Activation of Matrix Metalloproteinase (MMP)-9 by Membrane-Type-1 Matrix Metalloproteinase/MMP-2 axis Stimulates Tumor Metastasis

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An artificial receptor for pro-matrix metalloproteinase-9 (proMMP-9) was created by fusing tissue inhibitor of MMP-1 (TIMP-1) with type II transmembrane mosaic serine protease (MSP-T1). Expression of MSP-T1 in 293T cells induced binding of proMMP-9, which was processed by MMP-2 activated by Membrane-Type-1 MMP (MT1-MMP). HT1080 cells transfected with MSP-T1 gene produced activated MMP-9 in collagen gel, and addition of proMMP-2 to the culture augmented it, which resulted in intensive collagen digestion. These cells metastasized into chick embryonic liver more than control cells. Treatment of HT1080 cells with concanavalin A in the presence of exogenous proMMP-2 induced activation of not only proMMP-2 but also proMMP-9. Knock-down of MT1-MMP or TIMP-2 expression with small interfering (si) RNA suppressed activation of both proMMP-2 and proMMP-9. Transfection of TIMP-1 siRNA suppressed cell-binding and activation of proMMP-9, but not proMMP-2 activation. Knock-down of a disintegrin and metalloproteinase 10 (ADAM10) expression reduced cell-binding and processing of proMMP-9. These results suggest that proMMP-9 which binds to a receptor complex containing TIMP-1 and ADAM10 is activated by MT1-MMP/MMP-2 axis, and MMP-9 thus activated stimulates cellular proteolysis and metastasis.

Matrix metalloproteinase (MMP)-2 and MMP-9 are type IV collagenase/gelatinase, and their expression is often associated with tumor aggressiveness and a poor prognosis (reviewed in 1,2). Membrane-type-1 MMP (MT1-MMP) was first identified as an activator of latent MMP-2 (proMMP-2) (3). In addition to proMMP-2, a variety of substrates of MT1-MMP were identified, including extracellular matrix (ECM) proteins, cell adhesion molecules, cytokines, and others (4-7). ProMMP-2 is activated in a variety of tumor tissues, in which MT1-MMP is overexpressed. In contrast, proMMP-9 activation was detected in limited numbers of tumor samples, such as non-small cell lung carcinoma and colon carcinomas (8-10), and the active form of MMP-9 was predominantly present in colon carcinoma patients with metastases (8). ProMMP-9 can be processed *in vitro* by various proteases including MMP-2, MMP-3 and serine proteases, however, the molecular mechanism of proMMP-9 activation *in vivo* still remains to be solved (1).

In proMMP-2 activation mechanism, MT1-MMP/TIMP-2 complex serves as a receptor for proMMP-2, which is then processed by adjacent TIMP-2-free MT1-MMP (11-14). To mimic this model, TIMP-2 was fused with type II transmembrane mosaic serine protease (MSP) to create MSP-T2 as an artificial receptor for proMMP-2 (15). MSP-T2 served as a receptor for proMMP-2, and accelerated proMMP-2 activation by MT1-MMP (15). ProMMP-9 is known to bind to TIMP-1 in a similar manner as proMMP-2 binds to TIMP-2, which suggested model implicates TIMP-1 as a bridging molecule to tether proMMP-9 on cell surface for its processing (1,2).

In the present study, a TIMP-1 chimera protein with MSP, MSP-T1 was constructed, which served as an artificial receptor for proMMP-9 and induced proMMP-9 activation by MT1-MMP/MMP-2 axis. Stable expression of MSP-T1 in HT1080 fibrosarcoma cells stimulated proMMP-9 activation and metastasis. ProMMP-9 processing through TIMP-1-containing receptor and MT1-MMP/MMP-2 axis was demonstrated in concanavalin A (ConA)-treated HT1080 cells.

Materials and Methods

Cell culture. Human embryonic kidney 293T and fibrosarcoma HT1080 cells were obtained from American Type Culture Collection and cultured in DMEM (Sigma, St. Louis, MO, USA) supplemented with 5% fetal calf serum (FCS). HT1080 cells stably expressing MSP-T1 were generated by transfecting MSP-T1 plasmid, and transfected cells were selected under 5 μg/mL puromycin (Sigma). Type I collagen Cellmatrix Type I(P) was purchased from Nitta Gelatin (Osaka, Japan). Recombinant TIMP-1 and TIMP-2 proteins were gifts from the Daiichi Fine Chemical Co. Ltd (Takaoka,

Japan). A synthetic MMP inhibitor BB94 (batimastat) was a kind gift from the Kotobuki Pharmaceutical Co. Ltd (Nagano, Japan).

Antibodies. Monoclonal antibodies against MMP-9 (56-2A4), MT1-MMP (222-2D12), TIMP-1 (7-6C1) and TIMP-2 (67-4H11) were gifts from the Daiichi Fine Chemical Co. Ltd. An antibody against A disintegrin and metalloproteinase 10 (ADAM10) was purchased from R&D Systems (Mineapolis, MN, USA).

