Rapid Communication

Targeted Disruption of the Murine Tissue Factor Gene Results in Embryonic Lethality

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Tissue factor (TF) is an integral membrane glycoprotein that is believed to be the physiologic initiator of the blood coagulation cascade. Disruption of the mouse tissue factor gene leads to embryonic lethality between days E9.5-E11.5 of gestation. On E9.5, TF(-/-) embryos appear indistinguishable from their TF(+/-) and TF(+/-) littermates. By E10.5, TF(-/-) embryos are severely growth retarded, appear nearly bloodless, and are in most cases dead. Initial observations suggest that TF(-/-) embryos are dying of circulatory failure. Approximately 15% of the TF(-/-) embryos survive beyond E10.5, but none complete gestation. Heterozygotes appear normal and free of bleeding complications.

Preparation of a replacement vector and targeting the murine TF gene

The murine TF gene was cloned from a 129SV genomic library and sequenced. A 1.5-kb pgk-neomycin phosphotransferase gene (transcriptionally in the same orientation as the mTF gene) (Fig 1A). A 1.8-kb Sal I Xho I 1 kb-thymidine kinase (hsv-tk) gene (transcriptionally in the opposite orientation of the mTF gene) was inserted into a unique Sac I site in the vector (Fig 1A). Additionally, an extensive Southern analysis, which is not shown, confirmed the targeting event depicted in Fig 1A (data not shown).

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Fig 1. Targeted disruption of the mTF gene in ES cells. (A) Targeting construct consisting of a 5.9-kb Kpn I 129SV genomic fragment containing mTF exons 3-5 cloned into the Kpn I (K) site of bluescript KS(+). Inserted at the 5' end of the mTF gene fragment is a 1.8-kb hsv-TK gene. A 1.5-kb pgk-Neo expression cassette was inserted into an Xho I (X) site in exon 3. Homologous recombination with the mTF locus, introduces a new Sac I site into the mTF locus. Digestion with Sac I and Southern blot analysis with probe A, yields a wild-type allele at -12 kb and/or a targeted allele at -6.8 kb. Arrows indicate the PCR primers used in screening ES cell colonies for homologous recombination (see Materials and Methods). (B) Analysis of DNA preparations from embryos derived from heterozygote matings. Southern blot analysis using probe A is shown. Lanes 1 through 3 are TF(+/+), TF(+/-), and TF(-/-), respectively.

TF(+/-) ES cell clones were injected into 3.5-day postcoitus C57BL/6 blastocysts and then subsequently transferred to the uterine horns of 2.5 day postcoitus pseudopregnant recipient Swiss Webster mice. Thirteen male chimeras, 70% or better, were derived from these pregnancies. The chimeras were mated to C57BL/6 females. Chimeras derived from two independent ES cell clones had agouti offspring. Analysis of the tail DNA from these offspring confirmed the germline transmission of the targeted TF allele (Fig 1B).

Derivation of TF(+/-), TF(+/-), and TF(-/-) mouse embryonic fibroblasts (MEFs). TF(+/-) heterozygotic mice were interbred. Pregnant females were sacrificed 13.5 days postcoitus and the embryos harvested. The embryos were decapitated, eviscerated, minced,

**Table 1. Genotype of Embryos Derived From Timed Matings of TF(+/-) Heterozygotes**

<table>
<thead>
<tr>
<th>Day of Gestation</th>
<th>Litters</th>
<th>Total Embryos</th>
<th>(+/+</th>
<th>(+/-</th>
<th>(-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5</td>
<td>7</td>
<td>40</td>
<td>6</td>
<td>25</td>
<td>9    (23%)</td>
</tr>
<tr>
<td>10.5</td>
<td>5</td>
<td>41</td>
<td>9</td>
<td>25</td>
<td>7    (17%)</td>
</tr>
<tr>
<td>11.5</td>
<td>4</td>
<td>23</td>
<td>9</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>2</td>
<td>16</td>
<td>5</td>
<td>10</td>
<td>1    (6%)</td>
</tr>
<tr>
<td>13.5</td>
<td>4</td>
<td>28</td>
<td>6</td>
<td>21</td>
<td>1    (4%)</td>
</tr>
<tr>
<td>14.5</td>
<td>3</td>
<td>15</td>
<td>7</td>
<td>7</td>
<td>1    (7%)</td>
</tr>
<tr>
<td>15.5</td>
<td>3</td>
<td>21</td>
<td>9</td>
<td>11</td>
<td>1    (5%)</td>
</tr>
<tr>
<td>16.5</td>
<td>3</td>
<td>14</td>
<td>4</td>
<td>9</td>
<td>1    (7%)</td>
</tr>
<tr>
<td>17.5</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>18.5</td>
<td>1</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>212</td>
<td>58</td>
<td>133</td>
<td>21 (10%)</td>
</tr>
</tbody>
</table>

