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Gene-based continuous expression of FVIIa for the treatment of hemophilia

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TABLE OF CONTENTS

1. ABSTRACT

Qualitative or quantitative defects in the genes for coagulation factors VIII (FVIII) or IX (FIX) result in a life-threatening, bleeding phenotype (hemophilia A [HA] or B [HB], respectively). Although hemophilia treatment by clotting factor replacement is effective, a proportion of patients develop neutralizing antibodies (inhibitors) to the infused factor that complicate the disease management. For inhibitor patients, recombinant human activated coagulation Factor VII (rhFVIIa), when administered at therapeutic doses, has been shown to bypass the deficiency in FVIII or FIX and result in hemostasis. As an alternative to this protein infusion therapy, a gene-based approach for the treatment of hemophilia with inhibitors has been developed, using continuous expression of a transgene coding for FVIIa following viral-mediated delivery. This approach was validated in hemophilic mice and, notably, in dogs as a model that closely resembles the human disease. In particular, liver-directed FVIIa gene delivery in hemophilic dogs resulted in multi-year transgene expression that ameliorated the bleeding phenotype, without thrombotic complications. These data support the gene-based FVIIa expression as a novel bypass therapy for hemophilia with inhibitors.

2. INTRODUCTION

Blood coagulation is a homeostatic system aimed to prevent excessive loss of blood following injury by the formation of a clot localized to the site of injury. The highly orchestrated and multi-protein nature of the coagulation system makes it vulnerable to mutations with profound effects that can affect survival. Prime examples are hemophilia A (HA) and B (HB), caused by deficiency in circulating levels or activity of FVIII or FIX, respectively, that are characterized by bleeding diatheses with a range of severity (depending on the specific mutation). Although the current treatment by plasma-derived or recombinant factors has greatly improved the management and care of hemophilic patients, the development of neutralizing antibodies (inhibitors) to such factors in a subset of patients (~30% and ~5% of severe HA and HB, respectively) represents the most significant complication in disease management. In those cases, hemostasis in response to bleeds is successfully implemented by bolus infusion of recombinant human activated Factor VII (rhFVIIa) that can bypass the defects in FVIII or FIX. As an alternative to protein infusion for hemophilia treatment, gene delivery of a transgene coding for a functional copy of the missing factor may provide

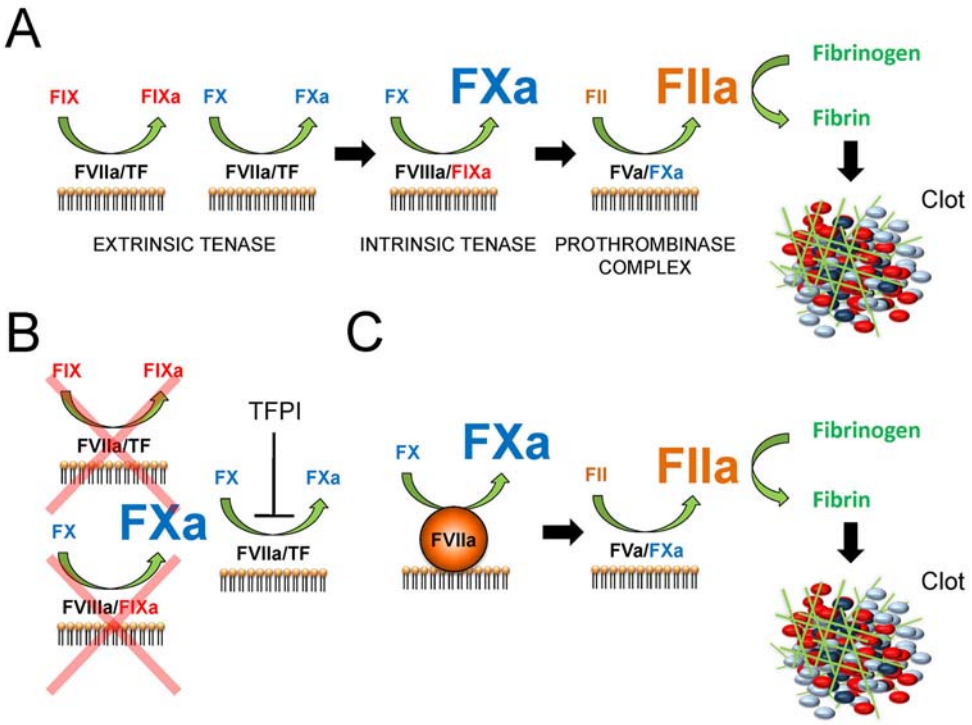

Figure 1. Simplified overview of hemostasis. (A) Schematic representation of coagulation reactions on negatively charged phospholipid membranes that lead to the conversion of prothrombin (FII) to thrombin (FIIa) and clot formation in hemostatically normal individuals. (B) In hemophilia, deficiency in FVIII or FIX results in a dysfunctional extrinsic/intrinsic tenase complex. The FXa produced by the FVIIa-TF complex cannot rescue normal thrombin generation since the FXa-FVIIa-TF ternary complex is rapidly shut down by TFPI. (C) Therapeutic administration of FVIIa in hemophilia results in *direct* FX activation and therefore thrombin generation that can bypass the need for FVIII/FIX. In all panels, calcium ions are omitted.

life-long amelioration of the disease. Specifically for inhibitor patients, delivery of a gene-based *activated* FVII has the potential to provide hemostasis regardless of the inhibitor status of the recipient, similar to that achieved by bolus rhFVIIa infusions. This review will focus on the existing biochemical and animal model data using activated FVII as a gene-based bypass for hemophilia.

3.1. Coagulation overview

Blood coagulation depends on interactions between soluble as well as membrane-bound components. Such interactions result in a cascade of proteolytic events that convert inactive precursors (zymogens) to catalytically active enzymes (or cofactors; Figure 1A). The initial step in blood clotting is provided by the exposure of tissue factor (TF, a 47 kDa transmembrane glycoprotein) following injury, that tethers Factor VIIa (FVIIa) on a negatively charged phospholipid (PL) membrane to form an enzymatic complex (TF-FVIIa-PL) (1). In the presence of calcium ions (Ca^{2+}), this complex converts small amounts of factors IX (FIX) and X (FX) to their active forms (FIXa and FXa, respectively). The FXa generated by TF-FVIIa-PL-Ca^{2+} (also referred to as extrinsic tenase complex), converts trace amounts of prothrombin to thrombin. Subsequently, thrombin converts small amounts of cofactors Factor V (FV) and FVIII, to their active form (FVa and FVIIIa, respectively) and augments activation of circulating platelets at the site of injury. The negatively charged PL membrane of activated platelets provides the proper milieu for the formation of the intrinsic tenase complex (FVIIIa together with FIXa and Ca^{2+}) that is responsible for the bulk of FXa generation during coagulation. FXa together with FVa and PL/Ca^{2+} form the prothrombinase complex that converts prothrombin to thrombin. Thrombin can then activate Factor XI (generating FXIa), which subsequently generates more FIXa that is channeled into the intrinsic tenase complex. The overall result is a self-amplifying, thrombin-generating loop, where the product (thrombin) catalyzes the cleavage of fibrinogen to fibrin, forming a mesh of blood cells and hence a hemostatic plug. Although not described here, the anticoagulant and fibrinolytic systems (that operate concurrently with ongoing coagulation), curb excessive hemostasis and initiate clot retraction prior to wound healing at the injury site.

3.2. The bleeding phenotype in hemophilia and its current treatment

Our current understanding of coagulation assigns important roles to prothrombin, FX, FV as well as FVIII and FIX. This is exemplified by the rarity of viable, complete deficiencies in prothrombin, FX and FV that are within the 1 in 1,000,000 to 2,000,000 births (2). In contrast to such rare events, defects in FVIII or FIX, resulting in abnormalities in circulating levels (either by

mutations in the coding sequence or in regulatory elements of their respective cistrons) or in activity (either enzymatic [for FIX] or cofactor [for FVIII]) are more common. Although the extrinsic tenase complex can generate the initial trace FXa amounts, tissue factor pathway inhibitor (TFPI) rapidly shuts down FXa production by this complex. As a result, the extrinsic tenase complex cannot compensate for deficiencies in FVIII or FIX that effectively translate in diminished clot formation (Figure 1B).

