Matrix Metalloproteinase 7 Mediates Mammary Epithelial Cell Tumorigenesis through the ErbB4 Receptor

Conor C. Lynch, Tracy Vargo-Gogola, Michelle D. Martin, Barbara Fingleton, Howard C. Crawford, and Lynn M. Matrisian

Abstract

To delineate the role of matrix metalloproteinase 7 (MMP7) in mammary tumorigenesis, MMP7 was expressed in the normal murine mammary gland cell line, c57MG. MMP7 markedly enhanced the growth rate of the c57MG cells in three-dimensional culture and promoted tumor formation in vivo. Subsequent investigation showed that MMP7 (a) up-regulated ErbB4 receptor levels, (b) solubilized the ErbB4 receptor cognate ligand heparin-bound epidermal growth factor, and (c) mediated the proteolytic processing of ErbB4 to yield a soluble intracellular domain (ICD) that localized to the cytoplasm and the nucleus. Furthermore, overexpression of the ErbB4 ICD in the c57MG cell line recapitulated the proliferative response of MMP7 in vitro and in vivo. These data indicate a novel mechanism for mammary epithelial cell transformation by MMP7. [Cancer Res 2007;67(14):6760-7]

Introduction

Matrix metalloproteinases (MMP) have been implicated in the invasion and metastasis of breast cancer due to their ability to degrade the extracellular matrix (1). However, in recent years, it has become apparent that the MMPs are also exquisite regulators of cell-cell communication by virtue of their ability to process many nonmatrix molecules, such as cytokines and growth factors (1). As a consequence, the MMPs can affect tumor progression on multiple levels, including tumor growth, apoptosis, angiogenesis, and immunoevasion.

In human breast cancer, the MMPs are predominantly expressed by the host stroma with the exception of MMP7, which is typically found in the tumor compartment (2). Immunohistochemical studies have shown that MMP7 is highly expressed during the early stages of human breast cancer (2, 3). Transgenic mice that express MMP7 in the mammary epithelium have been shown to develop areas of hyperplasia in the mammary gland by as yet unidentified mechanisms (4).

The aim of the current study was to delineate how MMP7 promotes mammary gland tumorigenesis. To address this, we used an immortalized mammary epithelial cell line, c57MG (5), which is nontransformed, contact inhibited in culture, anchorage dependent, requires serum for growth, does not express MMP7, and is unable to form tumors when injected s.c. into athymic mice. In addition, the c57MG cell line is syngeneic with the c57BL/6 mouse. Therefore, MMP7 expression in the c57MG cell line allows for the investigation of how MMP7 affects normal mammary epithelial cell behavior in vitro and the testing of potential mechanisms in vivo following orthotopic injection.

Materials and Methods

Cell culture and stable clone generation. c57MG were maintained using standard tissue culture conditions (5). Retroviral infection with the LXSN vector (6) was used to introduce full-length MMP7 (7) or vector controls into the c57MG cell lines, whereas Superfect (Qiagen) was used to transiently transfect the alkaline phosphatase–tagged heparin-bound epidermal growth factor (HBEGF-AP) plasmid (Dr. Rosalyn Adam, Children's Hospital, Boston, MA; ref. 8) into the cell lines according to the manufacturer's instructions. A luciferase-expressing construct (pGL4-Control, Promega) was cotransfected with the construct to normalize transfection efficiency. The intracellular domain (ICD) cyt-2 domain of ErbB4 fused with green fluorescent protein (GFP) or vector control plasmids containing GFP alone (Dr. Graham Carpenter, Vanderbilt University, Nashville, TN) were also introduced using Superfect. Stable cell lines were selected using 800 μg/mL G418 (Mediatech, Inc.) and the subsequent clones were maintained in medium containing 200 μg/mL G418. Phorbol 12-myristate 13-acetate (PMA) dissolved in 10 mmol/L DMSO (Sigma-Aldrich) treatments (100 ng/mL) were done in serum-free DMEM for 3 h before the collection of cell lysates. For MMP inhibition, cells were treated with medium containing 5 μmol/L BP-94 (obtained from Dr. Peter Brown, British Biotech, Oxford, United Kingdom) for 3 h before analysis, a neutralizing MMP7 antibody (R&D Systems) at a concentration of 2 μg/mL, or tissue inhibitor of metalloproteinase-3 (TIMP-3; Abcam) at a concentration of 0.5 μmol/L for indicated times.

