Downregulation of matriptase suppresses the PAR-2/PLCγ2/PKC-mediated invasion and migration abilities of MCF-7 breast cancer cells

JEONG-MI KIM[1*], JINNY PARK[2*], EUN-MI NOH[3], HYUN-KYUNG SONG[3], SANG YULL KANG[4], SUNG HOO JUNG[4], JONG-SUK KIM[1], HYUN JO YOUN[4] and YOUNG-RAE LEE[3,5]

[1]Department of Biochemistry, Jeonbuk National University Medical School, Jeonju, Jeollabuk 54896; [2]Department of Internal Medicine, Division of Hematology, Gil Medical Center, Gachon University College of Medicine, Incheon 405-760; [3]Department of Oral Biochemistry, School of Dentistry, Wonkwang University, Iksan, Jeollabuk 570-749; [4]Department of Surgery, Research Institute of Clinical Medicine, Jeonbuk National University Hospital, Jeonbuk National University and Biomedical Research Institute, Jeonju, Jeollabuk 560-182; [5]Integrated Omics Institute, Wonkwang University, Iksan, Jeollabuk 570-749, Republic of Korea

Received June 9, 2021; Accepted September 13, 2021

DOI: 10.3892/or.2021.8198

Abstract. Matriptases, members of the type II transmembrane serine protease family, are cell surface proteolytic enzymes that mediate tumor invasion and metastasis. Matriptase is highly expressed in breast cancer and is associated with poor patient outcome. However, the cellular mechanism by which matriptase mediates breast cancer invasion remains unknown. The present study aimed to determine the role of matriptase in the protein kinase C (PKC)-mediated metastasis of MCF-7 human breast cancer cells. Matriptase small interfering RNA-mediated knockdown significantly attenuated the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced invasiveness and migration of MCF-7 cells, and inhibited the activation of phospholipase C γ2 (PLCγ2)/PKC/MAPK signaling pathways. Matriptase-knockdown also suppressed the expression of MMP-9 and inhibited the activation of NF-κB/activator protein-1 in MCF-7 cells. Additionally, GB83 [an inhibitor of protease-activated receptor-2 (PAR-2)] inhibited PKC-mediated MMP-9 expression and metastatic ability in MCF-7 cells. Furthermore, downregulation of matriptase suppressed TPA-induced MMP-9 expression and invasiveness via PAR-2/PLCγ2/PKC/MAPK activation. These findings shed light on the mechanism underlying the role of matriptase in MCF-7 cell invasion and migration ability, and suggest that matriptase modulation could be a promising therapeutic strategy for preventing breast cancer metastasis.

Introduction

Breast cancer is a malignant tumor with a high mortality rate (1), which can be attributed primarily to invasion and metastasis. One of the primary approaches to treating breast cancer metastasis has been the development of effective anti-invasive agents (2,3). The initial steps of metastasis include cellular invasion through the degradation of the extracellular matrix (ECM), followed by the migration of cancer cells to other organs through the surrounding tissues (4,5). The ECM comprises collagens, laminins, glycoproteins, proteoglycans/glycosaminoglycans and fibronectin (6). It is degraded by extracellular proteases, of which MMPs play an important role in breast cancer (4,5).

Matriptases are members of the type II transmembrane serine protease (TTSP) family and are expressed in the epithelial compartments of all tissue types (7). Matriptase dysregulation is involved in various epithelial carcinomas, such as breast, prostate, colon, ovarian, uterine, cervical and skin cancers, where it is reportedly upregulated (8,9). Matriptase was first discovered in breast cancer cell lines, where it is highly expressed (10,11); however, despite its importance in breast cancer (12,13), the mechanism underlying its effects on breast cancer metastasis is unclear.

Protease-activated receptor-2 (PAR-2), a G protein-coupled receptor 11, induces various intracellular signaling pathways by activating endogenous serine proteinases, including matriptase (14-17). Previous studies have shown that matriptases are important activators of PAR-2 (16,18). When PAR-2 binds to a G protein, it produces diacylglycerol (DAG) and

Correspondence to: Professor Hyun Jo Youn, Department of Surgery, Research Institute of Clinical Medicine, Jeonbuk National University Hospital, Jeonbuk National University and Biomedical Research Institute, 567 Baekje daero, Deokjin, Jeonju, Jeollabuk 560-182, Republic of Korea
E-mail: yhj0903@jbnu.ac.kr

Professor Young-Rae Lee, Department of Oral Biochemistry, School of Dentistry, Wonkwang University, 460 Iksandae-ro, Iksan, Jeollabuk 570-749, Republic of Korea
E-mail: mindyr@wku.ac.kr

*Contributed equally

Key words: matriptase, protease-activated receptor-2, protein kinase C, MMP-9, invasion, breast cancer

activates canonical phospholipase C (PLC)/Ca^{2+}/protein kinase C (PKC) signaling or extracellular signal-regulated kinase-1/2 (19-21). Furthermore, PAR-2 levels are elevated in breast cancer, which plays a key role in regulating cellular migration by MAPKs (22,23).

MMPs are a family of zinc-dependent endopeptidases that consist of six subclasses: Collagenases, stromelysins, gelatinases, matrilysins, membrane-associated MMP and other MMPs (24). MMP-9 is involved in cancer cell infiltration and is directly associated with poor patient prognosis and the metastasis of breast cancer (25,26). Therefore, the regulation of signaling pathways to inhibit MMP-9 expression may play an important role in the treatment of various malignancies, including breast cancer (27-31). The expression of MMP-9 is induced by various stimuli, including cytokines, growth factors and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [10] (32-36). In particular, TPA is known to stimulate MMP expression by activating PKC in breast cancer cells (27,28,33). Furthermore, several studies have indicated that TPA activates PKC by activating PLC. In breast cancer invasion, TPA-induced MMP-9 expression is known to be induced by activation of NF-κB and activator protein-1 (AP-1) (37,38), transcription factors whose expression is regulated by MAPKs (39,40).

In the present study, the regulatory role of matriptase in TPA-induced MMP-9 expression, as well as invasion and migration, were investigated using MCF-7 breast cancer cells. Furthermore, to confirm the signaling mechanism of matriptase, the association between PAR2 and PLC/PKC was investigated. These results may provide a potential strategy for the treatment of breast cancer metastasis.

Materials and methods

Cell lines and culture. The human MCF-7 breast cancer cell line was purchased from the American Type Culture Collection. The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (antibiotic-antimycotic, 100X; Gibco; Thermo Fisher Scientific, Inc.), and maintained in a humidified incubator at 37˚C (5% CO_2).

Reagents. TPA (cat. no. P1585) and DMSO were obtained from Sigma-Aldrich (Merck KGaA). Matrigel was acquired from Corning, Inc. The PAR-2 antagonist (GB83) was purchased from Axon Medchem LLC, and BAPTA-AM was obtained from Invitrogen (Thermo Fisher Scientific, Inc.).