Since the genes for FVIII and FIX are located on the X chromosome, the FVIII or FIX deficiencies (called hemophilia) follow an X-linked pattern of inheritance. Incidence rates in the United States are 1 in 10,000 males (HA) and 1 in 35,000 (HB) males (3). Similar incidence rates exist worldwide (4). Based on this frequency, females homozygous for FVIII or FIX deficiency are extremely rare. Despite the differences in disease frequency, HA and HB are clinically indistinguishable and the severity of disease correlates with the circulating factor levels. Normal circulating levels of FVIII and FIX are 0.1-0.2 μg/ml and ~5 μg/ml, respectively; patients with <1% normal are classified as severe, those with 1-5% normal are moderate and those with 6-30% normal are mild. To put this in perspective, approximately 43% of hemophilic patients are classified as severe, 26% classified as moderate and 31% as mild (3). The most common clinical manifestation of hemophilia is musculoskeletal bleeding: severe hemophiliacs exhibit spontaneous bleeds into joints/muscles/soft tissues in addition to bleeds following minor trauma, whereas moderate or mild hemophiliacs show less frequent spontaneous bleeds or bleeds after surgery/major trauma, respectively (5). Recurrent bleeds in the joints accounting for 90% of all hemophilia bleeds (6) result in neovascularization, synovial hypertrophy and, eventually, a persistent inflammatory disorder called hemophilic synovitis, leading to hemophilic arthropathy, the major morbidity in hemophilic patients (7, 8).

Treatment of spontaneous bleeding episodes in hemophilia is by on-demand infusion of factor. Although viral inactivation procedures and donor screening processes helped to greatly reduce the risk of human immunodeficiency virus, hepatitis B and C transmission from the initial plasma-derived factor concentrates, such risk from yet unidentified or thermo-resistant or non-lipid-coated pathogens remains (9). Fortunately, the advent of recombinant protein production for coagulation factors virtually eliminated the risk of infectious disease transmission. It also augmented prophylaxis in disease management, aimed at prevention of spontaneous joint bleeds and preservation of joint function (5). The clinical benefit of prophylaxis vs. on-demand therapy on joint bleeds and delaying or preventing hemophilic arthropathy was originally suggested from observational studies that started in Sweden in 1958 with prophylactic administration of FVIII or FIX (10). These observations were further confirmed by other studies (11-16). While on prophylaxis, the only significant predictor of hemophilic arthropathy is the age of patients at which they are enrolled in such regimen (17) as it is most successful at a young age. Unfortunately, enrollment of large pediatric/young patient numbers on a prophylaxis regimen is hampered by common use of central lines that increases the incidence of central venous thrombosis and, notably, by financial considerations (18).

3.3. Development of neutralizing antibodies in hemophilia patients

Despite improvements in protein replacement therapy and further mechanistic insights in blood coagulation, the most challenging aspect of hemophilia management is the development of neutralizing alloantibodies to the infused factor (19), clinically referred to as “inhibitors”. These antibodies are polyclonal and high affinity, belonging predominantly to the IgG4 subclass (20). Inhibitor development is multifactorial and influenced by genetics (the underlying FVIII/FIX mutation or mutations in genes involved in regulation of the immune system), family history (21), as well as environmental, treatment-related reasons (such as age at first exposure to factor) or type of factor used (recombinant or plasma-derived) (20). Inhibitor patients represent ~30% and ~5% of severe HA and HB patients, respectively (20) and have a far more challenging clinical management than patients without inhibitors (22). They are categorized according to the magnitude of neutralizing activity (titer) in response to factor infusion, measured in Bethesda units (BU; one BU is the dilution of the patient plasma that neutralizes 50% of FVIII/FIX activity of normal plasma). Patients with titers ≤ 5 BU are low-responders whereas those with > 5 BU are high responders.

As expected, the neutralizing nature of inhibitors complicates hemostasis management and increases the disease mortality and morbidity (23). In the long-term, eradication of inhibitors by immune tolerance induction (ITI) might be considered to be the best option, especially for high-titer inhibitors where the success rate can reach ~ 60% (in the case of HA (24)) but carries a substantial cost (~ US $1 million per 5 year old (25)) and inconvenience to the patient from daily factor administration over a long period of time. For the treatment of acute bleeds in inhibitor patients, so-called “bypass” agents are used. Such agents rely on thrombin generation bypassing the need for FVIII or FIX and therefore effect hemostasis regardless of the inhibitor status of the recipient. Recombinant activated human Factor VII (rhFVIIa) has been a well-established hemostatic agent for inhibitor patients (26) and, following the introduction of rhFVIIa to home treatment programs, has resulted in improved preservation of joint function (27). To correct hemostasis, bolus infusion of 90-120 μg/kg of rhFVIIa every 2-3 hours, or a single high-dose of 270 μg/kg are necessary (28) that results in *direct* FXa and hence thrombin formation (Figure 1C).

Despite the clear hemostatic efficacy, rhFVIIa therapy has several disadvantages that prevent its long-term use in prophylaxis: (i) the short half-life of the protein that is ~2.5h (29); (ii) the need for frequent dosing to achieve hemostasis in the 90-120 μg/kg regimen that results in (iii) overall high cost of treatment. The latter is exemplified by the fact that following the introduction of rhFVIIa to the market in 1996, the annual cost of protein therapy for high-titer inhibitor patients increased >3 fold ($186,000 vs.

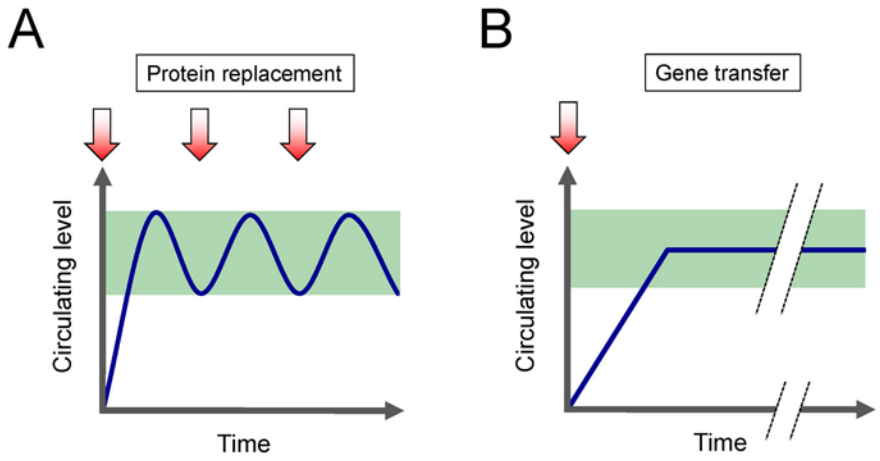

Figure 2. A comparison of protein replacement and gene therapy. Protein replacement therapy results in variable circulating levels (A) within the therapeutic range (green box) determined by the timing of administration. In contrast, gene transfer of the appropriate transgene (B) results in an initial rise in circulating levels that remain relatively stable with time, within the therapeutic range (green box).

$60,000) (30), suggesting a currently unmet clinical need for alternative therapeutic applications. In addition, the risk of FVIIa-mediated thrombosis from bolus dosing has been somewhat controversial: studies of rhFVIIa use for an approved indication (such as hemophilia) suggest a low rate of thromboembolic events (<1% (31, 32)). In contrast, another study suggested that off-label rhFVIIa use was associated with increased thromboembolic events; however such conclusions may have been affected by concomitant therapies or underlying complex disease (33). A new meta-analysis of 35 randomized clinical trials on off-label rhFVIIa administration for the treatment of bleeding (26 with patients and 9 with healthy volunteers) also suggests an increased risk of arterial but not venous thromboembolic events (34).

3.4. The concept of gene therapy for hemophilia

In contrast to repeated protein infusion where circulating levels display peaks and troughs, gene-delivery of a therapeutic transgene in an appropriate tissue offers the potential for its continuous expression within the therapeutic window of the disorder (Figure 2). On the other hand, this mode of delivery translates to more complex pharmacokinetics, where steady-state circulating levels of the therapeutic transgene product depend on the rate of synthesis, secretion, clearance and inactivation (if applicable).