Cell growth and proliferation assays. For cell proliferation assays in three dimensions, 1 × 10⁵ cells were plated into 48-well plates that were coated with 100 μL of a basement membrane matrix (Matrigel, BD Biosciences). Cells were retrieved from the three-dimensional matrix by treating with dispase (BD Biosciences). Viable cells were counted using a trypan blue exclusion assay every 2 days. All experiments were done in triplicate and repeated independently at least thrice. For MMP7 neutralizing antibody and HB-EGF treatments, cells were treated daily with either MMP7 neutralizing antibody or isotype control goat IgG at a concentration of 2 μg/mL or 10 ng/mL HB-EGF (R&D Systems) in normal growth medium or placebo control. Cell numbers were graphed using log₁₀ scale to identify the linear growth phase of the cells. The specific growth rate constant was calculated using the formula: μ = (log₁₀ Nₙ / log₁₀ N₀) / time (days), where μ is the specific growth rate constant and T₀ and Tₙ are early and late time points within the linear phase of the graph, respectively. The doubling time (Dₚ) in hours was calculated using the specific growth rate constant as follows: Dₚ = (log₂ / μ) × 24 h. For histologic analysis, cells growing in three-dimensional culture were fixed (10% formalin), dehydrated through...
an ethanol series and xlenes, parafin embedded, and sectioned. Sections were rehydrated by immersion in xlenes and an ethanol series ranging from 100% to 50%. The sections were subsequently stained with hematoxylin for 5 min, washed in tap water, and then counterstained in eosin for 2 min. After rinsing in water, the sections were dehydrated through the ethanol series (50−100%) and xlenes before being permanently mounted.

Reverse transcription-PCR. Total RNA was collected from cell lines grown in two-dimensional and three-dimensional conditions with Trizol (Invitrogen) as per manufacturer's instructions. For murine HB-EGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, the following primers were used after a standard reverse transcription reaction for cDNA generation: HB-EGF 5'-TTTGGA-GAGTCCTTTGCAGA-3' (sense) and 5'-TTTGGA-GAGTCCTTTGCAGA-3' (antisense) and GAPDH 5'-ACCA-CAGTCCATGCCATCAC-3' (sense) and 5'-CCACCCACCGCTATTGTTGTA-3' (antisense). PCR conditions for HB-EGF/GAPDH: 1 cycle of 95°C for 5 min and 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The expected bp product sizes for HB-EGF and GAPDH were 279 and 452 bp, respectively.

Immunoblotting and immunoprecipitation. Standard cytoplasmic, nuclear fractionation, and cell lysis protocols were used to isolate total proteins from cells growing in two-dimensional and three-dimensional culture. For immunoblotting, 50 μg of total protein isolated from confluent two-dimensional monolayers and three-dimensional cultures at day 10 were electrophoresed and transferred to nitrocellulose. The blots were processed using standard procedures as described (10). For quantitation, spot densitometry was done using an Alpha Innotech Fluorchem 8900. Densitometric values were expressed as a percentage of the control. The primary antibodies used were the following: α-MMP7 (1:10; ref. 11), α-ErbB1 to α-ErbB4 full-length receptors (1:1,000; Santa Cruz Biotechnology), α-ErbB4 ectodomain (1:500; Labvision), α-HB-EGF (1:500; R&D Systems), α-tumor necrosis factor (TNF)-α-converting enzyme (TACE; 1:1,000; Chemicon), α-GFP (1:1,000; Clontech), α-actin (1:1,000; Santa Cruz Biotechnology), and α-histone H4 (Santa Cruz Biotechnology). The ErbB4 ICD was immunoprecipitated as described (12) using a primary antibody directed to the ICD domain of ErbB4 (a kind gift from Dr. Graham Carpenter). HB-EGF was precipitated from 1 mL of conditioned medium at an ethanol series and xylenes, paraffin embedded, and sectioned. Sections were then fixed for 5 min at 4% paraformaldehyde in 1× PBS, dehydrated through ethanols, and embedded in paraffin and sectioned. Five-micrometer sections of the mouse mammary gland were dehydrated, H&E stained, and scored as positive or negative for cell growth. Animal surgeries and care protocols were approved by the Vanderbilt University Institute of Animal Care and Use Committee.