Plasmids. Expression plasmids for MT1-MMP, MSP-T1, MMP-2 and MMP-9 were constructed in pEAK8 vector (Edge BioSystems, Gaithersburg, MD, USA) as described previously [16-19]. The fusion gene for MSP-T1, which encodes amino acid residues 1 to 188 of MSP and 24 to 207 of TIMP-1, was constructed by replacing the fragment encoding TIMP-2 of MSP-T2 with that of TIMP-1 [25]. The human-TIMP-1 cDNA fragment encoding amino acid residues 24 to 207 was amplified by PCR using 5' and 3' primers with extra EcoRI and XbaI sites, respectively (GAATTCTGCACC TGTGTCCCA CCCCAC and TCTGATCAGGCTA TCT GGGACCGCAG, restriction enzyme site). The cDNA encoding mutant MMP-9 lacking C-terminal hinge and hemopexin-like domains (ΔMMP-9) was amplified by PCR using a primer TCTAGATCA ACCATAGAGGTGCC GATGCC (restriction site). The cDNA encoding MMP-9 mutants with amino acid substitution at Met^{41} and Phe^{88} with Val was amplified by PCR using mutation primers CTCGGGTGGCAGAGGTGCGTGGAGAGTCGA and CAGA CCTGGGCAGAGTCC AAACCTTTGAGG (mutated nucleotide), respectively.

RNA interference. RNA interference technology was used to generate specific knockdown of MT1-MMP, TIMP-1, TIMP-2, ADAM10 and MMP-9 mRNA transcription. Small interfering RNA (siRNA) was prepared by FASMAK Co., Ltd. (Kanagawa, Japan). The siRNA target sequences were as follows: MT1-MMP-I, CCAGAAGCTGAA GGTAGAA; MT1-MMP-II, GCGATGAAGTCTTCATTA; TIMP-1-I, TCAACCAG ACCACCTTATA; TIMP-1-II, GATGTATAAAGGGTTCCAA; TIMP-2-I, GGATCCA GTATGAGATCAA; TIMP-2-II, GAGATCAAGCAGA-TAAAGA; ADAM10-I, GATA TCCAGTCATGTTAAA; ADAM10-II, CTGGAATTATTACTGTTCA; MMP-9-I, CAC AACAUCACCUAUUGGA; MMP-9-II, GGAGUA-CUCGACCUGUACC. Scrambled oligo was used as a negative control. siRNA reverse transfection was done using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

Zymography. ProMMP-2, proMMP-9 and proMMP-9 mutants supernatants were prepared from 293T cells transfected with respective expression plasmids, as previously described [20, 21]. 293T cells cultured in a 24-well microplate were transfected with expression plasmids, cultured in DMEM supplemented with 5 % FCS for 24 h, and were incubated in 300 μL Opti-MEM (Invitrogen) containing proMMP-2 and proMMP-9. MMP-2 and MMP-9 in the supernatant were examined by mixing with a same volume of zymography sample buffer. For the detection of cell-bound MMP-2 and MMP-9, cells were washed twice with PBS, and then dissolved in 200 μL sample buffer by sonication. These samples were incubated at 37°C for 20 min, and subjected to gelatin zymography gel containing gelatin labeled with Alexa Fluor 680 (Molecular Probes Inc., Eugene, OR, USA). Gels were processed and monitored by a LI-COR Odyssey™ IR imaging system (Lincoln, NE, USA) as described previously [15].

Western blotting. Cell lysates or proteins precipitated from conditioned medium with 10% trichloroacetic acid (TCA) were analyzed by Western blotting with the indicated antibody. Goat anti-mouse antibody conjugated with Alexa Fluor 680 was used as a second antibody. The signal was monitored using a LI-COR Odyssey™ IR imaging system. Precision Plus Protein Standards were used as molecular weight standards (Bio-Rad, Hercules, CA, USA).

Chick embryo assay. The assay was done as originally described by Endo and colleagues [17]. Briefly, HT1080 cells stably transfected with pEAK control plasmid or MSP-T1 expression plasmid (1.0×10^5 cells per egg) were injected into the chorioallantoic membrane vein of the chicken eggs 11 days after fertilization, and incubated for a further 7 days. Ten eggs were used for the injection of each cells. The number of HT1080 cells that colonized liver was estimated by quantitative real-time PCR as described previously [15, 17].

Gelatin degradation. Alexa Fluor 680–labeled gelatin (150 ng/ 200 μL Opti-MEM) was incubated with HT1080 cells in a 24-well microplate for 1 h, separated on 10% SDS-PAGE and detected by a LI-COR Odyssey™ IR imaging system. [15]

