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Melanotransferrin induces human melanoma SK-Mel-28 cell invasion *in vivo*

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Abstract

The expression of melanotransferrin (MTf), a membrane-bound glycoprotein highly expressed in melanomas, is correlated with tumor vascularization and progression, suggesting a proinvasive function associated with MTf in malignant tumors. To test this hypothesis, we silenced MTf in human melanoma SK-MEL-28 cells using small interfering RNA (siRNA) and examined the plasmin activity and invasiveness of MTf-silenced melanoma. *In vitro*, the siRNA-mediated MTf knockdown inhibited by 58% the cell surface activation of plasminogen into plasmin. In addition, decreased expression of MTf in melanoma cells reduced cell migration. *In vivo*, we used a nude mice invasion model in which tissue factor (TF) induces vascular [^{125}I]-fibrin deposition following injection. Using this metastasis model, the invasive potential of MTf-silenced cells into the lungs was reduced by fivefold. Altogether, these findings strongly suggest that MTf overexpression in melanoma cells contributes to tumor progession by stimulating plasmin generation as well as cell migration and invasion.
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Keywords: Melanoma; Melanotransferrin; siRNA; Invasion; *In vivo* metastasis assay

Melanotransferrin (MTf, GenBank locus ID 4241) is a 97-kDa glycoprotein that shares substantial amino acid sequence similarity and iron-binding properties with transferrin, lactoferrin, and ovotransferrin. MTf exists as soluble (sMTf) and membrane-bound (mMTf) forms, depending on whether the protein possesses a GPI anchor. MTF was first identified on the surface of melanoma cells and is a marker for metastatic melanoma cells [1]. However, the expression of MTf is correlated with tumor vascularization and progression, suggesting a proinvasive function associated with MTf in malignant tumors [2]. Recently, we reported that both forms of MTf could stimulate the plasminogen activator (PA)-mediated activation of plasminogen (Plg) into plasmin [3,4]. We also demonstrated that sMTf is able to increase fibrinolysis [5].

To invade tissues, metastatic cells secrete proteases which are required for the degradation of the extracellular matrix [6]. A study has shown that plasmin promotes fibrin clot dissolution as well as cell migration and invasion through extracellular matrices when activated at the cell surface [7]. We therefore investigated whether MTf could modulate the invasiveness of tumor cells.

The ability of tumor cells to establish new metastatic colonies from blood vessels involves, eventually, the tumor cell implants in the capillary bed of an organ and proliferates into metastasis [8]. Clinical evidence supports the notion that metastasis aggressiveness involves the hemostasis system [9]. It has been shown that tumor-derived tissue factor (TF) is involved in the cancer hypercoagulable states [10]. TF is a single-chain, 263-amino acid, membrane-bound glycoprotein that binds active factor VII and initiates the plasma coagulation cascades [11]. This process produces a fibrin clot that serves as an anchor matrix for metastatic cells to invade tissues [12].

Here in the present study, we used small interfering RNA (siRNA) to block MTf gene expression in human SK-Mel-28 melanoma cells. Our results demonstrate that

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the downregulation of MTf in SK-Mel-28 cells reduces their ability to generate plasmin and to migrate. We also used an *in vivo* nude mice metastasis model, in which the injection of TF increased accumulation SK-Mel-28 cells in the lung. However, the siRNA-mediated MTf knockdown decreased this accumulation of SK-Mel-28 into the lung. Overall, these results provide evidence that mMTf expression facilitates coagulation cascade-dependent melanoma invasion *in vivo*.

Materials and methods

Materials. The L235 monoclonal antibody (mAb) against MTf was kindly provided by Biomarin Pharmaceutical (Novato, CA). Specific pathogen-free, male CD-1 mice as well as female athymic Crl:CD-1-nuBR nude mice were obtained from Charles River Laboratories (Lasalle, QC). Other biochemical reagents were from Sigma–Aldrich (Oakville, ON).

