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MT1-MMP regulates MMP-2 expression and angiogenesis-related functions in human umbilical vein endothelial cells

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ABSTRACT

Membrane type 1 (MT1)-MMP is a member of matrix metalloproteinases (MMPs) that regulates extracellular matrix remodeling. In addition, MT1-MMP also serves as a multi-functional protein. However, the functional role of MT1-MMP in human endothelial cells remains unclear. In this study we use real-time PCR and Western blotting to demonstrate for the first time that MMP-2 expression is regulated by MT1-MMP in human endothelial cells. Moreover, MMP-2 activity is also modulated by MT1-MMP. In addition we found that endothelial cells, ECM adhesion and human endothelial cell tube formation, which are known to be regulated by MMP-2, are blocked by MT1-MMP siRNA. These results suggest that MT1-MMP plays an important role in regulating angiogenesis in human endothelial cells.

1. Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent proteases, of which at least twenty have been identified in humans. Their major function is to modify the extracellular matrix (ECM), which is involved in regulating cell behaviors and modulating cell morphology [1,2]. MMPs are produced as zymogens and their active domains are covered by a fragment of pro-peptides. Most MMPs can be activated either by themselves or by other proteases [2,3].

MMP-2, also called 72 KDa gelatinase A, is a crucial regulator for cell migration. Many studies have illustrated that MMP-2 regulates cardiac rupture, abdominal aortic aneurysm, and vessel formation [1,2,4–7], indicating that MMP-2 plays important roles in cell migration and angiogenesis. As its name indicates, TIMP-2 is a member of the tissue inhibitors of matrix metalloproteinases (TIMPs) which inhibit MMPs' activity. However, it is also shown to be an activator of MMP-2. Membrane type 1 (MT1)-MMP, a member of membrane-type MMPs, is also involved in MMP-2 activation [8,9]. Therefore, the balance between MT1-MMP and TIMP-2 determines whether MMP-2 is activated or inhibited [9,10]. MT1-MMP and TIMP-2 also play important roles in tumor invasion, cell migration, and angiogenesis [11–14]. In part, MT1-MMP regulates these functions via MMP-2 activation [15]. Interestingly, other roles for MT1-MMP have recently been demonstrated. For instance, several intracellular signals are mediated by MT1-MMP, which indicates that MT1-MMP-regulated networks may be more complex than previously thought [16–18].

Angiogenesis, the formation of new blood vessels from preexisting vessel, is a complex process which involves the early production and release of angiogenic signals, endothelial cell migration, endothelial cell tube formation and the stabilization of new vessel [19–21]. Integrin-regulated endothelial cells-matrix adhesion also plays a role in angiogenesis [22] and since blood vessels are composed of endothelial cells, smooth muscle cells, and connective tissues; many studies have focused on investigating the role of endothelial cells in angiogenesis.

ECM remodeling is considered a key process in cellular behavior, including cell migration, cell adhesion, tube formation, and cell homing [22–24]. As mentioned above, these processes are also involved in angiogenesis. It has been previously demonstrated that integrins interact with MT1-MMP, thereby regulating cell behaviors [14,25,26], suggesting MT1-MMP might play important roles in regulating the angiogenesis processes.

Our previous studies have demonstrated that MMP-2 serves as a main mediator of endothelial cell invasion, which is a critical step

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for angiogenesis [7]. On the other hand, MMP-2 activity has been shown to be regulated by MT1-MMP [8,9]. Therefore, the role of MT1-MMP in endothelial cells was further investigated in this study. Herein, we demonstrate that MT1-MMP might regulate angiogenesis-related cell functions through modulating MMP-2 and TIMP-2 expression.

2. Materials and methods

2.1. Reagents

S1P, gelatin, and collagenase IV were obtained from Sigma–Aldrich (St. Louis, MO). Human umbilical vein endothelial cell (HUVEC) culture media, including medium 199 and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT) and endothelial growth medium (EGM) was from Cell Application (San Diego, CA). The growth factor reduced BD Matrigel™ matrix was from BD Biotech (Bedford, MA). Scrambled and MMP-2 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and MT1-MMP siRNA was purchased from Sigma–Aldrich.

