Long-term expression of murine activated factor VII is safe, but elevated levels cause premature mortality

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Intravenous infusion of recombinant human activated Factor VII (FVIIa) has been used for over a decade in the successful management of bleeding episodes in patients with inhibitory antibodies to Factor VIII or Factor IX. Previously, we showed that expression of murine FVIIa (mFVIIa) from an adeno-associated viral (AAV) vector corrected abnormal hemostatic parameters in hemophilia B mice. To pursue this as a therapeutic approach, we sought to define safe and effective levels of FVIIa for continuous expression. In mice transgenic for mFVIIa or injected with AAV-mFVIIa, we analyzed survival, expression levels, in vitro and in vivo coagulation tests, and histopathology for up to 16 months after birth/mFVIIa expression. We found that continuous expression of mFVIIa at levels at or below 1.5 μg/ml was safe, effective, and compatible with a normal lifespan. However, expression levels of 2 μg/ml or higher were associated with thrombosis and early mortality, with pathologic findings in the heart and lungs that were rescued in a low–factor X (low-FX) mouse background, suggesting a FX-mediated effect. The findings from these mouse models of continuous FVIIa expression have implications for the development of a safe gene transfer approach for hemophilia and are consistent with the possibility of thromboembolic risk of continuously elevated FVIIa levels.

Introduction
Hemophilia results from mutations in either of 2 genes, F8 or F9, which encode proteins in the intrinsic pathway of coagulation. The current approach to therapy is intravenous infusion of the missing or defective coagulation factor, but work by Hedner and colleagues has established that infusion of recombinant human activated Factor VII (rFVIIa; NovoSeven), a critical protein in the extrinsic pathway, can restore hemostasis through rFVIIa-catalyzed generation of thrombin in patients with antibodies to Factor VIII (FVIII) or Factor IX (FIX) (1–3). Based on the current understanding of the coagulation cascade, all patients with hemophilia could conceivably be treated with rFVIIa, a protein to which all such patients have immunological tolerance. This would simplify hemophilia management to a single product, but the high cost, short half-life, and ongoing concerns about risk of thrombosis with rFVIIa (4) have limited this approach.

In previous studies in hemophilic mice, we showed that a gene transfer approach could circumvent the issue of short half-life, because activated FVII can be continuously expressed from a donated gene introduced into the liver via an adeno-associated viral (AAV) vector (5). The engineered FVII construct contained a nucleotide sequence encoding RKRRKR (referred to herein as 2RKR) inserted between Arg152 and Ile153 — the normal site of cleavage — to allow secretion of activated FVII. Expression of such an engineered FVIIa improved hemostasis in hemophilia B (HB) mice following AAV-mediated gene transfer. To further pursue vector-mediated expression of FVIIa as a possible therapeutic approach, we sought to define the range of FVIIa levels that restore hemostasis and are safe when expressed continuously. As an experimental approach, we generated transgenic mice expressing a range of levels of murine FVIIa (mFVIIa), detected using an assay specific for mFVIIa that shows minimal cross-reactivity to mFVII. We categorized mice from different transgenic lines as low and high expressers based on plasma levels of mFVIIa and crossed them to HB mice. We carried out similar analyses of mice injected with an AAV vector expressing mFVIIa. We found that when mFVIIa was continuously expressed in transgenic mice, even low levels were sufficient to improve hemostasis in a mouse model of hemophilia; moreover, continuous expression of mFVIIa at levels up to 1,500 ng/ml (30 nM) was compatible with a normal lifespan in mice. Identical findings were observed in mice injected with doses of AAV-mFVIIa adequate to generate circulating mFVIIa levels of 300–800 ng/ml (6–16 nM), and overexpression of mFVIIa at levels greater than 2,000 ng/ml (40 nM) caused early mortality in both normal and hemophilic mice, with pathology predominantly in heart and lungs. We also demonstrated that crossing mFVIIA-overexpressing mice into a newly generated low–factor X (low-FX) mouse model (6) resulted in restoration of normal longevity and decreased thrombin generation, which indicates that the shortened lifespan of the high expressers is mediated through the coagulation cascade rather than another mFVIIA-mediated signaling event. These results establish ranges for hemostatic efficacy of continuously expressed mFVIIA in a mouse model and also define upper limits for safety for continuously expressed mFVIIA. They also demonstrate an association between continuously elevated levels of FVIIa and premature mortality.

Nonstandard abbreviations used: -a, activated; AAV, adeno-associated viral (virus); aPTT, activated partial thromboplastin time; FIX, Factor IX; FVII, Factor VII; FVIII, Factor VIII; FX, Factor X; h-, human; HB, hemophilia B; m-, murine; p-, plasmid; PT, prothrombin time; rh-, recombinant human; TAT, thrombin-antithrombin; TF, tissue factor; TTR, transthyretin.

