

Membrane Type 1-Matrix Metalloproteinase Cleaves Off the NH₂-Terminal Portion of Heparin-Binding Epidermal Growth Factor and Converts It into a Heparin-Independent Growth Factor

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Abstract

Epidermal growth factor (EGF) receptors (ErbB) and EGF family members represent promising targets for cancer therapy. Heparin-binding EGF (HB-EGF) is a member of the EGF family and is an important target for therapy in some types of human cancers. Processing of HB-EGF by proprotein convertases, and successively, by ADAM family proteases, generates a soluble growth factor that requires heparin as a cofactor. Although heparin potentiates HB-EGF activity *in vitro*, it is not clear how the heparin-binding activity of HB-EGF is regulated. Here, we show that membrane type 1-matrix metalloproteinase (MT1-MMP; MMP14), a potent invasion-promoting protease, markedly enhances HB-EGF-dependent tumor formation in mice. MT1-MMP additionally cleaves HB-EGF and removes the NH₂-terminal 20 amino acids that are important for binding heparin. Consequently, the processing of HB-EGF by MT1-MMP converts HB-EGF into a heparin-independent growth factor with enhanced mitogenic activity, and thereby, expression of both proteins costimulates tumor cell growth *in vitro* and *in vivo*. The ErbB family of receptors expressed in human gastric carcinoma cells play a role in mediating enhanced HB-EGF activity by MT1-MMP during invasive cell growth in collagen. Thus, we shed light on a new mechanism whereby HB-EGF activity is regulated that should be considered when designing HB-EGF-targeted cancer therapy. *Cancer Res*; 70(14); 6093–103. ©2010 AACR.

Introduction

Heparin-binding epidermal growth factor (HB-EGF), a member of the EGF family, transduces extracellular signals via ErbB receptors and plays a pivotal role in many physiologic and pathologic processes (1–3). Most HB-EGF-deficient mice do not survive beyond the neonatal stage and exhibit severe defects in multiple tissues and organs (4). HB-EGF is also expressed in a variety of human carcinomas such as pancreatic, esophageal, colon, gastric, ovarian, and bladder cancers (5), and targeting it therapeutically is under clinical evaluation (6). HB-EGF is synthesized as a proform of membrane-bound HB-EGF (pro-mHB-EGF), which contains a propeptide sequence that is eliminated by proprotein con-

vertases such as furin (7), as indicated in Fig. 1A. The ectodomain of mHB-EGF comprises an NH₂-terminal heparin-binding domain containing a core stretch of basic amino acids, an EGF-like domain, and a juxtamembrane domain (1). The ectodomain of mHB-EGF is shed by the action of proteases that belong to the ADAM (a disintegrin and metalloprotease) family (3), thereby releasing sHB-EGF from the cells. The importance of shedding of the sHB-EGF fragment was shown in mutant mice in which HB-EGF was replaced with a mutated HB-EGF that was resistant to cleavage by ADAMs. These mice exhibited phenotypes similar to HB-EGF-null mice indicating that most of the biological activity of HB-EGF is attributable to sHB-EGF (8).

HB-EGF has heparin-binding activity, and the binding of heparin enhances the mitogenic activity of HB-EGF (9). Therefore, heparin or heparan-sulfate proteoglycans (HSPG) are believed to activate HB-EGF activity *in vivo*. The heparin-binding domain of HB-EGF masks the ability of the EGF-like domain to bind its cognate ErbB receptors, and binding of heparin to the sequence releases this inhibition. The basic amino acid motif within the NH₂-terminal domain comprises the core sequence for heparin binding, and binding of heparin to this site might induce conformational changes in the EGF-like domain to an appropriate form that could bind the receptors (10); however, the precise molecular mechanisms by which this occurs are still unclear.

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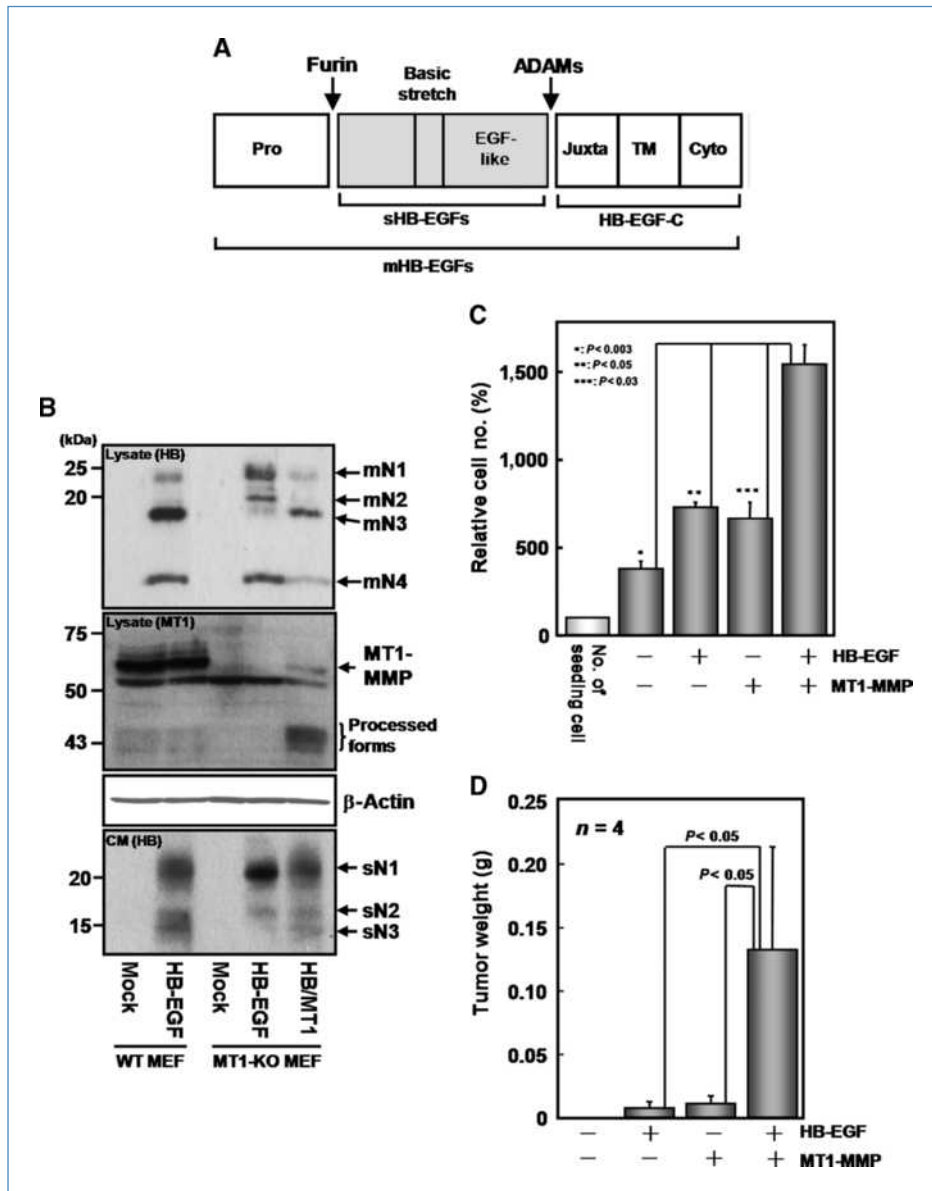