Western blot analysis. MCF-7 cells ($5x10^7$) were transfected with matriptase siRNA for 24 h. Additionally, cells ($7x10^5$) were treated with BAPTA-AM or GB83 for 1 h, and then incubated with TPA for 24 h at 37˚C. Total protein was extracted from cells using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) containing protease and phosphatase inhibitors (Calbiochem; Merck KGaA). The lysates were centrifuged at 16,000 x g for 10 min at 4˚C, and the protein concentrations were evaluated using the BioSpec-nano spectrophotometer (Shimadzu Corporation). The samples (20 µg) were separated by 10% SDS-PAGE and then transferred to Hybond™ polyvinylidene fluoride membranes (Cytiva). The membranes were blocked with 5% BSA (bovine serum albumin) or 5% skim milk buffers for 2 h at 4˚C, and then incubated with the following primary antibodies (all 1:2,500) overnight at 4˚C: Anti-β-actin (cat. no. A5441; Sigma-Aldrich; Merck KGaA); JNK (cat. no. 9252), p38 (cat. no. 9212), ERK (cat. no. 9102), IκB kinase α (IKKα; cat. no. 2682), IKKβ (cat. no. 2678), phosphorylated forms of PLCγ2 (cat. no. 3874), JNK (cat. no. 9251), p38 (cat. no. 9211), ERK (cat. no. 9101), c-Jun, IκBα (cat. no. 2859) and IKKαβ (cat. no. 2697) (all Cell Signaling Technology, Inc.). PLCγ2 (cat. no. SC-5283), p50 (cat. no. SC-7178), IκBα (cat. no. SC-371), MMP-9 (cat. no. SC-12759) and proliferating cell nuclear antigen (cat. no. SC-7907) (all Santa Cruz Biotechnology, Inc.). PKCα (cat. no. ab32376), PKCβ (cat. no. ab32026), PKCδ (cat. no. ab182126) and anti-sodium ATPase plasma membrane loading control (cat. no. ab76020) (all Abcam). Matriptase-specific antibodies were obtained from R&D Systems (cat. no. MAB3946). The blots were washed in TBS with 0.2% Tween-20 and then incubated with secondary HRP (horseradish peroxidase)-conjugated anti-mouse (cat. no. SC-2005) or anti-rabbit (cat. no. SC-2004) antibodies (1:2,500; both Santa Cruz Biotechnology, Inc.) for 1 h at 4˚C. Immunoreactive bands were detected using Luminol HRP Substrate Reagent (EMD Millipore) with a Mini HD6 Image Analyzer and Alliance 1D (UVItec Cambridge; Cleaver Scientific Ltd.). Immunoreactive bands were quantified using ImageJ software (Version 1.53k; National Institutes of Health).

RNA isolation and reverse transcription-quantitative (RT-q) PCR. RT-qPCR was performed using the StepOnePlus™ Real-time PCR System and SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Total RNA was isolated from MCF-7 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA concentration and purity were determined by absorbance at 260/280 nm. Complementary DNA was synthesized from 1 µg total RNA using the PrimeScript™ RT Reagent Kit (Takara Bio, Inc.) according to the manufacturer's instructions. The primers were: MMP-9 forward, 5'-CCTGGAGACCTGAGAACCAATCT-3' and reverse, 5'-CCACCCGAGTGTAACCATAGC-3'; and GAPDH forward, 5'-ATGGAAATCCCATCACCATCTT-3' and reverse, 5'-CGCCCCACTTGATTTTGG-3'. mRNA expression levels were normalized to those of GAPDH. The qPCR cycling conditions were as follows: Initial denaturation at 95˚C for 10 min, 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, followed by a melting curve ranging from 95˚C for 15 sec, 60˚C for 1 min, to 95˚C for 15 sec. Relative quantitation was performed using the comparative $2^{-\Delta\Delta Cq}$ method (41).

Small interfering RNA (siRNA) transfection and preparation of cytosolic and nuclear protein extracts. MCF-7 cells were transfected with 100 pmol matriptase siRNA or negative control siRNA (Shanghai GenePharma Co., Ltd.) using Lipofectamine® RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C (5% CO_2), and then incubated with TPA for 3 h at 37˚C. The sequences of human siRNA were as follows: Matriptase siRNA, 5'-GUGUCCAGAAGGUCUUCAATT-3' (sense) and 5'-UUGAAGACCUUCUGGACACTT (antisense); control siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). Cytoplasmic and nuclear extracts were prepared from

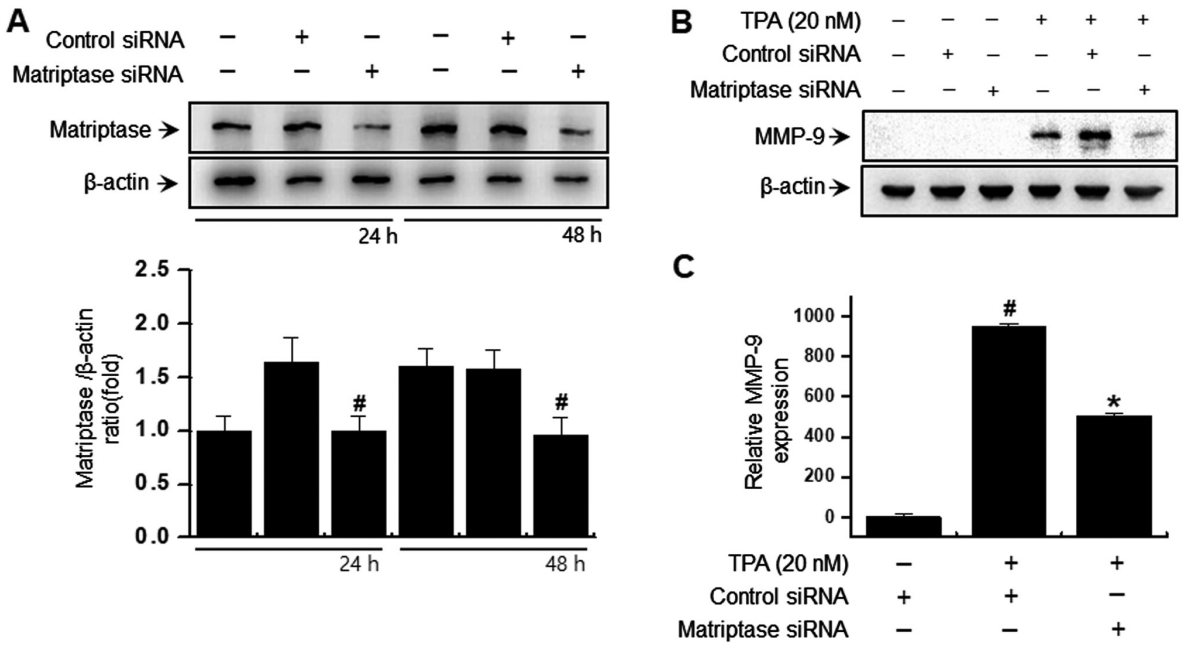

Figure 1. Effect of matriptase on the TPA-mediated expression of MMP-9 in MCF-7 cells. (A) MCF-7 cells were transfected with control and matriptase siRNAs for 24 and 48 h, and matriptase expression was confirmed by western blot analysis. (B) Transfected MCF-7 cells were treated with TPA for 24 h, and the levels of MMP-9 protein expression were detected by western blotting. (C) Total RNA was isolated from MCF-7 cells and MMP-9 mRNA levels were detected using reverse transcription-quantitative PCR. Data represent the mean ± SEM of three independent experiments. #P<0.01 vs. untreated control; *P<0.01 vs. TPA. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; siRNA, small interfering RNA.

the cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Dual-luciferase reporter assay. Cells transfected with matriptase siRNA were then transfected with the NF-κB/AP-1 luciferase reporter plasmid (Agilent Technologies, Inc.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. At 24 h post transfection, the cells were treated with 20 nM TPA for 4 h at 37˚C. Whole-cell lysates were prepared, and luciferase activity was measured using the Dual-luciferase Reporter Assay Kit (Promega Corporation) and Lumat LB 9507 Luminometer (Berthold Technologies GmbH & Co.KG). Relative Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Membrane fractionation. MCF-7 cells ($5x10^7$) were transfected with matriptase siRNA for 24 h. Additionally, $7x10^5$ cells were treated with BAPTA-AM or GB83 for 1 h and then incubated with TPA for 1 h at 37˚C. The cells were mixed with homogenization buffer (20 mM Tris-HCl, 2 mM EDTA, 5 mM EGTA, 5 mM DTT and protease inhibitor; pH 7.5) and homogenized using a sonicator (5 times for 10 sec, each at 10% amplitude) and incubated on ice for 30 min. To separate the soluble (cytosolic) and pellet (membrane) fractions, the cell lysate was centrifuged at 16,000 x g for 15 min at 4˚C. The pellet fraction was incubated in a solubilization buffer (homogenization buffer containing 1% NP-40) for 30 min on ice, and then centrifuged at 16,000 x g for 15 min at 4˚C.