Cell culture. Human melanoma SK-MEL-28 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in modified Eagle's medium (MEM) containing 1 mM Na pyruvate and 10% fetal bovine serum (FBS) under 5% CO_2/95% air atmosphere.

siRNA-mediated MTf knockdown. siRNA SK-Mel-28 cell transfection was performed with siRNA duplex sense r(gggcgaaguguacgaucaa)dTdT and antisense r(uugaucguacacuucgccc)dAdC using HiPerFect transfection reagent from Qiagen (Mississauga, ON). As a control, SK-Mel-28 cells were transfected with the same vector containing a inoperant duplex sense r(gcaacaagugugugggcaa)dTdT and antisense r(uugcccacacacuuguugc)dGdG. The siRNA transfection was performed according to the manufacturer's protocol. The cells were then incubated for 1–8 days. Seventy-two hour of incubation with siRNA was used for the MTf-knockdown in subsequent assays.

Fluorescence-activated cell sorting (FACS) analysis. SK-Mel-28 cells were suspended and incubated with either 1 g/ml mAb L235 or with non-specific, control immunoglobulin G (IgG). The cells were then washed and incubated in the dark with 1 μg/mL goat anti-mouse IgG-Alexa488 (Molecular Probes, Eugene, OR). After rewashes, the cells were analyzed by flow cytometry on a Becton–Dickinson FACSCalibur with a 488 nm argon laser. Cell surface expression levels of MTf were corrected for the background fluorescence intensity measured in the presence of non-specific IgG and were expressed as a percentage of the mean fluorescence intensities measured for the control and inoperant duplex.

Plasminolytic activity assay. The *in vitro* plasminolytic activity of SK-Mel-28 cells (control and MTf-silenced) was measured using a colorimetric plasmin activity assay as described in our earlier study [3]. In this assay, the cleavage of VLK–pNA results in a *p*-nitroanilide molecule that absorbs at 405 nm. The plasmin activity was monitored at 37 °C for 6 h at 405 nm using a ThermoMAX microplate reader (Molecular Devices, Sunnyvale, CA).

Cell migration assay. SK-MEL-28 cell migration was performed using Transwell filters (Costar, Corning, NY; 8 μm pore size) precoated with 0.15% gelatin as described in our earlier study [3]. Migrating cells were visualized at 100× magnification using a Nikon Coolpix 5000 digital camera (Nikon Canada, Mississauga, ON, Canada) attached to a Nikon TMS-F microscope (Nikon Canada). The average number of migrated cells per field was assessed by counting at least four random fields per filter.

Radial clot lysis assay. Radial clot lysis was assayed as described by Mosesson [13] , with minor modifications described in our earlier study [14]. Clots were visualized at 40× magnification using a Nikon Coolpix 5000 digital camera attached to a Nikon TMS-F microscope. The clot diameters were measured in order to quantify the fibrinolysis observed.

In vivo [^{125}I]-fibrin deposition assay. Male CD-1 mice were anesthetized by intraperitoneal (IP) injection of pentobarbital (50 mg/kg). Human plasma [^{125}I]-fibrinogen (2×10^6 CPM) was injected intravenously (IV) in the right jugular vein. Microthrombi were induced 5 min after the injection of fibrinogen by TF infusion (0.4 μg/kg). The mice were sacrificed 20 min after the TF infusion. Several organs (kidney, lung, heart, and brain) were excised and weighed. Organ-associated radioactivity, representing the level of [^{125}I]-fibrin deposition, was quantified using a gamma counter.

In vivo TF-induced metastasis assay. The TF-induced SK-Mel-28 cell metastasis assay was based on the method of Muto [15] with minor modifications. 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. Briefly, SK-Mel-28 cells were incubated with [^{3}H]-thymidine for 72 h. After the metabolic labeling, 2.5×10^5 SK-Mel-28 cells (control and MTf-silenced) were injected into the tail vein. Microthrombi were then induced by injection into the tail vein of 0.1 μg/kg TF as described [15]. The mice were sacrificed 24 h after TF injection and the lungs were excised and dissolved in solvent (Solvable, Perkins; Wellesley, MA) for 24 h. The amount of lung associated [^{3}H]-thymidine labeled SK-Mel-28 cells was measured by liquid-scintillation spectrometry.

Data analysis. Statistical analyses were performed using Student's paired *t*-test via GraphPad Prism (San Diego, USA). Significant difference was assumed for P values less than 0.05.

