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CONTRIBUTION OF HOST-DERIVED TISSUE FACTOR TO TUMOR NEOVASCULARIZATION

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Abstract

Objective—The role of host-derived tissue factor (TF) in tumor growth, angiogenesis and metastasis has hitherto been unclear, and was investigated in this study.

Methods—We compared tumor growth, vascularity and responses to cyclophosphamide (CTX) of tumors in wild type (wt) mice, or in animals with TF levels reduced by 99% (low-TF mice).

Results—Global growth rate of three different types of transplantable tumors (LLC, B16F1 and ES teratoma), or metastasis were unchanged in low-TF mice. However, several unexpected tumor/context-specific alterations were observed in these mice, including: (i) reduced tumor blood vessel size in B16F1 tumors; (ii) larger spleen size and greater tolerance to CTX toxicity in the LLC model; (iii) aborted tumor growth after inoculation of TF-deficient tumor cells (ES TF-/-) in low-TF mice. TF-deficient tumor cells grew readily in mice with normal TF levels, and attracted exclusively host-related blood vessels (without vasculogenic mimicry). We postulate that this complementarity may result from tumor-vascular transfer of TF-containing microvesicles, as we observed such transfer using human cancer cells (A431) and mouse endothelial cells, both *in vitro* and *in vivo*.

Conclusions—Our study points to an important, but context-dependent role of host TF in tumor formation, angiogenesis and therapy.

INTRODUCTION

Several experimental studies point to the antitumor effects of genetic and pharmacological blockade of the coagulation system including tumor-cell associated tissue factor (TF) [1;2], and often through interference with angiogenesis [3-6]. However, other reports suggest that TF

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expression by cancer cells is of little (or no) consequence for tumor aggressiveness [7;8]. For instance, experiments with murine embryonic stem (ES) cells with either intact (TF+/-) or disrupted (TF-/-) *TF* gene originally led Toomey *et al* to conclude that subcutaneous teratomas could be efficiently generated irrespectively of the TF status of tumor forming (ES) cells [7]. A possible explanation of this conundrum could lie in the observation that TF may be expressed by various subsets of host cells often present in the tumor mass, including, stromal fibroblasts, inflammatory cells and the endothelium, all likely involved in regulation of angiogenesis [9], but this possibility has not been explored directly.

The effects of TF on vascular cells could be mediated through localized procoagulant effects of this receptor, irrespectively of its cellular source, leading to the generation of factor Xa and thrombin, followed by the deposition of fibrin and activated platelets [2;10]. However, the effects of TF could also be more cell autonomous in nature, and driven by changes in intracellular signaling [11]. In this case the co-expression of TF and protease activated receptors (PARs) could trigger signals mediated by thrombin (*via* PAR-1), or by TF/VIIa complex [11-13], including interactions between the cytoplasmic tail of TF and PAR-2 receptor in endothelial cells [14]. Interestingly, deletion of the latter domain in transgenic mice altered (increased) their vascular responses in the context of tumors and developing retina [14]. However, the angiogenic consequences associated with the obliteration of the entire TF molecule in the host compartment of a growing tumor are still to be examined.

In this regard, studies on host-related TF have been hampered by the embryonic lethality associated with TF gene disruption in mice, and with limitations of the available mouse-specific pharmacological antagonists of this receptor. Moreover, such agents tend to target selectively some (procoagulant), but not all biological activities of TF [15], e.g. may not obliterate TF-dependent signaling [11]. One way to circumvent these difficulties is to take advantage of a unique strain of mice, in which the endogenous mouse TF gene (mTF) is substituted with a human minigene (hTF) giving rise to the expression of a severely hypomorphic TF phenotype (low-TF mice) [16]. Homozygous low-TF mice (mTF-/-/hTF+/+) are viable, but unlike their wild type (mTF +/+), or heterozygous (mTF+/-/hTF+/-) counterparts express only 1% of the expected TF activity, encoded exclusively by the hTF sequence [16]. We chose low-TF mice to examine the effects of host-TF on tumor angiogenesis.

Here we show that the growth of three different types of TF-expressing transplantable tumors was unchanged in low-TF mice, as compared to that in their TF-proficient counterparts. However, host TF does exert a number of context-dependent effects, including on blood vessel size in some, but not all tumors. Importantly, in the absence of tumor-related TF the growth of tumors in low-TF mice was completely aborted. We propose that TF plays a crucial role in tumor formation, and it can be shared between tumor and host compartment through exchange of membrane microvesicles.

MATERIALS AND METHODS

Cells and culture conditions

Lewis Lung Carcinoma (LLC), B16F1 melanoma and A431 human squamous cell carcinoma cells were purchased from American Type Tissue Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, Utah, USA) supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, CA, USA). Mouse brain endothelial cells (MBE) were cultured on 1% gelatin coated dishes in EGM2 medium, as previously described [17;18]. The labeling of A431 cell surface and related microvesicles with the PKH26 dye (Sigma, San Diego, CA) was conducted as previously described [6;19]. MTF+/- and TF-/- ES cells were generated as

detailed elsewhere [7], as were the wild type ES cells (R1) and their LacZ (C16) and YFP (YC5)-tagged derivatives, a generous gift of Dr. Andras Nagy, University of Toronto [20]. All ES cells were maintained on gelatinized (0.1%) culture plates in the presence of mitomycin C treated fibroblasts (MEFs; DR4 from American Tissue Culture Collection, Manassas, VA) containing 2ME, non essential amino acids, leukemia inhibitory factor (LIF/ESGRO, Millipore, Billerica, MA; 500 U), 15% pre-tested fetal bovine serum (Hyclone) and with frequent feeding and subculturing. The cells were passaged several times without MEFs before injection into mice [20].

