

## GENE TARGETING IN HEMOSTASIS. TISSUE FACTOR PATHWAY INHIBITOR

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### 1. ABSTRACT

Tissue Factor Pathway Inhibitor (TFPI) is a serine protease inhibitor of the Factor VIIa/Tissue Factor (FVIIa/TF)-initiated clotting cascade. Mice expressing a mutant form of TFPI, in which its Kunitz-1 domain has been deleted (TFPIKu1<sup>d/d</sup>), die prematurely in embryogenesis between E9.5dpc and birth. These results provide a rationale for the absence of TFPI-deficient patients. This early mortality can be ameliorated by an accompanying heterozygous or homozygous deficiency in FVII. Thus, diminishment of FVII activity precludes the requirement for TFPI-mediated inhibition of the FVIIa/TF pathway during embryogenesis.

### 2. INTRODUCTION

From studies dating back to the early 1950's, it was known that blood contained an endogenous component, which could inhibit Tissue Factor (TF)-initiated coagulation. However, it was not until renewed interest in the 1980's that the protein was isolated and characterized. Although it had been referred to by several names, such as the extrinsic pathway inhibitor (EPI) or lipoprotein-associated coagulation inhibitor (LACI), the International Society on Thrombosis and Hemostasis (ISTH) in 1991 recommended a globally recognizable nomenclature, Tissue Factor Pathway Inhibitor, or TFPI. While several anticoagulant proteins exist to down-regulate different reactions of the coagulation system (e.g., activated protein C, protein S, heparin cofactor II, and antithrombin III), TFPI is the only known physiological inhibitor of the FVIIa/TF complex.

TFPI is a serine protease inhibitor, comprised of an acidic amino-terminus, followed by three tandem Kunitz-type proteinase inhibitory domains (Ku) and a basic carboxy terminus (1). Inhibition of FVIIa/TF activity involves the formation of a quaternary complex between TFPI, FVIIa, TF, and Factor Xa (FXa) on a membrane surface. Thus, TFPI mediates feedback inhibition of the initiation complex of the extrinsic pathway, but not before some FX is activated. Kunitz domain 1 (Ku1) binds the FVIIa/TF complex and inhibits FVIIa at its catalytic site, while Kunitz domain 2 (Ku2) is responsible for FXa inhibition, albeit these domains do not have exclusive roles independent of other regions in the protein (2, 3). An inhibitory role for Kunitz domain 3 (Ku3) and the carboxy-terminus has yet to be elucidated, although positively charged residues in these regions are believed important for endothelial cell binding and optimal FXa inhibition (4).

### 3. TFPI EXPRESSION AND LOCALIZATION

The human *TFPI* gene, which spans approximately 70 kb, is located on human chromosome 2, mapped to the region 2q31-2q32.1 (5, 6). Several potential binding sites for transcription factors have been described in the TATA-less 5' flanking region of the human gene (7). They are GATA-2, SP1, and c-Myc. In addition, a number of negative regulatory elements have been identified. The GATA motifs likely regulate its expression by the GATA-2 transcription factor produced by endothelial cells. Primer extension and S1 nuclease protection analysis indicates multiple transcription initiation sites. Comparison of the gene sequence with that of the cDNA for TFPI indicates that the gene contains nine exons and that alternative

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splicing can occur, resulting in the absence of exon 2 in the 5' untranslated region of some messages. The three Kunitz domains are encoded by separate exons.

TFPI is synthesized mainly in endothelial cells and possibly in other cell types such as stimulated monocytes (8). Northern blot analyses has demonstrated that TFPI is expressed in the microvasculature of a number of tissues, in the order of placenta>lung>liver>kidney>heart>skeletal muscle=pancreas>brain (9).

