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GENE TARGETING IN HEMOSTASIS. FACTOR VII

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1. ABSTRACT

Factor VII (FVII), in concert with its natural cofactor and receptor, Tissue Factor (TF), initiates the process of blood coagulation following vascular injury. Mice completely deficient in FVII were generated *via* specific deletion of exons 2 to 8, thus deleting the entire coding region of the mature protein. In contrast to the early lethality observed in TF-deficient embryos (*TF*$^{-/-}$), embryos deficient in FVII (*FVII*$^{-/-}$) developed normally, without incidence of hemorrhage. However, *FVII*$^{-/-}$ neonates succumbed to either early intraabdominal or intracranial hemorrhage in later life.

2. FVII AND ITS ROLE IN COAGULATION

Blood coagulation, stimulated either through mechanical or pathological insult, is initiated and sustained by a sequence of specific proteolytic activations of plasma zymogens, ultimately resulting in fibrin formation. Specifically, TF, a sub-endothelial protein normally sequestered from blood, becomes exposed, thereby allowing its contact with the plasma protein, FVII. Binding of FVII to TF promotes the activation of TF-bound FVII to its active form (FVIIa). The FVIIa/TF complex catalyzes cleavage of Factor IX (FIX) and Factor X (FX) to activated Factor IX (FIXa) and activated FX (FXa), respectively. FXa then participates in "prothrombinase assembly" with activated FV (FVa), resulting in the conversion of prothrombin to thrombin, and ultimately, fibrin formation at the site of vascular injury. This FVIIa/TF-initiated cascade, also commonly referred to as the extrinsic pathway of blood coagulation, is believed to contribute significantly to the immediate initial requirement for thrombin generation. The cascade is quickly inhibited by the anticoagulant, tissue factor pathway inhibitor (TFPI), the anticoagulant function of which requires complex formation between itself, FVIIa, TF, and FXa.

3. PROTEIN BIOCHEMISTRY

FVII is initially synthesized in the liver in a precursor form, with a 38-residue leader sequence preceding the amino terminus of the mature protein. The first 20 amino acids, coding for the signal peptide, direct the nascent polypeptide to the rough endoplasmic reticulum (ER), translocation through the ER, and passage into the secretory pathway. The remaining 18 amino acids (propeptide) contributes to the control of Vitamin K-dependent gamma-carboxylation of specific glutamic acid residues in the N-terminus of the protein (1). Prior to secretion into the plasma, the subsequent maturation steps must occur: *viz.*, loss of the leader polypeptide, specific gamma-carboxylation of 10 glutamic acid residues *via* a vitamin K-dependent carboxylase, beta-hydroxylation at sequence position 63, and N-glycosylation at positions 145 and 322.

Following removal of the pre- and propeptide, the mature protein circulates as a single chain zymogen with a predicted molecular weight of approximately 45,500 for the human protein. The primary sequence of FVII displays strong homology to other vitamin K-dependent plasma proteins such as prothrombin, PC, FIX, and FX (2-5). Beginning at the amino-terminus, the protein is first comprised of a Gla domain, so named because of the 10

