

Tumorigenesis and Neoplastic Progression

Matriptase Is Involved in ErbB-2-Induced Prostate Cancer Cell Invasion

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Deregulation of both ErbB-2 signaling and matriptase activity has been associated with human prostate cancer (PCa) progression. In this communication, we investigated the roles of both ErbB-2 signaling in matriptase zymogen activation and matriptase in ErbB-2-induced PCa malignancy. In a human PCa cell progression model, we observed that advanced PCa C-81 LNCaP cells exhibited an aggressive phenotype with increased cell migration and invasion capacity; these cells concurrently showed both enhanced ErbB-2 phosphorylation and increased matriptase zymogen activation compared with parental C-33 LNCaP cells. Moreover, ErbB2 activation, both ligand-dependent (eg, epidermal growth factor treatment) and ligand-independent (eg, overexpression), was able to induce matriptase zymogen activation in this cell line. Inhibition of ErbB-2 activity by either the specific inhibitor, AG825, in epidermal growth factor-treated C-33 LNCaP cells or ErbB-2 knockdown in C-81 LNCaP cells, reduced matriptase activation. These observations were confirmed by similar studies using both DU145 and PC3 cells. Together, these data suggest that ErbB-2 signaling plays an important role in matriptase zymogen activation. ErbB-2-enhanced matriptase activation was suppressed by a phosphatidylinositol 3-kinase inhibitor (ie, LY294002) but not by a MEK inhibitor (ie, PD98059). Suppression of matriptase expression by small hairpin RNA knockdown in ErbB-2-overex-

pressing LNCaP cells dramatically suppressed cancer cell invasion. In summary, our data indicate that ErbB-2 signaling via the phosphatidylinositol 3-kinase pathway results in up-regulated matriptase zymogen activity, which contributes to PCa cell invasion. (Am J Pathol 2010, 177:3145–3158; DOI: 10.2353/ajpath.2010.100228)

Prostate cancer (PCa) is one of the most common malignancies among men in Western countries and the incidence is progressively rising within low-risk populations including China, Japan, and Taiwan.^{1,2} The death of patients with PCa is mainly due to hormone refractory and metastatic disease,³ and metastatic ability is believed to be due to cancer cells gaining the ability to degrade the extracellular matrix.⁴ Overexpression of ErbB-2 has been reported and is believed to participate in oncogenic signaling in metastatic prostate cancer.^{5–9} ErbB-2 signaling has been shown to induce several phenotypic changes associated with more aggressive disease such as increased cell motility,¹⁰ cell proliferation,¹¹ and/or invasive ability.¹² Although no direct ligand for ErbB-2 has yet been identified, ErbB-2 activation is known to occur in both a ligand-independent and ligand-dependent manner.^{13–16} Ligand-dependent ErbB-2 activation involves heterodimerization of the receptor with another ligand-activated ErbB receptor (epidermal growth factor [EGF] receptor/ErbB-1, ErbB-3, and ErbB-4).^{17,18} For example, in prostate cancer cells, the ErbB ligand EGF induces the formation of both ErbB-1/ErbB-1 homodimers and ErbB-

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1/ErbB-2 heterodimers.¹⁹ Ligand-independent ErbB-2 activation is largely thought to be due to ErbB-2 overexpression, resulting in spontaneous homodimerization and autoactivation.^{20,21} Clinical data show that ErbB-2 protein levels are elevated in a subset of patients with clinically significant prostate cancer, especially in the recurrent, hormone-refractory phase of the disease.^{6,8,22} ErbB-2 overexpression promotes the survival and growth of prostate cancer cells through the MAPK and/or phosphatidylinositol 3-kinase (PI3K)/AKT pathway in the absence of androgens,^{8,23} and abnormal ErbB-2 activation due to overexpression may serve as one of the key factors leading to prostate cancer recurrence during hormone ablation therapy, and/or to promote prostate tumors with a highly metastatic potential.^{8,24} Thus, ErbB-2 overexpression in patients with prostate cancer has been associated with poor prognosis.²⁵ The exact nature of the contribution made by elevated ErbB-2 protein levels to prostate cancer cell invasion during the cancer progression is, however, still not well-characterized.

Cell surface proteolysis by secreted or plasma membrane-anchored proteases has been strongly implicated in cancer cell invasion and metastasis due to their roles in the degradation of the Extracellular matrix, cell growth, adhesion, and migration.²⁶ Recently, several lines of evidence have shown that deregulation of type II transmembrane serine proteases may play a role in many diseases and, in particular, may enhance tumor growth, invasion, and metastasis.²⁷⁻³¹ One of the best characterized type II transmembrane serine proteases is the enzyme matriptase.³²⁻³⁶ In human cancer, matriptase is expressed in a variety of epithelial-derived tumors including prostate, breast, colon, stomach, and ovarian carcinomas.³⁵⁻³⁸ In the prostate, matriptase expression is increased in primary prostate cancers and in metastatic lesions.³⁹ Immunohistochemical analysis has shown that increased matriptase protein level is correlated with tumor grade and poor prognosis.⁴⁰ Moreover, a role for matriptase in carcinogenesis is further supported by data from animal models. Overexpression of matriptase in the skin of transgenic mice, driven by a keratin-5 promoter, induces *ras*-independent multistage carcinogenesis and promotes *ras*-mediated, carcinogen-induced tumor formation.⁴¹ Recently, matriptase has been suggested to be involved in the activation process of the pro-oncogenic and prometastatic factors pro-uPA and pro-HGF/SF.⁴²⁻⁴⁵ Despite these varied observations, little is known about the role of oncogenic signaling in matriptase activation or how this might be involved in enhanced cancer malignancy during the progression of prostate cancer.

In this study, we explore the possible role that ErbB-2 signaling may play in the deregulation of cell-surface proteolysis through modulation of matriptase zymogen activation, and how the ErbB-2-matriptase axis may promote an invasive phenotype in prostate cancer cells.

Materials and Methods

Materials

Lipofectamine 2000 transfection reagent and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), Dulbecco's

modified Eagle's medium, and RPMI 1640 culture media were obtained from Hyclone (Logan, UT). Protein assay kits were from Bio-Rad (Hercules, CA). Protein molecular weight standard markers and an anti-Myc epitope antibody (Ab) were obtained from Bioman Scientific Company, Ltd (Taipei, Taiwan). Polyclonal anti-ErbB-2 Ab (C-18) for immunoblotting and horseradish peroxidase-conjugated anti-rabbit IgG Ab were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine (pY100), anti-phospho-p44/p42 ERK1/2 (Thr202/Tyr204), rabbit polyclonal anti-phospho-ErbB-2 (Y877), rabbit polyclonal anti-phospho-ErbB-2 (Y1221/Y1222), rabbit polyclonal anti-phospho-ErbB-2 (Y1248), anti-phospho Akt, and anti-Akt Abs were obtained from Cell Signaling Technology (Beverly, MA), and a polyclonal anti-Erk1/2 Ab was purchased from Millipore (Millipore, CA). The mouse monoclonal anti-phosphotyrosine (4G10) Ab was purchased from Upstate Biotechnology (Lake Placid, NY). The Enhanced Chemiluminescence (ECL) detection system was purchased from Pierce (Rockford, IL). AG825, LY294002, and PD98059 were from Calbiochem (San Diego, CA). EGF, heregulin- β 1, and all other chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted.

Cell Culture

The human prostate carcinoma cell lines DU145, C-33, and C-81 LNCaP cells were gifts from Dr. Ming-Fong Lin at the Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center (Omaha, NE). C-33 cells, C-81 cells, and DU 145 cells were maintained in a regular medium consisting of RPMI 1640 supplemented with 5% FBS, 1% glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. HEK 293T cells were used for lentiviral production. HEK 293T cells and PC-3 cells were gifts from Dr. Pei-Wen Hsiao, Institute of Agricultural Biotechnology Research Center, Academia Sinica, Taiwan, and cultured in Dulbecco's modified Eagle's medium with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Transient Transfection with ErbB-2, Constitutively Active MEK, and Akt (Myr-Akt) cDNA Constructs

Cells were seeded in regular culture medium at a density of 4×10^5 cells for C-81 LNCaP cells and 6×10^5 cells for C-33 LNCaP cells per 60-mm dish. One day after plating, transient transfection with wild-type ErbB-2, constitutively active MEK, or Akt plasmids was performed by Lipofectamine 2000 reagent in Optimum-Minimum Essential Medium (Invitrogen, Carlsbad, CA) medium, according to the manufacturer's instruction. The transfected cells were harvested 48 hours after transfection. The ErbB-2 and a constitutively active MEK expression constructs were gifts from Dr. Ming-Fong Lin.^{46,47} The Myr-Akt plasmid was a gift from Dr. Ruey-Hwa Chen, Institute of Biological Chemistry, Academia Sinica, Taiwan.

