

***In vivo* inhibition of angiogenesis by a soluble form of melanotransferrin**

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Melanotransferrin (MTf), the membrane-bound human melanoma antigen p97, binds to plasminogen and stimulates its activation, thus regulating a crucial step involved in angiogenesis. In our study, a truncated soluble form of MTf (sMTf) inhibits, in a dose-dependent manner, the *in vitro* tubulogenesis of human umbilical vessel endothelial cells (HUVEC) induced by media conditioned with MTf-expressing cells. Following these results, we used the *in vivo* Matrigel™ plug angiogenesis assay to investigate whether truncated sMTf could inhibit neovascularization in mice. We found that basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and MTf-expressing cells strongly stimulate *in vivo* Matrigel™ neovascularization. However, subcutaneous (s.c.) administration of truncated sMTf inhibits both bFGF- and VEGF- as well as human melanoma SK-Mel-28-induced angiogenesis. This inhibition was dependent on the truncated sMTf concentration and reached maximal inhibition at 40 mg/kg/week. Furthermore, we found that a single s.c. injection of truncated sMTf into nude mice at 5 mg/kg produced a maximal blood concentration of ~40 nM, which is comparable with the level required to inhibit *in vitro* HUVEC tubulogenesis. Overall, our results strongly suggest that s.c. administration of truncated sMTf may provide a novel therapeutic strategy for the treatment of angiogenesis-related disorders.

Introduction

Melanotransferrin (MTf), also called human melanoma antigen p97, is a glycosylated protein and is part of the transferrin family (1,2). The transferrin family also contains serum transferrin (Tf), lactoferrin and ovotransferrin. There are two forms of endogenous MTf described to date; a membrane-bound form (mMTf), which is anchored to the cell membrane by a glycosylphosphatidylinositol-anchor, and a soluble secreted form (sMTf) (3). Expressed only slightly in normal tissues, MTf is present in much larger amounts in neoplastic cells such as melanomas and fetal tissues (2). Studies have shown that MTf is expressed in various tissues,

Abbreviations: bFGF, basic fibroblast growth factor; CHO, Chinese hamster ovary; EC, endothelial cells; Hb, hemoglobin; HUVEC, human umbilical vessel EC; mAb, monoclonal antibody; MTf, melanotransferrin; R/H, Ringer/HEPES; sMTf, soluble form of MTf; PA, plasminogen activator; s.c., subcutaneous; VEGF, vascular endothelial growth factor.

such as sweat gland ducts, salivary glands, liver endothelial cell (EC), brain endothelium and chondrocytes (4). Since MTf is an iron-binding protein that shares 39% protein sequence identity with Tf and lactoferrin (1), it was first thought that MTf could serve as an iron transporter; however, it was later shown that MTf played very little role in iron transport (5–7). We recently showed that both forms of MTf could bind plasminogen and stimulate its activation into plasmin by plasminogen activator (PA) (8,9), thus regulating cell motility, migration and invasion.

Angiogenesis, the process of new blood vessel growth from pre-existing capillaries, is involved in physiological and pathological processes such as embryonic development, wound healing, reproductive cycles, diabetic retinopathy, chronic inflammation, tumor growth and metastasis (10). This process involves EC proliferation, migration and morphogenic differentiation into capillary-like structures (tubulogenesis) (11). Thus, suppression of abnormal angiogenesis may provide therapeutic strategies for the treatment of angiogenesis-dependent disorders. In our laboratory, we have found that truncated sMTf could inhibit EC migration and tubulogenesis *in vitro*. In fact, truncated sMTf disturbs the regeneration of the free and active urokinase-type PA receptor (u-PAR) at the cell surface by the low-density lipoprotein receptor-related protein, which in turn reduces the EC capacity to generate plasmin from plasminogen (12).

In the present study, since MTf regulates a crucial step involved in angiogenesis, we used the *in vivo* Matrigel™ plug angiogenesis assay to investigate the ability of a human, truncated form of sMTf to inhibit neovascularization in mice. We show that MTf-expressing cells stimulate neovascularization of the Matrigel™ implant, which can be inhibited by the addition, into the matrigel, of the L235 monoclonal antibody (mAb) directed against MTf. Moreover, subcutaneous (s.c.) administration of a truncated form of sMTf inhibits SK-Mel-28- as well as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) induced *in vivo* angiogenesis. Altogether, our results strongly suggest that MTf participates in the neovascularization of solid tumors and that s.c. administration of truncated sMTf appears to be of potential interest as an antiangiogenesis treatment modality in clinical testing.

Materials and methods

Materials

Truncated human recombinant sMTf, which is produced by introducing a stop codon following the glycine residue at position 711 of full-length MTf cDNA (producing a 27 C-terminal amino acid deletion), and the L235 mAb were kindly provided by Biomarin Pharmaceutical (Novato, CA). Chinese hamster ovary (CHO) cells transfected with full-length human MTf cDNA (MTf-transfected cells) or with control vector (control cells) were from Dr Malcom Kennard of the University of British Columbia (Vancouver, BC). Recombinant human bFGF and VEGF-A165 were from Cedarlane

Laboratories Limited (Hornby, ON). Other biochemical reagents were from Sigma-Aldrich (Oakville, ON).

Cell culture

Cells were cultured under 5% CO₂/95% air atmosphere. Human umbilical vessel EC (HUVEC) were obtained from American Type Culture Collection (ATCC, Manassas, VA). HUVEC were cultured in EC basal medium-2 (EBM-2; Bullet kit, Clonetics, San Diego, CA). CHO cells (control and MTF-transfected) were cultured in Ham's F12 medium from Invitrogen (Burlington, ON) containing 10 mM HEPES and 10% calf bovine serum (Hyclone, Logan, UT). Human melanoma SK-Mel-28 cells were obtained from ATCC. SK-Mel-28 cells were grown in modified Eagle's medium from Invitrogen containing 1 mM Na pyruvate and 10% fetal bovine serum (Hyclone).