Hemophilia has been regarded as an ideal monogenic disorder to treat by gene transfer for several reasons: (i) wide therapeutic window; (ii) ability of a variety of cell types to synthesize biologically active clotting factors; (iii) availability of small and large animal models of disease; and (iv) existence of several *in vitro* assays for determination of efficacy (35). Gene-based approaches for hemophilia aim to introduce the therapeutic transgene (FVIII or FIX) via a vector (viral or plasmid) to a target organ/cell that subsequently expresses it. The premise is that the recipient will acquire the ability to synthesize their own FVIII or FIX, thus minimizing (or avoiding altogether) the need for frequent factor infusions. As such, a gene-based approach for hemophilia can potentially address three limitations of the current protein replacement therapy: first, it can provide stable levels of the therapeutic protein. Second, prophylactic factor infusions in the pediatric population necessitate placement of central catheters that is associated with venous thrombosis and infection (36, 37). Third, inhibitor development from factor infusion remains a significant complication for treatment and its eradication by ITI carries a substantial financial burden.

Hemophilia gene therapy clinical trials are described in detail elsewhere (38) and therefore will not be described in length here. However, since adeno-associated virus (AAV) is the most promising delivery vehicle for the work described below and has been used in clinical trials for hemophilia B (39, 40), it will be briefly discussed. Wildtype AAV is a single-stranded DNA, non-enveloped member of the *Dependovirus* genus of the *Parvovirinae* subfamily of *Parvoviridae* (41). Its genome (~4500 base pairs) contains the necessary viral coding sequences flanked by two inverted terminal repeats (ITRs). Entry of AAV in target cells is dependent on binding to cell surface receptors (42); subsequently, the single-stranded genome undergoes conversion to the transcriptionally active, double-stranded form. More than one hundred different serotypes of AAV have been described with different degree of identity in their capsid proteins (43). Despite the high sero-prevalence rate for some serotypes, AAV is considered non-pathogenic. Wild type AAV does not possess the ability to replicate and depends on the presence of a helper virus (usually adenovirus or herpes virus (41)). As a gene-delivery vehicle, recombinant replication-defective AAV-based vectors retain the ITRs but the remaining viral sequences are replaced by the cistron(s) that direct expression of the transgene(s) of interest. Despite its relatively small payload (~ 4.3 kb) imposing

constraints in the size of the therapeutic transgene, its ability for long-term transgene expression, make it a highly-desirable vehicle for gene transfer (42).

3.5. Immunologic considerations for hemophilia gene therapy with FVIII or FIX

Current gene therapy approaches for hemophilia pivot on the concept of gene addition with FVIII or FIX. Although these approaches aim for long-term hemostatic efficacy, the setting of gene transfer adds an extra layer of complexity in terms of immunological responses since two separate entities (the delivery vehicle and the therapeutic transgene product, with immunogenic potential) are introduced into the recipient. This is illustrated by clinical studies, where, for example, immunoreactivity to the AAV serotype 2 capsid in the form of T cell responses has been observed in the context of liver-directed gene transfer in hemophilia B patients (40, 44). This resulted in only transient FIX expression, due to a capsid-specific CD8+ T-cell response that cleared the transduced hepatocytes. Intramuscular delivery of AAV encoding for canine FIX in hemophilic dogs also demonstrated that higher vector dose per injection site (45) and higher local levels of FIX expression (46) are associated with an increased risk of inhibitor development. However, muscle delivery of AAV expressing canine FIX via the intravascular route or via afferent transvenular retrograde extravasation in the context of transient immunosuppresion, prevented antibody responses to canine FIX and resulted in long-lasting transgene expression (47, 48). Moreover, studies in animals have shown that expression of a transgene from a viral vector in the liver tends to promote tolerance to the transgene product (49-52). Based on these studies, the ultimate outcome on the recipient following gene transfer with FVIII or FIX may be difficult to predict. Clearly, an approach that minimizes an antigen-specific immune response while maintaining hemostatic efficacy is the most desirable.

3.6. Gene-based bypass therapy for hemophilia

In addition to the considerations explained in the previous Section, approaches using FVIII or FIX as therapeutic transgenes are not suitable for the significant number of inhibitor patients. However, existing strategies to bypass hemophilic inhibitors and provide hemostasis present a tantalizing concept in the context of gene therapy for hemophilia. The question is whether experience with protein-based "bypass" can be amalgamated with gene-transfer technology. Effectively, is it possible to generate a transgene other than FVIII or FIX that, when expressed following gene delivery to an appropriate tissue, has beneficial hemostatic effects? Successful gene candidate(s) must fulfill three prerequisites: (i) appropriate size that can be accommodated in the existing viral delivery vehicles; (ii) the protein product must have defined clinical hemostatic effects; and (iii) must not be immunogenic. Although hemostatically effective in hemophilia patients with inhibitors (53), activated prothrombin complex concentrates (aPCCs) function as a multicomponent product of prothrombin complex zymogens and their activation products (54). This is a feature that renders them unfavorable for gene-based delivery. In contrast, activated FVII (FVIIa), appears to fulfill all prerequisites set above. As such, an approach with a FVIIa *transgene* has unique features that differentiate it from FVIII and FIX gene-addition therapy on several levels. In terms of genetics, it relies on overexpression of an active form of a protein that is already present and functional in the patient's circulation. In terms of the protein product, this approach should avoid the issue of immunogenicity since patients will be tolerant to the expressed FVIIa. Moreover, based on a plethora of clinical data, the transgene product (FVIIa) should provide hemostasis regardless of the inhibitor status of the recipient. Lastly, much like rhFVIIa protein infusion, a gene-based FVIIa approach may improve hemostasis for other inherited multigenic coagulation defects (such as platelet defects), where protein replacement therapy does not exist or for FVII deficiency (55).

3.7. Development of a FVIIa transgene

FVII is a vitamin-K dependent protein synthesized in the liver as a single polypeptide (zymogen). Under physiological conditions, activation during coagulation results in cleavage at Arg152-Ile153 bond, generating a two-chain molecule (light and heavy chain). This molecule remains in a "zymogen" state and requires two events to obtain full catalytic activity: (1) interaction with TF and Ca^{2+} (56, 57); (2) salt-bridge formation between the newly-formed N-terminus of the heavy chain (Ile153) and Asp343. These events result in ordering of the active site and shift the equilibrium towards the catalytically-active form of FVIIa that, on negatively charged PL surface, activates FX and FIX (Figure 1A).

Clearly, demonstration that FVII can be engineered for secretion in a two-chain form is the critical prerequisite for FVIIa gene therapy. For this, a hypothesis was postulated that a transgene product with an engineered cleavage site at Arg152-Ile153 for an *intra*cellular protease (Figure 3A) would result in a secreted two-chain molecule (Figure 3B). Mammalian cells employ machinery for the conversion of pro-protein precursors to their mature form, as they traffic through the secretory pathway. The proteases involved in this processing are called proprotein convertases (PPCs), a family of seven subtilisin/kexin-like endoproteases (58). PPCs cleave at the C-terminus of single, paired or tetra basic amino acid residues within the consensus motif of $[R/K]\text{-}[X]_n\text{-}R/K]\downarrow$, where X is any amino acid, R/K indicates either Arg or Lys, *n* is the number of spacer amino acids (0, 2, 4 or 6 (59)) and the symbol "↓" indicates the location of cleavage. Therefore, the properties of PPCs make them ideal mediators of intracellular processing of an engineered FVIIa transgene to a two-chain form.

Furin is a PPC expressed in a broad range of tissues, localized mostly in the trans-Golgi network and is necessary for the processing of receptors, growth factors, hormones, matrix metalloproteases as well as FIX, FX, protein C and von Willenbrand factor (60). Following the hypothesis on engineering a FVIIa *transgene* (*vide supra*), three different furin cleavage sites inserted at Arg152-Ile153 of human FVII were tested: (i) from the human pro-