Results

siRNA-mediated MTf knockdown in SK-Mel-28 cells

Cell surface mMTf expression in SK-MEL-28 cells was determined by FACS using the mAb L235, which recognizes a conformational epitope on MTf, following siRNA-mediated MTf knockdown (Fig. 1). The intensity of the green fluorescence (Alexa488; FL1 detection) associated with detection of cell surface mMTf by the mAb L235 is much lower in MTf-silenced than in control SK-Mel-28 cells (Fig. 1A). The inhibition of mMTf expression by siRNA was optimal after 72 h, reaching a maximal inhibition of 70% (Fig. 1B) . Eight days after the siRNA knockdown of MTf, the mMTf cell expression inhibition was still about 45% of control levels. These results demonstrated that siRNA vectors significantly reduced mMTf protein expression. As control, SK-Mel-28 cells were also transfected with a inoperant duplex. The MTf expression in the SK-Mel-28 cells was unaffected by this duplex as with the transfected agent.

siRNA-mediated MTf knockdown reduces cell surface plasminogen activation and cell migration

We previously established that mMTf affects the activation of Plg as well as cell migration and invasion *in vitro* [3,16]. Here, we investigated whether siRNA-mediated MTf knockdown could modulate the SK-MEL-28 cell surface activation of Plg. The plasmin activity at the surface of control and MTf-silenced SK-Mel-28 cells was measured (Fig. 2A). The Plg activation initial velocity (v) was 48.3 ± 2.9 mOD/min for control cells and decreased to 20.7 ± 2.7 mOD/min for MTf-silenced cells, which represents 58% inhibition of plasmin generation at the cell surface. Since mMTf expression affects the activation of Plg *in vitro* and since Plg activation is required for tumor cell invasion and metastasis [17], the impact of mMTf knockdown on *in vitro* SK-Mel-28 cell migration was examined (Fig. 2B). The siRNA-mediated MTf

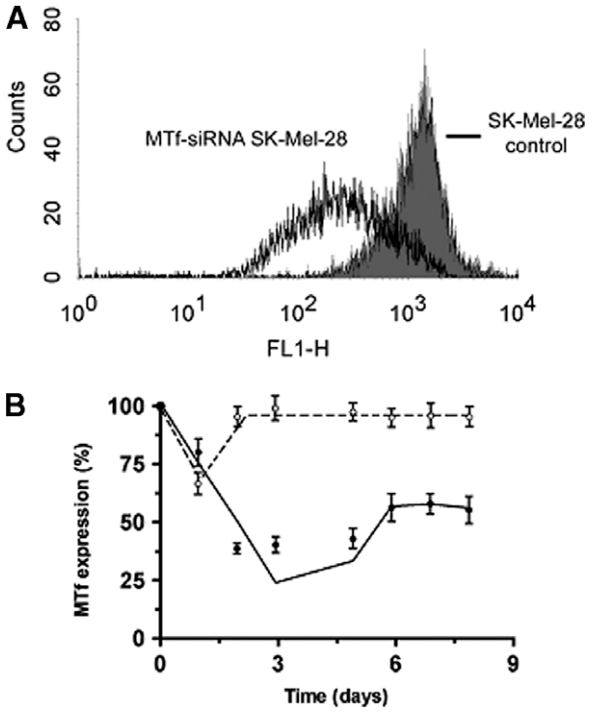

Fig. 1. siRNA-mediated MTf knockdown in SK-Mel-28 cells. (A) Flow cytometry analysis of cell surface MTf levels was performed as described in Materials and methods. Control Sk-Mel-28 (filled line) and MTf-silenced Sk-Mel-28 (empty line) cells were probed with anti-MTf mAb L235 and detected with goat anti-mouse IgG-Alexa488. (B) Flow cytometry analysis of cell surface MTf levels was performed at different times as described in Materials and methods. Cell surface expression levels of MTf in cells transfected with (●) siRNA duplex and (○) siRNA inoperant were corrected for the background fluorescence intensity measured in the presence of a non-specific IgG and were expressed as a percentage of mean fluorescence intensities compared to control cells and inoperant dulex exposed cells. Statistically significant differences, as compared to control conditions, are indicated by $^{***}P < 0.001$ (Student's t test) ($N = 3$).

