Activation of Matrix Metalloproteinase-2 (MMP-2) by Membrane Type 1 Matrix Metalloproteinase through an Artificial Receptor for ProMMP-2 Generates Active MMP-2

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Abstract

The suggested model for pro-matrix metalloproteinase-2 (proMMP-2) activation by membrane type 1 MMP (MT1-MMP) implicates the complex between MT1-MMP and tissue inhibitor of MMP-2 (TIMP-2) as a receptor for proMMP-2. To dissect this model and assess the pathologic significance of MMP-2 activation, an artificial receptor for proMMP-2 was created by replacing the signal sequence of TIMP-2 with cytoplasmic/transmembrane domain of type II transmembrane mosaic serine protease (MSP-T2). Unlike TIMP-2, MSP-T2 served as a receptor for proMMP-2 without inhibiting MT1-MMP, and generated TIMP-2–free active MMP-2 even at a low level of MT1-MMP. Thus, MSP-T2 did not affect direct cleavage of the substrate testican-1 by MT1-MMP, whereas TIMP-2 inhibited it even at the level that stimulates proMMP-2 processing. Expression of MSP-T2 in HT1080 cells enhanced MMP-2 activation by endogenous MT1-MMP and caused intensive hydrolysis of collagen gel. Expression of MSP-T2 in U87 glioma cells, which express a trace level of endogenous MT1-MMP, induced MMP-2 activation and enhanced cell-associated protease activity, activation of extracellular signal–regulated kinase, and metastatic ability into chick embryonic liver and lung. MT1-MMP can exert both maximum MMP-2 activation and direct cleavage of substrates with MSP-T2, which cannot be achieved with TIMP-2. These results suggest that MMP-2 activation by MT1-MMP potentially amplifies protease activity, and combination with direct cleavage of substrate causes effective tissue degradation and enhances tumor invasion and metastasis, which highlights the complex role of TIMP-2. MSP-T2 is a unique tool to analyze physiologic and pathologic roles of MMP-2 and MT1-MMP in comparison with TIMP-2. [Cancer Res 2008;68(21):9096–104]

Introduction

Recent studies have shown that members of the matrix metalloproteinase (MMP) gene family play a central role in the degradation of extracellular matrix (ECM) macromolecules under various physiologic and pathologic conditions (1–5). Membrane type 1 MMP (MT1-MMP, MMP-14) was the first member of the MT-MMP family to be discovered and was identified as the first physiologic activator of latent MMP-2 (proMMP-2; ref. 6). The role of MT1-MMP in pericellular proteolysis is not restricted to proMMP-2 activation because MT1-MMP is a functional enzyme that can also degrade a number of ECM components (7–10) and hence can play a direct role in ECM turnover. The MMP family is balanced by a family of tissue inhibitors of metalloproteinase (TIMP). TIMP-2 preferentially complexes with proMMP-2 (11) and plays a pivotal role in the MT1-MMP–mediated activation process (12–16). The suggested model implicates TIMP-2 as a bridging molecule, tethering proMMP-2 through binding between the COOH-terminal ends of proMMP-2 and TIMP-2 and binding between the NH2-terminal ends of MT1-MMP and TIMP-2. The propeptide of proMMP-2 is cleaved by an adjacent TIMP-2–free MT1-MMP between Asn167 and Leu168, generating an activated intermediate form that is further processed to the fully activated form by an intermolecular autocleavage when present at a sufficiently high concentration at the cell surface. ProMMP-2 activation was expected to occur only at low TIMP-2 concentrations relative to MT1-MMP, which would permit availability of active MT1-MMP to process the proMMP-2 bound in the ternary complex (12, 13, 17–19). Recent study showed that proMMP-2 is activated by MT1-MMP, which is mostly saturated with TIMP-2, and thus TIMP-2 inhibits cleavage of other direct MT1-MMP substrates even at the level that induces proMMP-2 activation (20). Furthermore, MT1-MMP generates TIMP-2–free active MMP-2 only in a narrow range of TIMP-2 concentration. Thus, TIMP-2 concentration dictates MT1-MMP substrate choice, proMMP-2 activation, or direct cleavage of substrates. The optimum TIMP-2 concentration to produce active MMP-2 is restricted to a narrow range, which has hampered the analysis of significance of MMP-2 activation in pathologic conditions.

