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GENE TARGETING IN HEMOSTASIS. FACTOR X

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TABLE OF CONTENTS

1. ABSTRACT

Blood coagulation Factor X (FX) is a vitamin K-dependent serine protease that plays a central role in blood clotting. Additionally, FX may exert other functions beyond coagulation but the precise role of this protein in these processes and the *in vivo* relevance remains to be further delineated. The development of gene knockout technology has allowed for a direct means to determine the physiological relevance of various proteins in a number of normal and pathological processes. Mice with a total deficiency of FX have been generated by targeted deletion of all exons encoding the mature FX protein. The genotypic distribution indicated that homozygous deficiency results in partial embryonic lethality at embryonic day (E) 11.5-12.5 with signs of massive bleeding but no histologically evident defects in the vasculature of these embryos or their yolk sac. The majority of those that survive to term die within 5 days, most frequently from intraabdominal bleeding. The remainder die between postnatal day (P)5 and P20 with intraabdominal, subcutaneous, or intracranial bleeding or a combination thereof. These observations underline the importance of FX function in embryonic and postnatal survival and confirm that these mice serve as effective models of the bleeding disorders observed in human FX deficiency. While the early *FX*$^{-/-}$ lethality impedes investigation of potential morphogenetic functions of FX *in vivo*, such studies may however become feasible through the availability of mice expressing mutant FX proteins, *e.g.*, EPR-1 binding site mutants, or of mice with conditional FX deficiency.

2. INTRODUCTION

Factor X (FX) was first identified in the 1950s as an essential coagulation component following the analysis of plasma from two patients suffering hereditary hemorrhagic disorders. Since these samples complemented plasmas with other already identified coagulation deficiencies, they defined a new coagulation component, the Stuart-Prower Factor, named after the index patients (1). Since then, the FX protein and gene have been isolated and characterized along with those of most other coagulation components. Analysis of the biochemical properties of these hemostatic factors and their interactions has led to the development of complex models of the coagulation system in which FX is a central component in a network of proteolytic reactions (2,3).

Hereditary FX deficiency is a rare autosomal recessive disorder occurring in many ethnic and racial groups with a frequency of approximately 1 in 500,000 individuals. About one-third of the patients have both decreased FX activity and antigen level, the others have low FX activity but normal antigen levels. The severity of the disorder parallels the degree of deficiency. Patients having greater than 15% of normal FX activity rarely suffer spontaneous hemorrhagic episodes although they may experience bleeding after surgery or trauma. In contrast, individuals with less than 1% normal FX activity are likely to experience severe spontaneous bleeding mainly in soft tissues.

3. BIOCHEMISTRY

3.1. Structure of the FX protein.

Factor X is a vitamin K-dependent serine protease whose protein and gene structure (4,5) are similar to that of coagulation Factor VII (FVII) (6,7), Factor IX (FIX) (8), and Protein C (PC) (9), suggesting that these genes arose by duplication of a common precursor. FX is

