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# Effect of tissue factor deficiency on mouse and tumor development

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**ABSTRACT** **Previous reports suggest that tissue factor (TF) may play an essential role in embryonic vascular development and tumor angiogenesis. To further examine this relationship, the morphology of fully developed TF-deficient embryos and the growth of TF-deficient teratomas and teratocarcinomas were analyzed. In a 129/Sv genetic background, TF null embryos do not survive beyond mid-gestation. In contrast, 14% of 129/Sv × C57BL/6 TF-deficient embryos escape this early mortality and survive to birth. On gross and microscopic inspection, these late gestation, TF-deficient embryos appear normal. The growth and vascularity of TF(+/+), TF(+/−), and TF(−/−) teratomas and teratocarcinomas are indistinguishable. Thus, tumor-derived TF is not required for tumor growth and angiogenesis and the combined data do not support an essential role for TF in embryonic vascular development.**

Tissue factor (TF) is an integral membrane glycoprotein that, when exposed to plasma, is a potent procoagulant. It is believed to be the physiological initiator of blood coagulation (1). TF is constitutively expressed by a variety of cells, most prominently in epithelial and central nervous system tissues (2–3). Although ordinarily absent from vascular endothelium and peripheral blood cells, TF expression on monocytes and endothelium can be induced by cytokines and inflammatory molecules (4–10).

In quiescent fibroblasts and epithelial cells, TF is a mitogen-inducible gene (11–15). This induction occurs rapidly and in the absence of protein synthesis, classifying TF as an immediate early gene. This raises the possibility that TF, like other immediate early genes (16), may have a growth-related function. Consistent with this notion are reports suggesting that TF is either directly or indirectly involved in signal transduction (17–19).

Most tumor cells express TF (20–21), and the hypercoagulable state seen in many cancer patients has been attributed in large part to this expression (22). In general, TF expression does not appear to correlate with malignant transformation (20, 22). However, several reports suggest a role for TF in tumor angiogenesis and metastasis. Zhang *et al.*(23), using sense and anti-sense TF cDNA constructs in Meth-A sarcoma cells, reported that TF influences tumor growth by regulating tumor angiogenesis. Tumor growth and angiogenesis were promoted by TF overexpression and inhibited by suppression of TF expression. Warfarin treatment of the recipient animals did not affect tumor growth, so this effect of TF was thought to be independent of its role in blood coagulation. Additional studies have shown that the metastatic potential of melanoma cells is dependent on TF expression (24–25). Whereas Mueller *et al.* and Fischer *et al.* found that TF-mediated enhancement of melanoma metastasis is related to its procoagulant function (24, 26), Bromberg *et al.* report that TF's action is unrelated to coagulant activity (25). Taken as a whole, the data suggest that TF plays an important role in tumor growth and metastasis although the mechanism of TF action is unclear.

TF gene disruption in mice has been accomplished by three separate laboratories (27–29) with similar, although not identical, phenotypes. Bugge *et al.* (27) reported that, in a 129/Sv × NIH Black Swiss background, TF deficiency causes catastrophic hemorrhaging into the yolk sac cavity between embryonic days (E) 8.5 and 9.5, with no TF(−/−) embryos surviving beyond E10.5. Carmeliet *et al.* (29) report that TF deficiency causes abnormalities of vascular pericytes, resulting in defective yolk sac vessel development and subsequent embryo wasting by E10.5. The genetic background in these studies was C57BL/6 × 129/Sv (P. Carmeliet, personal communication), and a small number of TF null animals apparently survived to later gestation.

In our studies (28) in the 129/Sv × C57BL/6 background, a small but significant proportion of TF(−/−) embryos survived beyond E10.5 into late gestation. The presence of these late gestation embryos left the role of TF in mouse development uncertain.

To further investigate the effect of TF deficiency on embryonic vascular development, late term TF(−/−) embryos were examined and the influence of genetic background on their phenotype was determined. In addition, tumor angiogenesis and growth was assessed in a TF(−/−) teratoma model. A similar tumor model was used by Ferrara *et al.* (30) to confirm the critical role of vascular endothelial growth factor in vessel development. The combined results of investigations in TF(−/−) embryos and TF(−/−) tumors, however, do not support an essential role for TF in embryonic vascular development or tumor angiogenesis.

## MATERIALS AND METHODS

**Polyclonal Antibody to Mouse TF.** Recombinant mouse soluble TF was kindly provided by Thomas J. Girard (Monsanto, St. Louis, MO). An inhibitory rabbit polyclonal antibody to mouse soluble TF was generated according to standard procedures (31).

