Gene-based FVIIa prophylaxis modulates the spontaneous bleeding phenotype of hemophilia A rats

Shannon M. Zintner,1 Juliana C. Small,1 Giulia Pavani,1 Lynn Dankner,1 Oscar A. Marcos-Contreras,1 Phyllis A. Gimotty,2 Mads Kjelgaard-Hansen,3 Bo Wiinberg,3 and Paris Margaritis1,4,5

1The Children’s Hospital of Philadelphia, Philadelphia, PA; 2Department of Biostatistics, Epidemiology and Informatics, The University of Pennsylvania, Philadelphia, PA; 3Global Research, Novo Nordisk A/S, Måløv, Denmark; 4The Raymond G. Perelman Center for Cellular and Molecular Therapeutics, The Children’s Hospital of Philadelphia, Philadelphia, PA; and 5Department of Pediatrics, Perelman School of Medicine, The University of Pennsylvania, Philadelphia, PA

Key Points

- We show how FVIIa prophylaxis in HA rats using rat FVIIa gene transfer and expression affects their bleeding phenotype.
- Rat FVIIa transgene levels ≥708 ng/mL (≥14 nM) reduce bleeds in HA rats, whereas levels >1250 ng/mL (>25 nM) eliminate them.

A sizable proportion of hemophilia inhibitor patients fails immune tolerance induction and requires bypass agents for long-term bleed management. Recombinant human-activated coagulation Factor VII (rhFVIIa) is an on-demand bypass hemostatic agent for bleeds in hemophilia inhibitor patients. Prophylactic use of rhFVIIa may enable sustained hemostatic management of inhibitor patients, but the critical relationship of rhFVIIa circulating levels and clinical outcome in that setting remains unclear. To address this in vivo, we used the rat hemophilia A (HA) model that exhibits spontaneous bleeds and allows longitudinal studies with sufficient statistical power. We simulated activated Factor VII (FVIIa) prophylaxis by adeno-associated virus (AAV) gene transfer of a rat FVIIa transgene. Compared with naive HA animals, rat FVIIa continuous expression affected the overall observed bleeds, which were resolved with on-demand administration of recombinant rat FVIIa. Specifically, although 91% of naive animals exhibited bleeds, this was reduced to 83% and 33% in animals expressing less than 708 ng/mL (<14 nM) and at least 708 ng/mL (≥14 nM) rat FVIIa, respectively. No bleeds occurred in animals expressing higher than 1250 ng/mL (>25 nM). Rat FVIIa expression of at least 708 ng/mL was also sufficient to normalize the blood loss after a tail vein injury. Continuous, AAV-mediated rat FVIIa transgene expression had no apparent adverse effects in the hemostatic system of HA rats. This work establishes for the first time a dose dependency and threshold of circulating FVIIa antigen levels for reduction or complete elimination of bleeds in a setting of FVIIa-based HA prophylaxis.

Introduction

One third of hemophilia A (HA) patients and 2% to 5% of hemophilia B (HB) patients develop inhibitory alloantibodies (inhibitors) that complicate factor replacement therapy.1 Moreover, frequent bleeds and joint damage, including cartilage and subchondral bone damage, ultimately lead to debilitating arthropathy.2 As such, inhibitor patients have an increased morbidity and an overall poor quality of life.2,3 Immune tolerance induction consisting of repeated injections of factor is a long-term strategy to eradicate inhibitors, but has a success rate ranging from 53% to 79%,4 with a low historical peak inhibitor titer being a good predictor of success.5,6 Unfortunately, roughly 16% of these patients experience inhibitor relapse.7 For individuals who fail immune tolerance induction, on-demand treatment with bypassing agents such as activated recombinant human Factor VII (rhFVIIa) or activated prothrombin complex concentrates are the only alternatives to manage bleeding.1,3,7

