Tissue factor pathway inhibitor (TFPI), a multivalent serine protease inhibitor with three Kunitz-like domains, is an endogenous inhibitor of TF-mediated coagulation. TFPI suppresses factor Xa generation by binding via its Kunitz 2 domain to factor Xa and via its Kunitz 1 domain to the TF:FVIIa catalytic complex (1). The formation of a quaternary TF:VIIa:TFPI:Xa complex dampens ongoing FXa generation. Additionally, TFPI also has been shown to regulate the internalization and degradation of TF:VIIa complex on cell surfaces (2-4).

TFPI is found in platelets, microvascular endothelium and stimulated blood monocytes (5-8). In our previous studies TFPI expression was demonstrated in vascular smooth muscle and endothelial cells in human atherosclerotic plaques (9, 10). In these plaques, levels of TFPI expression was inversely associated with the severity of the lesion. In carotid homogenates, TFPI increased to 2 to 3-fold in carotid homogenates. There was no difference in plasma TFPI levels or hemostatic measures (PT, aPTT and tail vein bleeding times) between these mice and their wildtype littermates. In a ferric chloride-induced model of carotid thrombosis, homozygotic transgenic mice demonstrated resistance to thrombotic occlusion compared to wildtype littermates. In transgenic mice 22% occluded within 30 minutes of application while 84% of wild type mice occluded within the same time frame (p <0.01). Heterozygotic transgenic mice had an intermediate thrombotic phenotype. Taken together, these data indicated that local VSMC-specific TFPI overexpression attenuated ferric chloride-induced thrombosis without systemic or hemostatic effects. Furthermore, this transgenic mouse model should prove useful for studying the role of TFPI in the development and progression of vascular disease.

Keywords
Tissue factor / factor VII, tissue factor pathway inhibitor (TFPI), transgenic animals

Introduction
Tissue factor pathway inhibitor (TFPI), a multivalent serine protease inhibitor with three Kunitz-like domains, is an endogenous inhibitor of TF-mediated coagulation. TFPI suppresses factor Xa generation by binding via its Kunitz 2 domain to factor Xa and via its Kunitz 1 domain to the TF:FVIIa catalytic complex (1). The formation of a quaternary TF:VIIa:TFPI:Xa complex dampens ongoing FXa generation. Additionally, TFPI also has been shown to regulate the internalization and degradation of TF:VIIa complex on cell surfaces (2-4).

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ed with local TF activity suggesting a local regulatory role. Furthermore, in human plasma, TFPI exists in small quantities (<5%) as a free full length protein, but is predominantly in association with lipoproteins (11-14). Lipoprotein-associated TFPI is a truncated form which has been shown to be less active in vivo than full length TFPI (14). Taken together, these findings suggest a potentially important role for locally active, full-length forms of TFPI.

To define the local role of TFPI in vascular disease, several studies have used gene transfer of TFPI in models of vascular injury (15-18). These studies are limited by the extent and duration as well as the lack of cellular and temporal regulation of transgene expression. To investigate the role of TFPI in arterial thrombosis, mice in which a murine TFPI transgene was expressed under the control of the murine SM22α promoter (19, 20) were generated in order to target transgene expression to smooth muscle cells in large arteries of transgenic mice.

### Materials and methods

#### Cell culture

Human vascular smooth muscle cells (HVSMC) (Clonetics, San Diego) were grown in SmBM with SmGM-2 supplement (Clonetics, San Diego). 293 and 3T3 cells (ATCC, Manassas, VA) were grown in Dulbecco’s modification of Eagle’s medium containing 10% FBS, 100 I.U./ml Penicillin, 100 μg/ml Streptomycin and 250 ng/ml Amphotericin B. All cell cultures were incubated at 37°C with 5% CO2.