**TF-Deficient Mice.** The generation of TF-deficient mice has been described (28). TF deficiency in different genetic backgrounds was achieved by intercrossing the original chimeric founders [derived from RW-4 129/SvJ embryonic stem (ES) cells] with C57BL/6 or by backcrossing to 129/SvEv (Taconic Farms).

**Histology.** E17.5 embryos were dissected free of maternal tissue. Tails were removed for genotyping, and the embryo was placed in 10% phosphate buffered formalin × 24 h, followed by 70% ethanol. Embryos were dehydrated, paraffin-embedded, and transversely sectioned from crown to rump according to standard protocols. Sections were subsequently stained with hematoxylin/eosin.

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Abbreviations: TF, tissue factor; E, embryonic day; ES, embryonic stem; Neo, neomycin; Hyg, hygromycin b; MEF, mouse embryonic fibroblasts; EC, embryonal carcinoma.

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**Cesarean Section.** To perform a Cesarean section in mice, two pregnant mice with due dates that are within 0–1 day of each other are required. One pregnant female serves as an embryo donor and the other serves as the foster mother. Timed matings were set up on 22 TF(+/−) females. Over a 1-month period, six pairs of females became pregnant so that one member of the pair could serve as a donor and the other could serve as the foster mother. Cesarean sections were performed on donor females on E18.5 according to standard protocols (32). Umbilical cords and later tail sections were taken for genotype determination. If bleeding occurred on removing the umbilical cord, the umbilical stump was cauterized. During all tail biopsies, the remaining tail tip was cauterized.

**Targeting of Both TF Alleles in ES cells.** The cloning of the murine TF gene and the construction of a TF gene targeting construct have been described (28). In this study, the original targeting construct was used in addition to a modified construct in which the 1.5-kb neomycin phosphotransferase gene (Neo) was replaced with a ≈2.0-kb hygromycin b phosphotransferase gene (Hyg). The Neo construct (see Fig. 2*A*) was linearized at a unique *Sal*I site, flanking the hsv-tk cassette (not shown) and introduced via electroporation into RW-4 and E14 ES cells. Electroporated ES cells were grown on irradiated mouse embryonic fibroblasts and selected with G418 (0.8 mg/ml) and ganciclovir (2 μM). Surviving colonies were screened for homologous recombination using PCR, with one primer derived from outside of the targeting construct in intron two and the other from within the Neo gene. Successful targeting of the first TF allele was verified for each clone by *Sac*I/*Sca*I digest and Southern analysis using probe A (Fig. 2). The second allele was targeted by transfecting a TF(+/−) ES clone (in both RW4s and E14s) with the Hyg construct and then plating the cells on gelatin-coated plates and selecting with G418 (0.4 mg/ml) and Hyg (0.3 mg/ml; Calbiochem). Surviving colonies were screened by *Sac*I/*Sca*I digest and Southern blot analysis using probe A (Fig. 2).

**Derivation of TF(+/+) and TF(−/−) Mouse Embryonic Fibroblasts (MEFs).** TF(−/−) ES cell clones were injected into 3.5-day postcoitus C57BL/6 blastocysts, which were subsequently transferred to the uterine horns of 2.5-day postcoitus, pseudopregnant recipient Swiss–Webster mice. On E13.5, the pregnancy was terminated, and the chimeric litter was collected. The embryos were eviscerated, minced, trypsinized, and plated onto tissue culture plates. After expansion of the primary MEFs, the fibroblasts were selected with G418 (0.7 mg/ml) and Hyg (0.2 mg/ml) for 1 week, and then intermittently thereafter. After selection, the surviving TF(−/−) fibroblasts were combined and immortalized using a 3T3 protocol (33). The derivation of TF(+/+) MEFs has been described (28). The TF(+/+) fibroblasts also were immortalized using a 3T3 protocol.

**Measurement of TF Activity and Western Analysis.** TF activity in the immortalized TF(+/+), TF(−/−) MEFs was determined in a one-stage clotting assay as described (28). For analysis by Western blot, fibroblasts were grown to confluence in a 24-well tissue culture plate. Cells were washed three times with PBS and then lysed by freezing and thawing in serum-free DMEM. An aliquot of the lysate was taken for protein concentration determination (Bio-Rad protein assay dye reagent). Protein concentrations were normalized, diluted in an equal volume of 2X sample buffer (250 mM Tris, pH 6.8/4% SDS/10% glycerol/0.006% bromophenol blue/2% β-mercaptoethanol) and boiled for 5 min. Samples were electrophoresed on a SDS/polyacrylamide gel (10%) and then transferred to nitrocellulose. The nitrocellulose filter was blocked overnight with blocking buffer (5% nonfat dry milk/10 mM Tris, pH 7.5/100 mM NaCl/0.1% Tween 20) at 4°C. The following day, the blocking buffer was decanted and replaced with blocking buffer containing anti-mouse soluble TF polyclonal antibody (1:1000). After a 1-h incubation, this solution was decanted and the membrane was washed (wash buffer: 10 mM Tris, pH 7.5/100 mM NaCl/0.1% Tween 20) for 30 min with buffer changes every 5 min. The membrane then was incubated for 1 h in blocking buffer containing a goat anti-rabbit alkaline phosphatase-conjugated antibody (Sigma). This was followed by another 30 min of washing and then detection using the Sigma Fast 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt/nitroblue tetrazolium alkaline phosphatase substrate solution (Sigma).