Genotypes were performed by Southern blot analysis of embryos or yolk sacs using probe A. None of these data represent resorbed embryos. The morning on which a vaginal plug is identified, is considered 0.5 days of gestation.
Fig 4A and B.

Fig 4.
Whole-mount and histologic analysis of TF(+/-) and TF(-/-) E9.5 and E10.5 embryos. (A) H&E-stained sagittal section of E9.5 TF(+/-) embryo. (B) H&E-stained sagittal section of E9.5 TF(-/-) embryo. (C) Photomicrograph of E10.5 TF(+/-) and TF (-/-) embryos. BA, branchial arch; AS, aortic sac; VW, ventricular wall; LCAC, left component of common atrial chamber.

Fig 3.
Whole-mount photomicrographs of TF(+/-), TF(+/-), and TF(-/-) embryos at E9.5 (original magnification × 16) and E10.5 (original magnification × 10).
and then trypsinized for 1 hour at 37°C. The trypsinized tissue was vortexed vigorously and plated onto tissue-culture plates in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS). These primary mouse embryonic fibroblasts were expanded and then frozen in FCS containing 10% dimethyl sulfoxide. DNA derived from head tissue was used in genotyping these embryos. Genotyping was performed by Southern blotting using an Sac I digest and probe A (Fig 1A).

**Measurement of TF activity.** TF(+/-), TF(+/-), and TF(-/-) mouse embryonic fibroblasts were plated into 60-mm tissue culture Petri dishes. Confluent plates were washed three times with phosphate-buffered saline and then (using a disposable cell scraper) harvested in volume of 0.5 mL serum-free DMEM. The cells were immediately flash frozen in an ethanol dry-ice bath. The cells were lysed by freezing and thawing (four times total). Aliquots from each cell lysate were taken and the protein concentrations determined (Bio-Rad Protein assay dye reagent, Hercules, CA). Protein concentrations were normalized by diluting in DMEM. To assay for TF, cell lysates were serially diluted in TBSA (0.05 mol/L Tris, pH 7.5, 0.1 mol/L NaCl, 0.1 mg/mL bovine serum albumin). Sixty microliters of the diluted lysate was added to a fibrin cup in a BBL Fibrosystem Fibrometer (Becton Dickinson, Cockeysville, MD). This was immediately followed by 60 µL of rabbit brain cephalin (Sigma, St Louis, MO) diluted 1:10 in 0.85% NaCl, and by 60 µL of 0.02 mol/L CaCl2. This mixture was incubated at 37°C for 30 seconds, and then 60 µL of reconstituted mouse plasma (Sigma) was added, the fibrometer started, and clotting time recorded.

**Histology.** Embryos and yolk sacs were dissected free of maternal tissue. One was used for genotyping while the other was fixed in 4% phosphate-buffered formalin. Fixed tissue was then dehydrated, paraffin embedded, and sectioned according to standard protocols. Sections were stained with hematoxylin and eosin (H&E).

## RESULTS

Targeted disruption of the mTF alleles was achieved by introducing the neomycin phosphotransferase gene (Neo) into exon 3 (Fig 1A and B). This places pgk-neo 76 amino acids from the amino terminus of mature mTF in the first Ig-like domain. Inactivation of the mTF alleles was verified by assessing TF activity in primary MEFs derived from TF(+/-), TF(+/-), and TF(-/-) embryos. As shown in Fig 2, TF(-/-) MEFs are completely devoid of TF activity. The TF activity in heterozygotic TF(+/-) MEFs ranges between 30% and 60% of TF(+/-) MEFs. There are no obvious morphologic or proliferative differences between the TF(+/-) and TF(-/-) MEFs. Bleeding times of TF(+/-) and TF(+/-) mice as assessed by tail biopsy are indistinguishable (data not shown). No phenotypic abnormalities were observed in heterozygotes.