Northern analysis

Expression of TF transcripts was analysed as described earlier [6]. The membranes were examined using the Typhoon 9410 Phosphoimager (Amersham Biosciences) and autoradiographed.

Mice

Low-TF mice were described previously [16]. For tumor studies the mice were backcrossed into either C57BL/6 (LLC, B16F1), or (ES-teratoma) severe combined immunodeficiency (SCID) background.

Tumor generation and analysis

Tumorigenic cells were collected and injected subcutaneously in 0.1 ml of phosphate buffered saline (PBS), at the following predetermined numbers: 7-10 × 10^5 (LLC), 2 × 10^6 (B16F1) and 10^7 (ES cells) under isoflurane anesthesia (1 – 3%). Tumor growth was monitored as described earlier [20]. All *in vivo* experiments were conducted approved by the institutional Animal Care Committees at McMaster and McGill Universities and in accordance with the Canadian Council of Animal Care (CCAC) guidelines.

Therapy

Cyclophosphamide (CTX; Procytox, ASTA Medica, Ltd.) was used according to previously published maximal tolerated dose (MTD) and low dose/metronomic (LD) protocols [21]. Under the MTD regimen the drug was injected at 150 mg/kg on days: 2, 4 and 6 post tumor cell inoculation, and then on days: 23, 25 and 27. Increase in mortality occurred after the first round of injections. For metronomic (LD) therapy the drug was injected at the dose of 150 mg/kg on day 2 post tumor inoculation and then daily at 25 mg/kg [21]. A monoclonal anti-human TF antibody (TF8-5G9; Centocor Inc.; 5 mg/kg) or vehicle was injected intraperitoneally into low-TF mice (hTF) harboring LLC tumors (mTF) 3 times a week beginning on day 2 post tumor cell inoculation.

Experimental metastasis

A single cell suspension of 10^5 LLC cells/0.2 ml PBS was injected into the pre-warmed dilated lateral tail veins of the respective wild type, or low-TF syngeneic C56BL/6 mice, as indicated. Mice were sacrificed after 28 days and autopsied. Lungs were excised preserved in Bouin's fixative, and surface nodules were counted under a dissecting microscope, as described [22].

Tissue staining

Microvasculature of tumors was assessed by immunostaining for endothelial (CD31/PECAM; CD105/endoglin), or pericyte markers (αSMA), as described elsewhere [23].

Data analysis

The experiments were repeated 2-5 times, mostly 3 times, with similar results. The number of experimental mice per group in all *in vivo* studies varied between 4-10 for each experiment. The results were expressed as mean +/- SD for each group. Unpaired Student t tests were used to examine the differences between groups. Probability values <0.05 were considered significant unless otherwise stated.

RESULTS

Host TF and blood vessel patterning in melanoma

In syngeneic mice, B16F1 mouse melanoma cells form rapidly growing tumors containing cuffs of viable malignant parenchyma clustering around distinctly large (15-30μm) blood vessels, in which endothelial layers are covered with well-differentiated pericytes, as determined by staining for CD31/PECAM (or CD105/endoglin) and alpha smooth muscle actin (αSMA), respectively (Fig. 1A). These host cells are known to express TF [13]. Injection of B16F1 cells into mice with intact TF levels (mTF+/-) or into their hypomorphic, low-TF counterparts resulted in indistinguishable rate of tumor formation (Fig. 1B), however the tumor vascular microarchitecture has undergone a significant rearrangement (Fig. 1C). Thus, in low-TF mice tumor blood vessels were still covered with pericytes, but for the most part were considerably smaller in size (mostly < 15μm in diameter) than those in TF-proficient mice (both mTF+/+ and mTF+/-).

Unchanged, growth, vascularity and metastasis of Lewis Lung Carcinoma (LLC) in low-TF mice

Similarly to B16F1 melanoma, LLC cells form aggressive tumors *in vivo*, but with vastly different vascular patterns. A defining feature in this case is the absence of large vessels and a dense network of small capillaries (<15μm), largely devoid of SMA-reactive pericytes (Fig. 2A). While the TF content of LLC tumors increases *in vivo*, possibly with some contribution of host TF (Fig. 2B), the latter does not influence LLC progression or angiogenesis, as tumor growth, experimental metastasis and vascular patterns were virtually identical in wild type (mTF+/+), heterozygous (mTF+/-) and low-TF mice (Fig. 2CD, data not shown). Tumor growth remained unchanged even when tumor-bearing, low-TF mice were treated with an anti-human TF antibody (TF8-5G9), which would selectively obliterate the residual host TF (encoded by the hTF transgene), but not tumor/LLC-related (mouse) TF [16] (Fig. 2C).