**Teratoma/Teratocarcinoma Production and Derivation of Embryonal Carcinoma (EC) Cell Lines.** Teratomas were produced (34) by s.c. injection of $5.0 \times 10^6$ ES cells in a volume of 0.1 ml into either SCID (C.B-17/IcrCrl-scid-bgBR) or nude (N:NIH-bg-nu-xidBR) female mice (Charles River Breeding Laboratories). After 4 weeks, mice were killed and tumors were removed and weighed. Tumors developed comparably in both nude and SCID mice, and the data were combined for this report. To derive EC cell lines (34), tumors then were minced finely and trypsinized for 30–45 min at 37°C. The trypsinized tissue was disrupted further by pipetting or vortexing. After allowing any large clumps of tissue to settle, the remaining tumor digest supernatant was plated on 10-cm Petri dishes in DMEM containing 10% fetal calf serum. After 1–2 weeks of culture, EC colonies became visible and were expanded. The EC cell lines were regenotyped and frozen. Teratocarcinomas were generated by s.c. injection of $2.0 \times 10^6$ EC cells in a volume of 0.1 ml into nude mice. After 4 weeks, mice were killed and the teratocarcinomas were removed and weighed. The data on teratoma/teratocarcinoma weights were analyzed using Student's *t* test.

## RESULTS

**Timed Matings.** Timed matings were extended from our original study to further assess the consequences of TF deficiency in fully differentiated embryos and in 1- to 2-day-old neonates (Table 1). Of 204 embryos examined between Ell.5 and E18.5, 3.0% were TF(−/−). This is statistically different from expected Mendelian values ($P < 0.001$). Based on Mendelian genetics, this value is ≈14% of the expected number of null embryos and is observed consistently in TF(+/−) mating pairs derived from independent ES cell lines. Of the TF(−/−) embryos surviving beyond E10.5, none appear to be lost during the latter half of gestation. By $\chi^2$ analysis, there was not a statistical difference between the number of TF(−/−) embryos at E13.5 and at E17.5 ($P > 0.1$). E17.5 TF(−/−) embryos showed no evidence of growth retardation (crown-to-rump lengths) and appeared, on gross inspection, indistinguishable from their littermates (Fig. 1). Transverse sections were taken from crown-to-rump of the TF(−/−) E17.5 embryos. Histologically, the cardiovascular system, cen-

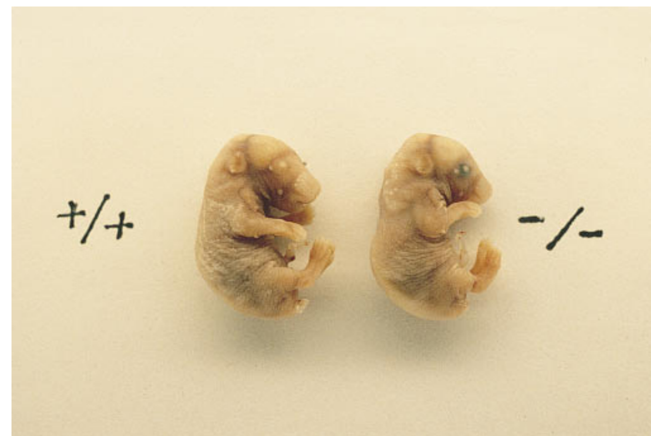


FIG. 1. Whole mount of TF(+/+) E17.5 embryo (*Left*) and TF(−/−) E17.5 embryo (*Right*). Tails have been removed for genotyping.