Figure 1. MT1-MMP cooperates with HB-EGF to stimulate the proliferation of MEFs. **A**, domain structure and processing sites of HB-EGF. Arrows, sites of pro-mHB-EGF cleavage by furin and ADAMs. **B**, mHB-EGFs and MT1-MMP were detected in MT1-MMP knockout (MT1-KO) or wild-type (WT) MEF cells by Western blotting with anti-FLAG (M2) antibody [Lysate (HB)], and anti-MT1-MMP antibody [Lysate (MT1)], respectively. sHB-EGFs was detected by Western blotting using anti-HB-EGF polyclonal antibody in serum-free CMs of both MEFs (34). β -Actin was detected as a loading control. **C**, synergistic effect of HB-EGF and MT1-MMP on MEF cell growth in collagen gels. MT1-KO cells expressing either exogenous MT1-MMP or HB-EGF, or both proteins simultaneously, were cultured in collagen for a week. Columns, mean of triplicate experiments; bars, SD. **D**, MT1-MMP and HB-EGF synergistically promote tumorigenesis. MT1-KO cells expressing either exogenous MT1-MMP or HB-EGF alone, or both proteins simultaneously, were injected into nude mice. Tumor weight was measured 6 wk after injections. Student's *t* test was used for statistical analyses.

The major heparin-like molecules in tissue, HSPGs, exist abundantly on the surface of most cells and in tissues (11). HSPGs are required for HB-EGF activity in cell culture conditions (12); however, they may act as a reservoir for HB-EGF in tissue and restrict the availability of HB-EGF to the receptors. Thus, the regulatory roles of HSPGs on HB-EGF activity *in vivo* and *in vitro* are not necessarily clear.

Membrane type 1-matrix metalloproteinase (MT1-MMP) is an integral membrane protease for which many substrates located in the vicinity of cells have been identified (13, 14). These are mostly extracellular matrix (ECM) proteins and cell surface molecules. To identify new substrates for MT1-MMP, we recently undertook an approach to survey proteins associating with MT1-MMP using a systemic whole cell

analysis (15, 16). Although HB-EGF was not identified as an MT1-MMP-associating protein directly, we noticed that some membrane proteins that have been reported to interact with HB-EGF, such as integrins $\alpha 3$ and $\beta 1$, CD63, and CD9 (17, 18), were identified in the assay. Because MT1-MMP might encounter HB-EGF through interaction with these proteins, MT1-MMP might cleave HB-EGF like other MT1-MMP-associating proteins (15, 16). Indeed, we observed that expression of HB-EGF and MT1-MMP in MT1-MMP-deficient cells induces the processing of HB-EGF. Furthermore, the expression of both proteins in cells synergistically enhances tumor growth in mice. MT1-MMP cleaves off the NH_2 -terminal portion of HB-EGF, and the cleavage negates the heparin-binding activity of HB-EGF and converts it from a heparin-dependent to a heparin-independent growth factor

resulting in its potentiation as a growth factor. Thus, the regulation of heparin-binding activity of HB-EGF by MT1-MMP is an important step for HB-EGF to promote tumor growth.

Materials and Methods

Cells and cell culture

32D cells expressing EGF receptor (DER cells) are EGF receptor (EGFR)-expressing derivatives of interleukin-3-dependent hematopoietic 32D cells. These cells respond to HB-EGF in suspension culture (10). Human gastric cancer TMK-1 cells were a gift from Dr. Tahara (Hiroshima University, Hiroshima, Japan), and STKM-1 and STKM-2 cells were from Dr. Yanoma (Kanagawa Cancer Center, Yokohama, Japan). Madin-Darby canine kidney (MDCK), buffalo rat liver (BRL), myelomonocyte WEHI-3, and gastric cancer MKN7 and MKN28 cells were provided by Health Science Research Resources Bank (Osaka, Japan). Mouse embryonic fibroblasts (MEF) obtained from MT1-MMP knockout or wild mouse whole embryos were established in our laboratory (19). MDCK, BRL, DER, and MEF cells were cultured in DMEM. Other cells were cultured in RPMI 1640. Both media (Sigma) were supplemented with 10 mmol/L of HEPES; 1.2 mg/mL of NaHCO₃, 2 mmol/L of glutamate, and 10% FCS (HyClone) were used as basal media. These cells were cultured in a humidified atmosphere of 5% CO₂/95% air.

Transfection of expression plasmids

These expression plasmids were transfected into cells using FuGENE6 (Roche-Diagnostics). Transfected cells were selected in the presence of 400 µg/mL of zeocine (Invitrogen).

Knockdown of MT1-MMP and HB-EGF mRNAs in STKM-2 cells

Small interfering RNAs targeting MT1-MMP (no. 1, 5'-ggaggacacggagaauuu-3'; no. 2, 5'-gcgauagaagucucacuaa-3') and HB-EGF (no. 1, 5'-ggaccaugucucggaaa-3'; no. 2, 5'-gagguuaugaugggaaa-3') were designed and prepared by B-Bridge, and transfection was carried out using Lipofectamine RNAi-MAX (Invitrogen). Cells were cultured for 36 hours, and seeded into collagen gels where cell growth was monitored. The knockdown efficiency of those mRNAs was analyzed by reverse transcription-PCR (RT-PCR). Primers to detect mRNAs for MT1-MMP, HB-EGF, and ErbB family genes are indicated in Supplementary Table S1.

Heparin-binding activity and growth factor activity of HB-EGFs

mHB-EGF fragments were applied to a HiTrap-Heparin column (GE Healthcare) on an AKTA-Purifier (GE Healthcare) and eluted with a linear gradient of NaCl. Each fraction was analyzed by Western blotting using an M2 antibody.

To measure growth factor activity of N1 and N3 fragments, DER cells (5×10^5 cells/mL) were plated in each well of a 96-well plate in DMEM containing 10% FCS. Purified mHB-EGF fragments (0.25 nmol/L each) or 5% (w/v) WEHI-3 CM as a source of interleukin-3 were added to the wells and the cells were

incubated in the presence or absence of heparin (50 µg/mL) for 30 hours at 37°C. The number of cells was counted using a hemocytometer under phase-contrast microscope.