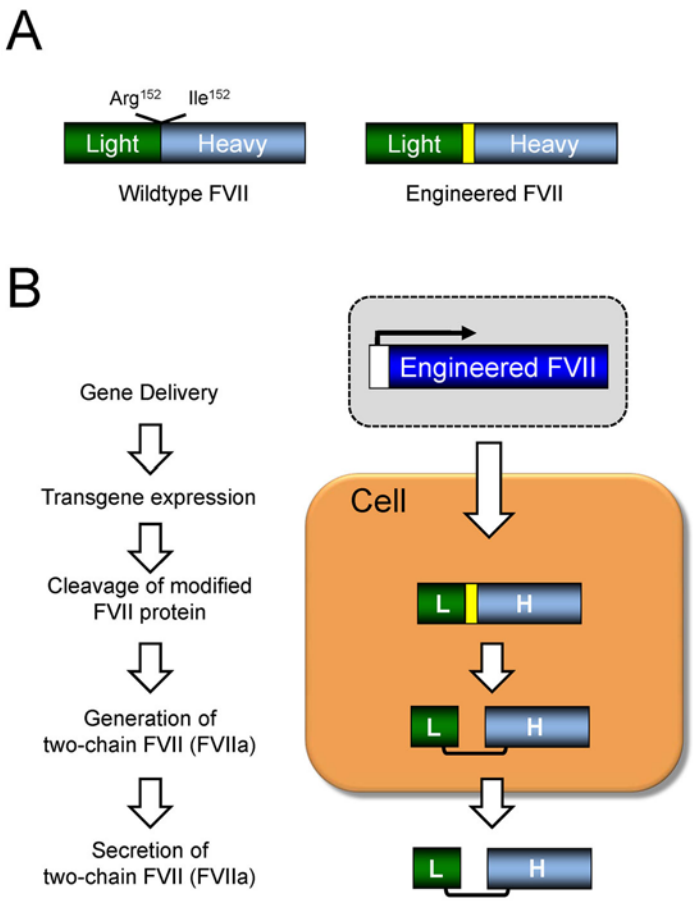

Figure 3. The essentials of FVIIa gene transfer. (A) In order to generate an engineered FVII transgene that would be secreted as two-chain FVIIa, a furin cleavage site (yellow box) was inserted at Arg152-Ile153, the normal site of cleavage of zymogen FVII. The light and heavy chains of FVII following cleavage are indicated. (B) To generate secreted FVIIa, the engineered FVII transgene is introduced via a vector into an appropriate tissue/cell type. Subsequently, the transgene product undergoes intracellular processing mediated by furin at the cleavage recognition sequence (yellow box), leading to the generation of a two-chain (light [L] and heavy [H]) molecule that is secreted into the circulation.

insulin receptor (PRPSRKRR (61)); (ii) from human FX (RKR, when inserted in the FVII sequence would become RRKR, the underline indicates Arg contributed by the human FVII sequence); and (iii) a duplication of RKR, termed 2RKR (RKRRKR). The availability of immunological reagents as well as methodology for purification of recombinant human FVII (62) allowed for the demonstration that biologically active, two-chain human FVII can be generated using either of the three tested cleavage sites. However, the 2RKR site offered the most favorable characteristics in terms of percentage cleaved material and biological activity. In particular, the FVII-2RKR protein was secreted almost exclusively in the two-chain form and had extrinsic activity of ~85% relative compared to rhFVIIa (63). N-terminal sequencing of the heavy chain of each FVIIa protein revealed the correct first residue (Ile) that is necessary for salt-bridge formation to Asp343 and catalytic activity. Interestingly, the utility of the 2RKR sequence has been subsequently demonstrated in generating activated FV (64) as well as a way to express two functional proteins from a single polypeptide in AAV vectors by furin cleavage at an appropriate position, generating two protein products (65).

One of the major advantages of mouse models of hemophilia lies in the established experimental methodology to investigate *in vivo* hemostatic responses in a systematic fashion. Unfortunately, human FVIIa interacts poorly with murine TF (66) and this may affect the outcome of hemostatic assays *in vivo*. Therefore, investigation of the hemostatic efficacy of FVIIa gene transfer in murine hemophilia models necessitates the use of a *homologous* transgene product. For this, it was necessary to initially purify and characterize recombinant murine FVII harboring the 2RKR furin cleavage site at Arg152-Ile153 (mFVII-2RKR [mFVIIa]). It was found that mFVII-2RKR had comparable activity to rhFVIIa (using human reagents) and was correctly cleaved at Arg152-Ile153, as confirmed by N-terminal sequencing of the heavy chains (63). More importantly, using an AAV serotype 2 vector directing expression of mFVII-2RKR from a liver-specific promoter, long-term normalization of

the clotting time (activated partial thromboplastin time [aPTT]) and significant reduction of blood loss following tail clip in hemophilia B mice was demonstrated (63). These results provided the first experimental evidence that FVIIa expressed continuously from a transgene can effect significant hemostatic improvements in a mouse model of hemophilia.

3.8. The safety profile of FVIIa gene transfer – lessons from a mouse hemophilia model

The fact that FVIIa is a *protease* lends a distinguishing feature to FVIIa gene therapy in terms of safety, compared to gene delivery of *zymogen* FVIII or FIX. Studies of rhFVIIa by bolus administration for an approved indication (such as hemophilia) suggest a low rate of thromboembolic events (<1% (31, 32)). However, the effects of *continuous* expression of FVIIa by gene transfer on coagulation (hemostatic or thrombotic) are governed by more complex pharmacokinetics than bolus protein administration. In order to better define the therapeutic window of this FVIIa gene delivery for hemophilia, a long-term efficacy and safety study using transgenic mice expressing mFVIIa from a liver specific promoter was initiated (67). Such mice were categorized into low (<1.5 μg/ml) and high (>2 μg/ml) expressers, crossed into the hemophilia B phenotypic background and monitored over time including several *in vivo* assays of hemostasis. The results of this study demonstrated that expression of mFVIIa in hemophilia B mice below 1.5 μg/ml resulted in hemostatic improvement. This was defined by hemostatic challenges of the micro- (cremaster muscle arteriole laser injury) or macrocirculation ($FeCl_3$ carotid artery injury or measuring blood loss after a tail clip). No thrombotic complications were observed as determined by long-term survival analysis and measurements of markers of pathological coagulation activation. In contrast, higher mFVIIa circulating levels (> 2 μg/ml) resulted in premature mortality (within months since birth). This particular cohort of mice exhibited elevated thrombin-antithrombin levels and histological examination revealed fibrin deposition in the lungs and the myocardium (67), tissues rich in TF (68, 69). The involvement of the coagulation system in these findings was further confirmed: mice on a low-FX genetic background (homozygous for the FX Friuli mutation in the mouse FX gene, exhibiting ~4% FX activity (70)) expressing ~ 4μg/ml of mFVIIa showed normal survival (67). However, it remains unclear whether effects mediated by pathways other than coagulation (via direct or indirect protease-activated receptor [PAR] activation by the TF-FVIIa complex) are dysregulated in mice overexpressing mFVIIa. Altogether, these findings defined the therapeutic window of FVIIa gene therapy approach for hemophilia and the risk of prothrombotic complications from continuously elevated circulating FVIIa levels.

3.9. Expression of FVIIa in a large animal model of hemophilia

3.9.1. Efficacy data

The mouse model of hemophilia has several limitations compared to the human condition: size (mouse weighs ~25g vs. ~75kg of the average person), genetics (mice are inbred whereas humans are outbred) and disease symptomatology (lack of spontaneous bleeds that are characteristic of hemophilia patients). In contrast to mice, the hemophilia dog model closely mimics the human condition (including the occurrence of spontaneous bleeds) and has been a good predictor of hemophilia treatments (protein or gene-based) (71-74). There are two colonies of hemophilia A dogs: the Chapel Hill (North Carolina, USA) dogs have a genomic DNA inversion that replaces exons 22-26 (75), similar to that is seen in 40-50% of severe hemophilia A patients (76); the dogs at the colony of Queen's University (Canada) have a RNA processing defect that results in aberrant splicing and transcription termination after exon 22 of canine FVIII (77). For hemophilia B, there are also two colonies of dogs: the Chapel Hill dogs exhibit a point mutation (GGG>GAG) that results in Gly379 substituted for Glu and a complete lack of circulating FIX (78); the dogs at Auburn University (Alabama, USA) have a deletion of nucleotides 772-776 and a C>T transition at nucleotide 777, resulting in <1% circulating FIX level (79).