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Prophylactic treatment with Factor VIII (FVIII) or IX (FIX) has been effective in preserving joint function in children, reducing hemorrhages in adults, and providing broader clinical benefits and improved quality of life. Limited data are available for prophylactic use of bypass agents in inhibitor patients. A meta-analysis of clinical data found that prophylaxis with activated prothrombin complex concentrates or rhFVIIa resulted in a significant decrease in the number of bleeds. However, no firm conclusions could be drawn in terms of superiority of one agent over the other, or for dosing regimens. Clearly, expanded prospective studies may address these issues and provide a more defined treatment. Experiments in hemophilia animal models could potentially address some long-standing questions in hemophilia prophylaxis with bypass agents. Specifically, for activated Factor VII (FVIIa) prophylaxis, the relationship of its circulating levels and prophylaxis with bypass agents. Specifically, for activated Factor VII (FVIIa) prophylaxis, the relationship of its circulating levels and clinical outcome in treated patients has not been defined. Here, we address this using HA rats that naturally exhibit bleeds requiring on-demand administration of human FVIII or FVIIa. This is a clinically relevant animal model whose genetics, size, and life cycle enable studies powered to distinguish small differences as a result of various treatments. We used adeno-associated virus (AAV) to deliver and continuously express a rat FVIIa transgene in HA rats, as a model of gene-based prophylaxis. Our aim was to identify the dose-dependency of steady-state level of expression of rat FVIIa with the ability to reduce or eliminate the bleeding phenotype in HA rats. Any observed bleeds would be treated with recombinant rat FVIIa. As such, our results could be useful in the design of future clinical studies of FVIIa prophylaxis in patients with HA.

Methods

Expression and purification of recombinant rat FVIIa

The DNA sequence of activated rat FVII (rat FVIIa), based on NCBI Gene ID 260320, had a PACE/Furin intracellular cleavage site (RKRRKR) inserted between the light and heavy chains of the zymogen form, a C-terminal HPC4 epitope, and a human prothrombin propeptide to ensure maximal γ-carboxylation of the Gla domain. This was codon-optimized based on the human and CHO codon usage table and generated by gene synthesis (Genscript, Piscataway, NJ). Human embryonic kidney cells (HEK293) over-expressing soluble human Furin were used to generate a stable cell line expressing rat FVIIa, which was purified from conditioned medium by chromatography, as previously described.

Animals

HA rats containing a 13-base pair deletion (FVIIIΔ13) and hemostatically normal rats on a Sprague-Dawley background were obtained from Tacoon Biosciences (Hudson, NY). AAV-treated animals were evaluated for bleeding events without prior knowledge of rat FVIIa transgene expression levels. Discoloration, visible swelling, lacerations, internal bleeding (identified by pale coloring and changes in behavior) were noted on a daily basis and treated with 270 μg/kg recombinant rat FVIIa for a maximum of 4 treatments. Pain was treated with 1 mg/kg Buprenorphine SR Lab every 72 hours, as needed. Animals with unresolved bleeds were euthanized by CO₂ inhalation.

AAV vector

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Children’s Hospital of Philadelphia, the Danish Animal Experiments Council, and the Novo Nordisk Ethical Review Council. An AAV plasmid encoding rat FVIIa (containing the RKRRKR sequence between Arg152-Ile153 and optimized for the rat codon usage table) under the control of a liver specific promoter/enhancer was used, as previously described. Recombinant serotype 8 AAV was obtained from the Vector Core at the University of Pennsylvania Center for Advanced Retinal and Ocular Therapeutics. AAV vector administration and blood collection is described in supplemental Methods.

Prothrombin time assay, rat FVII/a antigen determination, rotational thromboelastometry, fibrinogen, and thrombin-antithrombin level assays

These assays are described in supplemental Methods.

