

Tissue-restricted expression of thrombomodulin in the placenta rescues thrombomodulin-deficient mice from early lethality and reveals a secondary developmental block

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SUMMARY

The endothelial cell surface receptor thrombomodulin (TM) inhibits blood coagulation by forming a complex with thrombin, which then converts protein C into the natural anticoagulant, activated protein C. In mice, a loss of TM function causes embryonic lethality at day 8.5 p.c. (post coitum) before establishment of a functional cardiovascular system. At this developmental stage, TM is expressed in the developing vasculature of the embryo proper, as well as in non-endothelial cells of the early placenta, giant trophoblast and parietal endoderm. Here, we show that reconstitution of TM expression in extraembryonic tissue by aggregation of tetraploid wild-type embryos with TM-null embryonic stem cells rescues TM-null embryos from early lethality. TM-null tetraploid embryos develop normally during midgestation, but encounter a secondary developmental block between days 12.5 and 16.5 p.c. Embryos lacking TM develop lethal consumptive

coagulopathy during this period, and no live embryos are retrieved at term. Morphogenesis of embryonic blood vessels and other organs appears normal before E15. These findings demonstrate a dual role of TM in development, and that a loss of TM function disrupts mouse embryogenesis at two different stages. These two functions of TM are exerted in two distinct tissues: expression of TM in non-endothelial extraembryonic tissues is required for proper function of the early placenta, while the absence of TM from embryonic blood vessel endothelium causes lethal consumptive coagulopathy.

Key words: Thrombomodulin, Placenta, Trophoblast, Parietal endoderm, Tetraploid mouse, Knock-out mouse, Blood coagulation, Consumptive coagulopathy, Cardiovascular system, Lung development, Endothelial cell

INTRODUCTION

Thrombomodulin (TM) is an endothelial cell surface associated, glycosylated transmembrane receptor for activated coagulation factor IIa, the serine protease thrombin (Esmon, 1987; Esmon, 2000). Formation of the TM-thrombin complex alters the substrate specificity of thrombin, abolishing its procoagulant properties, and enhancing the conversion of protein C into the natural anticoagulant, activated protein C (Fuentes-Prior et al., 2000). In concert with protein S, activated protein C proteolytically cleaves activated coagulation factors Va and VIIIa, thereby inactivating the enzyme complexes that generate activated coagulation factors Xa and thrombin. The TM-protein C pathway therefore constitutes a physiological anticoagulant mechanism that controls the activation of the blood coagulation system by suppressing the propagation of thrombin generation.

Inherited defects in the pathway components protein C, protein S, and factor V, are among the most common recognized causes underlying familial prothrombotic disease in humans (Lane et al., 1996a; Lane and Grant, 2000). Left

untreated, the complete or near complete lack of protein C or protein S causes fatal consumptive coagulopathy shortly after birth, secondary to unfettered activation of the coagulation system (Marlar and Neumann, 1990). Carriers of the factor V *Leiden* mutation, which renders activated factor Va resistant to inactivation by activated protein C, do not exhibit severe coagulopathy after birth, but are at a greatly increased risk for venous thrombosis at a later stage in life (Rosendaal et al., 1995; Ridker et al., 1995). In contrast, complete or near complete TM-deficiency has not been recorded in humans (Lane and Grant, 2000), raising the possibility that a severe reduction or complete loss of TM function has more severe consequences than defects in protein C, protein S, and factor V, and may cause embryonic lethality, thus excluding carriers of such mutations from the population.

Genetic disruption of the protein C pathway in mice by elimination of the protein C gene or by introduction of the factor V *Leiden* mutation essentially replicates the phenotype observed in human carriers of the corresponding mutations. (Jalbert et al., 1998; Yin et al., 2000). Ablation of the TM gene causes early post-implantation embryonic lethality that

precedes the establishment of a functional cardiovascular system within the embryo (Healy et al., 1995). The first indications of abnormal development are observed at day 8.5 after fertilization (day 8.5 p.c.) and are manifest as an overall growth retardation. This initial phenotype is invariably followed by rapid and complete resorption of homozygous TM-null embryos within the next 10-12 hours. These findings demonstrated that, in mice, TM is absolutely required for the successful maintenance of pregnancy beyond day 8.5 p.c., and that the consequences of TM deficiency are indeed more severe than, and qualitatively different from, those caused by a lack of protein C or the factor V *Leiden* mutation.

The phenotype of TM-null embryos is set apart from that observed in all other described mouse models lacking coagulation factors or regulators of thrombin generation by three distinguishing hallmarks (Healy et al., 1995; Bi et al., 1995; Toomey et al., 1996; Carmeliet et al., 1996; Bugge et al., 1996; Cui et al., 1996; Lane et al., 1996b; Huang et al., 1997; Wang et al., 1997; Jalbert et al., 1998; Sun et al., 1998; Xue et al., 1998; Dewerchin et al., 2000; Ishiguro et al., 2000): First, embryonic death at E8.5 occurs 12-24 hours before the earliest time of phenotype onset in any of the other knock-out models. Second, while other factor-deficient embryos can be recovered from the implantation site for a prolonged period of time after the initial phenotypic manifestation of the defect, *TM*^{-/-} embryos invariably undergo resorption within 12 hours after phenotype onset. Third, a significant portion of mice lacking prothrombin (Sun et al., 1998; Xue et al., 1998), tissue factor (Toomey et al., 1996; Carmeliet et al., 1996; Bugge et al., 1996; Toomey et al., 1997), tissue factor pathway inhibitor (Huang et al., 1997), or factor V (Cui et al., 1996) escapes midgestational lethality, whereas TM-null mice exhibit a complete penetrance and a uniform onset of the lethal phenotype (Healy et al., 1995).

During the critical time window between E8.5-9.5, TM is expressed within endothelial cells of the visceral yolk sac and within the endothelial cell lineage of the embryo proper (Ford et al., 1993; Weiler-Guettler et al., 1996a). In addition, TM expression is observed in giant trophoblast cells and in parietal endoderm cells (Imada et al., 1987; Weiler-Guettler et al., 1996a), the outermost embryonic tissues of the designated early or yolk sac placenta (Cross et al., 1994). At later developmental stages, TM is expressed at particularly high levels in the developing outflow tract and atrioventricular valves of the heart, in branching airway epithelium of lung buds, in a subpopulation of neural cells, in keratinocytes, and in developing bone (Ford et al., 1993; Buck et al., 1996; Weiler-Guettler et al., 1996a).

The purpose of the present study was to test the hypothesis that the early lethality of TM-null embryos is a placental defect. To this end TM-null ES cells were aggregated with tetraploid wild-type embryos and the developmental potential of the resulting chimeras was determined. In these aggregation chimeras the trophoblast lineages as well as the parietal and visceral endoderm of the early placenta are derived from the tetraploid wild-type embryo, while the embryo proper, including blood vessels, are derived from TM-null ES cells (Nagy et al., 1993; Duncan et al., 1997). We show that TM expression that is limited to non-endothelial cells of the placenta is sufficient to rescue TM-null embryos from early midgestation lethality, and that a loss of TM function in the embryo proper causes a second developmental block at a later stage of pregnancy.

MATERIALS AND METHODS

Animals and embryonic stem cells

Thrombomodulin-deficient D3 TM-null ES cells described earlier were kindly provided by Dr A. Healy (Boston University, Boston, MA, USA) and R1-derived wild-type ES cells were kindly provided by Steven Duncan (Medical College of Wisconsin, Milwaukee, WI, USA). CD-1 mice for aggregation experiments were obtained from Charles River Laboratories (Portage, MI, USA). All animals were housed and the experiments were performed in the Oscar F. Peterson Animal Resource Center at the Medical College of Wisconsin, following standards and procedures approved by the Animal Care and Use Committee of the Medical College of Wisconsin (protocol # 318-97-1).