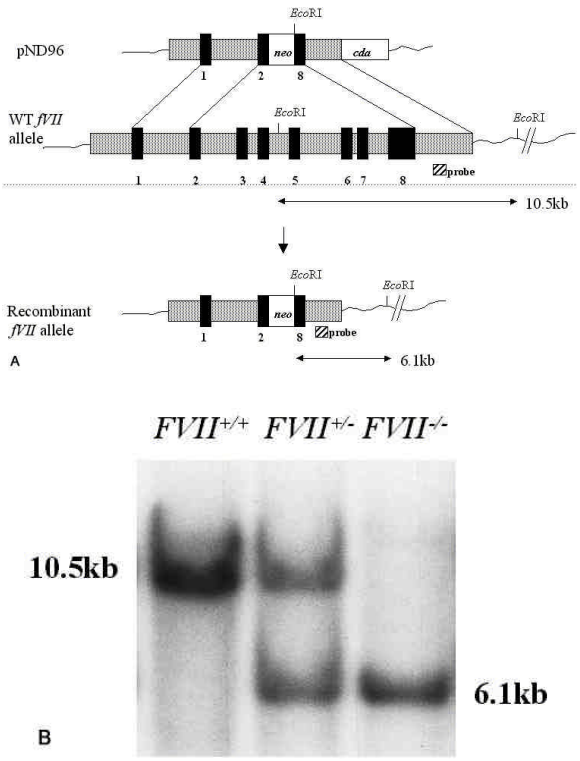

Figure 1. Targeting strategy for the disruption of the murine *FVII* gene. a) Following electroporation of the targeting vector, pND96, into RW4 embryonic stem cells, specific recombination at one of the *FVII* alleles would result in deletion of exons 2 to 8 (as indicated by the black bars) in the *FVII* gene. These exons would be replaced with the neomycin phosphotransferase gene cassette. Digestion of genomic DNA with *Eco*RI and hybridization with a genomic probe internal to the 3' flank resulted in the detection of a 10.5kb fragment for the *WT* allele, and a 6.1kb fragment for the mutated allele. b) Electrophoresis of *Eco*RI-digested genomic DNA and subsequent probing with a 3'-DNA fragment internal to the site of recombination resulted in the detection of a single10.5kb DNA band for the $FVII^{+/+}$, a 10.5 kb and a 6.1 kb band for the $FVII^{+/-}$, and a single 6.1 kb band for the $FVII^{-/-}$.

specific gamma-carboxylated glutamic acid residues located in this region. Full gamma-carboxylation of these residues is essential for Ca^{2+}-induced structural changes, which contribute to phospholipid binding and functional activity. The Gla domain is followed sequentially by two epidermal-like growth factor domains (EGF), an activation peptide region, and a catalytic domain containing the and His^{193}, Asp^{242}, and Ser^{344} catalytic triad residues common to all serine proteases. Once FVII is activated to FVIIa *via* cleavage between Arg^{152}-Ile^{153}, the resulting chains are connected by a single disulfide bond.

Both the cDNA and gene encoding FVII have been cloned and characterized from various species (6-12). A comparison of their translated sequences demonstrates some degree of homology. The activation cleavage site region, plus the downstream hexapeptide disulfide loop downstream, is highly conserved. The positions of the catalytic triad residues are conserved between human, mouse, bovine, and rabbit sequences. The human FVII gene is positively regulated primarily by a liver-specific transcription factor, hepatocyte nuclear factor-4 (HNF-4) (13). Indeed, mutation of the HNF-4 binding has resulted in FVII levels that were less than 1% of normal, resulting in a severe bleeding diathesis (14).

FVII is a zymogen in that it requires activation for manifestation of significant biological activity. Activation of FVII to FVIIa, by cleavage of the Arg^{152}-Ile^{153} bond, is Ca^{2+} and phospholipid-dependent, and requires interaction with TF. Thus, the role of TF is not limited to the localization of FVIIa activity at the site of vessel damage. Many regions of the FVII molecule have been implicated in the physical interaction with TF, including the Gla domain, the protease domain, Ca^{2+} binding sites outside of the Gla domain and the EGF domain. *In vitro*, FVII is most rapidly activated by FXa; however several other proteases, such as FIXa, FXIIa and thrombin can also activate FVII. FVII can also be subject to autoactivation by FVIIa, and this is dependent on the cytoplasmic domain of TF. The specific physiological protease, however, is not known. The resulting activated species has a half-life of approximately 2-3 hours, similar to its zymogen form.

The proteolytic activity of FVIIa towards its natural substrates, FIX and FX, increases by at least 100-fold compared to its inactive zymogen. FVIIa/TF activation of FIX involves the cleavage of the Arg^{145}-Ala^{146} and Arg^{180}-Val^{181} bonds, resulting in the release of the 10 kDa FIX activation peptide, and the generation of activated two-chain FIXa (45 kDa). FX is activated via cleavage of its Arg^{194}-$Ileu^{195}$ bond to produce a 34 kDa two-chain molecule. A short peptide (11 kDa) is also released during FX activation.