2.2. Cell culture

Human umbilical tubes were kindly provided by National Taiwan University Hospital (Taipei, Taiwan). Primary cultures of HUVECs have been described previously [27] and those cells from passages 2–5 were used in all experiments. Cells were incubated in a humidified incubator containing 5% CO_2 at 37 °C.

2.3. Reverse-transcription polymerase chain reaction (RT-PCR) and real-time PCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and complementary (c)DNA was acquired by reverse transcriptase (Thermo Scientific, Waltham, MA). Real-time PCR was carried out using the iCycler iQ real-time detection system (Bio-Rad Laboratories, Hercules, CA) with ABsolute SYBR Green Fluorscein (Thermo Scientific, Waltham, MA) as the fluorescent dye. For quantification, the target gene was normalized to GAPDH gene. Oligonucleotide primers for PCR were designed using Beacon Designer2 software (Premier Biosoft International, Palo Alto, CA).

2.4. SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting

The procedure was performed as previously described [7]. Antibodies used in this study were: MMP-2 (sc-10736), β-actin (sc-1616), goat anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP) (sc2005), goat anti-rabbit IgG-HRP (sc2004), which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); TIMP-2 antibody (MAB971) was from R&D system (Minneapolis, MN); MT1-MMP antibody (M3927) was from Sigma–Aldrich; and rabbit anti-goat IgG-HRP (31402) was from Pierce (Rockford, IL).

2.5. siRNA & electroporation

Scrambled (sc-37007), MMP-2 (sc-29398), and MT1-MMP (sc-41565) siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or from Dharmacon (Lafayette, CO). These siRNAs were introduced to HUVECs using an Amaxa HUVEC transfection kit (Gaithersburg, MD), according to the manufacturer's instructions. The mixtures were electroporated with the HUVEC-specific U-01 program.

2.6. Gelatin zymography

MMP-2 enzymatic activity was determined by gelatin zymography as described previously [7]. In brief, aliquots of appropriately diluted media from HUVECs transfected with MT1-MMP or MMP-2 siRNA were diluted 1:1 in a nonreducing sample buffer and

