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Timp-2 binding with cellular MT1-MMP stimulates invasion-promoting MEK/ERK signaling in cancer cells

Nor Eddine Sounni[1], **Dmitri V. Rozanov**[1], **Albert G. Remacle**[1], **Vladislav S. Golubkov**[1], **Agnes Noel**[2], and **Alex Y. Strongin**[1]

[1]Cancer Research Center, Burnham Institute for Medical Research, La Jolla, CA

[2]Laboratory of Biology of Tumors and Development, University of Liege, Liege, Belgium

Abstract

Both invasion-promoting MT1-MMP and its physiological inhibitorTIMP-2 play a significant role in tumorigenesis and are identified in the most aggressive cancers. Despite its antiproteolytic effects *in vitro*, clinical data suggest that TIMP-2 expression is positively associated with tumor recurrence, thus emphasizing the wide-ranging role of TIMP-2 in malignancies. To shed light on this role of TIMP-2, we report that low concentrations of TIMP-2, by interacting with MT1-MMP (a specific membrane receptor of TIMP-2), induce the MEK/ERK signaling cascade in fibrosarcoma HT1080 cells which express MT1-MMP naturally. TIMP-2 binding with cell surface-associated MT1-MMP stimulates phosphorylation of MEK1/2, which is upstream of ERK1/2, and the ERK1/2 substrate p90RSK. Consistent with volumes of literature, we confirmed that the activation of ERK stimulated cell migration. Both the transcriptional silencing of MT1-MMP and the inhibition of MEK1/2 reversed the signaling effects of TIMP-2/MT1-MMP while the active site-targeting MMP inhibitor GM6001 did not. Our data suggest that both the interactions of TIMP-2 with MT1-MMP, which activate the pro-migratory ERK signaling cascade,and the conventional inhibition of MT1-MMP's catalytic activity by TIMP-2, play a role in the invasion-promoting function of MT1-MMP. The TIMP-2-induced stimulation of ERK signaling in cancer cells explains the direct, as opposed to the inverse, association of TIMP-2 expression with poor prognosis in cancer.

Keywords

MT1-MMP; TIMP-2; cell migration; ERK; MEK

The MMP family includes 24 individual proteinases.[1] Among MMPs, MT1-MMP is the most relevant to cell locomotion, tumorigenesis and metastasis processes.[2,3] An enhanced expression of MT1-MMP is directly associated with aggressive malignancies.4 In tumors, MT1-MMP acts both as a growth factor and as an oncogene and, as a result, usurps tumor growth control.5–7 MT1-MMP is directly involved in the pericellular proteolysis of the matrix, in the activation of soluble MMPs, and in the cleavage of the cell receptors.[3] The presence of the transmembrane and the cytoplasmic domains distinguishes MT1-MMP from the soluble MMPs. The MT1-MMP zymogen also exhibits an N-terminal prodomain, a catalytic domain, a hinge and a C-terminal hemopexin (PEX) domain. To become active, the MT1-MMP zymogen requires proteolytic activation.[8,9] The N-terminal prodomain is removed by furin

Correspondence to: Alex Y. Strongin, Burnham Institute for Medical Research, 10901 North Torrey Pines Rd, La Jolla, CA 92037, USA. Tel: +858-795-5225, Fax: +858-795-5225, strongin@burnham.org .

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during the secretion pathway of MT1-MMP and the catalytic site of the emerging MT1-MMP enzyme becomes liberated.

The catalytic domain of MT1-MMP binds TIMP-2 with high affinity.[10] TIMP-2 is a member of a multigene family (TIMP-1 through −4) that binds noncovalently to active MMPs in a 1:1 molar ratio and inhibits their activity.[11] In contrast with TIMP-2, TIMP-1 does not inhibit MT1-MMP.10 MMP/TIMP balance, including the balance of MT1-MMP with TIMP-2, is a major factor in the regulation of the proteolytic activity of MMPs.1

TIMP-2 contains two domains. The inhibitory N-terminal domain binds the active site of MMPs including MT1-MMP, blocking the access of substrates to the catalytic site.[12] The C-terminal domain of TIMP-2 binds to the PEX domain of proMMP-2.[13,14] This binding mode is essential for the cell surface activation of proMMP-2 by MT1-MMP.[4,15] The peptide sequence of the PEX domain is conserved in the MMP family. This domain regulates the interactions of MT1-MMP with its cleavage targets including CD44 and the α integrins.[16–18] The PEX is also responsible for the formation of the MT1-MMP homodimers, the presence of which is essential for the activation of MMP-2.[19]

Our earlier data suggest that MT1-MMP's PEX binds the C-terminal domain of TIMP-2 in a manner similar to proMMP-2. It is likely that these interactions, rather than binding the N-terminal domain of TIMP-2 with the catalytic domain of MT1-MMP, are required for the induction of the Ras/Raf/ERK signaling cascade.[20] The stimulation of this cascade appears to play a significant role in the functionality of MT1-MMP. Because in our earlier work we used transfected cells, which overexpressed MT1-MMP, we limited the significance of our findings. To extend our results, we now show that TIMP-2 binding to MT1-MMP, which is naturally expressed by cancer cells, activates the intracellular cascade that involves MEK1/2, ERK1/2 and the downstream ERK substrate p90RSK. We show that the MT1-MMP/TIMP-2 complex stimulates cell locomotion via a mechanism that is independent of and in addition to the proteolytic activity of MT1-MMP.[21,22]

Material and Methods

Reagents

Unless otherwise indicated, reagents were purchased from Sigma (St. Louis, MO). Human 18.5 kDa myelin basic protein (MBP) was from Biodesign International (Saco, ME). The individual catalytic domain of MT1-MMP (MT1-CAT) was purified from the *E. coli* inclusion bodies and refolded to restore its catalytic activity.[23] Human TIMP-1, a rabbit anti-human antibody to the hinge (AB815) and a murine monoclonal antibody against the catalytic domain of MT1-MMP (3G4), a TMB/M substrate and a hydroxamate inhibitor (GM6001) were from Chemicon (Temecula, CA). The goat anti-human TIMP-2 antibody (AF971) was from R&D Systems (Minneapolis, MN). The rabbit antibodies to pERK1/2 Thr202/Tyr204 (D13.14.4E), pMEK1/2 Ser217/221 (41G9), p-p90RSK Ser-380 (9341), total ERK1/2 (137F7), total MEK1/2 (47E6), pSrc Tyr-416 (2101), total Src (2108) and a MEK inhibitor (U0126) were from Cell Signaling Technology (Danvers, MA). The secondary horseradish peroxidase (HRP)-conjugated antibodies were from GE Healthcare-Amersham (Piscataway, NJ). Rat tail Type I collagen was from Becton-Dickinson (San Diego, CA).