4. HUMAN FVII DEFICIENCY

Congenital FVII deficiency is a rare autosomal recessive disease affecting 1 in 500,000 with variable expression and high penetrance. The molecular/biochemical basis of the disease is usually genetically heterogeneous. The variant FVII molecules produced are altered in either their catalytic activity (FVII:C) (*via* defective zymogen activation, proteolytic activity, or affinity for TF) and/or their antigenicity (FVII:A) (such as those mutations which reduce transcriptional levels or enhance degradation). This heterogeneity has resulted in varying opinions regarding the clinical severity of the disease, but the general consensus is that bleeding can range from mild to severe. Bleeding is frequently of mucocutaneous type, but an entire array of hemophilic bleeding may also occur and interestingly, a hemorrhagic diathesis is not commonly reported in patients having a FVII:C level at >15% of normal. Only recently have patients with a complete FVII deficiency been identified (15, 16). Humans found to be significantly deficient in FVII (<10% functional activity) are highly susceptible to severe bleeding tendencies. In fact, central nervous system hemorrhage occurs in approximately 16% of FVII-deficient patients (17).

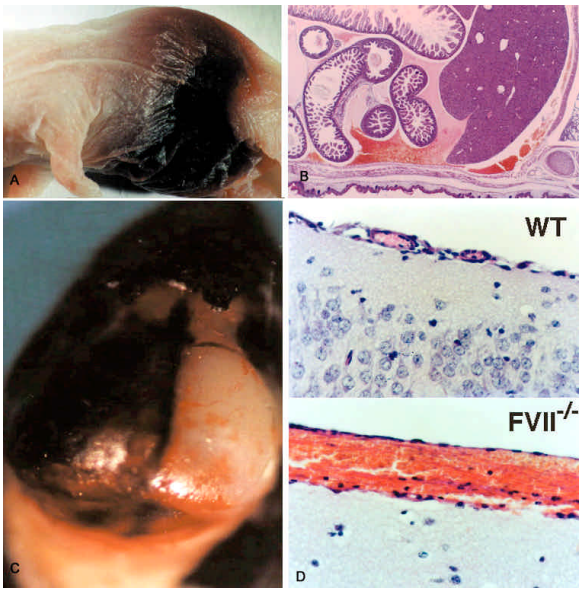

Figure 2. Fatal hemorrage in *FVII*$^{-/-}$ neonates. a) More than half of the *FVII*$^{-/-}$ neonates succumbed to intra-abdominal bleeding immediately following birth. b) By hematoxylin/eosin (H/E) staining of abdominal sections, widespread extravasation of red blood cells was observed, with concomitant anemia in the surrounding organs. c) Of the few *FVII*$^{-/-}$ surviving P0, the majority of the neonates sucumbed to intra-cranial hemorrhage, with the oldest surviving to 45 days. d) In comparison to a similarly aged *WT* brain section, unclotted blood was found subdurally (by H/E) between the arachnoid layer and the white matter (astertisk). "Reprinted by permission from Nature (390: 290-292) copyright (1997) Macmillan Magazines Ltd."

5. TARGETING OF THE *FVII* GENE IN MICE

The cloning and characterization of the *FVII* cDNA (8) and gene (9) from the murine 129SVJ strain facilitated the construction of the targeting vector, pND96 to be used to disrupt the *FVII* gene in mice (18), as shown in figure 1a. The vector was comprised of a neomycin phosphotransferase expression cassette (neo), flanked by a 5' 4.0 kb homologous segment and by a 3' 5.7 kb homologous segment, which extended from a site in the 3' untranslated region to a site external of the gene. A second gene encoding the bacterial cytosine deaminase enzyme (CDA) was cloned into the vector immediately following the 3' flank region. The replacement of one of the alleles *via* homologous recombination would result in a loss of exons 2 to 8 of the *FVII* gene and thus deletion of the entire coding region for the mature protein.