Capillary-like structure formation (tubulogenesis) assay

Phenol red Matrigel™ (50 µl; BD Bioscience, Mississauga, ON) was mixed with concentrated (16x, 10000 MWCO) cell conditioned media (4:1) or truncated sMTf (20 and 200 µg/ml), added to a 96-well plate and incubated for 10 min at 37°C. HUVEC were harvested by trypsinization. A total of 2.0×10^4 cells were resuspended in 100 µl medium (1% serum) and added to Matrigel™-coated wells. After 30 min incubation at 37°C, truncated sMTf (0, 10 and 100 nM) in fresh, serum-free medium was added over the cells. Cells were then incubated for 18 h at 37°C. After incubation, tubular structures were visualized at a 50x magnification using a Nikon TMS-F microscope. The length of the total capillary network was digitized and quantified using Northern Eclipse software (Empix Imaging, Mississauga, ON).

In vivo Matrigel™ plug angiogenesis assay

The Matrigel™ implantation assay was based on the method of Passaniti (13). Angiogenesis was considered to be indicated by the growth of blood vessels from s.c. tissue into a solid gel of Matrigel™ extracellular matrix. Specific pathogen-free, female athymic Crl:CD-1[®]-nuBR nude mice were obtained from Charles River Laboratories (Lassalle, QC). All mice used were 5–10 weeks of age. All animal studies were conducted in accordance with recommendations from the Canadian Council on Animal Care (CCAC) for care and use of experimental animals. Before injection, heparin was incubated with or without bFGF, VEGF or cells (SK-Mel-28, control and MTF-transfected CHO cells) for 5 min, then diluted into phenol red-free Matrigel™ on ice for a final concentration of 0.0025 units/ml heparin, 250 ng/ml bFGF, 200 ng/ml VEGF and 2×10^6 cells/ml. Then, mice were subcutaneously injected under anesthesia in the ventral midline region of the right flank with 0.5 ml of Matrigel™ alone or with Matrigel™ containing bFGF, VEGF or cells. After 7 days, the mice were euthanized and the Matrigel™ implant was harvested and washed with phosphate-buffered NaCl solution (PBS; 138 mM NaCl, 2.8 mM KCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4). Then, Matrigel™ implants were photographed with a digital Nikon Coolpix™ 5000 camera (Nikon Canada, Mississauga, ON). The remaining implants were immediately frozen and lyophilized overnight. The weight of each dry Matrigel™ implant was determined and the implants were resuspended in 0.4 ml of 0.1% Triton X-100 for 1 h, disrupted by vigorous pipetting and centrifuged at $14000 \times g$ for 15 min to remove particulates. The concentration of hemoglobin (Hb) in the supernatant was then determined directly by absorbance at 405 nm and compared with a standard curve of purified Hb, as described previously (14,15).

Procedures for testing efficacy of antiangiogenic molecules

Testing of antiangiogenic substances was initiated at the time of Matrigel™ implantation. First, treatment with either ε-amino-*n*-caproic acid (εACA; 35 mg/ml) as well as with mAb L235 or with non-specific, control IgG (25, 50 and 100 nM) was performed by diluting εACA or the antibodies directly in the phenol red-free Matrigel™ before implantation. Secondly, systemic treatment with various concentrations of truncated sMTf (2.5, 5.0 and 10 mg/kg/mouse; 4 times/week; over 7 days) was performed by s.c. injection at Day 0, 2, 4 and 6 post-implantation. Thirdly, to achieve maximal long-term treatment with truncated sMTf, an Alzet[®] (Cupertino, CA) osmotic pump (100 µl, 7-day delivery) was implanted subcutaneously in the dorsal infrascapular region to deliver various concentrations of truncated sMTf (1.42 and 2.85 mg/kg/mouse/day; over 7 days). In all experiments, Ringer/HEPES (R/H) solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM MgCl₂, 6 mM NaHCO₃, 5 mM HEPES, 2.8 mM glucose, pH 7.4) was used for control treatment.

Truncated sMTf pharmacokinetic parameters determination

Truncated sMTf was radio-iodinated by standard procedures using Na-[¹²⁵I] (Amersham Pharmacia Biotech, Baie D'Urfée, QC) and an Iodo-beads kit

from Pierce Chemical Co. (Rockford, IL). In a first set of experiments, female athymic Crl:CD-1[®]-nuBR nude mice (aged 5–10 weeks) received a single dose of 5 mg/kg truncated [¹²⁵I]-sMTf in an R/H solution by s.c. administration (100 µl; 10 µM; 1.2×10^6 CPM). In the next set of experiments, an Alzet[®] osmotic pump was implanted subcutaneously as described above to deliver 2.85 mg/kg/day truncated [¹²⁵I]-sMTf (100 µl over 7 days; 40 µM; 2.8×10^6 CPM). At the allotted times, blood samples (50 µl) were withdrawn from the tail vein into a heparinized Microvette[®] CB 300 (Sarstedt, Nümbrecht, GE) container. The concentration of truncated sMTf was then quantified by measuring blood-associated radioactivity using a gamma counter.

Data statistical analysis

Statistical analyses were made with Student's *t*-test when one group was compared with the control group. To compare two or more groups with each other, one-way analysis of variance (ANOVA) and Tukey's multiple comparison test as post-test were used. All statistical analyses were made using GraphPad Prism (San Diego, USA). Significance was assumed for *P*-values < 0.05.

Results

Truncated sMTf inhibits HUVEC tubulogenesis

In the present study, since MTF expression stimulates cell motility, migration and invasion (9), the *in vitro* morphogenic differentiation of EC into capillary-like structures (tubulogenesis) on Matrigel™ was examined under several conditions. Hence, we first examine the effects of Matrigel™-trapped MTF-expressing cell conditioned media on HUVEC tubulogenesis (Figure 1A). MTF is highly expressed by both MTF-transfected CHO and SK-Mel-28 cells, whereas control CHO and HUVEC cells do not express detectable levels of MTF (8,9,12). Moreover, the expression levels of secreted endogenous sMTf in MTF-transfected CHO and SK-Mel-28 cells is much lower than their mMTf expression. HUVEC cell growth on Matrigel produced a stabilized network of capillary-like structures. The tubulogenesis of HUVEC cells was stimulated ~2-fold by the addition of conditioned media from MTF-transfected cells and SK-Mel-28 cells nor control cells into the Matrigel™ (Figure 1A). We also determined the effects of exogenous truncated sMTf on this phenomenon. Interestingly, the addition in the medium of truncated sMTf (10–100 nM) during the assay inhibits both basal and MTF-induced tubulogenesis of HUVEC cells in a concentration-dependent manner (Figure 1A), without affecting cell proliferation (data not shown). Moreover, to determine whether the increase in HUVEC tubulogenesis, induced by the addition of conditioned medium from MTF-expressing cells, could be related to the presence of matrix-bound sMTf, truncated sMTf was added (1 and 10 µg) into the matrigel (Figure 1B). Matrigel™-trapped truncated sMTf induced the tubulogenesis of HUVEC in a concentration-dependent manner. Altogether, these results indicate that matrix-bound sMTf could induce capillary-like tube formation of EC and that truncated sMTf antagonized this effect.

