resistance		
	R306Q			
	R506Q			
	R679Q			
FXa				
zymogen-like FXa	I195L[a]	extends biological half-life of activated protease by limiting inhibition by serpins	early-phase clinic studies	179–183
A1AT				
KRK Serpin PC	P357K	specifically and irreversibly inhibits APC	efficacy in murine studies	184
	M358R			
	S359K			

ADA, anti-drug antibodies; TF, tissue factor.[a]FXa-I195L is typically referred to in the literature as I16L based on chymotrypsin numbering; here, for consistency with the other proteins, we have provided the numbering based on the mature zymogen.

hemophilia phenotype in mice,[185] as well as the clinical observation that protein C deficiency similarly improves the bleeding phenotype in HA patients.[186] Several clinical studies have demonstrated the safety of AAV gene therapy for A1AT deficiency,[187] suggesting that A1AT-KRK may be adaptable as a gene therapy for hemophilia with inhibitors.

The adoption of these engineered proteins as gene therapies would require specific preclinical studies to determine their safety and efficacy as transgenes. Because these bypassing therapies rely on promoting hemostasis by circumventing regulatory pathways, the continuous expression provided by gene therapy may open unique therapeutic windows for these proteins, as illustrated in Figure 2B.

No Such Thing as a Free Lunch: The Immunogenic Cost of Bioengineering

The potential advantages of bioengineered variants in gene therapy for hemophilia must be balanced against the potential risk of exacerbating the immunogenicity of the transgene. Small changes in the amino acid sequence of proteins can have a profound effect on their immunogenicity. We currently cannot predict with certainty how primary protein sequence changes may impact the response of the

immune system to therapeutic proteins or transgenes. Further complicating the balancing of immunogenic risks versus biological benefits is the emerging paradigm that gene therapy, and especially liver-directed gene therapy, is biased toward immune tolerance compared with other therapeutic approaches.[188–190] Immunogenicity data of an engineered protein therapy, therefore, may not be applicable for gene therapy with the same engineered variant. However, there is likely little enthusiasm for a transgene that has already demonstrated immunogenicity concerns as a protein therapy.

The recent clinical trial experience of two distinct bioengineered FVIIa variants as protein therapy with three and six amino acid substitutions (Table 4) that resulted in antibodies that cross-reacted to FVIIa-WT highlights the immunogenic risk of protein bioengineering.[191–193] These variants were designed to overcome some of the current limitations of FVIIa-WT protein therapy by improving its hemostatic ability and enhancing its half-life. The amino acid substitutions in NN1731 are in the heavy chain of FVIIa, whereas the substitutions in BAY 86-6150 are predominantly in the light chain (Table 4). Both variants demonstrated safety and efficacy in early-phase studies,[194–196] but ~10% of subjects in the phase 3 studies developed antibodies against the FVIIa variants: 1 of 10 subjects for BAY 86-6150 and 11 of 72 subjects for NN1731.[191,192] In contrast, there have not been reports of immunogenicity against recombinant FVIIa-WT in patients with hemophilia despite decades of clinical use, and the rate of anti-FVII antibodies in congenital FVII deficiency is lower than the rate of inhibitors in HB.[197] Alarmingly, the antibodies of 4 out of the 11 subjects in the NN1731 trial and the antibodies from the subject in the BAY 86-6150 trial cross-reacted with FVIIa-WT, suggesting that treatment with the variant FVIIa protein broke the previously established tolerance to endogenous FVIIa-WT.[191,192] The antibodies from the BAY 86-6150 subject were also notably neutralizing to both variant and FVIIa-WT.[191] It is plausible that this immunogenic risk was only apparent in the late-phase clinical trials as the number of subjects and exposure days increased.

A similar conclusion may be inferred from a recent report of the development of inhibitors in the most intensive-dosing cohort of HB subjects receiving subcutaneous FIX-R318Y/R338E/T343R (CB 2679d/ISU304). However, this outcome was not wholly unanticipated as earlier studies had suggested that subcutaneous administration of FIX-WT to HB patients was already associated with an increased immunogenic risk.[198] Combined, these disappointing clinical trial experiences with bioengineered proteins with $\geq$3 amino acid substitutions emphasize the immunogenic risk of this approach. The implication of these results for bioengineered transgenes is unknown.