Results

MSP-T1 functions as a receptor for proMMP-9. 293T cells transfected with MSP-T1 and/or MT1-MMP plasmids were incubated with proMMP-2 and proMMP-9, and cell-bound MMP-2 and MMP-9 were examined by gelatin zymography. ProMMP-9 bound to the cells expressing MSP-T1 more effectively than to control cells (Fig. 1A, Cells). Expression of MT1-MMP alone induced activation of proMMP-2, but not proMMP-9. Co-expression of MSP-T1 with MT1-MMP induced activation of both proMMP-2 and proMMP-9. To confirm that proMMP-9 activation is mediated by transfected cells, transfected cells were incubated with proMMP-2, and then supernatants from transfected cells were incubated with proMMP-9 (Fig. 1A, Sup). ProMMP-9 was not processed in the supernatant from cells expressing MSP-T1, MT1-MMP and MMP-2. Next, to examine the requirement of MMP-2 in proMMP-9 activation through MSP-T1/MT1-MMP, cells co-expressing MSP-T1 and MT1-MMP were incubated with proMMP-9 and 2-fold serially diluted proMMP-2 (Fig. 1B). ProMMP-9 activation was shown to depend on MMP-2 concentration added to the culture. Time course experiment demonstrated that proMMP-9 activation by the cells expressing MSP-T1/MT1-MMP/MMP-2 was induced at 0.2 h and reached a plateau at 1 to 2 h after incubation (Fig. 1C). ProMMP-9 binds to TIMP-1 through the carboxy-terminal hemopexin-like domain. ProMMP-9 mutant lacking hemopexin-like domain (ΔMMP-9) was compared with wild-type proMMP-9 for the binding and processing by the cells expressing MSP-T1 and MT1-MMP in the presence of proMMP-2 (Fig. 1D). Wild-type proMMP-9 bound to the cells expressing MSP-T1 and activated by the cells expressing MSP-T1 and MT1-MMP in the presence of proMMP-2. However, ΔproMMP-9 failed to bind to MSP-T1-expressing cells and was not activated by the cells expressing MSP-T1 and MT1-MMP. ProMMP-9 was shown to be cleaved *in vitro* by activated MMP-2 at the Glu^{40}-Met^{41} amide bond to generate activation intermediate form, which is converted to fully active form through the auto-cleavage at the Arg^{87}-Phe^{88} amide bond [22]. To confirm