#### Construction of pSM22α-mTFPI transgene

A murine TFPI transgene construct was made in the pGL3-basic vector (Promega, WI). The luciferase gene fragment in pGL3 was removed by specific restriction endonucleases, Nco I and Xba I. The mouse TFPI gene cDNA was PCR amplified from pBluescript KS II-mTFPI plasmid (gift from Dr. George Broze, Washington University) and then inserted into Nco I and Xba I sites of pGL3 vector, resulting in pGL3-mTFPI plasmid. The 441 bp mouse SM22α promoter fragment was excised at Kpn I and Xho I sites from pAdMCSloxp plasmid (gift from Dr. Elizabeth Nabel, National Institutes of Health) and cloned into the same restriction enzymes sites in the MCS of pGL3-mTFPI plasmid to yield pSM22α-mTFPI construct.

#### Transient transfection

Transient transfection of multiple cell lines was performed by using Lipofectamine (GIBCO-BRL, MD) according to the manufacturer’s instructions. Briefly, cells were seeded in 10 cm culture dishes with optimal culture medium and incubated overnight at 37°C with 5% CO2. The 70-80% confluent cells culture were transfected with 10 μg plasmid DNA and 100 μl Lipofectamine in 6 ml Opti-MEM overnight. The transfection medium was replaced with complete growth medium and cell culture was incubated additional 36 hours. The culture supernatant was collected and cells were washed twice in cold phosphate buffered saline (PBS) and harvested with lysis buffer (5).

#### Western blotting

The protein concentrations of cell lysates were determined using Bio-Rad Protein Assay Kit (BIO-RAD). 10 μg total protein of cell lysate or 30 μl of conditioned media from each cell culture were applied on 12% SDS-PAGE for size separation and then transferred to nitrocellulose membrane. The membrane was stained by 0.5% Ponceau S solution to verify the equal loading and transfer. Immunoblotting was performed by using rabbit anti-mouse TFPI antiserum (6) at 1:500 dilution and rabbit anti-β-actin antibody at 1:1000 (Sigma, St. Louis MO) in non-fat milk/Tris-buffered saline. After washes, the membrane was probed with a horseradish peroxidase conjugated anti-rabbit IgG antibody (Amersham Life Sciences) and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford). The membrane then was exposed to X-ray film (Kodak) and subsequently developed.

#### TFPI activity assay

Mouse TFPI activity was measured by Actichrome TFPI Activity Assay Kit (American Diagnostic Inc.) following the manufacturer’s instruction. Briefly, 20 μl supernatant of each cell culture were added into the wells of a micro-test plate and 20 μl of factor VIIa/TF complex then were added into the wells containing TFPI standards or samples. The micro-test plate was incubated at 37°C for 30 min. After incubation 20 μl of human factor X were added into each well and incubated an additional 15 minutes. 20 μl of EDTA were added and followed with 20 μl spectrozyme FXa substrate. Color development was read at 405 nm wavelength.

For mouse plasma and arterial TFPI activity, carotid arteries were harvested immediately post mortem and were homogenized in 50 mmol/L Tris HCl, pH 8.0 using a tissue homogenizer. The homogenate was centrifuged and supernatant was collected. Protein content in the supernatant was determined by using Bio-Rad Protein Assay Kit (BIO-RAD). TFPI activity in both the plasma and arterial homogenates was measured using a chromogenic assay (American Diagnostic Inc.). This assay is based on the measurement of the ability of TFPI to inhibit the catalytic activity of TF-VIIa complex to activate factor X to Xa and has been previously used with murine plasma (7).

#### Generation of transgenic mice

The pSM22α-mTFPI plasmid was double digested with Not I and Sal I to remove the plasmid sequence and gel purified using QIAquick Gel Extraction Kit (QIAGEN). The transgenic mouse core facility at Mayo generated the transgenic mice using standard procedures (21). The linearized construct was microinjected into fertilized eggs from C57B/6 mice. Viable embryos were...
re-implanted into the oviducts of pseudopregnant foster mothers. Mice carrying the transgene were identified by Southern hybridization analysis using mTFPI cDNA as probe. Founders were backcrossed and intercrossed to obtain homozygous transgenic mice. The transgenic lines were subsequently screened by Southern hybridization and PCR using 5'-CTTGCAGGTCTTCTTGTGGG-3' (in SM22a fragment) as forward primer and 5’-TTCTCGTTCCTTCACATCC-3' (in mTFPI cDNA fragment) as reverse primer. The selected heterozygotic transgenic mice (Tg/+ were intercrossed to obtain SM22α-mTFPI homozygotic transgenic (Tg/Tg) mice. These mice were subjected to further experiments.