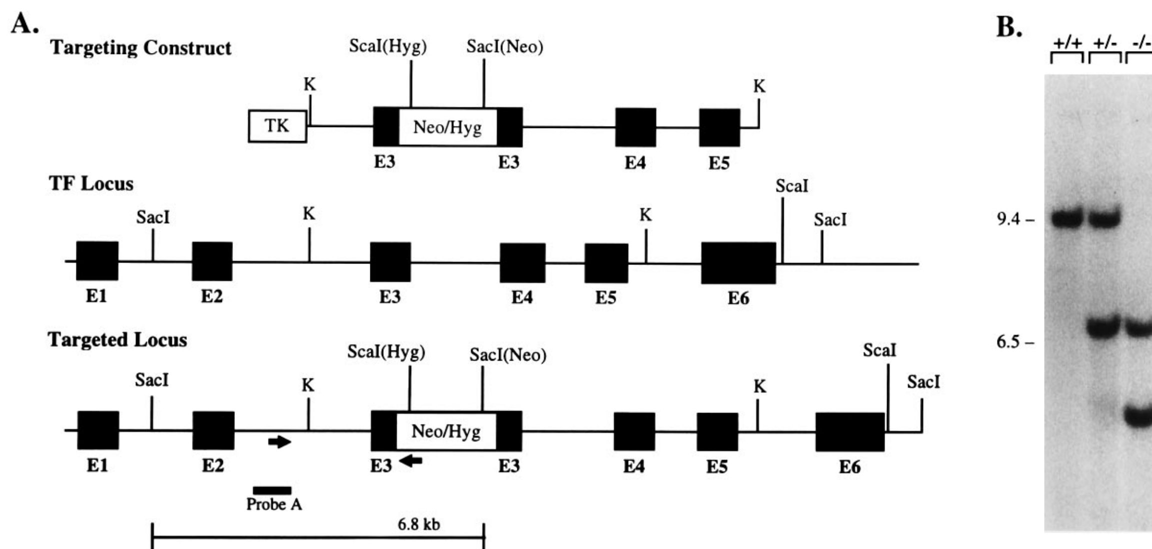


FIG. 2. Disruption of the mTF gene in ES cells. (*A*) Targeting constructs consist of a 5.9-kb *Kpn*I 129/Sv genomic fragment containing mTF exons 3–5 cloned into the *Kpn*I (K) site of pBluescript II KS(+). Inserted at the 5′ end of the mTF gene fragment is a 1.8-kb hsv-TK gene. Either a 1.5-kb pgk-Neo expression cassette or a ≈2.0-kb pgk-Hyg expression cassette was inserted into an introduced unique *Xho*I (X) site in exon 3. Homologous recombination with the mTF alleles introduces a new *Sac*I (for Neo construct) or *Sca*I (for Hyg construct) site into the mTF locus. Arrows indicate the PCR primers used in screening ES cell colonies for homologous recombination with the Neo construct. Digestion of genomic DNA with *Sac*I/*Sca*I yields a wild-type mTF allele at ≈11 kb and targeted alleles at ≈6.8 kb (Neo construct) and/or ≈5.8 kb (Hyg construct). (*B*) Southern blot analysis using probe A of *Sac*I/*Sca*I-digested DNA prepared from wild-type ES cells (+/+), ES cells targeted with the Neo construct (+/−), or ES cells targeted with both the Neo and Hyg constructs (−/−).

tral nervous system, lungs, gut, and urogenital and musculoskeletal systems appeared morphologically normal. Additionally, there was no evidence of hemorrhage or vascular abnormalities. Approximately 2% of P1-P2 (1–2 days postpartum) neonates were also TF(−/−). These pups suffered ubiquitous catastrophic hemorrhage (data not shown), and none survived the first week of life. Therefore, there were two distinct populations of TF(−/−) embryos, those that died by E10.5 and those that died at or around the time of birth.

**Cesarean Sections.** Gross and microscopic analyses failed to detect abnormalities in E17.5 TF(−/−) embryos, so it was conceivable that the trauma associated with vaginal delivery might contribute to the death of these animals during the perinatal period. To address this question, 42 offspring from TF(+/−) matings were delivered by Cesarean section at E18.5. Southern analysis of umbilical cord DNA showed that one pup was TF(−/−), which was later confirmed using tail DNA. This pup developed intraabdominal bleeding by P1-P2, which resolved by P5. At 3 weeks, the TF(−/−) mouse weighed 20% less (lacking tail) than its littermates but was active and thriving. At 4 weeks, the TF(−/−) mouse acutely developed ataxia and apparent blindness and died shortly thereafter. Subsequent autopsy showed a massive right cerebral hemorrhage (data not shown).

