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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Activation of Matrix Metalloproteinase (MMP)-2 By Membrane-type 1-MMP Through An Artificial Receptor For ProMMP-2 Generates Active MMP-2

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Abbreviations: MMP, matrix metalloproteinase; MSP, mosaic serine protease; SDS, sodium lauryl sulfate; TIMP, tissue inhibitor of metalloproteinase; MT, membrane type; DMEM, Dulbecco's Modified Eagle Medium; PAGE, polyacrylamide gel electrophoresis; ECM, extracellular matrix.

Summary

The suggested model for pro-matrix metalloproteinase-2 (proMMP-2) activation by membrane-type 1-matrix MMP (MT1-MMP) implicates the complex between MT1-MMP and tissue inhibitor of MMP (TIMP)-2 as a receptor for proMMP-2. To dissect this model and assess the pathological 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 MT1-MMP. Thus, MSP-T2 did not affect direct cleavage of a substrate testican-1 by MT1-MMP, whereas TIMP-2 inhibited it even at the level which stimulated 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 extra-cellular 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 physiological and pathological roles of MMP-2 and MT1-MMP in comparison with TIMP-2.

Introduction

Recent studies have demonstrated that members of the matrix metalloproteinase (MMP) gene family play a central role in the degradation of extra cellular matrix (ECM) macromolecules under various physiological and pathological 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 physiological activator of latent MMP-2 (proMMP-2) (6). The role of MT1-MMP in pericellular proteolysis is not restricted to proMMP-2 activation as 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 TIMPs. 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 MT1-MMP and TIMP-2 NH2-terminal ends. The propeptide of proMMP-2 is cleaved by an adjacent TIMP-2-free MT1-MMP between Asn^{37} and Leu^{38}, generating an activated intermediate form which is further processed to the fully activated form by an intermolecular auto-cleavage 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 which 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 analysis of significances of MMP-2 activation in pathological 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 a low level 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 ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) supplemented with 5% fetal calf serum. 293T cells express a negligible level 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 endogenous MT1-MMP, respectively. Three-dimensional collagen gel culture was performed as described previously (21).

Antibodies and recombinant proteins

Polyclonal antibody against MSP was prepared by injecting recombinant protein expressed in E. coli into rabbits with Freund's complete adjuvant. Monoclonal antibody against FLAG epitope (M2) was purchased from Sigma (St Louis, MO). Monoclonal antibodies against MT1-MMP (113-5B7) and TIMP-2 (11-19-03), and recombinant TIMP-1 and TIMP-2 were gifts from Daiichi Fine Chemical Co. Ltd. (Takaoka, Japan). Antibodies against extracellular signal-regulated kinase (ERK)2 and phospho-p44/42 MAPK (Thr202/Tyr204) (pERK) were purchased from BD Biosciences (San Jose, CA) and Cell Signaling Technology (Danvers, MA), 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, Gaithersburg, MD) as described previously (22-25).

The fusion gene for MSP-T2, which encodes amino acid residue 1 to 188 of MSP and 27-220 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) (GAATTCTGTGTGGCCCTGCCAGAAC and GAATTCAGCTAAGCCTGGAGG,

respectively), and the humanTIMP-2 cDNA fragment encoding amino acid residues 27-220 was amplified using 5' and 3' primers with an extra EcoRI and XbaI site, respectively (underlined) (GAATTCTGCAGCTGCTCCCCGGTG and TCTAGATTATGGGTCCTCGATGCT, respectively). The mouse TIMP-2 cDNA fragment corresponding to the same region was amplified using following primers (GAATTCTGCAGCTGCTCCCCGGTGCACC and TCTAGACTTACGGGTCCTCGATGTCAAG) 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 MMP-2-transfected 293T cells as previously described (19). Cells cultured in 24-well microplate were transfected with expression plasmids, cultured for 36 h, and were incubated with 100 μl proMMP-2 for 1 h. Activation state of MMP-2 in the supernatant was examined by mixing with a same volume of zymography sample buffer. For the detection of cell-bound MMP-2, cells were washed twice with PBS, and then dissolved in 200 μl sample buffer by sonication. For the assay of total MMP-2, 100 μl sample buffer was directly added to the culture previously incubated with proMMP-2, and then the mixture was homogenized by sonication. These samples were incubated for 20 min at 37°C and then subjected to gelatin zymography gel containing Alexa Fluor 680-labeled gelatin. Gels were processed, and monitored by a LI-COR Odyssey IR imaging system (Lincoln, NE) as described previously (26).

MMP-2 enzyme activity in supernatants was examined using Alexa Fluor 680-labeled gelatin or Fluorescence-Quenching Substrate for MMP. Briefly, supernatants (10 μl) were incubated with an equal volume of Alexa Fluor 680-labeled gelatin (2 μg/ml) for 1h, separated on 10% SDS-PAGE, and monitored by Odyssey as described previously. Fluorescence-Quenching Substrate MOCAc- Pro- Leu- Gly- Leu- A2pr(Dnp)- Ala-Arg-NH_2 (Peptide Inst., Osaka, Japan) (1

μM) was incubated with 100 μl supernatants in a total volume of 300 μl for 30 min, and the fluorescence was monitored according to 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 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 using anti-FLAG M2 antibody, as described previously (20).