knockdown decreased, by about 78%, the migration of SK-Mel-28 melanoma cells (Fig. 2C). These results strongly suggest that the siRNA-mediated MTf knockdown inhibits both cell surface Plg activation and cell migration in SK-Mel-28 melanoma cells. As a control, the migration of Sk-Mel-28 cells transfected with the inoperant duplex was also performed. The migration of Sk-Mel-28 cells transfected with the inoperant duplex is similar to that of untransfected cells.

Recombinant sMTf stimulates radial clot dissolution by plasminogen activators

During tumor cell migration, the invading cell needs to degrade the extracellular matrix [6]. Since melanoma aggressiveness is linked to the coagulation system, [18] we used a radial clot lysis assay to demonstrate the effect of recombinant sMTf on plasminogen activator (PA)-induced clot lysis (Fig. 3A). In this assay, the fibrin clot contains Plg. The addition of MTf to both PAs enhanced their fibrinolysis properties, leading to threefold increased dissolution of the fibrin clots. These results indicate that recombinant sMTf stimulates fibrin clot dissolution by PAs (Fig. 3B).

MTf stimulates fibrinolysis and SK-Mel-28 melanoma organ invasion

A previous study has shown that the injection of TF increases the formation of fibrin clots *in vivo* [15]. To evaluate the capacity of TF to induce fibrin deposition in organs, [^{125}I]-labeled fibrinogen is injected into mice. Circulating [^{125}I]-fibrinogen is then cleaved by thrombin into [^{125}I]-fibrin by the extrinsic coagulation pathway. As shown in Fig. 3A, the [^{125}I]-fibrin deposition in the *in vivo* assay is highest in the lung with 55×10^3 CPM/g of tissue, followed by the kidney with 41×10^3 CPM/g of tissue. In heart and brain, [^{125}I]-fibrin deposition was much lower at 4×10^3 CPM/g and 0.4×10^3 CPM/g of tissue, respectively. These results show that TF can increase [^{125}I]-fibrin deposition within several organs. We used this TF-induced clot *in vivo* model to investigate the link between clot deposition and the ability of SK-Mel-28 melanoma cells to invade various organs (Fig. 4A).

siRNA-mediated MTf knockdown reduces TF-induced lung metastases in nude mice

The Plg system is considered as the primary effector of cell invasion. During cell invasion, this system mediates destruction of the extracellular matrix by fibrinolysis. To determine whether MTf is involved during *in vivo*, TF-induced SK-Mel-28 melanoma invasion into lung, metabolically radiolabeled SK-Mel-28 cells were injected intravenously into mice that were preteated (or not) with TF. The melanoma cell invasion into the organs was assessed by measuring the infiltration of SK-Mel-28 cells into these organs in untreated and TF-treated mice. [^{3}H]-thymidine-labeled SK-Mel-28 cell accumulation was observed to increase in lung by threefold whereas the kidney, heart, and brain accumulation was similar to that in the control animals. Altogether, these results demonstrated a correlation between the levels of [^{125}I]-fibrin deposition and of SK-Mel-28 cell accumulation in the lung (Fig. 4B). However, siRNA-mediated MTf knockdown reduces by about 80% the lung invasion by TF-induced SK-Mel-28 cells in nude mice (Fig. 4C). This reduction of SK-Mel-28 cell metastasis in the lung observed with MTf-silenced cells indicates that MTf may be involved during melanoma cell lung invasion.