Host-related TF modulates toxic side effects of cyclophosphamide

Anti-cancer agents often trigger procoagulant events [24]. We asked whether host TF may play a role in this context, or in responses to other prototypic therapies such as administration of cyclophosphamide (CTX) at maximal tolerated doses (MTD), or at antiangiogenic/metronomic, low doses (LD), both previously validated in the LLC model [21]. As shown in Fig. 3A, the responses of LLC tumors to MTD or LD protocols of CTX were unaffected by the host TF status. Interestingly, MTD therapy led to some mortality, but only in TF-proficient (mTF+/-) tumor bearing mice and not in their low-TF counterparts (Fig. 3B). Moreover, we observed that untreated low TF (SCID) mice exhibit markedly larger spleens than their mTF+/+ or mTF+/- controls (Fig. 3CD). This was also the case in LLC tumor bearing mice, where low TF hosts contained more extensive lymphocytic infiltrates in areas surrounding white pulp. While CTX treatment reduced spleen size and cellularity in all mice, low TF mice retained greater amounts of spleen mass and white pulp nodules (Fig. 3E-I). Interestingly, these mice also had reduced fibrin deposition in their kidney (not shown). This may suggest that low levels of host TF may have a protective role against

some of the side effects of anticancer therapy, likely through a combination of coagulation-dependent and hematopoietic effects that remain to be elucidated.

Host TF expression is essential for growth of TF-deficient teratomas

In agreement with the aforementioned results subcutaneous injection of TF-expressing and tumorigenic embryonic stem (ES) cells led to unperturbed formation of aggressive and angiogenic teratomas in both wild type and low-TF mice (Fig. 4)[23]. In this case the mice were backcrossed to the SCID background to avoid immune rejection. It is of note that the model of ES teratoma afforded us a unique opportunity to interrogate TF depletion in both the host and tumor cell compartments simultaneously, e.g. by including TF-/- ES cells [7] and low-TF mice. Thus, we observed that both TF+/- and TF-/- ES cells grew as vascular teratomas in SCID mice with wild type levels of TF[7]. Remarkably, this process was selectively aborted in the case of TF-/- ES cells (but not TF+/- cells) injected into low-TF SCID mice (Fig. 4B). This result suggests that TF is essential for the malignant teratoma growth to occur, and this requirement can be met through TF contribution by either tumor, or host cells.

ES cell-derived teratomas recruit host blood vessels

The aforementioned result is novel and potentially important. However, totipotential capacity of ES cells to differentiate [25] may imply that tumor blood vessels may in this case originate from tumor (ES) cells and not through recruitment of host endothelium, a phenomenon known as vasculogenic mimicry [26]. Indeed, ES teratomas contain complex mixtures of histological elements including abnormal capillaries (Fig. 4C-E). To unequivocally distinguish tumor (ES) and host (mouse)-derived elements we used several molecular tags, including: yellow and green fluorescent proteins (YFP and GFP), as well as beta galactosidase (LacZ; Fig. 4C-H). Thus, TF-proficient (mTF+/+) ES cells (R1) tagged with LacZ were injected into untagged SCID mice, and we determined that all tumor-associated, CD31-positive endothelial cells originated from the LacZ-negative host compartment (Fig. 4F). We also generated teratomas from unlabelled ES cells that were injected into YFP/SCID mice harboring a constitutively expressed YFP transgene[23]. Again, staining for CD105 and YFP revealed dual positivity (host origin) of all tumor-associated blood vessels (Fig. 4G). Finally, YFP-labeled ES cells were used to generate tumors in untagged SCID mice and, again, we observed essentially no overlap between YFP and CD105 staining, except for a solitary blood vessel found in one section (Fig. 4H). This enforces the notion that ES teratomas, like many other cancers, rely on recruitment of the host-derived vasculature, which may serve as a source of TF to support growth of TF-/- tumors shown in Fig. 4B

Endothelial cell uptake of tumor-related TF-containing microvesicles

The aforementioned experiments (Fig. 4B) raise the possibility that TF activity may be shared between tumor and host cell compartments. One mechanism by which this could occur is through intercellular exchange of TF containing microvesicles (MVs) [27-29]. To test this, we employed the human epithelial carcinoma cell line, A431, which produces ample amounts of TF (Fig. 5A), which is also shed into conditioned medium as membrane MVs [28]. To test, whether this material could, indeed, be transferred to endothelial cells, the membranes of A431 cells were labeled with the fluorescent dye (PKH26) and their conditioned medium (containing MVs) was incubated with TF-negative mouse brain endothelial cells (MBEs). This led to a lasting acquisition by the latter cells of PKH26 fluorescence, human TF antigen expression (not shown) and TF-dependent procoagulant activity (Fig. 6AB), all derived from A431 tumor cells. These changes did not occur when A431-derived material was depleted from the MV fraction by high-speed centrifugation [28]. Moreover, host blood vessels in A431 xenografts in SCID mice were positive for both

human TF (Fig. 5 E-G) and endothelial markers (CD105), suggesting the intercellular transfer of TF taking place *in vivo* (Fig. 5H).