Transfection of small interfering RNA (siRNA)

RNA interference technology was used to generate specific knock-down of MT1-MMP, TIMP-1 and TIMP-2 mRNA transcription. Sense and anti-sense oligonucleotides were synthesized by Nippon EGT (Toyama, Japan). The sequences were as follows (27): MT1-MMP (NM_004995) target sequence, 5-AACCAGAAGCTGAAGGTAGAA-3; TIMP-1 (NM_003254) target sequence, 5-AATCAACCAGACCACCTTATA-3; TIMP-2 (NM_003255) target sequence, 5-AAGGATCCAGTATGAGATCAA-3. Scrambled oligo was used as negative control. siRNA reverse transfection was performed using Lipofectamine™ RNAiMAX (Invitrogen, Karlsbad, Germany) according to manufacturer's instruction. Briefly, 10 pmol of siRNA in 100 μl Opti-MEM (Invitrogen) was mixed with 1 μl Lipofectamine™ RNAiMAX in 24-well microplate, and incubated for 20 min. Then, the mixture was added to 2 x 10^5 cells in 500 μl culture medium, and culture was continued for 24 h.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Ltd., Osaka, Japan). cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) using oligo dT primer. qRT-PCR was carried out on a LineGene fluorescent quantitative detection system (BioFlux, Tokyo, Japan) using SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan). Specific primer pairs were as follows: MT1-MMP (sense 5'-GGAATAACCAAGTGATGGATGG-3'; anti-sense 5'- TTGTTTCCACGGAAGAAGTAGG), TIMP-2 (sense 5'- GCGGTCAGTGAGAAGGAAGTGG-3; antisense 5'-CTTGCACTCGCAGCCCATCTG-3') and glyceraldehyde-3-phosphate dehydrogenase (sense 5'-GTATGACTCCACTCACGGCAAA-3'; antisense 5'-CCATTCTCGGCCTTGACTGT-3').

Detection of ERK phosphorylation

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

Chick embryo assay

The assay was performed as originally described by Endo et al. (30). Briefly, U87 cells (1.0 x 10^6 cells per egg) were injected into the chorioallantoic membrane vein of the chicken eggs 11 days after fertilization and incubated for a further 7days. 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 qRT-PCR with species specific primers using 1μg 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 TCCCCTTCCTATGACATGAACTTAACCA. Serially diluted genomic DNA from U87 cells was used as a standard.

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. Co-expression of MSP with MT1-MMP stimulated proMMP-2 activation by enhancing production of active form of MT1-MMP (Fig. 1A). Immunoprecipitation experiments demonstrated complex formation between MSP and MT1-MMP (data not shown). Although the physiological significance remains to be elucidated, these results indicate that MSP interacts directly with MT1-MMP. This lead us to fuse 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 10^{-9} M order of proMMP-2 binding to the cells expressing MSP-T2 (Fig. 1D).

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 (31) (Fig. 2A). Expression of MT1-MMP alone showed a trace level of proMMP-2 processing, and co-expression 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 co-transfected 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 considerably lower MT1-MMP level in the presence of MSP-T2 than in the presence of TIMP-2.

Co-expression of TIMP-2 induced accumulation of MT1-MMP active form by inhibiting its auto-degradation, 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 N^{132}-L^{133} peptide bond (20). Recombinant MT1-MMP also digested testican-1 at the same site *in vitro* (data not shown). MT1-MMP was co-expressed with either TIMP-2 or MSP-T2, and the effect on proMMP-2 activation and cleavage of testican-1 was compared (Fig. 2D). Co-expression of TIMP-2 enhanced proMMP-2 activation but inhibited testican-1 cleavage by MT1-MMP. In contrast, co-expression 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

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 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, enzyme activity of processed MMP-2 was examined using labeled gelatin and fluorescent peptide substrate. Enzyme activity of MMP-2 processed via TIMP-2 was proportional to the level of active MMP-2 just before activation ratio reached plateau, and then decreased with the increase of TIMP-2 concentration. In contrast, 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 more than 90%, which significantly down-regulated proMMP-2 activation (Fig. 4A). Transfection of siRNA targeting TIMP-2 gene knocked down TIMP-2 protein and mRNA level by more than

85%, which also suppressed proMMP-2 activation. Transfection of MSP-mouseT2 plasmid into HT1080 cells clearly enhanced proMMP-2 processing. Co-transfection 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 demonstrated 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. Co-transfection 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 pre-incubated 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 h

after plating onto fibronectin-coated plate, ERK was activated equally in cells transfected with control or MSP-T2 plasmid, and addition of BB94 had no effect. Activation level of ERK was remarkably reduced in both control and MSP-T2-transfected cells at 6 h after plating, and addition of BB94 further attenuated it. Incubation of cells in medium containing proMMP-2 for 6 h 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 chorioallantoic membrane vein, and the number of cells metastasized to liver and lung was measured 7 days after injection. MSP-T2-transfected U87 cells metastasized more efficiently into liver by 2.5-fold 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 auto-degradation of MT1-MMP and induced accumulation of MT1-MMP active form (Fig. 1A). Although, the mechanism and physiological significance of interaction between MSP and MT1-MMP still remain to be elucidated, 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 co-expression of MT1-MMP. This indicates that MSP-T2 is located adjacent to MT1-MMP as expected. TIMP-2-dependent proMMP-2 activation required much higher level of MT1-MMP than those 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 which induced most efficient proMMP-2 activation (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 tri-molecular 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 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 (unpublished

data).

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 which 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 (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 co-localizes with active MT1-MMP on cell surface. An excess expression of MT1-MMP in HT1080 cells failed to cause apparent hydrolysis of collagen gel (unpublished data), 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 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 h 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 co-localize 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 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 was suggested recently for ovarian cancer cells that MMP-2 enhances adhesion of cells through cleavage of fibronectin and vitronectin (32). 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 demonstrate the importance of MMP-2 in tumor metastasis, and are consistent with previous reports on role of MMP-2 in primary growth and metastatic spread of tumors *in vivo* (33, 34). 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 roles of MMP-2 and MT1-MMP in physiological and pathological processes.

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Figure Legends

Fig. 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 co-transfected with MSP or control plasmid (400 ng) into 293T cells cultured in 24-well microplate. Thirty-six h after transfection, cells were incubated with 1 μM proMMP-2 for 1 h. Cell lysates were examined for MMP-2 activation by gelatin zymography (top panel), MT1-MMP expression by Western blotting with anti-FLAG M2 antibody (middle panels), and MSP expression with anti-MSP polyclonal antibody as indicated (bottom panel). ***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 24-well microplate. Thirty-six h after transfection, cells were incubated with 1 μM proMMP-2 for 1 h. Cell-bound proMMP-2 was analyzed by gelatin zymography (upper panel), and MSP-T2 expression was detected by Western blotting using anti-TIMP-2 antibody (lower panel).

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 was detected by gelatin zymography as indicated (top panels). MSP-T2 expression was detected by Western blotting as described above (bottom panel).

