Ex vivo and In vivo Delivery of Anti-Tissue Factor Short Interfering RNA Inhibits Mouse Pulmonary Metastasis of B16 Melanoma Cells

Mohammed Amarzguioui,¹ Qian Peng,²,³ Merete T. Wiiger,¹ Vlada Vasovic,² Eshrat Babaie,¹ Torgeir Holen,¹ Jahn M. Nesland,² and Hans Prydz¹

Abstract

Purpose: The coagulation trigger tissue factor has been implicated in tumor growth, angiogenesis, and metastasis. In this study, we explore the effects of ex vivo and in vivo delivery of short interfering RNA (siRNA) targeting tissue factor on B16 melanoma colonization of the lung in a murine model for metastasis. The purposes of this work are to establish a noncytotoxic in vivo model for investigation of tissue factor function and provide preclinical assessment of the therapeutic potential of tissue factor siRNA for prevention of metastasis.

Experimental Design and Results: C57BL/6 mice were evaluated for pulmonary metastases following tail vein injection of B16 cells transfected with either active or inactive siRNA. Mice receiving cells transfected with active siRNA had significantly lower numbers of pulmonary tumors compared with mice injected with control cells (transfected with inactive siRNA). The average time point at which the mice started to exhibit tumor-associated stress was also increased significantly from 22 days for the control group to 27 days for the experimental group (P = 0.01). In a therapeutically more relevant model, where the siRNA was delivered i.p. and the cells (untransfected) by tail vein injection, an inhibitory effect on metastasis was observed when the siRNA treatment was initiated either before or at the time of cell injection.

Conclusions: The results suggest that tissue factor has a crucial function in promoting lung metastasis of blood-borne tumor cells in the early stages of the tumor take process and further suggest that treatment with tissue factor siRNA may become a viable clinical strategy for prevention of tumor metastasis.

Tissue factor has long been recognized as the primary and most potent trigger of blood coagulation (1) and functions as a receptor for clotting factor VII (FVII; 2). More recently, tissue factor has also been implicated in various processes promoting cancer development and spreading. Many studies have reported that tissue factor plays a major role in cancer-driven angiogenesis (3–6), whereas tissue factor expression seems to correlate with progression/growth, invasion, and metastasis of various types of cancer cells (3, 4, 7–10). Tissue factor expression may thus be a clinically useful prognostic factor (7). The correlation of tissue factor expression with tumor progression and metastasis further suggests that inhibition of tissue factor may offer a potential therapeutic avenue. Blocking coagulation activity by either monoclonal anti-tissue factor antibodies (9) or tissue factor pathway inhibitor (8) has been reported to inhibit experimental lung metastasis. In the present study, we explored the potential of short interfering RNA (siRNA; ref. 11)–mediated sequence-specific disruption of tissue factor gene expression for inhibition of experimental metastasis. The purposes of this study were to (a) confirm the proposed involvement of tissue factor in promotion of metastasis through selective disruption of tissue factor expression in metastatic cells, (b) establish a new siRNA-based in vivo model for investigation of the role of tissue factor in metastasis, and (c) evaluate the therapeutic potential of siRNA as an antimetastatic agent in a clinically relevant model. Tissue factor expression was transiently modulated by ex vivo and in vivo administration of chemically synthesized siRNA in a murine model for blood-borne metastases characterized by lung colonization of i.v. delivered murine melanoma B16-F10 (B16) cells. Our experimental system is thus without complications due to potential differences in genetic background inherent in comparing the metastatic potentials of different lines of tumor cells.

siRNAs are 21- to 23-nucleotide double-stranded RNAs with two-nucleotide 3’ overhangs that mediate sequence-specific degradation of homologous mRNA (11, 12). The process, termed RNA interference, is highly conserved during evolution and mediated by a multicomponent ribonucleoprotein complex
(13). We have previously reported that human tissue factor (hTF) mRNA levels in cultured cells can be effectively down-regulated by liposome-mediated delivery of chemically synthesized siRNA (14). Here, we show that knockdown of murine tissue factor (mTF) expression in B16 cells in vitro by 70% to 80% results in a dramatic reduction in the incidence of pulmonary tumors 10 to 20 days after i.v. injection of siRNA-treated cells in C57BL/6 mice. Furthermore, we show that the siRNA can efficiently target and inhibit metastasis of cells when delivered i.p. before or at the time of, but not 4 days after, tail vein injection of cells. Our results therefore indicate that tissue factor has an important role in the early stages of the tumor take process and substantiate the therapeutic potential of siRNA targeting tissue factor for inhibition of certain blood-borne metastases.