**Preparation of RNA**

Total RNA was prepared by homogenization of mouse aorta, heart, brain, kidney, spleen, liver, skeleton muscle and lung using TRIzol (GIBCOBRL) method. Samples were electrophoresed using 1% agarose and 2.2 mol formaldehyde/L gel and stained with ethidium bromide in order to visualize the 28s and 18s ribosomal RNA and thereby to confirm the integrity of the RNA.

**Ribonuclease protection assay**

The forward primer 5'-AGCAGACAGCGGATTATG-3' (in mTFPI cDNA) and reverse primer 5'-AGGATCCTTCTTCTTGGATGATGATG-3' (in vector sequence down stream of mTFPI) were used for PCR to generate the template for RPA probe. PCR product was sequenced and subcloned into pBluescript SK (Stratagene) at EcoRI site. The template containing T7 promoter was obtained by incubated the plasmid with PvuI and XbaI. MAXIscript in vitro transcription kit (Ambion) was used to generate RNA probe. The 32P labeling 266 base long RNA probe was produced by T7 RNA polymerase according to the kit protocol. Ribonuclease Protection Assay was performed using RPA II Kit (Ambion). Briefly 10 ug of total RNA of mouse aorta or other tissues from tested transgenic mice were used to hybridize with 1 x 105 cpm RNA probe. Protected fragments were separated on 7.5% acrylamide/8M urea gel and then the gel was dried on filter paper. Autoradiography was performed at -70°C against X-OMAT film (Kodak) between intensifying screens. Specific bands were identified on the basis of their individual migration patterns in comparison with the molecular weight standards. The bands were quantified by densitometric analysis in arbitrary pixel units and were normalized to wildtype TFPI mRNA.

**In situ hybridization**

In situ hybridization was performed as modified method previously described (8). Briefly, the 214bp of 3’ untranslated region in pSM22α-mTFPI transgene construct was cloned into pBluescript SK vector and used as template to transcribe sense and antisense riboprobes using “Riboprobe In Vitro TranscriptionSystem” kit (Promega) and labeled with DIG-11-UTP. Frozen sections fixed for 20 minutes with 4% paraformaldehyde, washed and dehydrated 2 minutes each in 50%, 80%, 95%, and 100% ethanol, and then incubated with proteinase K for 15 minutes at room temperature (1 ug/ml, Sigma). Slides were then incubated in hybridization buffer for 1 hour at 50°C (3 mol/L NaCl, 0.3 mol/L sodium citrate, 10% dextran sulfate, 1X Denhardt solution, 2 mmol/L EDTA, 50% formamide, and 500 ug/ml herring sperm DNA). The probes were then applied in hybridization buffer to the tissue sections overnight at 50°C. Slides were washed and incubated with anti-DIG antibody conjugated to alkaline phosphatase for 2 hours at room temperature (Roche). The sections were stained overnight with 0.18 mg/ml BCIP, 0.34 mg/ml NBT (Roche), and 240 ug/ml levamisole (Sigma) to visualize the reaction.

**Real time RT-PCR for tissue factor**

Total RNA isolated from was treated with DNase to eliminate DNA contamination in samples. Reverse transcription (RT) was performed with 2ug treated total RNA from each separate aorta to generate first strand cDNA. To determine the level of expression of TF, real time polymerase chain reaction (PCR) quantification was performed using LightCycler system (Roche, Penzberg, Germany). The primer sequences for TF were forward 5'-CAGGTCAATCTTGGGAGCGAGT-3’ and reverse 5'-CAGGTCAATCTTGGGAGCGAGT-3’. The transcript for the constitutive gene product GAPDH was used for data normalization. The primer sequences for GAPDH were forward 5’-GAGTCAATCATGAGCTGCT-3’ and reverse 5’-CTTCTAGGGCTTCTTCTT-3’. Real time PCR mix was prepared according to the manufacturer instruction. PCR condition was programmed as follows: initial denaturation at 95°C for 10 min followed by 95°C for 10 sec, 60°C for 5 sec, and 72°C for 10 sec for 40 cycles. A no template mix used as negative control.