TIMP-2 purification

CHO cells deficient for dihydrofolate reductase (DHFR) were cultured in Minimum Essential Medium alpha (α-MEM) containing 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were electroporated with the human TIMP-2 cDNA in the bi-cistronic adenoviral vector that encoded methotrexate-resistant DHFR.[24] Stable transfectants were

selected using α-MEM supplemented with 500 nM methotrrexate. To amplify the DHFR-TIMP-2 gene number, the methotrexate levels in the medium were doubled each week to reach a 25 μM final concentration. TIMP-2 was purified from the 5-day culture medium (500 ml). The medium was concentrated 10-fold using a 400 ml ultrafiltration cell with a 10 kDa cut-off membrane (Amicon) and dialyzed against 20 mM Tris-HCl, pH 8.5, containing 1 mM $CaCl_2$. The samples were chromatographed on a HiTrap Q Sepharose Fast Flow column (GE Healthcare-Amersham in 20 mM Tris-HCl, pH 8.5, containing 1 mM $CaCl_2$. TIMP-2 was eluted with a 0–200 mM NaCl gradient. The purity of TIMP-2 was greater than 90%. The yield was 15% (5 mg TIMP-2 from 1 l medium).

Cells

Parental human fibrosarcoma HT1080 (HT cells) and breast carcinoma MCF-7 cells were from ATCC (Manassas, VA). HT1080 cells stably transfected with MT1-MMP in the pcDNA3-neo plasmid (HT-MT1 cells) and control cells transfected with the original pcDNA3-neo plasmid (HT-neo cells) were obtained and characterized earlier.[25] Human glioma U251 cells (U-MT/PDX cells) cotransfected with both MT1-MMP and PDX were isolated and characterized earlier.26,27 Cells were cultured in DMEM/FBS and 10 μg/ml gentamicin.

siRNA constructs

Selection of the siRNA and siRNAscr constructs was based on our previous extensive investigations.25,27,[28] We tested the transcription silencing efficiency of six MT1-MMP siRNA constructs (numbered 1 through 6) designed using the siRNA Designer software (www.promega.com/techserv/siRNADesigner). As controls, we evaluated several scrambled constructs (Supporting Information Table 1). The sequence of construct 1 was identical with that of MT1-MMP siRNA reported earlier.[29] The efficiency of MT1-MMP silencing was determined using FACS, immunoblotting and gelatin zymography. The siRNA construct 3 was the most efficient in silencing MT1-MMP. HT1080 cells in which MT1-MMP transcription was silenced (MT1-MMP-siRNA cells) using the construct 3 and a scrambled siRNA-transfected cell control (siRNAscr cells) were isolated earlier.25 No off-target effects of the constructs were observed. To avoid clone-specific effects, transfected cell lines were generated as pools of 3–6 positive cell clones. Transfected cells were grown in the presence of 1 μg/ml puromycin.

Cell surface biotinylation

Cells were surface-biotinylated for 30 min on ice in PBS containing 0.1 mg/ml EZ-Link NHS-LC-biotin (Pierce, Rockford, IL). The residual biotin was quenched using 100 mM glycine. Cells were lysed in 50 mM *N*-octyl-β-D-glucopyranoside in TBS supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and a protease inhibitor cocktail III (Calbiochem, San Diego, CA). Biotin-labeled proteins were precipitated using streptavidin-agarose beads. The precipitates were analyzed by immuno-blotting with the MT1-MMP antibody 3G4 or AB815 followed by the secondaryHRP-conjugated antibody and a TMB/M substrate.

Cell migration

The efficiency of cell migration was measured in wells of a 24-well, 8 μm pore size Transwell plate (Corning Costar, Lowell, MA). A 6.5 mm insert membrane was coated with 50 μl Type I collagen (100 μg/ml in DMEM) and then dried for 12 hr. The collagen coating was rehydrated in 0.5 ml DMEM for 30 min before the experiments. The inner chamber contained DMEM supplemented with 10% FBS as chemoattractant. Cells (1×10^4) were seeded in the outer chamber in serum-free DMEM. GM6001 (10 μM), U0126 (20 μM) and TIMP-2 (10–1000 ng/ml) or DMSO alone (0.01%) were added to both inner and outer chambers 15 min before plating the cells. Cells were allowed to migrate for 12 h. Cells were removed from the upper membrane

surface with a cotton swab. Cells that migrate to the membrane's undersurface were stained with 20% methanol-0.2 % Crystal Violet. The incorporated dye was extracted from cells using 0.25 ml SDS 1%. The A_{570} value of the extract was measured. Data are means ± SE from three individual experiments performed in triplicate. Statistical analysis was performed by the two tail unpaired *t* test. *p* values ≤0.05 were considered significant.

Gelatin zymography

Gelatin zymography of the serum-free medium samples was performed as described previously.[28,30] Where indicated, TIMP-2 (10–1000 ng/ml) and GM6001 (10 μM) alone or in combination were added to the cells.

Immunoblotting

Cells were grown in DMEM/FBS for 18 h. The medium was replaced with DMEM containing TIMP-2 (10–1000 ng/ml), GM6001 (10 μM) and U0126 (10 μM) alone or in combination and incubation was continued for an additional 15 min or 24 h. Cells were washed with PBS containing 2 mM sodium orthovanadate and lysed in 50 mM *N*-octyl-β-D-glucopyranoside in TBS supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, and a protease inhibitor cocktail. Insoluble material was removed by centrifugation (10,000g; 30 min). The supernatant aliquots (20 μg total protein) were analyzed by immunoblotting with pERK1/2, pMEK1/2, p-p90RSK, total ERK1/2, total MEK1/2, pSrc, total Src and MT1-MMP antibodies followed by the secondary HRP-conjugated anti-body and a TMB/M substrate.[25]

MT1-MMP proteolysis of MBP

The cleavage reactions (20 μl) were performed in 50 mM HEPES, pH 6.8, containing 10 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 50 μM $ZnCl_2$. MBP (4 μg, 11 μM) was incubated for 1 h at 37°C with MT1-CAT (50 nM; an enzyme-substrate ratio 1:200). The increasing concentrations of TIMP-2 were added to MT1-CAT before the addition of MBP and the samples were preincubated for 10 min on ice. The reactions were stopped by a 5xSDS sample buffer and analyzed using SDS-PAGE.

Densitometry and statistical analysis

Quantitative analysis was obtained by scanning the images. The band density was digitized using the Multi Gauge V3.0 Image Analysis software (Fuji). The results are presented as a ratio between the readings of the sample and those of the corresponding loading control. Statistical analysis was performed by the Student's *t* test. *p* values <0.05 were considered significant.

Results

Transcriptional silencing of MT1-MMP in HT1080 cells

The results we describe here represent the continuation of our published study in which we have used breast carcinoma MCF-7 cells which were stably transfected and, as a result, overexpressed MT1-MMP.[20] We now specifically selected HT1080 cells because they express MT1-MMP naturally. To silence MT1-MMP, we stably transfected HT1080 cells with the siRNA construct and isolated MT1-MMP-siRNA cells. As a control, we isolated siRNAscr cells, which were transfected with the scrambled construct (Supporting Information Table 1).