In the present study, a TIMP-2 chimera protein with mosaic serine protease (MSP) was constructed (MSP-T2), which functions as a receptor for proMMP-2, but no longer inhibits MMP, and generates TIMP-2–free active MMP-2 even in cells expressing low level of MT1-MMP. MSP-T2 enables us for the first time to examine the true enzyme activities of MT1-MMP and MMP-2.

Materials and Methods

Cell culture. Human embryonic kidney 293T, HT1080 fibrosarcoma, and U87 glioma cells were obtained from American Type Culture Collection and cultured in DMEM (Sigma) supplemented with 5% FCS. 293T cells express negligible levels of MT1-MMP, MMP-2, and TIMP-2 and were used for transfection experiments (20). In contrast, HT1080 and U87 cells express a high and a low level of endogenous MT1-MMP, respectively. Three-dimensional collagen gel culture was done as described previously (21).

Antibodies and recombinant proteins. Polyclonal antibody against MSP was prepared by injecting recombinant protein expressed in E. coli.
Monoclonal antibody against FLAG epitope (M2) was purchased from Sigma. Monoclonal antibodies against MT1-MMP (113-5B7) and TIMP-2 (11-19-03) and recombinant TIMP-1 and TIMP-2 were gifts from Daichi Fine Chemical Co. Ltd. (Takaoka, Japan). Antibodies against extracellular signal-regulated kinase (ERK)-2 and phospho-p44/p42 mitogen-activated protein kinase (Thr202/Tyr204; pERK) were purchased from BD Biosciences and Cell Signaling Technology, respectively.

**Plasmids.** Expression plasmids for MT1-MMP, FLAG-tagged MT1-MMP (MT1-MMP-FLAG), TIMP-2, and FLAG-tagged testican-1 (testican-1-FLAG) were constructed in pEAK8 vector (EdgeBio Systems) as described previously (22–25).

Figure 1. MSP-T2 acts as a receptor for proMMP-2. A, control plasmid or expression plasmid for MT1-MMP-FLAG (MT1-F; 100 ng) was cotransfected with MSP or control plasmid (400 ng) into 293T cells cultured in a 24-well microplate. Thirty-six hours after transfection, cells were incubated with 1 μmol/L proMMP-2 for 1 h. Cell lysates were examined for MMP-2 activation by gelatin zymography (top), MT1-MMP expression by Western blotting with anti–FLAG M2 antibody (middle), and MSP expression with anti-MSP polyclonal antibody (bottom), as indicated. B, schematic representation of MSP, TIMP-2, and MSP-T2. MSP-T2 was constructed by replacing the signal sequence of TIMP-2 with the NH2-terminal region of MSP containing cytoplasmic and transmembrane (Cyt/TM) domains. C, serially diluted expression plasmid for MSP-T2 was transfected into 293T cells cultured in a 24-well microplate. Thirty-six hours after transfection, cells were incubated with 1 μmol/L proMMP-2 for 1 h. Cell-bound proMMP-2 was analyzed by gelatin zymography (top), and MSP-T2 expression was detected by Western blotting with anti–TIMP-2 antibody (bottom). D, 293T cells transfected with MSP-T2 plasmid (100 ng) were incubated with serially diluted proMMP-2 for 1 h, and then cell-bound and unbound proMMP-2 were detected by gelatin zymography as indicated (top). MSP-T2 expression was detected by Western blotting as described above (bottom).

The fusion gene for MSP-T2, which encodes amino acid residues 1 to 188 of MSP and amino acid residues 27 to 220 of TIMP-2, was constructed as follows: The cDNA fragment encoding amino acid residues 1 to 184 of MSP was PCR amplified using 5′ and 3′ primers with an extra EcoRI site (underlined; GAATTCTGCTGTCCTCCGCTCGAAGG and GAATTCACTGCTGTCCTCCGCTGAGT, respectively), and the human TIMP-2 cDNA fragment encoding amino acid residues 27 to 220 was amplified using 5′ and 3′ primers with extra EcoRI and XbaI sites, respectively (underlined; GAATTCTGCTGTCCTCCGCTCGAAGG and TCTAGATATGGGTCCTCCTGATGGCTG, respectively). The mouse TIMP-2 cDNA fragment corresponding to the same region was amplified using the primers GAATTCTGCTGTCCTCCGCTCGAAGG and TCTAGACTTACGGGTCCTCGATG.
to generate MSP-mouseT2. Amplified MSP and TIMP-2 cDNA fragments were sequentially ligated into pEAK8 plasmid.