TFPI exists in three distinct intravascular pools, with varying structures and interestingly, with varying degrees of inhibitory efficiency (10). The most abundant pool of TFPI, also possessing enhanced inhibitory activity, is bound to the endothelial cell surface *via* heparin sulfate or other glycosaminoglycans. Localization of this potent form of TFPI is likely critical since its actions can be directed to an immediate site of injury at the endothelial surface. Infusion of heparin results in detachment of TFPI from the endothelial surface, which accounts for increased levels in plasma. A second source of TFPI is found in the plasma (approximately 2 nM) bound to lipoproteins (LDL>HDL>VLDL). Not surprisingly, TFPI plasma concentrations correlate directly with plasma LDL levels, such that levels are increased in diet-induced hypercholesterolemia, and decreased in those with abetalipoproteinemia (11). These latter patients however demonstrate no tendency for thrombosis. Lipoprotein-associated TFPI demonstrates weak anticoagulant properties (12), making their physiological relevance unknown. The predominant forms of plasma TFPI are approximately 34 and 41 kDa in size. This heterogeneity is the result of varying carboxy-terminal truncations and the formation of mixed disulfide complexes with apo AII. The mechanism of these various truncation events has not been elucidated. A third population of TFPI is located within platelets (8 ng/mL), and is released by thrombin or other platelet activators (13).

Animal studies have shown that TFPI is taken up by the liver and kidney followed by clearance mediated by the LDL-receptor-related protein (LRP) in a two step mechanism. First, TFPI binds to an unrelated receptor, such as glypican-3 (14), through its carboxy terminus, and is then transferred to LRP for internalization (15). The requirement for the carboxy terminus is reflected in the fact that full-length rTFPI is cleared much faster than carboxy-terminal truncated forms of the protein (16). Alternatively, a second clearance mechanism independent of FXa has been identified through which FXa is also removed from the circulation (17).

### 4. HUMAN TFPI DEFICIENCY

Defining TFPI deficiency is a complex problem since different populations of the protein exists in plasma, bound to the vessel wall but releasable upon heparin infusion, and in platelets. Thus, it is questionable whether TFPI deficiency can be assigned simply through a plasma assay for TFPI, or if other methods need to be employed to detect TFPI in the other pools.

At this time, no reports of TFPI deficiency have been documented. In addition, extensive screening of

plasma samples from patients with unexplained arterial or venous thrombosis has not elicited the discovery of any TFPI mutants. These results suggest that a deficiency in TFPI would not be compatible with embryonic survival, either due to its importance in hemostasis or another unknown function. In addition, the requirement for embryonic TFPI is underscored by the fact that murine TFPI plasma levels at E11.5 and E17.5dpc are 30% and 300%, respectively, of WT levels. Partial TFPI deficiency has been induced in rabbits via immunodepletion, resulting in a sensitization to disseminated intravascular coagulation (DIC) following endotoxin treatment or TF (18, 19).

### 5. SPECIFIC TARGETING OF THE *TFPI* GENE IN MICE

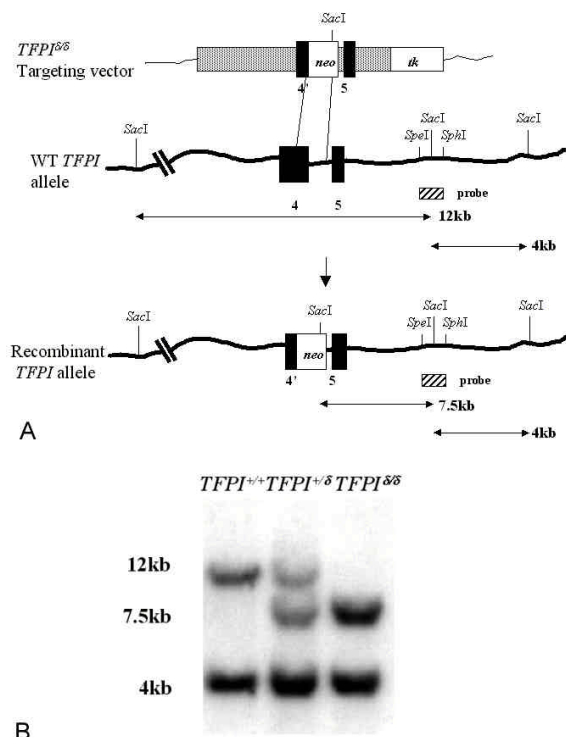
The targeting vector used to produce the in-frame TFPI gene disruption contained a 1.5kb PGK-neomycin phosphotransferase expression cassette replacing portions of exon 4 and intron D of the TFPI gene (20), as shown in figure 1a. Replacement of exon 4 and intron D would result in expression of a mature protein deleted in Ku1. A 1.8kb HSV-thymidine kinase expression cassette was inserted 3' of the *XhoI* restriction site in the pBluescript multiple cloning region. Following vector linearization with *NotI*, electroporation into 129SvJ RW4 and R1 embryonic stem (ES) cells, and positive and negative selection with gancyclovir and thymidine kinase, surviving cells were analyzed via Southern hybridization and by PCR amplification. For Southern analysis, genomic DNA was first digested with the restriction endonuclease *SacI*, and probed with a 1.2 kb probe internal to the site of recombination. This probe was isolated from a *SpeI/SphI* digest of the original targeting vector. The differential restriction pattern, as shown in figure 1b, for the wild-type allele results in the detection of a 12 kb fragment, whereas the mutant allele appears as a 7.5 kb fragment. Detection of a 4.0 kb fragment was also observed for both the wild-type and mutant alleles. Genotyping via PCR amplification required using three primers to detect both wild-type and mutant TFPI alleles: P<sub>F</sub> from exon 4 (GAGCTGGGGTCAATGAAACCGCTGC), P<sub>B1</sub> from exon 4 (ACACTCTTCCAGGTATCAAATCGG), and P<sub>B2</sub> from the neomycin gene (ACTTCCTGACTAGGGGAGGAGTAGA). Amplification of the wild-type allele produced a 170 bp fragment, whereas amplification of the mutant allele resulted in detection of a 330 bp fragment.