Generation homozygous TM-deficient R1 ES cells

Inactivation of the TM gene in R1 ES cells by insertion of a β -galactosidase gene cassette was performed as previously described (Weiler-Guettler et al., 1996b). Correctly targeted heterozygous ES cell clones (*TM*^{lacZ}) were identified by PCR (Fig. 1A,B). The sequence of the sense primer (P1, 5'-att tgg gaa gag aga agc tca gtt-3') was derived from a region of the endogenous TM promoter outside of the targeting vector sequence. The sequence of the antisense primer was derived either from the coding region of the *lacZ* gene (P2, 5'-atg tgc tgc aag gcg att aag ttg g-3'), resulting in a 2430 bp fragment, or the coding region of the endogenous *TM*-sequence (P3, 5'-ggc cct aaa tcc ata ctg ctg t-3'), yielding a 2751 bp fragment in the absence of the targeted insertion. Gene conversion of the *TM*^{lacZ} locus was induced by growing ES cells for 2 weeks in complete medium supplemented with 2.0 mg/ml geneticin (Mortensen et al., 1992; Weiler-Guettler et al., 1998) and homozygous TM-deficient clones (*TM*^{lacZ/lacZ}) were identified by Southern blot analysis as described.

Production of ES cell-tetraploid embryo aggregation chimeras

Aggregation of ES cells with tetraploid wild-type embryos was achieved essentially as described previously (Nagy et al., 1993; Duncan et al., 1997). Two-cell-stage embryos were isolated from wild-type CD-1 mice and fused by a single electric pulse (50 V, 40 μ seconds, DC) applied by a pulse generator (CF 100; Biochemical Laboratory Service, Budapest, Hungary). Tetraploid embryos were cultured for 20 hours and the resulting four-cell-stage embryos were aggregated with clusters of 10-15 *TM*^{lacZ/lacZ} ES cells after removal of the zona pellucida with acidic Tyrode's solution. The aggregates were cultured for 24 hours and subsequently transferred into the uteri of 2.5-day pseudopregnant CD-1 recipient mice. Culture media and reagents for tetraploid embryo aggregation were purchased from a commercial provider (Specialty Media, Phillipsburg, NJ).

Developmental assessment and genotyping of embryos

Embryos were assigned (1) a developmental age given as E8.0-E10.25, reflecting the developmental progress of the embryo in days, and (2) a Theiler stage reflecting the developmental stages reached by the embryo, based on morphological criteria (Kaufman, 1994). The developmental progress of the embryo was then correlated with the gestational age, with day 0.5 p.c. (post coitum) defined as the morning a vaginal plug was detected. Normally, Theiler stages 12-15 correlate with developmental ages of E8.0-10.25, and a pregnancy duration of 8.5-10.25 days. A delay or block of fetal development was defined as a discrepancy between Theiler stage/developmental age and pregnancy duration.

For genotyping of TM-null embryos retrieved after aggregation with tetraploid wild-type blastomers, genomic DNA was prepared from the upper third of the embryo only, to avoid contamination with wild-type visceral endoderm attached to the yolk sac at the hindgut. To determine the sensitivity of the PCR protocol employed to detect wild-type DNA in tetraploid embryos, known amounts of DNA isolated from in vitro cultured *TM*^{lacZ/lacZ} and wild-type ES-cells were

mixed at mass ratios ranging from 1:10 to 1:100000 ($TM^{+/+}$ DNA to $TM^{-/-}$ DNA) and the mixture was analyzed by PCR. Wild-type DNA was reliably detected when present at $\geq 0.1\%$ (w/w), and therefore the detection limit for the presence of wild-type cells in $TM^{lacZ/lacZ}$ embryos was estimated to be 0.1%.

Analysis of β -galactosidase gene expression immunohistochemistry and TM-ELISA

Detection of *lacZ* gene expression (β -gal) in whole-mount preparations of embryos, and immunohistochemical detection of TM in paraffin-embedded sections with the TM-specific monoclonal antibodies 34A and 201B (Kennel et al., 1988) were performed exactly as previously described (Weiler-Guettler et al., 1996a). Immunohistochemical detection of fibrin/fibrinogen was performed with a polyclonal rabbit anti-human fibrin(ogen) antibody (Dako, Carpinteria, CA, USA) and HRP-conjugated secondary goat anti-rabbit IgG antibody (Jackson Immuno Research, West Grove, PA, USA). HRP activity was visualized using DAB according to the manufacturer's instructions (Vector Laboratories, Burlington, CA, USA). For measurement of TM antigen in the embryo proper of E14.5 aggregation chimeras, embryos were dissected free of maternal tissue and extraembryonic membranes and disrupted in ice-cold 1 mM $NaNO_3$, 0.5% NP40, 10 μ g/ml PMSF, PBS. Homogenates were centrifuged for 10 minutes at 6000 rpm at 4°C, and TM levels in the supernatant were quantitated using a sandwich ELISA with monoclonal anti-mouse TM antibodies (a gift from S. Kennel, Oak Ridge National Laboratory, TN, USA). TM antigen was captured with immobilized 34A antibody, and detected through incubation with a biotinylated TM antibody 201B, followed by horseradish peroxidase (HRP) conjugation with ABC (Vector Laboratories, Burlington, CA, USA) reagent and assessment of HRP activity.

Morphometric analysis of lung and vasculature

Cellular glycogen content in lung epithelium was assessed by periodic acid/Schiff (PAS) staining. Sections were placed in 0.5% periodic acid for 10 minutes, briefly washed in H_2O , incubated in Schiff's reagent (Sigma, St. Louis, MI, USA) for 30 minutes at room temperature, washed in running tap water for 5 minutes, and counterstained with Hematoxylin. The percentage of less differentiated, PAS-positive lung tubules and lung buds relative to the total number of lung buds and airways was determined in comparative sections from TM^{lacZ} and $TM^{lacZ/lacZ}$ embryos. The area covered by less differentiated, PAS-positive lung epithelium relative to total lung area was determined using Metamorph software (Universal Imaging Corporation, West Chester, PA 19380, USA). Morphology of the yolk sac vasculature was assessed using photomicrographs of whole-mounts and counting the number of vessels with a diameter larger than 50 μ m per unit area, as well as the number of branching points. Capillary density was used as an indicator for vascular development in TM^{lacZ} and $TM^{lacZ/lacZ}$ embryos (Thurston et al., 1999). β -gal-positive vessel profiles per unit area were counted in 8 sections of abdominal subcutaneous tissue obtained from at least 6 embryos of each genotype. Values for TM^{lacZ} and $TM^{lacZ/lacZ}$ embryos were compared using Student's *t*-test.

RESULTS

Generation of homozygous TM-deficient ES cells

We have previously described the targeted inactivation of the *TM* gene in the D3 embryonic stem (ES) cell line and subsequent derivation of homozygous TM-null ES cells by selection in high concentrations of G418 (Healy et al., 1995; Healy et al., 1998). These D3-derived TM-null cells have been successfully employed to generate chimeric mice with a significant ES cell contribution to all major organ systems of

the adult animals (Healy et al., 1998). Pilot experiments with wild-type D3 ES cells, heterozygous and homozygous TM-deficient D3-derived ES cells indicated that the D3 cell line yielded only marginal success rates in attempts to generate entirely ES cell derived embryos after aggregation with CD1 tetraploid embryos (data not shown). In contrast, aggregation experiments conducted with the R1 ES cell line (Nagy et al., 1993) consistently yielded intact embryos at various developmental stages, including term (not shown).

Therefore, we inactivated the *TM* gene in R1 ES cells, following a previously described targeting strategy that causes a complete loss of TM function and places the bacterial β -galactosidase reporter gene (*lacZ*) under the transcriptional control of the endogenous TM promoter (Weiler-Guettler, 1996a; Weiler-Guettler, 1996b; Fig. 1A,B). Three independently derived ES clones carrying the desired TM^{lacZ} mutation were obtained and exposed to high G418 concentration in order to induce homozygosity of the altered *TM* locus (Fig. 1A,C). Homozygous $TM^{lacZ/lacZ}$ ES cell clones were established from two independent parental TM^{lacZ} clones, and two $TM^{lacZ/lacZ}$ clones derived from each parental TM^{lacZ} clone were subsequently employed in aggregation experiments.