Zymography and MMP-2 enzyme assay. ProMMP-2 supernatant was prepared from MSP-2–transfected 293T cells as previously described (19). Cells cultured in a 24-well microplate were transfected with expression plasmids, cultured for 36 h, and were incubated with 100 μL proMMP-2 for 1 h. The activation state of MMP-2 was analyzed by gelatin zymography (top), and MSP-T2 and MT1-MMP expression was detected by Western blotting with anti–TIMP-2 antibody (middle) and anti–FLAG M2 antibody (bottom), respectively.

30 h after transfection, cells were incubated with 100 μL proMMP-2 (0.1 μmol/L) for 1 h and then 100 μL of zymography sample buffer were added to stop the reaction. MMP-2 processing and MT1-MMP expression were examined by zymography and Western blotting, respectively, as described above.

C, control plasmid (100 ng) or expression plasmid for TIMP-2 (50 ng) or MSP-T2 (100 ng) was cotransfected into 293T cells cultured in a 24-well plate with serially diluted MT1-MMP plasmid (Control, TIMP-2, and MSP-T2, respectively). Thirty-six hours after transfection, cells were incubated with 100 μL proMMP-2 (0.1 μmol/L) for 1 h and 100 μL of zymography sample buffer were added to stop the reaction. MMP-2 processing and MT1-MMP expression were examined by zymography and Western blotting, respectively.

D, control plasmid or expression plasmid for MT1-MMP (400 ng) was cotransfected with mock or expression plasmid for TIMP-2 (200 ng) or MSP-T2 [400 ng; lanes (-), TIMP-2, and MSP-T2, respectively] into 293T cells cultured in 35-mm-diameter dishes in duplicate. Thirty-six hours after transfection, cells were incubated with either 0.4 mL of proMMP-2 (0.1 μmol/L) for 1 h or 1 mL of testican-1-FLAG sample for 6 h, after which MT1-MMP expression was examined by Western blotting, cell-associated MMP-2 by gelatin zymography, and cleavage of testican-1 by Western blotting, as described in Materials and Methods.

Figure 2. Processing of MSP-T2–bound proMMP-2 by MT1-MMP. A, control plasmid (C) or expression plasmid for MSP-T2 or MT1-MMP-FLAG (MT1-F; 100 ng each) was transfected into 293T cells in a 24-well microplate. Thirty-six hours after transfection, cells were incubated with 1.0 μmol/L proMMP-2 for 1 h. Cell-bound MMP-2 was analyzed by gelatin zymography (top), and MSP-T2 and MT1-MMP expression was detected by Western blotting with anti–TIMP-2 antibody (middle) and anti–FLAG M2 antibody (bottom), respectively. B, cells transfected with control plasmid, MSP-T2 plasmid, or MT1-MMP-FLAG plasmid (100 ng each) were incubated with proMMP-2 in the presence of mock, 2 μg/mL recombinant TIMP-1 or TIMP-2, or 1.0 × 10−6 mol/L BB94 [lanes (-), T1, T2, and BB, respectively] for 1 h, and cell-bound MMP-2 was examined by gelatin zymography (top). MSP-T2 and MT1-MMP expression was detected as described above (middle and bottom, respectively). C, control plasmid (100 ng) or expression plasmid for TIMP-2 (50 ng) or MSP-T2 (100 ng) was cotransfected into 293T cells cultured in a 24-well plate with serially diluted MT1-MMP plasmid (Control, TIMP-2, and MSP-T2, respectively). Thirty-six hours after transfection, cells were incubated with 100 μL proMMP-2 (0.1 μmol/L) for 1 h, and then 100 μL of zymography sample buffer were added to stop the reaction. MMP-2 processing and MT1-MMP expression were examined by zymography and Western blotting, respectively, as described above. D, control plasmid or expression plasmid for TIMP-2 (400 ng) or MSP-T2 (200 ng) or MMP-2 (50 ng) was cotransfected into 293T cells cultured in 35-mm-diameter dishes in duplicate. Thirty-six hours after transfection, cells were incubated with either 0.4 mL of proMMP-2 (0.1 μmol/L) for 1 h or 1 mL of testican-1-FLAG sample for 6 h, after which MT1-MMP expression was examined by Western blotting, cell-associated MMP-2 by gelatin zymography, and cleavage of testican-1 by Western blotting, as described in Materials and Methods.
to the manufacturer’s instruction. Cell-associated gelatin degradation activity was examined using Alexa Fluor 680–labeled gelatin as described previously (20).