Tail vein transection injury model

Prior to the tail vein transection (TVT) injury, blood samples were drawn for rat FVIIa expression analysis. The TVT injury was carried out in 8- to 10-week-old experimental and control animals under isoflurane anesthesia, as described in detail in supplemental Methods. Because the animals subjected to the TVT did not complete a 21-week observation period, their bleeding events are not reported.

Statistical analysis

Statistical tests were performed using GraphPad Prism v7.0c (Macintosh) and are described in the figure legends, where appropriate. A receiver operating characteristic curve was computed to identify the cutoff for transgene expression levels with optimal differentiation between bleeders and nonbleeders among AAV-treated HA rats (maximizing Youden’s index). Statistical differences were considered significant when P < .05.

Results

Purification of rat FVIIa and in vitro activity

We generated a HEK293-based stable cell line expressing rat FVIIa with a C-terminal HPC4 tag for immunoadfinity purification (Figure 1A). Recombinant rat FVIIa was purified exclusively in a 2-chain form (Figure 1B), similar to what we previously observed with human, mouse, and canine FVIIa engineered transgenes. Using a prothrombin time (PT) clotting assay, we found that rat FVIIa tissue factor-dependent activity was similar to rhFVIIa (Figure 1C; P > .05).

AAV delivery in normal rats results in expression of biologically functional rat FVIIa

We generated a serotype 8 AAV vector in which expression of rat FVIIa (AAV-rat FVIIa) was directed by a liver-specific promoter/enhancer (Figure 2A). Due to the fragile nature of HA rats, we performed a preliminary study in WT rats to identify a range of AAV vector doses resulting in quantifiable transgene expression. After 16 weeks of observation, we found that AAV-rat FVIIa administration resulted in transgene expression from 300 to 3000 ng/mL (Figure 2B). Analysis of functional transgene activity via a PT clotting assay showed that treatment with 4.6E13 vector genomes (vg)/kg (high dose) resulted in a significant reduction in clot times compared with 4.6E11 vg/kg (low dose; P < .0001; Figure 2C).
Characteristic bleeds in control and HA rats receiving AAV-rat FVIIa
The aim of our study was to define the relationship of rat FVIIa circulating levels and modulation of clinical phenotype that manifested as bleeds in HA animals. In an effort to identify rat FVIIa levels eliminating bleeds altogether, we included a dose of 1.2E14 vg/kg, in addition to the AAV doses tested in WT rats. HA animals were infused at 4 weeks of age and WT and HA untransduced animals served as controls. Because the HA rats exhibit their peak bleeding phenotype within the first 21 weeks of age,11 we observed
all animals for 17 weeks after AAV-administration. A total of 51 HA animals were enrolled, but 7 animals (3 HA controls and 4 that received AAV) that reached ethical endpoints because of bleeds were euthanized. The PT, rat FVIIa transgene expression, bleeds, complete blood counts and thrombin-antithrombin complex levels of the euthanized animals are shown in supplemental Figure 1 and supplemental Table 1. All animals included here completed the study and had their bleeding events resolved by the administration of rat FVIIa. Representative bleeds are shown in Figure 3.

**Rat FVIIa transgene expression modulates in vitro parameters and the bleeding phenotype in HA animals**

HA animals received AAV at escalating doses (4.6E11-1.2E14 vg/kg), resulting in a trend to increased average rat FVIIa transgene expression (supplemental Figure 2A). We then categorized HA animals receiving AAV-rat FVIIa (HA-AAV) as bleeders (>1 bleeding event) or nonbleeders and compared their associated average transgene expression level as a biomarker for a bleeder/nonbleeder phenotype (supplemental Figure 2B). The mean levels of FVIIa transgene expression were significantly different between bleeders and nonbleeders. The optimal cut point for differentiation between the groups was identified as 708 ng/mL (receiver operator characteristic analysis). Using this cut point, there were 15/20 (75%) bleeder animals that had rat FVIIa transgene expression lower than 708 ng/mL (true positives, sensitivity), and there were 10/13 (76.9%) nonbleeder animals that had rat FVIIa transgene expression of at least 708 ng/mL (true negatives, specificity). For all subsequent analyses, we classified the HA-AAV animals into 2 groups with either less than 708 ng/mL or at least 708 ng/mL (range of ~730 to ~2000 ng/mL) average rat FVIIa transgene expression (Figure 4A).