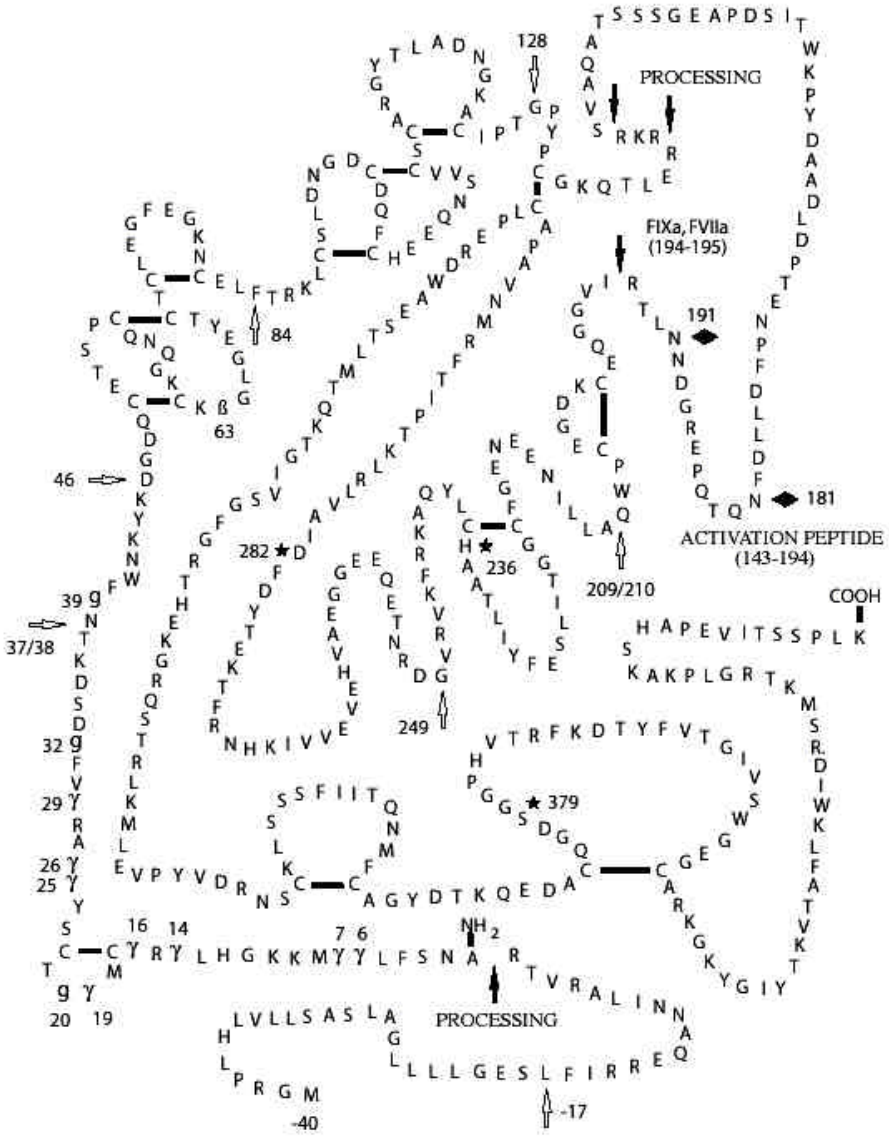

Figure 1. Structure of the human FX protein. The amino acid sequence is shown beginning with the signal sequence. The clear arrows represent the positions of introns in the gene. The positions of N-linked carbohydrate attachments are indicated by the filled diamonds. The asterisks illustrate the positions of the active site serine protease catalytic triad. The filled arrows represent positions of peptide bond cleavage in processing reactions or in conversion of FX zymogen to FXa. The latter step is catalyzed by either FIXa or FVIIa. γ=γ-carboxyglutamate.

synthesized primarily in the liver as a single chain precursor (10). In humans, the 488-amino acid single-chain FX undergoes several post-translational modifications (Figure 1). These steps include removal of the 23-amino acid signal peptide (amino acids -40 to –18; numbering starts with Ala^{1} at the amino terminus of the light chain of the mature zymogen found circulating in plasma) after translocation of the single chain precursor into the endoplasmic reticulum (11). The remaining propeptide (-17 to -1) contains a recognition site for the vitamin K-dependent γ-carboxylase which binds to the propeptide and carboxylates the first 11 glutamic acid residues in the adjacent Gla-domain (12,13). Following carboxylation, the 17-amino acid propeptide is cleaved exposing the amino terminal alanine found in the light chain of the mature protein (14,15).

Following removal of the signal peptide and propeptide maturation continues with excision of the tripeptide, Arg^{140}-Lys-Arg^{142}. These proteolytic modifications generate a two-chain zymogen consisting of a 139-amino acid light-chain linked to a 306-amino acid heavy-chain by a single disulfide bond between Cys^{132} and Cys^{302} (16-18). The light-chain includes the amino-terminal γ-carboxyglutamic acid (Gla) domain containing all 11 Gla residues (19). A short helical stack is present

downstream of the Gla domain, followed by two consecutive epidermal growth factor domains (EGF1 and EGF2) (20). The amino-terminus of the heavy-chain includes a 52-amino acid activation peptide, followed by the protease domain which contains the requisite active site triad of His^{236}, Asp^{282}, and Ser^{379} typical of serine proteases (21).

Other post-translational modifications include ß-hydroxylation of Asp^{63} in the EGF-1 domain (22) and glycosylation of 4 sites within the activation peptide (23,24). These include O-glycosidic linkages to Thr^{159} and Thr^{171}, and N-glycosidic linkages to Asn^{181} and Asn^{191} which appear to be necessary for efficient conversion of the zymogen FX to the activated form, FXa.

Maturation of FX also involves the formation of 11 disulfide bonds including 3 in the EGF-1 domain (Cys^{50}-Cys^{61}, Cys^{55}-Cys^{70}, and Cys^{72}-Cys^{81}), 3 in the EGF-2 domain (Cys^{89}-Cys^{100}, Cys^{96}-Cys^{109}, and Cys^{111}-Cys^{124}), and 4 in the catalytic domain (Cys^{201}-Cys^{206}, Cys^{221}-Cys^{237}, Cys^{350}-Cys^{364}, an Cys^{375}- Cys^{403}). In addition, following excision of the Arg^{140}-Lys-Arg^{142} tripeptide, the disulfide bond between Cys^{132} and Cys^{302} provides covalent linkage between the resulting 139 amino acid light chain and the 306 amino acid heavy chain that comprise the mature FX zymogen purified from plasma.

3.2. Structure of the FX gene

The gene encoding human FX is located on chromosome 13, closely linked to the *FVII* gene (25-27). The *FX* gene is 18 kb in length and is organized in 8 exons (4): exon 1 encodes the signal peptide; exon 2 encodes the propeptide and Gla domain; exon 3 encodes the aromatic amino acid stack domain; exons 4 and 5 each code for the EGF-like regions; exon 6 encodes the activation domain, and exons 7 and 8 encode the catalytic domain. The relationship of the exon-intron organization of the *FX* gene with the domain structure of the protein (5), as well as its linkage to the *FVII* gene, is identical in human and mouse (28).