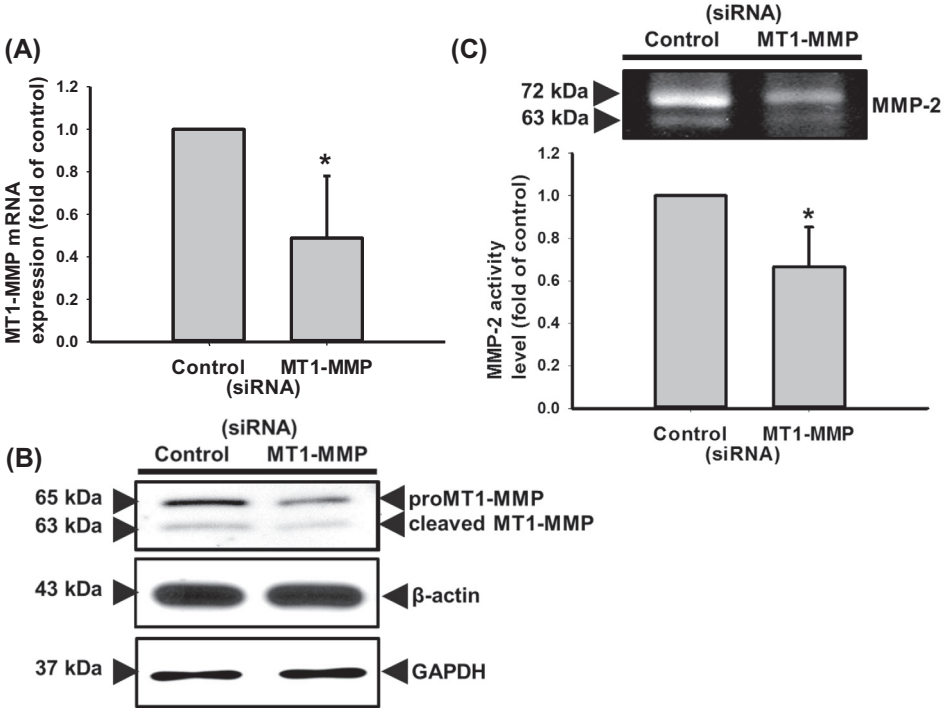

Fig. 1. MMP-2 activation was suppressed by knockdown of MT1-MMP. (A) HUVECs were transfected with MT1-MMP siRNA, followed by cell lysis to isolate RNA or protein. Subsequently, the expression of MT1-MMP was detected using real-time PCR (A) and Western blotting (B). Results were normalized to the expression level of GAPDH. (C) Condition media of scrambled or MT1-MMP siRNA groups were collected to perform the MMP-2 activity assay using a MMP-2-specific substrate gel. Results are shown as the mean ± SE ($n = 6$; $^*p < 0.05$ vs. the scrambled siRNA control).

electrophoresed under nonreducing conditions using a 10% polyacrylamide gel containing 0.1% gelatin for 120 min at 110 V. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 30 min to remove the sodium dodecyl sulfate. After incubation for 16 h at 37 °C in developing buffer, the gels were stained for 1 h with 0.25% Coomassie blue R 250 and destained with 7% acetic acid.

2.7. Over-expression of MMP-2,TIMP-2 and MT1-MMP

Plasmids for expression of full-length MMP-2, TIMP-2 and MT1-MMP were purchased from OriGene Technologies (Rockville MD, USA). These plasmids were introduced to HUVECs using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. Briefly, MMP-, TIMP-2 and MT1-MMP plasmids (50 nM) and lipofectamine 2000 were diluted in OPTI-MEM medium and incubated for 5 min. After incubation, plasmids and lipofectamine were mixed together and further incubated for 30 min, prior to adding to the cells. After 24 h, the cells were washed and cultured in complete growth media containing antibiotics.

2.8. Endothelial cell adhesion assay

Substrates (1:100 dilution matrigel, 1% (V/V) gelatin) were coated onto a 96-well plate for 30 min. One hundred microliters of cells (5×10^5/ml) were added to each well and incubated for 1 h at 37 °C. The dish was washed three times with CB and stained with 0.1% crystal violet (1% crystal violet, 10% EtOH, 89% double-distilled (dd) H_2O) for 30 min at room temperature. Stained wells were washed with CB, and 10% (V/V) acetic acid was then added to dissolve the crystal violet. The optical emission level was detected by an ELIZA reader at 595 nm.

2.9. Endothelial cell tube formation assay

The endothelial cell tube formation assay was modified from Langlois' method [12]. Briefly, Matrigel™ (BD Bioscience, Bedford, MA) was pre-coated on a 96-well plate for 30 min at 37 °C. Cells at 250 μl M199 (6×10^4 cell/ml) were added on top of the Matrigel™ and incubated for 48 h at 37 °C. The formation of capillary structures was observed by light microscope, and photographs were taken with a digital camera.

2.10. Statistical analysis

Significant differences between the control and treatment groups were analyzed by one-way analysis of variance (ANOVA) using the StatView program (Abacus Concept, Berkeley, CA). Each experiment was repeated at least three times. A value of $p < 0.05$ was considered to be a statistically significant difference, and the