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Figure 1
Transgenic construct for mFVIIa (mFVII-2RKR) and mFVII zymogen mice. The mFVIIa/mFVII cDNA is under control of the mTTR promoter separated by a synthetic intron. A polyadenylation signal (pA) from bovine growth hormone follows the 3′ untranslated region (UTR) for both cDNAs.

Results
Generation of transgenic mice expressing mFVIIa at a range of levels.
Our initial studies using AAV vector–mediated FVIIa gene transfer demonstrated the efficacy of continuously expressed mFVIIa in effecting hemostasis in a murine model of hemophilia (5). As a next step, we sought to determine the minimum levels of mFVIIa required for efficacy and the maximum levels that would be safely tolerated. We chose to analyze the safety and efficacy of a range of circulating mFVIIa levels using both transgenic and AAV-injected mice; compared with vector-treated mice, transgenic animals offer the advantages of decreased intragroup variation in mFVIIa levels among littermates, maintenance of and AAV-injected mice; compared with vector-treated mice, transgenic mice about 3,000 ng/ml (Figure 2A). PT progressively shortened as circulating mFVIIa levels increased (Figure 2B). The aPTT was restored to the WT range in hemophilic mice expressing low-range mFVIIa (P < 0.0001 versus WT; Figure 2C). As a surrogate measure of thrombin generation, we measured the levels of TAT. HB mice expressing low and high levels of mFVIIa showed a dose-dependent increase in TAT levels (P < 0.05, HB–low-mFVIIa versus HB–high-mFVIIa; Figure 2D). These data were further confirmed by shortening of clot formation times and increase in alpha angles on rotational thromboelastometry in citrated blood from HB mice with the mFVIIa transgene compared with nontransgenic HB littermates (data not shown). Similar results for in vitro clotting assays were obtained when mFVIIa transgenic mice were crossed with hemophilia A mice (data not shown). In contrast to HB-mFVIIa transgenic animals, HB mice transgenic for mFVII zymogen (expressing about 1,700 ng/ml total antigen and approximately 150 ng/ml mFVIIa) showed a modest reduction in PT, no improvement in aPTT, and no increase in TAT levels (Figure 2).

For comparison, another cohort of HB mice (n = 8) were injected via the portal vein with an AAV2 vector at a range of doses (3 × 1011–1.2 × 1012 vector genomes/mouse). This resulted in

Table 1
mFVII and mFVIIa levels in transgenic founders or WT controls

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>mFVII/mFVIIa total antigen (ng/ml)</th>
<th>mFVIIa antigen, biotinylated inhibitor ELISA (ng/ml)</th>
<th>PT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-mFVIIa Tg</td>
<td>6</td>
<td>4,250 ± 1,100</td>
<td>3,150 ± 800</td>
<td>11.9 ± 0.9</td>
</tr>
<tr>
<td>Low-mFVIIa Tg</td>
<td>6</td>
<td>2,000 ± 350</td>
<td>1,150 ± 600</td>
<td>15.2 ± 1.2</td>
</tr>
<tr>
<td>WTa</td>
<td>30</td>
<td>900 ± 450</td>
<td>150 ± 100</td>
<td>21.9 ± 1.0</td>
</tr>
<tr>
<td>mFVII Tg</td>
<td>5</td>
<td>2,050 ± 550</td>
<td>150 ± 50</td>
<td>20.8 ± 1.3</td>
</tr>
<tr>
<td>WTb</td>
<td>4</td>
<td>800 ± 300</td>
<td>100 ± 100</td>
<td>22.4 ± 0.7</td>
</tr>
</tbody>
</table>

Values are mean ± 1 SD. *Littermates of mFVIIa transgenic mice. **Littermates of mFVII transgenic mice.
mFVIIa levels of 300–800 ng/ml (mean, 510 ng/ml), which was adequate to reduce APTT, PT, and blood loss following a tail-clip assay and to dramatically improve long-term survival (Table 2). Thus, the results obtained from the AAV-injected mice closely resemble those obtained from the transgenic mice.