3.3. Activation of FX

During the initiation phase of blood coagulation, the zymogen, FX, is converted to its active form FXa by FVIIa bound to its cellular receptor, Tissue Factor (TF) (29). This initial burst of FX activation is limited by binding of Tissue Factor Pathway Inhibitor (TFPI) to FXa (30, 31) which then forms a quaternary complex with FVII and TF. During the propagation phase of blood coagulation, FX activation is sustained by FIXa in association with FVIIIa, acidic membrane phospholipid, and Ca^{2+}. Both TF/FVIIa or FVIIIa/FIXa activate FX by cleavage of the Arg^{194}-Ile^{195} bond, with release of a 52-amino acid activation peptide from the heavy-chain (32).

Zymogen FX can also be activated by non-hemostatic factors *in vitro*. FX has been shown to bind to the integrin Mac1 found on leukocytes (33). FX or fibrinogen bound to Mac-1 on monocytes leads to the release of cathepsin G-containing granules. Cathepsin G converts FX to its active form by cleavage of the Leu^{177}-Leu^{178} bond in the activation domain (34). While the binding and activation of FX bound to Mac-1 on monocytes provides a mechanism for activation of the coagulation system during an inflammatory response, the physiological significance of these interactions needs to be determined.

An additional FX-activating activity has been identified on certain tumors. Activation of the coagulation system and accumulation of thrombin catalyzed crosslinked fibrin are associated with the pathophysiology of cancer. While the most common procoagulant activity expressed on tumors is TF, a novel cysteine protease named cancer procoagulant (CP) has been identified on some tumors that activates FX (35,36). The primary cleavage site for CP in the activation peptide is Tyr^{163}-Asp^{164} (37), unlike that of FVII, FIX or cathepsin G. Interestingly, the only non-malignant tissue found to express CP are embryonic amnion and chorionic membranes (38). The significance of CP-mediated FX activation during embryonic development remains to be determined.

Since it is important to limit coagulant reactions to protect against the development of prothrombotic states, there are multiple mechanisms to inhibit FXa activity (39,40). Antithrombin III (ATIII) is a serpin that inhibits several coagulation proteases including thrombin, FIXa, FXa and FVIIa (when complexed with TF). Heparin enhances ATIII inhibitory activity 1000-fold by binding both ATIII and the serine protease thus increasing the probability of protease-serpin interaction (41). Additionally, heparin induces ATIII conformational changes that facilitate ATIII-protease complex formation (42,43). The importance of ATIII in regulating the coagulation system is underscored by the severe prothrombotic conditions suffered by patients with ATIII deficiency (44-46). Additionally, ATIII deficient mice exhibit severe embryonic prothrombotic pathologies (47). Since ATIII down-regulates several coagulation proteases, it is not possible to determine the relative importance of the ATIII-FXa interaction, specifically.

Protein Z (PZ) is a *gamma*-carboxylated plasma protein that is structurally similar to FVII, FIX, FX, and PC. However, it is missing the canonical sequence of serine proteases (48,49) and although the protein was first identified in 1977 (50), its function has remained elusive (51). Recently, it has been established that PZ, a 62 kDa glycoprotein, promotes inhibition of FXa (52). In the presence of Ca^{2+}, PZ promotes the formation of a tertiary complex that also includes FXa, phospholipids and the PZ-dependent protease inhibitor (ZPI) (52-54). Thus, PZ appears to inhibit thrombin generation by facilitating the interaction of FXa with ZPI, a 72 kDa member of the serpin family. Although, PZ-deficient mice do not appear to have spontaneous hemostatic phenotypes, PZ-deficiency does increase the severity of the thrombotic phenotype of mice carrying the FV-Leiden mutation (55). These results suggest that PZ plays a physiologically important role in shifting the hemostatic balance away from a prothrombotic state.

TFPI is a critical inhibitor of FVIIa/TF initiated coagulation(2). TFPI contains three tandem repeats that are structurally homologous to Kunitz domains typical of basic protease inhibitors (31,56). FXa binds to the second Kunitz unit while FVIIa interacts with the first domain. TFPI acts by first binding and inhibiting FXa in plasma (30,57). The TFPI/FXa complex then forms a tertiary structure with FVIIa/TF on phospholipid surfaces inhibiting both FXa and FVIIa/TF coagulant activity. Since FXa is required for TFPI activity, this mechanism of action permits a small burst of FXa (and thrombin) to be generated before coagulation initiation is inhibited. Once the FVIIa/TF pathway is shut down, FXa and thrombin generation are maintained by positive feedback loops involving thrombin activation of FV, FVIII, and FXI (and subsequently FIX) (58). The physiological importance of TFPI is demonstrated by the embryonic lethality of TFPI deficient mice (59). Interestingly, TFPI null mice are rescued by inactivation of the FVII gene suggesting that the critical role of TFPI is to inhibit the extrinsic pathway of coagulation initiation (60).

In vitro studies indicated that FXa is also inhibited by other major plasma protease inhibitors. These include α_1-antitrypsin, and α_2-macroglobulin. In addition, plasmin converts FXa from a procoagulant to a profibrinolytic factor (61). Plasmin catalyzes limited cleavage of FXa and eliminates its prothrombinase activity. Interestingly, the large plasmin-generated FX fragment interacts with tissue-type plasminogen activator and increases its rate of activation of plasminogen to plasmin. It remains to be determined if this activity is physiologically significant.