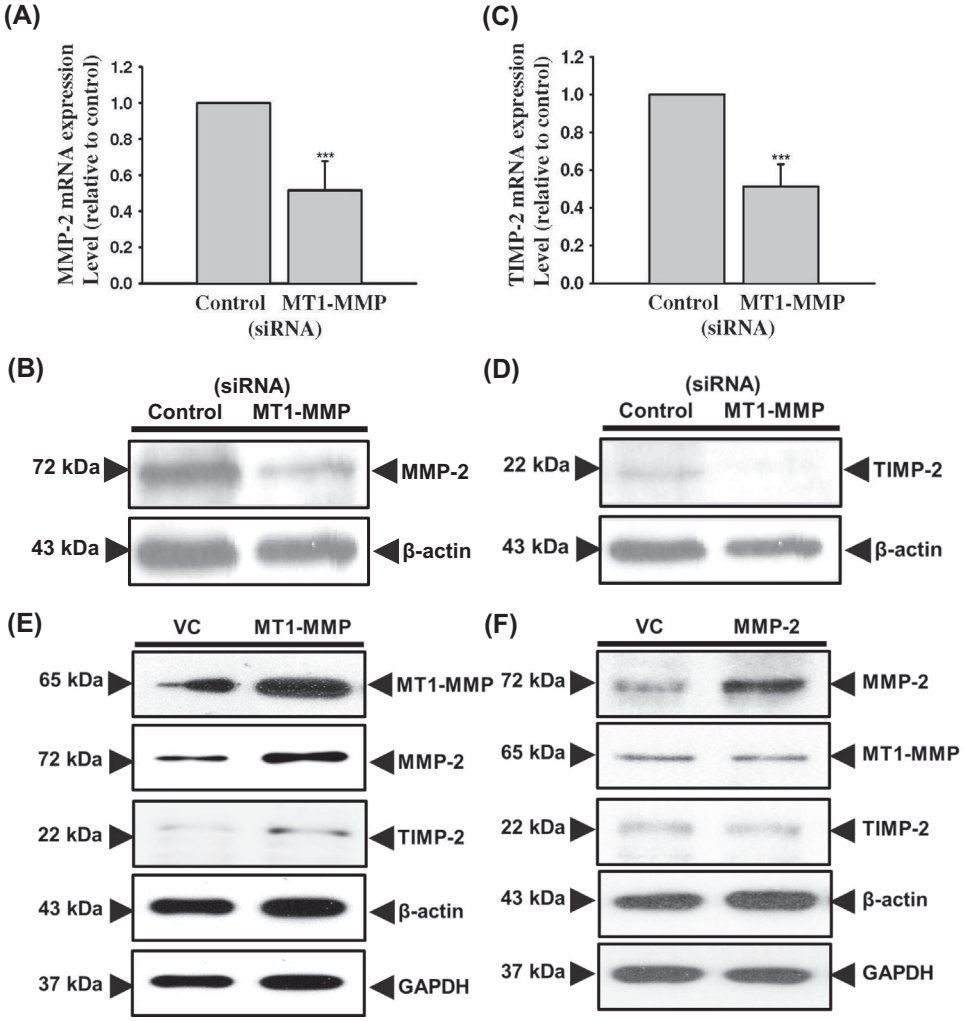

Fig. 2. MMP-2 and TIMP-2 expressions are downregulated by knockdown of MT1-MMP. MT1-MMP treated HUVECs were lysed to isolate RNA or protein. Then (A and B) MMP-2 and (C and D) TIMP-2 expression in HUVECs were analyzed using real-time PCR and Western blotting. Results are shown as the mean ± SE ($n = 3$; $^{***}p < 0.001$ vs. the scrambled siRNA control).

data are shown as mean ± SD ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared to the control).

3. Results

3.1. MMP-2 activity was down-regulated by knockdown of MT1-MMP

To investigate the role of MT1-MMP in endothelial cells, MT1-MMP siRNA were applied to HUVECs. As shown in Fig. 1, the expression level of MT1-MMP mRNA was reduced to 50% (Fig. 1A) and its protein expression (Fig. 1B) was eliminated upon MT1-MMP siRNA transfection.