We found that within the tiered HA cohorts that received AAV-rat FVIIa, animals expressing less than 708 ng/mL exhibited a PT of 22.6 ± 0.3 seconds, which was significantly different from HA control animals (P < .0001). Animals expressing at least 708 ng/mL also showed a significant reduction of their PTs to 17.2 ± 0.6 seconds (Figure 4B; P < .0001 vs HA). To further confirm the effect of rat FVIIa transgene expression on rat hemostasis, we performed whole-blood rotational thromboelastometry on samples from the HA-AAV and HA/WT control animals. We found that, after AAV infusion, transgene expression less than 708 ng/mL resulted in significant changes in the a angle and clot time compared with HA controls (41° ± 4° vs 24° ± 1.7° [P < .01] and 1095 ± 133 seconds vs 1480 ± 61 seconds [P < .05]; Figure 4C-D). Moreover, AAV-mediated transgene expression of at least 708 ng/mL resulted in an a angle and clot time that were similar to WT control values (58° ± 3° vs 59° ± 1.3°, and 588 ± 43 seconds vs 316 ± 11 seconds, respectively; P > .05; Figure 4C-D).

HA control animals experienced an average of 1.8 ± 0.4 bleeding events during the observation period (Figure 5A). Infusion of AAV-rat FVIIa in HA animals affected their bleeds in a transgene expression-dependent manner. Specifically, HA animals expressing less than 708 ng/mL experienced bleeding events similar to HA animals (1.5 ± 0.2 bleeds per animal [P < .05] vs HA; Figure 5A). In contrast, HA animals expressing at least 708 ng/mL exhibited significantly reduced bleeds per animal (0.6 ± 0.3 [P < .005] vs HA controls; Figure 5A). In addition to affecting the average bleeding events, rat FVIIa transgene expression resulted in a decrease in the proportion of HA animals that experienced bleeds (Table 1). Specifically, 91% of HA control animals experienced bleeds compared with 83% of animals in the less than 708 ng/mL cohort (P = 1.0). Importantly, only 33% of animals whose transgene expression was at least 708 ng/mL experienced bleeds (P < .05 vs
Interestingly, we found that none of the animals with an average transgene expression higher than 1250 ng/mL (\(25 \text{ nM; } n = 6\)) experienced any bleeds. Despite changes in the proportion of bleeding events in HA-AAV animals, we saw no significant changes in the number of recombinant rat FVIIa treatments required to resolve bleeding events across cohorts (Figure 5B). In addition, there was no significant difference in the location of bleeding events across cohorts (Table 2). Overall, our data in the HA rats have defined, for the first time, the relationship of rat FVIIa circulating levels and bleeding events. Rat FVIIa transgene expression of at least 708 ng/mL resulted in a significant reduction in bleeding events and the proportion of animals with bleeds. Moreover, rat FVIIa expression of more than 1250 ng/mL appears to be a threshold beyond which the observed bleeding phenotype in the HA rats is eliminated.