Immunohistochemical and immunofluorescence analysis. MMP7 and the ErbB receptors were detected using a standard immunohistochemistry protocol as described (10). For ErbB4 localization in vitro, vector control and MMP7-expressing cell lines were grown for 48 h and then fixed for 5 min at −20°C in 100% methanol. The cells were then washed twice in 1× PBS and incubated for 30 min in 3% blocking solution (3% milk in 1× PBS). Subsequently, the cells were washed and incubated with the primary antibody for 1 h at room temperature (ErbB4; 1:500). After washing the secondary antibody (Alexa Fluor 488 antirabbit diluted 1:1,000 in 3% blocking solution; Invitrogen) was added for 30 min at room temperature. The cells were then washed and aqueously mounted. For counterstaining of nuclei, 2 μmol/L 4,6-diamidino-2-phenylindole (DAPI) or 1 μg/mL propidium iodide was added to the mounting medium. A Zeiss LSM 510 META inverted microscope was used for confocal microscopy.

Analysis of MMP7 and TACE processing of ErbB4. A synthetic 12-amino acid residue (40 μmol/L) of the ErbB4 juxtamembrane cleavage site (provided by Dr. Graham Carpenter) was incubated either alone in 20 mmol/L HEPES (pH 7.2) or with 0.25 μmol/L MMP7 (Calbiochem) in MMP reaction buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L CaCl2 (pH 7.6)] or with 0.25 μmol/L TACE (Oncogene) in 20 mmol/L HEPES buffer (pH 7.2) in a final volume of 50 μL. All reactions were incubated at 37°C overnight. For cleavage assessment by matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry, 1 μL of each reaction was mixed with 1 μL of salicyl-aldehyde matrix and spotted on a sample plate. Analysis was done in conjunction with the Vanderbilt Proteomics Laboratory of the Mass Spectrometry Research Center.

Statistical analysis. Statistical significance was analyzed using a two-tailed Student's t test. P < 0.05 was considered significant.

Results

MMP7 promotes mammary epithelial cell growth in vitro and in vivo. Multiple c57MG vector control and MMP7-expressing cell lines were generated by retroviral transduction and examined by immunoblot analysis for MMP7 expression. Two vector control cell lines (L2 and L4) and two MMP7-expressing cell lines (M14 and M37) were randomly selected for investigation (Fig. 1A). Analysis of the cell growth under monolayer (two-dimensional) and three-dimensional culture conditions showed that MMP7 expression resulted in a slight but significant 0.24-fold shorter doubling time (D1) compared with the vector controls (data not shown). However, providing the cell lines with a three-dimensional basement membrane matrix that more closely mimicked the in vivo environment revealed that MMP7 had striking effects on the morphology (Fig. 1B) and growth of the normal mammary epithelial cells (Fig. 1C). MMP7 expression reduced the average doubling time (D1) ~2-fold (L2, 83.4 ± 11.9 h; L4, 63.3 ± 10 h; M14, 38.6 ± 2.66 h; and M37, 36.7 ± 1 h; P = 0.0007). The use of growth factor–depleted basement membrane matrix did not attenuate the proliferative effect of MMP7 (data not shown). The addition of a MMP7 neutralizing antibody that inhibits MMP7 activity by 50% at a concentration of 2 μg/mL showed a slight, but statistically significant, inhibition in the three-dimensional doubling time growth rate of the MMP7-expressing cells (D1, M14, 39.9 ± 1.4 h; M37, 39.6 ± 1.03 h; M14 α-MMP7, 46.9 ± 0.5 h; and M37 α-MMP7, 45.4 ± 0.5 h; P = 0.006), suggesting that MMP7 was directly responsible for the observed proliferative effect (Fig. 1C). The MMP7 neutralizing antibody had no effect on the growth of the vector controls (data not shown).