bFGF-induced neovascularization involves the PA/plasmin system

We used the Matrigel™ plug neovascularization assay to study the effect of MTF on the modulation of *in vivo* angiogenesis. This assay permits the determination of the extent of angiogenesis into the Matrigel™ implant by direct measurement of the amount of Hb present in the implant. Analysis of the Matrigel™ implants that did not contain growth factor showed very low pink hue coloration (Hb content), indicating that Matrigel™ itself was not angiogenic

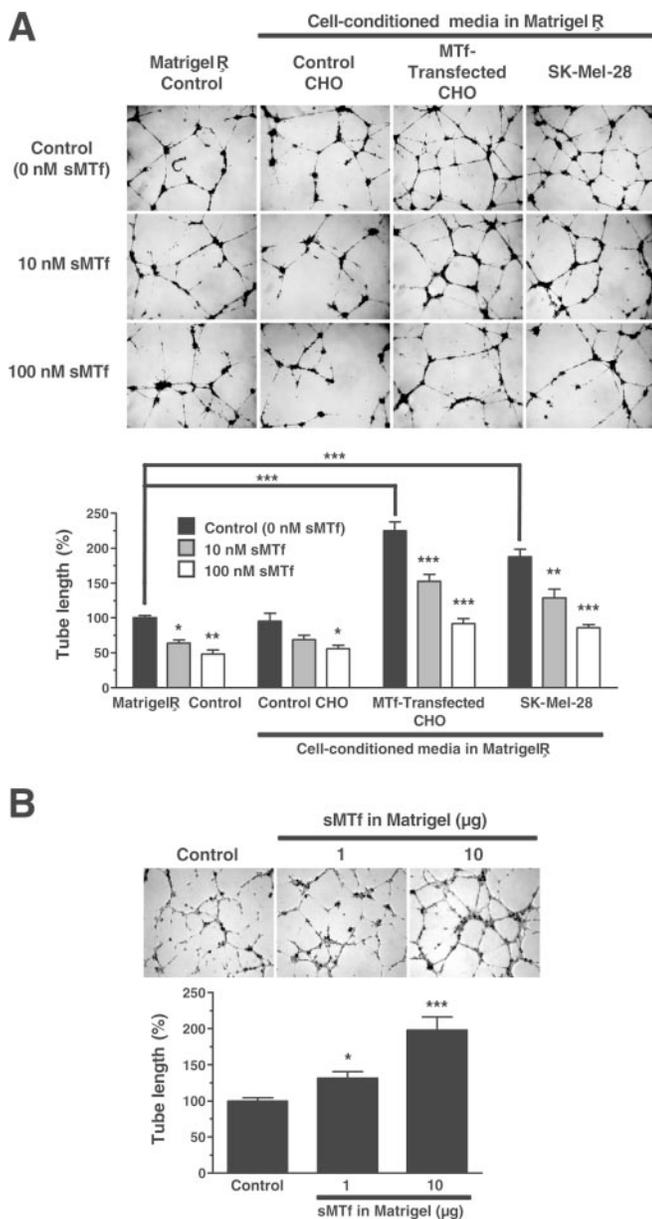


Fig. 1. Truncated sMTf inhibits HUVEC tubulogenesis. (A) HUVEC were grown onto Matrigel™-coated wells containing, or lacking, cell conditioned media from CHO (control and MTF-transfected) and SK-Mel-28 cells in the presence of various concentrations of truncated sMTf, as described in the Materials and methods section. Photos obtained from a representative experiment are shown in the upper panel. The tubulogenesis results shown in the lower panel were expressed as the percentage of capillary-like structures compared with Matrigel™ control conditions. (B) HUVEC were grown onto Matrigel™-coated wells containing, or lacking, truncated sMTf, as described in the Materials and methods section. Photos obtained from a representative experiment are shown in the upper panel. The tubulogenesis results shown in the lower panel were expressed as the percentage of capillary-like structures compared with Matrigel™ control conditions. Data represent the means ± standard error (SE) of results obtained from three different experiments. Statistically significant differences, as compared with control conditions (as well as between Matrigel™ control condition and other control conditions), are indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t*-test or ANOVA).

(Figure 2; upper panel). In contrast to Matrigel™ control condition, Matrigel™ implants containing bFGF showed strong neovascularization (Figure 2; upper panel). In the presence of bFGF alone, the amount of Hb was increased by

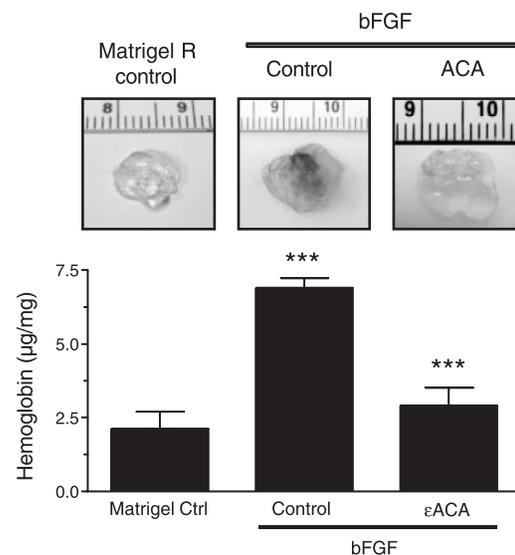


Fig. 2. bFGF-induced neovascularization involves the PA/plasmin system. bFGF-induced Matrigel™ plug *in vivo* angiogenesis assay containing, or lacking, εACA in nude mice, as described in the Materials and methods section. Photos obtained from representative samples are shown in the upper panel. Hb content from Matrigel™ implants are shown in the lower panel. Results in the lower panel are expressed as Hb content in dried Matrigel™ implant (µg Hb/mg dried implant). $n = 12$ for both control conditions, $n = 5$ for εACA condition and the means ± SE are shown. Statistically significant differences compared with respective control conditions are indicated as follows: *** $P < 0.001$ (Student's *t*-test).