Reagents

Anti-MT1-MMP monoclonal antibody was a gift from Daiichi Fine Chemical (Takaoka, Japan), and anti-FLAG (M2) monoclonal antibody was purchased from Sigma-Aldrich; HB-EGF polyclonal antibodies were from R&D Systems; EGFR and erbB4 neutralizing antibodies and β-actin monoclonal antibodies were from Millipore and Cell Signaling Technology; erbB2 neutralizing antibody was from Chugai Pharmaceutical. EGFR, erbB2, and erbB4 polyclonal antibodies were from Santa Cruz Biotechnology.

Results

MT1-MMP cooperates with HB-EGF to stimulate the proliferation of mouse fibroblasts

To test the possible cooperation between MT1-MMP and HB-EGF in cell growth, we used MEFs derived from wild-type and MT1-MMP-deficient (MT1-KO) mice (19) immortalized with SV40 T-antigen. These MEFs did not express endogenous HB-EGF that was detectable by Western blotting (Fig. 1A). Expression of HB-EGF in wild-type MEFs generated two major mHB-EGF fragments of 18 and 7 kDa (Fig. 1B, HB), whereas MT1-KO MEFs generated 25-, 21-, and 7-kDa fragments. Thus, MT1-MMP seems to affect the processing of HB-EGF. Additional expression of MT1-MMP in MT1-KO cells converted the processing pattern of HB-EGF to that of the wild-type cells (Fig. 1B, MT1); that is, the amount of the 25- and 21-kDa fragments was reduced, and the 18-kDa fragment was increased. We designated these membrane-anchored forms of NH₂-terminally processed fragments as mN1 to mN4 according to their molecular sizes. We infer that the 25-kDa (mN1) fragment corresponds to the mature membrane-bound HB-EGF produced following the processing of pro-mHB-EGF by proteases belonging to PCs and that the 7-kDa fragment (mN4) corresponds to the residual portion after ectodomain shedding by the ADAM family of proteases (Fig. 1B). The 21 kDa (mN2) and 18 kDa (mN3) likely correspond to mHB-EGF fragments produced by cleavage somewhere between the cleavage sites by PCs and ADAMs (Fig. 1B). In particular, MT1-MMP seems to cleave mN1 and mN2 fragments and generates the mN3 fragment. Soluble HB-EGF fragments (sN1, sN2, and sN3) detected in the culture medium (Fig. 1B, CM) are most likely the products of shedding of mN1, mN2, and mN3 by ADAMs, respectively. Indeed, the sN3 fragment was detected in medium obtained from cells that generated the mN3 fragment.

To evaluate the effect of MT1-MMP-dependent processing of HB-EGF on cell growth, MT1-KO MEFs expressing either HB-EGF or MT1-MMP, or expressing both proteins, were cultured in collagen gels and cell proliferation was measured (Fig. 1C). Expression of HB-EGF alone promoted cell growth by 1.8-fold whereas MT1-MMP alone promoted cell growth by 1.6-fold. However, coexpression of both proteins promoted cell growth by 4.5-fold (Fig. 1C). The cooperation of both

proteins was more apparent on tumor growth *in vivo*. MT1-KO MEFs formed a tumor-like aggregate when cells were implanted s.c. in syngeneic mice, and the effect of expression of either HB-EGF or MT1-MMP alone promoted tumor growth slightly (Fig. 1D). It is notable that the effect of HB-EGF alone was not that dramatic even in the HSPG-rich *in vivo* environment. In contrast, vigorous tumor growth (~12-fold) was observed when both proteins were coexpressed (Fig. 1D). Thus, HB-EGF and MT1-MMP cooperate to regulate cell growth in culture and tumor formation in mice, which correlates with the effect of MT1-MMP on the processing of HB-EGF.

MT1-MMP cleaves HB-EGF and converts it to a heparin-independent growth factor

We used MDCK cells that lack the expression of MT1-MMP to prepare processed mHB-EGF fragments for biochemical analysis. Processing of HB-EGF by MT1-MMP in the cells was similar to that observed in MT1-KO MEFs (Supplementary Fig. S1). HB-EGF with a FLAG-tag at the COOH terminus was expressed in the cells, and mHB-EGF fragments containing mN1, mN2, and mN4 were purified from the cells using anti-FLAG antibody (Fig. 2A). The purified proteins were incubated with a catalytic fragment of