**Materials and Methods**

**Sequence and preparation of siRNAs.** Twenty-one-nucleotide RNAs were synthesized in house, quantified, annealed, and quality controlled as described (14). siRNAs were designed against eight different sites within the coding region of mTF: mTF217i, mTF220i, mTF223i, mTF245i, mTF269i, mTF321i, mTF355i, and mTF395i. The siRNAs are named according to the position of the 5' nucleotide in the duplex target sites within mTF mRNA (accession no. M26071). A previously described siRNA (14), hTF167i, targeting hTF (accession no. M16553) and having no activity against mTF mRNA, was used as control. Sequences of all siRNA oligonucleotides are listed in Table 1.

**Cell culture and transfections.** B16-F10 cells (American Type Culture Collection, Manassas, VA; CRL-6475) were maintained in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 50 units/mL penicillin + 50 µg/mL streptomycin. The day before transfection, cells were counted and seeded at a density of 3.5 × 10⁴/cm². Cells were transfected with 100 nmol/L siRNA (0.1 mL/cm²) and complexed with LipofectAMINE 2000 (Invitrogen) as described (14). Briefly, liposomes and siRNA were diluted separately in equal volumes of serum-free medium or Opti-MEM (Invitrogen; 50-100 µL per µg RNA) at a v/v ratio of liposomes to RNA of 2.5:1 and then mixed together. The complexes were allowed to form at room temperature for 30 minutes and then diluted to the appropriate volume for either i.p. injection or transfection (see below). For time course experiments, transfected cells were subcultured on days 2 and 4 after transfection to maintain exponential growth.

**Preparation of cells for tail vein injections.** Cells were washed twice and detached with trypsin. Following serum inactivation of trypsin, cells were washed twice in PBS (140 mmol/L NaCl, 2.6 mmol/L KCl, 1.4 mmol/L KH₂PO₄, 8.1 mmol/L Na₂HPO₄, 2H₂O (pH 7.4)) and finally resuspended in PBS or serum- and calcium-free DMEM at 2.5 × 10⁶/mL. Viability of the injected cells was assessed by trypan blue staining and was typically >95%. Samples of the injected cells were collected for determination of the knockdown level at the time of injection. Northern analyses were used.

**Animal model and experiments.** Female C57BL/6 mice ages 4 weeks were obtained from Harlan Nederland (Horst, the Netherlands) and housed (10 mice per cage) for 10 to 14 days at the animal facility of the Norwegian Radium Hospital before initiation of the experiments. The mice were kept under specific pathogen-free conditions. Six-week-old mice were injected with 200 µL of 2.5 × 10⁶/mL B16-F10 cells in the tail vein. For some experiments, LipofectAMINE 2000–complexed siRNA was delivered in 1.0 mL i.p. After appropriate time intervals, mice were killed by cervical dislocation; their lungs were removed and rinsed; and the pulmonary metastases were counted and photographed. In experiments assessing the long-term benefits of siRNA treatment, the mice were inspected daily and sacrificed at the first indication of tumor-associated stress. In the case of s.c. tumor growth, B16-F10 cells (2.5 × 10⁵) suspended in 100 µL of PBS were implanted s.c. in the right flank of mice. The tumor volume was measured daily and calculated using the formula: (length) × (width)² × (π/6). All experiments were blinded to the investigator. The protocols of this study were approved by The National (Norwegian) Animal Research Authority, and all experiments on animals were conducted according to The National Ethical Committee’s Guidelines on Animal Welfare and the USPHS Policy on Humane Care and Use of Laboratory Animals.

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*Reference sequences for target positions: M26071 (mTF) and M16553 (hTF).
Tissue Factor siRNA Inhibits Pulmonary Metastasis

RNA isolation and Northern analysis. mRNA was isolated directly from cultured cells using Dynabeads oligo(dT)25 (Dynal, Oslo, Norway). Northern blots were hybridized with full-length CDNA probes for mTF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in PerfectHyb Plus (Sigma, St. Louis, MO) hybridization buffer according to the manufacturer’s instructions.