DISCUSSION

In this study we report several new findings related to the role of host TF in tumor angiogenesis and progression. Notably, we provide evidence that TF expression may be essential for tumorigenesis, at least in some settings, since a simultaneous depletion of this receptor from both tumor and host compartments led to a complete arrest of tumor formation. The nature of this effect remains presently unknown, but may include thrombin-dependent and/or independent, essential effects of TF on hemostasis, and/or on signaling in endothelial, stromal and/or tumor cells [11;13]. In spite of this uncertainty, our results may reconcile the long standing controversy, as to whether TF contributes to primary tumor growth and angiogenesis [3-6;30;31], or is irrelevant for these processes [7;8]. Using the same TF-/- ES cell lines [7] that have originally led to the latter notion we demonstrated that the removal of TF from cancer cells may, at least in some cases, be insufficient to block tumor formation, notably due to compensatory effects of the host-derived TF (and *vice versa*). We propose that this TF 'sharing' between tumor and host compartments could occur through the exchange of TF containing microvesicles, as recently described for inflammatrory cells and platelets [29]. In particular, we suggest that such TF transfer could contribute to procoagulant and proangiogenic reprogramming of endothelial cells in TF expressing tumors [9] (Fig 5E). In addition, our study provides an important validation of the widely used ES teratoma model, by documenting the absence of ES-derived blood vessels (vasculogenic mimicry) in these tumors.

Unlike ES cells, many cancer cell types express TF under influence of oncogenic alterations [6]. We postulate that in such a context tumor-derived TF may assume a dominant role in tumorigenesis and can be targeted therapeutically, as recently demonstrated by Versteeg [32] and others [3;6;33]. In these settings host-related TF makes a minimal quantitative contribution to local growth or metastasis, as documented by our experiments with several mouse tumor models (LLC, B16F1).

Interestingly, low levels of host TF led to qualitative re-patterning of the tumor microvasculature in B16F1 melanoma. We suggest that lack of similar effects in other tumors tested, is due to their *a priori* small caliber vasculature. It is unclear how host TF may impact blood vessel patterning. While thrombin and PAR-1 were implicated as regulators of vascular development [12;13], our injections of B16F1 cells into *PAR-1*-/- mice did not recapitulate the vascular patterns observed in low-TF mice (unpublished observation). While tumors in low-TF mice were of unchanged size, it is possible that vascular re-patterning may influence angiogenic pathways in a qualitative manner, and impact other vascular aspects of tumourigenesis, such as metastasis and therapeutic responses.

We were intrigued by the partial protection of LLC tumor bearing, low-TF mice from the toxicity of high doses (MTD) of CTX. This effect was unlikely due to a differential drug distribution, pharmacodynamics, or direct toxicity against tumor cells. In this regard, prothrombotic effects of anticancer chemotherapeutics are well established, including in the clinic [2;34]. These events may be attributed to endothelial injury [35;36], suppression of the protein C pathway [24], upregulation of TF and decrease in levels of the tissue factor pathway inhibitor (TFPI)[37]. Indeed, we observed a lower fibrin deposition in kidneys of CTX-treated tumor bearing low-TF mice (data not shown). These mice demonstrated larger spleens containing greater reserves of lymphocytic cells, relative to their wild type counterparts.

This finding may suggest a hitherto unappreciated role of TF in modulating hematopoietic toxicity of CTX.

Collectively, our findings reveal several novel properties of host TF. We suggest that a complex role of this receptor in tumor growth, angiogenesis, vascular patterning and drug toxicity has been previously obscured by exclusion of host TF from experimental studies. We observed that the effects of host-related TF are highly context dependent and different in tumors driven by aberrant differentiation (teratoma) or genetic aberrations (mouse melanoma, lung carcinoma). Therefore, defining a universal role for TF in cancer may prove elusive and targeting this receptor in various tumor settings may require a better understanding of cellular sources of TF and their specific contributions to the malignant process. Still, our study suggests that TF, in its cell-associated and microvesicular forms, may represent an attractive anticancer target, at least in some settings, especially as a regulator of tumor growth and/or modulator of anticancer drug toxicity.