**Analysis of hemostasis**

Prothrombin time and activated partial thromboplastin time were measured in citrated plasma using automated techniques (SCA Veterinary Coagulation Analyzer) from transgenic mice and control normal mice before any surgical interventions. Tail vein bleeding tests were performed on transgenic (n = 9) and nontransgenic (n = 9) mice as previously described (12). Briefly, mice were anesthetized with intraperitoneal phenobarbital (100 mg/kg) and placed in a restraining chamber. The distal 1 mm of the tails were amputated and immersed for 10 minutes in 1 ml of normal saline at 37°C. Blood loss was to be determined by measuring the absorbance of saline at 560 nm wavelength.

**Induction of carotid arterial thrombosis**

Mice used in these experiments were 8 to 12 weeks old, and about 23-25 g. FeCl3 induced vascular injury protocol was pre-
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Previously described (13). Briefly, mice were anesthetized with Ketamine (80 mg/kg) and Xylazine (5 mg/kg) intramuscularly. After the left common carotid artery was exposed, a flow probe (model 0.5 VB, Transonic Systems) was placed on the artery. The probe was connected to a flowmeter (Transonic model T106) and interpreted with a computerized data acquisition program (Windaq, DATAQ Instruments). A strip of filter paper saturated with 10% FeCl$_3$ solution was applied to the adventitial surface of the surgically exposed carotid artery for 3 min. Animal blood flow was then measured by the T106 Small Animal Blood Flow Meter for 30 minutes or until complete thrombus occurred.

**Rescue of TFPI deficient mice**

Tg/Tg mice were crossed with TFPI$^+/−$ mice (Gift from Dr. Broze) to generate Tg/+ TFPI$^+/−$ offspring. Tg/+ TFPI$^+/−$ mice were intercrossed and 100 newborn offspring were genotyped by PCR in the first 12 hours after birth. Briefly, the neonates were retrieved from the breeding pairs before they were 12 hours old and sacrificed under the IACUC approved methods by placing them in a CO$_2$ chamber. The bodies were kept in -80°C freezer until further test. Genotyping for the Tg was performed as described above. For wild-type TFPI and mutant alleles, PCR screening was performed as previously described (22).

**Statistical analysis**

Comparisons of TFPI activity and flow cessation were made using two-tailed nonpaired t tests. Comparisons of occlusion rates were made using chi-square analysis. A p value of < 0.05 was considered significant. Data are presented as mean +/- SEM.

**Results**

**Characterization of pSM22α-mTFPI**

Transfection of pSM22α-mTFPI into several different cell types demonstrated the specificity and activity of the construct. Western blotting of cell lysates and conditioned media from transfected and nontransfected HVSMC, 293, and 3T3 cells demonstrated that mTFPI expression and activity was predominantly associated with smooth muscle cells in vitro (Fig. 1).

**Characterization of transgenic mice**

Of almost 100 potential founders, six transgenic founders were identified by Southern blotting for the transgene and were not distinguishable from wildtype mice by appearance, body weight, behavior and fertility. Levels of mRNA for wildtype TFPI and transgenic mTFPI in aortas from founders were measured by ribonuclease protection assay (RPA) (Fig. 2). This assay allowed for simultaneous assessment of transgene and wildtype mRNA. One line (1661) showed the highest mRNA expression level of transgene compared with other tested. Three lines were negative for mRNA expression of transgene. The transgene mRNA level of line 1661 was about 4-fold higher (as measured by densitometry) than the level of endogenous TFPI mRNA in aortic homogenates. Thus, we chose line 1661 as our transgenic line to study. Transgenic RNA was also found in smooth mus-

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**Figure 1:** The expression of mTFPI transgene in transfected and nontransfected cells. Western blot analysis of TFPI in 30 ul of conditioned media (A) and 10 ug of total protein extract from cell lysates (B) of pSM22α-mTFPI (+) or control plasmid (-) transfected cells. Anti-mTFPI rabbit polyclonal antibody was used as primary antibody. C. TFPI activity in cell lysates from pSM22α-mTFPI (+) and control plasmid (-) transfected cells. HVSMC = Human vascular smooth muscle cell.