3.4. Activities of FX

3.4.1. Hemostasis

Blood coagulation FX plays a central role in blood clotting. When assembled on membrane surfaces in the presence of Ca^{2+}, FXa, in association with FVa, forms the enzyme component of the prothrombinase complex that catalyzes conversion of prothrombin to thrombin (62). This latter enzyme promotes thrombus formation by activating platelets and converting fibrinogen to fibrin, the major matrix component of a blood clot (for review, see reference (21)). In addition, thrombin triggers positive and negative feedback loops in the coagulation network. Thrombin activates FV, FVIII, and FXI necessary to maintain further thrombin activation after the FVII-TF coagulation initiation reactions are inhibited. In addition, thrombin binding to thrombomodulin on endothelial cells leads to PC activation which serves to inhibit and limit the coagulation reactions by inactivating FVa and FVIIIa. Thrombin also inhibits fibrinolytic activity by activating TAFI (63). Furthermore, thrombin interacts with G protein-coupled protease activated receptors (PAR1, PAR3 and PAR4) found on a variety of cells including platelets, fibroblasts, leukocytes and endothelial cells (64).

3.4.2. Non-hemostatic functions

FX may exert additional functions beyond coagulation. It stimulates proliferation of smooth muscle cells (SMCs) *in vitro* and *in vivo* and after balloon angioplasty in rabbits (65-67), possibly via the effector cell protease receptor-1 (EPR-1). Anti-EPR-1 antibodies as well as DX9065, a direct inhibitor of FXa, inhibited neointimal SMC proliferation in a rabbit arterial injury model (67). Furthermore, FXa stimulates an acute inflammatory response *in vivo*, presumably via EPR-1 (68). In addition, FXa has been shown to induce cytokine production and expression of adhesion molecules by human umbilical vein endothelial cells. However, the role of EPR-1 in the mechanism of FXa-mediated cell activation remains controversial (69). While one group reports that endothelial cell activation is mediated via an interaction among FXa, EPR-1 and PAR2 (70), others report that FXa activation of endothelial cells involves a pathway independent of EPR-1 (71). The precise role of FXa in these morphogenetic processes and the *in vivo* relevance remain to be further elucidated.

4. FACTOR X-DEFICIENT MICE

4.1. Construction of the FX gene deletion

To determine which *in vitro* FX activities have physiological significance, FX-deficient mice were generated by targeted gene disruption following homologous recombination in embryonic stem cells (72). The targeting vector contained a 4.0 kb FX 5' flanking region and extended from an XhoI site in intron 1 to the 3 end of the FVII gene (which is adjacent to the FX gene in a head-to-tail arrangement in mice as well as humans) (Figure 2). The FX 3' flank extended from an EcoRI site, located 1 kb 3' of the FX stop codon, to an ApaI site 7 kb further downstream. A neo^r cassette used for positive selection was cloned between the 5' and 3' flanking regions. A cytosine deaminase (cda) (73) cassette cloned downstream of the 3' FX flanking region was used for negative selection against random integrants. After electroporation of linearized targeting vector DNA into ES cells, homologous recombination between the 5' and 3' flanking regions of the targeting vector and murine chromosome led to the replacement of the entire coding sequence for the mature FX protein (exon 2 through exon 8) with the neo^r cassette.

Correctly targeted clones were aggregated with Swiss morula stage embryos yielding several chimeric animals. Three male chimeras originating from the same targeted clone transmitted the inactivated *FX* gene to their offspring which was detected by Southern blot analysis of tail-tip DNA. Intercrossing of these heterozygous FX-deficient *($FX^{+/-}$)* mice generated homozygous FX-deficient (*$FX^{-/-}$*) progeny for phenotypic analysis. As expected, *FX* RNA and FX antigen were undetectable in *$FX^{-/-}$* null offspring by RT-PCR and immunostaining of liver sections, respectively.

4.2. Viability of FX-deficient neonates

Homozygous FX-deficient animals were underrepresented among the offspring of heterozygous breeding pairs. Analysis of litters monitored continuously from birth revealed that only 15% of the neonates were *$FX^{-/-}$* (Table 1). Shortly after birth, dead *$FX^{-/-}$* pups were sometimes found