By using an immunoblotting analysis of the cell lysates we confirmed the near complete repression of MT1-MMP in MT1-MMP-siRNA cells. In turn, the scrambled siRNA construct did not exhibit any significant effect on MT1-MMP (Fig. 1). In both the original and the siRNAscr cells, MT1-MMP was represented only by the mature, 58 kDa, enzyme. Consistent

with the presence of MT1-MMP on cell surfaces, only siRNAscr cells activated the secretory, naturally produced, 68 kDa proMMP-2. As a result, the 64 kDa intermediate and the 62 kDa enzyme of MMP-2 were generated and detected by gelatin zymography. Because of the silencing of MT1-MMP, MT1-MMP-siRNA cells did not activate MMP-2 efficiently. Consistent with the silencing of MT1-MMP, the migration efficiency of MT1-MMP-siRNA cells was reduced when compared with parental HT1080 cells and siRNAscr cells. Inhibition of MT1-MMP by GM6001 decreased the migration efficiency of the original HT1080 and siRNAscr cells to the levels we recorded with the untreated MT1-MMP-siRNA cells. In turn, GM6001 did not cause any additional suppression in MT1-MMP-siRNA cells.

TIMP-2 induced ERK1/2 activation

To identify the role of naturally expressed MT1-MMP in ERK1/2 activation, MT1-MMP-siRNA and siRNAscr cells were coincubated for 15 min with TIMP-2 (100 ng/ml) alone, and with TIMP-2 (100 ng/ml) together with GM6001. The cells were lysed. An immunoblotting analysis determined the levels of both ERK1/2 phosphorylation and total ERK1/2 in the lysates (Fig. 2). As a control, we determined the levels of pSrc and total Src. TIMP-2 induced a significant level of ERK1/2 phosphorylation in siRNAscr cells. GM6001 did not reverse the stimulatory effect of TIMP-2 on phosphorylation of ERK1/2 in siRNAscr cells. As we expected,[20] TIMP-2 did not induce any significant activation of ERK1/2 in MT1-MMP-siRNA cells. In contrast with pERK1/2, TIMP-2 did not affect the levels of both pSrc and total Src in both MT1-MMP-siRNA and siRNAscr cells, thus supporting the specific activation of the ERK pathway.

TIMP-2 used in these tests was highly purified. Its purity was confirmed by both gel electrophoresis and immunoblotting. TIMP-2, at the molar ratio of MT1-CAT - TIMP-2 equal to 1:2, completely blocked the cleavage of MBP (a sensitive protein substrate of MT1-MMP) by MT1-CAT, thus indicating the high inhibitory potency of our TIMP-2. TIMP-2 (100 ng/ml) blocked the activation of MMP-2 in HT1080 cells (Supporting Information Fig. 1). These tests confirmed the inhibitory efficiency of TIMP-2 and suggested that TIMP-2 itself caused ERK1/2 activation in siRNAscr cells.

TIMP-2 stimulated cell migration

It has been demonstrated that excessively high, 2.5–10 μg/ml, nonphysiological concentrations of TIMP-2 repressed cell migration.[6,7,31–33] To determine if low concentrations of TIMP-2 affect cell migration, MT1-MMP-siRNA and siR-NAscr cells were allowed to migrate for 12 h in the presence of increasing, 10–1,000 ng/ml, concentrations of TIMP-2 (Fig. 3). In parallel, we coincubated the cells for 24 h with the same concentrations of TIMP-2. The concentrations of TIMP-2 we used are comparable with those of endogenous TIMP-2 measured using ELISA in the medium conditioned for 24 h by HT1080 cells (1×10^6/ml).[34]

We then determined the levels of pERK1/2 and total ERK1/2, and pSrc and total Src in the cells using immunoblotting analyses. We also examined the levels of active MT1-MMP by measuring the ability of the cells to activate MMP-2. No significant effects of TIMP-2 were observed in MT1-MMP-siRNA cells. In turn, we observed a significant increase of pERK1/2 at 50–1000 ng/ml TIMP-2 in MT1-MMP-siRNA cells. 50–100 ng/ml TIMP-2 induced the maximal level of ERK1/2 phosphorylation. The presence of the residual levels of MT1-MMP explains the minor increase in TIMP-2-induced ERK1/2 phosphorylation we observed in the incompletely silenced MT1-MMP-siRNA cells.

In contrast to MT1-MMP-siRNA cells, there was an obvious effect of TIMP-2 on the levels of MMP-2 activation, pERK1/2 and the migration efficiency in siRNAscr cells. The noticeable level of the activation of MMP-2 was observed at 10–50 ng/ml TIMP-2, while a further increase

in TIMP-2 levels inhibited MMP-2 activation by siRNAscr cells. In turn, both the efficiency of migration and the phosphorylation of ERK1/2 were at their highest levels at 50 ng/ml TIMP-2. Over 100 ng/ml TIMP-2 repressed the levels of MMP-2 activation and the migration efficiency but maintained the pERK1/2 levels in siRNAscr cells.

In contrast to pERK1/2, TIMP-2 did not significantly affect total ERK1/2, pSrc and total Src in siRNAscr cells, although some minor variations in the low levels of pSrc and total Src were observed (Fig. 3). A quantitative densitometry analysis did not reveal any significant TIMP-2-induced changes in the pSrc-total Src ratio in MT1-MMP-siRNA and siRNAscr cells (Fig. 3). Incubation for 24 h with GM6001 alone or in combination with TIMP-2 had no effect on the levels of pSrc and total Src in MT1-MMP-siRNA and siR-NAscr cells (Supporting Information Fig. 2).

These results suggest that the migration efficiency of siR-NAscr cells is controlled by the proteolytic and the nonproteolytic mechanisms, both of which employ TIMP-2 as a mediator. In the nonproteolytic mechanism, stimulation of ERK1/2 phosphorylation requires lower concentrations of TIMP-2 compared to those which are required for the quantitative inhibition of the proteolytic activity of MT1-MMP.

TIMP-2 stimulated phosphorylation of MEK1/2

To corroborate our results, we tested if MEK1/2, which is directly upstream of ERK1/2, is activated by TIMP-2. For this purpose, we coincubated cells for 24 h with TIMP-2 (50 ng/ml and 100 ng/ml), GM6001 and U0126 alone and in combination (Fig. 4). We then used immunoblotting to measure the levels of MEK and ERK in cell lysates. No significant effect of TIMP-2, GM6001 and U0126 was observed in MT1-MMP-siRNA cells. In contrast, a significant effect was evident in siRNAscr cells. TIMP-2 strongly increased the levels of both pMEK1/2 and pERK1/2 in siRNAscr cells. U0126 reversed the stimulatory effect of TIMP-2 on pMEK1/2 and pERK1/2 while GM6001 alone or in combination with 100 ng/ml TIMP-2 had no significant effect on MEK and ERK (Fig. 4).

Similar effects were observed with 50 ng/ml TIMP-2 (Fig. 4 and Supporting Information Fig. 3). These data demonstrated that the low, 50 ng/ml and 100 ng/ml, concentrations of TIMP-2 were comparably efficient in inducing the signaling in siRNAscr cells but not in MT1-MMP-siRNA cells. In agreement with these data, migration of siRNAscr cells was stimulated by TIMP-2 (50 ng/ml) and repressed by U0126. U0126 alone repressed the baseline migration rate of both MT1-MMP-siRNA and siRNAscr by 50%. TIMP-2 alone did not demonstrate any effect in MT1-MMP-siRNA cells.

To investigate the effect of GM6001, we measured the efficiency of migration of cells in the presence of 50 ng/ml TIMP-2 and GM6001 alone and in combination (Fig. 4). In siRNAscr cells, TIMP-2 (50 ng/ml) and GM6001 increased and inhibited migration, respectively. TIMP-2 and GM6001 had no significant effect in MT1-MMP-siRNA cells. If the inhibitors were used jointly, GM6001 had only a minor effect on the TIMP-2-induced migration stimulation in siRNAscr cells.