To determine whether MMP7 expression could promote growth of the c57MG cells in vivo, the vector control and MMP7-expressing cell lines were orthotopically injected into epithelia cleared mammary fat pads of syngeneic 3-week-old virgin C57BL/6 mice. After 3 weeks, histologic analysis indicated tumor formation in 42% of mice injected with either of the MMP7-expressing cell lines (Fig. 1D). No tumors were detected in any mice injected with vector control clones. Immunohistochemical staining for MMP7 showed that the majority of the cells in the identified tumor masses were positive for MMP7 expression (Fig. 1D).

MMP7 mediates ErbB4 cytoplasmic and nuclear localization. Mammary epithelial cell growth is well known to be influenced by the ErbB family of receptor tyrosine kinases (14). Therefore, the expression of ErbB family members in control and MMP7-expressing c57MG cells was examined by immunoblotting. Under three-dimensional culture conditions, the levels of ErbB4 were markedly enhanced in the MMP7-expressing cell lines compared with the vector controls, with only subtle increases being observed in the remaining ErbB family members (Fig. 2A). Interestingly, the ErbB4 immunoblots revealed the presence of prominent 80-kDa
fragments in the lysates of the MMP7-expressing cell lines. Concomitant with increased levels of the ErbB4 receptor, a marked increase in the amount of phosphorylated ErbB4 was also detected in the MMP7-expressing cell lines compared with the vector controls (data not shown). Consistent with our \textit{in vitro} observations, ErbB4 was detected in 100% of tumors that were generated by the MMP7-expressing cell lines \textit{in vivo} using immunohistochemistry (Fig. 2B).

The presence of an 80-kDa fragment of ErbB4 in MMP7-expressing cells suggested the possibility that MMP7 mediated ErbB4 processing. On ligand binding, ErbB4 is internalized and processed by TACE at a juxtamembrane site, resulting in the generation of a 120-kDa ectodomain fragment (15, 16). The remaining 80-kDa ICD is subsequently released from the cell membrane by \gamma-secretase and can translocate to the nucleus (17). Analysis of ICD localization in the vector control and MMP7-expressing cell lines using both confocal microscopy and cytoplasmic/nuclear fractionation revealed that the ICD was detectable in the cytoplasm and nucleus of the MMP7-expressing cell lines (Fig. 2C). Next, we determined if metalloproteinase activity was mediating the distinct cytoplasmic and nuclear localization pattern of the ErbB4 ICD in the MMP7-expressing cell lines. PMA has been shown to promote ErbB4 processing (12). Treatment of the vector control cell lines with PMA resulted in a striking increase of cytoplasmic and nuclear ErbB4 ICD compared with the nontreated vector controls, whereas no effect was observed in the PMA-treated MMP7-expressing cell lines (Fig. 2D). Conversely, treatment with a broad spectrum metalloproteinase inhibitor, BB-94, had no effect on the vector controls but reduced the amount of cytoplasmic and nuclear localized ErbB4 ICD in the MMP7-expressing cell lines (Fig. 2D). Collectively, these data suggested that MMP7 potentially mediated ErbB4 processing resulting in the cytoplasmic and nuclear localization of the ICD.