3-fold over the level found in control plugs containing no added growth factor (Figure 2; lower panel). The implication of the PA/plasmin system during the *in vivo* Matrigel™ plug neovascularization induced by bFGF was determined by adding into the implant the plasmin(ogen) inhibitor εACA during the assay. Lysine derivatives, such as εACA, are effective inhibitors of the PA/plasmin system (9,16,17). The addition of εACA (35 mg/ml) into the plug strongly inhibited by ~80% the angiogenic response elicited by bFGF, as indicated by the lower implant vascularization (Figure 2; upper panel) and lower Hb content (Figure 2; lower panel). These results suggest that plasmin generation from plasminogen is involved during bFGF-induced *in vivo* angiogenesis, indicating that this model is suitable for studying the impact of MTf on *in vivo* angiogenesis since MTf modulates plasmin generation by PA from plasminogen.

Subcutaneous, systemic truncated sMTf treatments inhibit bFGF- and VEGF-induced neovascularization

Given the important role of plasmin (18), a protein like MTf that targets the formation of plasmin and acts on the invasiveness capacity of EC and SK-Mel-28 cells as well as on EC tubulogenesis might be expected to affect *in vivo* angiogenesis. To test this hypothesis, Matrigel™ implant neovascularization was stimulated by bFGF and nude mice were treated systemically four times (Days 0, 2, 4 and 6 post-implantation; s.c. injection) with either truncated sMTf (10, 20 and 40 mg/kg; total treatment) or control R/H (Figure 3A and B). In the presence of bFGF alone, there was a robust angiogenic response as indicated by the strong pink hue distributed throughout the plug (Figure 3A). To obtain a more quantitative analysis, the Hb content within each plug was measured to assess the angiogenic index (Figure 3B).

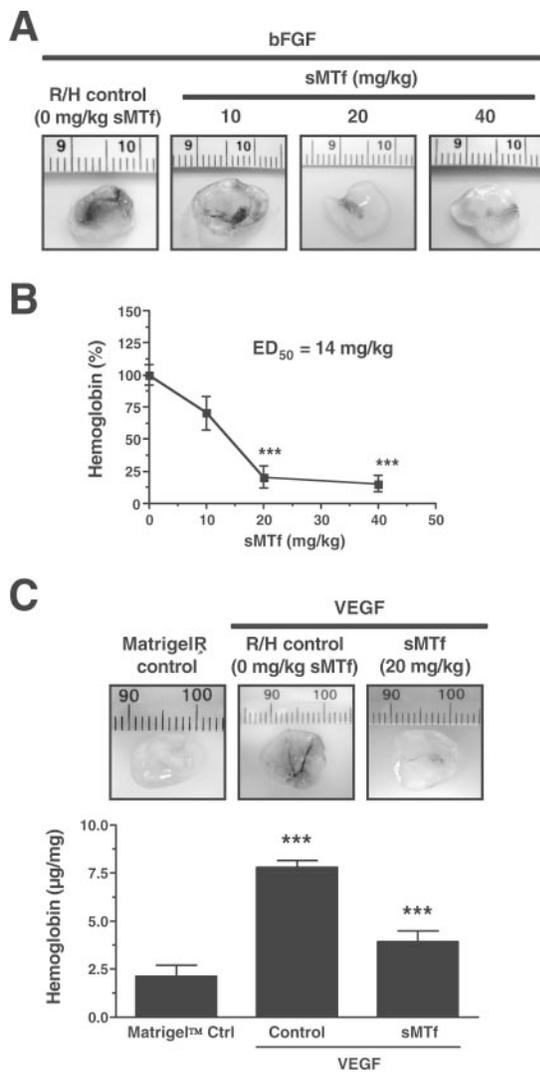


Fig. 3. Subcutaneous, systemic truncated sMTf treatment inhibits bFGF- and VEGF-induced neovascularization. bFGF- and VEGF-induced Matrigel™ plug *in vivo* angiogenesis assay in nude mice, as described in the Materials and methods section. (A) bFGF-induced Matrigel™ plug *in vivo* angiogenesis assay in nude mice treated systemically for 7 days with repeated s.c. injections of either R/H control solution or truncated sMTf (10, 20 and 40 mg/kg/week), as described in the Materials and methods section. Photos obtained from representative samples are shown. (B) Hb content from Matrigel™ implants containing bFGF in nude mice treated systemically for 7 days with repeated s.c. injections of truncated sMTf. Results were corrected for background Hb content measured under the Matrigel™ control condition and expressed as percentage inhibition compared with R/H control condition. $n = 10$ for R/H control condition; $n = 4$ for each other conditions and the means \pm SE are shown. (C) VEGF-induced Matrigel™ plug *in vivo* angiogenesis assay in nude mice treated systemically for 7 days with repeated s.c. injections of either R/H control solution or truncated sMTf (20 mg/kg/week), as described in the Materials and methods section. Photos obtained from representative samples are shown in the upper panel. Hb content from Matrigel™ implants are shown in the lower panel. Results in the lower panel are expressed as Hb content in dried Matrigel™ implant (μg Hb/mg dried implant). $n = 12$ for Matrigel™ control condition, $n = 5$ for each other conditions and the means \pm SE are shown. Statistically significant differences compared with respective control conditions are indicated as follows: *** $P < 0.001$ (Student's *t*-test).

When mice were treated with 10 mg/kg truncated sMTf, the amount of vascular development was reduced, whereas at 40 mg/kg the inhibition of vascularization was maximal, with a median effective dose (ED_{50}) at 14 mg/kg (Figure 3A

and B). Furthermore, to show the generality of the truncated sMTf-mediated angiogenesis inhibition, we characterized the effect of truncated sMTf s.c. treatment on *in vivo* Matrigel™ plug assay in the presence of another standard proangiogenic cytokine, for example, VEGF (Figure 3C). In contrast to Matrigel™ control condition, Matrigel™ implants containing VEGF showed strong neovascularization (Figure 3C; upper panel). In fact, the amount of Hb was increased by 3.5-fold in the presence of VEGF alone compared with control implants containing no added growth factor (Figure 3C; lower panel). When mice were treated systemically four times (Days 0, 2, 4 and 6 post-implantation; s.c. injection) with truncated sMTf (20 mg/kg; total treatment), the amount of vascular development within the Matrigel™ implant was significantly reduced by 68% (Figure 3C; lower panel). These results indicate that truncated sMTf inhibits both bFGF- and VEGF-induced angiogenesis, suggesting that s.c. administration of truncated sMTf may provide a novel therapeutic strategy for the treatment of angiogenesis-related disorders.