TIMP-2 stimulated phosphorylation of p90RSK

To elucidate if TIMP-2 binding with MT1-MMP stimulated the terminal events of the Ras/Raf/MEK/ERK cascade such as phosphorylation of the downstream effector of ERK1/2 p90RSK, we coincubated cells for 24 h with TIMP-2 (100 ng/ ml) alone and in combination with GM6001. The levels of p-p90RSK were estimated using immunoblotting of the cell lysates. We also used gelatin zymography to determine the ability of cellular MT1-MMP to activate MMP-2. Our results showed that both TIMP-2 and GM6001 were without effect in

MT1-MMP-siRNA cells (Fig. 5). As expected, GM6001 alone inhibited MT1-MMP and this event resulted in the detection of only proMMP-2 in the siRNAscr medium. GM6001 did not increase the level of pMEK1/2 in siRNAscr cells when compared with the untreated control. Compared to GM6001, TIMP-2 (100 ng/ml) was less efficient in inhibiting MMP-2 activation in siRNAscr cells. In contrast with GM6001, TIMP-2 up-regulated the levels of both pMEK1/2 and p-p90RSK in siRNAscr cells (Fig. 5).

TIMP-2 binding with overexpressed MT1-MMP stimulated the MEK/ERK pathway and cell migration

We used HT-MT1 cells, which overexpressed MT1-MMP, to recapitulate the effects we observed in siRNAscr cells, which produce MT1-MMP naturally. To assess cell surface-MT1-MMP, cells were surface-biotinylated. Biotin-labeled proteins were precipitated. The levels of MT1-MMP in the precipitates were determined by immunoblotting. Because of its self-degradation in HT-MT1 cells, MT1-MMP was predominantly represented by the 40–45 kDa inactive species but not by the full-length protease. Similar amounts of the full-length MT1-MMP were detected in the parental HT and HT-neo cells (Fig. 6). These data indicated that because of its complex with TIMP-2, MT1-MMP was largely inactivated and incapable of efficient self-proteolysis in HT1080 cells.[35]

MT1-MMP-overexpressing HT-MT1 cells activated proMMP-2 and generated active MMP-2 (Fig. 6). GM6001 fully inhibited MMP-2 activation by HT-MT1 cells while TIMP-2 (100 ng/ml) caused only a partial inhibition of MMP-2 activation. TIMP-2 (100 ng/ml) significantly up-regulated pERK1/2 in HT-MT1 cells. GM6001 did not reverse the stimulatory effects of TIMP-2 (Fig. 6). TIMP-2 (50 ng/ml) stimulated the migration efficiency of HT-MT1 cells (Fig. 6). U0126 alone did not affect the baseline migration efficiency in HT-MT1 cells. The pro-migratory effect of TIMP-2 was reversed by U0126, thus supporting the primary role of MEK/ERK in the pro-migratory TIMP-2-mediated pro-migratory pathway in HT1080 cells.[36]

TIMP-2 did not stimulate phosphorylation of MEK1/2 in breast carcinoma MCF-7 cells

We next determined if TIMP-2 was capable of activating MEK1/2 in MT1-MMP-deficient breast carcinoma MCF-7 cells. As controls we used parental HT1080 cells and glioma U251 cells (U-MT/PDX cells), which coexpressed MT1-MMP with PDX, an inhibitor of the MT1-MMP activator furin. Because of PDX, U-MT/PDX cells expressed both the proenzyme and the enzyme of MT1-MMP. Parental HT1080 cells exhibited only the MT1-MMP enzyme. No MT1-MMP was detected in MCF-7 cells (Fig. 6). These results were consistent with earlier observations.[20,30,37,38] TIMP-2 induced MEK1/2 activation in HT1080 cells but not in MCF-7 cells (Fig. 6).

Discussion

High levels of TIMP-2 positively correlate with an unfavorable prognosis in cancer patients.[39–44] There are additional reports that both aberrant hypermethylation of the TIMP-2 promoter[45,46] and TIMP-2 gene polymorphism[47–49] affect cancer risk. Clearly, more studies are required to determine the multiple, concentrationdependent effects of TIMP-2 and other TIMP species including TIMP-1 in tumor progression and metastasis.[50,51]

In a manner counterintuitive to the conventional thinking, we recently suggested that in addition to extracellular proteolysis, the MT1-MMP/TIMP-2 interactions control cell functions through an additional, unconventional mechanism.[20] TIMP-2 (but not structurally similar TIMP-1) binding to MT1-MMP induces, in a matter of minutes, an intracellular ERK1/2 signaling cascade. Our previous study, however, employed stably transfected, MT1-MMP-

overexpressing cells. In turn, we now proved that the TIMP-2/MT1-MMP-mediated signaling mechanism also takes place in fibrosarcoma HT1080 cells which express MT1-MMP naturally.

The concentrations of TIMP-2 in tissues and biological fluids are in the 10–220 ng/ml range.[52–56] The concentrations of endogenous TIMP-2 in the medium conditioned by HT1080 cells are also close to 100 ng/ml.[34] Previous studies by others employed TIMP-2 concentrations which were 10-to 500-fold higher when compared with the physiological range.[6,7,31–33] The physiological concentrations of TIMP-2 we used stimulated ERK signaling in HT1080 cells. We demonstrated that because of TIMP-2 binding with cell surface-associated naturally produced MT1-MMP and the subsequent stimulation of the intracellular signaling, the levels of pMEK1/2 that is directly upstream of ERK1/2, pERK1/2 itself and of the ERK1/2 substrate p-p90RSK increased in HT1080 cells. In turn, TIMP-2 binding with MT1-MMP did not affect pSrc. The TIMP-2/MT1-MMP-mediated signal generated a significant (2- to 3-fold) increase in the levels of pMEK/ pERK in the cells, thus suggesting the primary role of the TIMP-2/ MT1-MMP interactions in the activation of ERK. TIMP-2 did not induce the ERK/MEK pathway in the cells which exhibited a low level of MT1-MMP (e.g., MT1-MMP-deficient breast carcinoma MCF-7 cells). The small-molecule, active site-targeting, inhibitor GM6001 did not reverse TIMP-2/MT1-MMP-mediated stimulation of the MEK-ERK-p90RSK cascade. We also confirmed that TIMP-2/MT1-MMP-dependent stimulation of intracellular signaling promotes cell migration.

These data agree well with the mechanism in which the C-terminal domain of TIMP-2 binds directly with the PEX domain of MT1-MMP.[20] It is likely that this binding mode elicits intracellular signaling. Evidence suggests that this binding mode takes place in the formation of TIMP-2's complex with proMMP-2.[13,57] Because of the high homology between the PEX domains of MMP-2 and MT1-MMP, we believe that there is a structural rationale for a similar interaction of TIMP-2 with MT1-MMP.