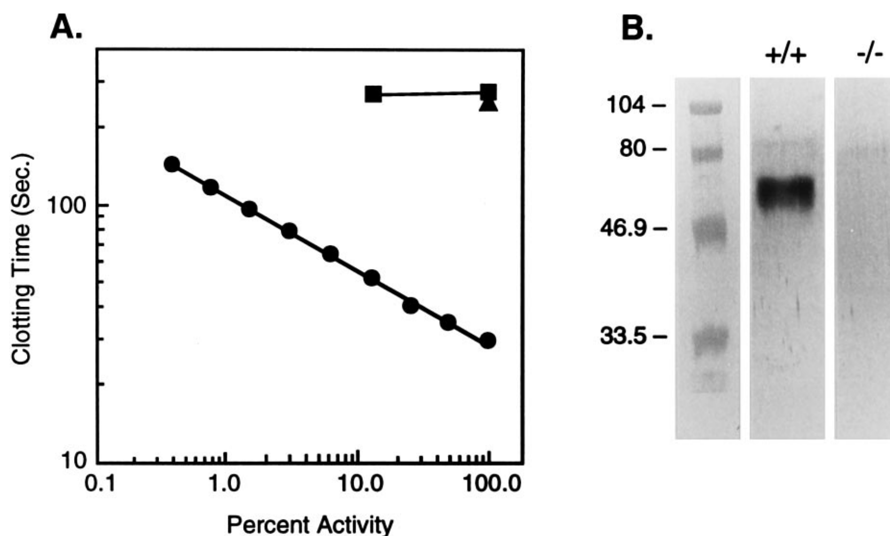


FIG. 3. Verification of TF gene disruption. (*A*) TF activity in TF(+/+) (●) or TF(−/−) (■) immortalized MEFs. Data are from a single representative experiment. Cell lysates were diluted serially in TBSA from 1:4–1:512, and TF activity was assessed in a one-stage clotting assay as described (28). The negative control has the cell lysate replaced with TBSA (50 mM Tris, pH 7.5/100 mM NaCl/0.1 mg/ml BSA) (▲). Data are plotted as percentage of activity vs. clotting time in seconds. The activity in a TF(+/+) cell lysate diluted 1:4 is designated as 100% activity. (*B*) Western blot analysis, using an anti-mouse soluble TF polyclonal antibody, of immortalized MEF cell lysates. Lane (+/+) is of a wild-type cell lysate, showing mTF migrating at ≈50 kDa, which is comparable to published data (12). Lane (−/−) is of a TF-deficient cell lysate, showing neither TF nor an aberrant protein product.

Table 1. Genetic background 129/SvJ × C57BL/6

| Day of gestation | Total | Genotype | | |
|---|---|---|---|---|
| | | +/+ | +/− | −/− (%) |
| 9.5 | 63 | 11 | 37 | 15 (23.8) |
| 10.0 | 15 | 5 | 6 | 4 (26.7) |
| 10.5 | 54 | 11 | 33 | 10 (18.5) |
| 11.5 | 23 | 9 | 14 | 0 |
| 12.5 | 16 | 5 | 10 | 1 (6.2) |
| 13.5 | 69 | 23 | 45 | 1 (1.4) |
| 14.5 | 15 | 7 | 7 | 1 (6.7) |
| 15.5 | 21 | 9 | 11 | 1 (4.7) |
| 16.5 | 14 | 4 | 9 | 1 (7.1) |
| 17.5 | 37 | 8 | 27 | 2 (5.4) |
| 18.5 | 9 | 3 | 6 | 0 |
| P1-2 | 108 | 26 | 80 | 2 (1.9) |
| P21 | 303 | 109 | 194 | 0 |
| C-section | 42 | 18 | 23 | 1 (2.4) |

Genotype of offspring derived from timed matings of TF(+/−) hemizygotes. Genotypes were performed by Southern blot analysis of embryo, yolk sac, umbilical cord, or tail DNA using probe A. The morning on which a vaginal plug is identified is considered 0.5 days of gestation. P1-2 indicates 1-2 days postpartum. C-section, Cesarean section performed on E18.5.

**Genetic Compensation.** The initial data on the phenotype of TF deficiency was generated in a 129/SvJ x C57BL/6 background (Table 1). To assess the contribution of genetic background on the TF(−/−) phenotype, the original chimeric founders were bred into the strain 129/SvEv (129/SvJ × 129/SvEv), and timed matings were performed. In this genetic background, all of the TF(−/−) embryos examined on E10.5 were either dead or dying and were characterized by pallor and severe growth retardation (Table 2). In contrast to the C57BL/6 background, no TF(−/−) animals in the 129/Sv background survived until E15.5–E17.5 ($P < 0.01$).