Discussion

In this study, we demonstrated the involvement of endogenous MTf in *in vitro* Plg activation and cell migration as well as *in vivo* invasion of melanoma cells by using siRNA-mediated MTf knockdown. When siRNA is introduced into mammalian cells, transitory sequence-specific

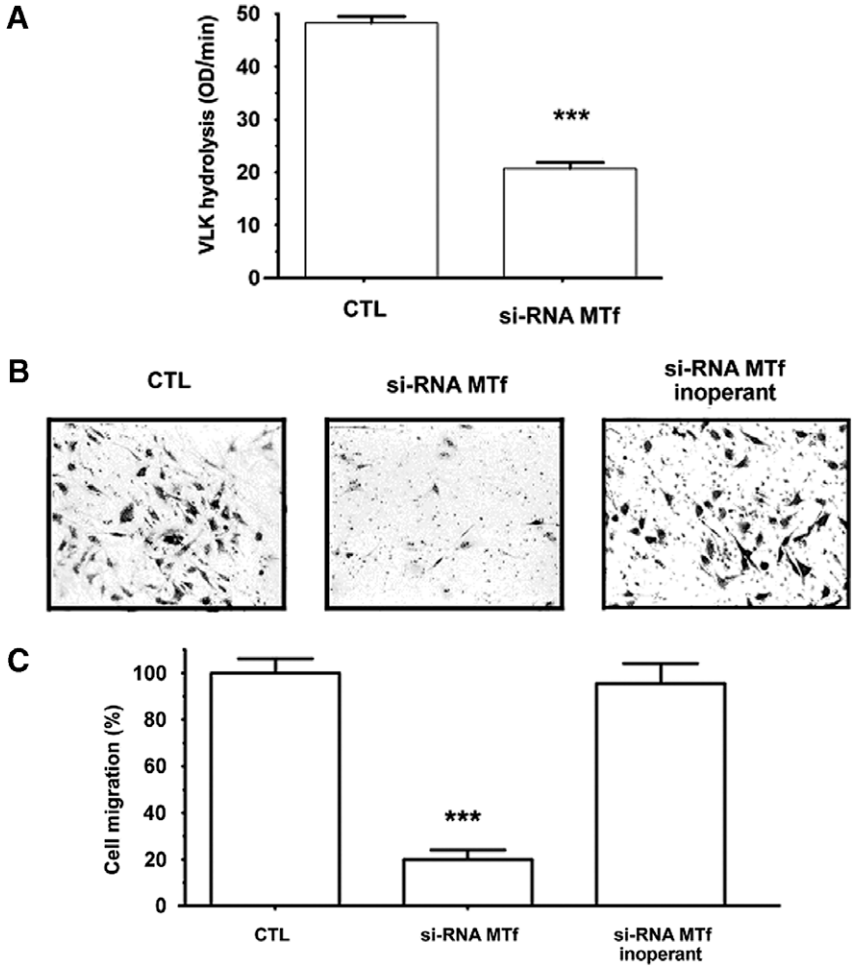

Fig. 2. siRNA-mediated MTf knockdown reduces cell surface plasminogen activation and cell migration. Effect of siRNA-mediated MTf knockdown on Sk-Mel-28 cell surface plasminolytic activity and migration. (A) The plasminolytic activity was measured for control and MTf-silenced Sk-Mel-28 cells as described in Materials and methods ($N = 8$). (B) SK-MEL-28 cell migration was performed using modified Boyden chambers with filters coated with gelatin. Cells that had migrated to the lower surface of the filters were fixed, stained, and counted as described in Materials and methods. Photos (original magnification 100×) obtained from a representative experiment are shown. (C) Cell migration quantification represent the means ± SEM of two independent experiments performed in triplicate. Statistically significant differences from control values are indicated by $^{***}P < 0.001$ (Student's t test) ($N = 6$).

destruction of endogenous target mRNAs occurs and gene expression is effectively suppressed [19].

The melanoma cell line SK-MEL-28, which highly expresses MTf, [3] has been used to investigate the involvement of MTf in Plg activation. The siRNA-mediated knockdown of MTf decreased Plg activation at the Sk-Mel-28 cell surface. This result is in agreement with our study, where the overexpression of MTf in Chinese hamster ovary (CHO) cells stimulated Plg activation by PA [16]. However, the use of siRNA to inhibit the expression of MTf provides the first evidence that a downregulation of MTf leads to a reduction in Plg activation at the cell surface. Interestingly, the disruption of MTf also led to decreased cell migration, suggesting that MTf do play a role in plasminogen activation by uPA or tPA. An earlier study showed that the activation of Plg at the cell surface is a crucial step during cell migration [20]. A recent study confirm using others siRNA that mMTf is involved in migration of Sk-Mel-28 cells *in vitro* [21]. Our study further characterized the involvement of mMTf *in vivo*. The TF by initiating blood coagulation creates a favorable site for the implantation of tumor cells, a crucial step for tumor aggressiveness [8] Here, ours results showed for the first time that the inhibition of the expression of mMTf antagonized the action of TF.