However, other amino acid differences and neo-epitopes in factor variants have not been associated with immunogenic concerns. As mentioned, most evaluations of therapeutic FVIII-BDD protein with its single neo-epitope have found it has a similar immunogenicity as full-length FVIII proteins,[81–83] although this conclusion is not unequivocal.[199] Interim analysis of the immunogenicity of a new B-domain-modified product with an alternative linker with a partial non-FVIII sequence (Figure 1B; Nuwiq, Antihemophilic Factor [Recombinant]) designed to facilitate expression in human cell lines suggests this strategy may have a lower inhibitor risk than other FVIII variants expressed from non-human cell lines.[200] Likewise, there have been no reports to date of the development of new anti-FVIII antibodies against BAY 94-9027 (Jivi, antihemophilic factor [recombinant], PEGylated-aucl), which is a recently approved EHL FVIII product with the substitution K1804C (in the A3 domain) introduced to allow site-specific pegylation.[201,202] There have also been no reports of anti-FIX antibodies in the $\geq$16 subjects who have received FIX-Padua gene therapy (P.E. Monahan et al., 2015, Congr. Int. Soc. Thromb. Haemost., abstract).[50]

Similarly, the risk of inhibitor development in patients with non-severe HA is highly dependent on the location of the disease-causing missense variation.[203] Several non-disease-causing polymorphisms in FVIII also do not increase the recognition of FVIII products by the immune system.[204] Likewise, discrepancies between endogenous FIX and FIX-therapeutics due to a common polymorphism at position 148 are not associated with an increased risk of an anti-FIX immune response with either recombinant protein or muscle-directed gene therapy.[134] Combined, these observations suggest that only specific discrepancies between endogenous factor and therapeutic products will be recognized as foreign; as such, there are likely low-risk regions where protein modifications are unlikely to change the immunogenicity.

There is no current gold standard experimental system to definitively define the immunogenic risk of an engineered protein or transgene, although regulatory guidance has provided some instruction.[205] Comprehensive studies utilizing multiple experimental approaches will likely have the most success in identifying problematic variants with immunogenic concerns during preclinical development. Comparative data using the WT variant as a control can define the relative immunogenic risk of new engineered variants compared with the WT. Potential approaches could include: (1) *ex vivo* studies measuring the recognition and response of patients' immune cells to the engineered protein;[193,204,206] (2) in silica studies to determine the likelihood of neo-epitope recognition by clinically relevant repertoires of HLA types, which could also suggest HLA alleles at particular high risk that may serve as clinical trial exclusion criteria;[193,206] (3) small-animal studies utilizing models tolerant to the WT variant to test whether therapeutic administration of the engineered variant will break an already established immune tolerance;[135] and (4) large-animal studies utilizing species-specific transgenes (or proteins) to avoid the xeno-protein response.[117,135,136,138,140,151]

In summary, the immunogenicity of an engineered protein or transgene likely depends on the number and/or location of neo-epitopes. Comprehensive preclinical evaluation using various experimental approaches may recognize immunogenic engineered variants that should not be developed further, but currently cannot provide definitive certainty about the risk of immunogenicity. The safest strategy is

likely to minimize the number of neo-epitopes and localize them to known or predicted low-risk regions of the protein.

Conclusions

The use of engineered FVIII and FIX transgenes has been essential for AAV-based gene therapy to advance to phase 3 studies for HA and HB, respectively. However, the vector dose-dependent hepatotoxicity remains a major safety and efficacy limitation for AAV gene therapy. The clinical experience of using FIX-Padua instead of FIX-WT for HB gene therapy exemplifies the ability of bioengineered transgenes to increase the potency of a given vector to address these concerns. Improved vector potency through protein engineering would also ease production and manufacturing costs.

Engineered non-FVIII and -FIX variants also have the potential to help treat patients with inhibitors, although these strategies have mostly been tested as protein therapeutics. The exception is FVIIa gene therapy (Figure 2), which has demonstrated safety and efficacy in large-animal hemophilia models, but not at clinically feasible vector doses to date. The appropriate therapeutic window of FVIIa transgene is also undefined.

The numerous alternative variants of FVIII and FIX (Table 3) with desirable biological properties already identified suggest that these proteins are highly amenable to bioengineering approaches. However, no preclinical study can currently reliably predict the immunologic costs of each strategy. Both *in silica* and *in vivo* studies are probably informative and complementary. Engineering strategies that minimize the number of neo-epitopes and prioritize changes to low-risk protein regions are the most likely to be successful. Biochemical characterization of the variant proteins will also likely be helpful. Engineered transgenes will likely have synergistic effects with other vector improvements, such as codon optimization, on the overall potency of the gene product. Codon optimization has increased transgene levels of both FVIII[207] and FIX,[207] although codon optimization is not without its own concerns, including the introduction of new open reading frames.[208] Disease-causing synonymous mutations have also been described in both FVIII and FIX.[209,210]

Gene therapy for hemophilia offers the promise of a lifelong cure after a single therapeutic administration. There is considerable optimism about fulfilling this promise with the commencement of phase 3 gene therapy studies for both HA and HB utilizing engineered transgenes, although barriers remain for widespread adoption. Hopefully, continued advancements will eventually expand gene therapy to all hemophilia patients worldwide.

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