To determine if ErbB4 was being processed in the vector control and MMP7-expressing cell lines in response to ligand binding, antibodies directed specifically to the extracellular domain and ICD of ErbB4 were used. Analysis of the conditioned medium from confluent two-dimensional cultures showed the presence of a 120-kDa fragment in the MMP7-expressing clones (Fig. 3A). Conversely, using immunoprecipitation, the ICD of ErbB4 was detected primarily in the lysates of the MMP7-expressing cells (Fig. 3A). TACE has been shown previously to generate the ectodomain of ErbB4 (12). Interestingly, TACE and MMP7 share several common substrates, such as TNF, Fas ligand, and receptor activator of nuclear factor-\(\kappa\)B ligand (10, 18, 19). Therefore, the possibility that MMP7 was responsible for the direct processing of ErbB4 was explored. Using a peptide sequence that mimicked the cleavable juxtamembrane domain of ErbB4, mass spectral analysis revealed that MMP7 was not capable of cleaving the peptide sequence. These results implied that TACE was potentially responsible for direct ErbB4 processing in the MMP7-expressing cell lines (Fig. 3B).

Immunoblot analysis identified mature TACE expression in the vector controls and MMP7-expressing cell lines (Fig. 3C). Short-term inhibition of MMP7 activity in the MMP7-expressing cells overnight using a neutralizing antibody confirmed that MMP7 was incapable of direct processing of ErbB4, whereas treatment with TIMP-3, an inhibitor of TACE and other MMPs, resulted in a robust decrease in the amount of ErbB4 ICD (Fig. 3D). These data suggest that the presence of TACE alone in the vector controls was insufficient for the processing of ErbB4 and required an additional event, such as activation of an upstream proteolytic cascade or solubilization of an ErbB4 ligand.

**HB-EGF solubilization is dependent on MMP7 activity.** ErbB4 is a cognate receptor for HB-EGF, a pluripotent cytokine that can mediate proliferation, differentiation, or apoptosis depending on the cellular context (20, 21). MMP7 has been shown previously to process HB-EGF to a soluble form (22). Under exponential two-dimensional culture conditions, HB-EGF mRNA was detected in the empty vector control and MMP7-expressing cell lines. However, soluble HB-EGF was detected only in the conditioned medium obtained from the MMP7-expressing cell lines (Fig. 4A and B) albeit at low levels. Given the difficulty in detecting soluble HB-EGF, we used an HBEGF-AP expression vector for further analysis. To identify the role of MMP7 in the solubilization of HB-EGF, the empty vector control and MMP7-expressing cell lines were transiently transfected with HBEGF-AP. Immunoblotting and
densitometry revealed no endogenous shedding of HBEGF-AP in the empty vector controls (Fig. 4C). However, soluble HBEGF-AP could be detected in the conditioned medium of the empty vector controls on the addition of recombinant active MMP7. Conversely, blocking MMP7 activity in the MMP7-expressing cell lines using a MMP7 neutralizing antibody reduced the amount of HBEGF-AP solubilization by 31%, whereas TACE and MMP inhibition with TIMP-3 had resulted in a 16% decrease in soluble HBEGF-AP (Fig. 4C). These data show that MMP7 is an important mediator of HB-EGF solubilization, a result that is consistent with previous reports (22).

Because MMP7 significantly enhanced cell growth under three-dimensional culture conditions, the expression of HB-EGF was analyzed via reverse transcription-PCR (RT-PCR). Under these conditions, only the MMP7-expressing cell lines were found to express HB-EGF (Fig. 4D). This suggests that MMP7 activity was important for HB-EGF expression in three-dimensional culture, potentially via the transcriptional regulation of the HB-EGF promoter by the ErbB4 ICD. However, whether the HB-EGF promoter is a target of the nuclear ErbB4 ICD remains to be determined. Next, we determined if the addition of exogenous HB-EGF to the vector controls would enhance the growth of the cells under three-dimensional conditions and found that the doubling time of the HB-EGF–treated cells was significantly shorter (D L2 HB-EGF, 53.6 ± 1.4 h; L4 HB-EGF, 51.4 ± 1 h; P = 0.0006; Fig. 4D). Collectively, these data show that MMP7, either directly or indirectly, is a key proteinase involved in the release of HB-EGF from the cell surface and that the resultant soluble form of HB-EGF increased cellular proliferation under three-dimensional culture conditions.