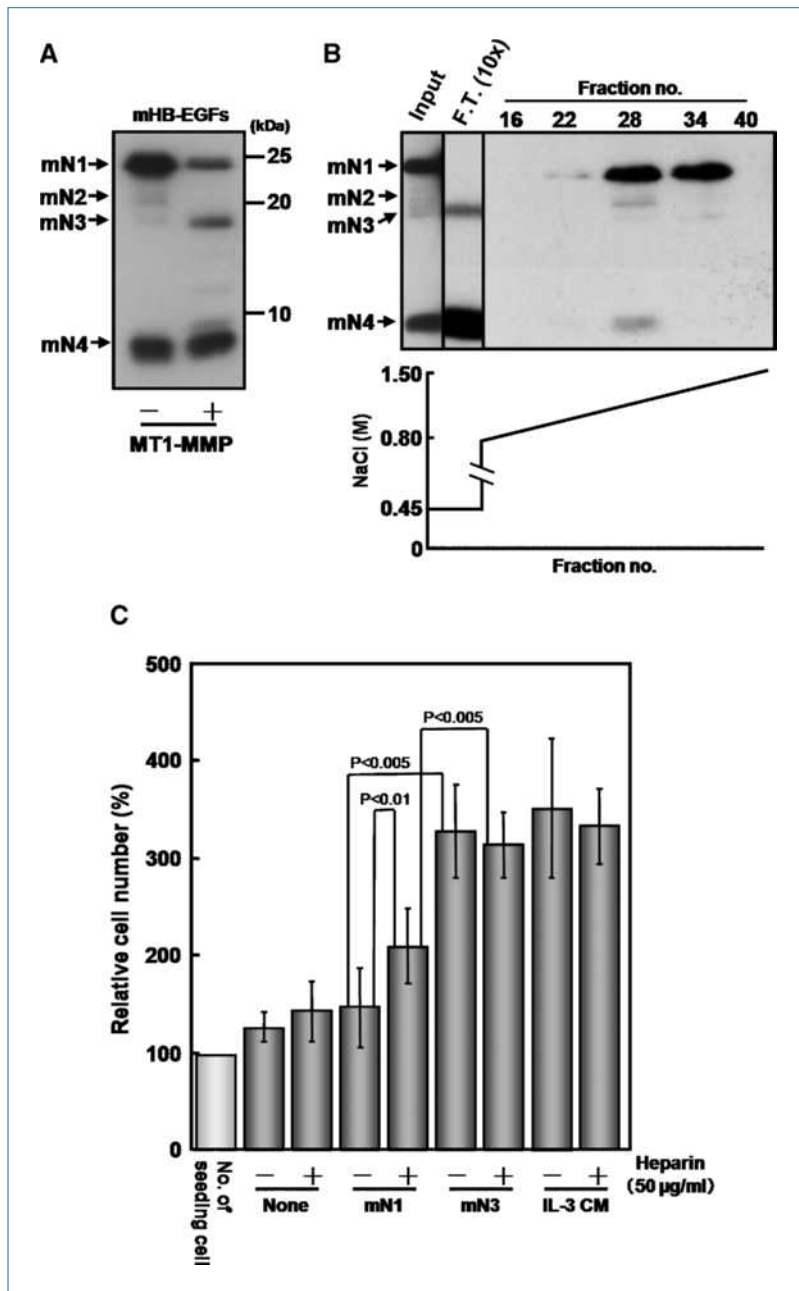
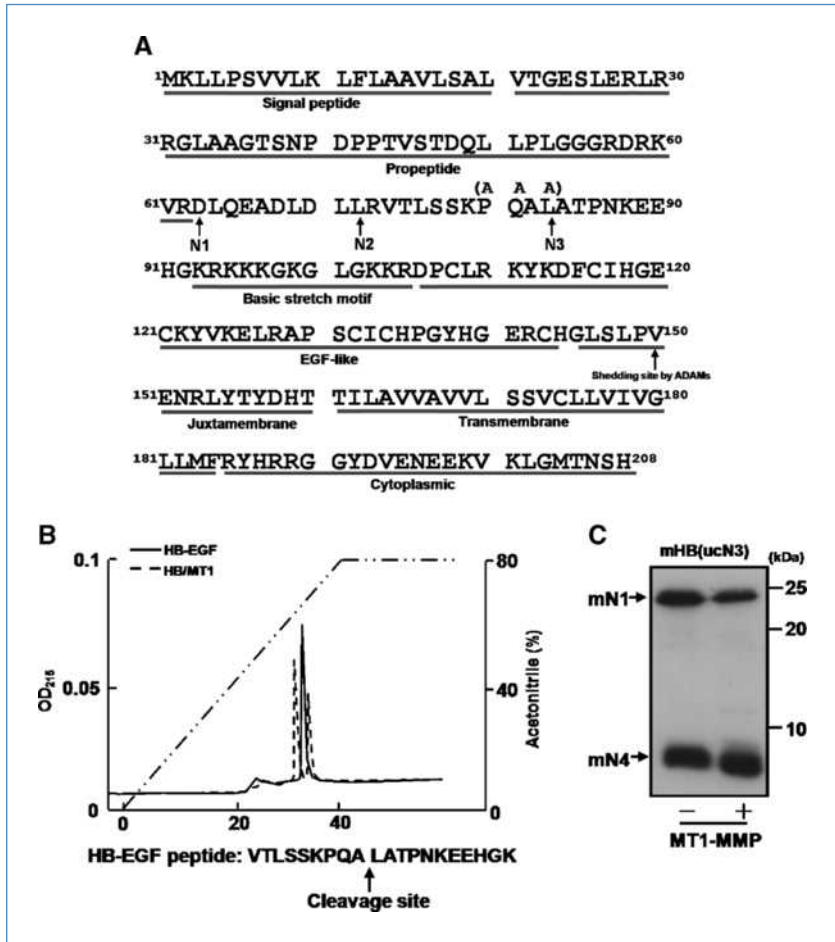


Figure 2. MT1-MMP cleaves HB-EGF and converts it into a heparin-independent growth factor. A, digestion of HB-EGF by MT1-MMP *in vitro* and analysis of the products by immunoblotting using an M2 antibody. B, mHB-EGF prepared from MDCK cells expressing HB-EGF and MT1-MMP was applied to a heparin HPLC and eluted with a linear gradient of NaCl. Original sample (Input), flow-through (FT), and fraction numbers are indicated (top). Arrowheads indicate mN1, mN2, mN3, and mN4 fragments. C, mitogenic activity of mHB-EGF fragments was analyzed using DER cells in the presence/absence of heparin. Columns, average values from experiments performed in triplicate. Student's *t* test was used for statistical analyses.

Figure 3. Analysis of amino terminal sequences of HB-EGF fragments. A, amino acid sequence of human proHB-EGF (1^M-208^H). The NH₂-terminal sequences of mN1, mN2, and mN3 fragments (arrows). The domain structure of HB-EGF is underlined. Alanine (A) substitutions in mHB-EGF are indicated above the mutated residues. The shedding sites by ADAM family proteases (arrows). B, a 20-mer polypeptide (HB-EGF peptide) spanning the expected cleavage site was prepared. The peptide was incubated with rMT1-MMP for 3 h at 37°C. The peptides were separated by reverse-phase HPLC on SepaxGP-C18 with 0.05% trifluoroacetic acid with a linear gradient of acetonitrile (0–80%, v/v). Each elution fraction was analyzed by MALDI-TOF mass spectrometry. MT1-MMP cleaved the HB-EGF peptide at a single site identified between A-83L. C, a mutant HB-EGF, HB(ucN3), containing alanine substitutions at the cleavage site was digested with MT1-MMP and analyzed as in Fig. 2A.



MT1-MMP to confirm whether MT1-MMP directly cleaves mN1 and mN2. MT1-MMP reduced the amount of the mN1 and mN2 fragments and converted them to mN3, whereas mN4 was not affected (Fig. 2A). To purify mHB-EGF fragments further, fragments obtained from the cells expressing both HB-EGF and MT1-MMP were applied to a heparin-sepharose column (Fig. 2B). The mN1 and mN2 fragments were retained in the heparin column and they were eluted in response to a linear concentration gradient of NaCl (Fig. 2B). We observed that mN3 and mN4 were mostly in the flow-through fraction (Fig. 2B). Thus, mN1 and mN2 fragments have heparin-binding activity, whereas the mN3 fragment does not. mN4 lacks all the extracellular domains, judging from its molecular size.

We next examined the mitogenic activity of two major forms of mHB-EGF fragments (mN1 and mN3) using a DER cell that could grow in a suspension culture. These cells have low levels of HSPGs on the cell surface (20). Interleukin-3 is a heparin-independent growth factor for the cells (21), and indeed, it stimulated the cells to grow without requiring heparin (Fig. 2C). In contrast, the mN1 fragment showed only weak mitogenic activity at the concentrations used in this assay, and this activity was enhanced by the addition of

heparin (Fig. 2C), as reported previously (10). In contrast, the mN3 fragment exhibited a potent mitogenic activity without requiring heparin and the activity was comparable to interleukin-3. The activity of mN3 was not further enhanced by the addition of heparin (Fig. 2C). Thus, processing of mHB-EGF by MT1-MMP converted HB-EGF to a heparin-independent growth factor and enhanced the mitogenic activity at the same time.