**Testican-1 cleavage.** The expression plasmid for testican-1-FLAG (10 μg) was transfected into 293T cells in a 10-cm-diameter dish, and culture medium was replaced with 10 mL of serum-free DMEM after 48 h. Conditioned medium was harvested after 24 h, diluted 10-fold with fresh serum-free DMEM, and used as testican-1-FLAG sample. Testican-1-FLAG (1 mL) was incubated with transfected cells in 35-mm-diameter dishes for 6 h, concentrated with trichloroacetic acid, and analyzed by Western blotting with anti–FLAG M2 antibody, as described previously (20).

**Transfection of small interfering RNA.** RNA interference technology was used to generate specific knockdown of MT1-MMP, TIMP-1, and TIMP-2 mRNA transcription. Sense and antisense oligonucleotides were synthesized by Nippon EGT. The sequences were as follows (27): MT1-MMP (NM_004995) target sequence, 5′-AACCAAGCTAAGGATGAG3′; TIMP-1 (NM_000254) target sequence, 5′-AATCAACCAGGATGATGAT3′; TIMP-2 (NM_000255) target sequence, 5′-AAGGATCCAGTATGAGATCAA3′. Scrambled oligo was used as a negative control. Small interfering RNA (siRNA) reverse transfection was done using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instruction. Briefly, 10 pmol siRNA in 100 μL Opti-MEM (Invitrogen) was mixed with 1 μL Lipofectamine RNAiMAX in a 24-well microplate and incubated for 20 min. Then, the mixture was added to 2 × 10⁵ cells in 500 μL culture medium, and culture was continued for 24 h.

**Quantitative real-time PCR.** Total RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Ltd.). cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen) using oligo dT primer. Quantitative real-time PCR was carried out on a LineGene fluorescent quantitative detection system (BioLux) using SYBR Green Real-time PCR Master Mix (TOYOBO). Specific primer pairs were as follows: MT1-MMP (sense, 5′-GGATACCAAGTGATGATG3′; antisense, 5′-TTGTTCACAGGAGAAGTAG3′), TIMP-2 (sense, 5′-GCGGTCAAGGAAGGAAGTGG3′; antisense, 5′-CTTCACTGCAGCCCATG3′), and glyceraldehyde-3-phosphate dehydrogenase (sense, 5′-GTATGACTCAGCAGCAAA3′; antisense, 5′-CCATTCTGCGCTT- GACTG3′).

**Detection of ERK phosphorylation.** U87 cells suspended in serum-free medium were plated onto a 12-well microplate coated with fibronectin. Cells were incubated in the presence or absence of 1 μmol/L proMMP-2 for 0.5 or 6.0 h and then lysed with SDS sample buffer for Western blotting with antibodies against pERK and ERK2 (28, 29).

**Chick embryo assay.** The assay was done as originally described by Endo and colleagues (23). Briefly, U87 cells (1.0 × 10⁶ per egg) were injected into the chorioallantoic membrane vein of the chicken eggs 11 d after fertilization and incubated for a further 7 d. Embryo livers and lungs were then dissected, and the total DNA was extracted. A 589-bp fragment of the human β-actin gene in U87MG cells that colonized liver and lung was amplified by quantitative real-time PCR with species specific primers using 1 μg total DNA as a template in 20 μl reaction volume as described above. The sequences of primers to amplify the human β-actin gene were AGGGCAGAGCCATCTATTGCTTACATT and TCCCCTTCCTGACTGTCTAAG. Serially diluted genomic DNA from U87 cells was used as a standard.

**Figure 3.** MMP-2 processed via MSP-T2 by MT1-MMP is active. Control plasmid or MT1-MMP expression plasmid (100 ng) was cotransfected with 2-fold serially diluted TIMP-2 or MSP-T2 plasmid into 293T cells cultured in a 24-well microplate. Thirty-six hours after transfection, cells were incubated with 0.1 μmol/L proMMP-2 for 1 h. Supernatant and cell-bound MMP-2 were analyzed by gelatin zymography (Sup. and Cell, respectively). Supernatants were also examined for gelatin degradation activity (Gelatin) and cleavage of peptide substrate for MMP (Peptide Substrate) as described in Materials and Methods. Lysates were also examined for expression of TIMP-2, MSP-T2, or MT1-MMP by Western blotting (bottom).
Results