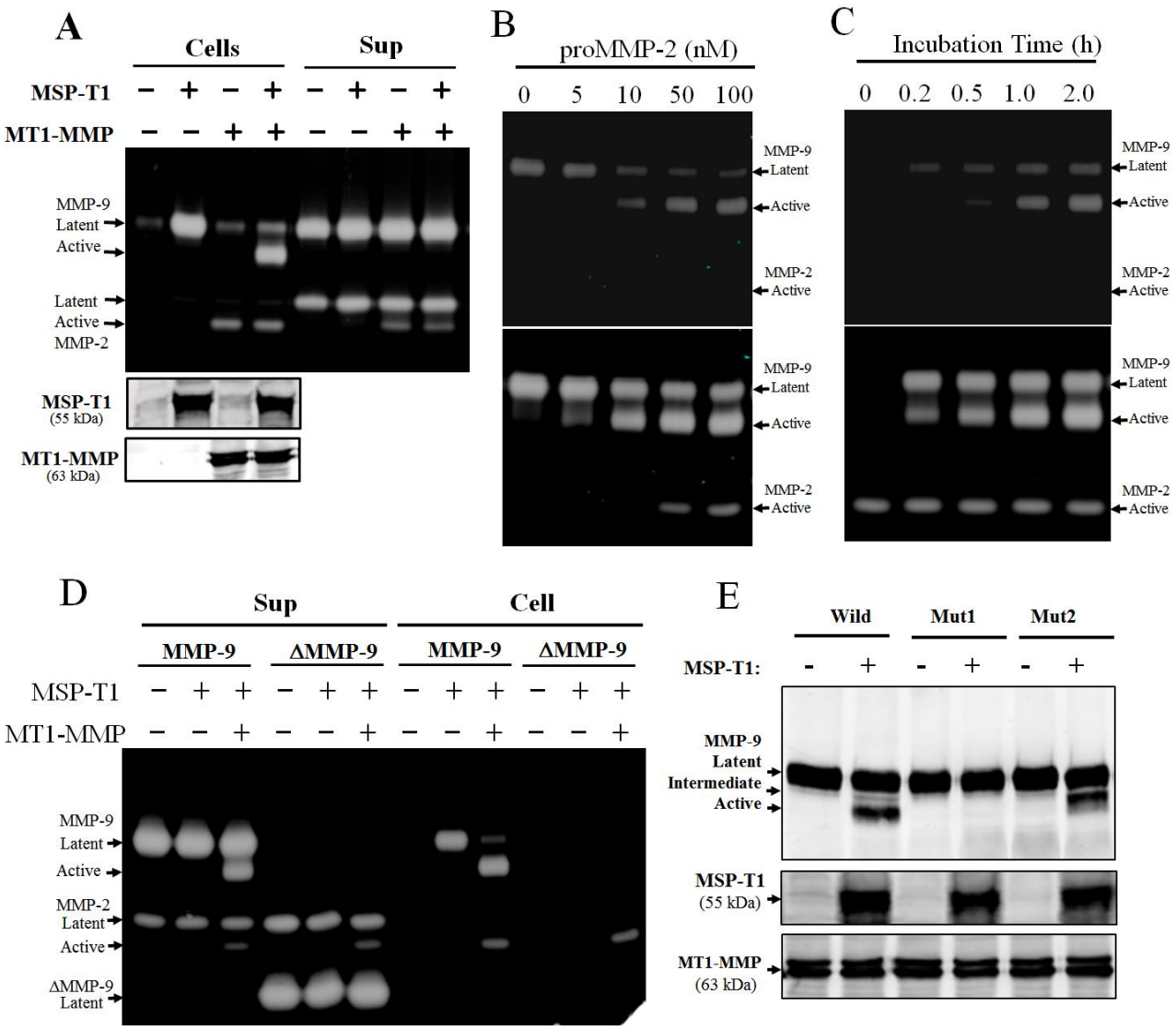

Fig. 1. ProMMP-9 activation by cells expressing MSP-T1, MT1-MMP and MMP-2. (A) Control plasmid or expression plasmid for MT1-MMP (200 ng) and/or MSP-T1 (300 ng) was transfected into 293T cell in a 24-well microplate. Twenty-four hours after transfection, cells were incubated in Opti-MEM containing proMMP-2 (100 nM) and proMMP-9 (20 nM) for 2 h. Cell-bound MMP-2 and MMP-9 were analyzed by gelatin zymography (Cells). Each cells were incubated with proMMP-2 for 2 h, and then the supernatant was incubated with proMMP-9 (20 nM) for further 2 h. ProMMP-9 processing was monitored by zymography (Sup). Expression of MSP-T1 and MT1-MMP was detected by Western blotting using anti-TIMP-1 and anti-MT1-MMP antibodies, respectively (lower panels). (B) 293T cells transfected with expression plasmids for MSP-T1 and MT1-MMP as described above were incubated with proMMP-9 (20 nM) and serially diluted proMMP-2 for 2 h, and then cell-bound MMP-2 and MMP-9 were subjected to gelatin zymography. Gels were processed and monitored by an IR imaging system with low (top) and high sensitivity (bottom). (C) 293T cells transfected as above were incubated with proMMP-2 (100 nM) for 1 h, and then proMMP-9 (20 nM) was added and incubated for the indicated period. Cell-bound MMP-2 and MMP-9 were detected as above. (D) 293T cells transfected with control, MSP-T1 and/or MT1-MMP plasmid as described above were incubated with proMMP-2 and wild-type proMMP-9 or pro MMP-9 for 2 h, and cell-bound (Cell) or supernatant MMP-2 and MMP-9 (Sup) were detected by gelatin zymography. (E) MT1-MMP plasmid (1 μg) was co-transfected with control or MSP-T1 plasmid (1 μg) into 293T cells cultured in 35 mm dish. Twenty-four h after incubation, 50 nM wild-type or mutant proMMP-9 with an amino acid substitution at Met^{41} (Mut 1) or Phe^{88} (Mut 2) was incubated with cells in the presence of proMMP-2 (50 nM) for 3 h. Supernatant was concentrated with TCA, and subjected to Western blotting using an antibody against MMP-9. Expression of MSP-T1 and MT1-MMP was detected by Western blotting as described above (lower panels).

proMMP-9 cleavage sites by the cells expressing MSP-T1 and MT1-MMP in the presence of proMMP-2, wild-type or mutant proMMP-9 with the amino acid substitution at Met^{41} (Mut1) or Phe^{88} (Mut2) was incubated with the cells expressing MSP-T1 and MT1-MMP in the presence of proMMP-2 (Fig. 1E). Wild-type proMMP-9 was converted to fully active form by these cells, but proMMP-9 mutant with amino acid substitution at Met^{41} (Mut1) was not processed. ProMMP-9 mutant with an amino acid substitution at Phe^{88} (Mut2) was cleaved to generate activation intermediate form. These results indicate that proMMP-9 is processed by the cells expressing MSP-T1, MT1-MMP in the presence of proMMP-2 through the cleavage at the same sites as *in vitro* activation by active MMP-2. The activation efficiency by these cells was dramatically improved compared with activation by MMP-2 *in vitro* [22].