MTf-expressing cells stimulate neovascularization, involvement of the PA/plasmin system

Since MTf overexpression stimulates cell motility, migration and invasion (9) and MTF-expressing cell conditioned media stimulate *in vitro* HUVEC tubulogenesis, we determine the effects of endogenous MTF protein expression on *in vivo* angiogenesis. Thus, we examined the impact of CHO (control and MTF-transfected) and SK-Mel-28 melanoma cells within the implant on *in vivo* angiogenesis using the Matrigel™ plug neovascularization assay (Figure 4A). Quantitative analysis of angiogenesis indicated that MTF-expressing cells (MTf-transfected CHO and SK-Mel-28) induced by ~ 6 -fold the neovascularization of the Matrigel™ implants, as demonstrated by the higher Hb content, whereas CHO control cell had no significant impact on Matrigel™ Hb content (Figure 4A; lower panel). In addition, MTF expression in CHO and SK-Mel-28 cells was unchanged following bFGF treatment. Also, bFGF secretion in CHO cells was unaffected by MTF transfection (data not shown). These results show that MTF expression in CHO and SK-Mel-28 cells could contribute to the increased angiogenic response in the *in vivo* Matrigel™ plug neovascularization assay. Moreover, we also determined the effect of ϵ ACA into the implant on the neovascularization induced by SK-Mel-28 cells (Figure 4B). The presence of ϵ ACA (35 mg/ml) into the plug strongly inhibited by $\sim 75\%$ the angiogenic response elicited by SK-Mel-28 cells, as indicated by the lower implant vascularization (Figure 4B; upper panel) and lower Hb content (Figure 4B; lower panel). These results indicate that the PA/plasmin system is involved during SK-Mel-28-induced *in vivo* angiogenesis.

L235 inhibits SK-Mel-28-induced neovascularization

We have previously shown that the mAb L235, which recognizes a conformational epitope on MTF, inhibits the binding of plasminogen on MTF-expressing cell, leading to the reduction of plasminogen activation into plasmin at their cell surface (8,9). The reduced capacity of these cells to generate plasmin from plasminogen in the presence of this mAb also leads to the inhibition of cell motility, migration and invasion (9). The mAb L235 was used to determine whether cell-induced Matrigel™ implant *in vivo*

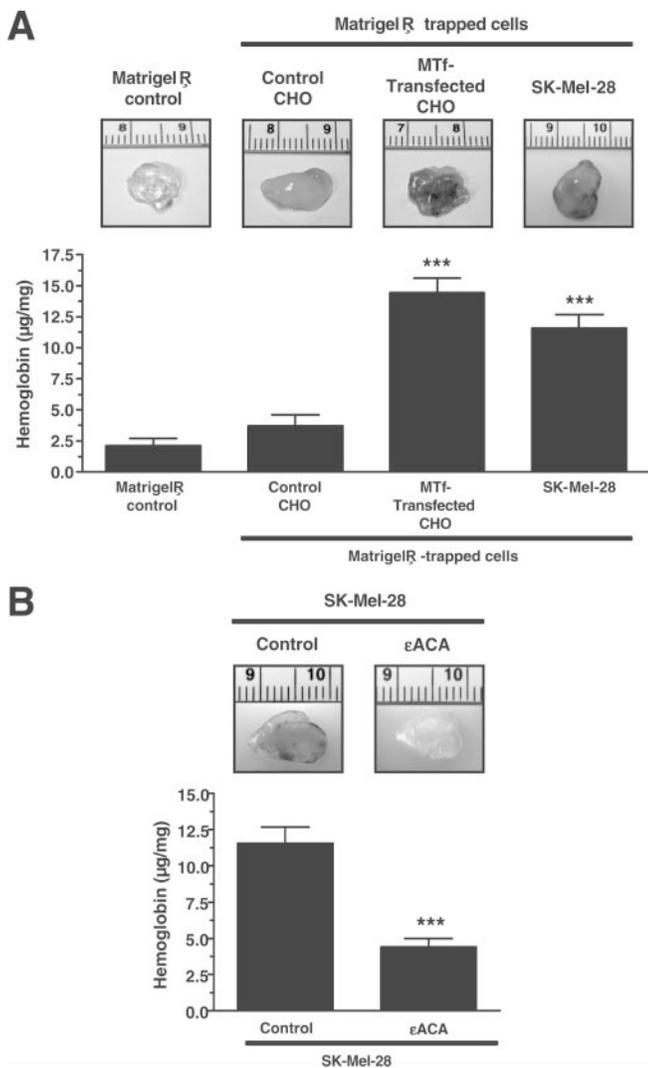


Fig. 4. MTF-expressing cells stimulate neovascularization, involvement of the PA/plasmin system. (A) Cell-induced MatrigelTM plug *in vivo* angiogenesis in nude mice, as described in the Materials and methods section. Photos obtained from representative samples are shown in the upper panel. Hb content from MatrigelTM implants containing, or lacking, CHO (control and MTF-transfected) and SK-Mel-28 cells are shown in the lower panel. $n = 12$ for MatrigelTM control condition, $n = 5$ for CHO cells conditions, $n = 7$ for SK-Mel-28 condition, (B) SK-Mel-28-induced MatrigelTM plug *in vivo* angiogenesis assay containing, or lacking, εACA in nude mice, as described in the Materials and methods section. Photos obtained from representative samples are shown in the upper panel. Hb content from MatrigelTM implants are shown in the lower panel. Results in the lower panel are expressed as Hb content in dried MatrigelTM implant (µg Hb/mg dried implant). $n = 7$ for SK-Mel-28 control condition, $n = 5$ for SK-Mel-28 + εACA condition and the means ± SE are shown. Statistically significant differences compared with respective control conditions are indicated as follows: *** $P < 0.001$ (Student's *t*-test).