**Expression of rat FVIIa in HA rats and their response to a hemostatic challenge**

To further confirm the hemostatic effects of AAV-mediated expression of rat FVIIa, we used a TVT injury on animals administered AAV-rat FVIIa or controls. The advantages of this assay are both a long observation period (45 minutes) and an additional hemostatic challenge by removal of the formed plug at the injured vein at regular intervals. However, the maximum tail diameter for this assay necessitated that animals be no older than 10 weeks. Moreover, because of the terminal nature of the procedure, bleeds in animals...
subjected to the TVT injury were not analyzed, as they did not complete a 21-week observation period. Nevertheless, we found that HA animals with rat FVIIa transgene expression of at least 708 ng/mL had an average of 1.92 ± 0.30 mL total blood loss, similar to WT animals (1.11 ± 0.17 mL; P > .05), but significantly lower than HA controls (13.69 ± 1.51 mL; P < .0001; Figure 6A). AAV-rat FVIIa-infused animals expressing less than 708 ng/mL saw a significant reduction in total blood loss (8.86 ± 1.05 mL) compared with HA controls (P < .0002), but not to the same extent as WT animals. The inverse relationship of blood loss and rat FVIIa expression was confirmed by analysis without binning of the AAV-infused animals according to their rat FVIIa transgene expression levels (Figure 6B). Collectively, these results suggest that circulating rhFVIIa levels in HA animals with rat FVIIa transgene expression of at least 708 ng/mL can normalize blood clotting in the face of an injury. These results further confirm the hemostatic effects of expressed rat FVIIa on the bleeding phenotype of HA animals.

**Safety of transgene expression in AAV-rat FVIIa infused HA rats**

The safety of continuous rat FVIIa transgene expression of AAV-treated HA animals was assessed on multiple levels. Complete blood counts, obtained weekly, showed animals had normal platelet, red and white blood cell, hematocrit, and hemoglobin levels compared with HA control animals (Figure 7A-E). In addition, fibrinogen levels remained stable throughout the study across the AAV-treated and control groups (Figure 7F). Despite variations in some points, comparable findings were observed in WT animals that received AAV-rat FVIIa (supplemental Figure 3). We also measured levels of thrombin-antithrombin complex in HA animals expressing more than 1250 ng/mL, as those would be at higher risk for aberrant coagulation activation, but did not find an increase above baseline (supplemental Figure 4A). A similar finding was observed in WT animals that received AAV-rat FVIIa (supplemental Figure 4B), even in animals with ~3000 ng/mL of rat FVIIa transgene expression. Overall, these data suggest that rat FVIIa transgene expression did not result in overt coagulation-related complications, even when expressed in a hemostatically normal environment.

**Discussion**

Despite the significant advancement in hemophilia care in terms of both protein replacement therapy and novel gene-based approaches, the major complication remains the management of bleeds in the face of inhibitory antibodies. Prophylaxis in inhibitor patients using bypass agents, including rhFVIIa, has been shown to be effective. However, no defined data exist on the relationship between circulating rhFVIIa levels and clinical benefit. Clearly, a better understanding of such relationship would affect patient care by facilitating a more focused prophylaxis treatment. Here, we used a unique rat model of HA that closely resembles human patients by exhibiting spontaneous bleeds requiring hemostatic treatment. Similar to our previous experiments in hemophilic mice and dogs, we simulated FVIIa prophylaxis in these rats, using AAV-mediated gene delivery of rat FVIIa, which was expressed at steady plasma levels. We demonstrate that transgene expression levels of at least 708 ng/mL (>14 nM) can significantly reduce the proportion of animals with bleeds from 91% (naive) to 33%, whereas levels higher than 1250 ng/mL (>25 nM) were sufficient to eliminate bleeds altogether. These

### Table 1. Bleeding in HA controls and AAV-rat FVIIa-infused HA rats expressing <708 ng/mL (HA-AAV <708) or ≥708 ng/mL (HA-AAV ≥708) of rat FVIIa

|                     | HA controls, n (%) | HA-AAV <708, n (%) | HA-AAV ≥708, n (%) |
|---------------------|--------------------|--------------------|--------------------|
| Animals with bleeds | 10 (91)            | 15 (83)            | 5 (33)             |
| Animals with no bleeds | 1 (9)            | 3 (17)            | 10 (87)            |
| Number of animals   | 11 (100)           | 18 (100)           | 15 (100)           |

*P < .05 vs HA controls (Fisher’s exact test).
†P < .05 vs HA-AAV <708 (Fisher’s exact test).
data define for the first time the relationship of FVIIa circulatory antigen levels and proportion of bleeds in HA animals in a setting that mimics FVIIa prophylaxis.