Low levels of mFVIIa improve in vivo clot formation in the microcirculation of hemophilic mice, but not in the macrocirculation of mFVIIa transgenic mice. To further analyze the hemostatic efficacy of varying mFVIIa levels, we used assays that allowed us to assess clot formation in a living mouse. A tail-clip bleeding test, in which the tail was severed at a diameter of 3 mm, was used to determine total blood loss during a 10-minute time period, as measured by OD575 nm of a saline solution into which the tail was submerged, following red blood cell lysis. Total blood loss and bleeding time were reduced in the HB-mFVIIa transgenic mice compared with hemophilic nontransgenic littermates (\( P < 0.05 \)), although not to the normal levels seen in WT littermates (data not shown). In contrast, both HB–low-mFVIIa and HB–high-mFVIIa mice (\( n = 3, 24 \) sites) displayed kinetics and clot volume indistinguishable from those of WT littermates (\( n = 5, 30 \) sites; Figure 3B), which suggests that relatively modest levels of mFVIIa (mean \( \sim \)1,000 ng/ml, or 20 nM, in low expressers) cause improved hemostasis in the microcirculation. This finding of efficacy at low-mFVIIa levels in the cremaster arteriole model is consistent with data we generated using an AAV-mFVIIa vector to correct hemostasis in hemophilic mice (Table 2). Finally, using a third in vivo test of thrombosis, the FeCl3–carotid artery injury model, we found no evidence of clot formation in either the high or the low expressers (Figure 3C).

In a second in vivo model, thrombus formation was assessed following a laser injury to a small arteriole (50 μm diameter) in the cremaster muscle by monitoring platelet accumulation at the site of injury (9). By assessing both kinetics of clot formation and the size of the formed thrombus, we determined that untreated HB littermates (\( n = 2, 14 \) injury sites) failed to form clots even after the extended time period of 10 minutes. In contrast, both HB–low-mFVIIa and HB–high-mFVIIa mice (\( n = 3, 24 \) sites) displayed kinetics and clot volume indistinguishable from those of WT littermates (\( n = 5, 30 \) sites; Figure 3B), which suggests that relatively modest levels of mFVIIa (mean \( \sim \)1,000 ng/ml, or 20 nM, in low expressers) cause improved hemostasis in the microcirculation. This finding of efficacy at low-mFVIIa levels in the cremaster arteriole model is consistent with data we generated using an AAV-mFVIIa vector to correct hemostasis in hemophilic mice (Table 2). Finally, using a third in vivo test of thrombosis, the FeCl3–carotid artery injury model, we found no evidence of clot formation in either the high or the low expressers (Figure 3C).

Continuous expression of mFVIIa at less than 1,500 ng/ml is associated with normal life expectancy, but expression at more than 2,000 ng/ml is associated with premature mortality. To assess the safety of continuous expression of mFVIIa, we determined survival in cohorts of mFVIIa transgenic, nonhemophilic mice as a function of circulat-
ing mFVIIa levels (Figure 4). Over a 16-month period of observation, survival in the low mFVIIa expressers was equivalent to that of nontransgenic WT littermates. Longer periods of observation, up to 24 months, showed the same result (data not shown), and these results are consistent with those obtained from AAV-injected mice (Table 2). However, for the high mFVIIa expressers, survival was reduced, with 50% of founders surviving at 16 months (P < 0.02 versus WT; Figure 4). Results were similar when the high expressers were crossed to HB mice: FIX levels less than 1% did not protect against premature mortality (data not shown). Backcrossing into C57BL/6 mice further reduced survival, with typically 20% of pups dying in the first 72 h after birth. Even among those surviving the first 72 h, lifespan was shortened compared with nontransgenic littermates, with 50% of N1 mice dead at 3 months (P < 0.001 versus WT) and 50% of N2 mice dead at 1 month (P < 0.0001 versus WT; Figure 4). Mortality rates on backcrossing were similar for 3 separate high-mFVIIa founder lines. The cause of the progressively earlier mortality on backcrossing was investigated, but not found:

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>mFVIIa (μg/ml)</th>
<th>PT (s)</th>
<th>aPTT (s)</th>
<th>OD575 nm A</th>
<th>Survival at 19 mo</th>
<th>Clots B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV2-mFVIIa</td>
<td>0.51 ± 0.19 C,D</td>
<td>27.9 ± 2.3 C</td>
<td>49.1 ± 16.3 C</td>
<td>0.7 ± 0.5 C,D</td>
<td>85% E</td>
<td>68 of 68 C</td>
</tr>
<tr>
<td>(n = 11)</td>
<td>(n = 11)</td>
<td>(n = 11)</td>
<td>(n = 14)</td>
<td>(n = 14)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>HB</td>
<td>0.16 ± 0.07</td>
<td>31.3 ± 3.5</td>
<td>66.3 ± 9.7 D</td>
<td>2.6 ± 0.7 D</td>
<td>45%</td>
<td>0 of 30</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 13)</td>
<td>(n = 11)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>WT</td>
<td>0.13 ± 0.11</td>
<td>29.9 ± 1.6</td>
<td>34.9 ± 2.6</td>
<td>0.3 ± 0.2</td>
<td>100%</td>
<td>45 of 45</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 15)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