**The Role of TF in Tumor Growth.** ES cells form teratomas when injected s.c. into syngeneic or immunocompromised hosts, and teratocarcinoma cell lines can be established from these primary tumors (see *Methods*; ref. 34). To directly assess the requirement of TF in tumor growth and angiogenesis, TF(−/−) ES cell lines were created in both RW4 and E14 ES cell lines (see *Methods*) (Fig. 2). Disruption of the mouse TF (mTF) alleles was verified by evaluating mTF activity in MEFs derived from chimeric embryos, which in turn were derived from TF(−/−) ES cells. Mouse TF is not detectable in TF(−/−) MEFs by functional assay or by Western blot analysis (Fig. 3). Teratomas and teratocarcinomas were generated in SCID or nude mice by s.c. injection of targeted ES cells and the subsequently derived EC cells. Tumor growth varied widely, however; in neither the teratomas nor the more malignant teratocarcinomas was tumor growth or tumor frequency affected by the absence of TF (Fig. 4). By *t* test, tumor weights were not statistically different. Moreover, the morphology and vascularity of TF(−/−) and TF(+/+) tumors were histologically indistinguishable (not shown).

Table 2. Genetic background 129/SvJ × 129/SvEv

| Day of gestation | Total | Genotype | | |
|---|---|---|---|---|
| | | +/+ | +/− | −/− |
| E10.5 | 18 | 2 | 8 | 8 (44%) |
| E15.5 | 6 | 4 | 2 | 0 |
| E16.5 | 62 | 16 | 46 | 0 |
| E17.5 | 58 | 19 | 39 | 0 |
| E15.5–E17.5 | 126 | 39 | 87 | 0 |
| P21 | 82 | 25 | 57 | 0 |

Genotype of offspring derived from timed matings of TF(+/−) hemizygotes. Genotypes were performed by Southern blot analysis of embryo, yolk sac, or tail DNA using probe A.

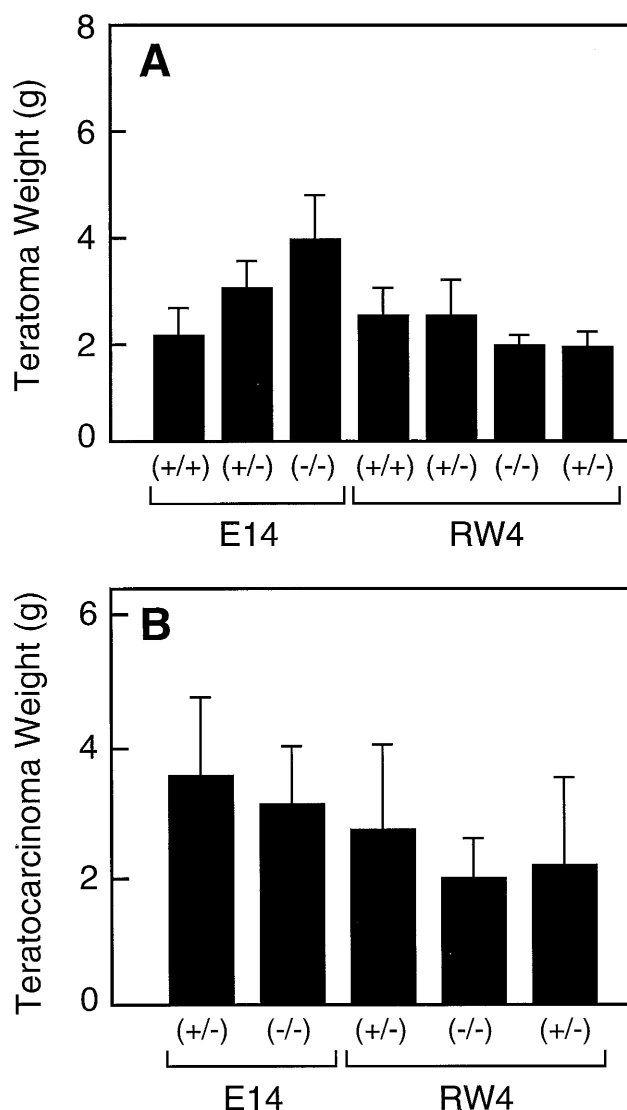


FIG. 4. ES and EC cell tumorigenesis in immunocompromised mice. (*A*) Mean ± SEM teratoma weights derived from s.c. injections of TF(+/+), (+/−), and (−/−) E14 ES cells ($n$ = 6, 15, and 20 respectively) and TF(+/+), (+/−), (−/−), and (+/−) RW4 ES cells ($n$ = 8, 9, 19, and 16, respectively). (*B*) Mean teratocarcinoma weights (± SEM) derived from s.c. injections of TF(+/−) and (−/−) E14 EC cells ($n$ = 7 and 8, respectively) or RW4 EC cells ($n$ = 4, 8, and 4). An additional control in the RW4 (last bar) injections was included. This cell line was targeted with both constructs and survived selection with both G418 and hygromycin. However, homologous recombination was achieved only with the Neo construct; the Hyg construct was nonhomologously integrated into the genome.