Five of 250 RW4 and 1 of 50 R1 ES cells were deemed positive for specific recombination at one of the two *TFPI* alleles. Individual colonies of cells were aggregated with C57Bl/6 blastocysts, and the resulting embryos implanted into pseudo-pregnant females. TFPI chimeric male mice, as assessed by greater than 80% coat color mosaicism, were bred with C57Bl/6 mice to generate *TFPI*Ku1<sup>dd</sup> animals. Mice heterozygous for the mutation appeared healthy and bred normally. These mice were then crossbred to produce *TFPI*Ku1<sup>dd</sup> mice.

### 6. PHENOTYPIC CHARACTERIZATION OF *TFPI*Ku1<sup>dd</sup> NEONATES AND EMBRYOS

Densitometric analyses of Western blots of *TFPI*Ku1<sup>dd</sup> plasma indicated that this mutant protein was

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**Figure 1.** Targeting strategy for the disruption of the murine *TFPI* gene. a) Following electroporation of the targeting vector into R1 or RW4 embryonic stem cells, specific recombination at one of the *TFPI* alleles would result in an in-frame deletion of part of exon 4 and intron D, and replacement with the neomycin phosphotransferase gene cassette. This mutation results in the expression of a mature protein deleted in Ku1. Digestion of genomic DNA with *SacI* and hybridization with a genomic probe resulted in the detection of a 12kb and 4kb fragment for the WT allele, and a 7.5kb and 4kb fragment for the mutated allele. b) Electrophoresis of *SacI*-digested genomic DNA and subsequent probing resulted in the detection of a 12kb and a 4kb DNA band for the *TFPI*<sup>+/+</sup>, and a 7.5kb and a 4kb band for the *TFPIKu1*<sup>ΔΔ</sup> mutation.

expressed at approximately 41% of normal wild-type TFPI plasma levels. As per deletion of Ku1, ligand-binding studies indicated that the protein could bind to FXa but not inhibit FVIIa/TF activity.

Breeding of *TFPIKu1*<sup>+/Δ</sup> animals resulted in the birth of *TFPIKu1*<sup>+/+</sup> and *TFPIKu1*<sup>+/Δ</sup> animals, but a severe under-representation of *TFPIKu1*<sup>ΔΔ</sup> neonates. Of the few neonates that were born, all died during or immediately following birth. Thus, to elucidate the cause of death, timed matings were established to examine embryos at different stages in development. At E8.5dpc, *TFPIKu1*<sup>ΔΔ</sup> embryos appeared normal and were present at the appropriate Mendelian distribution. However, small points of yolk sac hemorrhage were observed in a number of these embryos. By E9.5dpc, greater than 50% of the embryos exhibited abnormalities in both the yolk sac and the embryo, as shown in figure 2. Yolk sac abnormalities included anemia, a lack of vitelline vessels, swollen capillaries, coalescence of vessels to form pools of blood or