AAV2-mFVIIA was administered at 3 × 10^{11}–1.2 × 10^{12} vector genomes/mouse. Untreated HB and WT mice were used as controls. Values for mFVIIa, PT, aPTT, and OD575 nm are mean ± 1 SD. A Hemoglobin content following tail-clip assay. B Number of clots in the cremaster muscle following injury. C P < 0.01 versus HB. D P < 0.01 versus WT. E P < 0.05 versus HB.
we did not observe significant differences in platelet counts or TF levels (prepared from pulmonary and cardiac cell lysates) in the N1 and N2 mice (data not shown). Moreover, we did not observe any significant differences in TAT levels, clotting times (PT), or mFVIIa levels (data not shown).

Premature mortality in high FVIIa–expressing mice is associated with pathologic findings in heart and lungs. To investigate the causes of premature mortality, we carried out histopathologic studies of high FVIIa expressers on both normal and HB backgrounds. Results were identical for normal and hemophilic background in high-mFVIIa transgenic mice sacrificed or expiring at time points ranging from 2 to 6 months of age. Necropsy revealed no gross abnormalities. Histopathologic analyses of brain, liver, and kidney were also normal. Major pathologic findings were confined to the lungs and heart (Figure 5). The lungs of 4 of 8 mice examined showed increased fibrin deposition and intimal/smooth muscle proliferation in the pulmonary vasculature. In the hearts of all 8 mice examined, even in these relatively young mice aged less than 6 months, thrombi were present in coronary vessels and in the chambers of the heart. Occasional patchy inflammatory cell infiltrates were also noted. Immunohistochemical staining for fibrin showed increased fibrin deposition in the myocardium in a multifocal pattern. As a follow-up to these observations, we sacrificed 6 low-mFVIIa transgenic mice on a normal background at 18 months of age to search for evidence of pathologic findings in heart or lungs, but these tissues appeared normal even in these older low-mFVIIa transgenic mice (data not shown). None of these findings were observed in the 10 age-matched nontransgenic controls examined (P < 0.02 in lungs; P < 0.0001 in heart) or in the 6 low-mFVIIa transgenic mice examined up to 18 months of age (P = 0.085 in lungs; P < 0.0001 in heart).

**Figure 4**
Expression of high mFVIIa levels is associated with increased mortality after crossing the founder C57BL/6–SJL mice into C57BL/6 pure strain background. Kaplan-Meier survival plot of transgenic mFVIIa mice with low or high levels of transgene expression compared with WT littermates. Curves for high mFVIIa expressers exclude animals dying within 72 h of birth. Squares, WT (n = 10); filled circles, low-mFVIIa N1–N4 (n = 25); triangles, high-mFVIIa F0 (n = 6); diamonds, high-mFVIIa N1 (n = 17); open circles, high-mFVIIa N2 (n = 11). *P < 0.05 versus WT.

**Crossing to low-FX mice restores normal survival.** Although the mechanism of action of high-dose FVIIa is still controversial, the end product is the generation of FXa. In an effort to rescue the high-expressing mFVIIa transgenic mice, which had repeatedly succumbed at early time points, and to determine whether the premature mortality was mediated through FXa generation, we crossed these mice to mice with low levels of mFX activity. These mice are F10 knockouts and knockins for a F10 variant based on a human FX (hFX) mutation (F10<sup>Figurl</sup> ref. 10) that results in low FX activity (~5.5%) in homozygous mice without affecting survival (6). The targeted knock-in of the murine F10<sup>Figurl</sup> allele (F10<sup>Figurlm2Cam</sup>) allows expression of this variant from the endogenous promoter. To determine whether low mFX activity rescued the early mortality seen in mice overexpressing mFVIIa, we crossed these 2 mouse lines and analyzed littermates with high mFVIIa levels and low, mid-range, or normal mFX activity (about 3%, 50%, and 100%, respectively; Figure 6). The low-mFX mice were on a predominantly C57BL/6 background (backcrossed for 3 generations). Coagulation assays showed comparable levels of mFVIIa in all 3 groups as measured by ELISA (Figure 6A). As expected, mice with low mFX activity had prolonged PT compared with mice with mid-range or WT mFX activity (Figure 6B). TAT levels also varied among the 3 groups of mice, with markedly elevated levels in mFVIIa transgenic mice with 100% mFX activity, lower but still elevated levels in mice with 50% mFX activity, and levels close to those of nontransgenic WT littermates in mice with 3% mFX activity (Figure 6C). The consequences of these altered coagulation parameters are clear in the survival curves for these animals. Among mice expressing high levels of mFVIIa, survival at 11 months was 0% for mice with WT mFX activity (F1 and F2, P < 0.0001 versus WT), 18% for mice with 50% mFX activity (P < 0.0001 versus WT), and 100% for mice with 3% mFX activity (Figure 6D). Histologic analysis of tissues from 4 high-mFVIIa mice with either 100% or 50% mFX showed thrombi similar in distribution to those found in high-mFVIIa mice crossed to hemostatically normal mice (Figure 5 and data not shown).