TM protein was undetectable by ELISA in whole tissue extracts of E14.5 embryos derived from $TM^{lacZ/lacZ}$ ES cell tetraploid aggregations (Fig. 1D). Likewise, the expression pattern of the *lacZ* reporter gene in tetraploid embryos derived from $TM^{lacZ/lacZ}$ R1 ES cells was identical to that in mice derived from TM^{lacZ} targeted D3 ES cells (see below). This shows that, in R1 cells, the TM^{lacZ} mutation has disrupted *TM* gene function and placed the *lacZ* gene under the control of the endogenous TM promoter, exactly as described earlier for the TM^{lacZ} mutation in D3 ES cells.

Tetraploid aggregation rescues TM-null embryos from early midgestation lethality and reveals a second developmental block

A total of 253 and 96 aggregates of wild-type tetraploid embryos with $TM^{lacZ/lacZ}$ and TM^{lacZ} ES cells, respectively, were transferred into pseudopregnant recipient mice. Embryos were recovered for analysis between E10.5 and term, counting the day of transfer into pseudopregnant recipient mothers as

Table 1. Total number of embryos obtained from aggregation experiments

Embryonic age (d.p.c.)	$TM^{lacZ/lacZ}$				TM^{lacZ}		
	Total	Normal	Bleeding	Dead	Total	Normal	Dead
10.5	11	11 (8)	0	0	4	4 (1)	0
12.5	3	1	1	1	1	1	0
14.5	8	0	6	2	9	8	1
16.5	3	0	1	2	1	1	0
19.5	4	0	0	4	7	7	0
Total	29	12 (8)	8	9	22	21 (1)	1

Wild-type tetraploid embryos were aggregated with either TM-null ($TM^{lacZ/lacZ}$) or TM-expressing (TM^{lacZ}) ES cells. Embryos were isolated at pregnancy days as indicated. Embryos were phenotyped and staged on the basis of somite number (day 10.5 p.c. and 12.5 p.c.) or crown-rump length (14.5 p.c. to 19.5 p.c.), and morphological criteria. 'Normal' denotes embryos that did not exhibit any morphological anomalies and lacked signs of intrauterine bleeding. Figures in brackets are the number of embryos that were too small for the duration of pregnancy but appeared otherwise normal. No TM^{lacZ} embryo showed any sign of intrauterine bleeding.

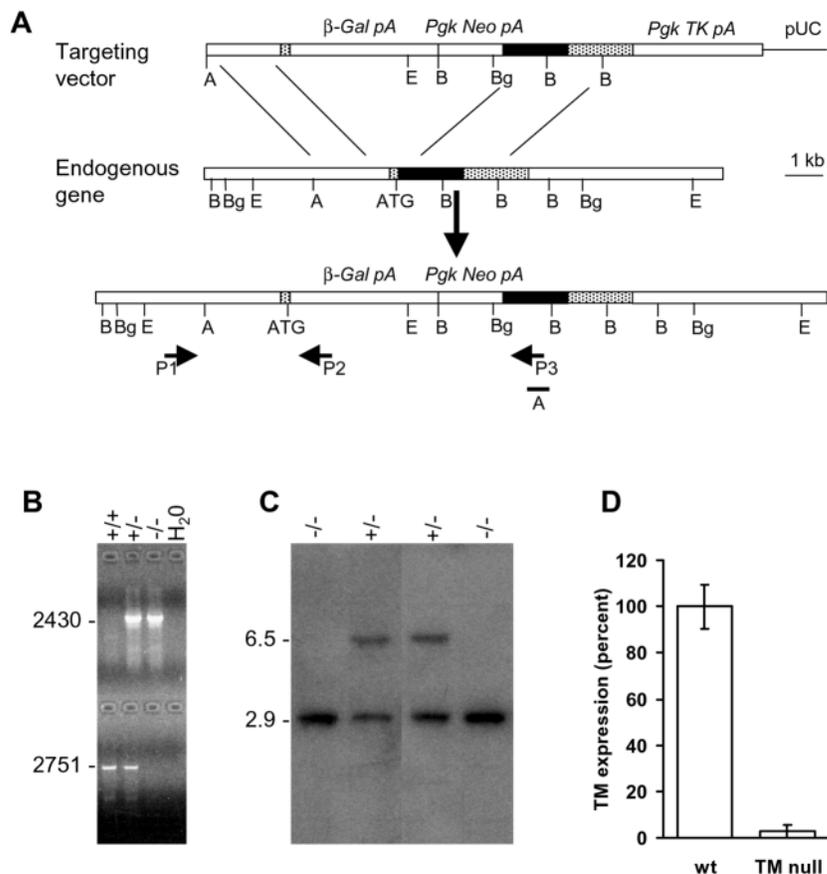


Fig. 1. Gene targeting of the thrombomodulin (*TM*) locus. (A) Crossover between the endogenous gene and *TM* sequences flanking the β -galactosidase (β Gal-*pA*) and the neomycin genes (*pgk Neo pA*) results in fusion of the *lacZ* gene to the promoter of the endogenous *TM* gene. The *TM* mRNA encoding region and the *TM* open reading frame are represented by stippled and black boxes. *pgk TK pA*, thymidine kinase gene expression cassette; A, *Apa*I; B, *Bam*HI; E, *Eco*RI; Bg, *Bg*III. (B) The structure of targeted ES cells (*TM^{lacZ}*) was analyzed by PCR, as described in Materials and Methods. (C) Following gene conversion, homozygous *TM*-deficient (*TM^{lacZ/lacZ}*) ES cells were identified using Southern blot hybridization. *Bam*HI-digested ES cell DNA was hybridized with probe A, as indicated in A, detecting a 6.5 kb and 2.9 kb fragment in wild-type (wt) and *TM^{-/-}* cells, respectively. (D) Absence of *TM* expression in E14.5 *TM^{lacZ/lacZ}* ES cell-derived embryos. *TM* antigen was determined by ELISA. *TM* antigen per total protein is undetectable in *TM^{lacZ/lacZ}* embryos ($n=4$). *TM* content of wild-type embryos ($n=8$) was arbitrarily set at 100%.

E2.5. The results are summarized in Table 1. The majority of *TM^{lacZ/lacZ}* embryos (86%) showed clear morphological evidence of developmental progress beyond Theiler stage 13 (E9), in contrast to the complete abortion, between days 8.5 and 9.5 p.c., of *TM*-null embryos obtained through natural mating. Rescue of *TM^{lacZ/lacZ}* embryos from early midgestational lethality was achieved with all 4 established *TM^{lacZ/lacZ}* ES cell lines, and the shown results are a composite of these experiments. The contribution of wild-type cells to *TM^{lacZ/lacZ}* embryos was less than 0.1% (see Materials and Methods), consistent with the absence of *TM* antigen in whole embryo extracts (see above). This demonstrates, that the embryo proper of *TM^{lacZ/lacZ}*-tetraploid aggregation chimeras was indeed exclusively derived from *TM^{lacZ/lacZ}* ES cells.