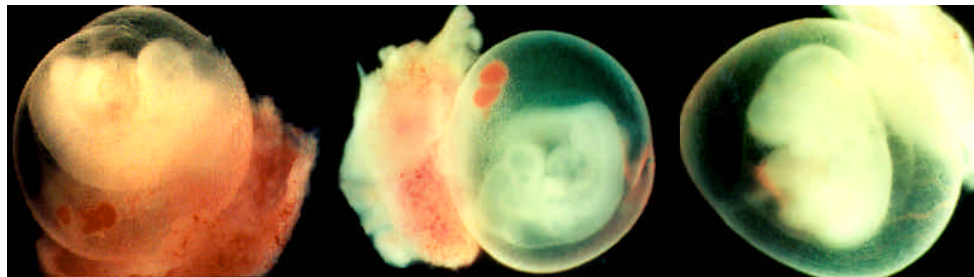
"blood lakes", and a bumpy, golfball-like appearance. Also, blood could often be observed floating freely between the yolk sac and the amnion layer. In general, the embryonic abnormalities appeared to be secondary to those exhibited in the yolk sac. These abnormalities included growth retardation, overall anemia, a faint heartbeat, enlarged blood-filled pericardia, anemia in the heart and other abnormal bleeding events found randomly in the embryo. Microscopic analyses indicated separation of endoderm and mesoderm layers in the yolk sac. By E10.0, a number of the *TFPIKu1*<sup>ΔΔ</sup> were necrotic in both the yolk sac and the embryo. These embryos were anemic, retarded in development, lacked a beating heart and blood flow within the yolk sac and embryo. The earliest point at which fibrin deposition was detectable was at E10.75dpc, found lining the interior of a single blood lake, as well as within the embryo (21). Yolk sac and embryo abnormalities were not absolute, and approximately 40% of the *TFPIKu1*<sup>ΔΔ</sup> embryos survived beyond E10.5dpc. Of the few embryos that survived to E17.5dpc, organ development appeared normal. Thus, TFPI does not appear to be required for organ development. However, these older embryos were afflicted with cranial and spinal hemorrhage, pallor, growth retardation, hepatic and intravascular fibrin deposition and other superficial anomalies such as the presence of shortened tails.

## 7. RESCUE OF EMBRYONIC MORTALITY ASSOCIATED WITH TFPI-DEFICIENCY VIA THE ADDITIONAL LOSS OF FVII OR FIBRINOGEN

To determine the role of FVII in TFPI-deficient early mortality, mice doubly heterozygous for the modified *TFPI* allele and for a deficiency of the *FVII* gene (22) were crossbred in an attempt to generate progeny deficient in both TFPI and FVII (*FVII*<sup>-/-</sup> *TFPIKu1*<sup>ΔΔ</sup>) (21). A murine model of total FVII deficiency has been generated via targeted deletion of the entire *FVII* gene. Briefly, *FVII*<sup>-/-</sup> mice developed normally through embryogenesis but suffered from severe perinatal bleeding, which greatly reduced their life span.

Double heterozygous matings (*FVII*<sup>+/Δ</sup> *TFPIKu1*<sup>+/Δ</sup> x *FVII*<sup>+/Δ</sup> *TFPIKu1*<sup>+/Δ</sup>) resulted in a total of 309 progeny, as shown in table 1. Considering that nine possible genotypes can be generated from a double heterozygous cross, all were present at their approximate Mendelian frequencies except *FVII*<sup>+/+</sup> *TFPIKu1*<sup>ΔΔ</sup> progeny (22% of expected), *FVII*<sup>+/Δ</sup> *TFPIKu1*<sup>ΔΔ</sup> progeny (52% of expected) and *FVII*<sup>-/-</sup> mice (65% of expected). The under-representation of FVII neonates was consistent with previous observations (22). *FVII*<sup>-/-</sup> mice were born at the expected frequency in all *TFPI* genotypic backgrounds. These mice succumbed to either intra-abdominal hemorrhage immediately following birth or later in life due to intra-cranial bleeding. Thus, the loss of TFPI activity (either 50% in *TFPIKu1*<sup>+/Δ</sup> or 0% in *TFPIKu1*<sup>ΔΔ</sup> animals) did not alter the phenotype of animals with a *FVII*<sup>-/-</sup> genotype.