It is highly likely that MT1-MMP itself cannot signal because its cytoplasmic tail is exceedingly short, because it does not exhibit any unusual sequences which are additional to the internalization signals and because the cytoplasmic tail-binding adaptor proteins have not been reliably identified.[58] It may well be that an interaction of the MT1-MMP•TIMP-2 with a cellular signaling receptor is required to elicit the signaling through the latter and that either the low density lipoprotein-related receptor protein-1 (LRP1)[59–63] or α3β1 in tegrin64 or both may play this signaling receptor role.

In sum, we suggest that both the interactions of TIMP-2 with MT1-MMP, which activate the signaling cascade, and the inhibition of the catalytic activity of MT1-MMP by TIMP-2 regulate the invasion-promoting function of MT1-MMP. The existence of this cascade in cancer cells supports a direct rather than an inverse association of TIMP-2 with a poor prognosis in certain cancer types. Non-MMP interactions may also affect the role TIMP-2 plays in malignancies. As a result, our hypothesis does not exclude a possibility that in some additional cancer types the interactions of the decreased levels of TIMP-2 with these non-MMP receptors counterbalance the pro-tumorigenic effects of the TIMP-2 binding with MT1-MMP.[51] Collectively, we believe that the evidence we acquired validate the high importance we place on the TIMP-2/MT1-MMP-mediated signaling in tumor biology. Our follow-on studies focused on the identification of the cellular receptor, which selectively interacts with the MT1-MMP/TIMP-2 complex, are now in progress.

Abbreviations

DMEM/FBS	Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum

ERK1/2	extracellular signalregulated kinases 1/2
HRP	horseradish peroxidase
MBP	myelin basic protein
MEK1/2	mitogen-activated protein kinases 1 and 2
MMP-2	matrix metalloproteinase-2
MT1-CAT	the individual catalytic domain of MT1-MMP
MT1-MMP	membrane tType-1 matrix metalloproteinase
p-p90RSK	phosphorylated p90RSK
p90RSK	90 kDa ribosomal S6 kinase
PDX	α1-antitrypsin Portland
pERK1/2	phosphorylated ERK1/2
PEX	hemopexin domain
pMEK1/2	phosphorylated MEK1/2
proMMP-2	the latent proenzyme of MMP-2
TIMP-2	tissue inhibitor of metalloproteinases-2

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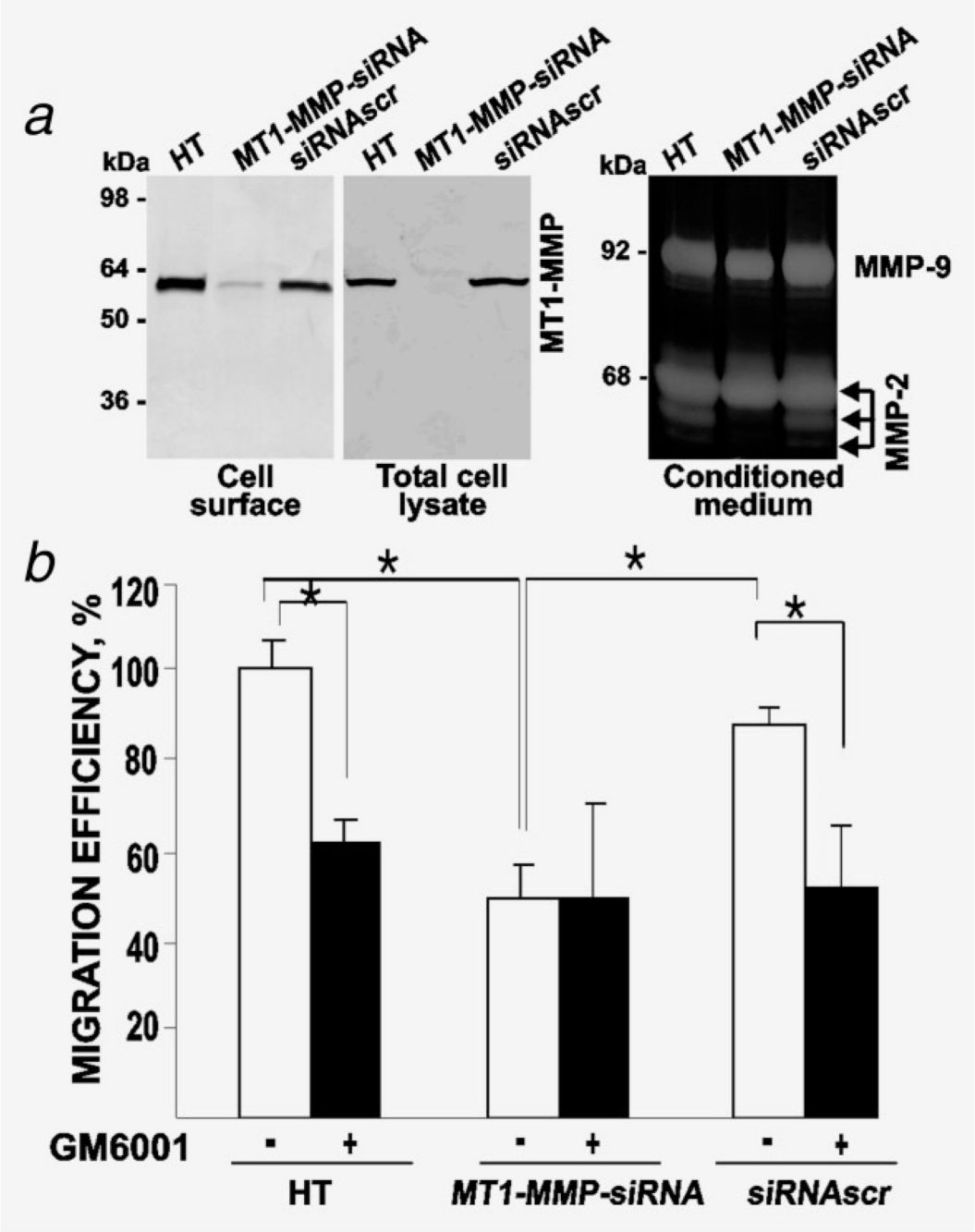

Figure 1.

Silencing of MT1-MMP inhibits cell migration. (*a*) Silencing of MT1-MMP in HT1080 cells. Left – Immunoblotting of the cell lysate and biotin-labeled samples of HT, MT1-MMP-siRNA and siRNAscr cells. The MT1-MMP antibodies 3G4 and AB815 were used to analyze cell lysate and biotin-labeled samples, respectively. Right – gelatin zymography of the medium from HT, MT1-MMP-siRNA and siRNAscr cells. (*b*) Migration efficiency of HT, MT1-MMP-siRNA and siRNAscr cells. Cells (1×10^4) were allowed to migrate for 12 h. Where indicated, GM6001 (10 μM) was added. A 100% number denotes the migration efficiency of the untreated HT cells (5000 cells = 100%). The data are from three independent experiments performed in triplicate. *$p < 0.05$.