The hemophilia dogs at Chapel Hill were used as models to demonstrate efficacy and establish safety of a FVIIa gene-based therapy in a large animal model. A critical feature of these animals is the frequency of spontaneous bleeds that they exhibit (5-6 per year (80)). This is a convenient endpoint of clinical relevance that is not available in hemophilic mice. However, modification of this frequency with continuous expression of FVIIa necessitates functional interaction(s) within the canine coagulation system. Therefore, the use of canine FVIIa as the therapeutic transgene would ensure species compatibility within this system and avoid potential immune responses to the introduced transgene product. The cDNA for canine FVII (cFVII) had recently been cloned (81) which allowed for the generation of the cFVII-2RKR (cFVIIa) transgene. An initial *in vitro* characterization of the cFVIIa recombinant protein demonstrated that the 2RKR sequence inserted at Arg152-Ile153 of cFVII yielded a molecule that could be secreted in a two-chain, biologically active form. In addition, AAV serotype 8 (AAV8)-mediated expression of cFVIIa (AAV8-cFVIIa) from a liver-specific promoter could correct the hemophilia A *in vitro* parameters in mice. Previous published reports with canine FVIII or FIX AAV8 gene delivery in hemophilic dogs suggested that a vector dose of 0.5-1E13 vector genomes (vg)/kg resulted in sustained expression of the transgene sufficient for phenotypic correction (82, 83). However, in the setting of FVIIa gene transfer, it was unknown whether such vector doses could result in similar efficacy given the substantially shorter half-life of FVIIa compared to FVIII or FIX (~2.5h vs. 8-12h [FVIII (84)] or ~18h [FIX (85)]). In that case, therapeutic expression would most likely necessitate higher administered vector doses than previously used for FVIII or FIX gene delivery. An initial administration of 2.06 E13 vg/kg of AAV8-cFVIIa in a hemophilia B dog resulted in absence of *in vitro* hemostatic effects, based on clotting assays with plasma (prothrombin time [PT]) or whole blood (whole blood clotting time [WBCT]) that were modestly affected. Expression of cFVIIa in that animal was below the limit of detection using an activity-based clotting assay. Despite

Table 1. Summary of *in vivo* studies of continuous expression of canine FVIIa in hemophilic dogs

Mode of delivery	Vector dose (vg/kg)	Total vector dose (vg)	Dog model (dog name)	Average cFVIIa expression (µg/ml)	Hemostatic effect (*in vivo*)
AAV8 viral vector	2.06E13	1.67E14	Hemophilia B (J10)	<0.5	No spontaneous bleeds (in 34 months, 15 expected bleeds[1])
AAV8 viral vector	6.25E13	1.25E15	Hemophilia A (J55)	~1.8	No spontaneous bleeds (in 18 months; 8 expected bleeds[1])
AAV8 viral vector	1.25E14	2.6E15	Hemophilia A (J57)	~2.0	No spontaneous bleeds (in 15 months; 7 expected bleeds[1])
AAV8 viral vector	1.25E14	2.5E15	Hemophilia A (E66)	~1.8	No spontaneous bleeds (in 12 months; 6 expected bleeds[1])

[1]Based on empirical data (80).

this somewhat disappointing finding, quite remarkably, *in vivo* phenotypic effects were observed: this dog did not experience any spontaneous bleeds for almost 3 years except for 3 *non-spontaneous* bleeds (after a hemostatic challenge) within the initial 8 months following AAV delivery (86). Although further confirmatory experiments are necessary, this observation suggested that phenotypic correction could occur even with "low-dose" AAV-cFVIIa vector doses.

The results from the initial HB dog (*vide supra*) prompted a dose escalation study in a cohort of three HA dogs of the Chapel Hill colony. In that cohort, administered AAV8-cFVIIa vector doses were in the range of 6.25-12.5 E13 vg/kg (3-6 fold higher than the HB dog previously described). This resulted in a sustained expression of cFVIIa at levels of ~2µg/ml and clearly measurable *in vitro* hemostatic effects (supra-physiological shortening of the PT and reduction of the hemophilic WBCT to ~30min [normal is ~14min, hemophilic WBCT is ~55min]). Moreover, none of the AAV-treated dogs exhibited spontaneous bleeds in a collective observation period of 3.75 years; in contrast, based on empirical (80) as well as concurrent observation of untreated hemophilia A dogs, the expected number of bleeds through that period would be 21 (cumulative for all the HA treated dogs). An additional consideration for a FVIIa gene-based approach of the experiments in hemophilic dogs expressing cFVIIa, is their *response* to bleeds. Although dogs treated with AAV8-cFVIIa did not exhibit spontaneous bleeds, it is unknown whether the increase in cFVIIa circulating levels following gene transfer can result in hemostasis in the event of a bleed following trauma. Recent advances in a non-invasive injury model (87, 88) certainly look promising in measuring *in vivo* hemostatic effects of continuously expressed cFVIIa following injury. Collectively, these results demonstrated that continuous expression of ~2µg/ml of cFVIIa resulted in long-term phenotypic improvement. A summary of the data from hemophilic dogs is shown in Table 1.

3.9.2. Safety data

A potential consequence of continuously elevated levels of circulating cFVIIa is the excessive activation of the endogenous coagulation system, as observed in hemophilic mice expressing > 2µg/ml of mFVIIa (67). As such, despite the observed improvements in hemostasis in the AAV8-cFVIIa treated dogs, an evaluation of the long-term safety profile of continuous cFVIIa expression was performed for multiple reasons: (i) a potential imbalance between hemostasis and thrombosis; (ii) liver and kidney dysfunction; and (iii) immune response to the expressed transgene. To evaluate the long-term effects on the coagulation system, several markers of an overactive coagulation system were monitored: platelet counts and fibrinogen levels (markers of a consumptive coagulopathy), assays of thrombin-antithrombin complex and D-dimer (elevated levels of which are markers of thromboembolic disease in dogs (89, 90)). However, despite variability in some markers, no evidence of an activated coagulation was seen. In terms of organ function (liver/kidney), serum chemistry analysis revealed values of markers within their respective normal range. Lastly, the potential for an immune response to either the viral delivery system (AAV) or the transgene itself (cFVIIa) was performed. Anti-AAV8 were detected in all hemophilia dogs treated with AAV-cFVIIa; a transient anti-cFVIIa IgG (non-inhibitory) was also measured from day 7 post-AAV administration that returned to baseline by day 28 (dog E66, Table 1). However, none of these immune responses were detrimental to the efficacy of this approach.

4. PERSPECTIVE

Although the data reviewed demonstrate the feasibility of hemophilia gene transfer with FVIIa, there are elements of this approach that require further refinement. For instance, the vector doses utilized in the dog studies were considerably higher than previously used for similar approaches with canine FVIII or FIX. Although this may reflect the intricacies of FVIIa as therapeutic transgene in terms of secretion/synthesis and clearance/inactivation, strategies that can lower the effective vector dose will be necessary. Potential avenues that can bring this to fruition include modifications on the DNA/RNA level in the delivery vector, the transgene itself, or both. For example, improved AAV vectors that obtain self-complementary form (as opposed to single-stranded) and direct higher transgene expression as well as modifications such as promoter and codon optimization have been described (91-93) Modification on the translated transgene product may provide an additional level of hemostatic improvement. Variants of human FVII with improved activity by either enhancing phospholipid binding or the catalytic function (94-97) have been described and data from use in humans exist (98). Based on these studies and the extended identity between human/mouse/canine FVII, the hemophilia mouse and dog models offer a suitable platform to investigate whether such modifications can result in improved, FVIIa gene-based variants. Lastly, given the protease nature of FVIIa, a gene therapy setting where FVIIa expression is continuous imposes additional safety concerns. However,

the experiments in mice and dogs described above suggest that the increased thrombotic risk depends on the level of transgene expression, something that may be controlled by appropriate viral vector dosing. Inducible expression systems that respond to orally bioavailable drugs may offer an additional layer of safety whereby FVIIa transgene expression may be induced "on demand". Such systems have been integrated with AAV for gene delivery of a secretable transgene product in non-human primates (99). Although the kinetics of FVIIa induction may not be fast enough for the treatment of acute bleeds and may require additional rhFVIIa administration, it is possible that low-level induction of FVIIa transgene expression on a regular basis may provide clinical benefits similar to those seen with prophylactic use of rhFVIIa (100).

In summary, data from initial experiments in hemophilic mice as well as subsequent experiments in hemophilic dogs have established the efficacy and safety of a FVIIa gene-based approach for hemophilia. This includes the demonstration of phenotypic improvement following hemostatic challenges in mice (63, 67) as well as correction of the bleeding diathesis in hemophilic dogs (86). As such, these results lend support for the further development and optimization of a FVIIa gene-based bypass therapy for the treatment of hemophilia complicated by inhibitors.