Immunoprecipitation

LNCaP cells (1×10^7 cells) were washed with PBS and a lysate was prepared by adding 1 ml of immunoprecipi-

tation assay buffer (50 mmol/L Tris-HCl, pH 7.8, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% deoxycholate, and 10 μ g/ml each of leupeptin, aprotinin, and phenylmethylsulfonyl fluoride) to the cells. The lysate was then centrifuged by using a microcentrifuge at 10,000 $\times g$ for 20 minutes. The supernatant was added to an anti-ErbB-2 Ab at 4°C for 1 hour. Protein-A/G-agarose beads (30 μ l; Calbiochem) were added to the lysate, and the mixture was incubated with shaking for 1 hour at 4°C. The beads were finally collected by centrifugation and washed three times with immunoprecipitation assay buffer. Proteins binding to the beads were eluted by adding 20 μ l of 2 \times electrophoresis sample buffer and analyzed by immunoblotting with an anti-phosphotyrosine Ab (4G10). After analysis the membranes were stripped and reblotted with an anti-ErbB-2 Ab to detect total protein levels.

Establishment of Stable ErbB-2-Overexpressing LNCaP Transfectants

Two days after transient transfection with the ErbB-2 plasmid or parent vector alone, the cells were treated with 400 μ g/ml Geneticin/G418 to select for stable integration of the plasmids. Stable pools of ErbB-2 or control transfected cells were maintained in a regular medium supplemented with 400 μ g/ml of Geneticin.

Lentiviral Particle Preparation and Infection for Small Hairpin RNA Delivery

Small hairpin RNAs (shRNA) in a lentiviral infection system were obtained from the National RNAi Core Facility of Academia Sinica, Nankang, Taiwan. shRNAs for the knockdown of ErbB-2 (shErbB-2, clone ID: TRCN 0000039879; shErbB-2u, clone ID: TRCN 0000303527), Akt1 (shAkt1-1, clone ID: TRCN 0000039795; Akt1-2, clone ID: TRCN 0000010174), Akt2 (shAkt2-1, clone ID: TRCN 0000265834; Akt2-2, clone ID: TRCN 0000255915), matriptase (shMTX, clone ID: TRCN 0000038053), or a negative control shRNA for knockdown of luciferase (shLuc) were all in the pLKO.1-puro vector and were packaged into lentiviral particles by using HEK 293T cells by co-transfection with pCMV Δ R8.91 and pMD.G, using Lipofectamine 2000, according to the recommended protocol. Conditioned medium from the transfected cells containing lentiviral particles was collected 24 and 48 hours after the addition of fresh medium. For lentiviral infection, cells were seeded at up to 70% confluence and cultured for 24 hours. Lentiviral infection was performed by adding 10% (v/v) of lentivirus-containing medium to the cell culture. Twenty-four hours after infection, transduced cells were selected by exposure to 1 μ g/ml puromycin for 3 days.

Western Blot

After washing twice with cold PBS (50 mmol/L potassium phosphate and 150 mmol/L NaCl, pH 7.2), the cells were lysed in a standard lysis buffer (1% Triton X-100 in PBS)

or a lysis buffer containing phosphatase and protease inhibitors (20 mmol/L HEPES [pH = 7.0], 0.5% NP-40, 2 mmol/L sodium vanadate, and 1 \times proteinase inhibitor cocktail; Roche Diagnostics GmbH, Mannheim, Germany). The lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatants were collected. Protein concentrations in the cell lysates were determined by Protein Assay solution (BioRad) with reference to a bovine serum albumin standard curve. Equal amounts of total protein were then mixed with Laemmli sample buffer for electrophoresis. Generally, 5% β -mercaptoethanol was added to the samples, which were then boiled for 10 minutes before loading on the gels; however, when blotting for matriptase or HAI-1, this was not done, because boiling disrupts matriptase-HAI-1 complexes, and reducing agents destroy the epitopes recognized by the monoclonal antibodies (mAbs).⁴⁸ Fifty micrograms of proteins per well were loaded and separated by SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes with a transfer buffer consisting of 25 mmol/L Tris-base, 192 mmol/L glycine, and 20% methanol. After protein transfer and between antibody incubations, the membranes were washed with Tris-Buffered Saline Tween-20 (TBST) wash buffer (20 mmol/L Tris-HCl [pH 7.5], 0.87% [w/v] NaCl, and 0.1% Tween 20). The membranes were blocked with 5% skim milk in TBST, and the specific proteins were recognized by primary antibodies and subsequently by secondary antibodies conjugated with Horseradish peroxidase (Jackson, PA) in 5% skim milk. The target proteins were revealed by enhanced chemiluminescent reagents (Pierce) and detected by X-ray films or a luminescent image analyzer with a CCD camera (LAS-4000; Fujifilm, Tokyo, Japan).

Wound Healing Assay

Cells for wound healing assays were cultured for 5 to 6 days in 60-mm Petri dishes until fully confluent. Wound gaps were made by scraping cell monolayers with a 10- μ l pipette tip, after which the cells were washed with PBS twice and fed with fresh culture medium. Images of wound gaps were taken by a light microscope (TS100; Nikon, Taipei, Taiwan) at 100 \times and the cells were incubated in a humidified, 37°C, 5% CO₂ incubator until subsequent images were taken. Widths of the wound and migratory distances were measured and calculated by using NIS-Elements D software (Nikon). Each observation was carried out in triplicate.

Transwell Invasion/Migration Assay

Transwell assays were performed according to previously described procedures with slight modification.⁴⁹ In brief, cell culture inserts (Millipore) were placed in the wells of 24-well plates (Corning Incorporated, Corning, NY). For an invasion assay, 10 μ g of Matrigel (BD Biosciences, San Jose, CA) diluted in 100 μ l distilled water was added to the filter of each insert, which was then air-dried overnight. The dried Matrigel was reconstituted

with 100 μ l serum-free RPMI 1640 medium for 1 hour before usage. No Matrigel was coated on filters for migration assay. Cells were serum-starved for 24 hours and then seeded in the upper chambers of transwell with 200 μ l serum-free RPMI 1640 medium. The lower chambers were filled with 600 μ l RPMI 1640 medium containing 10% FBS as a chemoattractant. After incubation for 48 hours, cells that had penetrated to the external surface of the filters were fixed in methanol for 10 minutes and stained with 1% crystal violet for 10 minutes. After washing with distilled water, the nonpenetrating cells on the inner surfaces of the upper chambers were wiped off by using cotton swabs. The cells that had penetrated were photographed (100 \times) and counted by light microscope (eight random 200 \times fields of each filter were observed). Each assay was carried out in triplicate.

Statistical Analysis

A mean \pm SE was calculated from three repeated groups in all experiments. A statistical significance between groups was determined by Student's *t*-test. A *P* value below 0.05 was considered as a significant difference between two groups.

Results

Androgen Refractory C-81 LNCaP Prostate Cancer Cells Exhibit Increased Motility and Invasive Behavior, and Have Higher Levels of ErbB2 and Matriptase Activation than the C-31 Prenatal Control Cells

Increased levels of both ErbB-2 and matriptase are correlated with more advanced human prostate cancer.^{6,8,40,50} This observation led us to wonder if increased ErbB-2 signaling might be mechanistically linked with some aspects of tumor progression by modulation of matriptase activity. We therefore set out to find a suitable experimental system that might allow us to evaluate this hypothesis. We started by evaluating the properties of a cell model based on the well known LNCaP prostate cancer cell line. Dr. Ming-Fong Lin at the Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center has developed two LNCaP sublines (C-33 and C-81 LNCaP cells) that have been shown to model prostate cancer tumor progression with increased ErbB-2 activity and prostate-specific antigen (PSA) secretion, and decreased PAcP expression and androgen dependence.^{51,52} We first examined the migratory/invasive abilities of the C-33 and C-81 LNCaP cells using modified Boyden chamber (transwell) assays, using uncoated or matrigel coated membranes. As shown in Figure 1A, C-81 LNCaP cells exhibited strong migration and invasion capability, compared with the parental LNCaP (C-33) cells. C-81 LNCaP cells therefore also model advanced human prostate cancer with respect to high cell migration and invasion capacity, compared with the C-33 LNCaP cells, which model early-stage cancer with low rates of cell migration and invasion. We then examined