neovascularization was directly dependent on plasminogen activation induced by the MTF expression in SK-Mel-28 tumor cell. Thus, we added various concentrations of mAb L235 into the MatrigelTM implants containing SK-Mel-28 cells (Figure 5A and B). The addition of L235 mAb into the MatrigelTM inhibited, in a concentration-dependent manner, the SK-Mel-28-induced neovascularization and reached a maximal angiogenesis inhibition of 70% at 100 nM. The ED₅₀ calculated for the inhibition of angiogenesis by L235

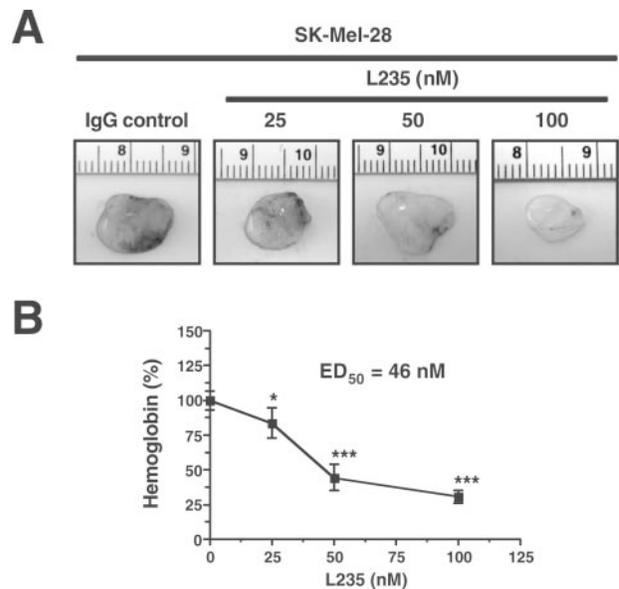


Fig. 5. L235 inhibits SK-Mel-28-induced neovascularization. SK-Mel-28-induced MatrigelTM plug *in vivo* angiogenesis assay containing, or lacking, the mAb L235, as described in the Materials and methods section. (A) Photos obtained from representative samples are shown. (B) Results were corrected for background Hb content measured in the MatrigelTM control condition and expressed as percentage inhibition compared with IgG control condition. $n = 12$ for IgG control condition, $n = 4$ for 25 and 100 nM conditions, $n = 9$ for 50 nM condition and the means ± SE are shown. Statistically significant differences compared with IgG control conditions are indicated as follows: * $P < 0.05$; *** $P < 0.001$ (Student's *t*-test).

is ~46 nM (Figure 5B). Because the mAb L235 does not cross-react with other species than human, this mAb only binds to MTF expressed by the SK-Mel-28 melanoma cells. Since L235 reduces MTF-induced plasminogen activation at the cell surface and specifically inhibits SK-Mel-28-induced *in vivo* neovascularization of the MatrigelTM implants, these results confirmed that MTF overexpression in these melanoma cells leads to an increased angiogenic response involving the PA/plasmin system.

Subcutaneous, systemic truncated sMTf treatments inhibit SK-Mel-28-induced neovascularization

To antagonize the proangiogenic effects linked to MTF overexpression, as observed in the presence of SK-Mel-28 cells, we used a truncated soluble form of MTF (sMTf) to inhibit MTF-induced MatrigelTM implant neovascularization (Figure 6). First, mice were treated systemically four times (Days 0, 2, 4 and 6 post-implantation; s.c. injection) with either truncated sMTf (10, 20 and 40 mg/kg; total treatment) or with control R/H (Figure 6A). In a second set of experiments, Alzet[®] osmotic pumps (100 µl, 7-day delivery) containing either truncated sMTf (10 and 20 mg/kg; total treatment) or control R/H were implanted subcutaneously on the same day as MatrigelTM plugs were implanted (Figure 6B). Quantitative analysis of angiogenesis indicated that s.c. systemic treatment with truncated sMTf antagonized neovascularization of the MatrigelTM implants, as demonstrated by a lower Hb content (Figure 6C). Moreover, continuous s.c. infusion of truncated sMTf using an Alzet[®] osmotic pump increased this antiangiogenic activity. In fact, the ED₅₀ was reduced from 17 mg/kg (repeated s.c.

injections) to 11 mg/kg when a continuous s.c. infusion was employed. These results show the efficacy of truncated sMTf in relatively low amounts as an inhibitor of the *in vivo* SK-Mel-28-stimulated angiogenesis.

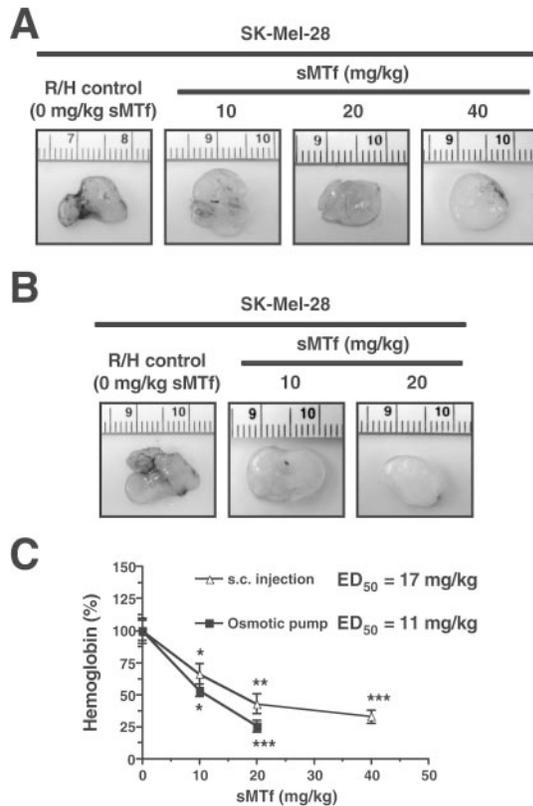


Fig. 6. Subcutaneous, systemic truncated sMTf treatments inhibit SK-Mel-28-induced neovascularization. (A) SK-Mel-28-induced Matrigel™ plug *in vivo* angiogenesis assay in nude mice treated systemically for 7 days with repeated s.c. injections of either R/H control solution or truncated sMTf (10, 20 and 40 mg/kg/week), as described in the Materials and methods section. Photos obtained from representative samples are shown. (B) SK-Mel-28-induced Matrigel™ plug *in vivo* angiogenesis assay in nude mice treated systemically by s.c. infusion using an Alzet® osmotic pump of either R/H control solution or truncated sMTf (10 and 20 mg/kg/week), as described in the Materials and methods section. Photos obtained from representative samples are shown. (C) Hb content from Matrigel™ implants containing SK-Mel-28 cells in nude mice treated systemically for 7 days with repeated s.c. injections or Alzet® osmotic pump s.c. infusion of truncated sMTf. Results were corrected for background Hb content measured under the Matrigel™ control conditions and expressed as percentage inhibition compared with R/H control condition. $n = 7$ for s.c. injection R/H control condition, $n = 5$ for 10 and 40 mg/kg/week conditions, $n = 7$ for 20 mg/kg/week condition, $n = 3$ for each Alzet® osmotic pump conditions and the means \pm SE are shown. Statistically significant differences compared with R/H control condition are indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t*-test).