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6. REFERENCES

1. N. Mackman: The role of tissue factor and factor VIIa in hemostasis. *Anesth Analg*, 108(5), 1447-52 (2009)

2. F. Peyvandi, S. Duga, S. Akhavan and P. M. Mannucci: Rare coagulation deficiencies. *Haemophilia*, 8(3), 308-21 (2002)

3. J. M. Soucie, B. Evatt and D. Jackson: Occurrence of hemophilia in the United States. The Hemophilia Surveillance System Project Investigators. *Am J Hematol*, 59(4), 288-94 (1998)

4. B. O'Mahony and C. Black: Expanding hemophilia care in developing countries. *Semin Thromb Hemost*, 31(5), 561-8 (2005)

5. N. I. Rodriguez and W. K. Hoots: Advances in hemophilia: experimental aspects and therapy. *Hematol Oncol Clin North Am*, 24(1), 181-98 (2010)

6. J. E. Handelsman: The knee joint in hemophilia. *Orthop Clin North Am*, 10(1), 139-73 (1979)

7. G. R. Jayandharan and A. Srivastava: The phenotypic heterogeneity of severe hemophilia. *Semin Thromb Hemost*, 34(1), 128-41 (2008)

8. E. C. Rodriguez-Merchan: Effects of hemophilia on articulations of children and adults. *Clin Orthop Relat Res*(328), 7-13 (1996)

9. J. Oldenburg, G. Dolan and G. Lemm: Haemophilia care then, now and in the future. *Haemophilia*, 15 Suppl 1, 2-7 (2009)

10. I. M. Nilsson, E. Berntorp, T. Lofqvist and H. Pettersson: Twenty-five years' experience of prophylactic treatment in severe haemophilia A and B. *J Intern Med*, 232(1), 25-32 (1992)

11. L. M. Aledort, R. H. Haschmeyer and H. Pettersson: A longitudinal study of orthopaedic outcomes for severe factor-VIII-deficient haemophiliacs. The Orthopaedic Outcome Study Group. *J Intern Med*, 236(4), 391-9 (1994)

12. W. Kreuz, C. Escuriola-Ettingshausen, M. Funk, H. Schmidt and B. Kornhuber: When should prophylactic treatment in patients with haemophilia A and B start?--The German experience. *Haemophilia*, 4(4), 413-7 (1998)

13. J. Panicker, I. Warrier, R. Thomas and J. M. Lusher: The overall effectiveness of prophylaxis in severe haemophilia. *Haemophilia*, 9(3), 272-8 (2003)

14. H. M. van den Berg, K. Fischer, E. P. Mauser-Bunschoten, F. J. Beek, G. Roosendaal, J. G. van der Bom and H. K. Nieuwenhuis: Long-term outcome of individualized prophylactic treatment of children with severe haemophilia. *Br J Haematol*, 112(3), 561-5 (2001)

15. T. T. Yee, K. Beeton, A. Griffioen, C. Harrington, A. Miners, C. A. Lee and S. A. Brown: Experience of prophylaxis treatment in children with severe haemophilia. *Haemophilia*, 8(2), 76-82 (2002)

16. M. J. Manco-Johnson, T. C. Abshire, A. D. Shapiro, B. Riske, M. R. Hacker, R. Kilcoyne, J. D. Ingram, M. L. Manco-Johnson, S. Funk, L. Jacobson, L. A. Valentino, W. K. Hoots, G. R. Buchanan, D. DiMichele, M. Recht, D. Brown, C. Leissinger, S. Bleak, A. Cohen, P. Mathew, A. Matsunaga, D. Medeiros, D. Nugent, G. A. Thomas, A. A. Thompson, K. McRedmond, J. M. Soucie, H. Austin and B. L. Evatt: Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*, 357(6), 535-44 (2007)

17. J. Astermark, P. Petrini, L. Tengborn, S. Schulman, R. Ljung and E. Berntorp: Primary prophylaxis in severe haemophilia should be started at an early age but can be individualized. *Br J Haematol*, 105(4), 1109-13 (1999)

18. A. D. Shapiro: Why is primary prophylaxis underutilized in the United States? *Haemophilia*, 9(6), 670-2 (2003)

19. M. Franchini, G. Targher, M. Montagnana and G. Lippi: Laboratory, clinical and therapeutic aspects of acquired hemophilia A. *Clin Chim Acta*, 395(1-2), 14-8 (2008)

20. M. Franchini and G. Lippi: Recent improvements in the clinical treatment of coagulation factor inhibitors. *Semin Thromb Hemost*, 35(8), 806-13 (2009)

21. S. C. Gouw and H. M. van den Berg: The multifactorial etiology of inhibitor development in hemophilia: genetics and environment. *Semin Thromb Hemost*, 35(8), 723-34 (2009)

22. J. M. Lusher: Inhibitor antibodies to factor VIII and factor IX: management. *Semin Thromb Hemost*, 26(2), 179-88 (2000)

23. M. Franchini, F. Manzato, G. L. Salvagno, M. Montagnana, M. Zaffanello and G. Lippi: Prophylaxis in congenital hemophilia with inhibitors: the role of recombinant activated factor VII. *Semin Thromb Hemost*, 35(8), 814-9 (2009)

24. D. Dimichele: The North American Immune Tolerance Registry: contributions to the thirty-year experience with immune tolerance therapy. *Haemophilia*, 15(1), 320-8 (2009)

25. A. B. Colowick, R. L. Bohn, J. Avorn and B. M. Ewenstein: Immune tolerance induction in hemophilia patients with inhibitors: costly can be cheaper. *Blood*, 96(5), 1698-702 (2000)

26. M. A. Siddiqui and L. J. Scott: Recombinant factor VIIa (Eptacog Alfa): a review of its use in congenital or acquired haemophilia and other congenital bleeding disorders. *Drugs*, 65(8), 1161-77 (2005)

27. N. S. Key, L. M. Aledort, D. Beardsley, H. A. Cooper, G. Davignon, B. M. Ewenstein, G. S. Gilchrist, J. C. Gill, B. Glader, W. K. Hoots, C. T. Kisker, J. M. Lusher, C. G. Rosenfield, A. D. Shapiro, H. Smith and E. Taft: Home treatment of mild to moderate bleeding episodes using recombinant factor VIIa (Novoseven) in haemophiliacs with inhibitors. *Thromb Haemost*, 80(6), 912-8 (1998)

28. G. Kenet and U. Martinowitz: Single-dose recombinant activated factor VII therapy in hemophilia patients with inhibitors. *Semin Hematol*, 45(2 Suppl 1), S38-41 (2008)

29. E. Erhardtsen: Pharmacokinetics of recombinant activated factor VII (rFVIIa). *Semin Thromb Hemost*, 26(4), 385-91 (2000)

30. M. N. Minno, G. D. Minno, M. D. Capua, A. M. Cerbone and A. Coppola: Cost of care of haemophilia with inhibitors. *Haemophilia* (2009)

31. T. Abshire: Safety update on recombinant factor VIIa in the treatment of congenital and acquired hemophilia. *Semin Hematol*, 45(2 Suppl 1), S3-6 (2008)

32. L. M. Aledort: Comparative thrombotic event incidence after infusion of recombinant factor VIIa versus factor VIII inhibitor bypass activity. *J Thromb Haemost*, 2(10), 1700-8 (2004)

33. K. A. O'Connell, J. J. Wood, R. P. Wise, J. N. Lozier and M. M. Braun: Thromboembolic adverse events after use of recombinant human coagulation factor VIIa. *JAMA*, 295(3), 293-8 (2006)

34. M. Levi, J. H. Levy, H. F. Andersen and D. Truloff: Safety of recombinant activated factor VII in randomized clinical trials. *N Engl J Med*, 363(19), 1791-800 (2010)

35. D. Dimichele, F. G. Miller and J. J. Fins: Gene therapy ethics and haemophilia: an inevitable therapeutic future? *Haemophilia*, 9(2), 145-52 (2003)

36. C. M. Bollard, L. R. Teague, E. W. Berry and P. A. Ockelford: The use of central venous catheters (portacaths) in children with haemophilia. *Haemophilia*, 6(2), 66-70 (2000)

37. J. M. Journeycake, C. T. Quinn, K. L. Miller, J. L. Zajac and G. R. Buchanan: Catheter-related deep venous thrombosis in children with hemophilia. *Blood*, 98(6), 1727-31 (2001)

38. N. O. Viiala, S. R. Larsen and J. E. Rasko: Gene therapy for hemophilia: clinical trials and technical tribulations. *Semin Thromb Hemost*, 35(1), 81-92 (2009)