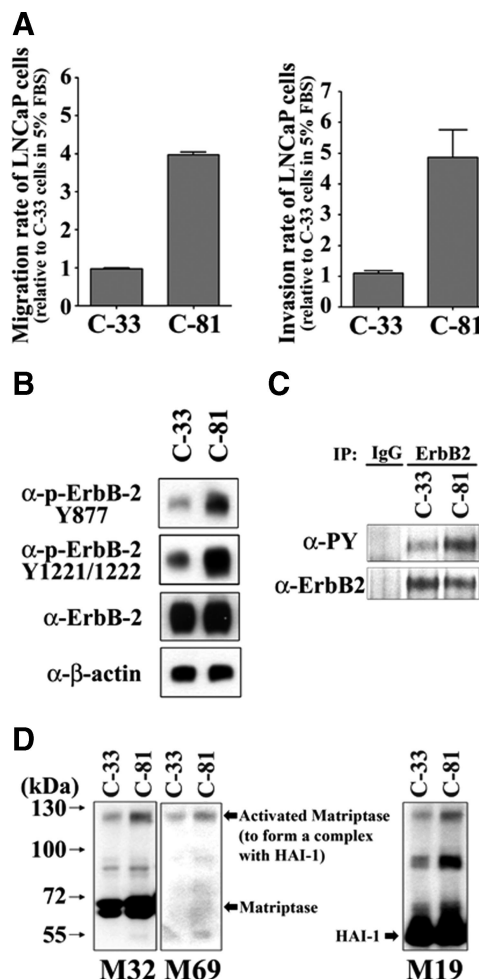


Figure 1. Migratory and invasive capabilities of different LNCaP cells and the activated levels of ErbB-2 and matriptase in those cells. **A:** Migratory and invasive capabilities of C-33 and C-81 LNCaP cells. After trypsinization, 4×10^5 of the cells were seeded in the upper chamber of each transwell coated without or with matrigel (30 μ g/cm²) in serum-free RPMI 1640 medium, and the lower chambers were filled with 5% FBS RPMI medium. Transwell migration assay was carried out for 24 hours. Migratory and invasive cells were fixed in methanol and stained with 0.25% crystal violet. Each assay was performed in triplicate for calculation of means \pm SE. **B:** Analysis of ErbB-2 expression and phosphorylation levels by immunoblotting assays. Cell lysates were collected with 0.5% NP-40 in HEPES buffer. The ErbB-2 tyrosine phosphorylation levels were detected by using anti-phospho-ErbB-2 Tyr877 and anti-phospho-ErbB-2 Tyr-1221/Tyr-1222 antibodies (Abs). Total ErbB-2 protein level was determined by using an anti-ErbB-2 Ab (C18). **C:** Whole cell lysates were immunoprecipitated with an anti-ErbB-2 antibody followed by immunoblotting with anti-pTyr (4G10) and anti-ErbB-2 Abs. **D:** Analysis of the levels of total and activated matriptase and HAI-1 in different LNCaP cells by immunoblotting assay. LNCaP cells were lysed in 1% Triton X-100 PBS and then collected under nonboiling and nonreducing conditions. The immunoblots for total matriptase, activated matriptase, and HAI-1 were conducted by using anti-total matriptase (M32), anti-activated matriptase (M69), and anti-HAI-1 (M19) mAbs.

the state of ErbB-2 and matriptase activation in the two sublines by immunoblot analysis. The activation of ErbB-2 was assessed by determining the degree of protein phosphorylation at specific tyrosine residues by using anti-phospho-ErbB-2 Tyr877 and anti-phospho-ErbB-2 Tyr-1221/Tyr-1222 Abs. The data generated showed that although both LNCaP sublines expressed similar levels of total ErbB-2 protein, the C-81 cells exhibited a significantly higher level of ErbB-2 activation compared with the C-33 cells (Figure 1B). The data were

confirmed by studies in which ErbB-2 was immunoprecipitated from the cells and then immunoblotted with an anti-phospho-Tyr antibody (Figure 1C).

To evaluate the levels of matriptase and its activated status within the LNCaP sublines, cell extracts were prepared and blotted with monoclonal antibodies that respectively recognize total matriptase (M32), the activated form of the enzyme (M69), and the cognate matriptase inhibitor HAI-1 (M19). The development and characterization of these antibodies has been described extensively in our previous publications,^{32,53,54} and their use allows simple assessment of overall levels of the enzyme and the degree of zymogen activation. The M32 antibody recognized the third LDLR domain of matriptase and detects levels of total matriptase as a band at 70 kDa, representing the latent zymogen, and a band at 120 kDa, representing the active enzyme in a complex with the inhibitor HAI-1. The M69 antibody is specific for the two-chain, active form of the enzyme, which is principally detected in the 120-kDa HAI-1-matriptase complex. The M19 antibody is specific to a region between the two kunitz domains of HAI-1 and detects this protein as a 50-kDa band representing free, uncomplexed HAI-1 and in the 120-kDa matriptase-HAI-1 complex. The data shown in Figure 1D demonstrate that although the overall level of matriptase is similar in the two LNCaP sublines, the level of matriptase activation appears to be significantly higher in the C-81 cells, compared with C-33 cells with the presence of a significantly more intense band at 120 kDa being detected by all three antibodies (Figure 1D). These data demonstrate that the C-81 cells are significantly more motile and invasive than the C-33 cells, and that both ErbB-2 and matriptase activation was also increased in the C-81 cells. This observation prompted us to explore the possible relationship between ErbB-2 activation, matriptase activation, and prostate cancer cell migration and invasiveness.

EGF-Induced ErbB2 Activation Stimulates Matriptase Zymogen Activation in C-33 LNCaP Prostate Cancer Cells

To begin to evaluate the possible effect of ErbB2 signaling on matriptase zymogen activation, we tested the ability of ErbB ligands to induce matriptase zymogen activation in PCa cells. LNCaP C-33 and C-81 cells were stimulated with one of two different Erb family ligands (EGF or heregulin- β 1), and their effects on matriptase activation were assessed by immuno-blot studies. As shown in Figure 2A, treatment with EGF but not heregulin- β 1 was able to dramatically induce matriptase zymogen activation in both cells with a reduced fold stimulation in C-81 LNCaP cells. The lower fold stimulation in C-81 cells may be due to a high basal level of activated matriptase in the cells. Thus, C-33 LNCaP cells were more sensitive to EGF stimulation on matriptase zymogen activation than C-81 LNCaP cells. To further examine the effect of EGF on matriptase zymogen activation and the role of ErbB-2 in this biological event, C-33 LNCaP cells were treated with EGF for different times. As shown in Figure 2B, after EGF administration, the activated level

of matriptase (indicated by the formation of 120-kDa matriptase/HAI-1 complexes) started to increase at 2 hours and decline after 24 hours. To confirm that this was an ErbB-2 mediated effect, cells were then treated with EGF for 2 hours in the presence or absence of a specific ErbB-2 inhibitor AG825. EGF-induced matriptase zymogen activation was dramatically reduced in the presence of the ErbB-2 inhibitor (Figure 2C), suggesting that ErbB-2 is indeed the receptor responsible for mediating EGF-inducing matriptase zymogen activation. Since shedding of matriptase into the extracellular environment is known to occur rapidly after activation, we determined if the decline in matriptase levels after prolonged EGF treatment was due to shedding of the activated enzyme. Levels of matriptase in the conditioned medium were assayed after 24-hour EGF treatment in the presence or absence of AG825. The data show that matriptase shedding was significantly increased after EGF-induced matriptase activation and that treatment with AG825 was able to inhibit EGF-induced matriptase shedding (Figure 2D). These data strongly suggest that ErbB-2 is involved in EGF stimulated matriptase zymogen activation in PCa cells. To further examine the role of ErbB-2 in matriptase zymogen activation of human PCa cells, C-81 LNCaP cells and PC-3 cells with high ErbB-2 activity (Figure 1, B and C, and Supplemental Figure S1 at <http://ajp.amjpathol.org>) were also treated with specific ErbB-2 inhibitor AG825. As shown in Figure 2E, treatment with AG825 efficiently inhibited matriptase zymogen activation in a dose-dependent manner. We then further addressed the role of ErbB-2 in prostate cancer cell migration and invasion. LNCaP C-81, PC3, and DU145 cells were treated with different concentrations of AG825 and cell migration and invasion assays were performed. The data presented in Figure 2, F and G, show that inhibition of ErbB-2 resulted in a reduction in the cell migration and invasion of these three PCa cells in a dose-response manner. These data suggest that ErbB-2 is involved in matriptase zymogen activation of PCa cells, as well as in their cell migration and invasion.