Pharmacokinetic characteristics of truncated sMTf in mice

To assess the pharmacokinetic characteristics of truncated sMTf in mice, blood drug levels was studied in female athymic Crl:CD-1®-nuBR nude mice. The mean blood concentrations of truncated sMTf after a single 5 mg/kg s.c. injection or a continuous 20 mg/kg s.c. infusion using an Alzet® osmotic pump were measured (Figure 7). The pharmacokinetic parameters derived from these data are summarized in Table I. After a single s.c. injection, truncated sMTf appeared rapidly in the circulation attaining a concentration maximum (C_{max}) of 41.0 ± 8.7 nM at 3 h. Next, using an Alzet® osmotic pump, truncated sMTf appeared more slowly in the circulation compared with the single s.c. injection and gained a C_{max} of 7.3 ± 1.9 nM at 144 h (Day 6). The terminal pharmacokinetics of truncated sMTf after a single s.c. administration were characterized by a slow elimination phase with a final steady state concentration (C_{last}) of 4.0 ± 1.5 nM, compared with 6.1 ± 1.6 nM when truncated sMTf is subcutaneously infused using an Alzet® osmotic pump. Also, the terminal half-life ($t_{1/2}$) of truncated sMTf was $\sim 7.6 \pm 0.9$ h. The high exposure of the vasculature to truncated sMTf was also reflected by the high area under the curve from 0.02 h to infinity ($AUC_{0.02-\infty}$) of 476.1 ± 67.7 nM·h for a single injection and 928.0 ± 59.8 nM·h for an Alzet® osmotic pump infusion. In addition, the truncated sMTf quantity delivered by an Alzet® osmotic pump infusion represents ~ 3.5 -fold the quantity of truncated sMTf delivered by a single s.c. injection. Thus, these results demonstrate that over a 7-day period treatment, the $AUC_{0.02-\infty}$ obtained with

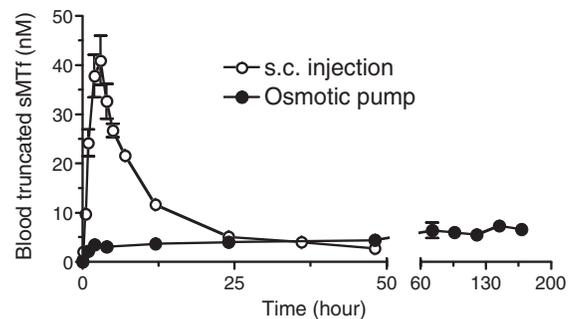


Fig. 7. Blood concentrations of truncated sMTf in nude mice. Female athymic Crl:CD-1®-nuBR nude mice received a single dose (open circle) of 5 mg/kg truncated sMTf in aqueous R/H solution by s.c. injection or by 7-day continuous s.c. infusion (closed circle) of 20 mg/kg truncated sMTf using an Alzet® osmotic pump, as described in the Materials and methods section. At the allotted times, blood was collected and the concentration of the compound was quantified by measuring blood-associated radioactivity using a gamma counter. Results were corrected for background radioactivity measured in untreated mice and expressed as truncated sMTf in blood (nM). The pharmacokinetic parameters derived from these data are summarized in Table I. For each conditions, $n = 3$ and the means \pm SE are shown.

Table I. Pharmacokinetic parameters for truncated sMTf in blood after s.c. administration to female athymic Crl:CD-1®-nuBR nude mice

Administration route	Truncated sMTf parameters ^a			
	t_{max} (h)	C_{max} (nM)	C_{last} (nM)	$AUC_{(0.02-\infty)}$ (nM·h)
Single s.c. injection (5 mg/kg)	3.0 ± 0.6	41.0 ± 8.7	4.0 ± 1.5	476.1 ± 67.7
Continuous s.c. infusion (20 mg/kg/week)	144.0 ± 36.7	7.3 ± 1.9	6.1 ± 1.6	928.0 ± 59.8

^aAreas under the curve from 0.02 h to infinity ($AUC_{0.02-\infty}$) were calculated using mean values. C_{max} (maximum concentration), t_{max} (time to maximum concentration) and C_{last} (steady-state concentration) were determined by inspection of the data using GraphPad Prism. Data are expressed as mean \pm SE ($n = 3$).

an Alzet[®] osmotic pump (928.0 nM·h) infusion is ~51% of that observed during a single s.c. injection (1808.8 nM·h). Furthermore, after a single s.c. dosing with 2.5 and 10 mg/kg of truncated sMTf, a dose-proportional relationship was observed in blood (data not shown). Overall, these results show that s.c. dosing of truncated sMTf can achieve blood concentrations that are in the same order as those required for the inhibition of *in vitro* HUVEC tubulogenesis.

Discussion

Many identified antiangiogenic molecules have the potential of becoming powerful weapons in the treatment of different diseases characterized or caused by excessive angiogenesis, such as cancer, psoriasis, arthritis, blindness, obesity, asthma, atherosclerosis and infectious disease (10). In this study, we have demonstrated for the first time that truncated sMTf inhibits *in vivo* angiogenesis and this can be achieved by s.c. dosing. This follows our previous work in which we demonstrated that this form of truncated sMTf potently inhibits angiogenesis-related mechanisms using *in vitro* models (8,9,12).