Fig. 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 24-well microplate. Thirty-six h after transfection,

cells were incubated with 1.0 μM proMMP-2 for 1 h. Cell-bound MMP-2 was analyzed by gelatin zymography (upper panel), and MSP-T2 and MT1-MMP expression was detected by Western blotting using anti-TIMP-2 antibody (middle panel) and anti-FLAG M2 antibody (lower panel), respectively. ***B,*** cells transfected with control plasmid, MSP-T2 plasmid or MT1-MMP-FLAG plasmids (100 ng each) were incubated with proMMP-2 in the presence of mock, 2 μg/ml recombinant TIMP-1, TIMP-2 or 1.0 x 10^{-6} M BB94 (lanes (-), T1, T2 and BB, respectively) for 1 h, and cell-bound MMP-2 was examined by gelatin zymography (upper panel). MSP-T2 and MT1-MMP expression was detected as described above (middle and lower panels, respectively). ***C,*** control plasmid (100 ng), expression plasmid for TIMP-2 (50 ng) or MSP-T2 (100 ng) was co-transfected into 293T cells cultured in 24-well plate with serially diluted MT1-MMP plasmid (panels Control, TIMP-2 and MSP-T2, respectively). Thirty-six h after transfection, cells were incubated with 100 μl proMMP-2 (0.1 μM) for 1 h, and then 100 μl of zymography sample buffer was 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 (C) or expression plasmid for MT1-MMP (400 ng) was co-transfected with mock, 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 h after transfection, cells were incubated with either 0.4 ml of proMMP-2 (0.1 μM) 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.

Fig. 3. MMP-2 processed via MSP-T2 by MT1-MMP is active.

Control plasmid (C) or MT1-MMP expression plasmid (100 ng) was co-transfected with 2-fold

serially diluted TIMP-2 or MSP-T2 plasmid into 293T cells cultured in 24-well microplate. Thirty-six h after transfection, cells were incubated with 0.1 μM proMMP-2 for 1 h. Supernatant and cell-bound MMP-2 was analyzed by gelatin zymography (panels, Sup. and Cell, respectively). Supernatants were also examined for gelatin degradation activity (panel, Gelatin) and cleavage of peptide substrate for MMP (panel, 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 panels).

Fig. 4. MSP-T2 stimulates proMMP-2 activation by MT1-MMP in HT1080 cells.

A, HT1080 cells in 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 x 10^5 cells were transfected through reverse transfection protocol with mock (-), 10 pg scrambled oligo (siC), siRNA targeting MT1-MMP (siMT1) or TIMP-2 (siTIMP-2) in 24-well microplate as described in Materials and Methods. MMP-2 activation was examined by incubating with 0.1 μM proMMP-2 sample (left panels). MT1-MMP and TIMP-2 protein level was examined by Western blotting of cell lysates with anti-MT1-MMP and anti-TIMP-2 antibodies as indicated, respectively. MSP-T2 expression was confirmed by Western blotting with anti-TIMP-2 antibody (right panel). ***B,*** HT1080 cells in 60 mm diameter dish were transfected with 2 μg control plasmid (C) or expression plasmid for MSP-T2 (MSP-T2), and incubated for 24 h. Then, 2 x 10^5 cells were suspended in 100 μl collagen mixture, which was polymerized on 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 μM 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

panel). A 20 μl drop of collagen mixture containing cells as prepared above was polymerized on the bottom of 24-well microplate, and cultured in 0.5 ml medium containing 1.0 μM proMMP-2 for 48 h (right panel). A dotted line indicates the border of collagen gel. Note that HT1080 cells expressing MSP-T2 caused intensive hydrolysis of collagen gel. Magnification = 100x.

Fig. 5. MSP-T2 enhances metastatic ability of U87 cells.

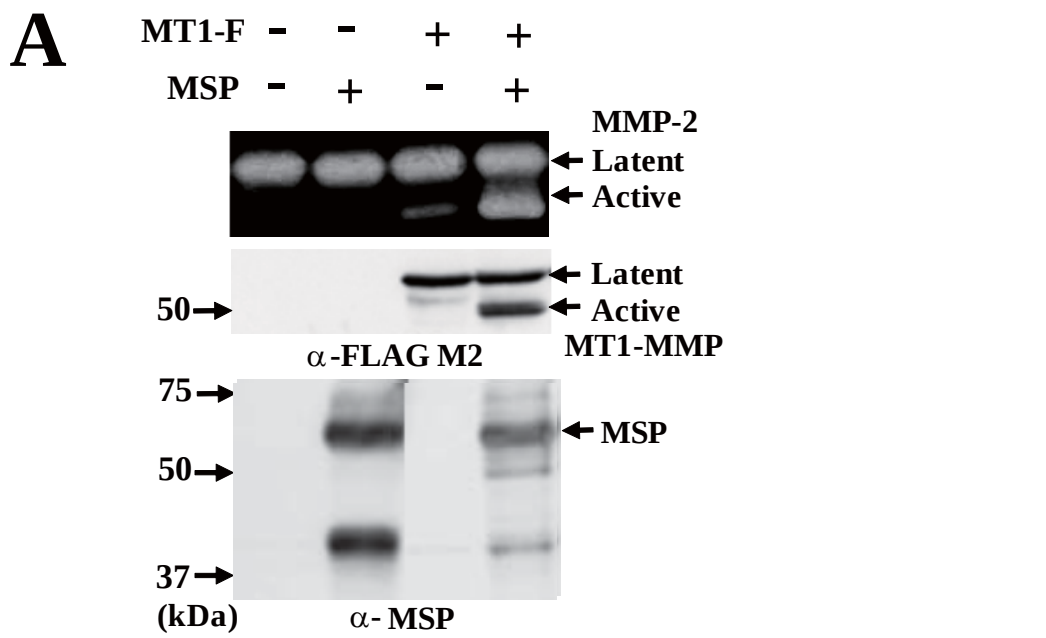
A, U87 cells in 10 cm diameter dish were transfected with 5 μg control plasmid (C) or MSP-T2 plasmid (MSP-T2), and incubated for 48 h. Transfected cells were re-plated onto 24-well microplate for transfection of mock (-), siRNA targeting MT1-MMP gene (siMT1) or scrambled oligo (siC) as described in Materials 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 using anti-TIMP-2 antibody (panel αTIMP-2). ***B,*** U87 cells transfected with control (C) or MSP-T2 (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 (C) or MSP-T2 (MSP-T2) plasmid were incubated in serum-free medium on fibronectin-coated plate in the presence or absence of 0.1 μM proMMP-2, and lysed for Western blotting to detect pERK and ERK2 at 0.5 h and 6.0 h after plating (panels pERK and ERK2, respectively). BB94 (1 μM) 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 panel). ***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 metastasized into liver and lung were analyzed as