Analysis of amino terminal sequences of HB-EGF fragments

The purified fragments were subjected to amino terminal sequence analysis. The mN1 fragment had a ⁶³DLQEA sequence that corresponds exactly to the cleavage site by PCs (7). The amino terminal sequence of the mN2 fragment was ⁷²LRVTL, and the mN3 fragment had an ⁸³LAXPN amino terminal sequence. The positions of the amino terminal residues of these fragments are indicated within the context of the full HB-EGF amino acid sequence (Fig. 3A). Although mN3 retains the core sequence for heparin binding, it can no longer bind heparin (Fig. 2B). Therefore, the NH₂-terminal sequence disrupted by MT1-MMP-dependent cleavage also plays a role in binding heparin.

A synthetic 20-mer polypeptide corresponding to amino acids 74 to 93 of HB-EGF was incubated with MT1-MMP *in vitro* and was cleaved at a single site between A-⁸³L, which is consistent with the NH₂-terminal sequence of mN3 (Fig. 3B). Amino acid sequences flanking the cleavage site fit the reported consensus ones (PXA-⁸³L) for cleavage sites by MT1-MMP. Substitution of three amino acids in the consensus sequence with alanine (PQA-⁸³L to AAA-⁸³A) made the peptide resistant to MT1-MMP (Supplementary Fig. S2). Mutant HB-EGF [HB(ucN3)] with the same substitutions at the cleavage site was no longer cleavable by MT1-MMP (Fig. 3C).

HB-EGF and MT1-MMP expressed in human gastric carcinoma cells support their invasive growth

We focused our attention on human gastric carcinoma cells to investigate the significance of MT1-MMP-dependent HB-EGF processing in tumor cell growth because gastric carcinoma is one of the most common malignancies in Japan and carcinoma cells are reported to overexpress HB-EGF (22) as well as MT1-MMP (23) compared with normal tissues. To examine whether MT1-MMP and HB-EGF contribute to their

cell growth, we first analyzed the expression of mRNAs encoding HB-EGF and MT1-MMP in five gastric carcinoma cell lines (Fig. 4A). MKN7, MKN28, and STKM-2 cells express both MT1-MMP and HB-EGF at significant levels, reflecting clinical situations and showing that these cells could grow in collagen gels (Fig. 4B). The growth of these cells was inhibited by an HB-EGF inhibitor (CRM197) derived from Diphtheria toxin (24), or a synthetic MMP inhibitor (MMI270; ref. 25; Fig. 4B), suggesting the contribution of HB-EGF and MT1-MMP to the growth of these cells in the collagen matrix. To confirm this further, we knocked down the expression of HB-EGF or MT1-MMP in STKM-2 cells using small interfering RNA. Knockdown efficiency was confirmed by RT-PCR (Supplementary Fig. S3). The growth of the cells was suppressed by treatment with either small interfering RNA targeting MT1-MMP or HB-EGF (Fig. 4C). Knockdown of either MT1-MMP or HB-EGF was also found to inhibit the invasion of the cells into the collagen (Fig. 4D). We also confirmed that MT1-MMP expressed in STKM-2 cells produced mN3 fragments in these cells (Supplementary Fig. S4). Both EGFR and ErbB2 are expressed in STKM-2 cells, as shown in Fig. 5A.