**Figure 2:** Autoradiogram of RPA analysis of mTFPI transgene and wildtype TFPI gene expression in mouse aortas from founder mice lines.
cle rich tissue such as lung, heart and kidney as previously described (data not shown) (20). TFPI activity of carotid artery homogenates from transgenic mice showed 2 to 3-fold increase over wildtype mice (Fig. 3). There was no difference in plasma TFPI activity between transgenic mice and control littermates. Arterial tissue factor mRNA (normalized to GAPDH) did not differ between transgenic and wildtype mice (Tg 0.082 ± 0.012, Wt 0.086 ± 0.01, p = NS). Localization of transgene expression was analyzed using in situ hybridization (Fig. 4). Anti-sense oligo probes hybridized with medial smooth muscle cells in the aortas of transgenic mice but not sense oligo probes. In aortas from wild type mice, by contrast, neither of these probes hybridized. These patterns of transgene expression confirmed that the regulatory elements conferred vasculature-specific transgene expression.

To determine if overexpression of mTFPI from VSMC results in hemostatic abnormalities, PT, aPTT and tail vein bleeding time were measured in the two groups. Prothrombin times and activated partial thromboplastin times were not different between the two groups (PT in transgenic mice 21.6 ± 0.6 sec. vs. normal mice 20.6 ± 0.7 sec.; aPTT in transgenic mice 65.4 ± 2.3 sec. vs normal mice 61.8 ± 7.5 sec.). The blood loss in tail vein bleeding tests were also similar between the groups (O.D. of blood loss in transgenic mice: 0.83 ± 0.37 vs. normal mice: 0.60 ± 0.20, p = NS). This suggests that overexpression of mTFPI from VSMC does not result in a hemostatic or bleeding abnormality.

**Analysis of ferric chloride-induced thrombosis**

To examine the role of overexpression of mTFPI from VSMC in regulating arterial thrombosis, a well-established model was used. Heterozygotic transgenic mice (Tg/+, n = 11), homozygotic transgenic mice (Tg/Tg, n = 9) and age and sex-matched control normal mice (+/+, n = 13) were studied following local application of FeCl₃. Baseline arterial blood flow was similar in the two groups. The flow patterns were recorded for 30 minutes or until complete occlusion, whichever happened earlier after initiation of injury. The flow reduction pattern in both groups has shown an initial rapid decline in the first 3 minutes. As time progressed, significant differences become manifest in the groups characterized by a shorter time to reach half of the initial flow (Tg/Tg:8.31 ± 2.28 min., Tg/+: 6.58 ± 1.74 min, +/-:2.25 ± 0.98 min. p < 0.04 for Tg/Tg vs +/-) in a gene-copy dependent manner (Fig. 5). The Tg/Tg mice had a lower rate of complete occlusion over 30 minutes compared to the wildtype mice (p <0.05). The heterozygotic mice had a similar degree of occlusion at 30 minutes as wildtype mice.

![Figure 3: Measurement of TFPI activity in mouse carotid arterial homogenates from transgenic (Tg+) and wildtype mice (wt). * = p <0.05.](image)

![Figure 4: Expression of mTFPI transgene in aorta prepared from transgenic and wildtype mouse demonstrated by in situ hybridization analysis. Aorta from transgenic mouse hybridized to DIG labeled antisense (A) but not to sense (B) RNA probe. Control aorta from wildtype mouse failed to hybridize to the same DIG labeled antisense (C) and sense (D) RNA probe.](image)
Attempts to rescue TFPI deficient mice

Intercrosses were established between Tg/+ and TFPI−/− mice and 100 offspring were produced for genotyping. As shown in the Table 1, of 6 possible genotypes (with the presence of Tg being represented solely as present or absent without regard to copy number) only 4 genotypes were observed. The 2 absent genotypes were those TFPI null mice which were determined to be embryonically lethal previously (22). These data suggest that TFPI expressed from this portion of the SM22α promoter cannot rescue TFPI null embryos.