Following linearization of the targeting vector at the unique *Not*I site, DNA was electroporated into murine RW4 ES cells and selection was applied upon the addition of G418 (gentamicin) and 5'-fluorocytosine. Colonies of cells surviving both selection processes were individually cultured and replica plated, and genomic DNA from each of the colonies was assessed for recombination at one of the *FVII* loci. Genomic DNA was digested with *Eco*RI and probed with a 1.2 kb DNA fragment isolated from the 3' homologous region of pND96. Southern hybridization resulted in the detection of a 10.5 kb band for the wild-type *FVII* allele and a 6.1 kb band for the recombinant allele, as shown in figure 1b. Of 500 colonies screened, 17 were identified to be heterozygous for the *FVII* gene mutation. Individual colonies of cells were aggregated with C57Bl/6 blastocysts, and the resulting embryos implanted into pseudo-pregnant females. The resulting FVII chimeric mice, as assessed by a mosaicism in coat color, were bred with C57Bl/6 mice to generate *FVII*+/- animals. Mice heterozygous for FVII appeared healthy and bred normally. These mice were then crossbred to produce *FVII*-/- mice.

6. PHENOTYPIC CHARACTERIZATION OF *FVII*$^{-/-}$ NEONATES

Genotypic analysis of 59 litters (totaling 520 births) sired from *FVII*+/- x *FVII*+/- crosses demonstrated that *FVII*-/- animals were being born, although were under-represented in their Mendelian frequency (18.4% observed instead of the 25% expected). More than half of the *FVII*-/- neonates succumbed to intra-abdominal bleeding within the first 24 hours following birth, as shown in figure 2a. Abdominal blood remained uniformly unclotted. Microscopically, hematoxylin and eosin (H&E) staining demonstrated general organ anemia with ensuing widespread extravasation of red blood cells into the peritoneal cavity, as shown in figure 2b. The majority of the remaining *FVII*-/- neonates died of severe intra-cranial hemorrhage, as shown in figure 2c. This subdural blood was also uniformly unclotted. Rarely, other abnormal bleeding events were observed, *i.e.*, bleeding into the stomach, intestines, and around the aorta. However, no gross developmental malformations were evident. H&E staining of brains harvested from these *FVII*-/- neonates confirmed this layer of unclotted blood between the arachnoid layer and white matter, in figure 2d, which leaked out freely following removal of the dura. Although vessel development surrounding the immediate area of the bleeding anomalies could not be analyzed, anti-von Willebrand factor staining and anti-alpha-actin staining of the base of these brains demonstrated normal vessel development. To date, the oldest *FVII*-/- animal has survived to 45 days. To verify the lack of FVII expression, *FVII* mRNA transcript levels were assessed in neonates genotypically identified as *FVII*-/-. Using the murine *FVII* cDNA as a probe against total RNA levels from neonatal livers, *FVII* mRNA was detected in both *FVII*+/+ and *FVII*+/- total RNA. Based on band intensity, the level of *FVII* mRNA from *FVII*+/- neonatal liver was approximately half that of wild-type. No *FVII* mRNA transcript was detectable from the *FVII*-/- liver.

Table 1. Mendelian distribution of embryos sired from *FVII*$^{+/-}$ x *FVII*$^{+/-}$ crosses

AGE	Calculated totals	*FVII*$^{+/+}$	*FVII*$^{+/-}$	*FVII*$^{-/-}$
E18.5dpc	44	10(11)	34(22)	21(11)
E14.5dpc	44	13(11)	29(22)	15(11)
E11.5dpc	30	10(8)	19(15)	9(8)
E10.5dpc	16	4(4)	12(8)	6(4)
E9.5dpc	10	6(3)	3(5)	4(3)

Numbers in parantheses are the expected number of embryos at that age. Calculated totals were obtained by assuming that the observed number of *FVII*$^{+/+}$ and *FVII*$^{+/-}$ were normal survival numbers and represented 3/4 of all genotypes.