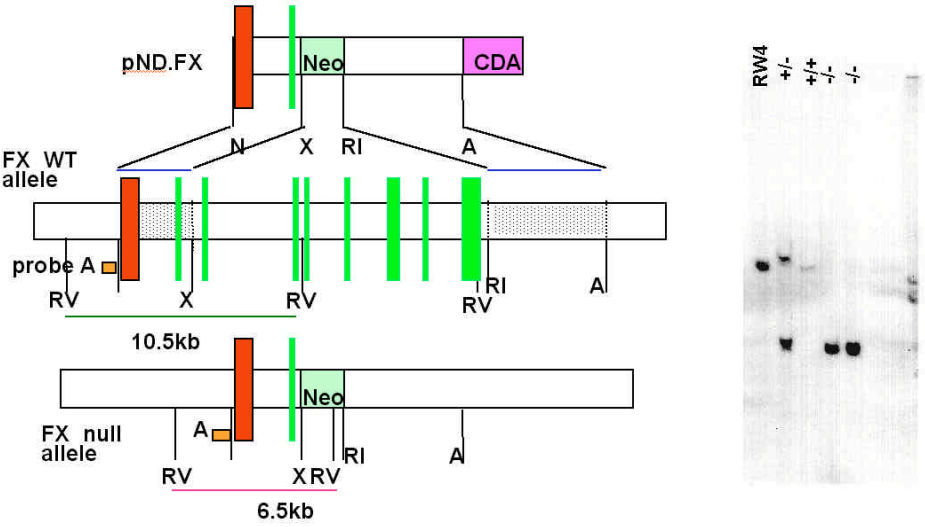

Figure 2. Strategy for targeted inactivation of the murine *FX* gene by homologous recombination in embryonic stem cells. **A**; The targeting vector p*PNC.FX*, the wild type *FX* allele and the targeted allele are schematically represented. The targeting vector contains the *neomycin resistance* gene (*neo*) and the *cytosine deaminase* (*CDA*) gene to allow for positive/negative selection of homologous recombination events. Transcription of the selection markers was in the same direction as the endogenous *FX* gene. Green boxes in the genomic structures represent exon sequences of FX; the red box represents exon 8 of FVII. Upon homologous recombination, the *neo* gene replaces a 18-kb genomic fragment comprising the sequences encoding the entire mature FX protein (exons 2-8). The expected restriction fragments of the wild type and the mutant allele are indicated with their relative size by underlining. Orange boxes under the genes represent the probes used for Southern blot analysis. Probe A is 0.4-kb PCR-generated fragment external to the targeting vector. **B**; Southern blot analysis of genomic DNA from tail tips of E17.5 embryos. DNA was digested with EcoRV and hybridized to the 5' flanking probe A giving a 10.5-kb band and a 6.5-kb band for the wild type and targeted allele, respectively.

partly consumed by their mother. Missing pups were therefore assumed to be $FX^{-/-}$ (Table 1). The $FX^{-/-}$ neonates developed fatal bleeding events, approximately 50% of them dying within the first day after birth (Figure 3), most frequently from intraabdominal bleeding (Figure 4A-C). The majority of the remaining $FX^{-/-}$ neonates died before postnatal day P5 and a approximately 10% died between P5 and P19 (Figure 3), showing intraabdominal, subcutaneous (Figure 4) or massive intracranial bleeding or a combination thereof. Microscopic analysis confirmed widespread blood in the peritoneal cavity (Figure 4B) or beneath the dermis (Figure 4E). Morphological analysis of H&E stained tissue sections (heart, lung, kidney, liver, brain) and immunostaining for vascular endothelial cells (vWF staining) or smooth muscle cells (α-actin staining) did not reveal abnormalities in organ or blood vessel development in the $FX^{-/-}$ neonates (not shown).

4.3. Viability and survival of FX-deficient embryos

To establish whether the partial loss of $FX^{-/-}$ animals occurred at or during birth or earlier during gestation, timed matings were set up to generate embryos at various developmental stages (Table 1). At E9.5, $FX^{-/-}$ embryos were present at the expected ratio (Table 1). They were macroscopically indistinguishable from their littermates**,** and the embryos (Figure 5A,B,) as well as their yolk sacs (not shown), displayed a normal vasculature. At E12.5, however, the percentage of non-resorbed $FX^{-/-}$ embryos was only 17% (different from expected Mendelian ratio: $p < 0.005$ by Chi-square analysis) (Table 1). At E9.5, few resorbtions were observed which were evenly distributed over the 3 genotypes (1 each for $FX^{+/+}$, $FX^{+/-}$, and $FX^{-/-}$). In contrast, 75% of the resorbtions at E11.5 (6 of 8) and 84% of the resorbtions at E12.5 (6 of 7) were $FX^{-/-}$. The degree of resorbtion at E11.5 and E12.5 varied from a totally disintegrated embryo within the remains of the yolk sac to an approximately normal-sized but white and avascular embryo (not shown). The non-resorbed $FX^{-/-}$ embryos grossly resembled their wild-type littermates (Figure 5). However, some embryos (1 at E11.5 and 1 at E12.5) showed overt signs of bleeding (Figure 5C,F), one of which had massive blood accumulated in the brain ventricles and along the central canal (Figure 5H,I). No evidence of brain or other tissue degradation was however observed. Histological analysis of yolk sac (Figure 5L,M) and placenta (not shown) of these embryos and immunostaining of the embryos for fibrin (not shown) did not reveal differences with the wild-type littermates.

Thus, there was the expected Mendelian frequency of live $FX^{-/-}$ embryos at E9.5 and E11.5. In contrast, beginning at E12.5, viable $FX^{-/-}$ embryos were recovered at a frequency of only 16-17%. Therefore, embryonic FX deficiency leads to partial embryonic lethality between E11.5 and E12.5.

The primary mechanism underlying the developmental block remains however unresolved. The

Table 1. Genotype distribution among the offspring of $FX^{+/-}$ breeding pairs

	+/+	+/-	-/-	Missing or no DNA	Total
Neonates *	77 (25%)	182 (60%)	40 (15%)	6	305
E9.5	30 (22%)	65 (47%)	35 (25%)	8 (6%)	138
E11.5	22 (19%)	67 (58%)	26 (23%)	0	115
E12.5 *	37 (32%)	59 (51%)	18 (17%)	1 (1%)	115
E14.5 *	51 (31%)	89 (54%)	26 (16%)	0	166
E17.5 *	82 (26%)	173 (55%)	50 (16%)	12 (3%)	317

Data represent the number of neonates or of non-resorbed embryos with the percentage of total in parentheses. (*) Difference from expected Mendelian distribution statistically significant ($p<0.005$) by Chi-square analysis.