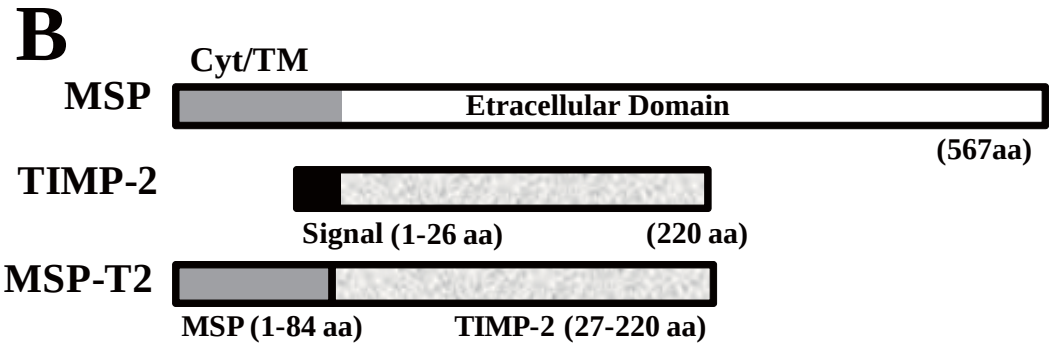
described in Materials and Methods. *P < 0.05.

Fig. 6. Schematic illustration of proMMP-2 activation mediated by MT1-MMP/TIMP-2 complex or MSP-T2.

A, MT1-MMP directly cleaves its substrate, but does not activate proMMP-2 in the absence of TIMP-2. *B*, in TIMP-2-dependent proMMP-2 activation, the majority of MT1-MMP is bound to TIMP-2, and functions as a receptor for proMMP-2. Since only a minor portion of MT1-MMP is free from TIMP-2 and involved in proMMP-2 activation, direct cleavage of substrate by MT1-MMP is very ineffective. *C*, MSP-T2 serves as a receptor for proMMP-2 without inhibiting MT1-MMP, and thus MT1-MMP can perform both proMMP-2 activation and direct cleavage of substrate.

Fig.1


A
MT1-F - - + +
MSP - + - +
MMP-2
Latent
Active
Latent
Active
MT1-MMP
50
α-FLAG M2
75
50
37
(kDa)
MSP
α- MSP


B
Cyt/TM
MSP
Etracellular Domain
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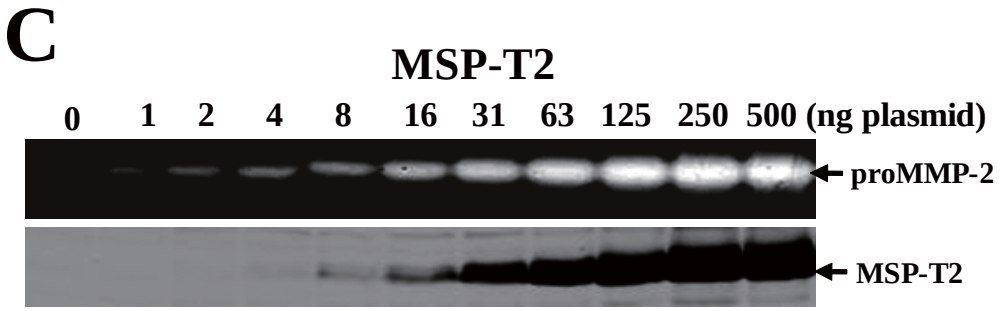
TIMP-2
Signal (1-26 aa)
(220 aa)
MSP-T2
MSP (1-84 aa)
TIMP-2 (27-220 aa)


C
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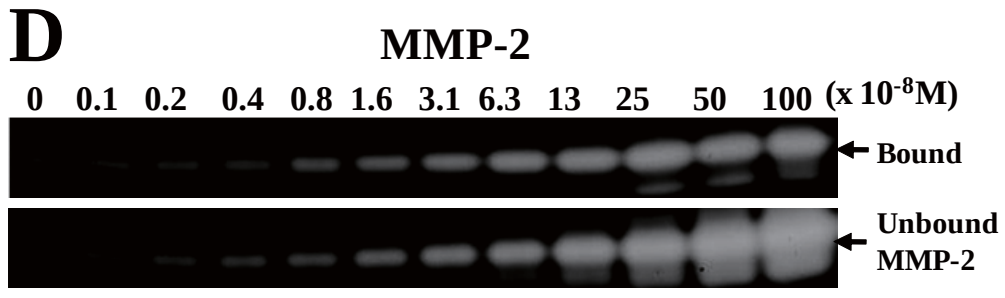
proMMP-2
MSP-T2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Bound
Unbound
MMP-2