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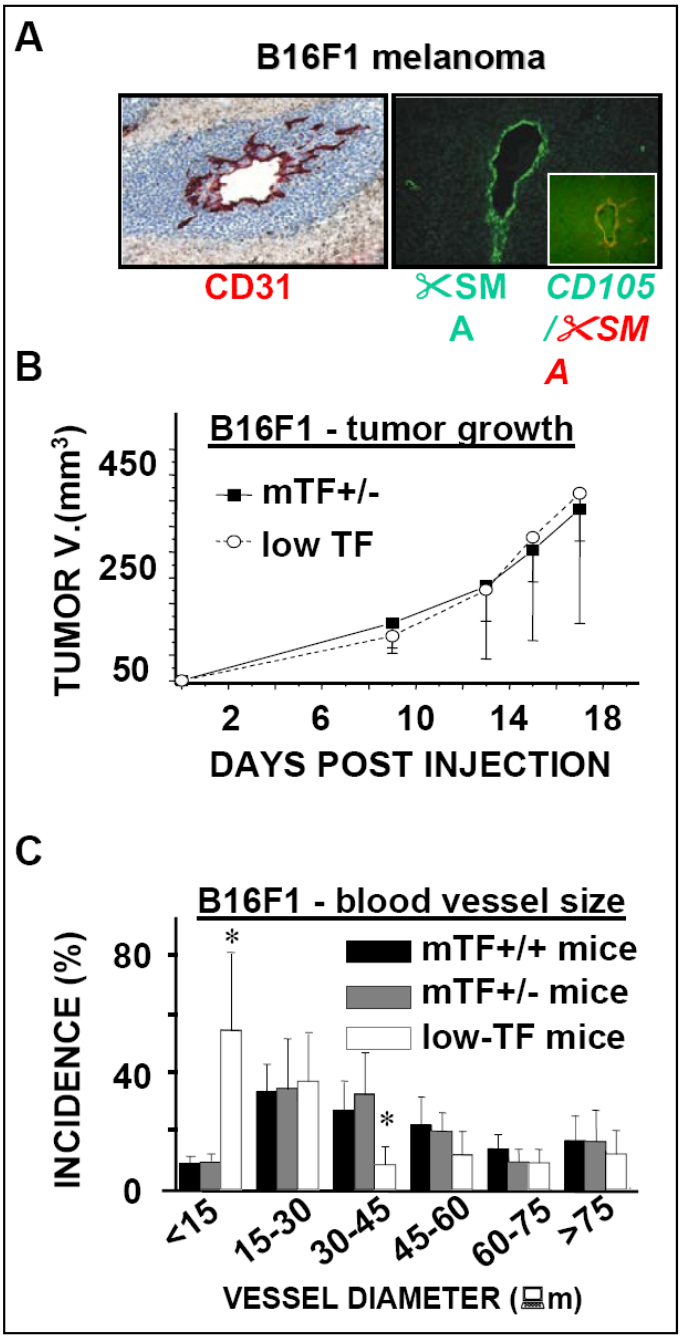

Figure 1. Growth and vascular patterning of B16F1 melanoma in low-TF mice

A. Distinct vascular pattern of B16F1 tumors in wild type C57BL/6 mice. Dilated blood vessels contain a well defined, CD31-positive endothelial lining (left panel) and a thick, continuous layer of αSMA-positive pericytes (right panel, green fluorescence). Inset, double immunofluorescent staining of B16F1 related blood vessels for endothelial (endoglin/CD105, green) and pericytic (αSMA, red) markers. **B.** Unchanged tumor growth kinetics of B16F1 tumors in TF-proficient (TF+/-) and low-TF mice (n = 5; mean∀ SD). The former mice represent crosses where one hypomorphic TF allele represents the *hTF* minigene and one (*mTF*) is endogenous to the wild type mouse. In low-TF mice both *TF* alleles are hypomorphic (*hTF*). **C.** Altered vascular architecture of B16F1 tumors in low-TF mice.

Diminution of larger vessels (30-45 μm in diameter) and preponderance of small capillaries (< 15 μm) in tumors growing in low-TF mice, but not in their wild type, or heterozygous (TF+/-) counterparts (αSMA staining).