**Discussion**

The development of rhFVIIa to effect hemostasis in hemophilic patients with inhibitors was a major advance in the management of what is currently the most common complication of treatment for hemophilia. However, the short half-life and substantial expense of the product remain impediments to more extensive use in the setting of hemophilia. Gene transfer offers the possibility of circumventing the short half-life, because expression from the donated gene is continuous. In addition, as a gene therapy product, FVIIa has the advantage that all hemophilic subjects are fully tolerant to it. Ongoing advances in AAV-mediated gene transfer suggest that successful clinical application will occur for other clotting factors (11, 12). We undertook the studies here in an attempt to define safe and efficacious levels of FVIIa in the setting of continuous expression.

The present study reports 2 major findings: first, that continuously expressed FVIIa demonstrates safety and hemostatic efficacy over a fairly broad range of levels and over the lifespan of the mouse, and second, that high-level continuous expression of FVIIa is associated with early mortality, by a mechanism dependent on FX. Previously, we showed that AAV-mediated gene transfer of an engineered FVIIa construct to the liver resulted in high expression of FVIIa in these animals. The long-term survival of these mice was 0% for mice with WT mFX activity (F1 and F2, P < 0.0001 versus WT), 18% for mice with 50% mFX activity (P < 0.0001 versus WT), and 100% for mice with 3% mFX activity (Figure 6D). Histologic analysis of tissues from 4 high-mFVIIa mice with either 100% or 50% mFX showed thrombi similar in distribution to those found in high-mFVIIa mice crossed to hemostatically normal mice (Figure 5 and data not shown).
in continuous expression of FVIIa and that hemophilic mice expressing mFVIIa for periods up to 6 months exhibited correction of plasma-based coagulation assays (PT and aPTT) and partial correction of the tail-clip bleeding time after vector infusion (5). We demonstrate that circulating levels of mFVIIa in the range of 500–1,500 ng/ml (10–30 nM) in hemophilic mice shorten the PT, correct the aPTT to the normal range, and modestly increase TAT levels. The levels seen in the low-mFVIIa transgenic mice closely approximated the levels obtained from AAV-mediated transduction, as seen in our previous study (5) and in data presented here (Table 2). These levels are similar to the peak therapeutic levels seen in patients infused with recombinant FVIIa (10–20 nM; ref. 13). Moreover, these mice exhibited a normal lifespan through the 16-month study duration, similar to AAV-treated mice (Table 2), and at necropsy showed no unusual pathology. In terms of efficacy, in vivo tests of coagulation provide a more nuanced view of mFVIIa-supported hemostasis in hemophilic mice than do standard plasma-based assays. Clot formation in the microvasculature, as measured in the cremaster arterioles after a laser injury, appeared indistinguishable from that seen in WT mice, and blood loss on the tail-clip assay was reduced compared with hemophilic nontransgenic littermates, although not to the levels seen in WT littermates. On the other hand, in the FeCl3 injury model of the carotid artery (macrocirculation), there was no clot formation in the transgenic mice, even after a prolonged period of observation.

A possible explanation for these discordant findings of clot formation in the micro- and macrocirculation may be due to differences in multiple factors, including the size of the vessel and/or the mechanism of clot formation in these settings. Chou et al. showed that clot formation in the microcirculation relies primarily on circulating TF, e.g., from microparticles (14). Clot formation in large diameter vessels, on the other hand, depends on exposure of vessel wall (subendothelial) TF (15). Additionally, the difference seen in the 3 injury models used here may be a reflection of the type of injury. For example, thrombus formation in the FeCl3 model appears to rely primarily on the glycoprotein VI–collagen pathway (16), whereas in the laser-induced injury model, initial thrombus formation relies on the TF-mediated pathway of thrombin generation (17). Finally, although we do not have direct evidence, we cannot exclude the possibility that continuous expression results in an increased extravascular pool of mFVIIa.