To invade tissues, metastatic cells secrete proteases which are required for the degradation of the extracellular matrix [6]. The fibrinolytic system is important in tumor spreading and involves the dissolution of the fibrin matrix [9]. Recently, we showed that recombinant sMTf could increase both tPA- and uPA-dependent fibrinolysis [3,14]. Therefore, the involvement of recombinant sMTf in PA-mediated fibrinolysis as well as the implication of MTf in the migration of Sk-Mel-28 cells is sufficient to suggest that MTf could be involved in melanoma invasiveness.

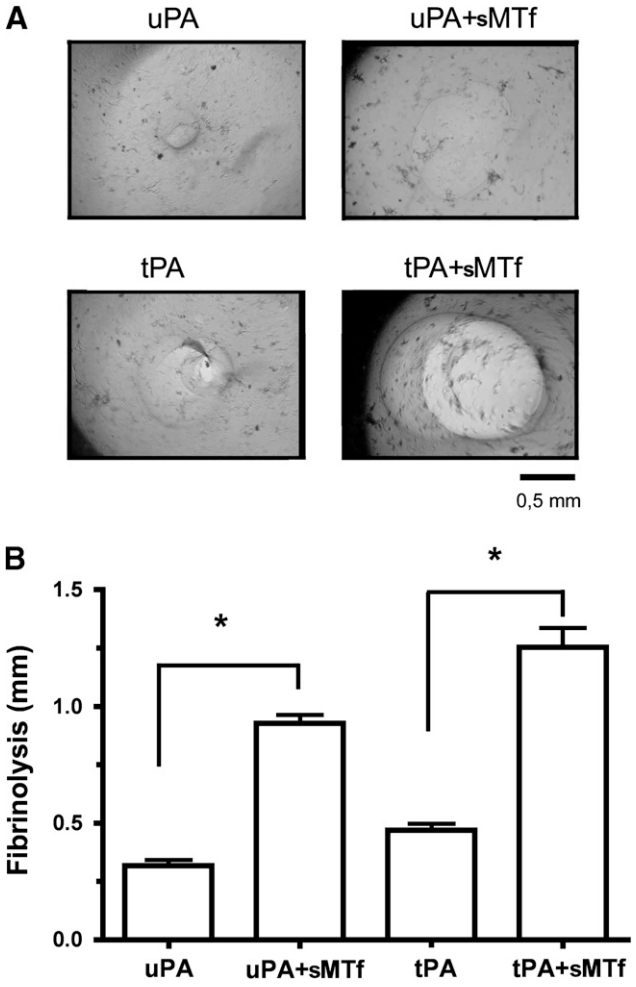

Fig. 3. Stimulation of fibrinolysis by MTf. (A) MTf was added to tPA or pro-uPA in a radial fibrinolysis assay. Fibrinolysis was performed as described in Materials and methods. Photos (original magnification 40×) obtained from a representative experiment are shown (upper panel) (field = 2 mm). (B) The clot diameters were measured (lower panel). Results represent means ± SEM of five different experiments. Statistically significant differences from control values are indicated by $^{*}P < 0.05$ (Student's t test).