Cellular invasion and migration assays. The invasion assay was carried out in 24-well chambers (pore size, 8 μm) coated with 20 μl Matrigel (diluted in DMEM) for 30 min at 37˚C; Matrigel Basement Membrane Matrix (Corning, Inc.) was rehydrated in 0.5 ml DMEM for 2 h immediately prior to experimentation. The top chamber was seeded with medium (0.5 ml; 10% FBS and 1% antibiotics) with $3x10^5$ resuspended cells transfected with matriptase siRNA, while the lower chamber was filled with medium containing TPA alone or combined with GB83. A migration assay was performed using chambers without Matrigel. Cells transfected with control and matriptase siRNA were added to the upper chamber and medium with TPA alone or with GB83 was added to the bottom chamber. Cells were allowed to invade/migrate to the lower membrane for 24 h (37˚C). After incubation, the cells on the upper membrane surface were removed with cotton swabs. The migrated/invasive cells were fixed with formaldehyde solution (3.6%) for 10 min, stained with crystal violet for 20 min (both at room temperature), and counted in five random fields per chamber at x10 magnification, using a Leica DM ILLED inverted microscope (Leica Microsystems).

Statistical analysis. Data are presented as the mean ± SD of ≥3 independent experiments. Statistical analysis was performed using ANOVA with Scheffe's post hoc test (SAS software, version 9.3: SAS Institute Inc.), and P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of matriptase suppresses TPA-induced MMP-9 expression in MCF-7 breast cancer cells. Western blotting and RT-qPCR were used to determine the effect of matriptase on TPA-induced MMP-9 expression. Intracellular matriptase expression was suppressed by transfection with matriptase siRNA (Fig. 1A), which inhibited the protein/mRNA

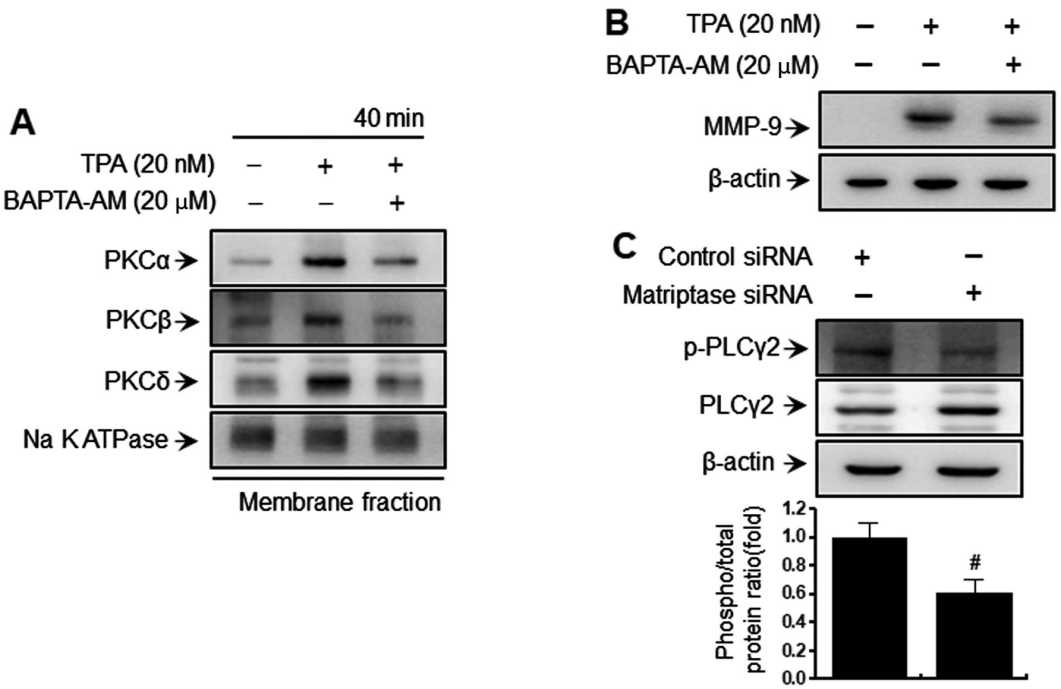

Figure 2. Effect of BAPTA-AM on TPA-induced PKC activation and MMP-9 expression, and the effect of matriptase on PLCγ2 phosphorylation, in MCF-7 cells. MCF-7 cells were treated with BAPTA-AM (20 μM) and TPA for (A) 40 min (to evaluate PKC activation) or (B) 24 h (to analyze MMP-9 expression). PKC isozyme and MMP-9 protein levels were detected by western blotting in MCF-7 cells. (C) MCF-7 cells were transfected with control and matriptase siRNAs for 24 h, after which PLCγ2 activation/levels of p-PLCγ2 were detected using western blotting. Data represent the mean ± SEM of three independent experiments. $^{\#}$P<0.01 vs. control siRNA-treated. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; siRNA, small interfering RNA; PLC, phospholipase C; p-, phosphorylated; Na K ATPase, sodium/potassium ATPase.

levels of TPA-induced MMP-9 (Fig. 1B and C). These results suggested that matriptase was involved in TPA-induced MMP-9 expression.

Downregulation of matriptase reduces PLCγ2 phosphorylation in MCF-7 breast cancer cells. Activation of PKC isozymes is mediated by DAG and Ca^{2+} (42). Therefore, intracellular calcium levels are important for MMP-9 expression and cellular metastasis through TPA-mediated PKC activation. In the present study, it was confirmed that an intracellular calcium chelator (BAPTA-AM) inhibited TPA-induced PKC activation (Fig. 2A) and MMP-9 expression (Fig. 2B). In addition, matriptase-knockdown suppressed PLCγ2 phosphorylation (Fig. 2C) in MCF-7 cells. These findings indicated that intracellular calcium levels are important for PKC-mediated MMP-9 expression, and that matriptase downregulation inhibited MMP expression and metastatic ability by regulating PLCγ2-mediated intracellular calcium levels.

Matriptase regulates TPA-induced PKC activation, as well as the MAPK and IKK signaling pathways in MCF-7 breast cancer cells. Previous studies have shown that PKC and the MAPK and IKK signaling pathways are involved in the expression of MMP-9 induced by TPA (38). In the present study, the effects of matriptase on PKC and the MAPK and IKK signaling pathways were confirmed. To determine whether matriptase affects PKC activation in TPA-induced MCF-7 breast cancer cells, membrane translocation levels of PKCα, PKCβ and PKCδ were evaluated. As shown in Fig. 3A, matriptase-knockdown attenuated TPA-mediated PKC membrane translocation. Furthermore, matriptase-knockdown reduced MAPK phosphorylation (p38, ERK and JNK) at 30 min post-TPA treatment (Fig. 3B), confirming the effect of matriptase on MAPK activation by TPA. In addition, matriptase-knockdown suppressed p-IKKαβ and p-IκBα levels and the degradation of IκBα in the cytoplasmic fraction, which confirms the role of matriptase on the NF-κB signal transduction cascade (Fig. 3C). These findings suggested that matriptase is involved in the activation of PKC and the MAPK and IKK signaling pathways through TPA-induced expression of MMP-9 in MCF-7 breast cancer cells.