Figure 5
Histological findings in lungs and hearts of high-mFVIIa transgenic mice compared with WT littermates. Intimal/smooth muscle proliferation was observed in the pulmonary veins (B and C) as well as fibrin deposition in the pulmonary bed (E and F) of mFVIIa transgenic mice, but neither was seen in WT mice (A and D). Thrombi were observed in the coronary artery (I and M) and ventricles (H and L) as well as inflammatory infiltrates in the myocardium (J and N) of mFVIIa transgenic mice, but none were observed in WT mice (G and K). Original magnification, ×20 (H and L); ×40 (G and K); ×100 (D, E, I, and M); ×200 (A–C, F, J, and N).
that may affect, or at least facilitate, the differences seen in the in vivo injury models described here. Elevated levels of extravascular FVIIa have been proposed as the explanation for the reduced number of bleeds observed in hemophilic patients undergoing daily infusions of rhFVIIa, both during and for 3 months after the infusions (18, 19). Additionally, hFVIIa activity of 34% has been reported in human lymph fluid that bathes the extravascular space (20). More pertinent to this study, the presence of mFVIIa has previously been demonstrated in the perivascular space outside dermal vessels even in the absence of tissue damage (21).

A critical question is the relationship of these findings to the frequency of bleeding in patients with hemophilia. The major morbidity of hemophilia stems from repeated bleeds into the joints that result in synovial hypertrophy, intense neovascularization, and inflammation (22). If indeed most of these bleeds begin as unsealed nicks in the microcirculation, then a normal-ized response to a hemostatic challenge in the microcirculation, as continuously expressed FVIIa seems to provide, could conceivably result in a reduced number of joint bleeds. A caveat in extrapolating these findings to humans is that mice generally have platelet counts 3- to 4-fold higher than do humans (23), which may result in a better outcome in this model, although the murine mean platelet volume is also smaller (one-half that of human platelets; refs. 24, 25), which compensates for the increased count. If, as has been proposed, most of the blood-borne TF comes from leukocyte-derived microparticles (14), the difference in platelet counts may be of limited concern in interpretation of results. The effect of continuously expressed FVIIa on frequency of joint bleeds will perhaps be better assessed in a canine model of hemophilia, where observation of animals over many years has established a baseline number of readily diagnosed bleeds, including joint bleeds, in untreated animals (26). Improvement of hemostasis in a large-animal model has previously been a strong predictor of efficacy of products in human subjects (27–29).

In addition to defining a range at which continuously expressed FVIIa appears to be safe and improves hemostasis, the other major finding in this study is the early mortality associated with high-level continuous expression of FVIIa. Although mice expressing lower levels of FVIIa exhibited normal lifespan and

Figure 6
Hemostatic differences in high-mFVIIa transgenic mice. (A) mFVIIa antigen; (B) PT measurements using hFX-deficient plasma; (C) TAT levels; and (D) Kaplan-Meier survival plot of high-mFVIIa transgenic mice in a background between ~3% and 100% mFX. (A–C) n = 3 (~3% mFX and high-mFVIIa ~3% mFX), 4 (high-mFVIIa), 6 (WT), 8 (50% mFX), 12 (high-mFVIIa 50% mFX). (D) Open squares, WT (n = 10); filled circles, high-mFVIIa F0 (n = 3); stars, high-mFVIIa F2 ~3% mFX (n = 8); diamonds, high-mFVIIa F2 50% mFX (n = 17); crossed-out squares, high-mFVIIa F1 100% mFX (n = 11); open circles, high-mFVIIa F2 100% mFX (n = 7). * P < 0.05 versus WT. Values are mean ± 1 SD.
absence of pathology (100% of mice alive and free of pathologic changes at 16 months), those expressing FVIIa in the range of 3,000 ng/ml showed early mortality, with 50% of founder mice surviving at 16 months. Several lines of evidence suggest that increased thrombin generation and resultant pathologic thrombus formation account for this finding. The most direct evidence comes from histopathologic studies of the high-FVIIa mice, which exhibited pathologic thrombi and increased fibrin deposition even at young ages. These findings are reminiscent of those described by Ameri et al. in mice transgenic for hFIX, although those animals exhibited altered histopathology and premature mortality with modest increases in FIX levels (30), whereas in our study continuous expression of mFVIIa appeared safe over a broad range of lower levels. Our finding of pathology primarily in the heart and lungs is consistent with the high levels of TF in these organs (31, 32) and supports the concept of TF forming extremely high levels of mFVIIa expression. We were not able to crossing to the low-mFX mice and the restoration of survival mortality with modest increases in FIX levels (30), whereas in our study we observed increased thrombin generation as the cause of the prematurity. However, we also show early mortality, characterized by thrombus formation and fibrin deposition in lungs and heart, as a complication of continuous expression at levels greater than 2,000 ng/ml (40 nM). This early mortality was rescued by crossing to mice with low circulating levels of FX. Our data suggest that the margin between the lowest effective dose (6 nM) and the highest safe dose (30 nM) is narrow, but this is characteristic of a number of drugs that alter coagulation.