Eight of 11 *TM^{lacZ/lacZ}* embryos retrieved at day 10.5 p.c. exhibited a delay in developmental progress relative to the gestational age, yet appeared morphologically intact. Cardiac contractions were apparent in

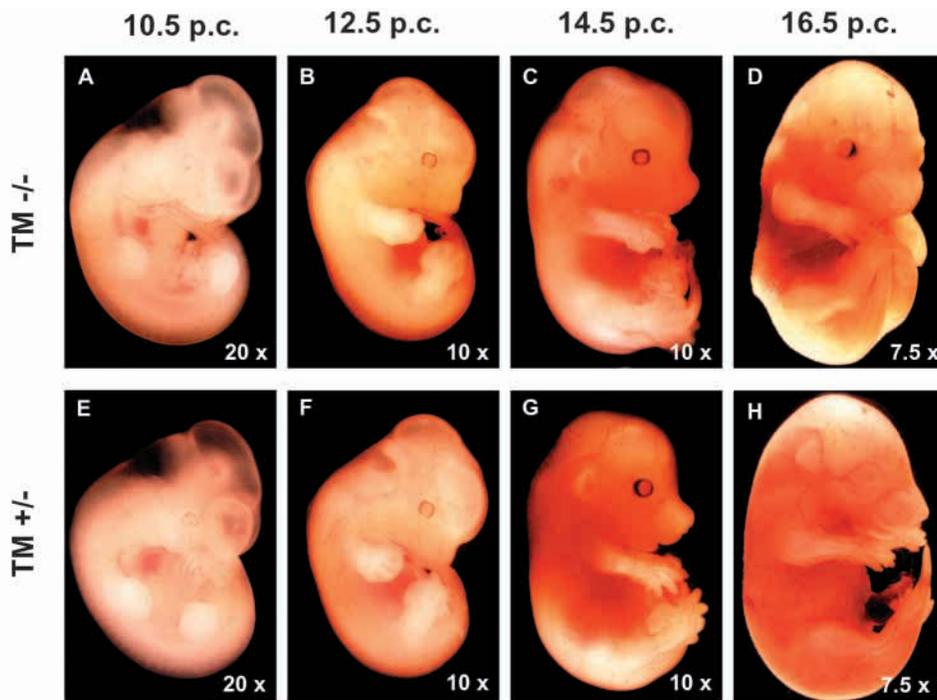


Fig. 2. *TM* expression in the placenta rescues *TM*-null embryos from midgestational lethality. (A-D) Aggregation of R1-derived *TM^{lacZ/lacZ}* ES cells with wild-type tetraploid embryos sustains development of *TM^{lacZ/lacZ}* embryos until day 16.5 p.c. The developmental stage reached by *TM^{lacZ/lacZ}* embryos is consistent with pregnancy duration (days p.c., shown on top of figure). (E-H) Control embryos derived from heterozygous *TM^{lacZ}* ES cells. Original magnifications are indicated at lower right.

Fig. 3. Intrauterine hemorrhage of TM-null embryos during the second half of gestation. *TM^{lacZ/lacZ}* embryos develop intrauterine hemorrhage, leading to intrauterine death. (A-C) Whole-mount *TM^{lacZ/lacZ}* tetraploid embryos, stage E14.5. In severe cases, the yolk sac vasculature is bloodless, but appears otherwise morphologically intact (A, arrows). Bleeding into subcutaneous tissue follows the pattern of cutaneous vessels and capillaries (B), and is frequently associated with marked subcutaneous edema (C, arrows). (D) Whole-mount X-gal staining of a *TM^{lacZ/lacZ}* embryo (E14.5). Blood pools are seen outside the microvasculature, indicating bleeding as opposed to vessel dilatation. (E,F) Hematoxylin and Eosin (H and E) stained sections, of paraffin embedded, whole-mount X-gal-stained embryos (E14.5). Blood cells are seen outside the subcutaneous microvasculature, and the surrounding tissue appears edematous (E). Blood pools in the peritoneal cavity (F, arrowheads; arrow indicates embryonic kidney). Original magnifications are indicated at lower right.

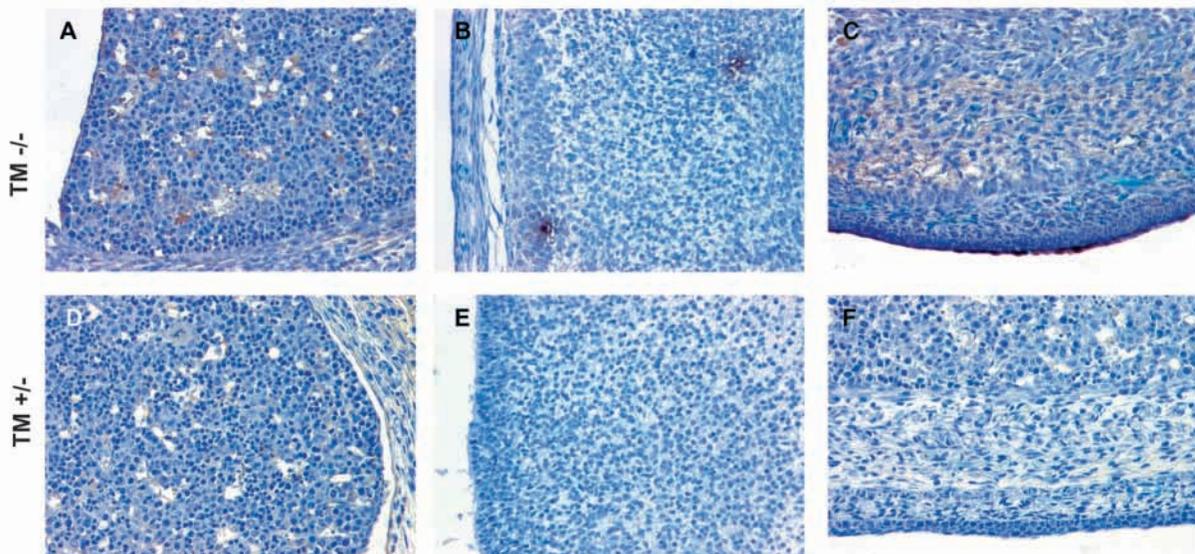
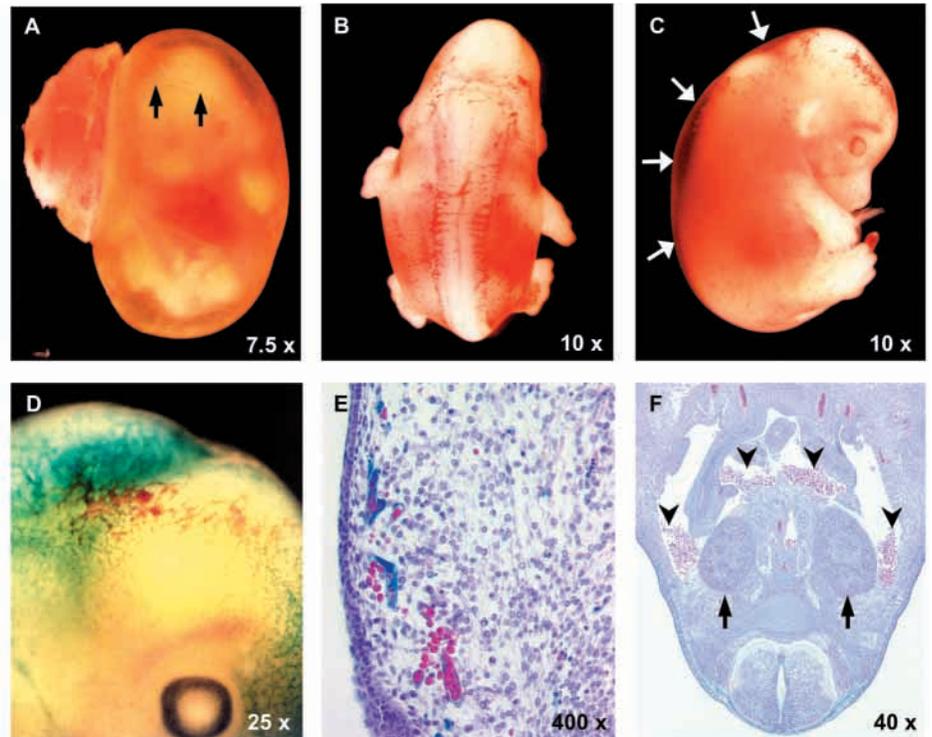


Fig. 4. Increased fibrin(ogen) deposition in E14.5 TM-null embryos. Fibrin(ogen) in paraffin sections of *TM^{lacZ/lacZ}* (A-C) or heterozygous *TM^{lacZ}* (D-F) embryos was detected with polyclonal anti-mouse fibrin(ogen) antibody. Hematoxylin counterstain. (A,D) Liver: increased fibrin(ogen) staining in hepatic blood vessels and sinus of TM-null mice. Note partial to complete occlusion of intrahepatic blood vessels. (B,E) Brain: pericapillary fibrin(ogen) staining in *TM^{lacZ/lacZ}*, but not in *TM^{lacZ}* embryos. (C,F) Skin: increased fibrin(ogen) staining in the subcutis of *TM^{lacZ/lacZ}* embryos accompanies widening of intracellular spaces, consistent with subcutaneous edema and plasma exudation. Original magnification of all images is 400 \times .