Matriptase-knockdown decreases TPA-mediated activation of NF-κB and AP-1 in MCF-7 breast cancer cells. To elucidate the mechanism by which matriptase inhibits TPA-induced MMP-9 expression, the effect of matriptase siRNA on TPA-induced NF-κB and AP-1 activation was evaluated using a luciferase reporter assay. First, western blot analysis was used to confirm that matriptase-knockdown suppressed p50 levels in the nuclear fraction (Fig. 4A). Furthermore, TPA induced the phosphorylation of c-Jun, a major subunit of AP-1, and matriptase-knockdown inhibited the phosphorylation of c-Jun (Fig. 4A). Also, matriptase siRNA treatment inhibited TPA-stimulated NF-κB/AP-1 binding in a luciferase assay (Fig. 4B and C). These findings demonstrate that matriptase regulated the expression of MMP-9 induced by TPA through the NF-κB and AP-1 pathways in MCF-7 breast cancer cells.

Matriptase-knockdown inhibits TPA-mediated migration and invasiveness of MCF-7 breast cancer cells. In previous study, upregulation of MMP-9 has been associated with

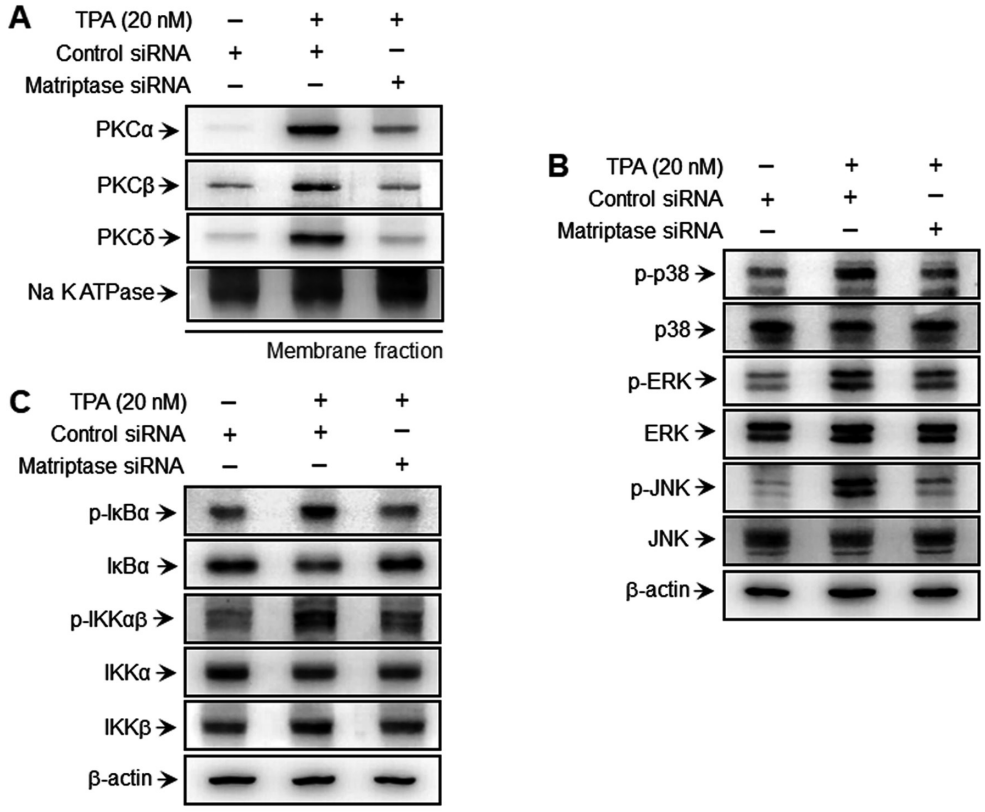

Figure 3. Effect of matriptase on TPA-induced PKC activation and MAPK signaling in MCF-7 cells. (A) MCF-7 cells were transfected with control and matriptase siRNAs for 24 h, followed by incubation with 20 nM TPA for 40 min. PKC isozyme levels in cell membrane fractions were analyzed by western blotting. (B) Transfected cells were treated with 20 nM TPA for 30 min and cell lysates were prepared for western blotting to evaluate the MAPK signaling pathway. (C) Additionally, transfected cells were treated with TPA, and after 4 h of incubation, cytoplasmic lysates were prepared; expression of upstream signaling molecules NF-κB, p-IκBα, IκBα, p-IKKαβ, IKKα and IKKβ were than analyzed via western blotting. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; siRNA, small interfering RNA; p-, phosphorylated; Na K ATPase, sodium/potassium ATPase.

the induction of cancer cell metastasis, including breast cancer (43). Therefore, the inhibitory effect of matriptase siRNA on the metastatic efficacy of MCF-7 cells was investigated using invasion (Fig. 5A) and migration (Fig. 5B) assays. TPA-induced invasiveness and migration were significantly reduced in cells treated with matriptase siRNA, compared with control siRNA- and TPA-treated cells.

Inhibition of PAR-2 suppresses TPA-induced PKC activation, MMP expression, as well as invasiveness and migration, in MCF-7 breast cancer cells. Matriptase induces PAR-2 activation (15); therefore, to investigate the effect of PAR-2-mediated breast cancer invasiveness, the effect of a PAR-2 inhibitor (GB83) on PKC activation and MMP-9 expression was evaluated in TPA-treated MCF-7 cells. GB83 (10 μM) was found to inhibit the expression of TPA-induced MMP-9 (Fig. 6A). It also attenuated TPA-mediated translocation of PKC to the membrane (Fig. 6B). Furthermore, invasion and migration assays revealed the inhibitory effect of GB83 on the metastatic properties of MCF-7 cells (Fig. 6C). These results suggested that PAR-2 was involved in PKC-mediated MMP-9 expression and metastatic ability in MCF-7 breast cancer cells.

Discussion

Breast cancer is a malignant tumor and the leading cause of mortality in women worldwide (2). The majority of breast cancer deaths result from metastasis to the bone, lung, liver, brain and kidney (1). The molecular mechanisms underlying cancer cell invasiveness and migration are complex; the initial event that provides biochemical and mechanical barriers to cancer cell migration is the proteolytic degradation of the ECM (4,44), which requires the activation and expression of MMPs, known to play a major role in breast cancer (43,44). Among the MMPs, MMP-9 activation is associated with tumor progression and invasion (45,46). Therefore, inhibition of the regulatory pathway involved in MMP-9 expression may be an important therapeutic strategy for preventing breast cancer metastasis. In the present study, matriptase was proposed as a signaling protein for inhibiting cellular metastasis through the regulation of MMP-9. Matriptase was first reported in 1993 to have novel gelatinolytic activity in breast cancer cells (47). Matriptase is one of the most well studied members of the TTSP family and is expressed in the epithelial compartments of all tissue types (7,48,49), where its dysregulation is associated with numerous types of cancer and poor patient outcomes therein (8,9). Furthermore, several studies have demonstrated that matriptase is highly expressed in MCF-7 breast cancer cells (10,11); in particular, matriptase is upregulated in breast cancer and increases the proliferation and invasiveness of breast cancer cells (11,50). A previous study demonstrated that inhibiting matriptase suppresses breast cancer progression using *in vivo*, *ex vivo* and *in vitro* approaches (50). However, the role and signaling mechanisms of matriptase in breast cancer