In contrast, the FVII genotype had a profound effect on the phenotype of *TFPIKu1*<sup>ΔΔ</sup> mice. *FVII*<sup>-/-</sup> *TFPIKu1*<sup>ΔΔ</sup> neonates were born at the expected Mendelian frequency but succumbed to the same fatal intra-abdominal or intra-cranial hemorrhaging as *FVII*<sup>-/-</sup> animals. Thus, the additional loss of factor VII rescued the intrauterine



**Figure 2.** Embryonic and yolk sac abnormalities in E9.5dpc *TFPIKu1<sup>dd</sup>* embryos. By E9.5dpc, greater than 50% of the embryos exhibited abnormalities in both the yolk sac and the embryo. Yolk sac abnormalities included anemia, a lack of vitelline vessels, swollen capillaries, coalescence of vessels to form pools of blood or "blood lakes", and a bumpy, golfball-like appearance. Also, blood could often be observed floating freely between the yolk sac and the amnion layer. In general, the embryonic abnormalities appeared to be secondary to those exhibited in the yolk sac. These abnormalities included growth retardation, overall anemia, a faint heartbeat, enlarged blood-filled pericardia, anemia in the heart and other abnormal bleeding events found randomly in the embryo. Microscopic analyses indicated separation of endoderm and mesoderm layers in the yolk sac.

**Table 1.** Mendelian distribution of *FVII/TFPI<sup>d</sup>* genotypes derived from cross-matings of *FVII<sup>+/-</sup>TFPIKu1<sup>+/-d</sup>* mice

	<i>FVII<sup>+/+</sup></i>	<i>FVII<sup>+/-</sup></i>	<i>FVII<sup>-/-</sup></i>	<i>TFPI<sup>+/+</sup></i>	<i>TFPI<sup>+/-</sup></i>	<i>TFPI<sup>dd</sup></i>	<i>TFPIKu1<sup>+/+</sup></i>	<i>TFPIKu1<sup>+/-d</sup></i>	<i>TFPIKu1<sup>dd</sup></i>
Postnatal <sup>a</sup>	24/23 (104%)	57/46 (124%)	50/46 (109%)	77/92 (84%)	5/23 (22%)	24/46 (52%)	25/23 (110%)	32/46 (70%)	15/23 (65%)
E17.5 <sup>a</sup>	17/14 (121%)	27/28 (96%)	38/28 (136%)	48/56 (86%)	4/14 (29%)	26/28 (93%)	10/14 (71%)	24/28 (86%)	11/14 (79%)

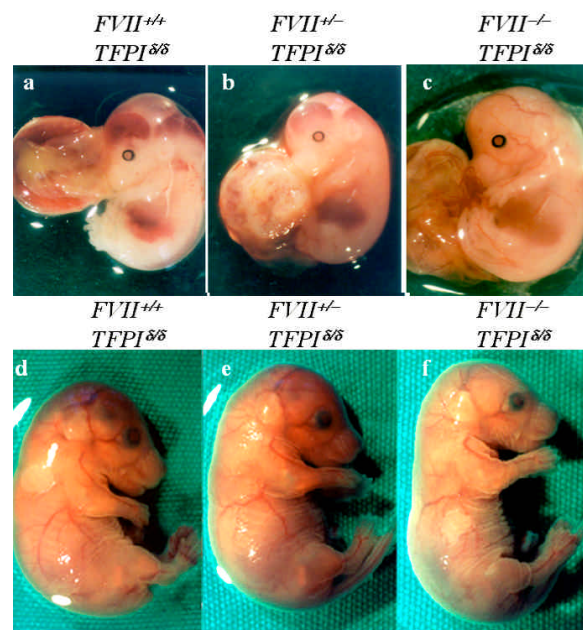
<sup>a</sup> The number of animals [exhibiting/expected (%)] of the indicated genotype out of a total of 370 postnatal and 231 E17.5 mice. These totals were obtained by assuming that the observed number of *FVII<sup>+/+</sup>TFPI<sup>+/+</sup>*, *FVII<sup>+/+</sup>TFPIKu1<sup>+/-d</sup>*, *FVII<sup>+/+</sup>TFPI<sup>dd</sup>*, and *FVII<sup>+/+</sup>TFPIKu1<sup>+/+</sup>* animals (which represents 9/16 of all possible genotypes from these matings) were normal survival numbers. Thus, the total of these four genotypes represented 16/9 of the total expected survivors.

lethality observed in singly deficient *TFPIKu1<sup>dd</sup>* embryos, suggesting that the TFPI-deficient phenotype was dependent on FVII. The presence of *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* neonates allowed for the measurement of TFPI activity in these animals carrying the mutated TFPI allele. This determination could not be made in the original TFPI study since the majority of the *TFPIKu1<sup>dd</sup>* embryos did not survive embryogenesis. Analysis of plasma samples collected from *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* embryos and neonates indicated that indeed, this particular gene modification resulted in a protein that could not inhibit FVIIa/TF activity.