**Characterization of TF(-/-) mice.** Heterozygous TF(+/-) mice derived from two independently targeted ES cell lines were independently intercrossed. Of 189 progeny, 71 (38%) were wild type TF(+/-) and 118 (62%) were heterozygous TF(+/-). Timed matings were initiated to establish the gestational age at which the TF(-/-) embryos were dying (Table 1). By chi-squared analysis, the oldest time points in which Mendelian ratios were approached (1:2:1) were at E9.5 and E10.5. The combined data between E11.5 and E18.5 is significantly different from Mendelian values (P < .001). Approximately 85% of the TF(-/-) embryos die before E11.5. The 15% that survive beyond E10.5 are alive and are not easily distinguished from their littermates, although none complete gestation.

By whole-mount (Fig 3B and D) and histologic analysis (Fig 4A and B), E9.5 TF(-/-) embryos appear indistinguishable from TF(+/-) or TF(+/-) littermates. The TF(-/-) E9.5 embryos contain red blood cells, suggesting that the embryonic and the extraembryonic vasculature of the yolk sac are connected. Cardiac development also appears unaffected.

By E10.5, the expected percentage of TF(-/-) embryos had decreased only slightly. However, by whole-mount analysis, (Fig 3A, C, and E) the majority of the TF(-/-) embryos are easily distinguishable from their littermates and in most cases appear dead. The most prominent characteristics of these embryos are that the embryo and yolk sac are nearly bloodless (Fig 4C), and that the embryos are severely growth and developmentally retarded. Although the E10.5 TF(-/-) embryos are smaller than a E9.5 embryo, they possess 25 to 30 pairs of somites (developmentally comparable to a E10.0-E10.25). It is likely, that most of the obvious developmental defects observed by E10.5, such as distention of the cardiac cavity and the lack of cephalic differentiation, are secondary to what appears to be circulatory failure and embryonic necrosis.

## DISCUSSION

TF's role as cofactor for factor VIIa and as an initiator of blood coagulation is well established. TF has been implicated in a variety of biological processes, from angiogenesis and tumor metastasis to vascular remodeling and signal transduction. How TF functions in these processes has not been shown. To determine the physiologic consequences of TF deficiency, a null mutation of the TF gene was studied in mice. The data clearly indicate that TF is essential for survival. TF(-/-) mice die primarily before E11.5. On E9.5, embryonic defects associated with TF deficiency are not apparent, but By E10.5, the embryos and yolk sac appear bloodless and suffer from severe growth retardation. At this stage of development, the embryo is dependent on a functioning yolk sac circulation and a functioning chorioallantoic placenta, both of which should be well established by E9.5. Mortality at this stage of development in other knockout mice has been attributed to inadequate development or maintenance of yolk sac and chorioallantoic circulation, defective hematopoiesis, or the loss of vascular integrity. In light of TFs putative effects on angiogenesis and its prominent role in coagulation, the apparent circulatory failure of TF(-/-) embryos could result from deficient vascular development, hemorrhage, or a combination of the two. A potential link between TF and hematopoiesis has not yet been reported. Interestingly, ~15% of the TF(-/-) embryos manage to escape this developmental bottleneck, although none survive gestation. Studies to determine whether different genetic backgrounds can influence this phenotype are in progress.

**NOTE ADDED IN PROOF**

After submission of this manuscript, Bugge et al reported that TF(-/-) mice die in utero due to massive hemorrhaging
from embryonic and extraembryonic vessels. This was manifested by extreme pallor in E10.5 TF(-/-) embryos, and by the pooling of red blood cells in the yolk sac cavity of E9.5 TF(-/-) embryos. We have also observed similar pooling of red blood cells in the yolk sac cavity of E10.0 TF(-/-) embryos. Out of 4 E10.0 TF(-/-) embryos, 3 appeared to have large pools of red blood cells in the yolk sac cavity. This is consistent with the observation of Bugge et al, and supports the notion that TF deficiency is resulting in a loss of vascular integrity.

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REFERENCES


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