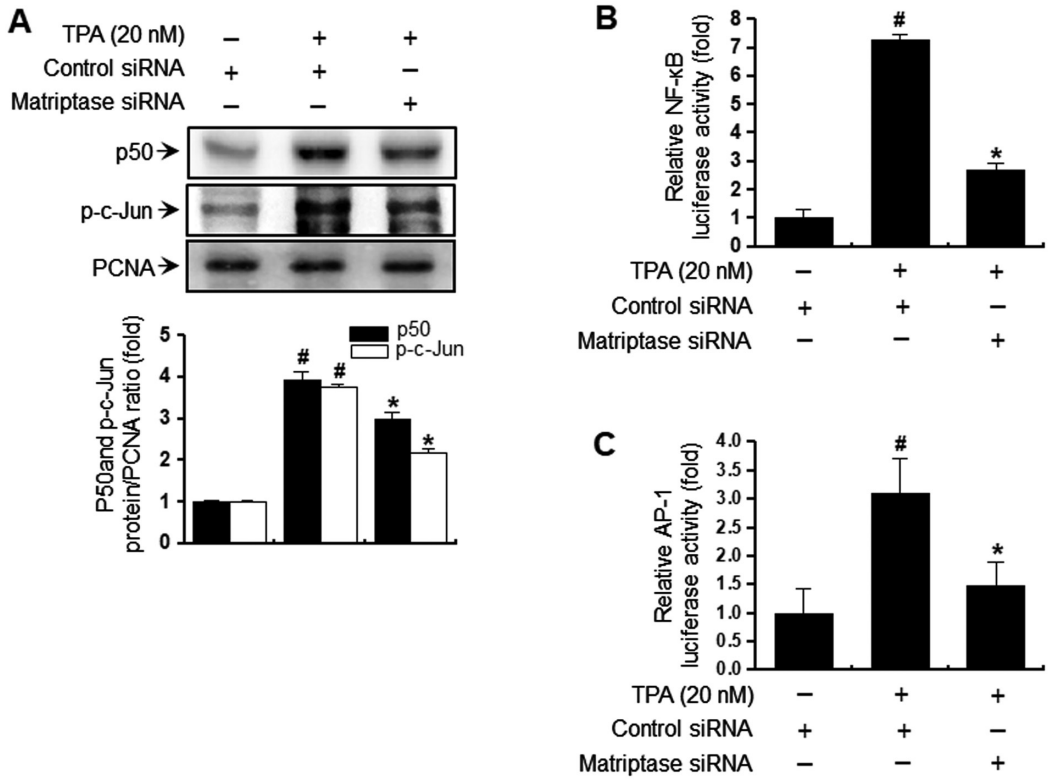

Figure 4. Effect of matriptase on TPA-induced NF-κB and AP-1 activation in MCF-7 cells. (A) MCF-7 cells were transfected with control and matriptase siRNAs for 24 h, followed by incubation with 20 nM TPA. After 4 h of incubation, western blot analysis was performed to determine the nuclear levels of p50 and AP-1 (p-c-Jun) subunits. (B) NF-κB-Luc or (C) AP-1-Luc reporter and the *Renilla* luciferase thymidine kinase reporter vector were co-transfected into MCF-7 cells. Cells transfected with matriptase siRNA for 24 h with TPA and promoter activity of NF-κB and AP-1 was measured with dual-luciferase reporter assays. Data represent the mean ± SEM of three independent experiments. $^{\#}$P<0.01 vs. untreated control; *P<0.01 vs. TPA. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; siRNA, small interfering RNA; p-, phosphorylated; PCNA, proliferating cell nuclear antigen; AP-1, activator protein-1.

metastasis were previously unclear. Therefore, the aim of the present study was to identify the regulatory role of matriptase in TPA-induced MMP-9 expression and invasion/migration in MCF-7 breast cancer cells. The findings show that inhibition of matriptase expression inhibited TPA-induced increases in MMP-9 expression, cellular invasiveness and migration (Fig. 1 and 5).

The present study demonstrated the role of matriptase in breast cancer metastasis by identifying its effects on MCF-7 breast cancer cell invasiveness, as well as the underlying mechanisms. Matriptase mediates multiple intracellular signaling pathways by cleaving the activation site of PAR-2, a G protein-coupled receptor (16,17). PAR-2 signaling produces DAG and activates the PKC-mediated NF-κB signaling pathway (19,22). Furthermore, the binding of PAR-2 to G protein induces canonical PLC/Ca2+/PKC signaling (19). Moreover, activation of PAR-2-induced MAPK signaling plays an important role in regulating the migration of breast cancer cells (22,23). These findings suggest that the PAR2-mediated signaling pathway is important in breast cancer cell metastasis. The current study results confirmed that that inhibition of PAR-2 in MCF-7 cells suppressed PKC activation, MMP-9 expression and cellular invasiveness (Fig. 6). In addition, inhibition of matriptase regulated MMP-9 expression and invasiveness mediated by Par-2/PLCγ2/PKC or Par-2/MAPK.

The activation of PKC is highly associated with increased invasiveness in breast cancer (51). TPA increases the invasiveness of breast cancer cells by activating MMP-9 through PKC (52,53). TPA also activates novel (δ, ε, η and θ) and conventional (α, βI, βII and γ) PKC isozymes by binding the C1 domains of these isoforms (54). The effect of TPA is similar to that of DAG, a natural activator of the PKC isoform. TPA-mediated activation of PKC involves the translocation of PKC isoforms to the plasma membrane, resulting in modulation of gene expression, proliferation, apoptosis, differentiation and malignant transformation of cancer cells (54,55).

PLCγ2 is a member of the phosphoinositide-specific PLCs and enhances PKC activation by catalyzing the degradation of phosphatidylinositol-4,5-bisphosphate in DAG and inositol-3,4,5-trisphosphate (IP_3). IP_3 induces an increase in intracellular calcium levels (42,55,56). A variety of cell signaling pathways act downstream of PKC isozymes, such as those of Ras/Raf/MAPK, PI3K/Akt and the transcription factors NF-κB, AP-1 and STAT-3 (57).

Our previous study demonstrated that the activation of PKCα, PKCβ and PKCδ by TPA mediates the expression and secretion of MMP-9 (58). Therefore, the current study confirmed that intracellular calcium is required for TPA-induced PKC activation and MMP-9 expression and invasiveness (Fig. 2A and B). In addition, inhibiting matriptase expression was found to reduce the expression of p-PLCγ2 (Fig. 2C). Furthermore, the study revealed that inhibition of matriptase expression reduced the TPA-induced membrane localization of PKCα, PKCβ and PKCδ in MCF-7 cells (Fig. 3A). These findings indicate that inhibiting matriptase expression modulates PKC-mediated MMP-9 expression