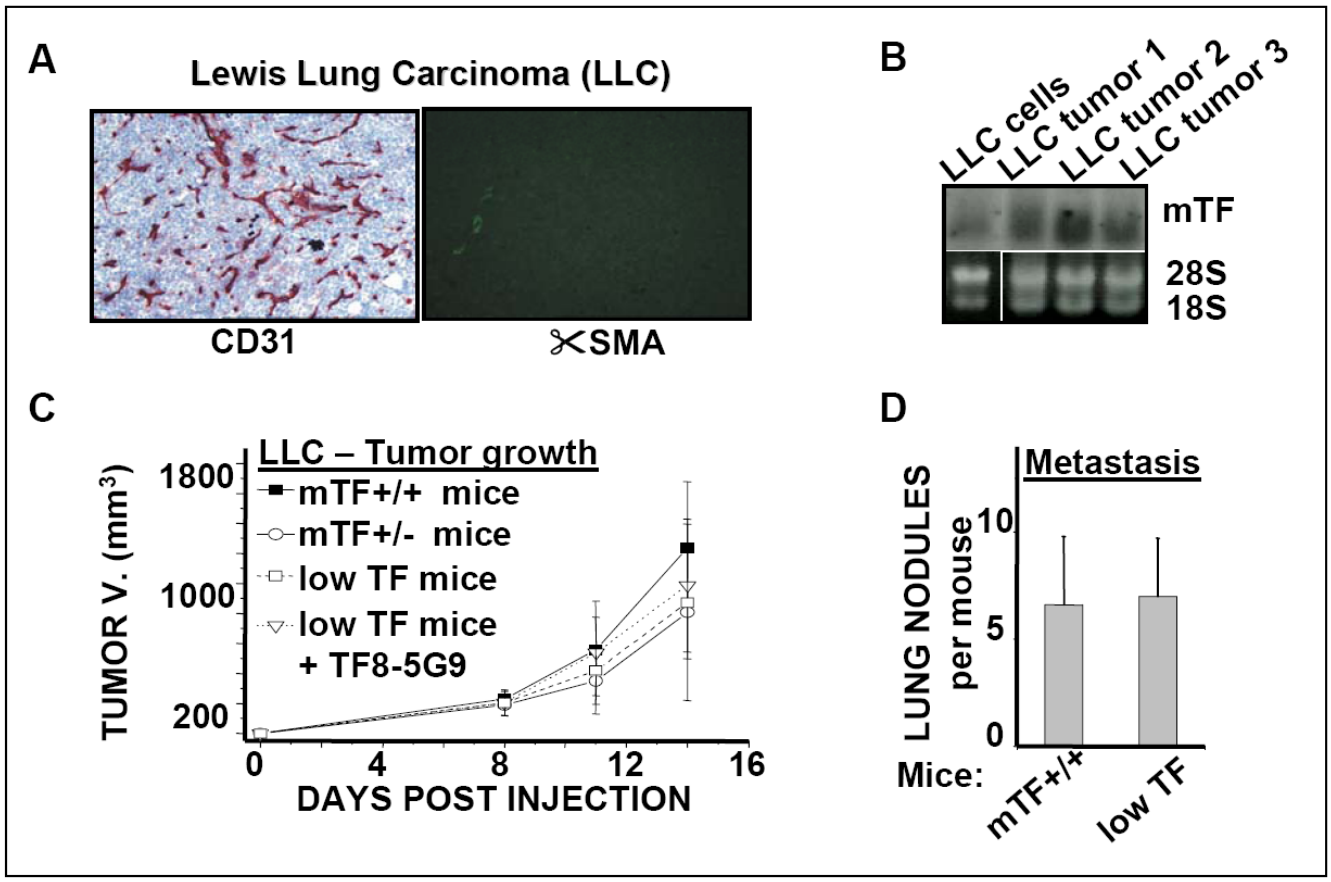

Figure 2. Unchanged growth and metastasis of LLC tumors in low-TF mice

A. Preponderance of small, CD31-positive and αSMA-negative capillaries within LLC tumors in wild type C57BL/6 mice. **B.** Increased expression of TF mRNA *in vivo*. **C.** Unchanged growth kinetics of LLC tumors in untreated low-TF mice and in the presence of the host-TF-specific (anti-hTF) neutralizing antibody (TF8-5G9; n = 5, mean ∀ SD). **D.** Indistiguishable capacity of LLC cells to form experimental lung nodules in wild type (wtTF) and low-TF mice (n = 7, mean ∀ SD).