## DISCUSSION

Tissue factor deficiency in mice results in exsanguination at midgestation between embryonic days 9.5 and 10.5. Whether the loss of vascular integrity is due to a failure in hemostasis or a failure in vasculature development is not clear (27, 29). Midgestation is characterized by the formation of the vitelline and chorioallantoic circulation and by increasing intravascular pressure. Of interest, a large number of engineered mouse mutations cause embryonic lethality during this period (30, 35–45). As with the TF knockout, the dying embryos are often characterized by pallor, growth retardation, distended pericardia, yolk sac abnormalities, and massive hemorrhage (38–

40). In the absence of additional evidence in viable animals, it is difficult to determine if specific embryonic abnormalities, changes in yolk sac architecture, or a loss of vascular integrity are primary events causing mortality or are secondary events due to morbidity. For this reason, we sought additional evidence pertaining to the role of TF in embryogenesis and vascular development.

Genetic background has been shown to influence mutant phenotypes (45, 46); therefore, we examined its contribution to the TF-deficient phenotype. In the 129/Sv background, TF(−/−) embryos uniformly die at midgestation whereas in the C57BL/6 background, two populations of TF(−/−) embryos are observed, one that dies at midgestation and another that dies at birth. Before birth, these full term TF(−/−) embryos appeared normal, and one TF(−/−) embryo delivered by Cesarean section lived 4 weeks. It is clear that a measure of genetic compensation is being observed in the C57BL/6 background, which alters the time of death but not the hemorrhagic phenotype. Whether this compensatory effect is derived transplacentally or is embryo specific remains to be determined. These studies suggest that TF is not essential for normal cell proliferation or cell viability but is instead necessary to survive major challenges to embryonic vascular integrity at midgestation (E9.5–E10.5) and at birth.

The putative role of TF in angiogenesis was assessed in ES cell-derived TF(−/−) teratoma and EC models. No relationship between tumor growth and the presence or absence of tumor-derived TF was detected. This result contrasts with the previous study of Zhang *et al.* (23), who used the transfection of sense and antisense TF cDNA constructs to manipulate TF expression in Meth-A sarcoma cells and reported a direct association between tumor TF expression and *in vivo* tumor growth. Whether this discrepancy is due to the differing tumor models or potential cellular effects of sense/antisense TF RNA unrelated to TF expression is not clear.

Using the same teratoma model, it recently has been shown that the tumor angiogenesis and growth of teratomas produced by vascular endothelial growth factor (−/−) ES cells are dramatically reduced (30). This directly correlated with the inability of vascular endothelial growth factor-deficient embryos to develop a normal vasculature (30, 42). TF deficiency, on the other hand, is compatible with normal embryogenesis and does not affect tumor growth. These results do not support an essential role for TF in angiogenesis and vascular development.