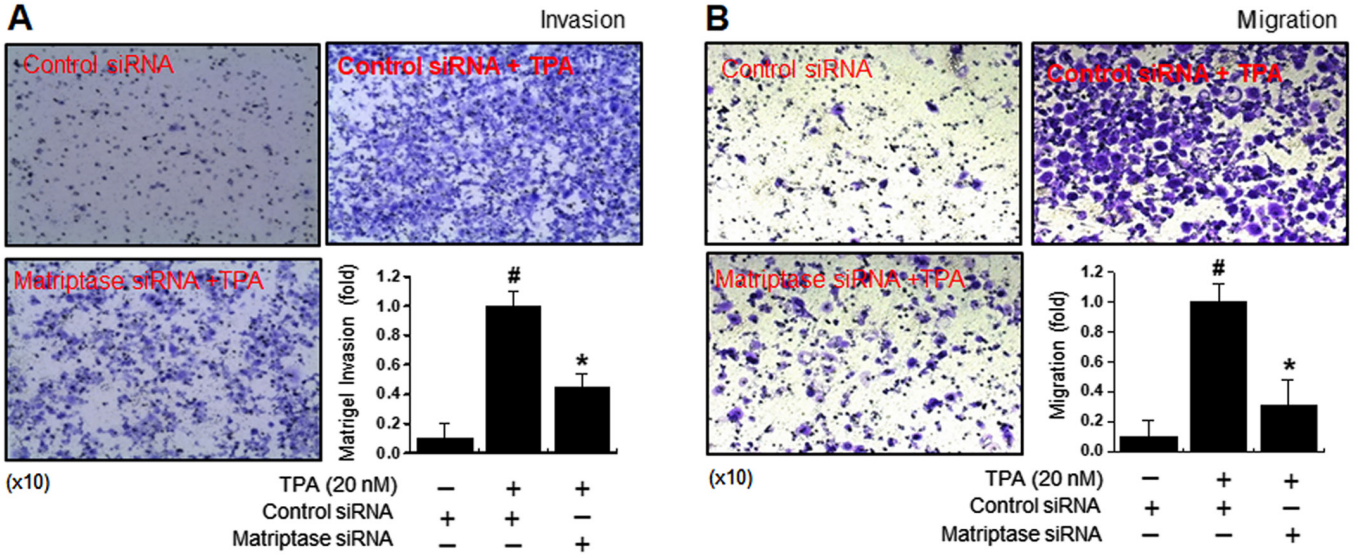

Figure 5. Effect of matriptase on the TPA-mediated invasiveness and migration of MCF-7 cells. (A) MCF-7 cells ($3x10^5$) were transfected with control and matriptase siRNAs, and seeded into the upper chamber of a Matrigel-coated Transwell insert; TPA was added to the lower chamber. (B) Transfected cell lysates were subjected to migration analyses. At 24 h post-incubation, invasive and migratory cells were stained and counted; magnification, x10. Data represent the mean ± SEM of three independent experiments. #P<0.05 vs. untreated control; *P<0.05 vs. control + TPA. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; siRNA, small interfering RNA.

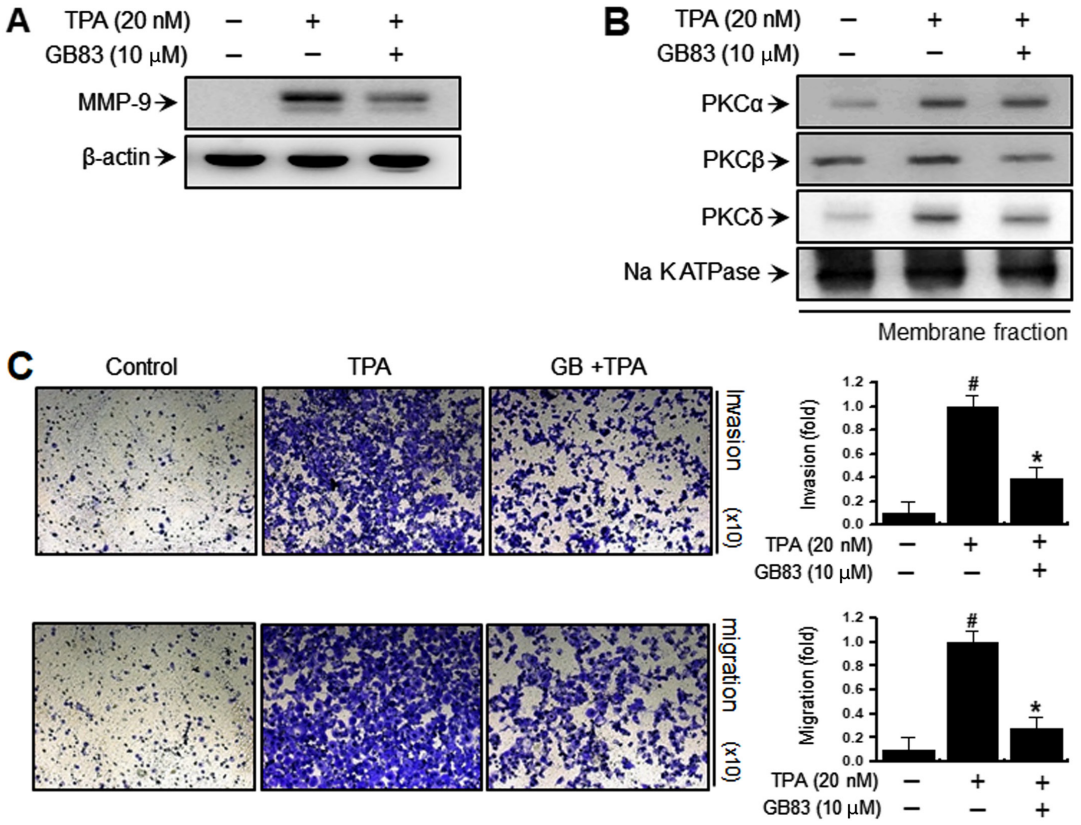

Figure 6. Effect of the PAR-2 inhibitor (GB83) on TPA-induced PKC activation, MMP-9 expression and invasiveness/migration in MCF-7 cells. MCF-7 cells were treated with GB83 and TPA for 24 h (to analyze MMP-9 expression and invasion/migration) or 40 min (to evaluate PKC activation). Western blot analysis was used to detect (A) MMP-9 and (B) PKC isozyme levels in cell membrane fractions. (C) GB83 inhibited TPA-induced invasiveness and migration of MCF-7 cells. Data represent the mean ± SEM of three independent experiments. #P<0.05 vs. untreated control; *P<0.05 vs. control + TPA. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; Na K ATPase, sodium/potassium ATPase.

and metastasis in MCF-7 breast cancer cells by inhibiting PLCγ2 activation, and regulating PLCγ2-mediated calcium levels.

To investigate the TPA-induced PKC downstream signaling cascade for TPA-induced MMP-9 expression, the expression of three MAPKs, and the DNA binding capacity of transcription

factors, were also investigated. These MAPKs (ERK, p38 and JNK) are upstream modulators of NF-κB, and activate MMP-9 expression (59). MAPKs are expressed in MCF-7 breast cancer cells and their activation can be confirmed by analyzing their phosphorylation (60). MAPKs are required for the activation of NF-κB and AP-1, which requires IκB kinase, MAPKs and PI3K/Akt, depending on the cell type in question (39,61,62). Herein, matriptase-knockdown suppressed the phosphorylation of p38, ERK, JNK and IKK following TPA treatment (Fig. 3B and C). NF-κB and AP-1 are important for the expression of MMP-9 in MCF-7 cells, and the MMP9 gene promoter contains NF-κB and AP-1 binding sites (63). The present study revealed that inhibition of matriptase expression inhibited TPA-induced MMP-9 expression by inhibiting NF-κB and AP-1 activation in MCF-7 breast cancer cells (Fig. 4).