MT1-MMP was reported to be an activator of MMP-2 activation [28]; therefore, we introduced MT1-MMP siRNA in HUVECs to test whether MMP-2 activity was affected by MT1-MMP. Results showed that MMP-2 activation was attenuated by MT1-MMP knockdown (Fig. 1C). These results indicate that MT1-MMP is involved in MMP-2 activation in HUVECs.

3.2. MMP-2 and TIMP-2 expression were inhibited by MT1-MMP siRNA

Recent studies have indicated that MT1-MMP plays important roles in several cellular signaling pathways [16–18]. To investigate whether MT1-MMP affects MMP-2 expression, MT1-MMP siRNA was applied. Results showed that both MMP-2 mRNA and protein expression levels were down-regulated by MT1-MMP siRNA (Fig. 2A and B). In addition, it was also revealed that both mRNA and protein expressions of TIMP-2 were significantly suppressed by MT1-MMP siRNA (Fig. 2C and D). To further confirm the relationship between MT1-MMP and MMP-2, over-expression of MT1-MMP was applied. Results showed that both MMP-2 and TIMP-2 were augmented upon over-expression of MT1-MMP (Fig. 2D). These results confirm that MT1-MMP is a regulator for MMP-2 and TIMP-2 expression.

3.3. MT1-MMP and TIMP-2 expression were not affected by MMP-2 siRNA

To verify whether MMP-2 also affects MT1-MMP and TIMP-2 expressions, MT1-MMP and TIMP-2 expressions were determined following MMP-2 siRNA transfection in HUVECs. Results showed that both MMP-2 mRNA (Fig. 3A) and MMP-2 activities (Fig. 3B) were suppressed by MMP-2 siRNA transfection. However, we demonstrated that neither MT1-MMP (Fig. 3C) nor TIMP-2 (Fig. 3D) mRNA expression were affected by knockdown of MMP-2 in HUVECs. Furthermore, over-expressed MMP-2 cannot induce either MT1-MMP or TIMP-2 expression (Fig. 2F). These results suggest that MT1-MMP is an up-stream regulator of MMP-2 expression in HUVECs. These results also suggest that TIMP-2 expression is regulated by MT1-MMP, but not MMP-2.

3.4. Angiogenesis-related cell functions were reduced by knockdown of MT1-MMP expression

Next, we examined whether angiogenesis-endothelial cell functions were regulated by MT1-MMP. It has been reported that MT1-MMP is involved in cell-matrix adhesion [28]. Therefore, an adhesion assay was performed to determine the role of MT1-MMP in HUVECs. We investigated the role of MT1-MMP in HUVECs-gelatin and matrigel adhesion. The results demonstrate that MT1-MMP siRNA suppressed endothelial cell adhesion to both gelatin (Fig. 4A) and matrigel (Fig. 4B).

Endothelial cell tube formation, another important characteristic in the angiogenesis process, was also determined. In the absence of treatment with S1P, a known induction factor for endothelial cell tube formation, there was no significant difference between the control and MT1-MMP siRNA groups (Fig. 4C, Upper panel). Following S1P stimulation, tube formation was observed in the scrambled siRNA group, but not in the MT1-MMP siRNA group (Fig. 4C, Lower panel). These results indicate that MT1-MMP plays critical roles in S1P-induced tube formation in HUVECs.