The ErbB4 ICD recapitulates MMP7 growth effects in vitro and in vivo. The ErbB4 receptor can mediate a wide range of effects, including apoptosis, differentiation, and proliferation (21). Treatment of the MMP7-expressing cell lines with γ-secretase

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Figure 2. ErbB4 expression, processing, and localization in MMP7-expressing cells. A, immunoblot analysis for ErbB receptor expression in the vector control (L2 and L4) and MMP7-expressing cell lines (M14 and M37). Actin was used as a loading control. Arrow, full-length ErbB4 at 200 kDa; arrowhead, the presence of an 80-kDa band in the MMP7-expressing cell lines (sc-283 antibody was used for direct immunoblotting). The molecular weight markers (in kDa) are labeled beside each panel. B, immunohistochemical staining for ErbB family members in tumors generated by orthotopic injection of the MMP7-expressing cell lines. Representative photomicrographs of negative rabbit isotype control (IgG), ErbB1, and ErbB4. Magnification, ×100. Tumors (n = 8) generated by the MMP7-expressing cell lines were scored as being positive or negative for the presence of the ErbB receptors using immunohistochemistry (IHC). C, ErbB4 localization (green) in the vector control and the MMP7-expressing cell lines was examined using confocal microscopy. Magnification, ×200. Side panels represent orthogonal views of the cells on the X to Z axes. Propidium iodide (red) was used as a nuclear stain. Cytoplasmic (actin control) and nuclear (histone H4 control) fractionation also revealed the presence of the 80-kDa ErbB4 ICD in each of these compartments. D, the localization of the ErbB4 receptor in the vector control cells (L2/L4) or MMP7-expressing cells (M14/M37) was examined on treatment with vehicle (DMSO), PMA (100 ng/mL for 1 h), or BB-94 (5 μmol/L for 3 h). All photomicrographs are at ×200 magnification. Arrows, cytoplasmic ErbB4; arrowheads, nuclear ErbB4.
inhibitors significantly reduced the three-dimensional growth rate of the MMP7-expressing cell lines, whereas no effect on the growth rate of the empty vector controls was observed (Supplementary Fig. S1). However, γ-secretase inhibition may also have interfered with the signaling of several other molecules such as members of the Notch family (23). Therefore, to specifically determine whether the ErbB4 ICD was capable of promoting cell growth, polyclonal and clonal vector control GFP and ICD-GFP (cyt-2 isoform)–expressing c57MG cell lines were generated. The cell lines were tested for GFP fluorescence (Fig. 5A). The ICD-GFP fusion product (105 kDa) and GFP alone (35 kDa) were detected in the ICD-GFP polyclonal cell lines by immunoblot analysis, suggesting that the ICD-GFP–fused protein may undergo proteolysis (Fig. 5A, inset). The overexpression of the ErbB4 ICD was also confirmed using immunofluorescence (data not shown). Analysis of the ICD-expressing and control cell line growth revealed that the ICD of ErbB4 significantly shortened the doubling time of the c57MG cells (D, GFPpoly, 122.8 ± 40.3 h; GFP 1, 74.1 ± 22.2 h; GFP 5, 76.5 ± 11.2 h; ICDpoly, 61.6 ± 3 h; ICD 1, 47.2 ± 3 h; and ICD 9, 67.5 ± 3.6 h; P = 0.0042; Fig. 5B).

The contribution of the ErbB4 ICD to c57MG tumorigenicity was determined via the orthotopic injection of the ICD-GFP and control GFP polyclonal cell lines into the cleared mammary fat pads of syngeneic C57BL/6 mice. After 3 weeks, histologic analysis using GFP fluorescence and immunohistochemical detection of the ErbB4 ICD revealed that no tumors formed in the control GFP injected mammary fat pads, whereas 90% of the ICDpoly injected mammary fat pads had evidence of tumor growth (Fig. 5C).