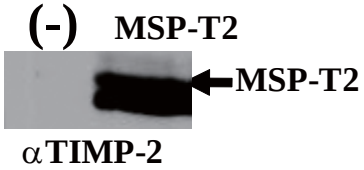

(-) MSP-T2
MSP-T2
αTIMP-2

Fig.2

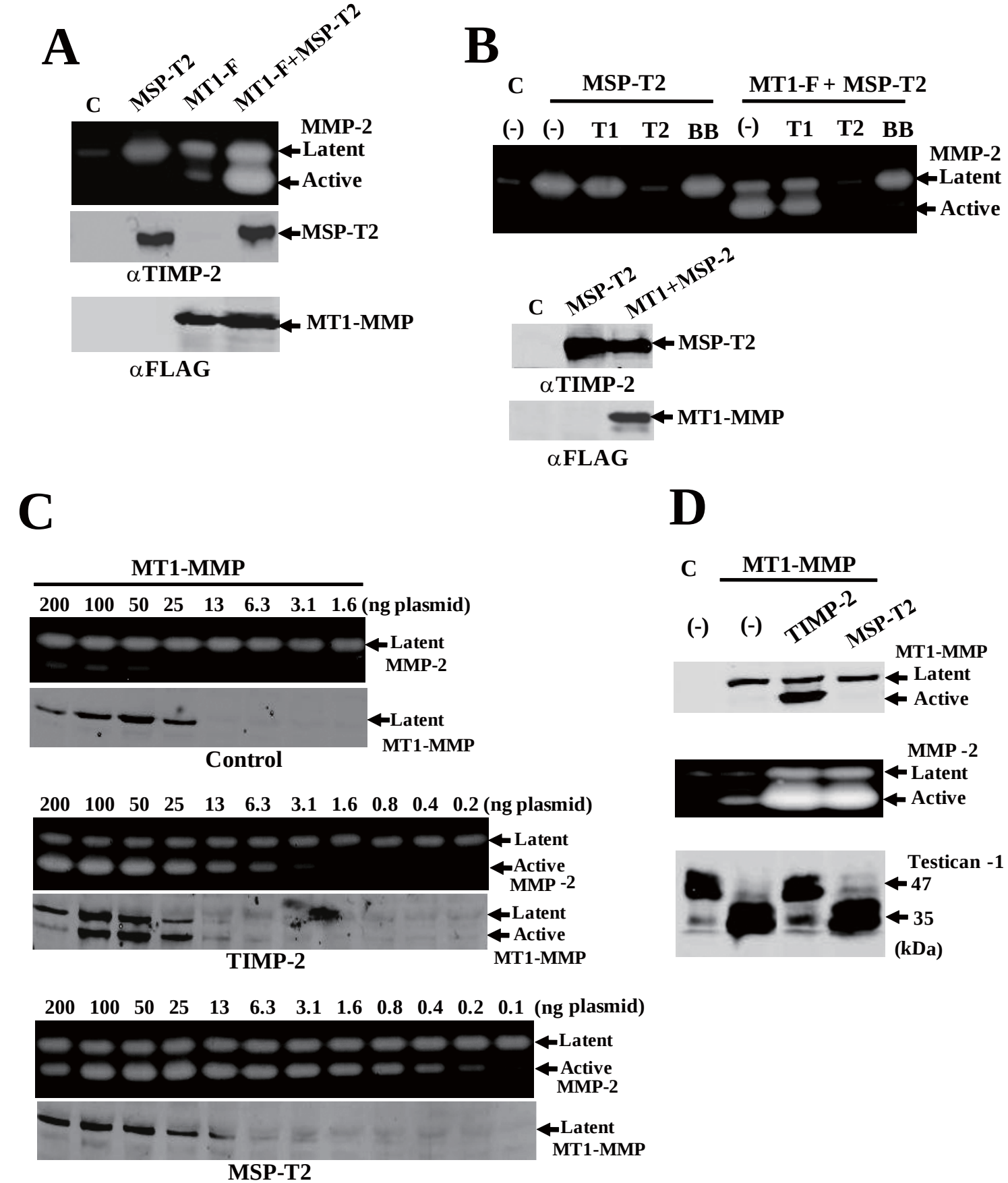
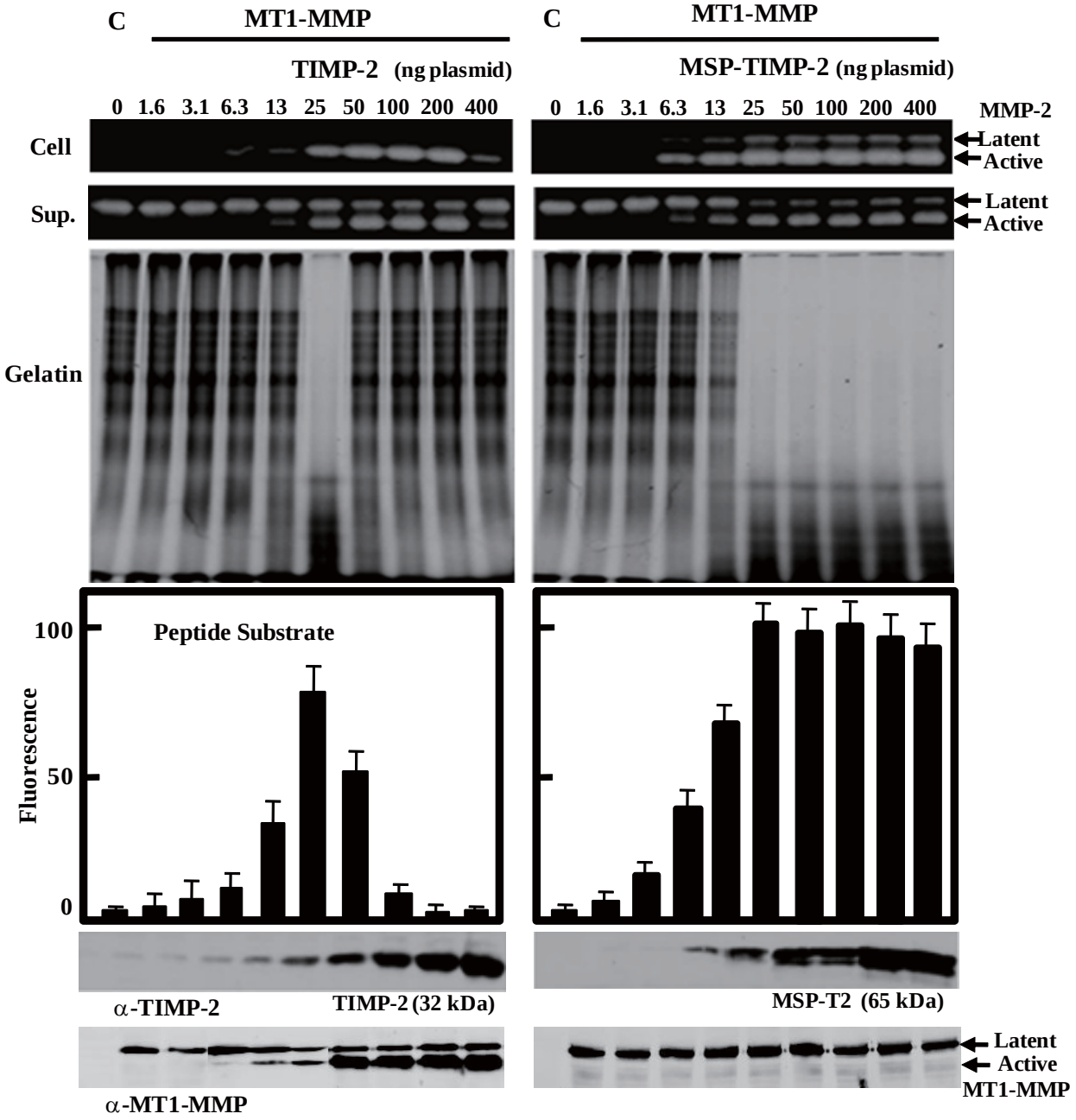