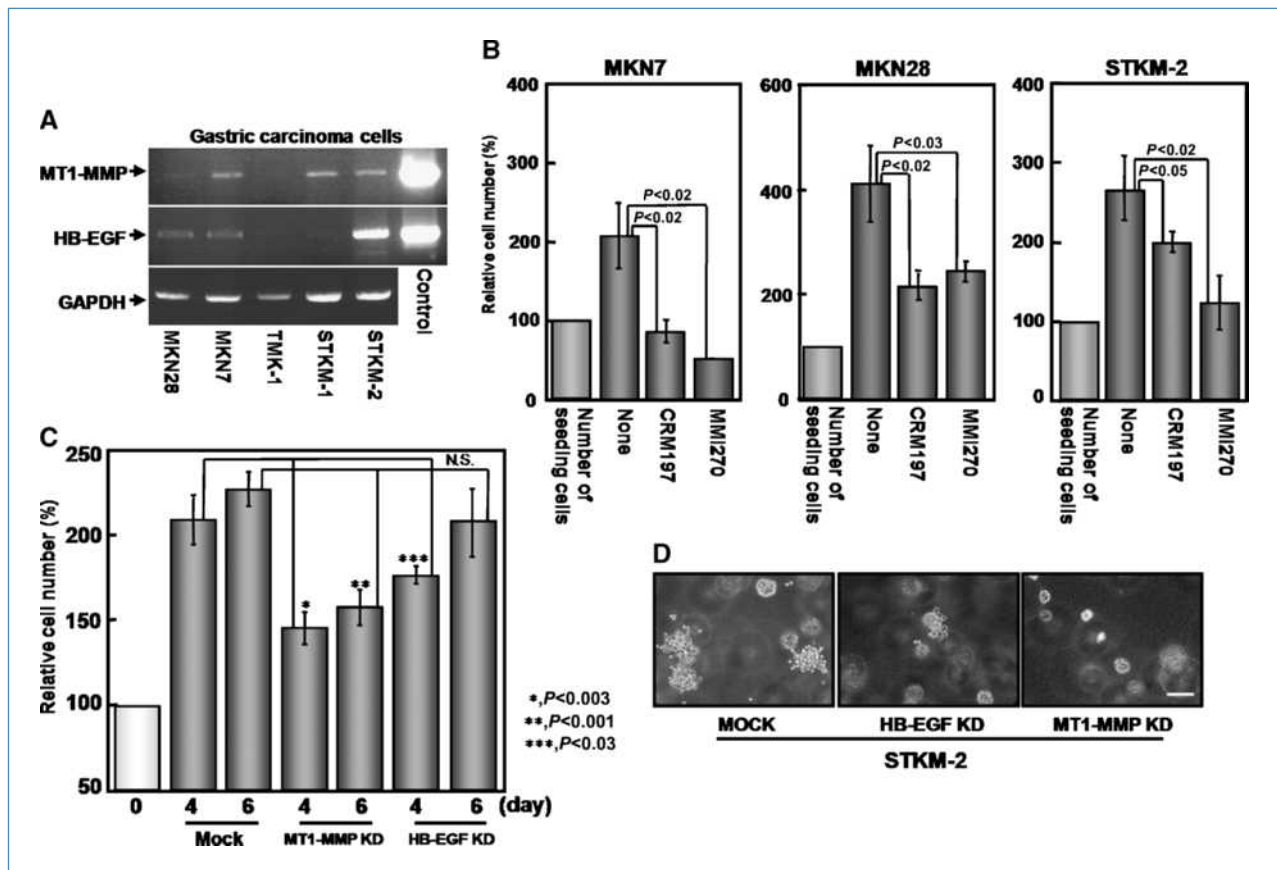
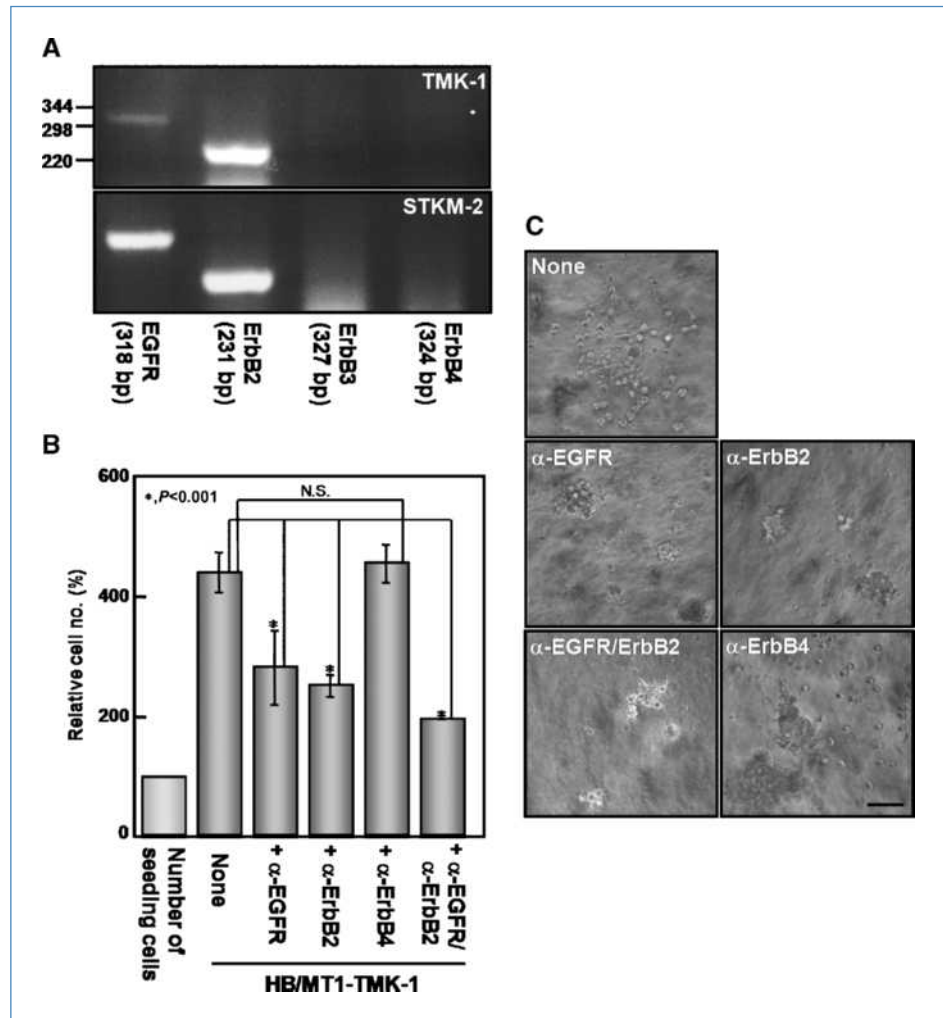


Figure 4. HB-EGF and MT1-MMP expressed in human gastric carcinoma cells are supporting their invasive growth. A, expression of HB-EGF and MT1-MMP mRNAs in human gastric cancer cells was analyzed by RT-PCR. GAPDH mRNA was used as an internal control and cDNAs for MT1-MMP and HB-EGF were used as positive controls. B, inhibition of tumor cell growth by CRM197 (5 μ g/mL) and MMI270 (5 μ g/mL). These cells were cultured for 4 d in the presence/absence of the indicated inhibitors. The results represent the average of experiments performed in triplicate. The Student's *t* test was used for statistical analyses. C and D, comparison of cell growth activity (C) and morphology (D) of STKM-2 in collagen gels after incubation for 4 d.

Figure 5. ErbB receptor-mediated signals regulate invasive growth of human gastric cancer cells. A, expression of ErbB receptors in TMK-1 and STKM-2 cells was analyzed by RT-PCR. B, effect of neutralizing antibodies against ErbB receptors on TMK-1 cell growth expressing HB-EGF and MT1-MMP. HB/MT1-TMK-1 cells were incubated with the indicated antibodies, anti-EGFR (5 μ g/mL), anti-ErbB2 (10 μ g/mL), anti-ErbB3 monoclonal antibodies (5 μ g/mL), and the combined anti-EGFR + anti-ErbB2 monoclonal antibodies treatment. Student's *t* test was used for statistical analyses. The results represent the average of experiments performed in triplicate. C, effects of neutralizing antibodies to the ErbB receptors on invasive morphology of the TMK-1 cells induced by HB-EGF and MT1-MMP coexpression in collagen gels (bar, 100 μ m).



Processing of HB-EGF by MT1-MMP is essential for the promotion of invasive growth of gastric carcinoma cells

Among the gastric carcinoma cells tested in Fig. 4A, TMK-1 is unique in that the cells lack the expression of both genes. Therefore, we used this cell line to reconstitute the processing of HB-EGF by MT1-MMP by expressing both proteins and observed the effect on cell growth in collagen gels. Expression of HB-EGF alone in TMK-1 cells gave rise to the generation of several processed fragments but the mN3 fragment was not detected, whereas coexpression of HB-EGF and MT1-MMP in these cells led to the specific generation of mN3 (Fig. 6A). However, mN3 was not produced following the coexpression of MT1-MMP and the processing mutant of HB(ucN3) (Fig. 6A).

We next examined the effect of coexpression of HB-EGF and MT1-MMP on the proliferation of TMK-1 cells grown in collagen matrix. Control mock-transfected cells multiplied 1.4-fold under these conditions during this period (Fig. 6B). Expression of MT1-MMP alone did not stimulate cell growth, whereas expression of HB-EGF alone enhanced cell growth by

2-fold. Coexpression of MT1-MMP and HB-EGF enhanced cell growth by 4-fold (Fig. 6B). This effect was not observed when MT1-MMP was coexpressed with the mutant HB-EGF, HB(ucN3) (Fig. 6B). We confirmed that HB(ucN3) retained heparin-dependent mitogenic activity similar to mN1 and mN2 as shown in Supplementary Fig. S5. Thus, processing of HB-EGF by MT1-MMP is important to potentiate the mitogenic activity of HB-EGF on cell growth in the collagen environment.