MSP-T2 functions as a receptor for proMMP-2. Among candidate molecules identified using an expression cloning strategy, which interact with MT1-MMP (23), the type II transmembrane protein MSP was selected to tether TIMP-2 on the cell surface. Coexpression of MSP with MT1-MMP stimulated proMMP-2 activation by enhancing production of active form of MT1-MMP (Fig. 1A). Immunoprecipitation experiments showed complex formation between MSP and MT1-MMP (data not shown). Although the physiologic significance remains to be elucidated, these results indicate that MSP interacts directly with MT1-MMP. This led us to fuse the cytoplasmid/transmembrane domain of MSP with TIMP-2 so as to tether TIMP-2 onto the cell surface adjacent to MT1-MMP (Fig. 1B). MSP-T2 was expressed in 293T cells, and binding of proMMP-2 was examined (Fig. 1C). ProMMP-2 bound to the cells expressing MSP-T2 in proportion to the level of MSP-T2 expression. Kinetics analysis indicated a 10 mol/L order of proMMP-2 binding to the cells expressing MSP-T2 in proportion to the level of endogenous TIMP-2 (ref. 30; Fig. 2A). Immunoprecipitation experiments showed complex formation between MSP and MT1-MMP (data not shown). MT1-MMP was examined in 293T cells, which express a negligible level of endogenous TIMP-2.

Figure 4. MSP-T2 stimulates proMMP-2 activation by MT1-MMP in HT1080 cells. A, HT1080 cells in a 60-mm-diameter dish were transfected with 2 μg control plasmid or MSP-mouseT2 plasmid and were harvested for transfection of siRNA after 24 h. Then, 2 × 10^6 cells were transfected through reverse transfection protocol with mock (−), 10 pg scrambled oligo (siC), or siRNA targeting MT1-MMP (siMT1) or TIMP-2 (siTIMP-2) in a 24-well microplate, as described in Materials and Methods. MMP-2 activation was examined by incubating with 0.1 μmol/L proMMP-2 sample (left). MT1-MMP and TIMP-2 protein levels were examined by Western blotting of cell lysates with anti–MT1-MMP and anti–TIMP-2 antibodies, respectively, as indicated. MSP-T2 expression was confirmed by Western blotting with anti–TIMP-2 antibody (right). B, HT1080 cells in a 60-mm-diameter dish were transfected with 2 μg control plasmid or expression plasmid for MSP-T2 and incubated for 24 h. Then, 2 × 10^6 cells were suspended in 100 μL collagen mixture, which was polymerized on a 24-well microplate. Cells in collagen gel were cultured in 0.5 mL culture medium for 24 h, and then medium was replaced with 100 μL of 0.1 μmol/L proMMP-2. After 1-h incubation, supernatants were mixed with an equal volume of zymography sample buffer, and collagen gels containing cells were dissolved in 200 μL sample buffer, and both were then analyzed by gelatin zymography (left). A 20-μL drop of collagen mixture containing cells as prepared above was polymerized on the bottom of a 24-well microplate and cultured in 0.5 mL medium containing 1.0 μmol/L proMMP-2 for 48 h (right). Dotted line, border of collagen gel. Note that HT1080 cells expressing MSP-T2 caused intensive hydrolysis of collagen gel. Magnification, ×100.

MSP-T2 enhances MT1-MMP–mediated proMMP-2 activation. The effect of MSP-T2 expression on proMMP-2 processing by MT1-MMP was examined in 293T cells, which express a negligible level of endogenous TIMP-2 (ref. 30; Fig. 2A). Expression of MT1-MMP alone showed a trace level of proMMP-2 processing, and coexpression of MSP-T2 clearly stimulated it. Binding of proMMP-2 to the cells expressing MSP-T2 alone was inhibited by the addition of recombinant TIMP-2 but not by TIMP-1 or BB94, and activation of bound proMMP-2 by MT1-MMP was suppressed by BB94 (Fig. 2B). To titrate the MT1-MMP requirement for proMMP-2 activation via TIMP-2 or MSP-T2, serially diluted MT1-MMP plasmid was cotransfected with control, TIMP-2, or MSP-T2 plasmid and proMMP-2 activation by these cells was examined (Fig. 2C). ProMMP-2 activation was induced at a considerably lower MT1-MMP level in the presence of MSP-T2 than in the presence of TIMP-2. Coexpression of TIMP-2 induced accumulation of MT1-MMP active form by inhibiting its autodegradation; however, MSP-T2 expression did not show such an effect, indicating that MSP-T2 does not inhibit MT1-MMP enzyme activity. Cells expressing MT1-MMP cleave testican-1 at the N132–L133 peptide bond (20). Recombinant MT1-MMP also digested testican-1 at the same site in vitro (data not shown). MT1-MMP was coexpressed with either TIMP-2 or MSP-T2, and the effects on proMMP-2 activation and cleavage of testican-1 were compared (Fig. 2D). Coexpression of TIMP-2 enhanced proMMP-2 activation but inhibited testican-1 cleavage by MT1-MMP. In contrast, coexpression of MSP-T2 stimulated proMMP-2 activation but had no effect on testican-1 cleavage. This indicates that MSP-T2 does not inhibit MT1-MMP.