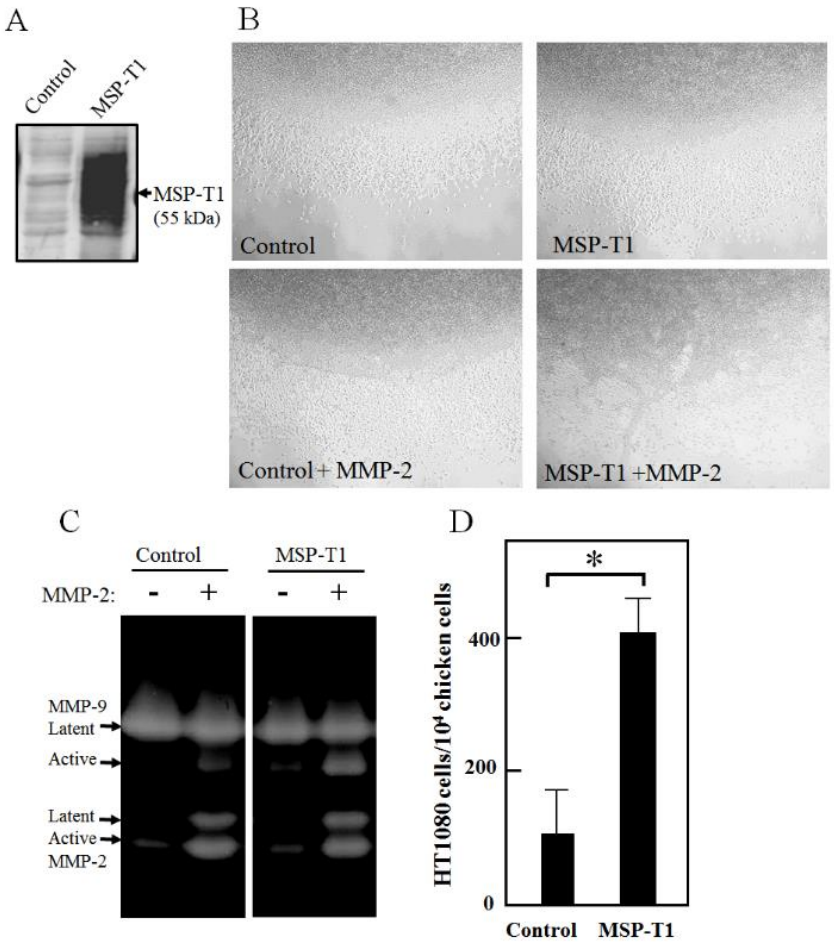

Fig. 2. Stable transfection of MSP-T1 into HT1080 cells. (A) HT1080 cells stably transfected with control or MSP-T1 plasmid were subjected to Western blotting using an antibody against TIMP-1. (B) HT1080 cells transfected with control or MSP-T1 plasmid (2 x 10^5 cells) were suspended in 100 μl collagen mixture. A 20 μl drop of collagen mixture containing cells was polymerized on the bottom of a 24-well microplate, cultured in DMEM supplemented with 5% FCS for a day, and then in Opti-MEM with or without 50 nM proMMP-2 for 2 days. Cells in collagen culture were photographed under a phase contrast microscope. (C) Cells cultured in collagen gel as described above were dissolved in a sample buffer, and subjected to gelatin zymography. (D) HT1080 cells transfected with control or MSP-T1 plasmid were injected into the chorioallantoic membrane vein of the chicken eggs, and cells that metastasized into liver were analyzed as described in Materials and Methods. *, P < 0.05.

Expression of MSP-T1 in HT1080 cells. HT1080 cells were stably transfected with MSP-T1 plasmid, and cultured in collagen gel in the presence or absence of exogenously added proMMP-2 to examine proMMP-9 activation and degradation of collagen gel (Fig. 2). HT1080 cells migrated out of collagen gel, but collagen degradation was not obvious in the culture of both cells without exogenous proMMP-2. In the presence of exogenously added proMMP-2, collagen gel was degraded intensively by the cells expressing MSP-T1, and moderately by control cells (Fig. 2B). Gelatin zymography analysis showed that endogenous proMMP-2 was activated equally by the cells transfected with control or MSP-T1 plasmid, but proMMP-9 processing was detected only in the cells expressing MSP-T1 in the absence of exogenously added proMMP-2. Addition of proMMP-2 to the culture produced a large amount of active MMP-2, which resulted in a significant proMMP-9 processing in control cells and more dramatic effect in MSP-T1-expressing cells (Fig. 2C). Metastatic ability of HT1080 cells expressing MSP-T1 was analyzed by chick embryo system. MSP-T1-expressing HT1080 cells metastasized into liver more efficiently by 4-fold than control cells (Fig. 2D).

Activation of proMMP-9 by HT1080 cells. HT1080 cells cultured in collagen gel in the presence of exogenous proMMP-2 activated proMMP-2 and slightly proMMP-9. To induce more efficient proMMP-2 activation in HT1080 cells, cells were treated with 2-fold serially diluted ConA in the presence of exogenous proMMP-2 (Fig.3A). ProMMP-2 activation was induced even at low ConA concentration. ConA treatment induced cell-binding and processing of proMMP-9 in HT1080 cells, but they required higher ConA concentration than proMMP-2 activation.

As shown in Fig. 1, activation of proMMP-9 by the cells expressing MSP-T1 and MT1-MMP required a considerable concentration of proMMP-2. Thus, requirement of MMP-2 in proMMP-9 activation by ConA-treated HT1080 cells was examined (Fig.3B). Addition of exogenous proMMP-2 to ConA-treated HT1080 cells induced activation of proMMP-2 and proMMP-9 depending on proMMP-2 concentration. Time course experiment demonstrated that proMMP-9 was processed to activation intermediate form in 1 h and to fully active form in 2 to 3 h (Fig. 3C).

The effect of MMP inhibitors on proMMP-9 activation by ConA-treated HT1080 cells was examined (Fig. 3D). Addition of TIMP-2 or BB94 interfered with activation of both proMMP-2 and proMMP-9 by ConA-treated HT1080 cells. Cells treated with TIMP-1 processed proMMP-2 to activation intermediate form, and failed to activate proMMP-9. These results suggest that proMMP-2 activation by MT1-MMP is associated with proMMP-9 processing in ConA-treated HT1080 cells.