7 of these embryos at the time of recovery and no signs of necrotic changes or hemorrhage were observed. In addition, 4 of these embryos had proceeded to a developmental stage (Theiler stage 14, E9.0-9.5) that is never reached by TM-deficient embryos obtained from natural mating. The remaining three *TM^{lacZ/lacZ}* embryos recovered at day 10.5 p.c. had reached a developmental stage that was consistent with the

gestational age, and they were indistinguishable from control embryos derived from aggregation experiments with *TM^{lacZ}* ES cells (Fig. 2A,E).

The developmental progress of all *TM^{lacZ/lacZ}* embryos retrieved at days 12.5 and 14.5 p.c. was consistent with the gestational age (Theiler stages 20-22, Fig. 2B,C,F,G). Conversely, the developmental progress and gestational age

were congruent only in one of the three $TM^{lacZ/lacZ}$ embryos obtained at day 16.5 p.c. (Fig. 2D,H). This embryo displayed minor signs of bleeding, but was viable as indicated by cardiac contractions. The other two embryos were necrotic and partially degraded. Similarly, 4 $TM^{lacZ/lacZ}$ embryos retrieved at term (day 19.5 p.c.) were found in advanced stages of resorption. Distinct morphological hallmarks (eyelid fusion or whisker rudiments) suggested that all $TM^{lacZ/lacZ}$ embryos recovered dead at day 16.5 p.c. or term had reached at least Theiler stages 20 (E12) before dying. Control experiments with TM^{lacZ} ES cells yielded 7 normally developed embryos at term, including 3 live-born pups.

These results show, first, that expression of TM limited to non-endothelial embryonic cells in the placenta is sufficient to overcome the lethal developmental defect that causes the resorption of completely TM-deficient embryos derived from natural mating. Second, despite rescue from this primary defect, TM deficiency within the embryo proper is incompatible with survival to term and causes death in the second half of gestation before E16.5.

TM-null embryos succumb to fatal hemorrhage in the second half of pregnancy

Although overall developmental progress was normal in $TM^{lacZ/lacZ}$ embryos retrieved between day 14.5 and 16.5 p.c., embryos exhibited either varying degrees of hemorrhage (7 of 11 embryos) or were dead (e.g. lacked cardiac contractions; 4 embryos). Two mice recovered at E14.5 showed massive bleeding into the yolk sac cavity that had drained the entire yolk sac vasculature (Fig. 3A). Blood within the yolk sac cavity did not clot, consistent with consumption of coagulation factors. Extensive subcutaneous bleeding was observed in the skin over the head, the back, the tip of the tail, and the snout (Fig. 3B). Edematous subcutaneous swelling was associated with bleeding in the skin in five cases (Fig. 3C). Pericardial or peritoneal effusion occurred in two embryos. Bleeding was mostly restrained to perivascular spaces, as judged by the presence of red blood cells outside the β -gal-positive embryonic vasculature (Fig. 3D,E). The latter observation indicates that the macroscopically observed blood pools did not simply represent hyperemic and dilated vasculature, and suggests a petechial, rather than ecchymotic, bleeding. In addition, histological examination of the liver revealed subcapsular hemorrhage and necrosis of individual hepatocytes. Bleeding into the peritoneal (Fig. 3F) and pleural cavity as well as into the first ventricle of the brain was noticed in one embryo, which was alive when retrieved from the uterus. None of the 8 TM^{lacZ} ES cell-derived embryos retrieved alive at day 14.5 p.c. displayed any sign of hemorrhage.

To assess whether embryonic hemorrhage was associated with an activation of the coagulation system, deposition of fibrin(ogen) was examined by immunostaining of sections from embryos which were alive when isolated from the uterus (heartbeat, pulsatile blood flow). Fibrin(ogen) staining in the liver of $TM^{lacZ/lacZ}$ embryos was increased, compared to TM^{lacZ} embryos. In $TM^{lacZ/lacZ}$ embryos, fibrin(ogen) deposits were most abundant in the liver interstitium and within blood vessels, while in liver sections of TM^{lacZ} embryos fibrin(ogen) was located mostly intracellularly (Fig. 4A,D). Enhanced perivascular fibrin(ogen) staining was observed in the brain of

$TM^{lacZ/lacZ}$, but not in TM^{lacZ} embryos (Fig. 4B,E). Increased fibrin(ogen) staining was occasionally observed in subcutaneous capillaries of $TM^{lacZ/lacZ}$ embryos (not shown). Fibrin(ogen) deposition was augmented in subcutaneous tissue and was associated with widening of extracellular spaces, consistent with plasma exudation and subcutaneous edema (Fig. 4C,F). Fibrin(ogen) deposition in the lung, the heart, large vessels, kidneys, gonads and intestine was unremarkable and was indistinguishable from that observed in TM^{lacZ} embryos.

These observations demonstrate that TM-deficient embryos succumb to fatal intrauterine hemorrhage between E12 and 16. The bleeding diathesis of $TM^{lacZ/lacZ}$ embryos is associated with increased fibrin(ogen) deposition, consistent with excessive activation and consumption of coagulation factors as the underlying cause of the observed bleeding diathesis.

Normal development of the placental and embryonic vasculature in the absence of TM

The presence of the TM^{lacZ} mutation in embryonic, but not in maternal, blood vessels of tetraploid aggregation chimeras was exploited to examine whether the absence of TM from endothelial cells of embryonic origin affected the establishment of the vascular plexus within the labyrinth layer of the placenta. Whole mount staining for β -galactosidase revealed a well elaborated vascular tree at the embryonic side of the placenta, with embryonic vessels protruding well into the placenta tissue (Fig. 5A). β -gal-positive embryonic vessels were in close proximity to maternal blood sinuses (Fig. 5 B,C), indicating the establishment of an intact labyrinthine layer. No overt thrombosis was observed in either maternal blood spaces or embryonic vessels. The lumen of TM-deficient embryonic vessels were generally patent and filled with embryonic red blood cells, indicating that the fetal circulation was functional. Embryonic vascular integrity appeared preserved, as judged from the mutually exclusive confinement of nucleated embryonic and anucleated maternal erythrocytes to their respective vascular compartments. Of note, labyrinth formation also occurred if the embryo itself was delayed/arrested in developmental progress, indicating that the establishment of the labyrinthine layer is at least partially independent of the fate of the embryo proper. To determine whether TM expression remains restricted to giant trophoblast and parietal endoderm cells during midgestation, placenta tissue from wild-type mating was obtained at day 10.5 p.c. and immunohistochemically stained. Strong TM expression was detected on giant trophoblast cells and parietal endoderm (Fig. 5D,E), but not on diploid trophoblast cells.