MMP-2 processed by MT1-MMP via MSP-T2 is active. The enzyme activity of MMP-2 processed by MT1-MMP via TIMP-2 and
MSP-T2 was compared (Fig. 3). ProMMP-2 was processed to the active form by MT1-MMP dependent on the expression level of TIMP-2 or MSP-T2 in a similar manner, except that TIMP-2 at the highest expression level was less effective than the lower levels. Next, the enzyme activity of processed MMP-2 was examined using labeled gelatin and fluorescent peptide substrate. The enzyme activity of MMP-2 processed via TIMP-2 was proportional to the level of active MMP-2 just before activation ratio reached a plateau, and then decreased with the increase in TIMP-2 concentration. In contrast, the enzyme activity of MMP-2 processed through MSP-T2 was totally dependent on the level of active MMP-2 and remained high after the maximal activity was reached.

**MSP-T2 enhances proMMP-2 activation by HT1080 cells.** HT1080 cells express endogenous MT1-MMP and activate proMMP-2. Transfection of siRNA targeting MT1-MMP gene suppressed MT1-MMP protein and mRNA synthesis by >90%, which significantly down-regulated proMMP-2 activation (Fig. 4A). Transfection of siRNA targeting TIMP-2 gene knocked down

![Figure 5](https://example.com/figure5.png)

**Figure 5.** MSP-T2 enhances metastatic ability of U87 cells. A, U87 cells in a 10-cm-diameter dish were transfected with 5 μg control plasmid or MSP-T2 plasmid (MSP-T2) and incubated for 48 h. Transfected cells were replated onto a 24-well microplate for transfection of mock (-), siRNA targeting MT1-MMP gene (siMT1), or scrambled oligo (siC) as described in Materials and Methods. BB94 was added to the culture of mock-treated cells (BB94). After incubation with proMMP-2, cell-bound MMP-2 was analyzed by gelatin zymography. Expression of MSP-T2 was examined by Western blotting with anti–TIMP-2 antibody (α-TIMP-2). B, U87 cells transfected with control plasmid or MSP-T2 plasmid were incubated with or without proMMP-2 for 1 h in duplicate. Cell-associated MMP-2 was examined by gelatin zymography, and cell-associated gelatin degradation activity was examined by incubating with Alexa Fluor 680–labeled gelatin for 1 h as described in Materials and Methods. C, U87 cells transfected with control plasmid or MSP-T2 plasmid were incubated in serum-free medium on a fibronectin-coated plate in the presence or absence of 0.1 μmol/L proMMP-2 and lysed for Western blotting to detect pERK and ERK2 at 0.5 and 6.0 h after plating (pERK and ERK2, respectively). BB94 (1 μmol/L) was added to the culture of cells transfected with MSP-T2 plasmid (+BB94). Cells cultured with proMMP-2 were also analyzed by gelatin zymography (bottom). D, U87 cells transfected with control or MSP-T2 plasmid as described above were injected into the chorioallantoic membrane vein of the chicken eggs, and cells that metastasized into liver and lung were analyzed as described in Materials and Methods. *, P < 0.05.
TIMP-2 protein and mRNA level by >85%, which also suppressed proMMP-2 activation. Transfection of MSP-mouseT2 plasmid into HT1080 cells clearly enhanced proMMP-2 processing. Cotransfection of MT1-MMP siRNA, but not TIMP-2 siRNA, significantly inhibited it. These results indicate that TIMP-2 can be replaced with MSP-T2 for MT1-MMP–mediated proMMP-2 activation in HT1080 cells.