7. PHENOTYPIC CHARACTERIZATION OF *FVII*$^{-/-}$ EMBRYOS

To determine whether a percentage of *FVII*$^{-/-}$ embryos were lost due to an embryonic pathology, timed matings were established between *FVII*$^{+/-}$ x *FVII*$^{+/-}$ crossings, and embryos were harvested at various stages from E9.5dpc through birth (*ca.*, E18.5 dpc). Genotypic analysis of yolk sacs or embryonic tissues demonstrated that *FVII*$^{-/-}$ embryos were present at their expected Mendelian frequency (*ca.*, 25%) at each of the embryonic ages assessed, as shown in table 1. Thus, the under-representation of *FVII*$^{-/-}$ postnatally was likely due to the death and consumption of *FVII*$^{-/-}$ neonates by the parents, a phenomenon that has been observed in other gene knockout studies resulting in early neonatal death. In contrast to *TF*$^{-/-}$ embryos, *FVII*$^{-/-}$ embryos were alive and appeared normal and healthy, with normal vasculature in both the embryo and yolk sac, as evidenced by histological analysis. Measurement of FVII plasma proteolytic activity from embryos collected at various stages (E11.5 dpc, 14.5 dpc, 18.5 dpc) demonstrated that there was no detectable FVII activity present in plasma collected from *FVII*$^{-/-}$ embryos. In contrast, *FVII*$^{+/+}$ embryos demonstrated 39.2% + 14.6% of adult WT levels at E18.5 dpc, 2.2% + 1.1 % of adult WT levels at E14.5 dpc and 0.2% + 0.1% of adult WT levels at E11.5 dpc. Embryos heterozygous for FVII demonstrated activity levels of approximately 50% of that seen in the age-matched WT embryos (20.6% + 10.8% at E18.5 dpc, 1.0% + 0.6% at E14.5 dpc, 0.1% + 0.1% at E11.5 dpc).

Since the functioning of FVII and TF had been considered indistinct from one another, such differing phenotypes for the two knockout models seemed unlikely. Additionally, doubt was placed on the fact that FVIIa/TF proteolytic activity was not required for embryonic development. To address this issue, an attempt to mimic FVII deficiency using biochemical methods was undertaken. E8.5 dpc to E10.5 dpc WT embryos of varying backgrounds were cultured in the presence of the recombinant FVIIa-inhibitor, rNAPc2. In addition, embryos were injected intracardially with the inhibitor. Even at rNAPc2 concentrations 100- to 1000-fold higher than levels capable of inducing bleeding in neonatal mice, rNAPc2-treated embryos never developed the vascular abnormalities or bleeding phenomenon as observed in *TF*$^{-/-}$ embryos (19-21). The requirement for FVIIa/TF proteolytic activity and subsequent fibrin formation was also examined by injecting thrombin into E9.5 dpc embryos. At doses 100- to 1000-fold higher than levels capable of inducing lethal thrombosis in adult mice, fibrin deposition was never observed in embryonic or yolk sac vessels, areas in which fibrin localization would be expected if it was required during vascular growth.

A plausible argument for the survival and normal development of FVII-deficient embryos is the placental transfer of maternal FVII. Since humans, with as little as 1-5% FVII survive embryogenesis, the question arises of the threshold of FVII required for embryonic survival. Additionally, the ability to detect maternally-derived FVII is marred by the limit of detection of currently available assays. Several lines of argument, however, do not support the hypothesis of the involvement nor requirement for maternal FVII in hemostasis. First, supra-physiological levels of human r-FVII were intravenously injected into pregnant mice and r-FVIIa distribution was measured using a human FVII-specific antibody, which did not cross-react with murine FVII. By ELISA, no human FVII was detectable at E9.5 dpc. Beyond E10.5 dpc, human FVII was present at less than 0.1% of maternal r-FVIIa levels at supraphysiological plasma levels (5 micrograms/mL). Using an anti-human FVII antibody, immunoreactivity was only observed in maternal endothelial cells within decidual tissue and on synctiotrophoblasts at the maternal-fetal interface, but not within any embryonic tissue. Secondly, FVII maternal transport has also been investigated in pregnant rats using [125I]-r-FVIIa, and, again, no FVII transfer into the embryo was observed (22). The final and most direct argument against the involvement of maternal FVII in embryonic hemostasis stems from the development of embryos deficient in both FVII and TFPI (*FVII*$^{-/-}$ *TFPIKu1*$^{delta/delta}$) (23). In this study, the additional loss of FVII resulted in a complete amelioration of the coagulopathy observed in embryos functionally deficient in the anticoagulant, TFPI. Thus, if transfer of maternal FVII to the embryo does occur, these levels alone (without the contribution of FVII from the embryo) are insufficient to cause the coagulopathy in *TFPIKu1*$^{delta/delta}$ embryos.