Enabled by continuing advances in systems biology and by the ability to transfer any gene of interest, novel therapeutic approaches to genetic disorders based on transfer of genes other than those affected by mutations will continue to be explored (36, 37). Based on the FVIIa model, it will be crucial to carefully define the boundaries of safety and efficacy over prolonged periods of time and to scrutinize the effect of continuous transgene expression on disease pathophysiology.

Methods

Generation of FVIIa and FVII transgenic mice. The 2.3-kb fragment containing the mFVII-2KR transgene (mFVIIa, containing the full mFVII untranslated region) with a synthetic intron from the AAV-hAAT-mFVII (5) was partially digested and purified with SacI and XbaI. A KmI linker fragment containing SacI and XbaI sites was ligated to a KmI-digested plasmid containing the liver-specific mTTR promoter (38) driving expression of hFIX (pTTR-hFIX; ref. 8). The intron-mFVIIa fragment was ligated to the pTTR vector. To generate pTTR-mFVII (zymogen), pTTR-mFVIIa and pAAV-hAAT-mFVII (containing the full mFVII untranslated region) were partially digested with ClaI/XbaI to release mFVIIa and mFVII, respectively, and the ClaI/XbaI mFVII fragment was ligated into digested pTTR vector. Sequencing was performed to confirm the presence or absence of the 2KR sequence in mFVII cDNA. Purified plasmid preparations were generated for each construct by standard cesium chloride gradient purification. The fragments for microinjection were released by HindIII restriction enzyme digestion, isolated by agarose gel electrophoresis, recovered by ethanol precipitation, and purified with an EndoFree spin column (Sigma-Aldrich) according to the manufacturer’s instructions. DNA fragments were dissolved in injection buffer (10 mM Tris and 0.1 mM EDTA, pH 7.5) and adjusted to the appropriate concentration. DNA was injected into a minimum of 150 fertilized eggs at a concentration of 5 ng/μl using standard microinjection techniques (Transgenic and Chimeric Mouse Facility, University of Pennsylvania) (39). Genomic DNA was extracted from tail biopsies or blood using tissue and blood DNA extraction kits (Qiagen). Transgenic pups were identified by a TTR-specific forward primer (5’-GCGAGGGATTCAGCAGCCTGG-3) and a cDNA-specific reverse primer (5’-CCCAACATTCCTTCTCCTTCTCTTCTTTGCGTCTTGG-3’), with a PCR program of 95°C for 2 min, 30 cycles of 94°C for 30 s, 62°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 20 min.
Experimental animals and AAV administration. All procedures and animal care were approved by the IACUC at the Children’s Hospital of Philadelphia. The transgenic founders were hybrid F1 generation resulting from a cross between C57BL/6 and SJL mice (The Jackson Laboratory). Transgenic mice were further backcrossed to pure C57BL/6 mice, resulting in early mortality (vide infra); thus all studies were conducted in F0 and N1–N4 mice, using nontransgenic congenic littermates as controls. Murine platelet counts were determined as previously described (40). AAV vector was prepared as previously described (5) and administered via the portal vein as previously described (41).

Tail-clip assay (severe bleeding model). For tail-clip assays, tails were prewarmed at 37°C for 2 min and then cut at a diameter of 3 mm and immersed in prewarmed saline. Time to cessation of bleeding was recorded if less than 10 min; otherwise, the experiment was terminated at 10 min and the tail cauterized to stop bleeding. The blood-containing saline was centrifuged at 520 g for 10 min at 4°C. Subsequently, 6 ml lysis buffer (10 mM KHCO3, 150 mM NH4Cl, and 1 mM EDTA) was added to the red blood cell pellet, and the lysate proceeded for 10 min at room temperature, after which the samples were centrifuged as described above and OD at 570 nm of the supernatants was measured. For all assays, nontransgenic littermates served as controls.

FeCl3 carotid artery model. The carotid arteries of adult mice were exposed, a Doppler flow probe (model 0.5SVB; Transonic Machinery Systems) was placed on the surface of the exposed artery, and a baseline blood flow measurement was recorded. Subsequently, a 2 mm2 piece of Whatman No. 1 paper soaked in a 15% solution of FeCl3 was applied to the adventitial surface of the exposed artery for 2 min, after which it was removed and carotid artery blood flow was recorded. Time to carotid artery occlusion was defined as the time from initiation of arterial injury until the onset of stable occlusion (42).