Statistical analysis. Student’s t test for independent samples was done for pairwise comparisons of mean values of variables. In calculating two-tailed significance levels for equality of means, equal variances were assumed for the two populations.

Results

In vitro characterization of siRNA. siRNA targeted to different sites within the same mRNA can display widely differing silencing efficiencies (14). Our previous experience from targeting of hTF with siRNA suggested the need for extensive screening of mTF to identify siRNAs of sufficient activity to be useful in our experiments (14). We designed eight siRNAs specific for mTF, targeting sites located within 200 bp corresponding to the region harboring the best siRNA targets in hTF (14). LipofectAMINE 2000–mediated transfection of B16 cells with 100 nmol/L siRNA showed a highly variable efficiency of the different target sequences (Fig. 1A), consistent with our previous observations (14). The most effective siRNA (mTF223i) consistently depleted mTF mRNA by ~80%, 24 hours after transfection. This was one of three overlapping siRNAs targeted to the region corresponding to the target sequence of the best hTF siRNA (hTF167i). Although highly active against its intended target (14, 15), the siRNA hTF167i contained multiple mismatches against the mTF sequence (Fig. 1B) and had no effect at all on mTF expression in cultured B16 cells (Fig. 1C). The level of knockdown achieved with mTF223i in cell culture suggested that this siRNA would be a good candidate for in vivo experiments. We next estimated the duration of silencing. In a subculturing time course experiment with cells in the exponential growth phase, expression of mTF recovered gradually from days 3 to 5 (Fig. 1D), in a manner resembling that previously reported for hTF (14, 15). Silencing of mTF expression during this window had no or little effect on proliferation, as measured by the levels of the nontargeted internal control housekeeping gene GAPDH (Fig. 1D). Lack of effect of mTF silencing on proliferation within this window has also been shown independently by total protein measurements on transfected cells 5 days after transfection (data not shown).

The observed window of reduced mTF expression was considered sufficient to attempt in vivo experiments without any further optimization of the siRNA composition (e.g., by chemical modification) for increased stability (15). For the in vivo experiments, we adopted an established model in which tail vein injection of B16 cells into C57BL/6 mice results in lung colonization within 10 to 14 days. We considered this to be sufficiently short for the underlying molecular processes to be amenable to functional modulation by a single administration of chemically synthesized siRNA (11, 14). Initially, B16 melanoma cells and a highly metastatic M4 clone of the primary K-1735 melanoma (16), both expressing high levels of tissue factor (10), were evaluated. In preliminary experiments, the M4 cells exhibited a higher metastatic tendency than B16 cells, coinciding with a 2- to 2.5-fold higher relative (normalized to GAPDH) expression of tissue factor mRNA compared with B16 cells (data not shown). These data were consistent with the previously reported correlation of the metastatic tendency of melanoma cells with their levels of tissue factor expression (8, 10). Although the M4 cells displayed a higher metastatic tendency than B16 cells, the former proved relatively refractory to liposome-mediated transfection under standard transfection conditions (only 40-50% knockdown in cell culture; data not shown). B16 cells were therefore chosen for our test system.

The day before injection, cells were transfected with a control siRNA against hTF (hTF167i) or with either of the two most active siRNAs targeting mTF (mTF223i and mTF321i). A total of three independent and blinded experiments were done, with at least five mice in each experimental group and harvesting time point. Mice were sacrificed on day 10 in the first experiment, on days 10 and 15 in the second experiment, and on days 15 and 20 in the third experiment (Table 2). On day 10 after injection, we observed on average 107 small but distinct blackish lung tumors, clearly derived from the melanin-rich B16 cells, in the control group of mice receiving hTF167i-transfected cells (Fig. 2A, left). In contrast, an average of only 10 tumors was observed in mice injected with mTF223i-transfected cells. Furthermore, 7 of 12 mice in the test group were completely free of tumors at this time point compared with none of 12 mice in the control group (P < 0.01). The difference in tumor development in mice treated with active and inactive siRNA was still evident on days 15 and 20 after injection (Fig. 2). Although the size and number of tumors in mice injected with mTF223i-transfected cells increased gradually from days 10 to 20, the increase was not by far as dramatic as that observed in the control mice (Fig. 2). On day 15, test-treated mice still exhibited a relatively moderate amount of small distinct tumors. Control-treated mice, however, had many (>500) confluent tumors, covering a substantial surface of the lungs (Fig. 2A, right). On day 20, lungs of control mice were visibly enlarged and completely covered with tumor tissue (Fig. 2B).