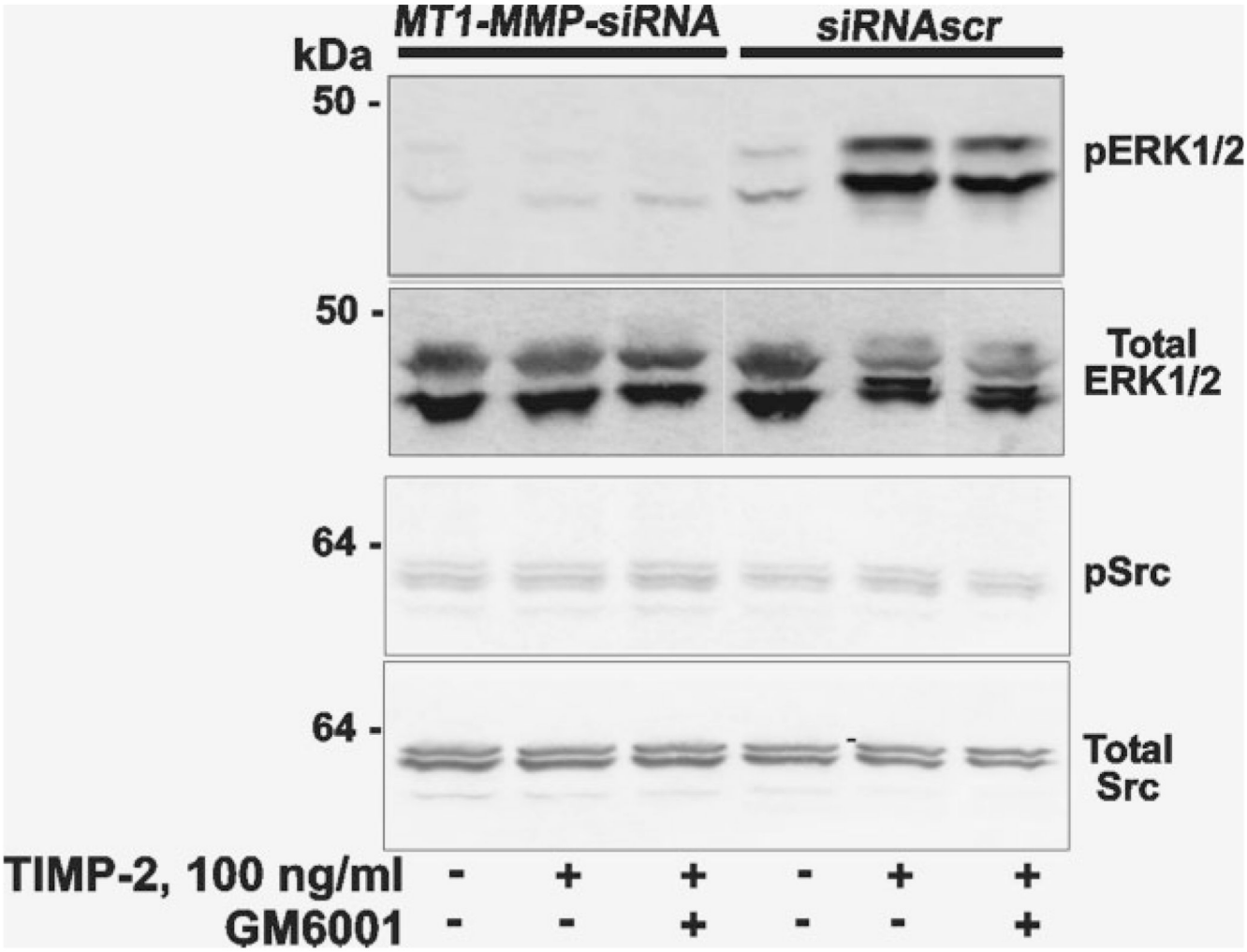

Figure 2.
TIMP-2 binding with MT1-MMP activates ERK1/2. MT1-MMP-siRNA and siRNAscr cells were incubated for 15 min in DMEM. Where indicated, TIMP-2 (100 ng/ml) alone and in combination with GM6001 (10 μM) was added. Cell lysates were analyzed by immunoblotting for pERK1/2, total ERK1/2, pSrc and total Src.

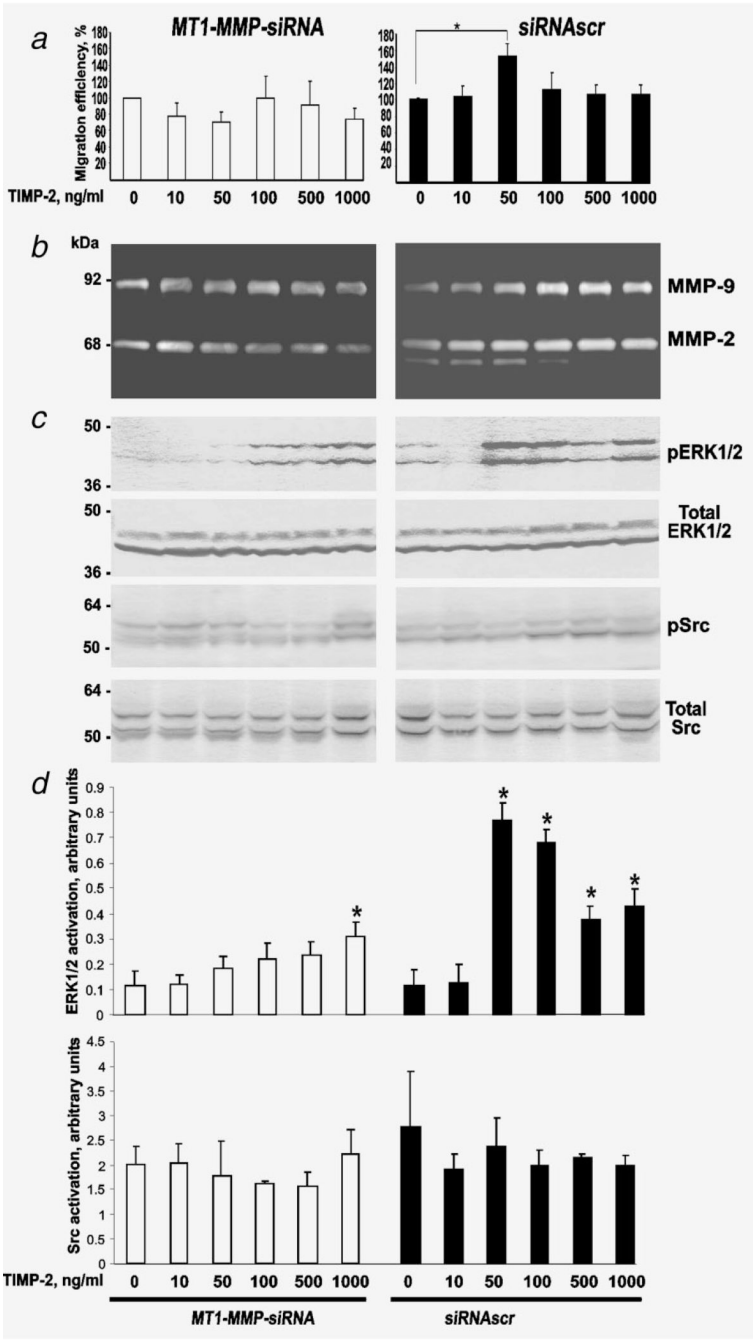

Figure 3.