8. POTENTIAL FUNCTIONS OF FVII/TF OUTSIDE OF HEMOSTASIS

The hemostatic role of the TF/FVIIa complex is relatively clear in that it initiates the sequence of events leading to coagulation. However, several lines of evidence have implicated its function beyond hemostasis. First, TF may play a hemostasis-independent role in blood vessel development (19, 20). Secondly, TF is structurally similar to the cytokine receptor superfamily and has been shown to induce cytosolic Ca^{2+} fluxes in various cultured cell lines following interaction with FVIIa (24). Third, FVIIa proteolytic activity and TF binding has been shown to activate the MAP kinase pathway (25) and upregulation of the transcription of the early growth response gene-1 (egr-1) (26). Interestingly, the cytosolic domain of TF is not required for this activation, nor does it involve the generation

of FXa. The two latter points suggest that the interaction and/or the proteolytic activity of FVIIa and TF may initiate a signal transducing mechanism, either independently or in concert with other receptors. Individuals on average possess approximately 1% of their total FVII antigen level as FVIIa (27). The means by which this small percentage of FVIIa exists in the absence of injury is not known. As well, whether it functions separately from hemostasis is also not known.

Interestingly, other *in vitro* substrates for the catalytic complex have also been identified. FVIIa/TF generates a form of FV with reduced specific activity, resistance to complete activation by thrombin and increased sensitivity to the anticoagulant, activated protein C (aPC) (28). FVIII can also be cleaved by FVIIa/TF to produce an unactivatable FVIII form (29). The *in vivo* physiological significance of these processes is not known.

In addition to the results observed in the mouse FVII-deficiency study, the recent identification of humans completely deficient in FVII provides additional evidence that FVII, although critical for hemostasis, is not required for normal embryonic development. A major caveat to this proposal is that embryos deficient in TF, the sole known receptor and cofactor for FVIIa, do not survive embryogenesis (19-21). At E8.5 dpc, $TF^{-/-}$ embryos are present at their expected Mendelian distribution, and they appear to be indistinguishable from their WT and $TF^{+/-}$ littermates. By E9.5 dpc, embryonic wasting is well evident, although appearing with variable onset and penetrance. Necrosis likely stems from ischemia secondary to deficient yolk sac circulation. Microscopically, $TF^{-/-}$ yolk sacs appeared to lack vitelline vessels but demonstrated enlarged capillaries. The entire yolk sac structure often had a "golf-ball"-like appearance. In addition, coalescence of vessels resulted in the appearance of "blood lakes", possibly due to a loss of contacts between endoderm and mesoderm layers of the yolk sac (19). This disturbance in yolk sac circulation resulted in secondary abnormalities, such as enlarged pericardia, developmental delay, overall anemia in the yolk sac and embryo, and aplasia. Although each of these separate TF deficiency state studies, using three distinct ES cell lines and targeting strategies, observed the same pathology, only one of the three groups afforded a possible mechanism for embryonic death. Between E8.5 dpc and E11.5 dpc, the blood islands appear to fuse to produce the visceral yolk sac vasculature, and the resulting challenge to its structural integrity is likely significant as blood begins to flow and intravasculature pressure increases. A possible decrease in mesenchymal cell population (as determined by decreased anti-alpha-actin immunostaining and electron microscopy), normally found surrounding vessels which likely provides structural support, was observed (19). Thus, in the reduction of such structural support, vessels may demonstrate increased fragility. How a lack of TF could contribute to decreased mesenchymal cell populations is still unknown. An alternative argument in understanding the early mortality of these TF-deficient embryos suggests simply that a lack of TF impedes fibrin deposition, which may be required as vessels grow (20). This explanation however, is not supported by the fact that fibrinogen-deficient mice survive embryogenesis and neonatal life (30, 31).