To confirm that the observed delay in tumor development was indeed due to the reduction in the level of tissue factor expression in targeted cells rather than a result of some unspecific effects of the siRNA in question, we included, in the third experiment, an additional experimental group consisting of cells treated with another active mTF siRNA. This siRNA (mTF321i) was unrelated in sequence to mTF223i and only slightly less active in cell culture experiments, typically resulting in 70% knockdown in vitro (Fig. 1A). This group of mice also developed significantly less tumors than the control group of mice (Table 2; Fig. 2B). Thus, our experiments show that a single liposome-mediated transfection of B16 cells with active mTF siRNA in vitro results in a target sequence specific delay in development of pulmonary tumors of i.v. injected cells. This delay is directly attributable to the transient knockdown of tissue factor expression.

The rate of tumor development in control-treated mice and the gradual emergence of tumors in mice injected with mTF siRNA-treated cells suggested that the protective effects of siRNA in this model were transient. The window of protection was subsequently examined. Mice injected with either control- or test-transfected cells were inspected several times daily and sacrificed at the first indication of tumor-associated stress. The average time point at which the mice first became symptomatic increased significantly (P = 0.01) from 22 days for the control group (n = 5) to 27 days for the mice injected with active...
mTF223i siRNA (n = 6). These data confirmed our expectation that treatment with mTF siRNA delayed but did not abolish tumor development.

The above pulmonary metastasis model cannot delineate which processes in tumor metastasis and progression are influenced by tissue factor. We decided to investigate whether tissue factor had an effect on localized tumor growth rate by evaluating the growth of tumors following s.c. injection of cells transfected with either mTF223i or hTF167i. Each experimental group consisted of 10 mice. The difference between the two growth curves (Fig. 3) indicated an initial and transient delay in the growth of tumors from cells treated with active siRNA compared with control-transfected cells, with a significant difference in the average tumor size (volume) on day 8 (P = 0.03) and day 9 (P = 0.05). Upon further incubation, the difference in the size of tumors in the two groups diminished gradually, so that after day 13, no effect of siRNA was seen.

In the above experiments, the siRNA was delivered to the cells ex vivo. We next addressed the question of whether siRNA could inhibit pulmonary metastasis of tail vein injected B16 cells if delivered separately from the cells. We anticipated that multiple injections of liposome-complexed siRNA would be required to achieve an inhibitory effect. Because multiple tail vein injections are technically challenging, we developed a model involving multiple i.p. injections of complexed siRNA and tail vein injection of the melanoma cells. The day before tail vein injection of cells, the mice were injected i.p. with 100 μg of siRNA. Two additional doses of 50 μg siRNA were