A
C
MSP-T2
MT1-F
MT1-F+MSP-T2
MMP-2
Latent
Active
MSP-T2
αTIMP-2
MT1-MMP
αFLAG
B
C
MSP-T2
MT1-F + MSP-T2
(-) (-) T1 T2 BB (-) T1 T2 BB
MMP-2
Latent
Active
C
MSP-T2
MT1+MSP-2
MSP-T2
αTIMP-2
MT1-MMP
αFLAG
C
MT1-MMP
200 100 50 25 13 6.3 3.1 1.6 (ng plasmid)
Latent
MMP-2
Latent
MT1-MMP
Control
200 100 50 25 13 6.3 3.1 1.6 0.8 0.4 0.2 (ng plasmid)
Latent
Active
MMP -2
Latent
Active
MT1-MMP
TIMP-2
200 100 50 25 13 6.3 3.1 1.6 0.8 0.4 0.2 0.1 (ng plasmid)
Latent
Active
MMP-2
Latent
MT1-MMP
MSP-T2
D
C
MT1-MMP
(-) (-) TIMP-2 MSP-T2
MT1-MMP
Latent
Active
MMP -2
Latent
Active
Testican -1
47
35
(kDa)

Fig.3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MT1-MMP
TIMP-2 (ng plasmid)
0 1.6 3.1 6.3 13 25 50 100 200 400
C
MT1-MMP
MSP-TIMP-2 (ng plasmid)
0 1.6 3.1 6.3 13 25 50 100 200 400
MMP-2
Cell
Latent
Active
Sup.
Latent
Active
Gelatin
Peptide Substrate
100
50
0
Fluorescence
α-TIMP-2
TIMP-2 (32 kDa)
MSP-T2 (65 kDa)
α-MT1-MMP
Latent
Active
MT1-MMP