Recently, we show that MTf is a cell surface receptor for plasminogen that stimulates plasmin generation by PA (9). Since plasminogen activation is crucial during angiogenesis, cell conditioned media were tested for their capability to induce angiogenesis in an *in vitro* Matrigel[™] tubulogenesis assay. Under our conditions, MTf-expressing cell conditioned media, trapped in extracellular Matrigel[™] matrix, induced HUVEC tubulogenesis. Moreover, truncated sMTf trapped in Matrigel[™] matrix also induced tubulogenesis of HUVEC. The present result is in agreement with another study from Sala *et al.* (19) that used the chick embryo CAM assay to show that gelatin-trapped sMTf could induce angiogenesis and that sMTf has the capacity to induce HMEC-1 cell chemotactic migration. However, in our assay, the addition of truncated sMTf antagonized basal EC tubulogenesis as well as the EC tubulogenesis induced by conditioned media from MTf-expressing cells, indicating that truncated sMTf has potent antiangiogenesis activity *in vitro*.

To evaluate angiogenesis *in vivo* and to probe the potential use of a truncated sMTf as an antiangiogenic therapeutic, we have utilized the Matrigel[™] plug neovascularization mouse model (13). It has been noted that, in melanoma, several angiogenic cytokines are expressed, including bFGF, VEGF, IL-8 and GM-CSF, which mediates autocrine and paracrine control of melanomas (20). Moreover, both bFGF and VEGF strongly and rapidly stimulate the induction of the PA/plasmin system in ECs from the microvasculature (21). The absence of tumor cells in the model using growth factors suggests that truncated sMTf could have direct antiangiogenic activity on ECs. Since truncated sMTf decreases EC cell surface plasminogen activation by affecting the expression of receptors involved in plasmin generation at the cell surface (12), truncated sMTf could thus interfere with the PA/plasmin system that is upregulated by these angiogenic factors.

Proangiogenic effects of MTf are mediated by stimulation of cell surface plasminogen activation (8,9). Previous studies showed that the L235 mAb inhibits MTf-expressing cell invasiveness capacity by reducing MTf-induced plasminogen binding and activation into plasmin at their cell surface (22). Using the L235 antibody and εACA, the *in vivo*

proangiogenic response elicited by SK-Mel-28 was specifically related to MTf expression and involved the PA/plasmin system. Therefore, as was seen in the case of melanoma cells, MTf overexpression within the extracellular matrix could stimulate angiogenesis within the cell's microenvironment by stimulating the PA/plasmin system. Although caution is necessary when extrapolating *in vitro* data to explain *in vivo* findings, previous *in vitro* investigations have suggested that truncated sMTf could antagonize MTf-stimulated angiogenesis-related processes (8,9,12). Various recent therapeutic approaches have aimed at the suppression of neovascularization and cancer cell growth by the use of soluble proangiogenic receptors. For example, the soluble FGF receptor (sFGFR), lacking transmembrane and cytoplasmic domains, inhibits vascularization and cancer growth by a dominant negative effect on cellular signaling and function (23,24). Also, the soluble platelet-derived growth factor-β receptor has the ability to interfere with PDGF signaling and PDGF-induced cell proliferation (25). Moreover, the soluble VEGF receptor is an antiangiogenesis-dependent tumor antagonist (26), whereas soluble u-PAR antagonizes cancer progression by inhibition of angiogenesis (27). In our experiments, the addition of the truncated analog sMTf prevented MTf-induced cell migration, motility, invasion, tubulogenesis and *in vivo* angiogenesis, suggesting that truncated sMTf could decrease plasminogen activation at the cell surface of EC and/or tumor cell by a competitive mechanism. In fact, the exogenous sMTf could antagonize the proangiogenic effect of MTf-expressing cell by breaking the equilibrium between mMTf and endogenous sMTf, which in turn reduces the plasminogen activation at the cell surface. Thus, limiting the proangiogenic activity of MTf by truncated sMTf may provide a method for decreasing the vascularization of tumors and therefore limiting tumor progression or growth. Nevertheless, we cannot exclude the possibility that truncated sMTf could also modulate other mechanisms involved during angiogenesis, such as the recruitment of inflammatory cells. Altogether, these observations indicate that a truncated sMTf could be used as an antiangiogenesis biological therapy.

In addition, systemic s.c. treatment with low amounts of truncated sMTf inhibited SK-Mel-28- as well as bFGF- and VEGF-induced neovascularization. The ED₅₀ values for truncated sMTf are lower compared with other antiangiogenic molecules. In fact, the angiogenesis inhibitor ZD6474, an agent that selectively targets VEGFR-2 and epidermal growth factor receptor (EGFR) tyrosine kinase activity, significantly inhibited gastric tumor growth and dissemination at 100 mg/kg/day (28), whereas the farnesyltransferase inhibitor ABT-100 showed significant reduction in tumor vascularity between 6.25 and 50 mg/kg/day (29). Our study provides further evidences that the antiangiogenic activity of truncated sMTf demonstrates significant potential clinical application.

Furthermore, *in vivo* antiangiogenic efficacy data correlate with the capacity of truncated sMTf to attain blood levels that are (i) in excess of *in vitro* concentration values needed for the inhibition of HUVEC tubulogenesis; (ii) sufficient to almost totally block the MTf-induced HUVEC tubulogenesis; (iii) in excess of *in vivo* sMTf concentration measured in human serum; and (iv) sufficient to demonstrate antiangiogenic effects in a bFGF-, VEGF- and cell-driven Matrigel[™] implant neovascularization model. Also, the use of Alzet[®]

osmotic pump infusion technique provided a higher therapeutic efficacy as compared with repeated s.c. injections, emphasizing the advantages of constant infusion rather than pulse-dose drug inputs for antiangiogenic effects. Our results are also consistent with data on angiostatin dosing patterns in mice, where continuous infusion of the drug had a dramatically improved antiangiogenic effect over twice-daily administration of the same dose (30). Moreover, the final steady state concentration of truncated sMTf is greater than the concentration of endogenous sMTf present in human serum (0.017–0.046 nM) (2,31–33), indicating that serum truncated sMTf modulation could be easily achieved. On the basis of this favorable preclinical profile, truncated sMTf appears to be of potential interest as an antiangiogenesis treatment modality in clinical testing.

In conclusion, our *in vitro* and *in vivo* data demonstrate a proangiogenic activity associated with human MTF overexpression, supporting the hypothesis that MTF may contribute to angiogenesis during melanoma progression. This MTF-induced *in vivo* angiogenesis is inhibited by systemic s.c. treatment with truncated sMTf, strongly indicating that truncated sMTf could be considered as a potential antiangiogenic agent.

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