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**Fig. 1.** *In vitro* characterization of mTF siRNA activity. **A,** screening of the activity of multiple siRNA targeting mTF mRNA. Northern analysis of B16 cells 24 hours after LipofectAMINE 2000–mediated transfection with 100 nmol/L of each of eight different siRNA targeting mTF mRNA. Mock-transfected cells were used as control. GAPDH expression served as loading and normalization control. Cleavage fragments of higher mobility than full-length mTF mRNA result from the action of active siRNA. **B,** partial sequence alignment of human and murine tissue factor, comparing the target sequences (underlined) of mTF223i and hTF167i siRNA. **C,** specificity of siRNA-mediated silencing. Experiments were done as in (A). Cells transfected with the mismatched siRNA hTF167i, targeting hTF, exhibited the same level of mTF expression as mock-transfected cells. **D,** persistence of mTF silencing (black columns) and proliferation (hatched columns) following transfection of B16 cells with 100 nmol/L mTF223i. Cells were subcultured 1 and 3 days after transfection to maintain exponential growth and harvested after 1, 3, 4, and 5 days. Expression of tissue factor mRNA was normalized to GAPDH and standardized to levels in mock-transfected cells (black columns). For proliferation, mock-standardized GAPDH levels (hatched columns) are indicated.
delivered 3 and 6 days after tail vein injection of the cells, and the mice were sacrificed on day 10. The number of metastases in lung tissue was 5-fold lower ($P = 0.047$) in mice treated with active mTF223 siRNA ($n = 4$) compared with a control group receiving inactive hTF167 siRNA ($n = 5$; Fig. 4A). We next investigated whether siRNA would have any inhibitory effect on pulmonary tumor development if treatment were delayed sufficiently to allow attachment of the circulating tumor cells to the lungs. Initiation of multiple siRNA treatments 4 days after delivery of cells (with additional treatments on days 7 and 10 and harvest on day 15) did not result in any inhibitory effect on tumor development: mock control group, 302 ± 73 (average ± SD) tumors; hTF167i group, 296 ± 57 tumors; mTF223i group, 280 ± 191 tumors ($n = 10$ mice per group). This suggested that the presence of siRNA, and consequently, the efficient depletion of tissue factor during the first few days after tail vein injection of B16 melanoma cells is crucial for inhibition of pulmonary metastasis. Because of the time window for siRNA knockdown, a single administration of siRNA might be sufficient to achieve an inhibitory effect. Varying doses of complexed siRNA were therefore given i.p. within an hour of tail vein injection of the cells. A dose-dependent inhibitory effect on the development of lung tumors was observed, reaching levels of statistical significance for doses of 50 and 100 μg siRNA (Fig. 4B). The level of inhibition (5-fold) was similar to that obtained with multiple injections of siRNA (Fig. 4A), which further supports our conclusion that the optimal window of tissue factor depletion is immediately after delivery of the cells. Presumably, depletion of tissue factor in circulating tumor cells adversely affects their ability to give rise to distant tumors. This is consistent with a mechanism in which tissue factor primarily promotes blood-borne metastases through adhesion of the tumor cells (9).

Because tissue factor has an important function in initiation of blood coagulation, siRNA-mediated silencing of tissue factor in normal cells may in theory have deleterious effects on hemostasis. We decided to test this by evaluating the level of

| Table 2. Incidence of pulmonary tumors in mice injected (i.v.) with B16 cells that were transfected ex vivo with different siRNA |
|---|---|---|---|
| SiRNA | day 10 | day 15 | day 20 |
| hTF167i | 107 ($n = 12$)* | >500 ($n = 11$) | >500 ($n = 5$) |
| mTF223i | 10* ($n = 12$) | 33* ($n = 11$) | 74* ($n = 8$) |
| mTF321i | ND | 16* ($n = 5$) | 41* ($n = 5$) |

Abbreviation: ND, not determined.

*The average number of tumors and the total number of mice ($n$) included in the analysis are given for each experimental group and harvesting time point.

$P < 0.001$, level of significance of the differences in tumors for test (mTF223i and mTF321i) and control (hTF167i) groups.

*$P < 0.001$, level of significance of the differences in tumors for test (mTF223i and mTF321i) and control (hTF167i) groups.

Fig. 2. Effect of siRNA on pulmonary tumor development. A, lungs from mice injected with hTF167i- and mTF223i-transfected B16 cells, harvested on day 10 (left) and day 15 (right). Representative pair of samples from day 10 together with all day 15 samples from the same experiment. B, lungs from mice harvested on day 20, in an experiment incorporating three experimental groups (B16 cells treated with hTF167i, mTF223i, or mTF321i siRNA).
transfected with either hTF167i or mTF223i siRNA. The growth curves differed significantly on day 8 ($P = 0.03$) and day 9 ($P = 0.05$).