39. H. Jiang, G. F. Pierce, M. C. Ozelo, E. V. de Paula, J. A. Vargas, P. Smith, J. Sommer, A. Luk, C. S. Manno, K. A. High and V. R. Arruda: Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. *Mol Ther*, 14(3), 452-5 (2006)

40. C. S. Manno, G. F. Pierce, V. R. Arruda, B. Glader, M. Ragni, J. J. Rasko, M. C. Ozelo, K. Hoots, P. Blatt, B. Konkle, M. Dake, R. Kaye, M. Razavi, A. Zajko, J. Zehnder, P. K. Rustagi, H. Nakai, A. Chew, D. Leonard, J. F. Wright, R. R. Lessard, J. M. Sommer, M. Tigges, D. Sabatino, A. Luk, H. Jiang, F. Mingozzi, L. Couto, H. C. Ertl, K. A. High and M. A. Kay: Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med*, 12(3), 342-7 (2006)

41. K. E. Brown: The expanding range of parvoviruses which infect humans. *Rev Med Virol*, 20(4), 231-44 (2010)

42. J. Wang, S. M. Faust and J. E. Rabinowitz: The next step in gene delivery: Molecular engineering of adeno-associated virus serotypes. *J Mol Cell Cardiol* (2010)

43. G. Gao, L. H. Vandenberghe and J. M. Wilson: New recombinant serotypes of AAV vectors. *Curr Gene Ther*, 5(3), 285-97 (2005)

44. F. Mingozzi, M. V. Maus, D. J. Hui, D. E. Sabatino, S. L. Murphy, J. E. Rasko, M. V. Ragni, C. S. Manno, J. Sommer, H. Jiang, G. F. Pierce, H. C. Ertl and K. A. High: CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med*, 13(4), 419-22 (2007)

45. R. W. Herzog, P. A. Fields, V. R. Arruda, J. O. Brubaker, E. Armstrong, D. McClintock, D. A. Bellinger, L. B. Couto, T. C. Nichols and K. A. High: Influence of vector dose on factor IX-specific T and B cell responses in muscle-directed gene therapy. *Hum Gene Ther*, 13(11), 1281-91 (2002)

46. V. R. Arruda, J. Schuettrumpf, R. W. Herzog, T. C. Nichols, N. Robinson, Y. Lotfi, F. Mingozzi, W. Xiao, L. B. Couto and K. A. High: Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. *Blood*, 103(1), 85-92 (2004)

47. V. R. Arruda, H. H. Stedman, V. Haurigot, G. Buchlis, S. Baila, P. Favaro, Y. Chen, H. G. Franck, S. Zhou, J. F. Wright, L. B. Couto, H. Jiang, G. F. Pierce, D. A. Bellinger, F. Mingozzi, T. C. Nichols and K. A. High: Peripheral transvenular delivery of adeno-associated viral vectors to skeletal muscle as a novel therapy for hemophilia B. *Blood*, 115(23), 4678-88 (2010)

48. V. R. Arruda, H. H. Stedman, T. C. Nichols, M. E. Haskins, M. Nicholson, R. W. Herzog, L. B. Couto and K. A. High: Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model. *Blood*, 105(9), 3458-64 (2005)

49. A. Follenzi, M. Battaglia, A. Lombardo, A. Annoni, M. G. Roncarolo and L. Naldini: Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. *Blood*, 103(10), 3700-9 (2004)

50. F. Mingozzi, Y. L. Liu, E. Dobrzynski, A. Kaufhold, J. H. Liu, Y. Wang, V. R. Arruda, K. A. High and R. W. Herzog: Induction of immune tolerance to coagulation factor IX antigen by *in vivo* hepatic gene transfer. *J Clin Invest*, 111(9), 1347-56 (2003)

51. G. P. Niemeyer, R. W. Herzog, J. Mount, V. R. Arruda, D. M. Tillson, J. Hathcock, F. W. van Ginkel, K. A. High and C. D. Lothrop, Jr.: Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood*, 113(4), 797-806 (2009)

52. L. Xu, C. Gao, M. S. Sands, S. R. Cai, T. C. Nichols, D. A. Bellinger, R. A. Raymer, S. McCorquodale and K. P. Ponder: Neonatal or hepatocyte growth factor-potentiated adult gene therapy with a retroviral vector results in therapeutic levels of canine factor IX for hemophilia B. *Blood*, 101(10), 3924-32 (2003)

53. J. Astermark, S. M. Donfield, D. M. DiMichele, A. Gringeri, S. A. Gilbert, J. Waters and E. Berntorp: A randomized comparison of bypassing agents in hemophilia complicated by an inhibitor: the FEIBA NovoSeven Comparative (FENOC) Study. *Blood*, 109(2), 546-51 (2007)

54. P. L. Turecek, K. Varadi, H. Gritsch and H. P. Schwarz: FEIBA: mode of action. *Haemophilia*, 10 Suppl 2, 3-9 (2004)

55. G. Mariani and F. Bernardi: Factor VII Deficiency. *Semin Thromb Hemost*, 35(4), 400-6 (2009)

56. J. R. Bjelke, O. H. Olsen, M. Fodje, L. A. Svensson, S. Bang, G. Bolt, B. B. Kragelund and E. Persson: Mechanism of the Ca2+-induced enhancement of the intrinsic factor VIIa activity. *J Biol Chem*, 283(38), 25863-70 (2008)

57. D. M. Martin, C. W. Boys and W. Ruf: Tissue factor: molecular recognition and cofactor function. *FASEB J*, 9(10), 852-9 (1995)

58. D. F. Steiner: The proprotein convertases. *Curr Opin Chem Biol*, 2(1), 31-9 (1998)

59. M. Rholam and C. Fahy: Processing of peptide and hormone precursors at the dibasic cleavage sites. *Cell Mol Life Sci*, 66(13), 2075-91 (2009)

60. K. Nakayama: Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem J*, 327 (Pt 3), 625-35 (1997)

61. J. B. Denault and R. Leduc: Furin/PACE/SPC1: a convertase involved in exocytic and endocytic processing of precursor proteins. *FEBS Lett*, 379(2), 113-6 (1996)

62. R. Toso, M. Pinotti, K. A. High, E. S. Pollak and F. Bernardi: A frequent human coagulation Factor VII mutation (A294V, c152) in loop 140s affects the interaction with activators, tissue factor and substrates. *Biochem J*, 363(Pt 2), 411-6 (2002)

63. P. Margaritis, V. R. Arruda, M. Aljamali, R. M. Camire, A. Schlachterman and K. A. High: Novel therapeutic approach for hemophilia using gene delivery of an engineered secreted activated Factor VII. *J Clin Invest*, 113(7), 1025-31 (2004)

64. R. Toso and R. M. Camire: Role of Hirudin-like factor Va heavy chain sequences in prothrombinase function. *J Biol Chem*, 281(13), 8773-9 (2006)

65. S. B. Foti, R. J. Samulski and T. J. McCown: Delivering multiple gene products in the brain from a single adeno-associated virus vector. *Gene Ther*, 16(11), 1314-9 (2009)

66. L. C. Petersen, P. L. Norby, S. Branner, B. B. Sorensen, T. Elm, H. R. Stennicke, E. Persson and S. E. Bjorn: Characterization of recombinant murine factor VIIa and recombinant murine tissue factor: a human-murine species compatibility study. *Thromb Res*, 116(1), 75-85 (2005)

67. M. N. Aljamali, P. Margaritis, A. Schlachterman, S. J. Tai, E. Roy, R. Bunte, R. M. Camire and K. A. High: Long-term expression of murine activated factor VII is

safe, but elevated levels cause premature mortality. *J Clin Invest*, 118(5), 1825-34 (2008)

68. T. A. Drake, J. H. Morrissey and T. S. Edgington: Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am J Pathol*, 134(5), 1087-97 (1989)

69. R. A. Fleck, L. V. Rao, S. I. Rapaport and N. Varki: Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. *Thromb Res*, 59(2), 421-37 (1990)

70. S. J. Tai, R. W. Herzog, P. Margaritis, V. R. Arruda, K. Chu, J. A. Golden, P. A. Labosky and K. A. High: A viable mouse model of factor X deficiency provides evidence for maternal transfer of factor X. *J Thromb Haemost*, 6(2), 339-45 (2008)

71. K. M. Brinkhous, H. Sandberg, J. B. Garris, C. Mattsson, M. Palm, T. Griggs and M. S. Read: Purified human factor VIII procoagulant protein: comparative hemostatic response after infusions into hemophilic and von Willebrand disease dogs. *Proc Natl Acad Sci U S A*, 82(24), 8752-6 (1985)