Analysis of proMMP-9 activation mechanism by siRNA. To confirm the association of MT1-MMP with proMMP-9 processing, MT1-MMP siRNA was transfected into HT1080 cells, and proMMP-9 activation was examined (Fig. 4). Knock-down of MT1-MMP expression suppressed activation of not only proMMP-2 but also proMMP-9 (left panel). MSP-TIMP-1 was shown to act as a receptor for proMMP-9, which facilitated proMMP-9 activation by MT1-MMP/MMP-2 axis. This suggest a possibility that TIMP-1

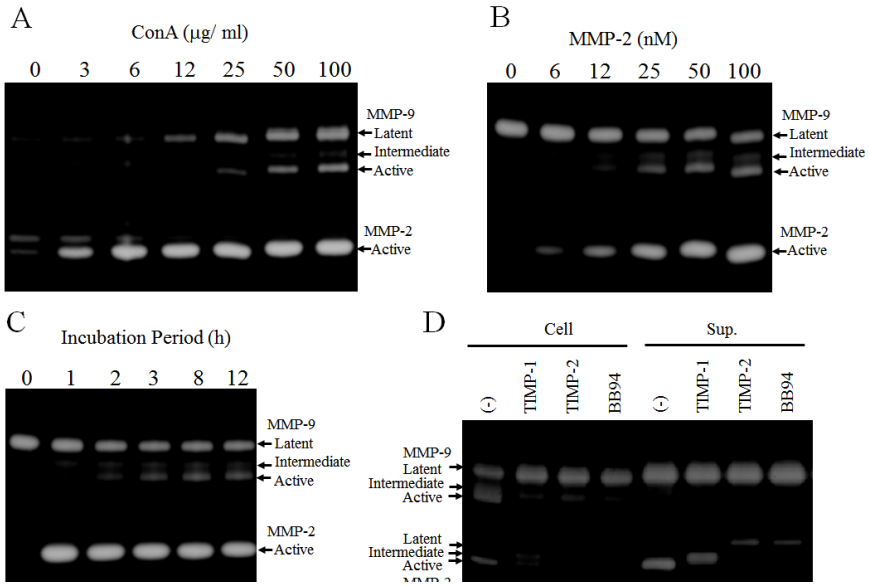

Fig. 3. Induction of proMMP-2 and proMMP-9 activation in HT1080 cells. (A) HT1080 cells were cultured in DMEM supplemented with 5% FCS for 24 h, and then in Opti-MEM containing proMMP-2 (50 nM) and a serially diluted ConA for 12 h. Cell-bound MMP-2 and MMP-9 were analyzed by gelatin zymography. (B) HT1080 cells cultured as above were incubated in Opti-MEM containing ConA (50 μg/ml) and a serially diluted proMMP2 for 12 h, and cell-bound MMP-2 and MMP-9 were analyzed by gelatin zymography. (C) HT1080 cells cultured as above were incubated in Opti-MEM containing ConA (50 μg/ml) and proMMP-2 (50 nM) for the indicated period, and cell-bound MMP-2 and MMP-9 were analyzed by gelatin zymography. (D) HT1080 cells cultured as above were incubated in Opti-MEM containing ConA (50 ng/ml) and proMMP-2 (50 nM) with mock (-), TIMP-1 (10μg/ml), TIMP-2 (10 μg/ml) or BB94 (1 μM) for 12 h. Cell-bound and supernatant MMP-2 and MMP-9 were analyzed by gelatin zymography.

acts as a part of proMMP-9 receptor and induces its processing in ConA-treated HT1080 cells. To test it, TIMP-1 expression was down-regulated by the transfection of siRNA, and proMMP-9 activation was examined. Knock-down of TIMP-1 did not alter proMMP-2 activation, but suppressed binding of proMMP-9 to the cells and subsequent activation in ConA-treated HT1080 cells (middle panel). Knock-down of TIMP-2 did not alter proMMP-9 binding but interfered with activation of both proMMP-2 and proMMP-9. These results suggest that TIMP-1 serves as a part of proMMP-9 receptor, which induces subsequent proMMP-9 processing. ADAM10, which is expressed at a considerable level in HT1080 cells, is known to be inactivated by TIMP-1 [23]. Transfection of ADAM10 siRNA did not affect proMMP-2 activation, but suppressed cell-binding and processing of proMMP-9 (right panel).

Contribution of MMP-9 to gelatin degradation by HT1080 cells. In order to examine the contribution of MMP-2 and MMP-9 to ECM degradation by HT1080 cells, MMP-9 expression was knocked-down through siRNA transfection, and gelatin degradation by these cells was monitored (Fig. 5). Gelatin was not significantly degraded by ConA-treated HT1080 cells in the absence of exogenous proMMP-2. Addition of proMMP-2 to ConA-treated cells generated active MMP-2 and MMP-9, which resulted in