Discussion

In this study, the development of a transgenic mouse with VSMC-targeted overexpression of mTFPI was used to investigate the role of vascular TFPI expression in thrombosis. TFPI

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<th>Genotype</th>
<th>TFPI wt (%)</th>
<th>TFPI Tg (%)</th>
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<th>Obs/E</th>
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expression from the smooth muscle cell-specific promoter SM22α resulted in the overexpression of mTFPI in a tissue-specific and local manner. Local TFPI overexpression did not affect hemostasis but significantly attenuated ferric chloride-induced arterial thrombosis. Taken together, these studies support the importance of local regulation of intravascular thrombosis.

Tissue factor within the atherosclerotic plaque has been described as a key initiator of arterial thrombosis (10). Many cells in the atherosclerotic plaques, such as monocytes, foam cells and endothelial cells express TF. Plaque rupture leads to exposure of TF to factor VII/VIIa in circulating blood and activation of TF-dependent coagulation (23). As an extrinsic regulator of TF-dependent coagulation pathway, TFPI bound TF-FVIIa complex via Xa to prevent further production of factor IXa and Xa. The formation of the quaternary complex, TF-FVIIa-TFPI-FXa, dampens ongoing coagulation and modulates thrombosis. Our group has shown that TFPI is expressed from medial vascular smooth muscle cells (VSMC) and endothelial cells (EC) in normal human coronary arteries and from EC, VSMC and macrophages with human atherosclerotic plaques (9, 10). Additionally, it was demonstrated that TFPI expression is associated with reduced TF activity within plaques. In spite of this apparent modulation, TF activity predominates within plaques. To further define the importance of this imbalance, exogenous recombinant TFPI (rTFPI) has been used to inhibit intravascular thrombosis in several animal models (24-26). The inhibition was achieved at the dose far higher than those present physiologically in plasma and is associated with prolongation in PT. Thus systemic administration of high dose rTFPI can effectively prevent intravascular thrombosis but with a risk of bleeding.

Gene transfer TFPI to vascular wall has been another approach to raise the local TFPI levels (15, 16, 27). The advantage of this strategy is that the locally expressed TFPI can inhibit local TF activity in vivo without concomitant prolongation of PT and PTT. However, the main limitations of this approach are the limited extent and duration of transgene expression (15, 27). Similarly, non-uniform expression of the transgene throughout the experimental period and between animals is often seen. Finally, vascular gene transfer targets multiple cell-types which complicates further analysis of role of TFPI in vascular disease. The model presented here eliminates the disadvantages of somatic gene transfer. Genetically altered transgenic mice can constitutively overexpress TFPI. Furthermore, the SM22α promoter directs the transgene expression in VSMC and allows the study of the role of locally derived TFPI in thrombosis.

This transgenic mouse will allow for the study of VSMC-derived TFPI in disease and development. It is, however, limited by the strength of the selected promoter. These studies indicate that the effects of the transgene expression were greater in mice homozygotic for the transgene. Since the activity of TFPI in carotid arteries of transgenic mice was about 2- to 3-fold higher than nontransgenic mice, we hypothesized that the yolk sac abnormality identified in TFPI null mice might be rescued by transgene expression from the SM22α promoter which was known to be active during development (28). The lack of rescue suggest that TFPI expression in the transgenic mice was not temporally, spatially or of a sufficient level to correct the hemostatic abnormality of the TFPI-/- mice.

In summary, a transgenic mouse was generated to overexpress TFPI in VSMC-specific manner. Local, tissue specific and functional overexpression of TFPI was demonstrated which inhibited local arterial thrombosis. These mice provide a unique resource to study the TF pathway in vascular disease. We are currently studying the role of TFPI in the initiation, development and progression of atherosclerosis using genetically altered mice.

Acknowledgements

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