Interestingly, genotypic and phenotypic (macroscopic and histological) analysis of embryos sired from double heterozygous matings demonstrated the effects of FVII gene dosage on the TFPI-deficient phenotype. *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* and *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* embryos were present at their expected frequencies at all embryonic ages examined (E9.5dpc, E14.5dpc, E17.5dpc), whereas *TFPIKu1<sup>dd</sup>* embryos succumbed to early mortality following E9.5dpc, as per the original study (20). At this stage, *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* demonstrated similar yolk sac, but not embryonic abnormalities, as those seen in the *FVII<sup>+/+</sup>TFPIKu1<sup>dd</sup>* embryos. By E14.5dpc, the coagulopathy and concomitant hemorrhage observed in the *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* embryos appeared less severe than those singly-deficient TFPI embryos that survive to this age, whereas *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* embryos demonstrate no coagulopathy nor hemorrhagic phenotype at this stage, as demonstrated in figure 3a-c, nor at E9.5dpc. Consistent with postnatal findings, *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* embryos did not reveal any degree of hemorrhage immediately prior to birth

(E17.5dpc), as seen in figure 3d-f. In contrast, while *FVII<sup>-/-</sup>TFPIKu1<sup>dd</sup>* were present at their expected Mendelian distribution, they demonstrated a severe systemic coagulopathy leading to hemorrhage and ischemia, resulting in their death during or immediately following birth. The fact that only 60% of the expected number of these mice were observed following birth was likely due to the fact that these neonates were immediately consumed following death, a phenomenon observed in the original *FVII* gene inactivation study. Overall, decreasing FVII levels resulted in a "normalization" of the *TFPIKu1<sup>dd</sup>* embryos, based on a lessening of the frequency and severity of the coagulopathic phenotype, and ultimately in an increase in embryonic and neonatal lifespan.

Recent studies have shown that all of the factors required for FVIIa/TF-mediated fibrin formation are present as early as E7.5dpc (23), prior to the formation of a functional circulatory system. However, formation of fibrin, in itself, is not required for embryogenesis (24, 25). Although FVII mRNA has been detected in E9.5dpc embryos, these studies demonstrate that physiologically/pathologically relevant levels of this protein are expressed and are functional at this time. However, in no way does its presence imply that FVIIa/TF proteolytic activity is required during embryogenesis. This is an important consideration to take into account. Whether the mortality associated with TFPI deficiency is due to uncontrolled fibrin generation is unlikely. The fact that fibrin deposition was observed in only one blood-lake and within only one *FVII<sup>+/+</sup>TFPIKu1<sup>dd</sup>* E10.75 embryo examined suggests that the presence of fibrin is an effect but not a cause of the phenotype. The more probable cause of death would be



**Figure 3.** Gene dosage effects of FVII in E14.5dpc and E17.5dpc *TFPIKu1<sup>d/d</sup>* embryos. Significant cranial hemorrhage and edema can be observed in the few *TFPIKu1<sup>d/d</sup>* embryos that survive to a) E14.5 and d) E17.5dpc. The pathology is less severe in *FVII<sup>+/-</sup>TFPIKu1<sup>d/d</sup>* (b,e) and is absent in *FVII<sup>-/-</sup>TFPIKu1<sup>d/d</sup>* (c,f) at both time points.

either the toxic effects of the coagulation enzymes themselves, or the consumption of clotting factors, resulting in diminished generation of FXa and/or thrombin. It is very likely that the presence of thrombin and FXa are required in this regard for several other functions related to cell signaling processes in which they are involved. However, the exact mechanism by which FVIIa/TF activity produces early death remains unclear and will continue to elude researchers until methods can be developed to precisely evaluate markers of DIC in murine embryonic samples.