We also noted an alteration in cell morphology during the growth of TMK-1 cells in collagen gels following the coexpression of HB-EGF and MT1-MMP. Mock-transfected cells formed compact spheroids whose morphology was not significantly altered by the expression of MT1-MMP (Fig. 6C). Expression of HB-EGF led to the generation of slightly larger spheroids (Fig. 6C). However, we observed a dramatic change in cell morphology following coexpression of MT1-MMP and HB-EGF (Fig. 6C). The cells did not form spheroids, but rather showed spindle-like mesenchymal morphology and spread out invasively into the collagen gel as observed with STKM-2 cells. In contrast, coexpression of HB(ucN3) and MT1-MMP did not lead to an invasive cell morphology (Fig. 6C). The

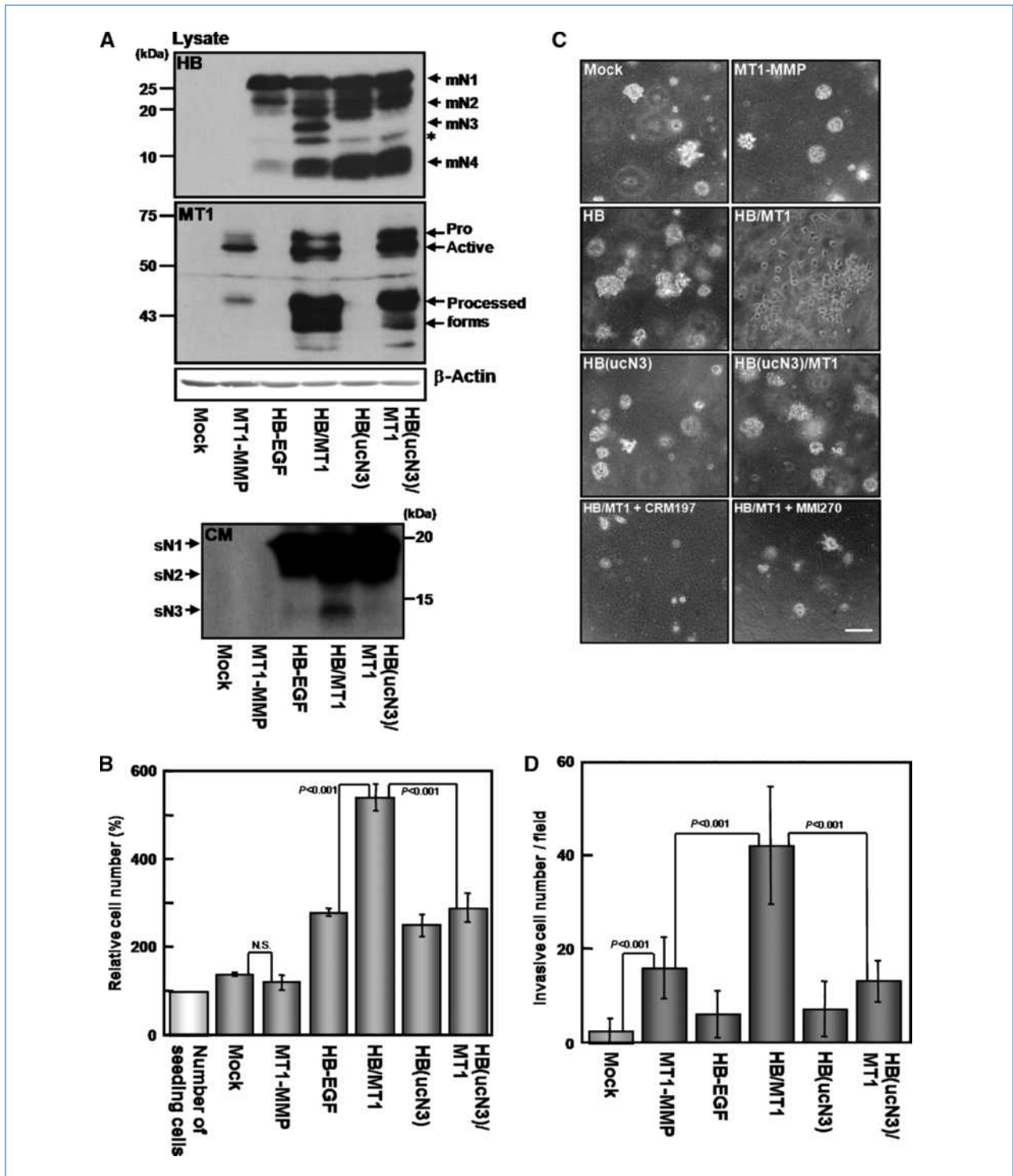


Figure 6. Processing of HB-EGF by MT1-MMP promotes invasive growth of TMK-1 cells in collagen gels. **A**, detection of HB-EGFs (HB) and MT1-MMP (MT1) in TMK-1 cells by Western blotting. HB-EGF was detected by Western blotting using a M2 antibody and MT1-MMP was detected with anti-MT1-MMP antibody. CMs were obtained from mock and transfectants. sHB-EGFs in CMs were analyzed by Western blotting using anti-HB-EGF antibody. sHB-EGF concentration was estimated to be ~1.5 ng/mL according to an ELISA assay using the antibody (data not shown). β -Actin was used as a loading control. *, cytoplasmic HB-EGF. **B**, effect of HB-EGF processing by MT1-MMP on TMK-1 cell growth in collagen gels. Mock and transfectants were cultured for 8 d. **C**, morphology of cells grown in collagen gels under light microscopy (bar, 100 μ m). **D**, invasion of the cells into collagen was analyzed using Transwell chambers using 10% FCS as a chemoattractant. The data represent average values from experiments performed in triplicate. Student's *t* test was used for statistical analyses.

invasive growth of TMK-1 cells expressing HB-EGF and MT1-MMP was nearly completely inhibited by exposure to either MMI270 or CRM197 (Fig. 6C). The invasive property of the cells into collagen was further confirmed by an assay using transwell chambers, as shown in Fig. 6D. Expression of MT1-MMP alone enhanced cell invasion significantly, but the expression of HB-EGF or HB(ucN3) alone had little effect on invasion. We observed a major enhancement of invasion following the coexpression of MT1-MMP and HB-EGF. This effect was not observed when MT1-MMP was coexpressed with HB(ucN3) (Fig. 6D). The results again indicate that the cleavage of HB-EGF between A-⁸³L by MT1-MMP markedly changed growth factor activity.