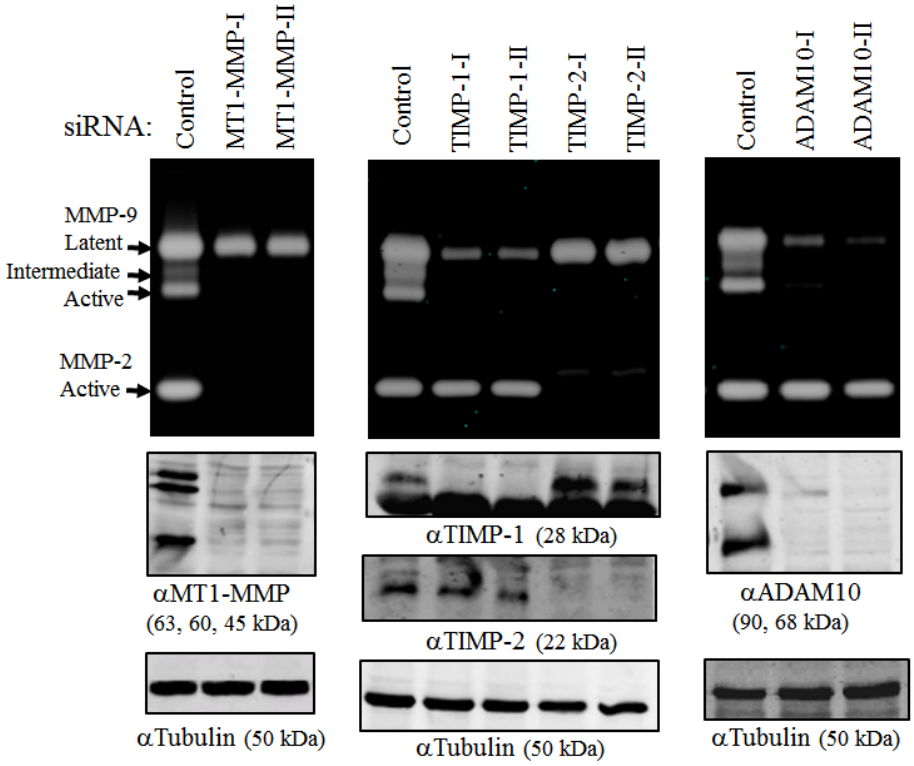

Fig. 4. Suppression of proMMP-9 processing by siRNA. HT1080 cells transfected with control RNA or siRNA for MT1-MMP (MT1-MMP-I, MT1-MMP-II), TIMP-1 (TIMP-1-I, TIMP-1-II), TIMP-2 (TIMP-2-I, TIMP-2-II) or ADAM10 (ADAM10-I, ADAM10-II) were cultured in Opti-MEM containing ConA (50 ng/ml) and proMMP-2 (50 nM) for 12 h. Cell-bound MMP-2 and MMP-9 were analyzed by gelatin zymography. The expression of each protein was analyzed by Western blotting.

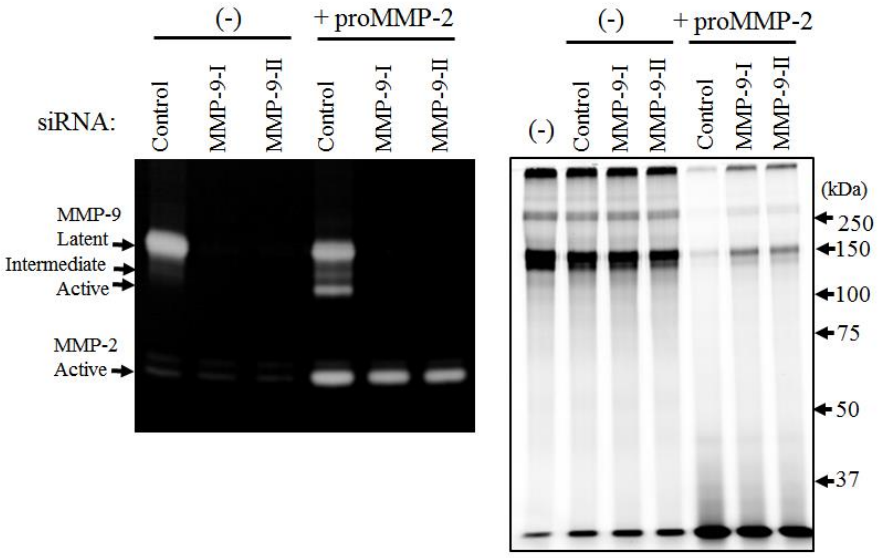

Fig. 5. Gelatin degradation by MMP-2 and MMP-9 in HT1080 cells. HT1080 cells transfected with control RNA or MMP-9 siRNA (MMP-9-I, MMP-9-II) were cultured in Opti-MEM containing ConA (50 ng/ml) with or without proMMP-2 (50 nM) for 12 h. MMP-2 and MMP-9 bound to cells were analyzed by gelatin zymography (left panel). These cells were incubated with fluorescence-labeled gelatin (-) for 1 h, and gelatin degradation was monitored as described in Materials and Methods (right panel).

intensive gelatin degradation. Knock-down of MMP-9 expression in these cells considerably reduced gelatin degradation by ConA-treated cells. These results indicate that not only MMP-2 but also MMP-9 activated by HT1080 cells contributes to gelatin degradation.