We have previously demonstrated the therapeutic effects of continuous FVIIa expression via gene transfer in hemophilia models in vivo, including HA dogs that exhibit spontaneous bleeds.12,15,17,18 Specifically, in 3 HA dogs, we showed that expression of ~2000 ng/mL (~40 nM) of canine FVIIa via AAV delivery eliminated spontaneous bleeds.17 Unfortunately, these studies offered limited information with regard to FVIIa expression thresholds and phenotype outcome. This information is critical for further clinical development of either protein or gene-based FVIIa prophylaxis regimens for patients with hemophilia. The novelty of our data presented here stems from the unique properties of the HA rat not easily available to other large animal models (dogs20,21 or sheep22). First, HA rats exhibited spontaneous bleeds that could be life-threatening, but were treated with rat FVIIa (mimicking human patients). Second, HA rats allowed us to quickly obtain large cohort data with statistical power to discern clinical changes resulting from treatment. Specifically, the 33 AAV-treated HA rats here provide a highly detailed description on how bleeding can be modulated by different circulatory antigen levels of rat FVIIa. Because of the clinical relevance of the animal model, we believe that our data reported here for the first time can now be incorporated as FVIIa antigen target levels in future clinical studies.

Our data are also first to demonstrate the AAV serotype 8-mediated therapeutic expression of rat FVIIa in the clinically relevant HA rat model. Unfortunately, we were unable to determine the levels of the rat FVIIa active protease (ie, uninhibited by endogenous serpins [eg, antithrombin]), which may be lower than reported by our total antigen enzyme-linked immunosorbent assay. Nevertheless, we did not observe any apparent dysregulation of the hemostatic system from continuous rat FVIIa transgene expression (up to ~2000 ng/mL (~40 nM)), even in WT rats exhibiting rat FVIIa transgene expression up to 3000 ng/mL (60 nM). This further corroborates the safety of continuous FVIIa expression in HA rats, at least within the confines of the experimental observation period. These data mimic those in HA dogs expressing canine FVIIa at comparable levels after AAV gene transfer.17 Here, it is unclear why the maximum levels of transgene expression were different between WT and HA animals. Empirical evidence in hemophilia dogs suggests that hemostatic correction during AAV administration may result in increased transgene expression.23 It is possible that the normal hemostatic environment has provided WT rats a similar benefit to AAV transduction and transgene expression vs HA rats. The viral vector doses used in HA rats were on par with our previous experiments in HA dogs.17 For a human application, refinements in vector and transgene design may allow for a reduction of the AAV vector dose within the range compatible with minimal toxicity observed in patients with hemophilia (≤2E12 vg/kg).24,25 However, recent FVIII gene therapy clinical data suggest that vector doses up to 6E13 vg/kg, close to those used here, could result in sustained transgene expression when combined with courses of glucocorticoids.26

Table 2. The number of bleeds classified by type that were observed in the HA controls and AAV-rat FVIIa-infused HA rats expressing <708 ng/mL (HA-AAV <708) or ≥708 ng/mL (HA-AAV ≥708) of rat FVIIa.

| Bleed type  | HA controls, n (%) | HA-AAV <708, n (%) | HA-AAV ≥708, n (%) |
|-------------|--------------------|--------------------|--------------------|
| Rear limbs  | 10 (50)            | 10 (37)            | 4 (44.5)           |
| Front limbs | 5 (25)             | 13 (48)            | 4 (44.5)           |
| Lacerations | 2 (10)             | 1 (4)              | 0 (0)              |
| Eyes        | 2 (10)             | 2 (7)              | 0 (0)              |
| Other       | 1 (5)              | 1 (4)              | 1 (11)             |
| Total bleeds| 20 (100)           | 27 (100)           | 9 (100)            |