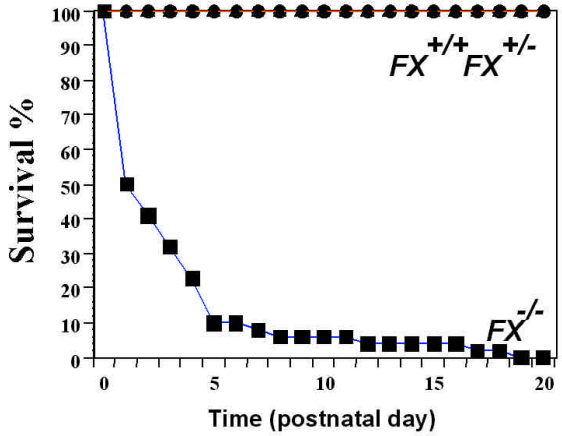

Figure 3. Survival of $FX^{+/+}$, $FX^{+/-}$ and $FX^{-/-}$ littermates from $FX^{+/-}$ intercrosses after birth.

non-resorbed $FX^{-/-}$ embryos grossly resembled their wild-type littermates. However, one E11.5 and one E12.5 $FX^{-/-}$ embryo showed signs of bleeding. No evidence of brain or other tissue degradation was observed. Histological analysis of yolk sac and placenta of these embryos did not reveal differences with their wild-type littermates. However, potential vascular defects occurring fulminantly between E11.5 and E12.5 cannot be excluded, as degeneration and necrosis of the vast majority of recovered, resorbed-$FX^{-/-}$ embryos prevented histological analysis of the vasculature and of organ structure. The proportion of $FX^{-/-}$ embryos remained constant (16%) beyond E12.5 (Table 1) and they were macroscopically and microscopically indistinguishable from their wild-type littermates.

Coagulant activity measurements in plasma from E17.5 embryos confirmed the absence of FX in the $FX^{-/-}$ embryos. FX levels were gene-dosage dependent with $FX^{+/-}$ embryos expressing approximately one-half the FX activity of their wild-type littermates. Surprisingly, $FX^{-/-}$, and to a lesser extent $FX^{+/-}$, embryos also displayed somewhat decreased levels of FVII, FIX, FXI and FXII, which seemed, however, not associated with decreased message levels, as verified by semi-quantitative RT-PCR, and which returned to normal levels in adult $FX^{+/-}$ heterozygotes. It is unlikely that reduced FVII, FIX, or FXI activity is responsible for the partial embryonic lethality associated with FX deficiency since total deficiencies in these factors do not affect embryonic development (74-77). However, it is conceivable that FX affects the expression of some yet undetermined factor involved in embryonic development. At this stage, there are no explanations for these very complex findings.

5. PERSPECTIVE

Interestingly, deficiencies of several other coagulation factors functionally related to FX result in at least partial embryonic lethality. TF deficiency results in defective yolk sac vessel development and vitello-embryonic circulation at E8.5, leading to death of the embryos at E10.5 (78-80). This suggests a role of TF beyond its key function in coagulation, which appears to be independent from its ligand, FVII, as suggested by the finding that FVII deficiency does not result in embryonic lethality (74). However, the absence of a developmental block in $FVII^{-/-}$ embryos might result from maternal FVII transfer below current limits of detection that could be sufficient to initiate TF/FVIIa function (74), independent of the role of this complex in hemostasis (60). Approximately 50% of embryos deficient in FV (81) die at mid-gestation with bleeding and vascular abnormalities in the yolk sac, while those embryos surviving the developmental arrest, continue to term but suffer fatal postnatal hemorrhage. Prothrombin-deficient embryos suffer a similar fate with one-half to three-fourths of the embryos succumbing before E10.5, possibly some loss of these embryos during late gestation, and fatal neonatal bleeding among the embryos that survive to birth (82, 83). While it has been reported that prothrombin deficiency results in abnormalities in the yolk sac vasculature at mid-gestation (83), another group did not observe such vascular defects (82). The more severe embryonic lethality of TF deficiency as compared to that of the other factors further suggests additional functions of TF beyond its classical involvement in the coagulation cascade.

The similarities among the embryonic phenotypes of FV-, FX-, and prothrombin-deficient mice suggest that FV/FX mediated activation of prothrombin provides a critical embryonic function at mid-gestation. However, it seems unlikely that this essential function only involves clot formation, since fibrinogen-deficient embryos (84) as well as NF-E2-deficient embryos that produce few platelets (85) develop normally. Furthermore, deficiency in the anticoagulant component, thrombomodulin (Tm), a cell-surface cofactor of thrombin involved in activation of