Fig.4

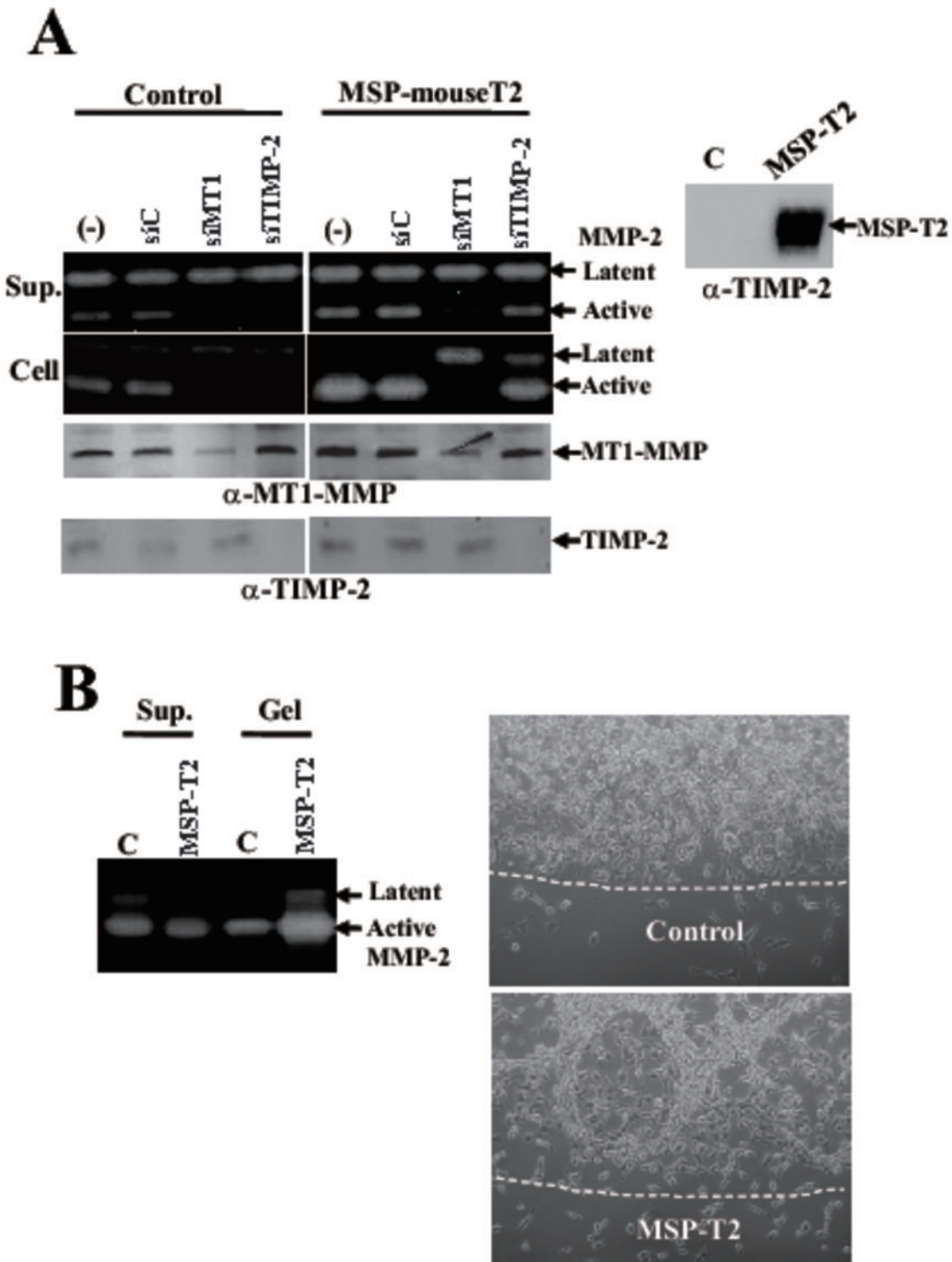
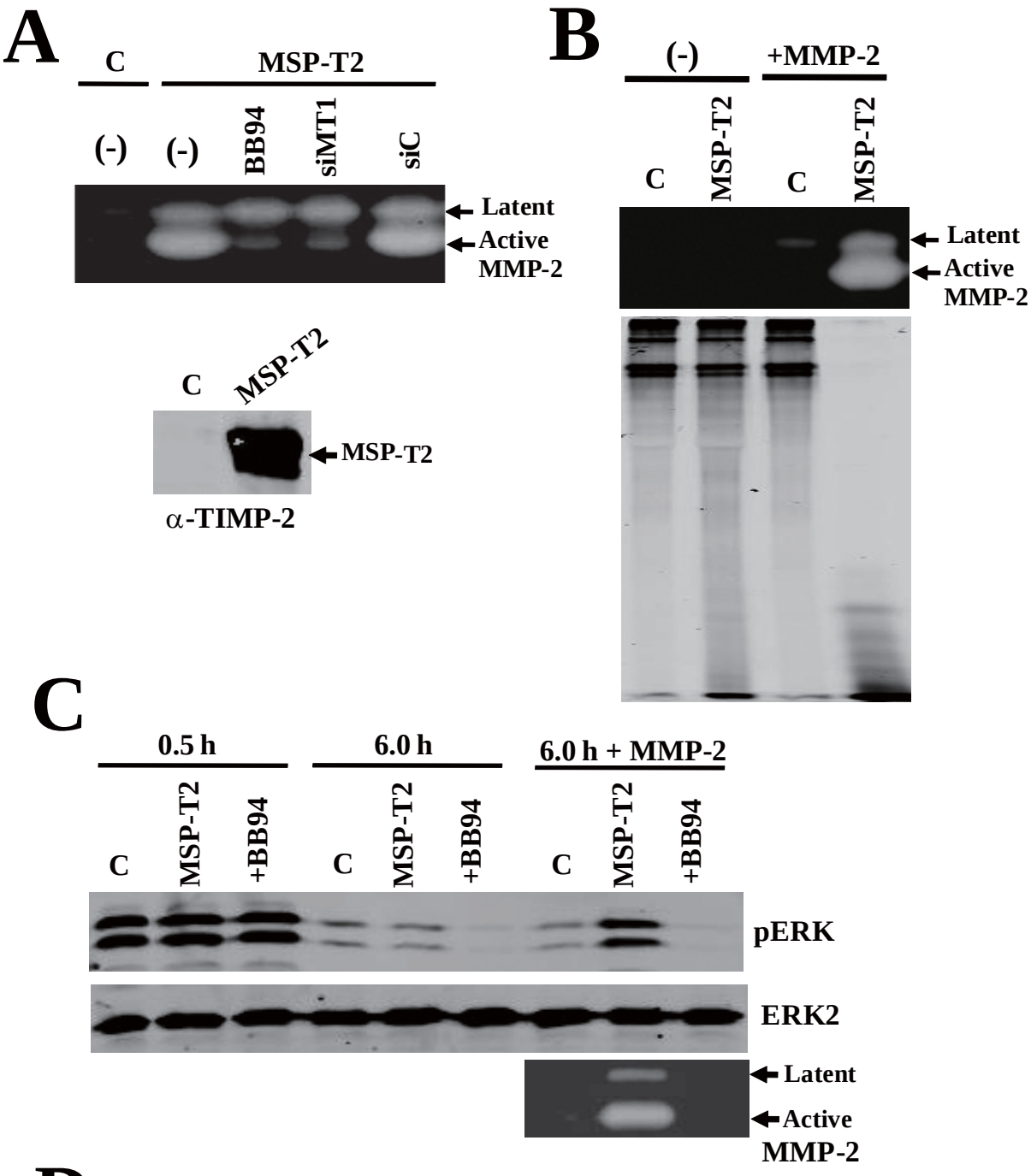

A
Control
MSP-mouseT2
(-)
siC
siMT1
siTIMP-2
(-)
siC
siMT1
siTIMP-2
MMP-2
Sup.
Latent
Active
Cell
Latent
Active
MT1-MMP
α-MT1-MMP
TIMP-2
α-TIMP-2
C
MSP-T2
MSP-T2
α-TIMP-2
B
Sup.
Gel
C
MSP-T2
C
MSP-T2
Latent
Active
MMP-2
Control
MSP-T2

Fig.5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C
MSP-T2
(-)
(-)
BB94
siMT1
siC
Latent
Active MMP-2
C
MSP-T2
MSP-T2
α-TIMP-2
B
(-)
+MMP-2
C
MSP-T2
C
MSP-T2
Latent
Active MMP-2
C
0.5 h
6.0 h
6.0 h + MMP-2
C
MSP-T2
+BB94
C
MSP-T2
+BB94
C
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+BB94
pERK
ERK2
Latent
Active MMP-2