There was no statistically significant difference in the location distribution among the 3 groups (Fisher’s exact test, P = .933)

Figure 6. Tail vein transection to test for efficacy of expressed rat FVIIa. (A) Total volume of blood loss (milliliters) during a 45-minute period after tail vein injury. Each symbol represents an individual animal in each designated animal cohort. (B) The data in panel A are plotted as a function of transgene expression (ng/mL) vs total volume of blood loss (mL). Each symbol represents an individual animal. Gray horizontal bar indicates the range of blood loss values of WT control animals. Dashed lines indicate the 708 and 1250 ng/mL levels. ****P < .0001; **P < .01, using a 1-way ANOVA with Tukey’s multiple comparison test. All data are presented as mean ± SD.
Figure 7. Safety in HA animals overexpressing rat FVIIa. (A–E) Complete blood counts in HA, WT, HA-AAV <708 (transgene expression in nanograms per milliliter) and HA-AAV $\geq$708 (transgene expression in nanograms per milliliter) are shown: red blood cells (RBC), white blood cells (WBC), platelets (PLT), hemoglobin (Hb), and hematocrit (HCT) were monitored weekly throughout the study. (F) Fibrinogen (FIB) levels were monitored weekly in the same animal cohorts as in (A–E). The following symbols are used for each group in all panels: ▼, WT controls (n = 12); ▲, HA controls (n = 11); ●, HA – AAV $\geq$708 (transgene expression in nanograms per milliliter; n = 15); ■, HA-AAV <708 (transgene expression in nanograms per milliliter; n = 18). Arrow indicates time of AAV administration. No statistical differences were observed among the experimental groups (repeated measures 1-way ANOVA with Tukey’s multiple comparison test). All data are presented as mean ± SD.
Current clinical data on the efficacy FVIII or FIX gene therapy for hemophilia are encouraging, but no data exist on inhibitor patients. A transgene-specific immune response may also be of concern in young patients where factor exposure is lower.37 Data from animal models suggest that liver gene therapy may simulate immune tolerance induction, and thus offer inhibitor eradication and phenotypic correction.23-28 Although future studies may validate these data in humans, a FVIIa-transgene approach may also be a suitable gene therapy alternative for inhibitor patients. Our experiments did not directly evaluate the efficacy of FVIIa gene therapy in the presence of FVIII inhibitors. Although the mechanism of action of high-dose rhFVIIa in hemophilia remains unclear,29 it results in direct generation of activated FX. Therefore, although it will require validation, we believe that our data in the HA rats would be similar even in the presence of inhibitors. Lastly, our data do not show differences in the number of hemostatic treatments (recombinant rat FVIIa) in case of bleeds between naive HA or AAV-treated animals. However, animals with circulatory rat FVIIa antigen levels more than 1250 ng/mL did not require hemostatic management because of their lack of bleeds. If these observations could be extrapolated to human patients receiving gene-based FVIIa prophylaxis, it is conceivable that some patients may still need treatment of breakthrough bleeds. However, this on-demand treatment has also been documented for protein prophylaxis with either rhFVIIa or activated prothrombin complex concentrates.30-32

A major complication in hemophilia is the development of hemophilic arthropathy (HAR) as a result of recurrent bleeding in the joint space.33,34 Although FVIII protein replacement can treat spontaneous bleeds, 90% of patients will still present with joint disease by 30 to 40 years of age. Untreated patients can see HAR development as early as 17 months of age.34,35 In addition to bleeding, HAR manifests from multiple pathologic processes, including inflammation, neovascularization, and cartilage/bone changes.36 FVIIa can exert anti-inflammatory effects via the TF/PAR1/endothelial protein C receptor (EPCR) axis37 and EPCR is expressed in the synovial space and is involved in the anti-inflammatory effects of activated protein C.38 Because HA rats have been used in assessing joint bleeding and arthropathy,39,40 this work provides a framework to investigate the pathological changes and the involvement of inflammatory pathways in a setting of gene-based rat FVIIa prophylaxis. This would address a critical gap in knowledge for hemophilia inhibitor patients, as rhFVIIa prophylaxis can reduce joint bleeds, but no systematic data exist with regard to HAR.31