Real-time wide-field intravital microscopy. The cremaster muscles of adult mice were exposed, stretched, and pinned across the intravital microscopy tray. The rat anti-CD41 (murine platelet glycoprotein complex Ibb/Ila) Alexa Fluor 555–labeled antibody (Invitrogen) was infused at a dose of 10 μg/mouse. Immediately after infusion of the antibody, a laser-induced injury was performed on the vessel wall of the cremasteric arterioles (9). The injuries were performed using a pulse-nitrogen dye laser applied through the micropoint laser system (Photonic Innovations). Fluorescence data were captured digitally for up to 10 ms per event for 300 frames. The amount of platelet accumulation in the developing thrombi was determined as the sum of all pixel values of the platelet-specific signal and expressed as relative fluorescent units, an arbitrary unit in which the integrated platelet fluorescence intensity is reflected.

Protein expression and purification, antibody production, and development of ELISAs. mFVIIa was purified, and polyclonal antibodies against purified mFVIIa were raised in rabbits as previously described (5). A fraction of the antibody was labeled with HRP according to the manufacturer’s instructions (Roche). Total mFVIIi/mFVIIia protein was measured by a sandwich ELISA using purified mFVIIi protein as a standard and the rabbit anti-mFVIIi capture and detecting (HRP-labeled) antibody. To measure the levels of mFVIIa in mouse plasma, we developed a second ELISA with minimal cross-reactivity to mFVIIi zymogen. Briefly, diluted murine plasmas were first incubated with an excess of a biotinylated active site probe to-phenylalaninyl-t-prolyl-t-arginine chloromethyl ketone (Haematologic Technologies) for 30 min at 4°C, after which the mixture was loaded onto a plate precoated with the rabbit anti-mFVII antibody and incubated at 4°C for 1 hour. The bound fraction of the biotinylated inhibitor was detected after a 30-min incubation at room temperature with HRP-strep-tavidin (BD Biosciences – Pharmingen). OD at 405 nm was measured, and protein concentrations were calculated against a standard curve constructed from serial dilutions of purified mFVIIa protein. As a third method, mFVIIa levels were extrapolated from a standard curve generated by spiking known amounts of purified mFVIIa into normal mouse plasma followed by clotting assay. PTs from diluted plasma samples were plotted, and mFVIIa levels were estimated against the standard curve. Levels of mFVIIa expression determined by PT standard curve and inhibitor-based ELISA were in excellent agreement (r > 0.93), whereas the total antigen ELISA reported higher total antigen as a result of endogenous mFVII present in mouse plasma samples or mFVIIa complexed with serpins. TF levels were assayed in cell lysates from pulmonary and cardiac C57BL/6 or SJL tissues by Western blotting using primary antibodies raised in 2 different species, i.e., goat anti-mTF (Santa Cruz Biotechnologies Inc.) and sheep anti-hTF (Haematologic Technologies) (43).

TAT and clotting assays. All reagents were prewarmed to 37°C before use. Mouse plasma for clotting assays was collected into one-tenth volume of 3.8% sodium citrate solution from the tail following a small snip. The first drop was discarded. PTs were measured by adding 50 μl citrated mouse plasma diluted 1:40 to 50 μl hFVII- or hFIX-deficient plasma (bioMérieux), and the time to clot formation was recorded with a fibrometer (BBB Fibro-system; Becton Dickinson) after adding 200 μl rHTF with calcium (Innovin; Dade Behring). aPTTs were measured by adding 50 μl undiluted citrated mouse plasma to 50 μl hFIX-deficient plasma (bioMérieux) and 50 μl aPTT reagent (bioMérieux). The mix was incubated at 37°C for 3 min before 50 μl of 25 mM CaCl2 was added, and time to clotting was recorded with a fibrometer. The TAT complex assay was performed on citrated mouse plasma using the Enzygnost TAT micro kit (Dade Behring) following the manufacturer’s protocol. Rotational thromboelastometry was performed using citrated whole blood, as previously described (44).

Histology and immunohistochemistry. Tissues obtained at necropsy were fixed in 10% formalin and paraffin embedded according to standard protocols. Tissues were sectioned and stained with H&E. In addition, immunohistochemistry was performed using fibrin/fibrinogen polyclonal antibody (Dako) following the manufacturer’s protocol.

Statistics. A 2-tailed Student’s t test was used for statistical analysis. Kaplan-Meier plots and analysis were performed using the JMP 6.0 software package (SAS Institute). Statistical analysis on the pathology findings was performed using Fisher’s exact test. A P value less than 0.05 was considered significant.

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