![Figure 3](image-url) **Figure 3.** MMP7 mediates ErbB4 processing via TACE. A, immunoblot analysis of conditioned medium from confluent vector control (L2 and L4) and MMP7-expressing (M14 and M37) cell lines for the presence of the soluble extracellular domain of ErbB4. Arrow, the soluble 120-kDa ErbB4 fragment. The T47-14 cell line was used as a positive control for ErbB4 shedding. The soluble 80-kDa ICD of ErbB4 was detected by immunoprecipitation and immunoblot analysis using antibodies directed toward the ICD domain of ErbB4. Arrowhead, ICD. B, A 12-residue peptide (YPWTGHSTLPQH) representing the cleavable juxtamembrane domain of the ErbB4 receptor (residues 646–657 of the JmA isoform) was incubated either alone or with TACE or with MMP7. Arrowhead, the full-length ErbB4 peptide; arrow, the processed ErbB4 peptide. m/z, mass to charge ratio. C, immunoblot analysis of mature TACE levels in the vector control and MMP7-expressing cell lines. D, analysis of ErbB4 ICD generation in the MMP7-expressing cell lines that were serum starved overnight in the presence or absence of 100 ng/mL recombinant active MMP7, 2 μg/mL of a MMP7 neutralizing antibody, or 0.5 μmol/L TIMP-3. Actin was used as a loading control.
Discussion

MMP7-expressing transgenic mice showed enhanced mammary gland proliferation and tumorigenicity (4). In the current study, we present a mechanism through which MMP7 can mediate these effects (Fig. 6). MMP7, HB-EGF, and ErbB4 have been shown to associate with the heparin sulfate proteoglycan–rich cell adhesion molecule CD-44 on the cell surface (22). MMP7 can process HB-EGF to an active form that subsequently interacts with the ErbB4 receptor (22). Binding of soluble HB-EGF to ErbB4 results in receptor activation and processing by TACE and γ-secretase to yield an active ErbB4 ICD (24). The ErbB4 ICD can mediate the effects of HB-EGF in two distinct but not mutually exclusive ways:

(a) by activating signaling pathways via the receptor tyrosine kinase domain and/or (b) via translocation to the nucleus to facilitate the expression of growth-related target genes. Using either of these pathways, the ErbB4 ICD has the potential to promote the proliferation and tumorigenesis of the c57MG cell lines, thus recapitulating the effects of MMP7.

The possibility that MMP7 mediates ErbB4 processing and tumorigenesis via alternative means also exists. For example, our data showed that the levels of other ErbB family members were also up-regulated, albeit not to the same extent as ErbB4. Therefore, the possibility that MMP7 processes other growth factors to soluble forms or that MMP7 proteolytically activates other proteinases that in turn promote cell proliferation cannot be ignored. However, the data presented herein shows that MMP7 mediates the processing of ErbB4 and that the generation of the ErbB4 ICD in response to upstream MMP7 activity is a principle mechanism through which MMP7 mediates mammary gland tumorigenesis.

MMP7-solubilized HB-EGF promotes cell growth via ErbB4. MMP7-solubilized HB-EGF enhanced c57MG proliferation under three-dimensional culture conditions that more closely mimicked the in vivo environment. Previous studies have shown that under two-dimensional conditions, although HB-EGF stimulates ErbB4 activation, there is no increase in cell proliferation (25). Our findings suggest that the three-dimensional environment is required for manifesting the proliferative effects of MMP7 and HB-EGF.