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1. Nemerson, Y. (1988) *Blood* **71,** 1–8.
2. Fleck, R. A., Vijaya Mohan Rao, L., Rapaport, S. I. & Varki, N. (1990) *Thromb. Res.* **59,** 421–437.
3. Drake, T. A., Morrissey, J. H. & Edgington, T. S. (1989) *Am. J. Pathol.* **134,** 1087–1097.
4. Niemetz, J. & Morrison, D. C. (1977) *Blood* **49,** 947–956.
5. Gregory, S.A., Morrissey, J. H. & Edgington, T. S. (1989) *Mol. Cell. Biol.* **9,** 2752–2755.
6. Schwager, I. & Jungi, T. W. (1994) *Blood* **83,** 152–160.
7. Nawroth, P. P., Handley, D. A., Esmon, C. T. & Stern, D. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3460–3464.
8. Drake, T. A. & Pang, M. (1989) *Infect. Immunity* **57,** 507–512.
9. Bevilacqua, M. P., Pober, J. S., Majeau, G. R., Fiers, W., Cotran, R. S. & Gimbrone, M. A., Jr. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4533–4537.
10. Colucci, M., Balconi, G., Lorenzet, R., Pietra, A. & Locati, D. (1983) *J. Clin. Invest.* **71,** 1893–1896.
11. Lau, L. F. & Nathans, D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1182–1186.
12. Hartzell, S., Ryder, K., Lanahan, A., Lau, L. F. & Nathans, D. (1989) *Mol. Cell. Biol.* **9,** 2567–2573.
13. Ranganathan, G., Blatti, S. P., Subramaniam, M., Fass, D. N., Maihle, N. J. & Getz, M. J. (1991) *J. Biol. Chem.* **266,** 496–501.
14. Bloem, L. J., Chen, L., Konigsberg, W. H. & Bach, R. (1989) *J. Cell. Physiol.* **139,** 418–423.
15. Cui, M., Parry, G. C. ., Oeth, P., Larson, H., Smith, M., Huang, R., Adamson, E. D. & Mackman, N. (1996) *J. Biol. Chem.* **271,** 2731–2739.
16. Herschman, H. R. (1991) *Annu. Rev. Biochem.* **60,** 281–319.
17. Zioncheck, T. F., Roy, S. & Vehar, G. A. (1992) *J. Biol. Chem.* **267,** 3561–3564.
18. Rottingen, J., Enden, T., Camerer, E., Iversen, J. & Prydz, H. (1995) *J. Biol. Chem.* **270,** 4650–4660.
19. Camerer, E., Rottingen, J., Iversen, J. & Prydz, H. (1996) *J. Biol. Chem.* **271,** 29034–29042.
20. Callander, N. S., Varki, N. & Rao, L. V. M. (1991) *Cancer* **70,** 1194–1201.
21. Falanga, A., Iacoviello, L., Evangelista, V., Belotti, D., Consonni, R., D'Orazio, A., Robba, L., Donati, M. B. & Barbui, T. (1995) *Blood* **86,** 1072–1081.
22. Vijaya Mohan Rao, L. (1992) *Cancer Metastasis Rev.* **11,** 249–266.
23. Zhang, Y., Deng, Y., Luther, T., Muller, M., Ziegler, R., Waldherr, R., Stern, D. M. & Nawroth, P. P. (1994) *J. Clin. Invest.* **94,** 1320–1327.
24. Mueller, B. M., Reisfeld, R. A., Edgington, T. S. & Ruf, W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11832–11836.
25. Bromberg, M. E., Konigsberg, W. H., Madison, J. F., Pawashe, A. & Garen, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8205–8209.
26. Fischer, E. G., Ruf, W. & Mueller, B. M. (1995) *Cancer Res.* **55,** 1629–1632.
27. Bugge, T. H., Xiao, Q., Kombrinck, K. W., Flick, M. J., Holmback, K., Danton, M. J. S., Colbert, M. C., Witte, D. P., Fujikawa, K., Davie, E. W. & Degan, J. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6258–6263.
28. Toomey, J. R., Kratzer, K. E., Lasky, N. M., Stanton, J. J. & Broze, G. J., Jr. (1996) *Blood* **88,** 1583–1587.
29. Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Vlaenderen, I. V., Demunck, H., Kasper, M., Breier, G., Evrard, P., Muller, M., Risau, W., Edgington, T. & Collen, D. (1996) *Nature (London)* **383,** 73–75.
30. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. & Moore, M. W. (1996) *Nature (London)* **380,** 439–442.
31. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
32. Hogan, B., Beddington, R., Costantini, F. & Lacy, E. (1994) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab. Press, Plainview, NY).
33. Darnell, J. E., Lodish, H. & Baltimore, D. (1986) *Molecular Cell Biology* (Scientific American Books, New York), p. 1040.
34. Robertson, E. J. (1987) *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (IRL, Washington, DC).
35. Li, E., Bestor, T. H. & Jaenisch, R. (1992) *Cell* **69,** 915–926.
36. Davis, A. C., Wims, M., Spotts, G. D., Hann, S. R. & Bradley. A. (1993) *Genes Dev.* **7,** 671–682.
37. Warren, A. J., Colledge, W. H., Carlton, M. B. L., Evans, M. J., Smith, A. J. H & Rabbitts, T. H. (1994) *Cell* **78,** 45–57.
38. George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. & Hynes, R. O. (1993) *Development* **119,** 1079–1091.
39. Yang, J. T., Rayburn, H. & Hynes, R. O. (1993) *Development* **119,** 1093–1105.
40. Dumont, D. J., Gradwohl, G., Fong, G., Puri, M. C., Gertsenstein, M., Auerbach, A. & Breitman, M. L. (1994) *Genes Dev.* **8,** 1897–1909.
41. Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. & Akhurst, R. J. (1995) *Development* **121,** 1845–1854.
42. Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. & Nagy, A. (1996) *Nature (London)* **380,** 435–439.
43. Connolly, A. J., Ishihara, H., Kahn, M. L., Farese, R. V., Jr. & Coughlin, S. R. (1996) *Nature (London)* **381,** 516–519.
44. Cui, J., O'Shea, K. S., Purkayastha, A., Saunders, T. L. & Ginsburg, D. (1996) *Nature (London)* **384,** 66–68.
45. Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C., Barnard, J. A., Yuspa, S. H., Coffey, R. J. & Magnuson, T. (1995) *Science* **269,** 230–234.
46. Sibilia, M. & Wagner, E. F. (1995) *Science* **269,** 234–238.