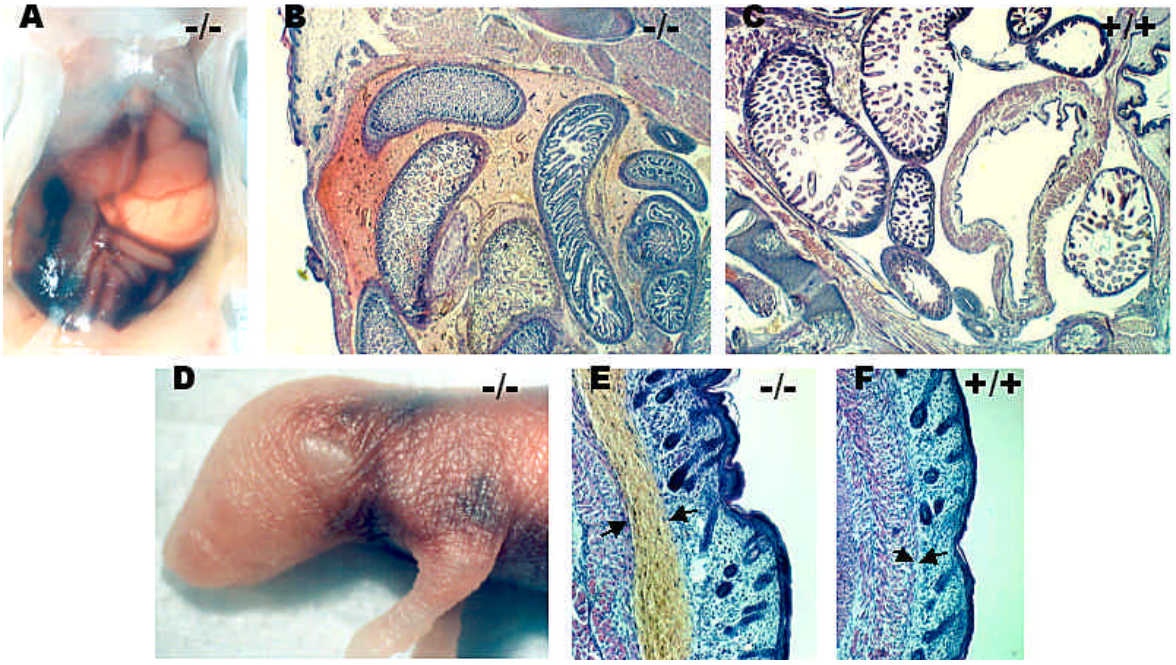

Figure 4. Postnatal bleeding in *FX*$^{-/-}$ neonates. **A-F**; Intracranial (A), intraabdominal (B) or subcutaneous (D,E) bleeding in two separate 1-day old *FX*$^{-/-}$ animals compared to their healthy *FX*$^{+/+}$ littermates (C,F).