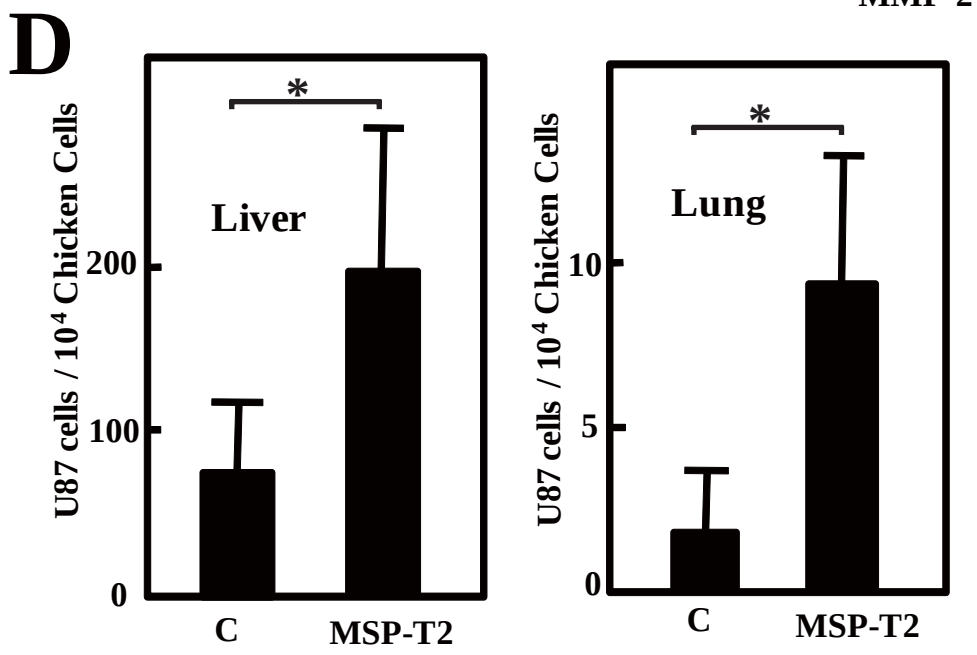
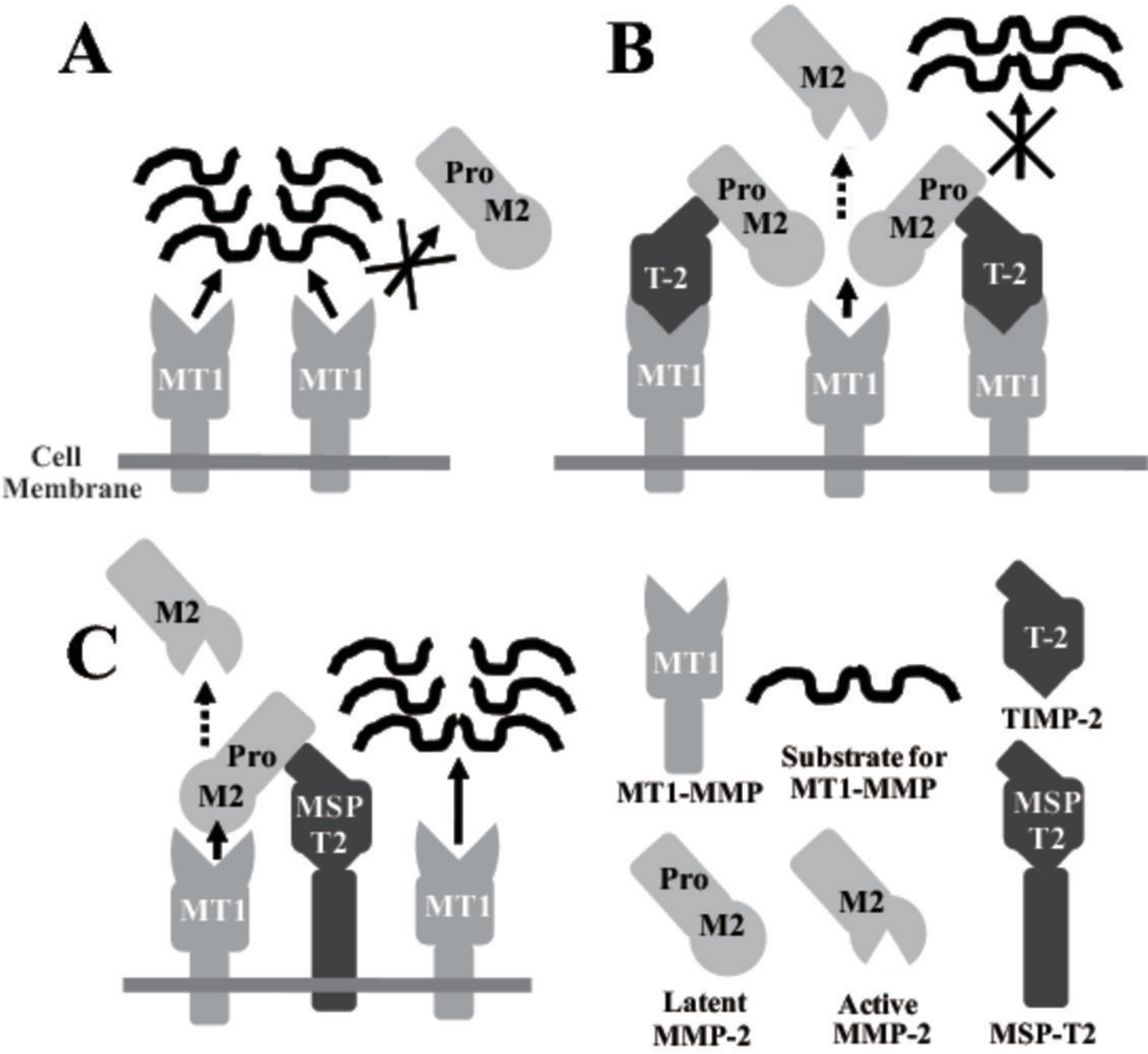

D
Liver
U87 cells / 10⁴ Chicken Cells
200
100
0
*
C
MSP-T2
Lung
U87 cells / 10⁴ Chicken Cells
10
5
0
*
C
MSP-T2

Fig. 6


A
Pro
M2
MT1
MT1
Cell
Membrane
B
M2
Pro
M2
Pro
M2
T-2
T-2
MT1
MT1
MT1
C
M2
Pro
M2
MSP
T2
MT1
MT1
MT1
MT1-MMP
Substrate for
MT1-MMP
T-2
TIMP-2
MSP
T2
Pro
M2
M2
Latent
MMP-2
Active
MMP-2
MSP-T2