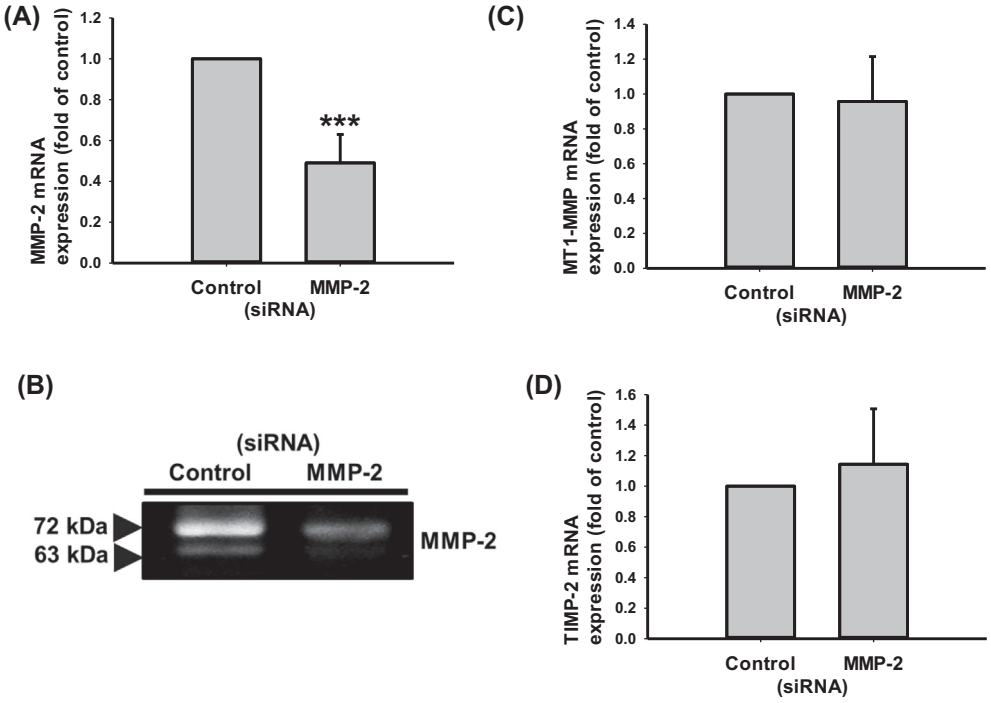

Fig. 3. The expressions of MT1-MMP or TIMP-2 are not regulated by MMP-2 siRNA. After transfection with MMP-2 siRNA for 48 h, HUVECs were lysed, and condition media were collected. (A) MMP-2 mRNA and (B) activity were determined using real-time PCR and substrate gel assay, respectively. (C) MT1-MMP and (D) TIMP-2 mRNA expressions were analyzed using real-time PCR. Finally, the acquired results were normalized by the expression levels of GAPDH. Results are shown as the mean ± SE ($n = 6$; $^{***}p < 0.01$ vs. the scrambled siRNA control).