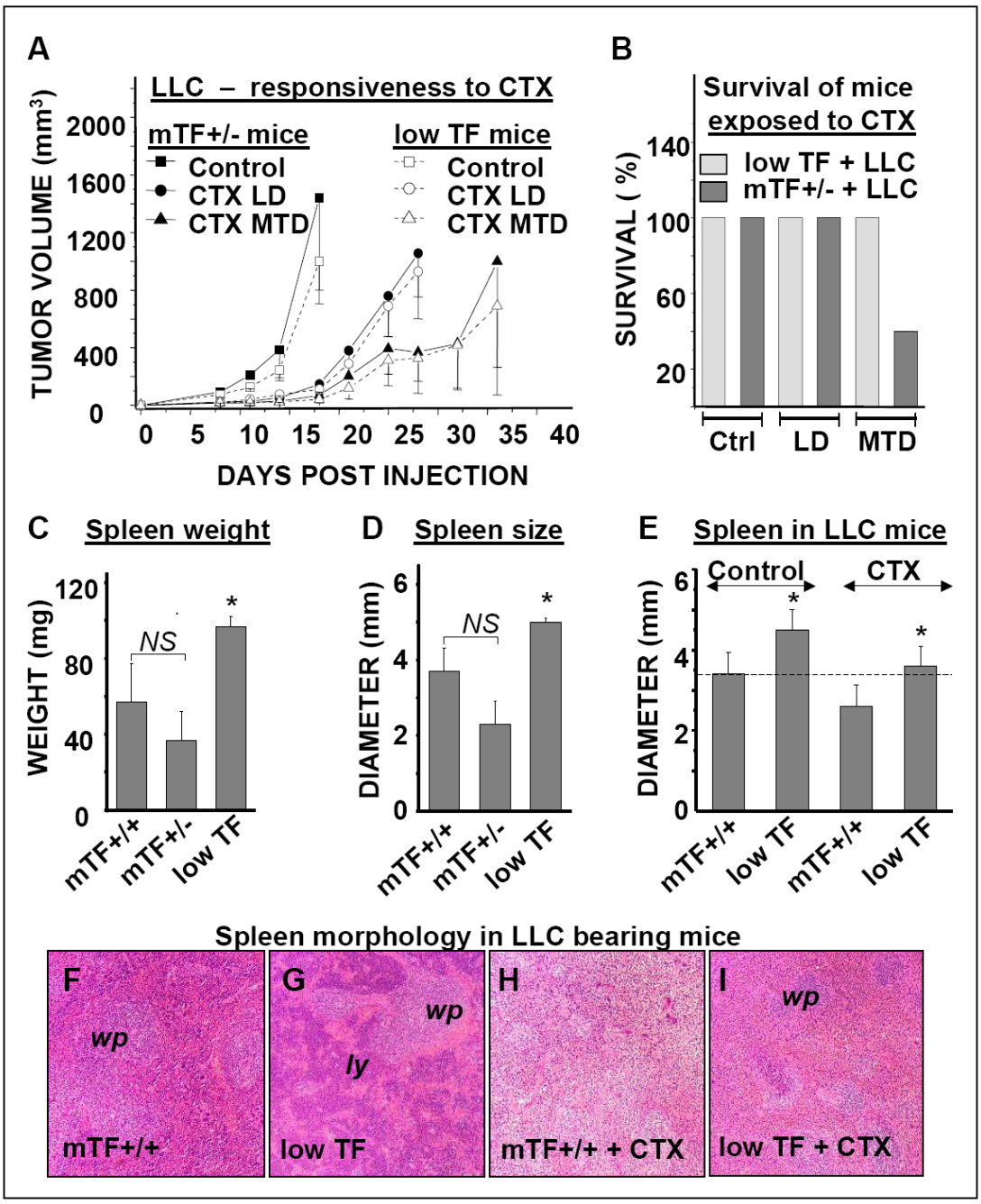

Figure 3. Unchanged antitumor responses, diminished chemotherapy-related mortality and abnormal spleens in low-TF mice harboring LLC tumors

A. Both maximal tolerated (MTD) and low/metronomic (LD) regimens of cyclophosphamide are similarly effective in TF-proficient (TF+/-) and low-TF mice. **B.** Treatment-related mortality of LLC bearing mice subjected to MTD regimen was averted in low-TF mice (n = 5 mice/group, mean ∀ SD; representative experiment out of 2 similar repeats). **C-D.** Spleen weight and diameter in mTF+/+, mTF+/- and low TF in untreated (SCID) mice. **E-I.** Spleen diameter * - p # 0.05; (E) and morphology (F-I) in LLC bearing mice 24 hours after the last dose of MTD CTX *vs* controls. Noticeable are lymphocytic infiltrates (ly) in low TF mice with retention of white pulp (wp) – H&E staining.

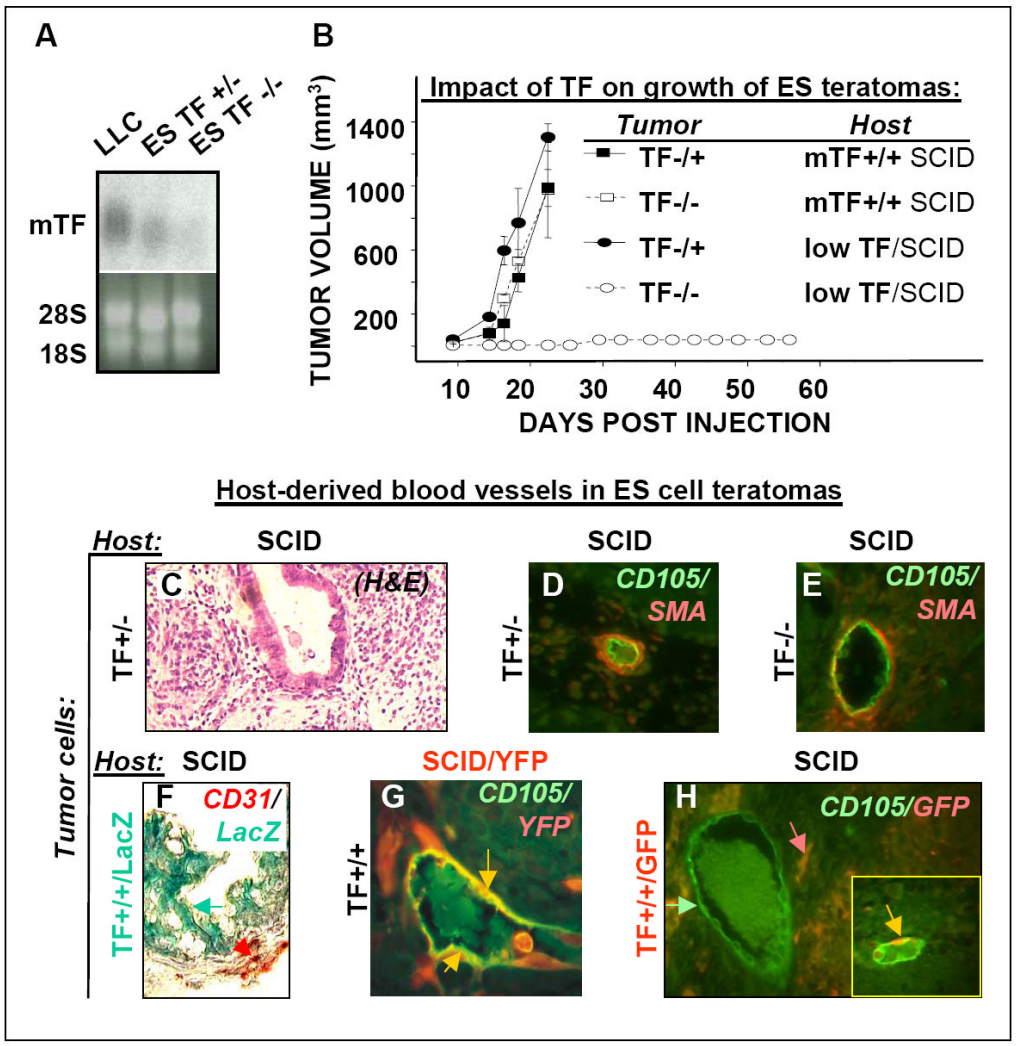

Figure 4. Complementary roles of host- and tumor-related tissue factor during formation of ES cell derived teratomas