Next, mock- and MSP-T2–expressing HT1080 cells were embedded in collagen gel matrix and cultured for 2 days in the presence of proMMP-2 (Fig. 4B). Collagen gel culture of HT1080 cells enhanced activation of proMMP-2 (compare Fig. 4A and B) and activated MMP-2 accumulated in MSP-T2–transfected cells (Fig. 4B). Control HT1080 cells showed invasive growth in collagen gel, and some cells invaded out of the gel. In contrast to control cells, MSP-T2–transfected cells caused intensive hydrolysis of collagen gel, and many cells migrated out of the gel.

**MSP-T2 enhances metastatic ability of U87 cells.** Real-time PCR analysis showed that U87 glioma cells express up to 10-fold lower MT1-MMP mRNA level than HT1080 cells (data not shown), and significant proMMP-2 binding and activation was not observed under control conditions (Fig. 5A). Expression of MSP-T2 in U87 cells induced binding and activation of proMMP-2. Cotransfection of siRNA targeting MT1-MMP or treatment of transfected cells with BB94 abrogated activation, but not binding, of proMMP-2. Unlike control U87 cells, cells expressing MSP-T2 showed efficient gelatin degradation activity when preincubated with proMMP-2 (Fig. 5B). These results indicate that MSP-T2 stimulates MT1–MMP–mediated proMMP-2 activation in U87 cells, which amplifies proteolytic activity.

Previously, we reported that lysis of ECM by MT1-MMP at cell-ECM adhesions induces sustained ERK activation by promoting focal adhesion turnover (28, 29). Activation of ERK in U87 cells transfected with control or MSP-T2 plasmid was examined (Fig. 4C). At 0.5 hour after plating onto a fibronectin-coated plate, ERK was activated equally in cells transfected with control or MSP-T2 plasmid, and addition of BB94 had no effect. The activation level of ERK was remarkably reduced in both control and MSP-T2–transfected cells at 6 hours after plating, and addition of BB94 further attenuated it. Incubation of cells in medium containing proMMP-2 for 6 hours significantly recovered ERK activation only in cells expressing MSP-T2, on which active MMP-2 accumulated.

U87 cells transfected with control or MSP-T2 plasmid were injected into the chick embryo choroidallantoic membrane vein, and the number of cells that metastasized to liver and lung was measured 7 days after injection. MSP-T2–transfected U87 cells metastasized more efficiently into liver and lung by 5.2-fold than control cells. These results indicate that MSP-T2–enhanced MMP-2 activation by MT1-MMP contributes to metastasis of U87 cells into chick embryonic liver and lung.

**Discussion**

In this study, an artificial receptor for proMMP-2 MSP-T2 was constructed by fusing transmembrane/cytoplasmic domain of type II transmembrane protein MSP with TIMP-2. *MSP* gene was cloned by expression cloning strategy, the product of which enhanced MT1-MMP–mediated proMMP-2 activation. Immunoprecipitation experiments showed direct interaction of between MT1-MMP and MSP, which might block autodegradation of MT1-MMP and induced accumulation of MT1-MMP active form (Fig. 1A). Although the mechanism and physiologic significance of interaction between MSP and MT1-MMP still remain to be elucidated, the transmembrane/cytoplasmic domain of MSP was expected to tether TIMP-2 onto cell surface adjacent to MT1-MMP. Actually, proMMP-2 bound to MSP-T2 on cell surface was processed to fully active form by coexpression of MT1-MMP. This indicates that MSP-T2 is located adjacent to MT1-MMP as expected. TIMP-2–dependent proMMP-2 activation required a much higher level of MT1-MMP than that induced by MSP-T2 (Fig. 2C). These results suggest that the majority of MT1-MMP is bound to TIMP-2 and functions as a receptor for proMMP-2 in TIMP-2–dependent proMMP-2 activation as illustrated in Fig. 6. This is consistent with the fact that TIMP-2 interfered with direct cleavage of testican-1 by MT1-MMP even at the level that induced most efficient proMMP-2 activation (ref. 20; Fig. 2D). Previously we have shown that proMMP-2 activation takes place under the condition where MT1-MMP is almost saturated with TIMP-2, and suggested that trimolecular complex formation may be a dynamic process in which TIMP-2 may transiently interact with MT1-MMP, proMMP-2, and activated MMP-2 (20). Furthermore, MMP-2 activated by MT1-MMP via MSP-T2 is free from TIMP-2 and active, whereas TIMP-2–free MMP-2 is generated via MT1-MMP/TIMP-2 complex at a very narrow range of TIMP-2 concentration. MSP-T2 enhanced not only proMMP-2 activation by MT1-MMP but also those by all MT-MMPs except for MT4-MMP.3