Enhanced ErbB-2 Activation Resulting from Overexpression of the Receptor Results in Enhanced Matriptase Zymogen Activation

To more directly explore the role of ErbB-2 in matriptase zymogen activation, we set out to test the effect of increased ErbB-2 expression on matriptase activation. LNCaP C-33 cells were transiently transfected with a wild-type ErbB-2 expression vector or with the vector alone as a control. Overexpression of ErbB-2 resulted in increased ErbB-2 activity, as indicated by receptor tyrosine phosphorylation levels at the specific residues Tyr877 and Tyr-1221/Tyr-1222. Interestingly, this increase in receptor activation was also associated with a concomitant increase in matriptase zymogen activation, with enhanced formation of the 120 kDa matriptase/HAI-1 complex (Figure 3A). A similar result was also obtained by using DU145 cells demonstrating that overexpression of

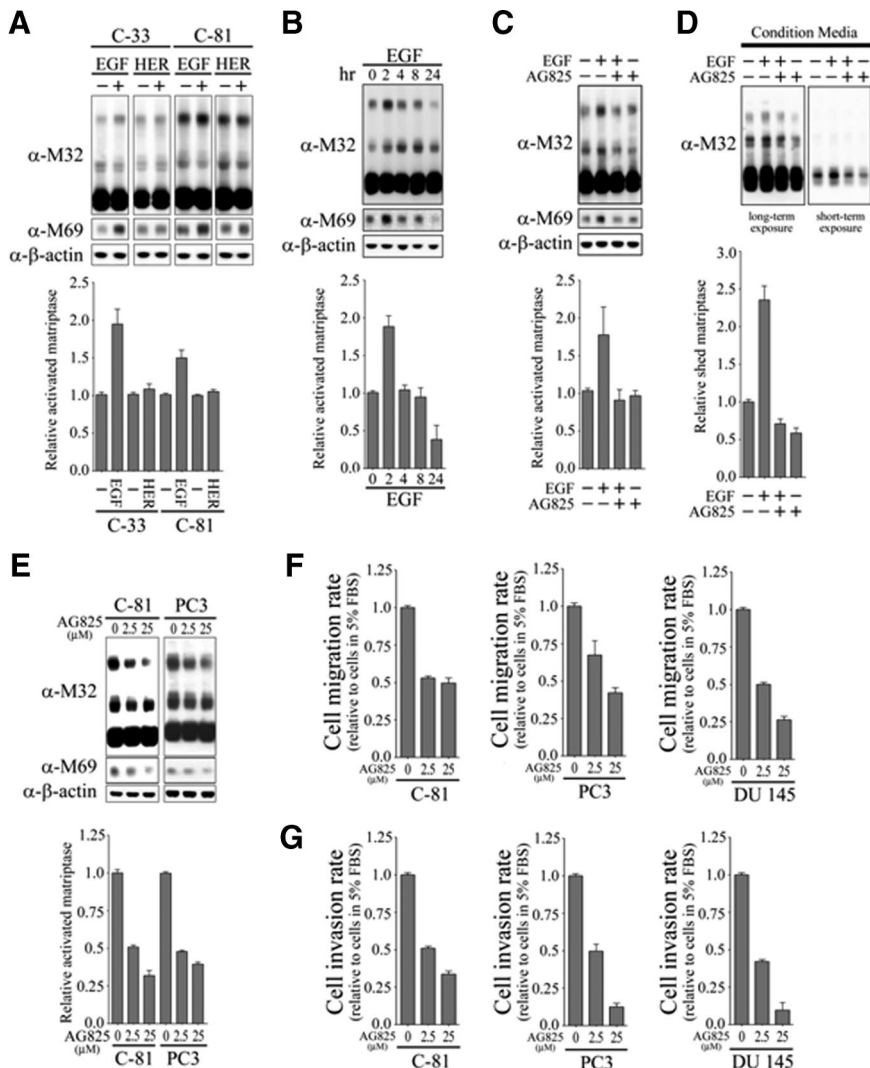


Figure 2. Effects of EGF and heregulin- β 1 on matriptase zymogen activation and shedding in C-33 LNCaP cells. **A:** Effects of EGF and heregulin- β 1 on matriptase zymogen activation in C-33 LNCaP cells. LNCaP C-33 cells were seeded at a density of 1×10^6 per well in each 6-cm dish. Two days after plating, cells were starved with serum-free medium for 36 hours. After treatment with 20 ng/ml EGF or 40 ng/ml heregulin- β 1 for 2 hours, cell lysates were collected under nonboiling and nonreducing conditions and immunoblotted for matriptase and activated matriptase with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. **B:** Kinetics of EGF-stimulated matriptase activation in C-33 LNCaP cells. Starved cells were treated with 20 ng/ml EGF for 0, 2, 4, 8, or 24 hours. Cell lysates were then prepared and used to assay total and activated matriptase with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. **C:** Serum-starved cells were treated with or without 20 ng/ml EGF in the presence or absence of 2.5 μ mol/L AG825 for 2 hours. The immunoblots were performed as described in **B**. **D:** Conditioned media were collected from the cells treated with or without 20 ng/ml EGF in the presence or absence of 2.5 μ mol/L AG825 for 24 hours. The immunoblots were performed as described in **B**. The levels of secreted matriptase were quantitated by using a densitometer and normalized to their respective cell numbers. **E:** The effect of AG825 on matriptase activation in C-81 LNCaP cells (**left panel**) and PC-3 cells (**right panel**). Starved cells were treated with AG825 at 0, 2.5, and 25 μ mol/L for 24 hours. The immunoblots were performed as described in **B**. The effects of AG825 on cell migration (**F**) and invasion (**G**). After cell seeding into transwells, LNCaP C-81, PC3, and DU145 cells were treated with the indicated concentrations of AG825. Cell migration and invasion assays were performed, according to the protocol, as described in Figure 1A.

ErbB-2 further promotes matriptase zymogen activation (Figure 3B). Thus, enhanced ErbB-2 activity caused by enforced overexpression of the receptor resulted in increased matriptase zymogen activation in PCa cells. These data raise the possibility that the enhanced ErbB-2 activation resulting from ErbB-2 overexpression, which has been shown to occur in a subgroup of human PCa patients, may lead to deregulation of matriptase activation, which in turn may participate in cancer progression and enhanced malignancy.

Suppression of ErbB-2 Protein Levels by shRNA Mediated Knockdown Results in Suppressed Matriptase Activation

To further establish a causal relationship between ErbB-2 levels and matriptase zymogen activation by using an independent approach, we tested the effect of suppressing ErbB2 protein levels on matriptase activation in the LNCaP C-81 cells. As we demonstrated in Figure 1, ErbB2 activity is much higher in these cells compared with the C-33 subline, and matriptase zymogen activation

is also very high in these cells. Transduction of the C-81 cells with an ErbB-2 targeting lentiviral vector resulted in a greater than 70% reduction in ErbB-2 protein levels compared with cells transduced with a control virus (Figure 3C, left panel). Concomitant with ErbB-2 knockdown, matriptase activation was also significantly suppressed, as shown by a reduction in the level of the 120 kDa matriptase/HAI-1 complex (Figure 3C, right panel). To control for potential off-target effect of the ErbB-2 shRNA and to further confirm the role of ErbB-2 in inducing matriptase zymogen activation, we confirmed our observation through the use of another independent shRNA construct (shErbB-2u), which is designed to target a specific ErbB-2 sequence located in the 3' UTR of ErbB-2, and through the use of a "rescue" strategy in which the levels of ErbB-2 expression were increased by transfection with an shRNA insensitive expression construct that does not contain the sequences targeted by shErbB-2u. As shown in Figure 3D, the reduction of ErbB-2 expression induced by shErbB-2u resulted in the inhibition of matriptase zymogen activation. This reduced matriptase activation was rescued by stable ErbB2 over-

expression with the shErbB-2u-resistant construct (Figure 3D). Taken together, these data strongly suggest that ErbB-2 is involved in the regulation of matriptase zymogen activation in PCa cells.

Signaling Through the ERK1/2 Pathway Does Not Appear to Be Involved in ErbB-2-Induced Matriptase Activation

We were next interested in trying to define the downstream signaling pathways responsible for ErbB-2 induced matriptase activation. The ERK1/2 pathway is known to be important in mediating downstream signaling from ErbB-2 in human prostate cancer cells.⁴⁷ To explore the role of the ERK1/2 pathway in ErbB-2-induced matriptase activation, we established stable pools of ErbB-2-overexpressing C-33 LNCaP cells. ErbB-2 activity in the transfectants was dramatically increased, as indicated by receptor tyrosine phosphorylation (Figure

4A). Consistent with our results from the transient transfection experiments described above (Figure 3A), this increased ErbB-2 activity was accompanied by enhanced matriptase activation. Using these cells, we tested the effect of various concentrations of the MEK inhibitor PD98059 on ERK1/2 activity and on matriptase zymogen activation. Interestingly, although the MEK inhibitor efficiently suppressed ErbB-2-induced ERK1/2 activity in a dose-response manner (Figure 4A, left panel), the compound had no significant effect on the level of matriptase activation (Figure 4A, right panel). To further analyze the possible role of ERK1/2 in matriptase zymogen activation in PCa cells, we transfected C-33 LNCaP cells with a constitutively activated MEK expression construct. As shown in Figure 4B, although expression of constitutively activated MEK resulted in abundant ERK1/2 activation, indicated by immunoblotting analysis with an anti-phosphoMAP kinase Ab, the increased ERK1/2 activity had no effect on matriptase activation. Together with the inhibitor studies shown in Figure 4A, these data strongly suggest that signaling through the ERK1/2 pathway is not responsible for ErbB-2-induced matriptase zymogen activation in PCa cells.