In addition to blood-borne FIX, extravascular FIX may be equally important in clot formation in hemophilia, as previous studies in mice have shown.41,42 Remarkably, functional coagulation factors are present in the synovial fluid, outside the vascular bed.43 It is conceivable that both intravascular and extravascular pools of infused coagulation factors can influence the resolution of joint bleeds and subsequent tissue remodeling in hemophilia, effects that may be pronounced in a prophylaxis setting. In mice, infused rhFVIIa appears to be transported to the extravascular space via an EPCR-mediated mechanism,44 where it persists for extended periods of time. Our laboratory has shown in hemophilic mice that the interaction of mouse FVIIa with EPCR enhances its hemostatic capacity in vivo.14,19 Interestingly, it has been postulated45 that extravascular rhFVIIa may explain the clinical benefits of rhFVIIa prophylaxis that remained for months after such prophylaxis ended.29 Such effects may be EPCR-dependent, including joint bleeds (EPCR is expressed in the synovial space).46 Therefore, a FVIIa gene-based approach may further facilitate an extravascular pool of FVIIa, potentially resulting in therapeutic circulatory FVIIa levels lower than those obtained through bolus infusion. An additional novelty of our data here is that they provide a platform to investigate whether extravascular rat FVIIa and its interaction with endogenous EPCR can affect bleeding and, as discussed earlier, HAR development. Unfortunately, we did not have tissue samples to shed light in the extravascular presence of expressed rat FVIIa. However, experiments are currently underway, and these data can potentially have mechanistic and translational ramifications.

In conclusion, data presented here are first to use gene therapy in a novel animal model of HA that recapitulates hallmarks of the human disorder with significant research advantages compared with other available models. In this model, we address a long-standing question in the treatment of HA with FVIIa regarding the relationship of circulating levels of the protein and clinical outcome. Although rat FVIIa transgene expression levels did not define a bleeder/nonbleeder phenotype on an individual animal basis, we demonstrate the dose-dependency of circulating rat FVIIa antigen levels and proportion of animals without bleeds. Overall, our data suggest that FVIIa antigen level thresholds can exist for reduced or complete elimination of bleeding events in a setting of FVIIa HA prophylaxis. Clinical observations will need to validate our data through appropriately designed protein and/or gene-based prophylaxis studies. We believe that our approach combined with the animal model and obtained data will be useful in future studies toward that goal.

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Authorship

Contribution: S.M.Z. designed and performed research, analyzed data, and wrote the manuscript; J.C.S. performed research and analyzed data; G.P., L.D., and O.A.M.-C. performed research; P.A.G. performed data; G.P., L.D., and O.A.M.-C. performed research; P.M. designed research; M.K.-H. and B.W. designed research; P.M. obtained funding; and all authors commented on the final manuscript.

Conflict-of-interest disclosure: B.W. is an employee of Novo Nordisk A/S. M.K.-H. is currently an employee of Ascendia Pharma A/S. P.M. receives research funding through a competitive grants from the Bayer Hemophilia Awards Program and salary (spouse) from Bristol-Myers Squibb and CSL Behring. The generation of rat FVIIa used technology licensed to Spark Therapeutics, Inc. (Spark). The Children’s Hospital of Philadelphia as an institution also holds equity in Spark. The remaining authors declare no competing financial interests.

Correspondence: Paris Margaritis, The Children’s Hospital of Philadelphia, 5024 Colket Translational Research Building, 3501 Civic Center Blvd, Philadelphia, PA 19104; e-mail: margaritis@email.chop.edu.
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