tissue factor mRNA in the two tissues within the vasculature that are known to express the highest amounts of tissue factor (kidney and lung). Tissues from three mice within each experimental group were harvested for Northern blot analyses 3 days after i.p. delivery of 75 μg of complexed siRNA (hTF167i or mTF223i). A mock complexation buffer treatment group was included in the experiment. The relative levels of GAPDH-normalized mTF mRNA were 100 ± 10% (average ± SD), 113 ± 3%, and 108 ± 5% for lungs from mock-treated, mTF223i-treated, and hTF167i-treated animals, respectively. Marginal increases of GAPDH-normalized mTF mRNA were 100 ± 10% (average ± SD), 113 ± 3%, and 108 ± 5% for lungs from mock-treated, mTF223i-treated, and hTF167i-treated animals, respectively. Marginal decreases relative to mock treatment were observed for the kidneys, but the differences were not statistically significant when comparing the mTF223i group (69 ± 10%) to either the mock (100 ± 24%; $t$ test, $P = 0.12$) or control hTF167i siRNA (80 ± 11%; $t$ test, $P = 0.32$) groups. Thus, these analyses showed no significant difference in tissue factor mRNA levels in the two most biologically relevant tissues within a time frame after delivery that should be optimal for detecting knockdown. Furthermore, the mice injected with siRNA targeting murine tissue factor did not show any signs of bleeding or thrombosis that would indicate functional disturbance of hemostasis. Published data indicate that tissue factor expression levels would have to be reduced much more than is likely to occur in a therapeutic situation to affect hemostasis. Transgene expression contributing only 1% of normal tissue factor activity is sufficient to rescue knockout mice from embryonic lethality (17).

**Discussion**

In this study, we have shown that transient knockdown of tissue factor mRNA in the B16 cells in vitro by 70% to 80% (Fig. 1A) dramatically reduced the number of pulmonary tumors on days 10, 15, and 20 after injection of siRNA-transfected cells (Fig. 2). This knockdown was also associated with a significantly delayed onset of tumor-associated symptoms. The effect was target sequence-specific and directly attributable to the knockdown because two different siRNAs targeting the murine version of tissue factor produced similar results. Treatment of cells with active siRNA also resulted in an initial inhibition of localized (s.c.) tumor growth, but the effect was of a more transient nature, as the tumor volumes only differed significantly between control and experimental group of mice on days 8 and 9 after injection (Fig. 3). The moderate effects on localized growth are consistent with in vitro proliferation experiments (Fig. 1D), and our previous data with human cells exhibiting stable tissue factor knockdown indicated a very marginal effect of tissue factor knockdown on proliferation compared with the effects on colony formation and in vivo metastasis (18).

In vitro data indicated that tissue factor knockdown lasted for only about 5 days. This window of depletion was sufficient to produce striking differences in the number of lung tumors on days 10 to 20 after injection between mice receiving inactive and active siRNA, suggesting that tissue factor has an important role at an early stage of the tumor take process. This is further supported by the results from experiments in which siRNA and cells were delivered separately. Although i.p. injection of siRNA either the day before or immediately after tail vein injection of cells resulted in significant inhibition in the number of lung tumors (Fig. 4), no inhibitory effect was seen when siRNA treatment was initiated 4 days after injection of the cells. Furthermore, when siRNA treatment was initiated within the optimal window of protection, no further inhibitory effects of additional later siRNA injections were observed. Combined, the above observations suggest that the inhibitory effects of tissue factor siRNA are not merely due to growth inhibition but most likely also due to an inhibition of the adherence of circulating tumor cells to the lung tissue.
Multiple mechanisms for tissue factor–mediated metastasis and angiogenesis have been suggested. Protease-activated receptor 2 plays an important role as a mediator of tissue factor–promoted cell migration and metastasis (19–21), whereas vascular endothelial growth factor figures prominently in tissue factor–dependent enhancement of angiogenesis (3, 5). We have previously shown that tissue factor functions as a receptor for FVII (2). The metastatic potential of tissue factor seems to be dependent on a functional interaction with its ligand because the process is inhibited by introduction of either mutated tissue factor or FVIIa (22, 23), or the use of monoclonal antibodies that disrupt their interaction (9). Furthermore, blocking coagulation at the level of tissue factor, FXa, or thrombin, inhibited metastasis in severe combined immunodeficient mice (24), whereas a possible synergy between tissue factor and the thrombin receptor protease-activated receptor 1 in promoting metastasis has been reported (25). Hence, the metastatic and growth-promoting properties of tissue factor seem to be related to its function in blood coagulation. Formation of a complex between tissue factor and activated FVII (FVIIa) triggers a substantial activation of blood coagulation. Formation of a complex between tissue factor and the thrombin receptor protease-activated receptor 1 in promoting metastasis has been reported (25). Hence, the metastatic and growth-promoting properties of tissue factor seem to be related to its function in blood coagulation. Formation of a complex between tissue factor and activated FVII (FVIIa) triggers a substantial activation of blood coagulation.

We thank Per W. Schulze for taking pictures of the lungs.

References
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