ErbB-2 Stimulation of Matriptase Activation Is Dependent on Phosphatidylinositol 3 Kinase Signaling

In addition to the MAPK pathway, ErbB-2 is known to signal through the PI3 kinase pathway and so we next set out to determine whether PI3 kinase signaling is respon-

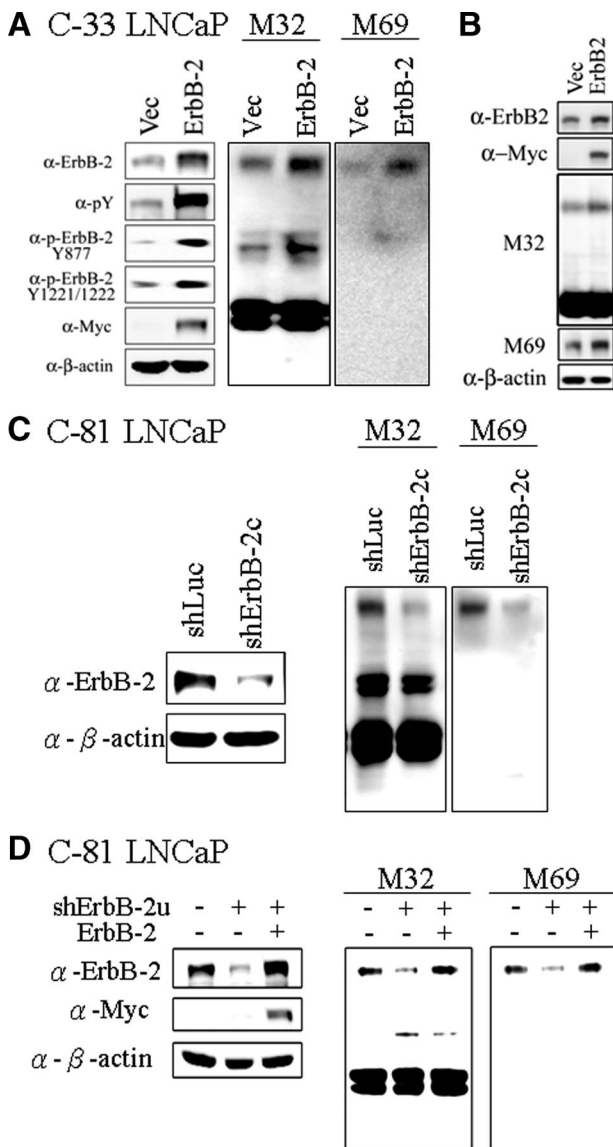


Figure 3. Effects of ErbB-2 level and activity on matriptase zymogen activation in PCa cells. **A:** Effects of ErbB-2 overexpression on matriptase activation in C-33 LNCaP cells. LNCaP C-33 cells were transiently transfected with ErbB-2 cDNA by Lipofectamine 2000 reagents, and control cells were transiently transfected with vector alone. Two days after transfection, cells were harvested for Western blot analysis. The separated proteins were transferred to a NC membrane and detected by immunoblotting with anti-pTyr (PY100), anti-ErbB-2 (C18), anti-c-Myc, and anti- β -actin (AC-15) Abs. For analyses of matriptase and HAI-1, nonreduced and nonboiled cell lysates were collected with Triton 1% X-100 in PBS, and immunoblotted with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. **B:** Effects of ErbB-2 overexpression on matriptase activation in DU 145 cells. DU 145 cells were transiently transfected with ErbB-2 cDNA, and the immunoblots were performed as described in **A**. **C:** Effects of ErbB-2 knockdown on activation of matriptase in C-81 LNCaP cells. Cells were seeded at a density of 1.2×10^6 per well in 6-cm dishes. One day after plating, cells were infected with lentiviral particles containing ErbB-2 shRNA for 24 hours. Control cells were infected with lentiviral particles containing luciferase shRNA. Three days after selection with 1 μ g/ml puromycin, cells were harvested for Western blot analysis. For analyses of the p-Tyr and protein levels of ErbB-2, cell lysates were collected and detected by anti-pTyr (PY100) and anti-ErbB-2 (C18) Abs. For analyses of matriptase and HAI-1, nonreduced and nonboiled cell lysates were collected with Triton 1% X-100 in PBS. Matriptase and HAI-1 were detected by immunoblotting with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. Equal protein loading was verified by blotting the membranes with an anti- β -actin Ab (AC-15). **D:** Effects of ErbB-2 re-expression on matriptase activation in ErbB-2-knockdown cells. C-81 LNCaP cells were infected with viral particles with control luciferase shRNA or ErbB-2u shRNA for 3 days. The ErbB-2u shRNA was designed to target a specific sequence located in the 3' UTR of ErbB-2. The ErbB-2- knockdown cells were transfected with control vector or ErbB-2 cDNA and cultured for 3 days. Cell lysates were collected and assayed by using an anti-ErbB-2 Ab (C18). For analyses of matriptase, cell lysates were collected as described in Figure 1D to detect the levels of total and activated matriptase by immunoblotting with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. Equal protein loading was verified by blotting the membranes with an anti- β -actin Ab (AC-15).

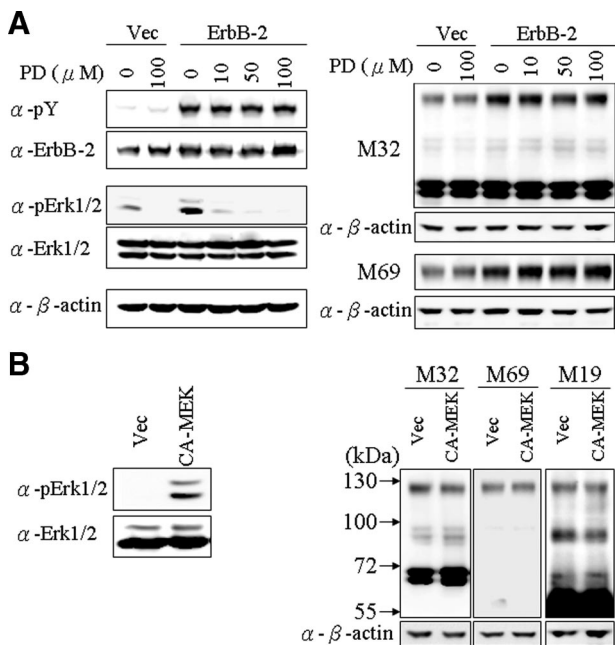


Figure 4. Role of MEK/ERKs in ErbB-2-induced matriptase activation in C-33 LNCaP cells. **A:** Stable ErbB-2-overexpressing C-33 LNCaP cells were seeded at a density of 6×10^5 per well in a 6-well plate. Two days after plating, cells were treated with PD98059 at concentrations of 0, 10, 50, and 100 $\mu\text{mol/L}$; control transfected cells were treated with concentrations of 0 and 100 $\mu\text{mol/L}$ PD98059. Treatment was carried out for 24 hours, and then cells were harvested for Western blot analysis. Cell lysates were collected with 0.5% NP-40 in HEPES buffer. The p-Tyr and protein levels of ErbB-2 were analyzed by immunoblotting with anti-pTyr (PY100) and anti-ErbB-2 (C18) Abs. The phosphorylation and protein levels of Erk1/2 were detected by anti-phosphoErk (Thr202 and Tyr204 of Erk1; Thr185 and Tyr187 of Erk2) and anti-Erk1/2 Abs. Nonreduced and non-boiled cell lysates were used for immunoblots to detect the levels of total and activated matriptase with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. Equal loading was evaluated with an anti- β -actin Ab. **B:** Effects of a constitutively active MEK on matriptase activation in C-33 LNCaP cells. Cells were seeded at a density of 1.2×10^6 per well in 6-cm dishes. Two days after plating, cells were transiently transfected with a constitutively active MEK (CA-MEK) cDNA and harvested 48 hours after transfection; control cells were transfected with vector alone. Cell lysates were collected with 0.5% NP-40 in HEPES buffer. The phosphorylation and protein levels of Erk1/2 were analyzed by immunoblotting with an anti-phosphoErk (Thr202 and Tyr204 of Erk1; Thr185 and Tyr187 of Erk2) and anti-Erk1/2 Abs. Nonreduced and non-boiled cell lysates were used to assay the activation status and total matriptase levels with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. An anti- β -actin (AC-15) Ab was used to evaluate protein loading.

sible for ErbB-2 stimulated matriptase zymogen activation in PCa cells. To do this we again made use of the stable ErbB-2-overexpressing C-33 LNCaP cells, treating them with different concentrations of the PI3 kinase-specific inhibitor LY294002. As shown in Figure 5A, treatment with LY294002 efficiently inhibited ErbB-2-induced PI3 kinase activity, as indicated by the suppression of the phosphorylation of its downstream target Akt. Suppression of PI3K activity also simultaneously blocked ErbB-2-induced matriptase zymogen activation in a dose-dependent manner (Figure 5B). To further examine the possible role of Akt in matriptase zymogen activation in PCa cells, we transiently transfected C-33 LNCaP and DU145 cells with a constitutively active Akt1 (Myr-Akt) and control plasmids. As shown in Figure 5C, the expression of the constitutively active Akt enhanced the level of activated matriptase. Since several Akt iso-