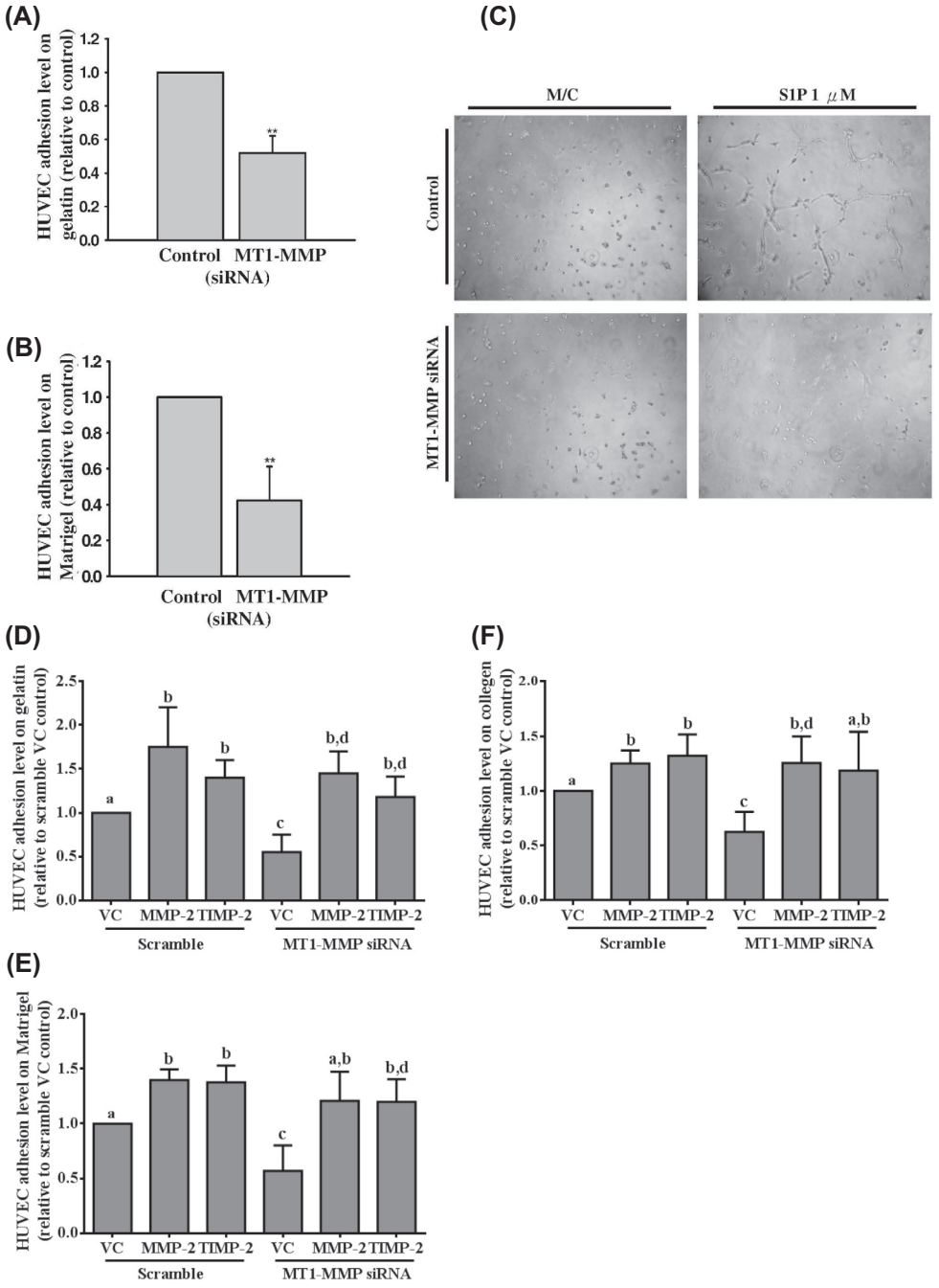

Fig. 4. MT1-MMP-regulated endothelial cell adhesion was mediated through activating MMP-2 and TIMP-2. HUVECs, transfected with scrambled or MT1-MMP siRNA, were seeded on (A) gelatin (1% W/W) and (B) Matrigel (1:100 dilution) for 1 h at 37 °C. The dishes were stained with crystal violet, and the absorbance detected using an ELIZA reader. (C) HUVECs transfected with scrambled or MT1-MMP siRNA were cultivated in serum-free medium overnight and then treated with the control vehicle (M/C) or 1 μM S1P for another 48 h at 37 °C. Tube formation assay was performed by microscope and recorded with a digital camera. HUVECs, transfected with scrambled or MT1-MMP siRNA followed by transfecting plasmids encoding MMP-2 or TIMP-2, were seeded on (D) gelatin (1% W/W), (E) Matrigel (1:100 dilution), and (F) collagen (2% W/W) for 1 h at 37 °C. The dishes were stained with crystal violet, and the absorbance detected using an ELIZA reader. VC, vehicle control. Results are shown as the mean ± SE ($n = 3$; $^{**}p < 0.01$ vs. the scrambled siRNA control). Different numbers above the bars indicate that those groups significantly differ from each other ($p < 0.05$).