TIMP-2 binding with MT1-MMP stimulates cell migration. (*a*) Migration efficiency of MT1-MMP-siRNA and siRNAscr cells. Cells (1×10^4) were allowed to migrate for 12 h in DMEM containing the indicated concentrations of TIMP-2. A 100% number denotes the migration efficiency of the untreated cells. The data are from three independent experiments performed in triplicate. *$p < 0.05$. (*b*) Gelatin zymography of the medium conditioned by MT1-MMP-siRNA and siRNAscr cells. Cells (1×10^5) were incubated for 24 h in 0.2 ml DMEM containing the indicated concentrations of TIMP-2. (*c*) Immunoblotting of cell samples. Cells (5×10^5) were incubated for 24 h in 1 ml DMEM containing the indicated concentrations of TIMP-2. Cell lysates were analyzed by immunoblotting for pERK1/2, total ERK1/2, pSrc and total Src.

(*c*) and (*d*), Densitometric analysis. Mean ± S.E. of densitometric readings normalized to the corresponding loading control (pERK1/2 versus total ERK, and pSrc versus total Src). *$p <$ 0.05. These experiments were repeated three times with comparable results.

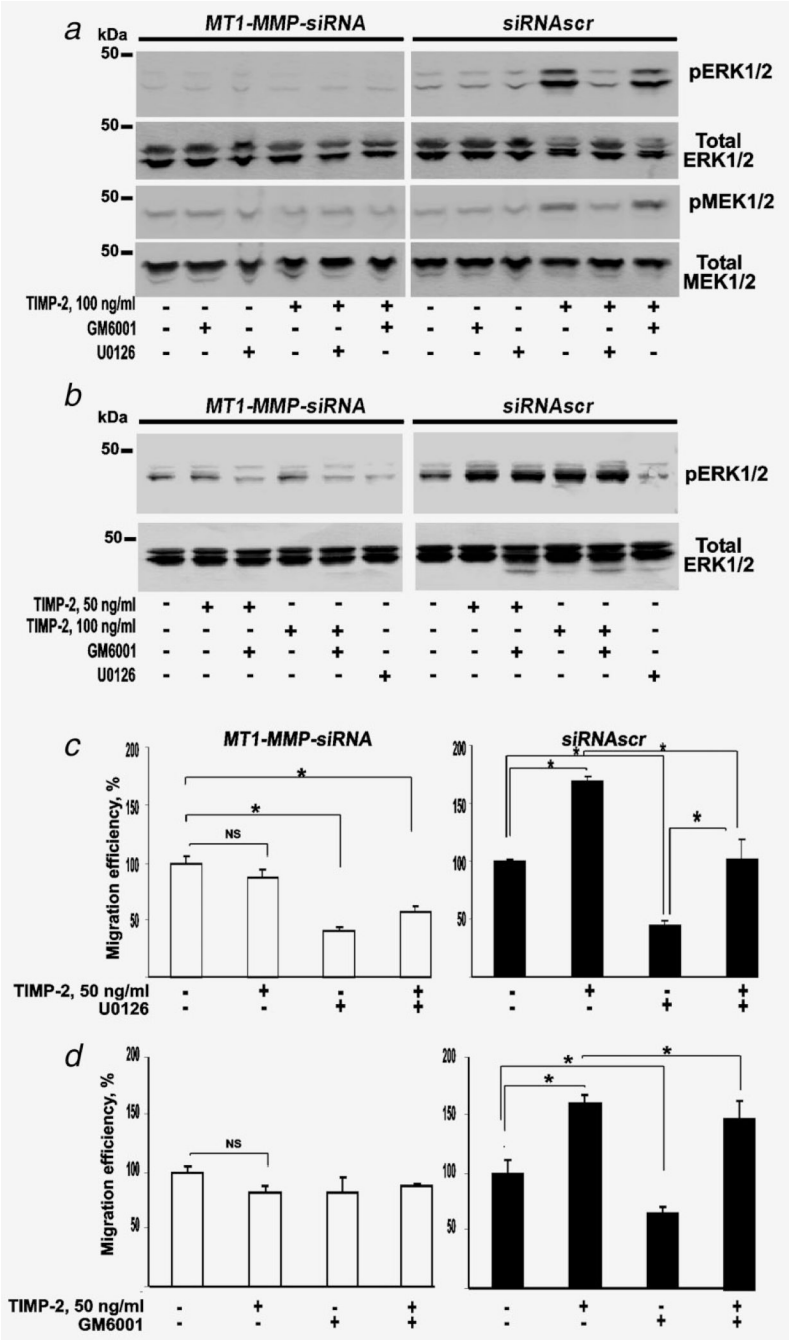

Figure 4.

TIMP-2 binding with MT1-MMP activates MEK1/2 upstream of ERK1/2. (*a*) MT1-MMP-siRNA and siRNAscr cells were incubated for 24 h in DMEM supplemented with TIMP-2 (100 ng/ml), GM6001 and U0126 (10 μM each) alone and in combination. Cell lysates were analyzed by immunoblotting for pERK1/2, total ERK1/2, pMEK1/2 and total MEK1/2. (*b*) Cells were incubated for 24 h in DMEM supplemented with TIMP-2 (50 ng/ml or 100 ng/ml) and GM6001 and U0126 (10 μM each) in combination or alone. Cell lysates were analyzed by immunoblotting for pERK1/2 and total ERK1/2. (*c*) Migration efficiency of MT1-MMP-siRNA and siRNAscr cells. Cells (1×10^4) were allowed to migrate for 12 h. Where indicated, TIMP-2 (50 ng/ml) and U0126 (20 μM) alone or in combination were added. (*d*) Cells where

incubated with TIMP-2 (50 ng/ml) and GM6001 (10 μM) alone or in combination. A 100% number denotes the migration efficiency of the untreated cells. The data are from three independent experiments performed in triplicate. *$p < 0.05$. These experiments were repeated three times with comparable results.