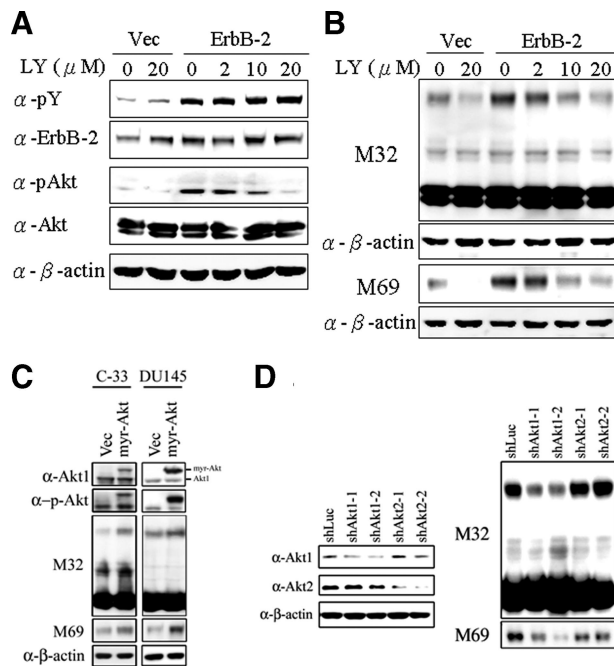


Figure 5. Role of PI 3 kinases/Akt in ErbB-2-induced matriptase activation in C-33 LNCaP cells. Stable ErbB-2-overexpressing C-33 LNCaP cells were seeded at a density of 6×10^5 per well in a 6-well plate. Two days after plating, cells were treated with LY294002 at concentrations of 0, 2, 10, and 20 $\mu\text{mol/L}$; control cells were treated with concentrations of 0 and 20 $\mu\text{mol/L}$ PD98059. Treatment was carried out for 24 hours, and then cells were harvested for Western blot analysis. **A:** Cell lysates were collected with 0.5% NP-40 in HEPES buffer and evaluated by immunoblots for p-Tyr and protein levels of ErbB-2, performed as described previously. The phosphorylation and protein levels of Akt were detected by anti-phosphoAkt (Ser473) and anti-Akt1/2 Abs. **B:** Nonreduced and nonboiled cell lysates were used for immunoblotting to detect the activation status and total matriptase with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. An anti- β -actin (AC-15) Ab was used to evaluate protein loading. **C:** Role of a constitutively activated Akt (Myr-Akt) in matriptase zymogen activation in C-33 LNCaP cells and DU 145 cells. LNCaP C-33 cells and DU 145 cells were transiently transfected with Myr-Akt and control plasmids by using Lipofectamine 2000. Two days after transfection, cell lysates were collected and analyzed by immunoblotting with anti-phosphoAkt (Ser473), anti-Akt1/2, anti-total matriptase (M32), and anti-activated matriptase (M69) Abs, respectively. **D:** Effect of Akt1 or Akt2 knockdown on activation of matriptase in LNCaP C-81 cells. Cells were seeded at a density of 1.2×10^6 per well in 6-cm dishes. One day after plating, cells were transfected with Akt1 shRNAs (shAkt1-1 and shAkt1-2), Akt2 shRNAs (shAkt2-1 and shAkt2-2), and control luciferase shRNA with Lipofectamine 2000. Cell lysates were collected for analysis of the levels of Akt1 and Akt2 protein by immunoblot with anti-Akt1 and anti-Akt2 Abs. For analyses of matriptase, cell lysates were collected and immunoblotted with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. Equal protein loading was assessed with an anti- β -actin Ab (AC-15).

forms are known to play distinct roles in tumor cell growth, invasion, and migration,^{55,56} and Akt1 and Akt2 are the forms predominantly expressed in PCa cells,^{57,58} we set out to identify which Akt isoform was involved in regulating matriptase zymogen activation by using small-interfering RNA technology to specifically knock down Akt1 and Akt2 and examining the effect on matriptase zymogen activation. As shown in Figure 5D, Akt1 but not Akt2 appears to be important for matriptase zymogen activation in PCa cells. These data indicated that signaling through the PI3 kinase/Akt1 pathway is responsible for ErbB-2-induced matriptase zymogen activation in these cancer cells.

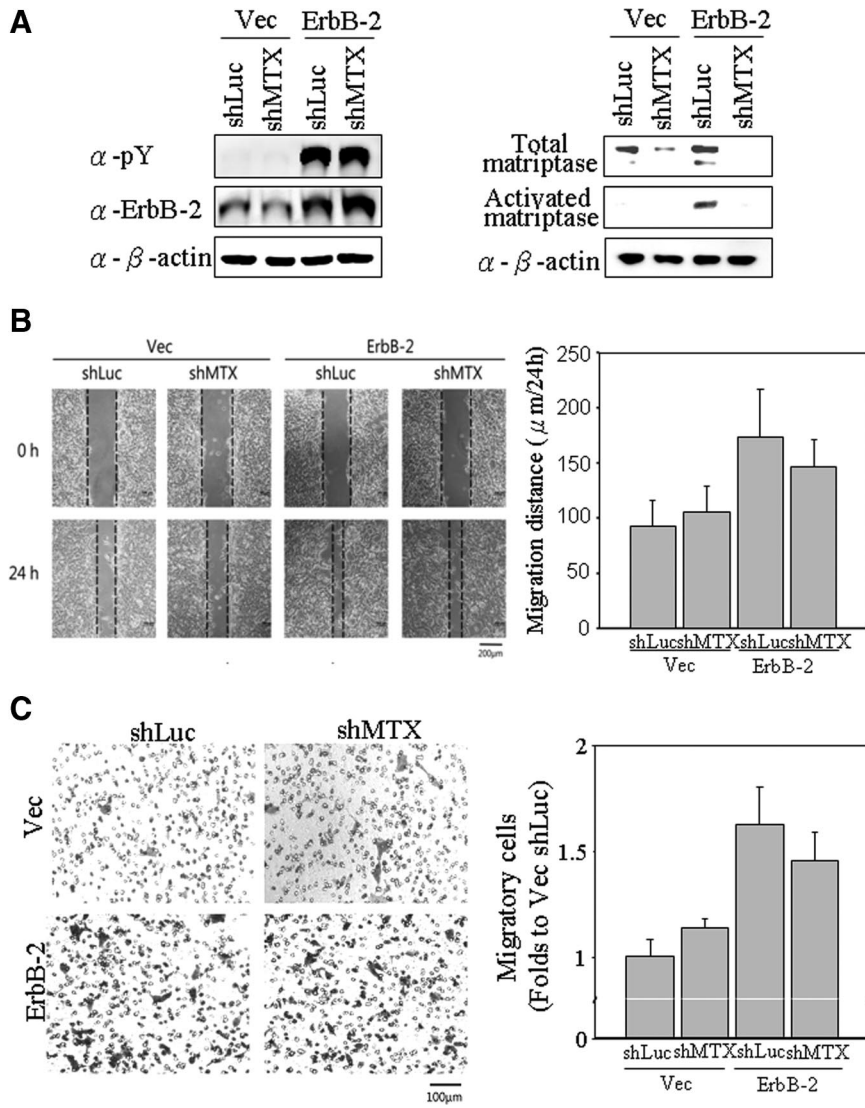


Figure 6. Effects of matriptase knockdown on ErbB-2-promoted cell migration of prostate cancer cells. Cells were seeded at a density of 1.2×10^6 per well in 6-cm dishes. Cells were infected by lentiviral particles with shRNAs specific to matriptase for 24 hours, selected by $1 \mu\text{g/ml}$ puromycin for 72 hours and then harvested for Western blot analysis. **A:** Cell lysates were collected with 0.5% NP-40 in HEPES buffer. The p-Tyr and protein levels of ErbB-2 were detected by anti-pTyr (PY100) and anti-ErbB-2 (C18) Abs. Nonreduced and nonboiled cell lysates were used for immunoblotting to detect the activation status and total matriptase with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. Loading was analyzed with an anti- β -actin mAb (AC-15). **B:** Effects of matriptase knockdown on ErbB-2-promoting cell motility by wound-healing assays. Wounds with widths of approximately $250 \mu\text{m}$ were made by scraping by using $10\text{-}\mu\text{l}$ pipette tips. Cells were incubated for 24 hours for wound-healing assay. Images were captured by a light microscopy with a magnification of $100\times$. The **dotted lines** define the edges of the wounds. Migratory distances (widths at 0 hours to widths at 24 hours) were analyzed by a NIS-Elements D software (Nikon) and are represented as means \pm SE calculated from triplicates; a statistically significant difference ($*P < 0.05$) was observed between Vec/shLuc and ErbB-2/shLuc. **C:** Effects of matriptase knockdown on ErbB-2-promoting cell motility by transmigration assays. After trypsinization, 1×10^5 cells were seeded with serum-free RPMI 1640 medium in each of the upper chambers, and the lower chambers were filled with 10% FBS RPMI 1640 medium. Transwell migration assay was carried out for 48 hours. Migratory cells were fixed in methanol and stained with 1% crystal violet, and images were captured by a light microscopy (original magnification, $\times 100$). Amounts of migratory cells on each filter were counted from eight random fields (original magnification, $\times 200$). Each assay was performed in triplicate for calculation of means \pm SE; a statistically significant difference, $*P < 0.05$ was observed between Vec shLuc and ErbB-2 shLuc.