Most importantly, the availability of these doubly deficient embryos and mice allows for some conclusions to be reached regarding maternal transfer of factor VII. The previous biochemical evidence was an indirect approach at addressing this issue, because the argument always arrived to whether all of the embryonic factor VII was inhibited by addition of rNAPc2 in the culture media and through intra-cardiac injections. In conjunction with the TFPI-deficiency, removal of factor VII, by crossing with the FVII gene-targeted mice, addressed this issue directly. If transfer of maternal FVII to the embryo does occur, the level of maternal FVII present in early-stage embryos is insufficient to cause a coagulopathy in those expressing the *TFPIKu1<sup>d/d</sup>* genotype, since the loss of embryonic FVII prevents intrauterine mortality in these embryos. Thus the rescue of the TFPI-deficient embryos with a concomitant loss in factor VII is the strongest and most direct argument against significant maternally transferred factor VII.

Studies have also been initiated to examine the effects of fibrinogen deficiency on the TFPI-deficient phenotype. Preliminary results indicate that a deficiency in

fibrinogen also rescues the embryonically lethal phenotype associated with a TFPI deficiency in mice (personal communication, J. Degen). In contrast to the *TFPIKu1<sup>d/d</sup>* study, *TFPIKu1<sup>d/d</sup>Fib<sup>-/-</sup>* embryos are present at their expected Mendelian frequency at E18.5dpc and immediately following birth. Although a significant percentage dies in the early neonatal period, a small percentage of these doubly-deficient animals can reach breeding age without incident.

## 8. PERSPECTIVE

It is clear through human clinical data, plus the generation of mice deficient in TFPI, that a deficiency in the anticoagulant TFPI is not compatible with normal embryogenesis and postnatal life. In mice, alterations in TFPI-related mortality can be significantly manipulated via the additional loss of FVII or fibrinogen, suggesting that the cause of death in *TFPIKu1<sup>dd</sup>* is related to its role in anticoagulation, and not to any as yet unknown function.

## 9. REFERENCES

1. Wun, T.C., Kretzmer, K.K., Girard, T.J., Miletich, J.P. & G.J. Broze Jr.: Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. *J Biol Chem* 263: 6001-6004 (1988)
2. Girard, T.J., Warren, L.A., Novotny, W.F., Likert, K.M., Brown, S.G., Miletich, J.P. & G.J. Broze Jr.: Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. *Nature* 338: 518-520 (1989)
3. Higuchi, D.A., Wun, T.C., Likert, K.M. & G.J. Broze Jr.: The effect of leukocyte elastase on tissue factor pathway inhibitor. *Blood* 79: 1712-1719 (1992)
4. Wesselschmidt, R., Likert, K., Girard, T., Wun, T.C. & G.J. Broze Jr: Tissue factor pathway inhibitor: the carboxy-terminus if required for optimal inhibition of factor Xa. *Blood* 79: 2004-2010 (1992)
5. Girard, T.J., Eddy, R., Wesselschmidt, R.L., MacPhail, L.A., Likert, K.M., Byers, M.G., Shows, T.B. & G.J. Broze Jr: Structure of the human lipoprotein-associated coagulation inhibitor gene. Intron/exon gene organization and localization of the gene to chromosome 2. *J Biol Chem* 266:5036-41 (1991)
6. Van der Logt, C.P., Reitsma, P.H. & R.M. Bertina: Intron-exon organization of the human gene coding for the lipoprotein-associated coagulation inhibitor: the factor Xa dependent inhibitor of the extrinsic pathway of coagulation. *Biochem* 30:1571-1577 (1991)
7. Petit, L., Lesnik, P., Dachet, C., Hugou, I., Moreau, M., Chapman, J. & M. Rouis M: The promoter of human tissue factor pathway inhibitor gene: identification of potential regulatory elements. *Thromb Res* 95:255-62 (1999)
8. Van der Logt, C.P.E., Dirven, R.J., Reitsma, P.H & R.M. Bertina: Expression of tissue factor and tissue factor



## The Generation of TFPI-deficient mice through Gene Targeting

pathway inhibitor in monocytes in response to bacterial lipopolysaccharide and phorbol ester. *Blood Coagul Fibrinol* 5: 211-220 (1994)

9. Bajaj, M.S., Kuppuswamy, M.N., Manepalli, A.N. & S.P. Bajaj: Transcriptional expression of tissue factor pathway inhibitor, thrombomodulin and von Willebrand factor in normal human tissues. *Thromb Haemost* 82:1047-52 (1999)