In all $TM^{lacZ/lacZ}$ embryos retrieved at day 10.5 p.c. the yolk sac vasculature appeared grossly normal (Fig. 6A). Pulsatile blood flow in yolk sac vessels was observed in 9 embryos that exhibited visible cardiac contractions at the time of dissection. No grossly detectable thrombosis was observed. A morphometric analysis of yolk sac vascular architecture was performed to detect more subtle alterations, such as those described in mice lacking the inhibitor of the coagulation system, tissue factor pathway inhibitor (Huang et al., 1997). Vascular density (number of vessels per section), size and distribution of yolk sac vessels, branching pattern (number of branches on vessels with $>50 \mu\text{m}$ diameter per unit length), and capillary branching (branching

points per unit area) were the same in the yolk sac of $TM^{lacZ/lacZ}$ and TM^{lacZ} embryos (data not shown). Likewise, the morphology of arteries, veins and capillary beds in the embryo proper of $TM^{lacZ/lacZ}$ mice retrieved from E10.5 to 14.5 was indistinguishable from that observed in TM^{lacZ} mice (Fig. 6B,C,D). The average capillary density in subcutaneous tissue of the lower abdominal wall of $TM^{lacZ/lacZ}$ embryos was similar to that measured in TM^{lacZ} controls (22.5 ± 7.3 and 24.6 ± 3.9 capillaries per field, respectively; Fig. 7C,D). Cardiac development in $TM^{lacZ/lacZ}$ embryos was unremarkable at all stages examined (E10.5–14.5 p.c.). The heart lacked necrotic changes, was lined with an intact endocardium, and formation of the aortico-pulmonary spiral septum (Fig. 6C) and cardiac valves (Fig. 6D), where high TM expression during mouse development has been previously demonstrated (Ford et al., 1993; Weiler-Guettler et al., 1996a), was normal.

TM is not required for normal organogenesis

A crucial function of TM during lung development has been proposed, based on the spatially and temporally highly regulated expression of TM in branching lung epithelium and in mesenchymal tissue surrounding differentiated airways (Ford et al., 1993; Buck et al., 1996; Weiler-Guettler et al., 1996a). Analysis of histological sections of E10.5–14.5 $TM^{lacZ/lacZ}$ embryos showed that lung development proceeds normally during this developmental period in the absence of TM. Morphogenic patterning of large blood vessels accompanying central airways and establishment of a capillary network in mesenchyme surrounding peripheral lung buds proceeded normally in the absence of TM. The number of glycogen rich (PAS positive) lung buds and immature lung epithelium normalized to the number of further differentiated (PAS negative) airway tubules were identical in TM-null and TM-expressing embryos (ratio PAS-positive/PAS-negative airway tubules: 9.0 and 8.9 for $TM^{lacZ/lacZ}$ and TM^{lacZ} embryos, respectively; Fig. 7A,B). No quantitative differences were observed in the maturation of lung bud epithelium into differentiated airway epithelium, reflected in the area of glycogen-rich immature airway epithelium compared to the total lung area (percentage of PAS-positive area: $5.5\% \pm 1.3\%$ and $7.5\% \pm 4.0\%$ in $TM^{lacZ/lacZ}$ and TM^{lacZ} embryos, respectively).

Likewise, other sites where TM is expressed during development appeared normal upon histological examination. In areas not affected by subcutaneous bleeding (see above), overall skin architecture and organization of the basal keratinocyte layer was normal (Fig. 7C,D). β -galactosidase staining was observed in keratinocytes other than those of the basal cell layer. Hair follicles of the skin and the vibrissae showed normal architecture and beginning hair formation consistent with the gestational age of the embryo. The leptomeninges were well elaborated and vascularized, and were generally free of thrombi. Chondroblast formation and early bone morphology were indistinguishable between TM^{lacZ} and $TM^{lacZ/lacZ}$ embryos. The male urogenital tract, including testis rudiments, formed normally. Development of the female reproductive tract could not be studied since the R1 ES cell line is genetically male. All other abdominal and thoracic organs were present, positioned correctly, and appeared morphologically unremarkable.

DISCUSSION

Our results show that the selective reconstitution of TM function in trophoblast and parietal endoderm cells of the early placenta prevents the early embryonic lethality of TM-deficient mice. Rescued embryos develop normally through midgestation, but subsequently succumb to lethal intrauterine hemorrhage. These findings demonstrate that a loss of TM function disrupts mouse embryogenesis at two different developmental stages, and document a crucial role for TM in two different tissues. Expression of TM in non-endothelial cells of the placenta is required for proper function of the early placenta, while the absence of TM from blood vessel endothelium causes excessive activation of the embryonic blood coagulation system.

Immunohistochemical detection of TM antigen in wild-type mice and analysis of β -galactosidase expression in heterozygous TM^{lacZ} mice consistently demonstrate that, until day 10.5 p.c., TM expression is restricted to giant trophoblast and parietal endoderm cells of the early placenta, and blood vessel endothelium of the embryo proper. In aggregation chimeras between $TM^{lacZ/lacZ}$ ES cells and tetraploid wild-type embryos, giant trophoblast and parietal endoderm cells are the only sites where tetraploid embryo aggregation restores TM expression. Consequently, it is the surface of these cells where TM expression must be required for the successful maintenance of pregnancy. In contrast, the absence of TM from embryonic blood vessel endothelium is compatible with proper function of both the yolk sac and chorioallantoic placenta. These observations provide direct and unambiguous experimental evidence that the early lethality of TM-null embryos is caused by a placental defect in giant trophoblast cells and/or parietal endoderm. The current results are entirely consistent with our earlier finding that the growth arrest of the TM-null embryo proper can at least in part be overcome by in vitro culture of mutant embryos (Healy et al., 1995) after removal of extraembryonic membranes.

In mice, primary giant trophoblast cells, parietal endoderm and Reichert's membrane, a layer of extracellular matrix separating the latter cell types (Muntener and Hsu, 1977), form the early yolk sac placenta as the major route for the exchange of nutrients, blood gases, and waste products between the embryo proper and the mother. With continued embryonic growth, this route of maternal-embryonic exchange becomes insufficient, and the chorioallantoic placenta develops (Cross et al., 1994). Defects in parietal endoderm or in secondary giant trophoblast cells may cause a malfunction of the early placenta and lead to embryonic lethality at or before day 9.5 p.c. (Williamson et al., 1997; Riley et al., 1998; Smyth et al., 1999). A failure to establish the chorioallantoic placenta leads to a later developmental block after day 9.5 p.c. (Li et al., 1992; Guillemot et al., 1994; Uehara et al., 1995; Kwee et al., 1995; Luo et al., 1997; Morasso et al., 1999). Since TM-null embryos are resorbed before day 9.5 p.c., the demise of TM-null embryos must be attributed to a defect of the early placenta rather than a failure of chorioallantoic placenta formation. The early placenta of TM-null mice is at least initially (i.e. until E8) functional, and no readily apparent morphological abnormalities of trophoblast derivatives, parietal endoderm, or Reichert's membrane are evident in the placenta at E8.5 (Healy et al., 1995), suggesting that cell lineage establishment and