Potential Role of Matriptase in ErbB-2-Induced Prostate Cancer Progression

Several studies have recently shown that ErbB-2 overexpression is associated clinically with metastatic potential in prostate cancer,⁵⁰ and that matriptase is a potential oncoprotein related to prostate cancer progression.⁴⁰ In the light of our data demonstrating the relationship between ErbB-2 activity and matriptase activation, we wondered if matriptase might play a role in ErbB-2-induced prostate cancer malignancy. To begin to explore this possibility, we decided to evaluate the role of matriptase in various aspects of the pathogenic behavior of control or ErbB-2 overexpressing C-33 LNCaP cells through the use of lentiviral-shRNA mediated matriptase knockdown. As shown in Figure 6A, transduction of the control (Vec) or ErbB-2 overexpressing (ErbB-2) C-33 LNCaP cells with the matriptase-targeting (shMTX) lentiviral constructs suppressed matriptase levels to less than 20% of the level in cells transduced with a control lentivirus (shLuc). As seen before, the cells overexpressing ErbB-2

exhibit high levels of activated matriptase. Transduction with the matriptase targeting shRNA construct also dramatically suppressed the levels of activated matriptase in these cells. The effect of this matriptase knockdown on ErbB-2-inducing cell motility, migration, and invasion was then evaluated as shown in the following experiments.

Matriptase Does Not Contribute to ErbB-2-Induced Cell Motility of Prostate Cancer Cells

To determine whether matriptase plays a role in ErbB-2-induced cell motility, we performed wound healing and transwell migration assays by using the control and ErbB-2-overexpressing LNCaP cells. Results from the wound healing assays showed that the ErbB-2-overexpressing LNCaP cells exhibited greater motility than control cells (Figure 6B, Vec shLuc versus ErbB-2 shLuc), consistent with the notion that ErbB-2 overexpression is clinically associated with malignant progression in prostate cancer.⁵⁰ Reduction in matriptase activity, however, had no

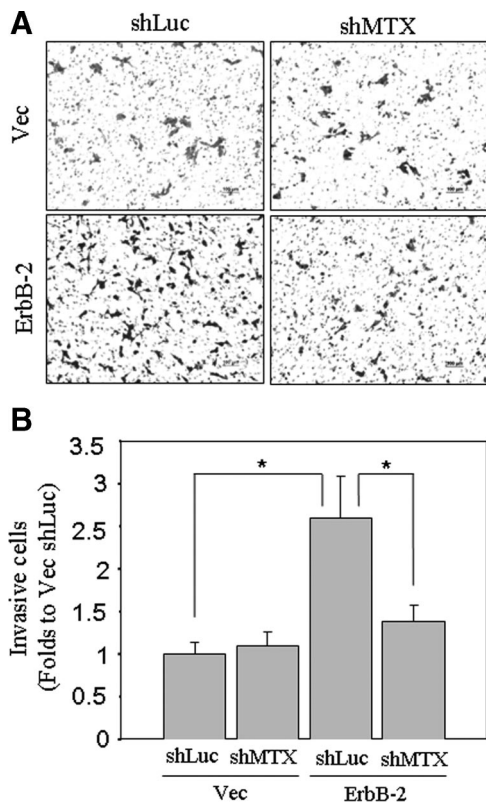


Figure 7. Invasion assay of ErbB-2-overexpressing LNCaP cells with or without matriptase knockdown. Matriptase knockdown was performed as described in Figure 6A. For cell invasion assays, each filter insert was coated with 30 $\mu\text{g}/\text{cm}^2$ matrigel. After trypsinization, 1×10^5 cells were seeded with serum-free RPMI 1640 medium in each insert chamber, and lower chambers were filled with 10% FBS RPMI 1640 medium. **A:** Transwell invasion assays were carried out for 48 hours. Invasive cells were fixed in methanol and stained with 1% crystal violet. Images were captured by a light microscopy (original magnification, $\times 100$). **B:** Numbers of invasive cells on each filter were counted from eight random fields (original magnification, $\times 200$). Each assay was performed in triplicate for calculation of means \pm SE; statistically significant differences, $*P < 0.05$ were observed between Vec/shLuc and ErbB-2/shLuc, as well as ErbB-2/shLuc and ErbB-2/shMTX.

significant affect on the motility of the ErbB-2-overexpressing LNCaP cells (Figure 6B, ErbB-2 shLuc versus ErbB-2 shMTX). Similarly, results from the transwell migration assays failed to demonstrate any effect of reduced matriptase levels/activity on ErbB-2-promoted transmigration of prostate cancer cells (Figure 6C, ErbB-2 shLuc versus ErbB-2 shMTX). Taken together, these data suggest that matriptase is most likely not involved in ErbB-2-induced cell motility in prostate cancer.

Matriptase Is Involved in ErbB-2-Induced of Prostate Cancer Cell Invasion

The invasive ability of cancer cells allows them to penetrate through the ECM, which is a determinant step for metastasis.³⁰ To examine the role of matriptase in ErbB-2-induced cancer cell invasion, we made use of the widely used matrigel-coated Boyden chamber invasion assay to assess the invasive properties of the cells. ErbB-2 overexpression greatly enhanced the invasive ability of the C-33 LNCaP cells (Figure 7, A and B, Vec

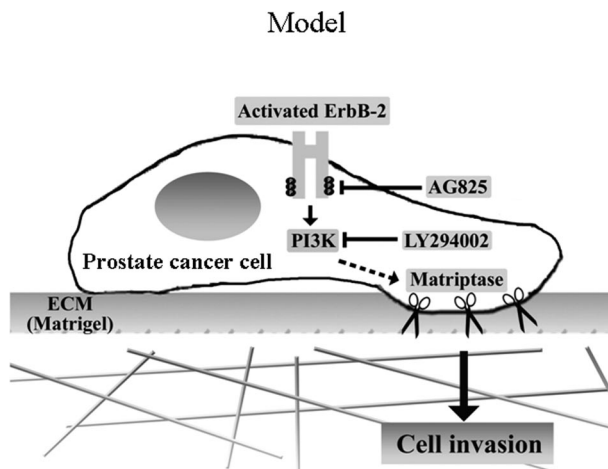


Figure 8. A model for the role of matriptase in ErbB-2-driven prostate cancer cell invasion. ErbB-2 hyperactivation by EGF stimulation or receptor overexpression results in the up-regulation of matriptase zymogen activation, at least in part via PI 3 kinase, leading to enhanced prostate cancer cell invasion.

shLuc versus ErbB-2 shLuc). Interestingly, shRNA-mediated matriptase knockdown almost completely abrogated the increased invasive potential of the ErbB-2 overexpressing cells (Figure 7, A and B, ErbB-2 shLuc versus ErbB-2 shMTX), suggesting that matriptase plays an important role in ErbB-2 stimulated invasiveness of human prostate cancer cells.

Discussion

Cancer metastasis is the driving cause of poor prognosis in prostate cancer. In this report, hyperactivation of ErbB-2 was shown to result in prostate cancer cells having increased invasive and migratory phenotypes. Activation of ErbB-2 by treatment with EGF or by overexpression also resulted in the up-regulated matriptase zymogen activation, mediated at least in part through the PI3 kinase pathway. Furthermore, we found that suppression of matriptase levels/activation was sufficient to block ErbB-2-induced prostate cancer cell invasion, while not affecting cancer cell motility. These observations suggest a mechanism by which dysregulation of these two proteins might interact to drive malignant progression in prostate cancer. A tentative model for this interaction is presented in Figure 8. Enhanced ErbB-2 signaling resulting from receptor overexpression and/or increase levels of ErbB ligands leads to enhanced down-stream signaling through the PI3 kinase pathway, resulting in increased matriptase zymogen activation and increased PCa cell invasion (Figure 8). Our data suggest that aberrant ErbB-2 activation or overexpression promotes prostate cancer progression, at least in part due to up-regulation of matriptase activation promoting cell invasion, consistent with the clinical observations that dysregulation of ErbB-2 by EGF-related signaling or overexpression, as well as dysregulation of matriptase activity all contribute to prostate cancer progression to an invasive phenotype.