3.5. Knockdown of MT1-MMP expression-induced angiogenesis-related cell functions were rescued by over-expressing of MMP-2 and TIMP-2

To further confirm whether MT1-MMP-regulated angiogenesis was mediated through MMP-2 and TIMP-2, HUVECs were transfected with MT1-MMP siRNA, followed by over-expressing MMP-2 and TIMP-2 and an adhesion assay was then assessed. The results show that MT1-MMP-suppressed endothelial cell adhesion on gelatin (Fig. 4D), collagen (Fig. 4E) and matrigel (Fig. 4F) was rescued by over-expression of MMP-2 and TIMP-2. These results indicate that MT1-MMP-regulated endothelial cell adhesion was mainly through activating MMP-2 and TIMP-2.

4. Discussion

Recently, MT1-MMP has been implicated in important roles in caspase-dependent mechanisms, Src-related functions, and the

induction of cell growth via a MAPK-dependent pathway [16–18]. Interestingly, we have shown that reduction of MT1-MMP inhibited MMP-2 expression at both the mRNA and protein levels (Fig. 2A and B). This study demonstrates for the first time that MT1-MMP regulates MMP-2 expression. That is, MT1-MMP regulates not only MMP-2 activation but also MMP-2 expression. In contrast, MT1-MMP expression was not affected by knockdown of MMP-2 (Fig. 3C). A few questions have been raised following these observations, including what are the signaling mechanisms, and which form of MT1-MMP regulates MMP-2 activity or MMP-2 expression. Indeed, MT1-MMP has been demonstrated to be a multifunctional factor interacting with several molecules, such as integrins, cell surface receptors, and signalingfactors [14,19,29,30]. Moreover, by regulating intracellular pathways, MT1-MMP serves as a critical modulator for corneal neovascularization [31], suggesting that MT1-MMP regulates MMP-2 expression via an integrin-dependent, EGFR transactivation, or intracellular signaling mechanism. On the other hand, TIMP-2 expression was also decreased by transfection with MT1-MMP siRNA (Fig. 2C and D), but not with MMP-2 siRNA in HUVECs (Fig. 3D). This result suggests that MMP-2 is not an up-stream modulator of TIMP-2 expression.

Hotary et al. [32] previously reported that cancer cell growth is reduced in MT1-MMP-deficient cells (MT1-MMP$^{-/-}$). These results are consistent with our observation that there were fewer live cells in the MT1-MMP knockdown group compared to the control group in HUVECs (data not shown). To exclude the possibility of different cell numbers being used in the experiments, we seeded the same number of live cells in each condition. Endothelial cell adhesion on various substrates was dramatically reduced after transfection with MT1-MMP siRNA (Fig. 4). Our result corresponds to Seiki's observation that MT1-MMP regulates cell-ECM adhesion in human breast carcinoma MCF-7 [14]. It has been shown that activated-MMP-2 regulates cell adhesion receptor expressions and indirectly interacts with integrins to modulate cell-ECM adhesion [33,34]. Hence, we propose that MT1-MMPmediates MMP-2 activation and thereby down-regulating endothelial cells adhesion to ECM. Indeed, we have shown that knockdown of MT1-MMP-suppressed endothelial cell adhesion was rescued by over-expressing MMP-2 and TIMP-2 (Fig. 4D–F).

Over-expression of MT1-MMP was indicated to cooperate with S1P to induce tube formation in HUVECs [12]. In this study, we further examined this effect by knockdown of MT1-MMP. Our results show that S1P-induced endothelial cell tube formation is attenuated by the introduction of MT1-MMP siRNA (Fig. 4D–F). To sum up, we proposed that MT1-MMP is a critical modulator of tube formation.

In conclusion, MMP-2 activity, endothelial cell adhesion, and endothelial cell tube formation were all down-regulated in the presence of MT1-MMP siRNA. Furthermore, our results indicated that MT1-MMP is an up-stream regulator for MMP-2 expression. In addition, MT1-MMP also regulates TIMP-2 expression. This is the first study to demonstrate that MT1-MMP regulates MMP-2 and TIMP-2 expression. Since MMP-2 activation, endothelial cell adhesion, and endothelial cell tube formation are necessary for vessel formation, these results suggest that MT1-MMP plays an important role in the angiogenesis process.

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