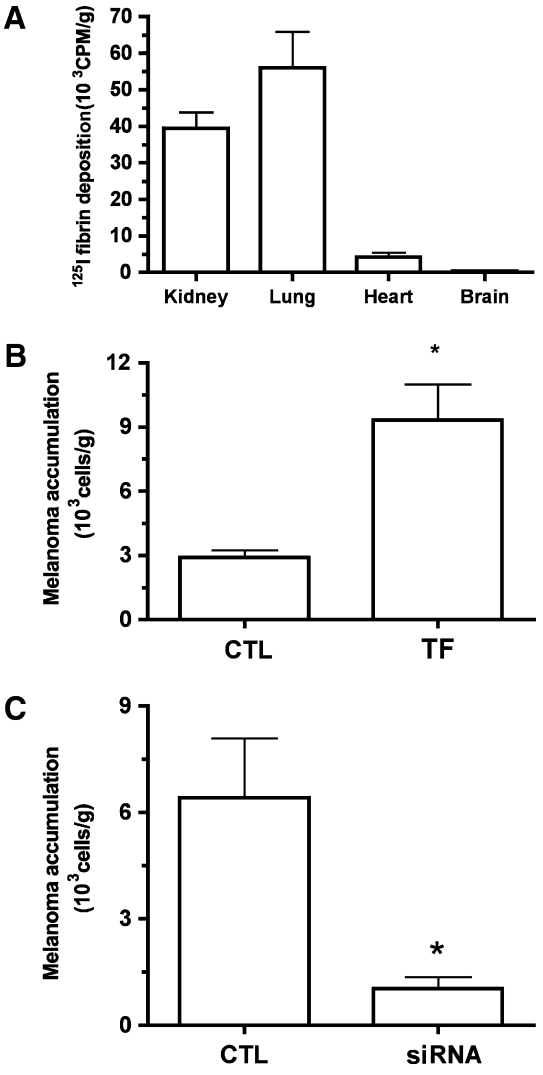

Fig. 4. siRNA-mediated MTf knockdown reduces TF-induced lung metastases in nude mice. (A) Fibrin clot deposition was performed in mice. Mice were treated with vehicle or with TF by i.v. injection. After 5 min [^{125}I]-fibrinogen was injected and [^{125}I]-fibrin deposition was detected at 20 min using a gamma counter. Several organs (kidney, lung, heart, and liver) were excised to monitor fibrin deposition. Data represent means ± SEM of five different experiments. (B) Cultured human melanoma SK-MEL-28 cells were radiolabeled with [H^3]-thymidine for 72 h. TF and cells were injected into nude mice via the tail vein. Radiolabeled SK-Mel-28 cells were measured in the lung with or without Tf injection. Lung-associated radioactivity was determined for controls and for TF-induced SK-Mel-28 metastasis. Data represent means ± SEM of five different experiments. (C) Effect of siRNA-mediated MTf knockdown on SK-Mel-28 cells lung metastasis. Lung-associated radioactivity was determined in control and MTf-silenced SK-Mel-28 metastasis. Data represent means ± SEM of five different experiments. Statistically significant differences from control values are indicated by $^{*}P < 0.05$ (Student's t test).

To verify this hypothesis, we determined whether TF-induced fibrin clot deposition leading to the stimulation of melanoma invasion was dependent on MTf expression. The TF is known to activate thrombin which cleaved fibrinogen into fibrin. Fibrin polymerized and formed a clot with other adhesive proteins, such as vitronectin, laminin, and fibronectin, fibrin forms a provisional matrix [22]. This TF model shows that the fibrin clot accumulates in the lung. This is in agreement with another study showing that fibrin clot has a tendency to deposit in the lung because of this tissue's ability to serve as a filter for fibrin clots [23]. Early and high metastatic formation is typical in human melanomas [24]. Also, thrombin has been shown to strongly enhance metastasis *in vivo* [12]. In the present study, human melanoma Sk-Mel-28 cells demonstrate increased TF-induced melanoma invasion into the lung. This finding corroborates another study where the presence of fibrin clots markedly increased the incidence of spontaneous macroscopic metastasis of melanoma into the lung [25]. Using siRNA-mediated MTf knockdown, we showed that MTf suppression reduced the TF-induced cell invasion, suggesting that MTf is directly implicated in melanoma cell metastasis.

In conclusion, we have shown for the first time that MTf gene expression knockdown using siRNA inhibits invasiveness of malignant human melanoma cells in nude mice. We

are also reporting that the loss of mMTF expression in SK-MEL-28 cells reduces Plg activation at the cell surface which, in turn, affects cell migration. Collectively, our findings suggest that endogenous mMTf can be considered as a potential therapeutic target for future therapy aimed at blocking MTf-expressing tumor cell tissue invasion.

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