Although ErbB-2 has been shown to be overexpressed in several carcinomas, including breast and ovarian cancers, the assessment of ErbB-2 overexpression in prostate cancer is controversial with published frequencies for ErbB-2 overexpression in primary carcinomas ranging from 0% to 100%.^{59–63} The discrepancy in reports of ErbB-2 expression in prostate cancer may be due to tissue selection and preparation, antibodies, methods, and the various scoring systems used. In 2000, Signoretti et al⁸ used the standardized Food and Drug Administration-approved Dako immunohistochemical assay to stain prostate cancer tissues and used the absolute scoring system to define the ErbB-2 protein overexpression as samples with $\geq 10\%$ of tumor cells with complete membrane staining. Their results showed that elevated expression of ErbB-2 protein was observed in 25% of untreated primary tumors, 59% of localized tumors after total androgen ablation therapy, and 78% of metastatic tumors with bone metastasis. A similar result was also found by Osman et al.⁶ Together, this suggests that the protein level of ErbB-2 could be elevated in a subgroup of recurrent, hormone-refractory prostate cancer. In prostate tumor tissue, the results from the chromogenic in situ hybridization showed that ErbB-2 gene is not amplified, as reported by several groups.^{8,63–66} Thus, it is clear that ErbB-2 gene is not amplified in prostate carcinomas although the protein level may be elevated in a subgroup of patients, especially with metastasis.^{6,8} During the progression of human prostate cancer cells, ErbB-2 activity increases with no significant protein alteration.⁵¹ In this study, our data showed that increased ErbB-2 activity resulting from EGF stimulation or receptor overexpression induced matriptase zymogen activation, leading to prostate cancer cell invasion.

Several lines of evidence support the hypothesis that ErbB-2 and matriptase are both related to cancer metastasis and malignancy.^{44,67,68} The expression level of ErbB-2 has been reported to be associated with the progression of prostate cancer to a hormone-refractory state after androgen deprivation therapy in patients,^{6,7} and clinically, prostate tissues with ErbB-2 overexpression exhibit high metastatic potential.^{5,6,8,9,50,69} These findings are further supported by the fact that ErbB-2 signaling can induce cell proliferation and PSA secretion in prostate cancer cells,^{46,47} and can accelerate progression to androgen independence in castrated animals.⁷⁰ In this study, we also show, using a model system of prostate cancer progression (Figure 1A; the C-33 cells compared with the C-81 cells), that progression is associated with increased ErbB-2 and matriptase activities (Figure 1, B–D). This observation is consistent with the finding that tissue levels of ErbB-2 and matriptase are elevated following the progression of prostate cancer in patients.^{6,7,40} Thus, the LNCaP cell model also imitates the progression of human prostate cancer from a low to high capability for cell migration and invasion.

The activation of ErbB-2 can occur in a ligand-dependent manner or a ligand-independent manner. ErbB-2 possesses a functional tyrosine kinase domain, but no direct acting ligand for the receptor has been identified to date, and it is believed that ErbB-2 needs to form a

heterodimer with another ligand-activated ErbB receptor to be activated in a ligand-dependent fashion.^{13–16} In prostate cancer cells, we found that EGF but not heregulin- $\beta 1$ was able to induce matriptase zymogen activation and that ErbB-2 kinase activity was important for EGF-induced matriptase activation and shedding. This is the first report, therefore, to show that matriptase activation is induced by EGF and that this activation is most likely mediated through epidermal growth factor receptor/ErbB2 heterodimers in prostate cancer cells. It is not clear why heregulin- $\beta 1$ had no significant effect on matriptase activation (Figure 2A). One possible explanation may relate to the physiological function of heregulin- $\beta 1$ as a paracrine differentiation factor, which is able to promote differentiation of hormone-sensitive PCa cells, to inhibit cell growth,^{71–73} or to induce PCa cell apoptosis, although it can increase the formation of ErbB-2/ErbB-3 heterodimers.⁷⁴ Recent reports have also shown that heregulin is mainly present in normal human adult prostate and benign prostatic hyperplasia with little expression in advanced PCa cells.^{72,73} The data suggest that ErbB-2 mediates signaling from a growth-promoting factor, EGF, but not a differentiation-induced factor, heregulin- $\beta 1$, to enhance matriptase zymogen activation. On ligand stimulation, ErbB-2 exhibits differentially physiological roles dependent on what type of ligand stimulation.

It has been reported that ligand-independent ErbB-2 activation is principally due to overexpression in prostate cancer, which is associated with the cancer progression. Our data demonstrate that enhanced ErbB-2 activation resulting from overexpression leads to increased matriptase activation, via a PI3 kinase mediated pathway, thereby increasing the proteolytic capacity and invasive potential of the cells, and so providing a possible mechanism for the enhanced pathogenesis associated with ErbB-2 overexpression (Figures 5 and 7). This observation provides a molecular linkage between two oncogenic proteins, ErbB-2 and matriptase, which contributes to cancer cell invasion. ErbB-2-induced matriptase activation may directly increase digestion of extracellular matrix proteins, such as fibronectin and laminin,⁷⁵ and activate uPA, matrix metalloproteinase (MMP)-3, PAR-2, and proHGF,^{45,76} all culminating in enhanced cancer cell invasion and cancer metastasis. Thus, matriptase may represent a good candidate for targeted therapy to prevent cancer cells from invading adjacent tissues and metastatic dissemination, particularly in the context of ErbB-2- or EGF-promoting cancer malignancy.

It has been shown that PI3 kinase signaling, a signaling pathway activated by ErbB-2, is involved in the stimulation of PCa cell invasion,⁷⁷ through the up-regulation of several MMPs, including membrane type 1 matrix metalloproteinase, MMP-2, and MMP-9.^{78,79} In this study, our data demonstrate that matriptase activity was up-regulated by via ErbB-2/PI3 kinase/Akt1 signaling (Figures 3, 5, and 6), and that this was important for ErbB-2-promoted cell invasion (Figure 7). These findings suggest that matriptase may be an important recipient of signaling from ErbB-2/PI3 kinase/Akt1 resulting in an increase in proteolytic activity, which promotes prostate cancer cell

invasion. This is a particularly appealing possibility since, based on other evidence, matriptase is believed to be a key regulatory protease, responsible for initiating a cascade of proteolytic activity, increasing the activity of several down-stream enzymes.^{45,48,80}

In addition to acting through the PI3 kinase pathway, ErbB-2 is known to signal through the MAP kinase pathway, and both PI3 kinase and Erk1/2 mediating ErbB-2 signaling are believed to participate in the transformation of prostate cancer from an androgen-sensitive phenotype to a hormone-refractory state.^{23,47,81} However, no involvement of Erk1/2 was detected in ErbB-2-induced matriptase activation (Figure 4), leading us to conclude that PI3 kinase, and not Erk1/2, is an important downstream mediator of ErbB-2 induced matriptase zymogen activation and prostate cancer cell invasion.

Matriptase zymogen activation requires two proteolytic processes.⁵⁴ The first proteolytic cleavage occurs at Gly149 of the SEA domain and is required to generate the 70-kDa latent form of matriptase. The second proteolytic cleavage at Arg614 occurs in the protease domain of latent matriptase and is required to initiate serine protease activity. The second cleavage is believed to occur through an autoactivation process and is a determinant step for matriptase activation.⁵⁴ It is an intriguing possibility that ErbB-2/PI3 kinase signaling may promote matriptase autoactivation by posttranslational modification, such as phosphorylation, of the protease or some other protein.^{82,83} The ErbB-2-induced PI3 kinase pathway may directly phosphorylate serine/threonine residues on the intracellular regions of matriptase and/or HAI-1, which then initiate the autoactivation process. Analysis of the matriptase and HAI-1 sequences for potential Akt-phosphorylation sites using the NetPhosK prediction engine⁸⁴ failed to find any consensus sites (RXRXXS/T) within the intracellular regions of the proteins. Akt has, however, been reported to phosphorylate nonconsensus sequences,^{85–87} and so it is possible that Akt can modulate the proteolytic activity through action on noncanonical sites on either matriptase or HAI-1. Another possibility is via indirect downstream kinase signaling of Akt to phosphorylate the Ser3 or Ser24 residue of matriptase and up-regulate the protease activity. Alternatively, ErbB-2/PI3 kinase signaling may cause the recruitment of matriptase and HAI-1, and/or activate other regulatory proteins resulting in the formation of activation foci, the locus of zymogen activation.⁸⁸ Thus, ErbB-2/PI3 kinase signaling may serve as an organizer to coordinate cell surface proteases for tissue remodeling or to abnormally up-regulate them to promote cancer cell invasion. More experiments are needed to clarify the molecular mechanisms for matriptase zymogen activation.

In conclusion, our results indicate that ErbB-2 activation by EGF or overexpression can stimulate matriptase zymogen activation, which contributes to prostate cancer cell invasion. The results may explain, in part, the observation that ErbB-2 overexpression or increased matriptase activity during PCa progression is clinically associated with a high metastatic potential and poor prognosis. These data provide compelling evidence for a functional linkage from ErbB-2 signaling to matriptase activity in cell invasion and

strongly suggests that matriptase could be a valuable target for the development of novel therapeutic approaches to prevent or suppress prostate cancer cell invasion and metastasis.

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