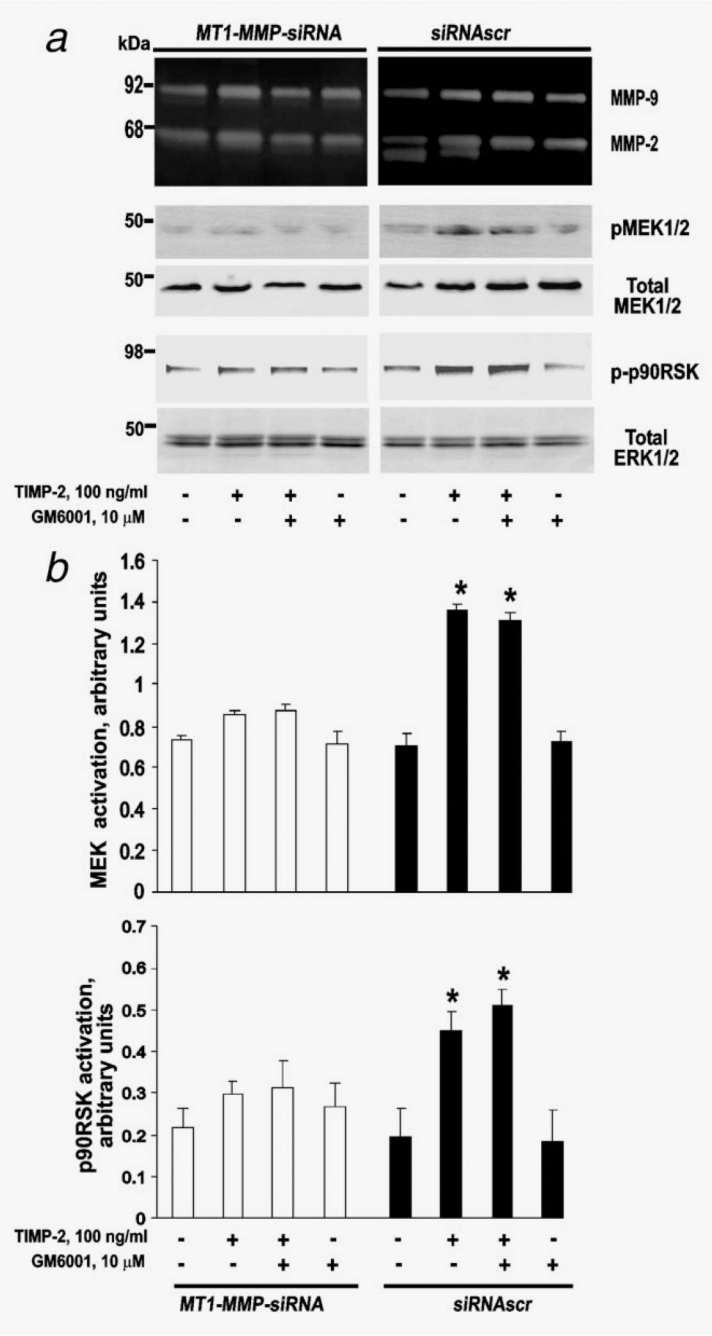

Figure 5.

TIMP-2 binding with MT1-MMP activates ERK1/2 substrate p90RSK. (*a*) MT1-MMP-siRNA and siRNAscr cells were incubated for 24 h in DMEM supplemented with TIMP-2 (100 ng/ml) and GM6001 (10 μM) alone or in combination. Medium aliquots were analyzed by gelatin zymography (upper panel). Cell lysates were analyzed by immunoblotting (bottom panels) for pMEK1/2, total MEK1/2, p-p90RSK and total ERK1/2 (loading control). (*b*) Densitometric analysis. Mean ± S.E. of densitometric readings normalized to the corresponding loading control (pMEK versus total MEK, and p-p90RSK versus total p90RSK). *$p < 0.05$. These experiments were repeated three times with comparable results.

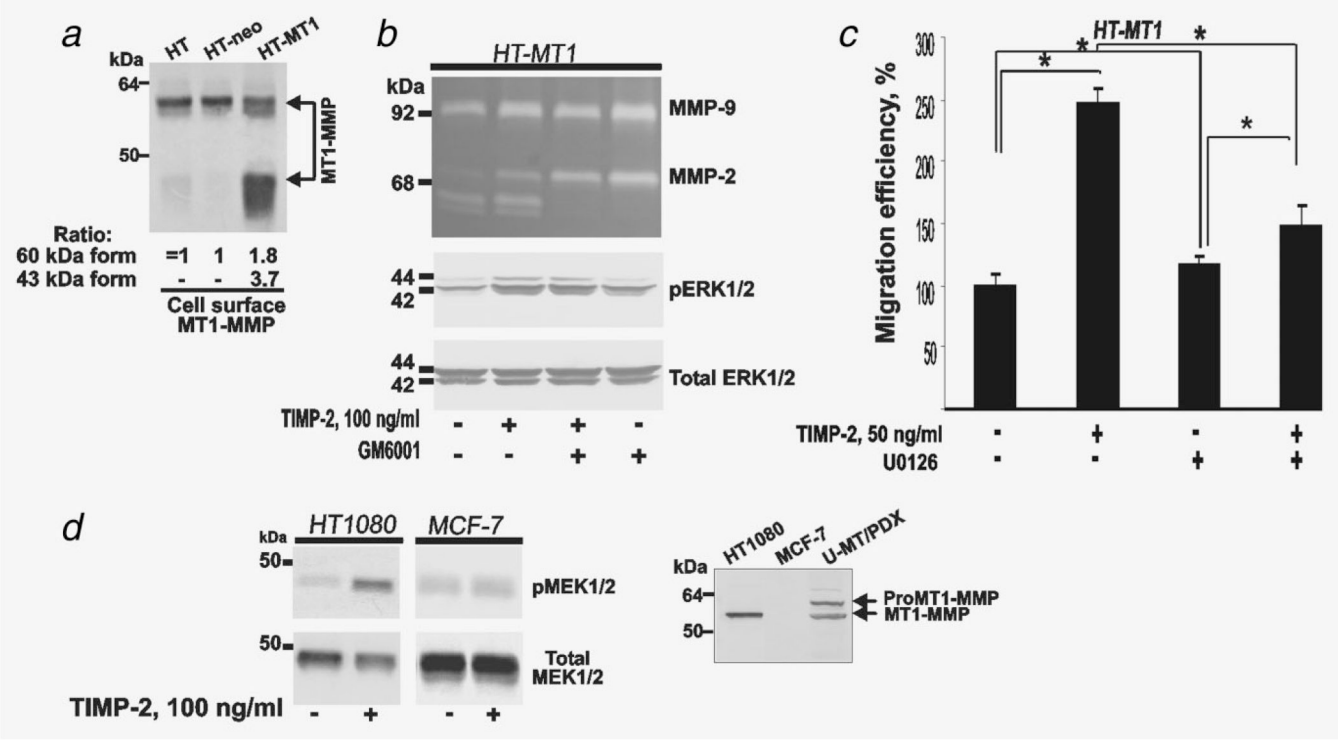

Figure 6.

TIMP-2 binding with overexpressed MT1-MMP activates ERK1/2 and stimulates cell migration. (*a*) Cell surface expression of MT1-MMP. HT, HT-neo and HT-MT1 cells (2.5×10^6 cells each) were surface labeled with membrane-impermeable biotin and then lysed. The lysates (1 mg total protein each) were precipitated with streptavidin-agarose beads. The precipitates were analyzed by immunoblotting with the MT1-MMP antibody AB815. The MT1-MMP bands were scanned and the images were digitized. The ratio of the band density is shown at the bottom of the panel (HT cells = 100%). (*b*) TIMP-2 binding activates ERK1/2. HT-MT1 cells were incubated 24 h in DMEM supplemented with TIMP-2 (100 ng/ml) and GM6001 (10 μM) alone or in combination. Medium was analyzed by gelatin zymography (upper panel). Cell lysates were analyzed by immunoblotting for pERK1/2 and total ERK1/2 (bottom panels). (*c*) Migration efficiency of HT-MT1 cells. Cells (1×10^4) were allowed to migrate for 12 h. Where indicated, TIMP-2 (50 ng/ml) and U0126 (20 μM) alone or in combination were added to the cells. (*d*) TIMP-2 activates MEK1/2 in HT1080 cells but not in MT1-MMP-deficient breast carcinoma MCF-7 cells. Left - HT1080 and MCF-7 cells were incubated 24 h with TIMP-2 in DMEM. Cell lysates were analyzed by immunoblotting for pMEK1/2 and total MEK1/2. Right - HT1080, MCF7 and U-MT/PDX cell surface-biotinylated proteins were analyzed by immunoblotting with the MT1-MMP antibody 3G4.