A. Expression of TF mRNA by TF+/- and TF-/- ES cells. **B.** Tumor growth upon inoculation of TF-/- and TF+/- ES cells into TF-proficient (SCID) or low-TF/SCID (TF-deficient) strains. Only a simultaneous diminution of TF expression in both tumor and host cells led to obliteration of teratoma formation. **C.** Morphology of ES-derived teratomas (H & E staining). **D.** Comparable expression of markers of endothelial (CD105) and pericytic (αSMA) cells in blood vessels in TF+/- and TF-/-teratomas growing in SCID mice. **F.** Non-overlapping staining for ES cell (LacZ) and endothelial (CD31) markers in LacZ expressing wild type teratomas **G.** Co-expression of host (YFP) and endothelial (CD105) markers in wild type teratoma growing in the strain of SCID mice constitutively expressing the YFP transgene (SCID/YFP) [23]. Arrows point to cells expressing both markers. **H.** Non-overlapping expression of ES cell associated (YFP) and endothelial (CD105) markers in tumors originating from YFP expressing ES cells in SCID mice (see text). Inset - rare blood vessels containing markers of ES cells (YFP) in the endothelium.

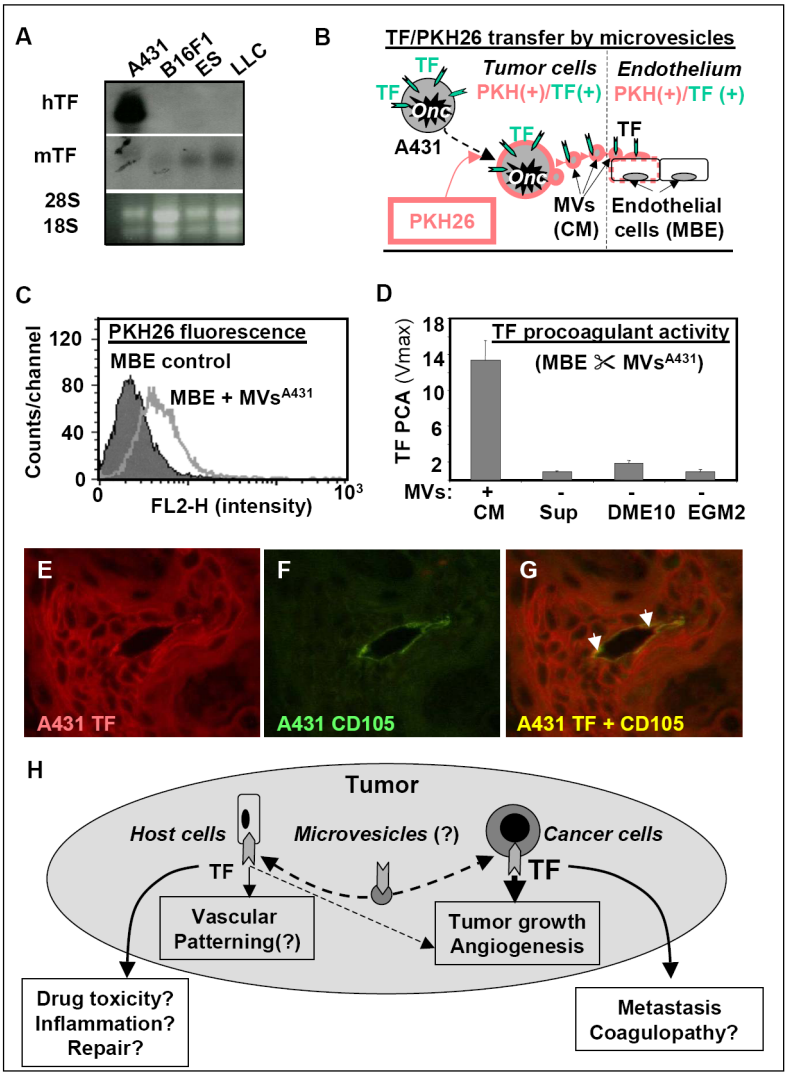

Figure 5. Microvesicle-mediated sharing of TF between tumor and endothelial cells

A. TF gene expression by human and murine tumor cell lines. **B.** Fluorescent labeling of microvesicles (MVs) derived from A431 cancer cells using the PKH26 dye and (**C**) their transfer to cultured mouse brain endothelial cells (MBE). **D.** Procoagulant reprogramming of MBE cells by the uptake of tumor cell derived TF, *via* MV transfer. CM – A431 conditioned media containing TF-rich MVs, Control treatments: Sup – A431 CM depleted of MVs; DME10 – A431 growth medium (DMEM with 10%FBS); EGM2 – endothelial growth medium; **E-G.** Uptake of human TF (red) *in vivo* by mouse endothelial cells stained for CD105 (green) within A431 xenografts in SCID mice (G – overlay). **H.** Proposed consequences of TF sharing between tumor and host compartments.