Normal development and survival of $FVII^{-/-}$ embryos suggests a new delineation of TF function independent of FVII proteolytic activity or binding. The contribution of TF to other processes is not surprising considering findings that TF binds extracellularly to other proteins *in vitro* besides FVII/FVIIa, such as plasminogen (Pg) (32). The binding site of Pg to TF is distinct from the binding site for FVII/FVIIa and is mediated through the lysine binding sites in kringles 1-3 of Pg. Although TF appears to upregulate Pg activation by urokinase-type plasminogen activator (uPA) in a dose-dependent and saturable manner, TF also inhibits Pg binding to endothelial cells, as well as inhibiting the fibrinolytic activity of uPA-activated Pg. These secondary effects may appear to be counterintuitive. However, TF binding and inhibition of uPA-activated Pg may be a means of inhibiting plasmin activity following vessel injury. TF binding may also localize Pg to the vessel surfaces to mediate its role in tissue remodeling.

More recently, attention has turned to the role of the cytoplasmic domain of TF, which has no apparent homology with any known sequence motifs. The presence of at least one serine residue in this region suggests that possible phosphorylation of this site could mediate a transcriptional signal (33). Indeed, it has been proposed that the cytoplasmic tail may contribute to processes such as metastasis (34) and the upregulation of other genes, such as vascular endothelial growth factor (VEGF) (35). In addition, the first intracellular TF ligand, actin-binding protein 280 (ABP-280) has been identified (36, 37). ABP-280, or filamin-1, is known to play a critical role in stabilizing the cortical actin cytoskeleton and in cell motility. It also regulates activation of stress-activated protein kinases in response to TNF-alpha and lysophosphatidic acid (38). Binding of ABP-280 to TF results in the reorganization of the actin cytoskeleton followed by phosphorylation of a central non-receptor tyrosine kinase, focal adhesion kinase (FAK). Serine to alanine substitution in the cytoplasmic tail of TF completely abolishes ABP-280 binding, while replacement of the extracellular domain of TF has no effect. These newly discovered signaling pathways may give insight into the role of TF in vasculogenesis, independent of FVII, during embryonic and possibly, tumor development.

9. HUMAN CLINICAL DATA AND THE SELECTION OF POTENTIAL *IN VIVO* MUTATIONS IN MICE

The first suggestions of a causal relationship between FVII levels and risk of cardiovascular disease stemmed from the findings in the Northwick Park Heart study which demonstrated that elevated levels of FVII were related to fatal myocardial infarctions (MI) (39). Since then, there have been studies which both support (PROCAM) (40, 41) and conflict with (Edinburgh Artery Study) (42), the notion that FVII (higher or lower levels) is a risk factor in coronary disease. Because of such controversy, attention has focused on particular polymorphisms, for example the $Arg^{353}Gln$ mutation in exon 8, which codes for the catalytic region of the protein, and the A2 mutation in the FVII promoter. These mutations independently result in lowered FVII/FVIIa levels. Interestingly, the frequencies of the hetero- or homozygous alleles for either of these point mutations appear higher in populations who have not had myocardial infarcts as

compared to those who have. *In vivo* murine studies of these kinds of mutations may lead to a further understanding of the role of FVII in cardiovascular disease.

10. PERSPECTIVE

In contrast to murine TF deficiency, a FVII deficiency results in normal embryonic development in mice, with no adverse hemostatic effects of the loss of the procoagulant protein. However, the majority of *FVII*$^{-/-}$ neonates succumbed to intraabdominal bleeding within the first days following birth. The remaining percentage of animals die of intracranial hemorrhage later in life, with the oldest *FVII*$^{-/-}$ animal surviving to 45 days.

11. ACKNOWLEDGMENTS

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