The primarily aim of the present study was to identify the MMP-regulated signaling mechanism for the matriptase-induced inhibition of cellular metastatic capacity. Inhibition of matriptase expression was found to attenuate TPA-induced MMP-9 expression and invasiveness by blocking NF-κB and AP-1 activation through the PAR-2/PLCγ2 and PKC/MAPK signaling pathways in MCF-7 breast cancer cells. Therefore, to the best of our knowledge, the present study is the first to demonstrated that MCF-7 breast cancer cell invasiveness is mediated by inhibiting MMP-9 expression through modulation of the PAR-2/PLCγ2-mediated PKC signaling pathway, induced by matriptase. These findings suggest that inhibiting matriptase may have potential therapeutic value in the treatment of breast cancer metastasis. Furthermore, these findings are expected to pave the way for *in vivo* and clinical studies to determine the efficacy of matriptase in preventing breast cancer metastasis.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology, Republic of Korea (grant nos. 2013R1A1A1059747 and 2013R1A1A2007181).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HJY, YRL and JP designed the study and confirmed the authenticity of all the raw data. JMK, EMN and HKS performed the experiments. SYK and JSK analyzed the data. SHJ contributed to data analysis and interpretation, and critically revised the manuscript. YRL drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Redig AJ and McAllister SS: Breast cancer as a systemic disease: A view of metastasis. J Intern Med 274: 113-126, 2013.
2. Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. CA Cancer J Clin 64: 9-29, 2014.
3. Leber MF and Efferth T: Molecular principles of cancer invasion and metastasis (review). Int J Oncol 34: 881-895, 2009.
4. Jiang WG, Sanders AJ, Katoh M, Ungefroren H, Gieseler F, Prince M, Thompson SK, Zollo M, Spano D, Dhawan P, *et al*: Tissue invasion and metastasis: Molecular, biological and clinical perspectives. Semin Cancer Biol 35 (Suppl 1): S244-S275, 2015.
5. van Zijl F, Krupitza G and Mikulits W: Initial steps of metastasis: Cell invasion and endothelial transmigration. Mutat Res 728: 23-34, 2011.
6. Theocharis AD, Skandalis SS, Gialeli C and Karamanos NK: Extracellular matrix structure. Adv Drug Deliv Rev 97: 4-27, 2016.
7. Szabo R and Bugge TH: Type II transmembrane serine proteases in development and disease. Int J Biochem Cell Biol 40: 1297-1316, 2008.
8. Murray AS, Varela FA and List K: Type II transmembrane serine proteases as potential targets for cancer therapy. Biol Chem 397: 815-826, 2016.
9. List K, Bugge TH and Szabo R: Matriptase: Potent proteolysis on the cell surface. Mol Med 12: 1-7, 2006.
10. Oberst M, Anders J, Xie B, Singh B, Ossandon M, Johnson M, Dickson RB and Lin CY: Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo. Am J Pathol 158: 1301-1311, 2001.
11. Bergum C, Zoratti G, Boerner J and List K: Strong expression association between matriptase and its substrate prostasin in breast cancer. J Cell Physiol 227: 1604-1609, 2012.
12. Tuhkanen H, Hartikainen JM, Soini Y, Velasco G, Sironen R, Nykopp TK, Kataja V, Eskelinen M, Kosma VM and Mannermaa A: Matriptase-2 gene (TMPRSS6) variants associate with breast cancer survival, and reduced expression is related to triple-negative breast cancer. Int J Cancer 133: 2334-2340, 2013.
13. Parr C, Sanders AJ, Davies G, Martin T, Lane J, Mason MD, Mansel RE and Jiang WG: Matriptase-2 inhibits breast tumor growth and invasion and correlates with favorable prognosis for breast cancer patients. Clin Cancer Res 13: 3568-3576, 2007.
14. Rattenholl A and Steinhoff M: Proteinase-activated receptor-2 in the skin: Receptor expression, activation and function during health and disease. Drug News Perspect 21: 369-381, 2008.
15. Bao Y, Hou W and Hua B: Protease-activated receptor 2 signalling pathways: A role in pain processing. Expert Opin Ther Targets 18: 15-27, 2014.
16. Sales KU, Friis S, Konkel JE, Godiksen S, Hatakeyama M, Hansen KK, Rogatto SR, Szabo R, Vogel LK, Chen W, *et al*: Non-hematopoietic PAR-2 is essential for matriptase-driven pre-malignant progression and potentiation of ras-mediated squamous cell carcinogenesis. Oncogene 34: 346-356, 2015.
17. Wojtukiewicz MZ, Hempel D, Sierko E, Tucker SC and Honn KV: Protease-activated receptors (PARs)-biology and role in cancer invasion and metastasis. Cancer Metastasis Rev 34: 775-796, 2015.
18. Bocheva G, Rattenholl A, Kempkes C, Goerge T, Lin CY, D'Andrea MR, Ständer S and Steinhoff M: Role of matriptase and proteinase-activated receptor-2 in nonmelanoma skin cancer. J Invest Dermatol 129: 1816-1823, 2009. Rothmeier AS and Ruf W: Protease-activated receptor 2 signaling in inflammation. Semin Immunopathol 34: 133-149, 2012.
19. Rothmeier AS and Ruf W: Protease-activated receptor 2 signaling in inflammation. Semin Immunopathol 34: 133-149, 2012.
20. Lidington EA, Steinberg R, Kinderlerer AR, Landis RC, Ohba M, Samarel A, Haskard DO and Mason JC: A role for proteinase-activated receptor 2 and PKC-epsilon in thrombin-mediated induction of decay-accelerating factor on human endothelial cells. Am J Physiol Cell Physiol 289: C1437-C1447, 2005.