10. Broze, G.J. Jr, Lange, G.W., Duffin, K.L. & L. Macphail: Heterogeneity of plasma tissue factor pathway inhibitor. *Blood Coag Fibrin* 5: 551-559 (1994)

11. Novotny, W.F., Brown, S.G., Miletich, J.P., Rader, D.J. & G.J. Broze Jr.: Plasma antigen levels of the lipoprotein-associated coagulation inhibitor in patient samples. *Blood* 78: 387-393 (1991)

12. Hansen, J.B., Huseby, K.R., Huseby, H.E., Ezban, M. & A. Nordoy: Tissue factor pathway inhibitor in complex with low density lipoprotein isolated from human plasma does not possess anticoagulant function in tissue factor-induced coagulation in vitro. *Thromb Res* 85: 413-425 (1997)

13. Novotny, W.F., Girard, T.J., Miletich, J.P. & G.J. Broze Jr.: Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein associated coagulation inhibitor. *Blood* 72: 2020-2025 (1988)

14. Mast, A.E., Higuchi, D.A., Huang, Z.F., Warshawsky, I., Schwartz, A.L. & G.J. Broze Jr.: Glypican-3 is a binding protein on the HepG2 cell surface for tissue factor pathway inhibitor. *Biochem J* 327: 577-583 (1997)

15. Warshawsky, I., Broze, G.J. Jr. & A.L. Schwartz: The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. *Proc Natl Acad Sci USA* 91: 6664-6668 (1994)

16. Warshawsky, I., Bu, G., Mast, A., Saffitz, J.E., Broze, G.J. Jr. & A.L. Schwartz AL: The carboxy terminus of tissue factor pathway inhibitor is required for interacting with hepatoma cells *in vitro* and *in vivo*. *J Clin Invest* 95:1773-81 (1995)

17. Ho, G., Toomey, J.R., Broze, G.J. Jr. & A.L. Schwartz: Receptor-mediated endocytosis of coagulation factor Xa requires cell surface-bound tissue factor pathway inhibitor. *Blood* 78: 387-393 (1996)

18. Sandset, P.M., Warn-Cramer, B.J., Maki, S.L. & S.I. Rapaport: Immunodepletion of extrinsic pathway inhibitor sensitizes rabbits to endotoxin-induced intravascular coagulation and the generalized Shwartzman reaction. *Blood* 78: 1496-1502 (1991)

19. Sandset, P.M., Warn-Cramer, B.J., Rao, L.V.M., Maski, S.L. & S.I. Rapaport: Depletion of extrinsic pathway inhibitor (EPI) sensitizes rabbits to disseminated intravascular coagulation induced with tissue factor: evidence supporting a

physiologic role for EPI as a natural anticoagulant. *Proc Natl Acad Sci USA* 88: 708-712 (1991)

20. Huang, Z.F., Higuchi, D., Lasky, N. & G.J. Broze Jr.: Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. *Blood* 90: 944-951 (1997)

21. Chan, J.C.Y., Carmeliet, P., Moons, L., Rosen, E.D., Huang, Z.F., Broze, G.J. Jr, Collen, D. & F.J. Castellino: Factor VII deficiency rescues the intrauterine lethality in mice associated with a tissue factor pathway inhibitor deficit. *J Clin Invest* 103: 475-482 (1999)

22. Rosen, E.D., Chan, J.C.Y., Idusogie, E., Clotman, F., Vlasuk, G., Luther, T., Jalbert, L.R., Albrecht, S., Zhong, L., Lissens, A., Schoonjans, L., Moons, L., Collen, D., Castellino, F.J. & P. Carmeliet: Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature* 390: 290-294 (1997)

23. Suh, T.T., Holmback, K., Jensen, N.J., Daugherty, C.C., Small, K., Simon, D.I., Potter, S.S. & J.L. Degen: Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. *Genes Dev* 9, 2020-2033 (1995)

24. Ploplis, V.A., Wilberding, J., McLennan, L., Liang, Z., Cornelissen, I., DeFord, M.E., Rosen, E.D. & F.J. Castellino: A total fibrinogen deficiency is compatible with the development of pulmonary fibrosis in mice. *Am J Pathol* 157:703-708 (2000)

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