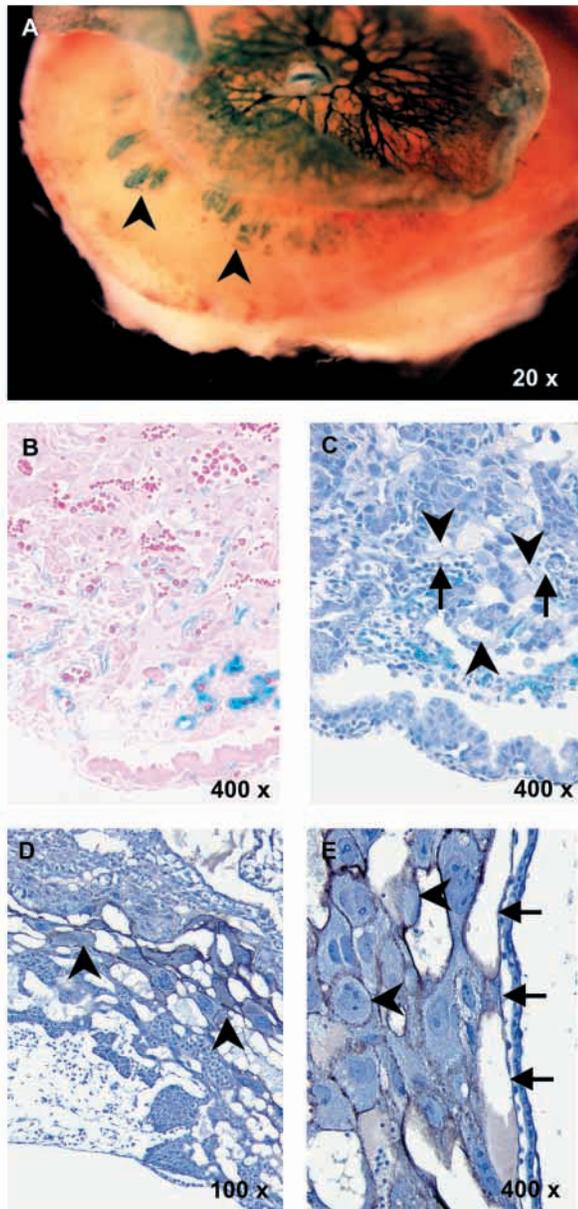


Fig. 5. TM expression in the placenta: Normal labyrinthine layer formation in the absence of TM and high TM expression in giant trophoblast cells and parietal endoderm. (A) Microphotograph of a whole mount placenta of a $TM^{lacZ/lacZ}$ tetraploid embryo obtained at day 10.5 p.c. and stained for β -gal. Embryo-derived, TM-deficient blood vessels form an elaborate vascular tree at the embryonic side of the placenta, and protrude well into the labyrinthine layer of the placenta (arrowheads). (B,C) Histological sections of paraffin embedded, E10.5 p.c. placenta of a $TM^{lacZ/lacZ}$ tetraploid embryo and stained for β -gal. Consecutive sections stained with Eosin (B) and Hematoxylin (C). Embryonic β -gal-positive vessels form an elaborate vascular network among trophoblast cells (B), contacting maternal blood lacunae, identified by anuclear erythrocytes (C, arrowheads; embryonic vessels marked by arrows). (D,E) Immunohistochemical detection of TM in the E10.5 p.c. placenta. Brown-black HRP reaction product identifies TM antigen, Hematoxylin counterstain. TM expression is detected in giant trophoblast cells (arrowheads in D and E) and parietal endoderm (arrows in E), but is absent from diploid trophoblast (D). Original magnifications indicated at lower right.

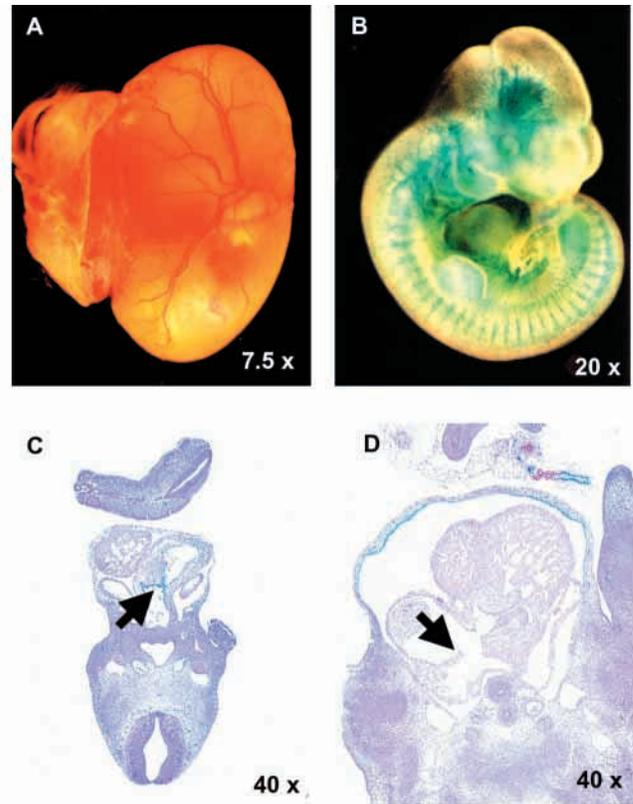


Fig. 6. Normal cardiovascular development in TM-null embryos. Normal vascular morphogenesis in the yolk sac and embryo proper in the absence of TM. (A) Microphotograph of E14.5 $TM^{lacZ/lacZ}$ yolk sac. (B) Whole-mount E10.5 $TM^{lacZ/lacZ}$ embryo stained for β -gal. (C,D) Histological sections of paraffin embedded, whole-mount $TM^{lacZ/lacZ}$ embryos obtained at day 10.5 p.c. stained for β -gal (C) and 12.5 p.c. (D), H and E stain. Note intact endocardium, normal formation of the aorticopulmonary spiral septum (arrow in C) and cardiac valves (arrow in D). Original magnifications are indicated at lower right.

differentiation of the above cell populations does not depend on TM.

Despite the rescue from early midgestation lethality by aggregation with tetraploid wild-type embryos, TM-deficient mice suffer from a hemorrhagic diathesis that is incompatible to survival beyond E16. This bleeding phenotype observed in TM-deficient aggregation chimeras is almost identical to that described in mice lacking the substrate of the TM-thrombin complex, protein C (Jalbert et al., 1998). This demonstrates that potential alternate pathways for the activation of protein C (Freysinet et al., 1991; Rezaie, 1998) probably have no physiological relevance during embryogenesis. In both animal models, bleeding occurs as early as E12.5 in multiple tissue sites, including subcutaneous tissue and brain ventricles. Hemorrhage is associated with increased perivascular fibrin(ogen) deposition, consistent with consumptive coagulopathy as the underlying cause of death. Protein C-deficient embryos survive until birth, while no TM-null embryo developed beyond E16. The somewhat more severe secondary bleeding phenotype of TM-null mice may reflect protein C-independent anticoagulant activities of TM (Dittman and Majerus, 1990; Koyama et al., 1991; Wang et al., 1998), but