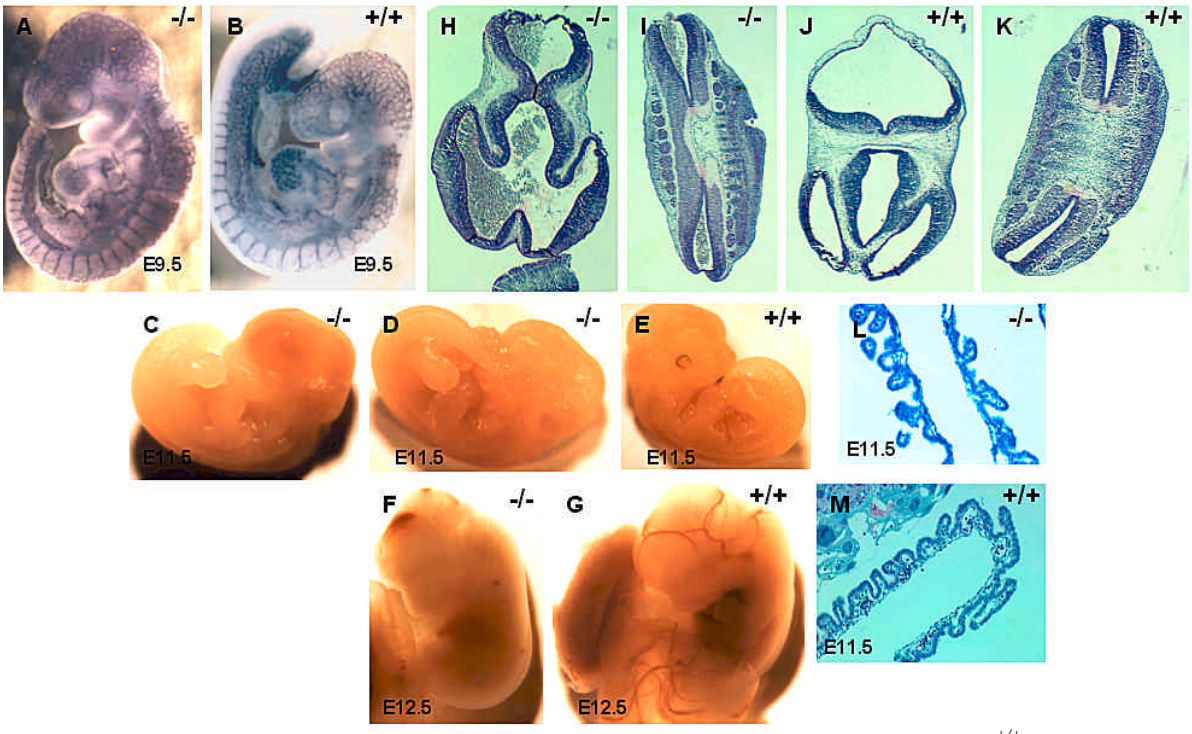

Figure 5. Embryonal development of *FX* targeted mice. **A,B**; PECAM staining of E9.5 *FX*$^{-/-}$ and *FX*$^{+/+}$ embryos, revealing normal vascular development. **C-G**; Whole embryos at E11.5 (C-E) or E12.5 (F,G) with normal gross morphology but indicating the occurrence of bleeding events in some of the *FX*$^{-/-}$ embryos (C,F). **H-K**; H&E stained sections of the *FX*$^{-/-}$ embryo shown in panel C (H,I) and of the *FX*$^{+/+}$ embryo shown in panel E (J,K) indicating massive blood collection in the brain ventricles (H) and central canal (I) of the *FX*$^{-/-}$ embryo. **L,M**; H&E stained sections of the yolk sac of the *FX*$^{-/-}$ embryo shown in panel C (L) and of the *FX*$^{+/+}$ embryo shown in panel E (M), indicating normal yolk sac vascular structures.

PC or TAFI, causes a total embryonic arrest at E8. 5 (86). The earlier and more severe consequences of Tm deficiency suggest that it is involved in a critical thrombin-independent process. Alternatively, maternal transfer of thrombin may satisfy early Tm-mediated requirements for thrombin in FV-, FX-, and prothrombin-deficient embryos.

In contrast, while approximately 50% of embryos lacking the thrombin receptor, PAR-1, die at E9.5 to E10.5 (87), surviving PAR-1-deficient embryos display a transient growth retardation, but are born and develop into fertile adults with no apparent hemostatic defect. PAR-1 is a G-coupled receptor found on platelets, fibroblasts and a variety of other cells, suggesting perhaps that FV/FX activation of prothrombin leading to thrombin-mediated intracellular signaling by PAR-1 could be involved in a critical developmental process.

While FV, FX, FII, and PAR-1 deficiencies all result in partial mid-gestational embryonic lethality, the specific time at which this occurs is not identical. FX-based embryonic lethality occurs between E11.5 and E12.5. In contrast, prothrombin and PAR-1 deficiencies result in developmental arrest between E9.0 and E11.5. While these differences might reflect strain variations, animal husbandry issues, and/or different criteria for classifying developmental phenotypes, it is also possible that these deficiencies may affect separate processes during embryonic development. For instance, in addition to its role in activating prothrombin, FXa has been shown to promote proliferation of smooth muscle cells, and to mediate intracellular signaling of leukocytes and activation of endothelial cells (8-10,12). Therefore, the essential role of FX in development may be independent of its function in the prothrombinase complex.

The cause of the partial embryonic lethality of FX deficiency between E11.5 and E12.5 remains enigmatic. If FX or FXa performs a critical developmental function, then total FX deficiency should result in 100% lethality. Conceivably there might be some maternal transfer of FX to the embryo providing sufficient FX to rescue some embryos *in utero*. Detection of trace amounts of FVIIa in embryos of pregnant females injected with 10-fold higher then normal concentration of FVIIa has been reported (74). However, these FVII concentrations were comparable to physiological concentrations of the structurally related FX, suggesting that low levels of maternal FX may transfer into the embryo. Alternatively, the requirement for FX during development may not be absolute. Stochastic processes could create a developmental threshold such that only a fraction of the embryos require a FX-mediated function to pass a particular developmental stage. Under such circumstances, only a fraction of the FX null embryos arrest at the particular embryonic stage; the remaining FX-deficient embryos survive to birth and succumb to hemostatic challenges as neonates.

The reduction of some coagulant activities in $FX^{-/-}$ embryos raises the possibility that the partial embryonic lethality associated with FX deficiency, results as a secondary effect of altered expression of some other activity. It is unlikely that reduced FVII, FIX, or FXI activity is responsible, since total deficiency in either of these factors does not affect embryonic development (74-77). However, it is conceivable that FX affects the expression of some yet undetermined factor involved in embryonic development.

In summary, FX deficiency in mice, similar to FV and prothrombin deficiency previously described (81-83), was found to cause embryonic lethality in about one third of the $FX^{-/-}$ embryos between E11.5-12.5, at least in part due to bleeding. No evidence of vascular defects was observed. However, most arrested $FX^{-/-}$ embryos could not be studied histologically because of rapid degradation and resorbtion. The remaining $FX^{-/-}$ embryos survived to term but succumbed after birth with severe intraabdominal, subcutaneous and/or intracranial bleeding. No apparent abnormalities in blood vessel or organ development were detected in the $FX^{-/-}$ neonates, suggesting that the failure to generate sufficient thrombin to support normal hemostasis caused neonatal death. The lethal phenotype of the $FX^{-/-}$ mice illustrates the importance of FX function in embryonic and postnatal survival. The early $FX^{-/-}$ lethality impeded investigation of potential morphogenetic functions of FX *in vivo*. Such studies may however become feasible through the availability of mice expressing mutant FX proteins, *e.g.*, EPR-1 binding site mutants, or of mice with conditional FX deficiency.

6. ACKNOWLEDGEMENTS

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