In addition to MMP7, TACE can solubilize HB-EGF to yield an active form of the cytokine (26). Although TACE was observed in both the vector controls and MMP7-expressing cell lines, no soluble HB-EGF was detected in the vector controls, suggesting that MMP7 activity was essential for the expression and solubilization of HB-EGF. Higher levels of TACE in the MMP7-expressing cell lines may be explained by a recent report that has documented increased TACE levels in response to EGF ligands (27), thus suggesting that MMP7-solubilized HB-EGF may stimulate also TACE expression. Based on these data, we propose that MMP7 and TACE work independently to mediate ErbB4 signaling, whereby MMP7 activity is required for the solubilization of ErbB4 ligands, such as HB-EGF, and TACE is required for generating the ectodomain of ErbB4.

The ICD of ErbB4 mediates the tumorigenic effect of MMP7. Although the possibility of the shed ectodomain of ErbB4 having a functional consequence in proliferation cannot be ruled out, in the current study, we showed that the ICD of ErbB4 promotes cell growth. This may be achieved via two potential means. First, the ICD of ErbB4 can chaperone factors, such as signal transducer and activator of transcription 5A (STAT5A), to the nucleus (31) and that crossing STAT5A-deficient animals into a transducer and activator of transcription 5A (STAT5A), to the nucleus (31) and that crossing STAT5A-deficient animals into a model of mammary gland tumorigenesis (WAP-TAg) results in a significantly slower tumor onset, suggesting that the nuclear STAT5A may promote tumor progression (32). Analysis of the localization of the ErbB4 ICD in the MMP7-expressing cell lines suggested that the cytoplasmic and nuclear forms may mediate the observed proliferative effects.
Recent studies have shown that the JmA-cyt-2 isoform of the ErbB4 receptor is overexpressed in ductal and lobular human breast cancers and that the transforming potential of the receptor depends on metalloproteinase processing of the receptor and the kinase activity of the ICD independent of ligand activation of the receptor (33). Although tumor cells may have evolved mechanisms to escape ligand dependence, our observations with a normal mammary gland epithelial cell line indicated that MMP7-mediated solubilization of ErbB4 cognate ligands, such as HB-EGF, is essential for the generation of the ErbB4 ICD.

The significance of MMP7 and ErbB4 expression in human breast cancer. MMP7 expression strongly correlates with early-stage human breast cancer (2, 3). The expression of MMP7 in a normal mammary gland cell line, c57MG, resulted in a marked increase in the proliferation and tumorigenesis of the cells that was consistent with our earlier observations (4). Determining the molecular mechanisms through which MMP7 mediated these effects revealed that HB-EGF solubilized by MMP7 activated the ErbB4 receptor, which in turn led to the generation of the ErbB4 ICD that could promote proliferation in vitro and in vivo. Therefore, our data suggest that the aberrant expression of MMP7 can contribute to the progression of early-stage human breast cancer.

ErbB4 is also frequently overexpressed in early-stage primary human breast cancers (34). However, given the pleiotropic effects of the ErbB4 receptor (21), studies have shown opposing conclusions as to the prognostic significance of ErbB4 expression in terms of disease-free survival (35–37). Our findings show that the ICD of ErbB4 alone can promote the tumorigenicity of normal mammary gland epithelial cells; therefore, clinical studies examining the correlation between ErbB4 expression and disease-free survival in human breast cancer should be reevaluated with careful selection of antibodies to distinguish between the full-length receptor and ICD.

Because MMP7 and ErbB4 are commonly overexpressed by the epithelial cells during the early stages of human breast cancer, we propose that MMP7 solubilization of ErbB4 ligands, such as HB-EGF, stimulates ErbB4 activation. Processing of the ligand-bound ErbB4 receptor by TACE subsequently leads to increased levels of the ErbB4 ICD in the cytoplasm and nucleus. Continual activation of this
mechanism can subsequently accelerate tumor growth. Our in vivo data are consistent with this hypothesis and point to both MMP7 and ErbB4 as potent effectors of mammary gland tumorigenesis.

Acknowledgments

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Conor C. Lynch, Tracy Vargo-Gogola, Michelle D. Martin, et al.


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