Activation of proMMP-2 by HT1080 cells was shown to be mediated by MT1-MMP and TIMP-2 using siRNA targeting each gene, and TIMP-2 could be replaced with MSP-T2 (Fig. 4A). HT1080 cells expressing MSP-T2 caused intensive hydrolysis of collagen gel in the presence of MMP-2, which we had never experienced before (Fig. 4B). Collagen gel culture of HT1080 cells enhanced proMMP-2 activation to the extent that no additional effect of MSP-T2 expression on proMMP-2 activation was evident in culture supernatant. However, more activated MMP-2 was associated with MSP-T2–expressing cells than control cells, which might cause intensive hydrolysis of collagen gel. MMP-2 digests not only basement membrane components, including type IV collagen, laminin, and fibronectin, but also gelatins that are generated by the action of collagenases, and plays a key role in the degradation of fibrillar collagens. MT1-MMP is a membrane-bound collagenase (7), and thus combination of MT1-MMP and MMP-2 on surface of cells expressing MSP-T2 may result in intensive hydrolysis of collagen gel. TIMP-2 dictates substrate choice of MT1-MMP depending on its concentration, and thus direct cleavage of substrates by MT1-MMP may be less effective in cells producing active MMP-2 (ref. 20; Fig. 2D). In contrast, MSP-T2 can generate active MMP-2 without affecting direct cleavage of substrates by MT1-MMP, and active MMP-2 colocalizes with active MT1-MMP on cell surface. An excess expression of MT1-MMP in HT1080 cells failed to cause apparent hydrolysis of collagen gel, which was observed with MSP-T2 expression, suggesting that cell-surface active MMP-2 plays an essential role in it.

U87 cells express up to 10-fold lower level of MT1-MMP mRNA than HT1080 cells, and proMMP-2 was not significantly processed by them. Expression of MSP-T2 induced MT1-MMP–mediated proMMP-2 activation in U87 cells, which amplified proteolytic activity, and the effect of MSP-T2 was evident when proMMP-2 was...
added in culture (Fig. 5). We reported that lysis of ECM by MT1-MMP at cell-ECM adhesions promotes focal adhesion turnover, sustained ERK activation, and subsequent cell migration (28, 29). ERK activation in U87 cells was remarkably reduced at 6 hours after plating, which was recovered by expression of MSP-T2 in the presence of MMP-2. Lysis of ECM by U87 cells might not be enough to support effective turnover of focal adhesions due to a low MT1-MMP level. MMP-2 activated and anchored on cell surface through MSP-T2 may colocalize with MT1-MMP at focal adhesion and promote lysis of ECM and subsequent focal adhesion turnover and sustained ERK activation. Expression of MSP-T2 in U87 cells enhanced metastatic ability of cells in chick embryo, which should be due to induction of MMP-2 activation and anchoring of active MMP-2 on cell surface. Active MMP-2 on cell surface may stimulate not only extravasation and local invasion but also proliferation of tumor cells in tissues. Another function of MMP-2 suggested recently for ovarian cancer cells was that MMP-2 enhances adhesion of cells through cleavage of fibronectin and vitronectin (31). In our in vitro experiment, MMP-2 activated via MSP-T2 may digest fibronectin and enhance integrin-mediated adhesion and induce effective turnover of focal adhesions and subsequent ERK activation (Fig. 5C). In our previous experiment using HT1080 cells, fibronectin digestion to induce ERK activation was solely mediated by MT1-MMP (28, 29); however, MMP-2 was shown to do it in this study. ERK activation may contribute to cell migration as well as proliferation (28, 29). These results show the importance of MMP-2 in tumor metastasis and are consistent with previous reports on the role of MMP-2 in primary growth and metastatic spread of tumors in vivo (32, 33). It should be noted that MMP-2 needs MT1-MMP as an activator, and collaboration between MT1-MMP and MMP-2 causes effective degradation of ECM as described above. TIMP-2 regulates direct cleavage of substrates by MT1-MMP, MMP-2 processing, and activity of processed MMP-2 in a complex manner. MSP-T2 can mediate proMMP-2 activation by MT1-MMP without blocking active MMP-2 and MT1-MMP, and thus will be a valuable tool to analyze the roles of MMP-2 and MT1-MMP in physiologic and pathologic processes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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