72. K. M. Brinkhous, J. L. Sigman, M. S. Read, P. F. Stewart, K. P. McCarthy, G. A. Timony, S. D. Leppanen, B. J. Rup, J. C. Keith, Jr., P. D. Garzone and R. G. Schaub: Recombinant human factor IX: replacement therapy, prophylaxis, and pharmacokinetics in canine hemophilia B. *Blood*, 88(7), 2603-10 (1996)

73. K. High: Gene transfer for hemophilia: can therapeutic efficacy in large animals be safely translated to patients? *J Thromb Haemost*, 3(8), 1682-91 (2005)

74. S. L. Murphy and K. A. High: Gene therapy for haemophilia. *Br J Haematol*, 140(5), 479-87 (2008)

75. J. N. Lozier, A. Dutra, E. Pak, N. Zhou, Z. Zheng, T. C. Nichols, D. A. Bellinger, M. Read and R. A. Morgan: The Chapel Hill hemophilia A dog colony exhibits a factor VIII gene inversion. *Proc Natl Acad Sci U S A*, 99(20), 12991-6 (2002)

76. D. Lakich, H. H. Kazazian, Jr., S. E. Antonarakis and J. Gitschier: Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. *Nat Genet*, 5(3), 236-41 (1993)

77. C. Hough, S. Kamisue, C. Cameron, C. Notley, S. Tinlin, A. Giles and D. Lillicrap: Aberrant splicing and premature termination of transcription of the FVIII gene as a cause of severe canine hemophilia A: similarities with the intron 22 inversion mutation in human hemophilia. *Thromb Haemost*, 87(4), 659-65 (2002)

78. J. P. Evans, K. M. Brinkhous, G. D. Brayer, H. M. Reisner and K. A. High: Canine hemophilia B resulting from a point mutation with unusual consequences. *Proc Natl Acad Sci U S A*, 86(24), 10095-9 (1989)

79. A. E. Mauser, J. Whitlark, K. M. Whitney and C. D. Lothrop, Jr.: A deletion mutation causes hemophilia B in Lhasa Apso dogs. *Blood*, 88(9), 3451-5 (1996)

80. K. E. Russell, E. H. Olsen, R. A. Raymer, E. P. Merricks, D. A. Bellinger, M. S. Read, B. J. Rup, J. C. Keith, Jr., K. P. McCarthy, R. G. Schaub and T. C. Nichols: Reduced bleeding events with subcutaneous administration of recombinant human factor IX in immune-tolerant hemophilia B dogs. *Blood*, 102(13), 4393-8 (2003)

81. M. B. Callan, M. N. Aljamali, P. Margaritis, M. E. Griot-Wenk, E. S. Pollak, P. Werner, U. Giger and K. A. High: A novel missense mutation responsible for factor VII deficiency in research Beagle colonies. *J Thromb Haemost*, 4(12), 2616-22 (2006)

82. H. Jiang, D. Lillicrap, S. Patarroyo-White, T. Liu, X. Qian, C. D. Scallan, S. Powell, T. Keller, M. McMurray, A. Labelle, D. Nagy, J. A. Vargas, S. Zhou, L. B. Couto and G. F. Pierce: Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood*, 108(1), 107-15 (2006)

83. L. Wang, R. Calcedo, T. C. Nichols, D. A. Bellinger, A. Dillow, I. M. Verma and J. M. Wilson: Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated, liver-directed gene therapy. *Blood*, 105(8), 3079-86 (2005)

84. J. S. Powell, D. J. Nugent, J. A. Harrison, A. Soni, A. Luk, H. Stass and E. Gorina: Safety and pharmacokinetics of a recombinant factor VIII with pegylated liposomes in severe hemophilia A. *J Thromb Haemost*, 6(2), 277-83 (2008)

85. D. A. Roth, C. M. Kessler, K. J. Pasi, B. Rup, S. G. Courter and K. L. Tubridy: Human recombinant factor IX: safety and efficacy studies in hemophilia B patients previously treated with plasma-derived factor IX concentrates. *Blood*, 98(13), 3600-6 (2001)

86. P. Margaritis, E. Roy, M. N. Aljamali, H. D. Downey, U. Giger, S. Zhou, E. Merricks, A. Dillow, M. Ezban, T. C. Nichols and K. A. High: Successful treatment of canine hemophilia by continuous expression of canine FVIIa. *Blood*, 113(16), 3682-9 (2009)

87. R. H. Behler, M. R. Scola, T. C. Nichols, M. C. Caughey, M. W. Fisher, H. Zhu and C. M. Gallippi: ARFI ultrasound for *in vivo* hemostasis assessment postcardiac catheterization, part II: pilot clinical results. *Ultrason Imaging*, 31(3), 159-71 (2009)

88. R. H. Behler, M. R. Scola, T. C. Nichols, D. A. Bellinger and C. M. Gallippi: ARFI ultrasound for *in vivo* hemostasis assessment postcardiac catheterization, part I: preclinical studies. *Ultrason Imaging*, 31(3), 153-8 (2009)

89. E. Dewhurst, S. Cue, E. Crawford and K. Papasouliotis: A retrospective study of canine D-dimer concentrations measured using an immunometric "Point-of-Care" test. *J Small Anim Pract*, 49(7), 344-8 (2008)

90. O. L. Nelson: Use of the D-dimer assay for diagnosing thromboembolic disease in the dog. *J Am Anim Hosp Assoc*, 41(3), 145-9 (2005)

91. A. C. Nathwani, J. T. Gray, J. McIntosh, C. Y. Ng, J. Zhou, Y. Spence, M. Cochrane, E. Gray, E. G. Tuddenham and A. M. Davidoff: Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood*, 109(4), 1414-21 (2007)

92. A. C. Nathwani, J. T. Gray, C. Y. Ng, J. Zhou, Y. Spence, S. N. Waddington, E. G. Tuddenham, G. Kemball-Cook, J. McIntosh, M. Boon-Spijker, K. Mertens and A. M. Davidoff: Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood*, 107(7), 2653-61 (2006)

93. Z. Wu, J. Sun, T. Zhang, C. Yin, F. Yin, T. Van Dyke, R. J. Samulski and P. E. Monahan: Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol Ther*, 16(2), 280-9 (2008)

94. S. B. Harvey, M. D. Stone, M. B. Martinez and G. L. Nelsestuen: Mutagenesis of the gamma-carboxyglutamic acid domain of human factor VII to generate maximum enhancement of the membrane contact site. *J Biol Chem*, 278(10), 8363-9 (2003)

95. G. L. Nelsestuen, M. Stone, M. B. Martinez, S. B. Harvey, D. Foster and W. Kisiel: Elevated function of blood clotting factor VIIa mutants that have enhanced affinity for membranes. Behavior in a diffusion-limited reaction. *J Biol Chem*, 276(43), 39825-31 (2001)

96. E. Persson, H. Bak and O. H. Olsen: Substitution of valine for leucine 305 in factor VIIa increases the intrinsic enzymatic activity. *J Biol Chem*, 276(31), 29195-9 (2001)

97. E. Persson, H. Bak, A. Ostergaard and O. H. Olsen: Augmented intrinsic activity of Factor VIIa by replacement of residues 305, 314, 337 and 374: evidence of two unique mutational mechanisms of activity enhancement. *Biochem J*, 379(Pt 2), 497-503 (2004)

98. J. Moss, B. Scharling, M. Ezban and T. M. Sorensen: Evaluation of the safety and pharmacokinetics of a fast-acting recombinant FVIIa analogue, NN1731, in healthy male subjects. *J Thromb Haemost* (2008)

99. P. Chenuaud, T. Larcher, J. E. Rabinowitz, N. Provost, B. Joussemet, H. Bujard, R. J. Samulski, D. Favre and P. Moullier: Optimal design of a single recombinant adeno-associated virus derived from serotypes 1 and 2 to achieve more tightly regulated transgene expression from nonhuman primate muscle. *Mol Ther*, 9(3), 410-8 (2004)

100. B. A. Konkle, L. S. Ebbesen, E. Erhardtsen, R. P. Bianco, T. Lissitchkov, L. Rusen and M. A. Serban: Randomized, prospective clinical trial of recombinant factor VIIa for secondary prophylaxis in hemophilia patients with inhibitors. *J Thromb Haemost*, 5(9), 1904-13 (2007)

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