The extracellular portion of HB-EGF shed by ADAM activity is the entity that conveys the mitogenic activity of HB-EGF. However, recent studies have shown that the residual portion of HB-EGF following ectodomain shedding could have additional stimulatory effects on the cells (26, 27). An mN4 fragment translocates to the perinuclear region where it interacts with regulators of transcription factors and modulates cellular functions in cooperation with ErbB receptor-mediated signals elicited by the extracellular portion of HB-EGF (28). Thus, the NH₂- and COOH-terminal fragments of HB-EGF may cooperate to regulate cell function after being processed by ADAMs. Therefore, we examined whether the shed fragments were sufficient to elicit invasive cell growth or if the mN4 COOH-terminal fragment plays a role in the mitogenic response of cells expressing HB-EGF and MT1-MMP. To test this, we prepared conditioned medium (CM) from TMK-1 expressing HB-EGF either alone or together with MT1-MMP, and examined the effect of these media on parental TMK-1 cells that did not express both proteins. We confirmed the presence of sN3 fragments in CM from cells expressing MT1-MMP and HB-EGF by Western blotting (Fig. 6A, CM), whereas the sN3 fragment was not detected in CM from mock-transfected cells or cells expressing MT1-MMP, HB-EGF, or HB(ucN3)/MT1-MMP. Only CM prepared from cells expressing both MT1-MMP and HB-EGF stimulated the growth of TMK-1 cells in collagen gels (Supplementary Fig. S6). Thus, the sN3 fragment contained in CM is important to stimulate the invasive growth of TMK-1 cells. The COOH-terminal mN4 fragment might regulate other cell functions rather than the invasive growth in collagen.

Stimulation of ErbB receptors mediates the invasive growth of gastric tumor cells

To identify the receptors responsible for transducing the signals mediated by HB-EGF and MT1-MMP, we examined the expression of ErbB family members in TMK-1 and STKM-2 cells by RT-PCR (Fig. 5A). We detected the expression of EGFR (ErbB1) and ErbB2 but not ErbB3 and ErbB4 mRNAs in these cells. We next used neutralizing antibodies to EGFR, ErbB2, and ErbB4 to test whether these receptors were used for the growth of TMK-1 cells expressing MT1-MMP and HB-EGF. Anti-EGFR and anti-ErbB2 suppressed cell growth by 40% and 50%, respectively (Fig. 5B), and these

antibodies also inhibited the invasive property of the cells (Fig. 5C). EGFR and ErbB2 could also form a heterodimer and treatment of the cells with a combination of anti-EGFR and anti-ErbB2 antibodies resulted in a more efficient inhibition of growth (Fig. 5B). Finally, it is important to confirm that sN3 has the potential to stimulate the receptor. The CM containing the sN3 fragment (Fig. 5A) indeed induced phosphorylation of EGFR efficiently in TMK-1 cells (Supplementary Fig. S7).

Discussion

HB-EGF and MT1-MMP have been independently implicated in malignant tumor growth and invasion, and both proteins have been recognized as potential targets for cancer therapy (29–32). The present study shows that HB-EGF is a new substrate of MT1-MMP and the growth factor activity of HB-EGF is greatly enhanced by its processing. Interestingly, removal of the NH₂-terminal fragment of HB-EGF by MT1-MMP converted HB-EGF into a potent mitogen that does not require heparin as a cofactor (Fig. 2C).

NH₂-terminal processing of HB-EGF by metalloproteinases has been reported to occur at multiple sites (7, 33). Among them, MMP7 is reported to have processing activity against HB-EGF. Therefore, we examined whether MMP7 could cleave HB-EGF and generate mN3–HB-EGF. MMP7 generated mN2-like fragments and increased the amount of mN4-like fragments, although it did not generate any mN3-like fragments (Supplementary Fig. S8).

The NH₂-terminal heparin-binding sequence suppresses growth factor activity of HB-EGF, and binding of heparin to the sequence releases the suppression at least *in vitro* as reported previously (10). *In vivo*, HSPGs are abundantly present in tissues and are expected to activate HB-EGF like heparin (11). Indeed, HB-EGF binds to HSPGs and elimination of HSPGs from the cell surface by treating cells with heparitinase diminishes the cell's response to HB-EGF (12). To our surprise, however, we observed that exogenous expression of HB-EGF alone in MT1-KO MEFs was not sufficient to promote tumor growth in mice (Fig. 1D), whereas coexpression of MT1-MMP together with HB-EGF enhanced tumor growth synergistically. These results indicate that HSPGs in tissues are not sufficient to activate HB-EGF, and that MT1-MMP is required to induce the full mitogenic activity of HB-EGF. However, the abundance of HSPGs in tissue might suggest the alternative possibility that HSPGs play a negative regulatory role on HB-EGF *in vivo*, which may be released by the MT1-MMP cleavage of the HSPGs binding domain. For example, HSPGs may trap sN1- and sN2–HB-EGFs *in vivo* and prevent them from accessing their target cells, whereas sN3 could move through HSPG-rich tissues. This role may not be readily detectable in culture.

We observed that both proteins were coexpressed in most gastric carcinoma cells (Fig. 4A) and that the growth of cells in culture exhibited dependence on both proteins as evaluated by knockdown experiments or by using inhibitors (CRM197 and MMI270). Cooperative action between HB-EGF and MT1-MMP is mediated by the processing of HB-EGF between

the A-⁸³L by MT1-MMP. This was proven by demonstrating that the uncleavable mutant HB(ucN3) against MT1-MMP could not cooperate with MT1-MMP to stimulate cell growth and invasion (Fig. 6). Soluble HB-EGF fragments shed by ADAM proteases act as ligands for ErbB receptors (1), and the gastric carcinoma cells expressed EGFR and ErbB2 (Fig. 5A). The growth-promoting effect of HB-EGF and MT1-MMP on TMK-1 cells is mediated by the EGFRs as the effect was suppressed by neutralizing antibody to each receptor (Fig. 5B and C).

In conclusion, our present study uncovered the crucial role of MT1-MMP as a regulator of HB-EGF activity. Thus, although MT1-MMP is important for tumor invasion, as has been reported, it also promotes tumor growth by directly modulating HB-EGF activity. The specific HB-EGF inhibitor CRM197 is currently being evaluated in a phase I clinical trial in patients with ovarian carcinoma (6), and the present study suggests that combination therapy targeting the HB-EGF/ErbB pathway and the proteolytic activity of MT1-MMP would provide a better therapeutic outcome for the treatment of malignant gastric carcinomas, and that detection

of the sN3 fragment would be a good biomarker for selecting appropriate patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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