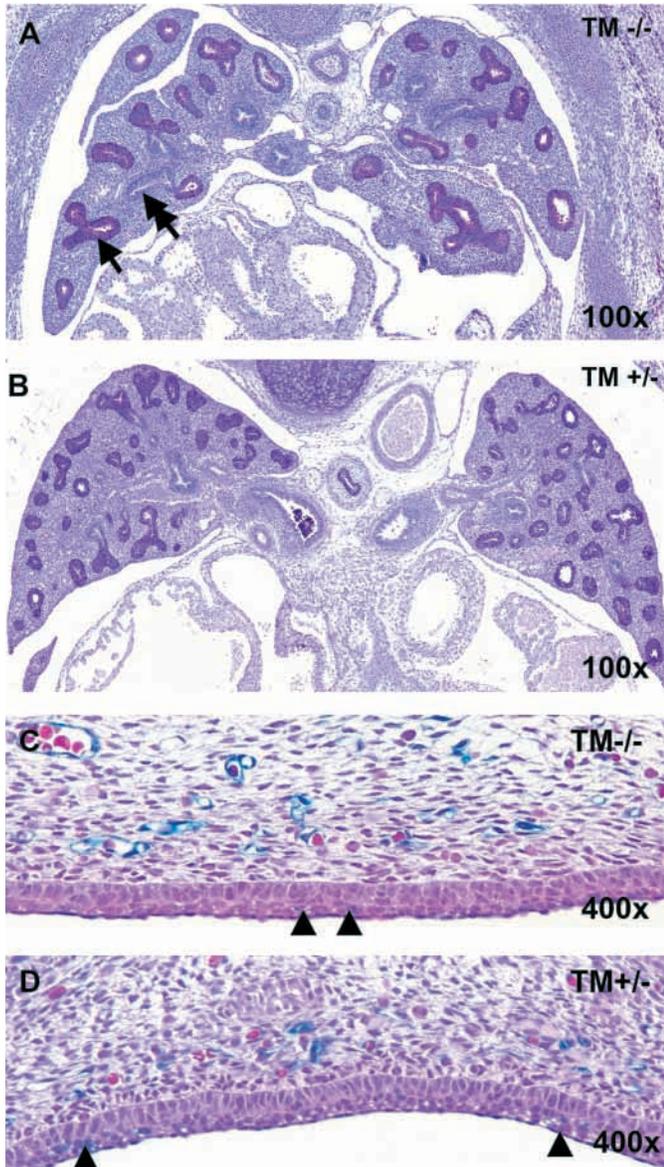


Fig. 7. Lung and skin morphology in $TM^{lacZ/lacZ}$ and TM^{lacZ} . (A,B) High glycogen content in less differentiated lung epithelium was detected using periodic acid/Schiff (PAS) reagent. (A) Extensive branching has occurred in the lung of an E14.0 $TM^{lacZ/lacZ}$ embryo, and numerous PAS-positive lung buds (arrow) are apparent. Some airways lack glycogen (double arrow), indicating that they are lined by differentiated lung epithelium. (B) Lung morphology in a TM^{lacZ} embryo is comparable to the lung of a $TM^{lacZ/lacZ}$ embryo, indicating that TM is not necessary for lung branching and airway differentiation. The increased number of lung tubules in the lung of the E15.0 TM^{lacZ} corresponds with its slightly advanced stage, compared to the E14.0 $TM^{lacZ/lacZ}$ embryo. (C,D) Histological sections showing skin of the lower abdominal wall in a $TM^{lacZ/lacZ}$ (C) and a TM^{lacZ} (D) embryo. Several layers of keratinocytes are present independent of the genotype. Lack of TM expression in keratinocytes (indicated by β -gal staining, arrowheads) does not alter skin morphogenesis. Vascularisation of the subcutaneous tissue is comparable in $TM^{lacZ/lacZ}$ and TM^{lacZ} mice. Original magnification is indicated at the lower right.

i.e., antithrombin (AT) and Tissue Factor Pathway Inhibitor (TFPI). AT deficiency causes extensive subcutaneous and intracranial bleeding, and results in severe thrombotic tissue damage in the myocardium and liver. These defects occur around day 14.5 p.c., and mutant embryos do not survive beyond E16 (Ishiguro et al., 2000). About one half of mice lacking TFPI develop a lethal thrombotic diathesis after day 12.5 p.c., and bleeding is most prominent in the brain and tail (Huang et al., 1997). In all of the above mouse models with disrupted anticoagulant mechanisms, hemorrhagic defects occur within a common time window, in which hemostatic system activity must be effectively controlled by natural anticoagulant mechanisms. However, the precise site of bleeding, fibrin deposition, and severity of hemorrhage in individual organs is rather distinct in these animals, suggesting that the basis of organ specific differences in the control of blood coagulation (Weiler-Guettler et al., 1998; Rosenberg and Aird, 1999; Christie et al., 1999) is already established during intrauterine development.

In approximately half of TFPI-null mice, defective blood vessel development in the yolk sac causes embryonic lethality before manifestation of the above bleeding diathesis (Huang et al., 1997). The defect in TFPI-deficient mice can be corrected by elimination of coagulation factor VII, suggesting that abnormal vascular development in TFPI-null embryos is caused by a lack of inhibition of TF-dependent thrombin generation (Chan et al., 1999). However, abnormal vascular development is also observed in mice lacking key components of the thrombin generating clotting system, including TF (Carmeliet et al., 1996; Bugge et al., 1996; Toomey et al., 1997), factor V (Cui et al., 1996), and prothrombin (Sun et al., 1998; Xue et al., 1998). Apparently, proper blood vessel development depends on thrombin generation that must be precisely balanced by inhibitory mechanisms, such as the TFPI pathway.

The rescue of TM-null embryos from early lethality made it possible to examine if inhibition of thrombin generation and/or activity by TM is necessary for vascular development during embryogenesis, akin to the function of TFPI. The architecture of blood vessels in the yolk sac and in the embryo proper of TM-null embryos appears morphologically unremarkable, vascular integrity is maintained, and vascular

could also be explained by differences in the genetic background of completely ES cell-derived TM-null embryos compared to protein C-deficient mice derived from intercrosses of mice with a mixed genetic composition.

Irrespective of the above similarities, disruption of the TM-protein C interaction by elimination of TM causes a placental defect and a much earlier death than the absence of protein C (Healy et al., 1995; Jalbert et al., 1998). This discrepancy is likely explained by the fact, that maternal protein C is readily available for interaction with TM expressed on placental surfaces exposed to maternal blood. In the embryo proper, on the other hand, embryonic protein C interacts with endothelial associated TM and inactivation of either blood coagulation regulator has almost identical consequences. However, our experiments do not rule out the possibility that TM function in the placenta is protein C independent, and therefore causes a defect that is not observed in protein C-deficient mice.

Lethal hemorrhage is also observed in mice lacking two other natural inhibitors of thrombin generation and activity,

density in selected tissues is unaltered. Vascularization and airway branching, which are synchronized during lung development (Warburton et al., 2000), proceed normally in the absence of TM. Heart morphogenesis and endocardium formation was not altered in TM-null mice, demonstrating that the lack of endocardium formation observed in E8.5 TM-null embryos (Healy et al., 1995) cannot be attributed to a loss of TM-function. Together, these results show that TM expression in the developing vasculature is not required for blood vessel morphogenesis. Likewise, the detailed morphological survey of other organs where TM is expressed during development in non-endothelial cells did not reveal any abnormalities, indicating that, at least until E15, the function of TM within the embryo proper is limited to the control of hemostasis.

Although the above experiments do not directly address the mechanism of TM function in the placenta, our results are compatible with the hypothesis that localized TM expression in trophoblast and/or parietal endoderm serves to control the activation of the coagulation system, as in blood vessel endothelium. Genetic elimination of the initiator of the blood coagulation mechanism, tissue factor, indeed rescues TM-deficient embryos from early midgestation lethality (author's unpublished observations), suggesting that increased thrombin formation in the microenvironment of the fetomaternal interface contributes to embryonic lethality.

TM expression has also been documented in trophoblast cells of the human placenta (Maruyama et al., 1985; Fazel et al., 1998). The conserved expression pattern of TM in mice and humans and the similar type of placentation in both species (Pijnenborg et al., 1981; Cross et al., 1994) suggests that a severe reduction of TM function and the ensuing activation of the blood clotting mechanism might be associated with fetal wastage in both species. Suppression of the protein C pathway in women (Brenner and Blumenfeld, 1997; Kupferminc et al., 1999; Tal et al., 1999) as well as other inherited or acquired thrombotic risk factors (Rand et al., 1997; Blumenfeld and Brenner, 1999) has indeed been associated with adverse pregnancy outcome. Pregnancy complications in patients with prothrombotic risk factors have mainly been linked to the formation of blood clots in placental blood vessels and sinuses, eventually leading to intrauterine growth retardation and, in the most severe case, fetal loss (Out et al., 1991; Salafia et al., 1995; Kupferminc et al., 1999). Placental thrombosis is indeed observed in the majority of these patients, but its severity does not always correlate with pregnancy outcome (Lockshin et al., 1985; Salafia and Cowchock, 1997). Similarly, the extent of intravascular thrombosis or extravascular fibrin clots in the mouse placenta does not correlate with the presence or absence of TM. Furthermore, TM must exert its critical function on non-endothelial cells of the placenta, whereas a loss of TM function in blood vessel endothelium of the yet-to-develop chorioallantoic placenta does not impede developmental progress through midgestation. These results suggest that intravascular thrombosis might not be the only mechanism through which excessive activation of the coagulation system could cause placental insufficiency. Our observations, therefore, raise the possibility that activated coagulation factors, generated at the fetal-maternal interface, may affect placental growth and function through a mechanism disparate from vascular thrombosis.

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