Discussion

Activation of proMMP-2 by MT1-MMP is a feature of the malignant phenotype of various tumors [4-7]. Tumor specific proMMP-9 activation is restricted to a limited numbers of tissues, and the molecular mechanism of proMMP-9 activation *in vivo* still remains unsolved [8-10]. ProMMP-2 and proMMP-9 binds to TIMP-2 and TIMP-1 through the carboxy-terminal domain of each molecule, respectively [1, 2]. Formation of tri-molecular complex consisting of proMMP-2/TIMP-2/MT1-MMP is the initial step for proMMP-2 activation by MT1-MMP [11-13]. Previously, MSP-T2 was constructed as an artificial receptor for proMMP-2, which accelerated proMMP-2 activation by MT1-MMP [15]. Assuming the similar scenario in proMMP-9 activation process, in which binding of proMMP-9 to the receptor containing TIMP-1 is the initial step for activation, MSP-T1 was created as an artificial receptor for proMMP-9. Indeed, MSP-T1 served as a receptor for proMMP-9, which was effectively processed by MT1-MMP/MMP-2 axis (Fig. 1). Membrane-bound serine proteases, such as MSP and matriptase were negative as a cell-surface proMMP-9 activator, when co-expressed with MSP-T1 (data not shown). Toth *et al.* reported activation of proMMP-9 by membrane fraction from cells expressing MMP-2 and MT1-MMP, however, incubation of proMMP-9 with these cells failed to activate proMMP-9, which was consistent with the present study [24]. Co-expression of MSP-T1 with MT1-MMP induced dramatic proMMP-9 activation in the presence of proMMP-2. ProMMP-9 is activated by these cells through the cleavage at the site identical with those by active MMP-2 *in vitro* [22]. These results suggest proMMP-9 activation process, in which proMMP-9 is anchored to cell surface through TIMP-1-containing receptor, and subsequently processed by MMP-2 activated and anchored on cell surface by MT1-MMP. HT1080 cells in collagen gel did not show detectable proMMP-9 processing, but addition of proMMP-2 induced significant proMMP-9 processing. ConA-treatment of HT1080 cells caused more efficient proMMP-2 activation than collagen culture, and induced cell-binding and processing of proMMP-9 in the presence of exogenously added proMMP-2 (Fig. 3). Knockdown of MT1-MMP or TIMP-2 expression, which is essential for proMMP-2 activation, suppressed processing of not only proMMP-2 but also proMMP-9. Knockdown of TIMP-1 expression down-regulated cell-binding and processing of proMMP-9, but did not affect proMMP-2 activation. These results demonstrate that TIMP-1 is involved in cell-binding of proMMP-9, which is subsequently processed by MMP-2 activated by MT1-MMP. ADAM10 is known to be inactivated by TIMP-1, suggesting the possibility that ADAM10 acts as a cell-surface binding molecule of TIMP-1 [23]. Knockdown of ADAM10 expression by siRNA reduced both binding and processing of proMMP-9. These results may suggest that ADAM10 is one of receptors for TIMP-1 and ADAM10/TIMP-1 complex acts as a receptor for proMMP-9, which is subsequently processed by MT1-MMP/MMP-2 axis. However, reconstitution of proMMP-9 receptor by co-expressing ADAM10 and TIMP-1 was not successful so far (data not shown). Actually, ADAM10 and TIMP-1 protein expression was not significantly affected by ConA-treatment of HT1080 cells (data not shown). The cell surface event induced by ConA-treatment seems to be complicated, and the mechanism by which ADAM10 participates in proMMP-9 processing still remains unsolved. Several TIMP-1 biding molecules such as CD44, CD63 and integrins were reported, which may function as proMMP-9 receptors by forming complexes with TIMP-1 [23]. Association of CD44 and MT1-MMP with proMMP-9 activation in osteoclasts and formation of proMMP-9/CD44/TIMP-1 complex were reported, although the molecular mechanism and physiological significance of them in relation to proMMP-9 processing still remain to be solved [25, 26].

Expression of MSP-T1 in HT1080 cells stimulated proMMP-9 activation by MT1-MMP/MMP-2 axis, which in turn enhanced metastatic ability *in vivo* and collagen degradation *in vitro*. These results demonstrated for the first time that activation of proMMP-9 stimulates tumor metastasis. Addition of proMMP-2 to ConA-treated HT1080 cells generated active MMP-2, which consequently processed endog-

enous proMMP-9. MMP-2 and MMP-9 cleaves various substrates, such as ECM molecules, membrane proteins, cytokines, growth factors and so on, that regulate key signaling pathways in cell growth, migration, invasion and angiogenesis [1, 2]. Membrane-bound MMP-9 thus activated by MT1-MMP/MMP-2 axis may have advantage over pericellular proteolysis. Participation of MMP-9 to coordinate action of MT1-MMP and MMP-2 farther facilitates pericellular proteolysis for tumor invasion and metastasis. ProMMP-2 is often abundantly expressed by stromal cells located adjacent to malignant epithelial cells, and functions as a mediator of tumor-stromal interaction by being activated by tumor-specific MT1-MMP, which may subsequently activate proMMP-9. In conclusion, proMMP-9 which is anchored though a receptor complex containing TIMP-1 is activated by MT1-MMP/MMP-2 axis, and MMP-9 thus activated contributes to pericellular proteolysis for tumor invasion and metastasis in collaboration with MT1-MMP and MMP-2.

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Disclosure Statement

The authors have no conflict of interest.

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