21. van der Merwe JQ, Moreau F and MacNaughton WK: Protease-activated receptor-2 stimulates intestinal epithelial chloride transport through activation of PLC and selective PKC isoforms. Am J Physiol Gastrointest Liver Physiol 296: G1258-G1266, 2009.
22. Su S, Li Y, Luo Y, Sheng Y, Su Y, Padia RN, Pan ZK, Dong Z and Huang S: Proteinase-activated receptor 2 expression in breast cancer and its role in breast cancer cell migration. Oncogene 28: 3047-3057, 2009.
23. Jiang Y, Yau MK, Lim J, Wu KC, Xu W, Suen JY and Fairlie DP: A potent antagonist of protease-activated receptor 2 that inhibits multiple signaling functions in human cancer cells. J Pharmacol Exp Ther 364: 246-257, 2018.
24. Stetler-Stevenson WG, Hewitt R and Corcoran M: Matrix metalloproteinases and tumor invasion: From correlation and causality to the clinic. Semin Cancer Biol 7: 147-154, 1996.
25. Itoh Y and Nagase H: Matrix metalloproteinases in cancer. Essays Biochem 38: 21-36, 2002.
26. Brinckerhoff CE and Matrisian LM: Matrix metalloproteinases: A tail of a frog that became a prince. Nat Rev Mol Cell Biol 3: 207-214, 2002.
27. Lin CW, Hou WC, Shen SC, Juan SH, Ko CH, Wang LM and Chen YC: Quercetin inhibition of tumor invasion via suppressing PKC delta/ERK/AP-1-dependent matrix metalloproteinase-9 activation in breast carcinoma cells. Carcinogenesis 29: 1807-1815, 2008.
28. Lee SO, Jeong YJ, Kim M, Kim CH and Lee IS: Suppression of PMA-induced tumor cell invasion by capillarisin via the inhibition of NF-kappaB-dependent MMP-9 expression. Biochem Biophys Res Commun 366: 1019-1024, 2008.
29. Saito N, Hatori T, Murata N, Zhang ZA, Ishikawa F, Nonaka H, Iwabuchi S and Samejima H: A double three-step theory of brain metastasis in mice: The role of the pia mater and matrix metalloproteinases. Neuropathol Appl Neurobiol 33: 288-298, 2007.
30. Castellano G, Malaponte G, Mazzarino MC, Figini M, Marchese F, Gangemi P, Travali S, Stivala F, Canevari S and Libra M: Activation of the osteopontin/matrix metalloproteinase-9 pathway correlates with prostate cancer progression. Clin Cancer Res 14: 7470-7480, 2008.
31. Kanayama H: Matrix metalloproteinases and bladder cancer. J Med Invest 48: 31-43, 2001.
32. Gum R, Wang H, Lengyel E, Juarez J and Boyd D: Regulation of 92 kDa type IV collagenase expression by the jun aminoterminal kinase- and the extracellular signal-regulated kinase-dependent signaling cascades. Oncogene 14: 1481-1493, 1997.
33. Newton AC: Regulation of protein kinase C. Curr Opin Cell Biol 9: 161-167, 1997.
34. Zeigler ME, Chi Y, Schmidt T and Varani J: Role of ERK and JNK pathways in regulating cell motility and matrix metalloproteinase 9 production in growth factor-stimulated human epidermal keratinocytes. J Cell Physiol 180: 271-284, 1999.
35. Hozumi A, Nishimura Y, Nishiuma T, Kotani Y and Yokoyama M: Induction of MMP-9 in normal human bronchial epithelial cells by TNF-alpha via NF-kappa B-mediated pathway. Am J Physiol Lung Cell Mol Physiol 281: L1444-L1452, 2001.
36. Weng CJ, Chau CF, Hsieh YS, Yang SF and Yen GC: Lucidenic acid inhibits PMA-induced invasion of human hepatoma cells through inactivating MAPK/ERK signal transduction pathway and reducing binding activities of NF-kappaB and AP-1. Carcinogenesis 29: 147-156, 2008.
37. Noh EM, Park YJ, Kim JM, Kim MS, Kim HR, Song HK, Hong OY, So HS, Yang SH, Kim JS, *et al*: Fisetin regulates TPA-induced breast cell invasion by suppressing matrix metalloproteinase-9 activation via the PKC/ROS/MAPK pathways. Eur J Pharmacol 764: 79-86, 2015.
38. Kim JM, Noh EM, Kwon KB, Kim JS, You YO, Hwang JK, Hwang BM, Kim BS, Lee SH, Lee SJ, *et al*: Curcumin suppresses the TPA-induced invasion through inhibition of PKCα-dependent MMP-expression in MCF-7 human breast cancer cells. Phytomedicine 19: 1085-1092, 2012.
39. Karin M: The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270: 16483-16486, 1995.
40. Madrid LV, Mayo MW, Reuther JY and Baldwin AS Jr: Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J Biol Chem 276: 18934-18940, 2001.
41. Schmittgen TD and Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108, 2008.
42. Isakov N: Protein kinase C (PKC) isoforms in cancer, tumor promotion and tumor suppression. Semin Cancer Biol 48: 36-52, 2018.
43. Duffy MJ, Maguire TM, Hill A, McDermott E and O'Higgins N: Metalloproteinases: Role in breast carcinogenesis, invasion and metastasis. Breast Cancer Res 2: 252-257, 2000.
44. Woessner JF Jr: Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5: 2145-2154, 1991.
45. Scorilas A, Karameris A, Arnogiannaki N, Ardavanis A, Bassilopoulos P, Trangas T and Talieri M: Overexpression of matrix-metalloproteinase-9 in human breast cancer: A potential favourable indicator in node-negative patients. Br J Cancer 84: 1488-1496, 2001.
46. Yousef EM, Tahir MR, St-Pierre Y and Gaboury LA: MMP-9 expression varies according to molecular subtypes of breast cancer. BMC Cancer 14: 609, 2014.
47. Shi YE, Torri J, Yieh L, Wellstein A, Lippman ME and Dickson RB: Identification and characterization of a novel matrix-degrading protease from hormone-dependent human breast cancer cells. Cancer Res 53: 1409-1415, 1993.
48. Fan B, Brennan J, Grant D, Peale F, Rangell L and Kirchhofer D: Hepatocyte growth factor activator inhibitor-1 (HAI-1) is essential for the integrity of basement membranes in the developing placental labyrinth. Dev Biol 303: 222-230, 2007.
49. Uhland K: Matriptase and its putative role in cancer. Cell Mol Life Sci 63: 2968-2978, 2006.
50. Zoratti GL, Tanabe LM, Varela FA, Murray AS, Bergum C, Colombo É, Lang JE, Molinolo AA, Leduc R, Marsault É, *et al*: Targeting matriptase in breast cancer abrogates tumour progression via impairment of stromal-epithelial growth factor signalling. Nat Commun 6: 6776, 2015.
51. Zhang J, Anastasiadis PZ, Liu Y, Thompson EA and Fields AP: Protein kinase C (PKC) betaII induces cell invasion through a Ras/Mek-, PKC iota/Rac 1-dependent signaling pathway. J Biol Chem 279: 22118-22123, 2004.
52. de Vente JE, Kukoly CA, Bryant WO, Posekany KJ, Chen J, Fletcher DJ, Parker PJ, Pettit GJ, Lozano G and Cook PP: Phorbol esters induce death in MCF-7 breast cancer cells with altered expression of protein kinase C isoforms. Role for p53-independent induction of gadd-45 in initiating death. J Clin Invest 96: 1874-1886, 1995.
53. Kim S, Han J, Lee SK, Choi MY, Kim J, Lee J, Jung SP, Kim JS, Kim JH, Choe JH, *et al*: Berberine suppresses the TPA-induced MMP-1 and MMP-9 expressions through the inhibition of PKC-α in breast cancer cells. J Surg Res 176: e21-e29, 2012.
54. Barry OP and Kazanietz MG: Protein kinase C isozymes, novel phorbol ester receptors and cancer chemotherapy. Curr Pharm Des 7: 1725-1744, 2001.
55. Newton AC: Protein kinase C as a tumor suppressor. Semin Cancer Biol 48: 18-26, 2018.
56. Wilde JI and Watson SP: Regulation of phospholipase C gamma isoforms in haematopoietic cells: Why one, not the other? Cell Signal 13: 691-701, 2001.
57. Koivunen J, Aaltonen V and Peltonen J: Protein kinase C (PKC) family in cancer progression. Cancer Lett 235: 1-10, 2006.
58. Kim JM, Noh EM, Kwon KB, Kim JS, You YO, Hwang JK, Hwang BM, Kim BS, Lee SH, Lee SJ, *et al*: Curcumin suppresses the TPA-induced invasion through inhibition of PKCα-dependent MMP-expression in MCF-7 human breast cancer cells. Phytomedicine 19: 1085-1092, 2012.
59. Chakraborti S, Mandal M, Das S, Mandal A and Chakraborti T: Regulation of matrix metalloproteinases: An overview. Mol Cell Biochem 253: 269-285, 2003.
60. Qi M and Elion EA: MAP kinase pathways. J Cell Sci 118: 3569-3572, 2005.
61. Yao J, Xiong S, Klos K, Nguyen N, Grijalva R, Li P and Yu D: Multiple signaling pathways involved in activation of matrix metalloproteinase-9 (MMP-9) by heregulin-beta1 in human breast cancer cells. Oncogene 20: 8066-8074, 2001.
62. Whitmarsh AJ: Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. Biochim Biophys Acta 1773: 1285-1298, 2007.
63. Eberhardt W, Huwiler A, Beck KF, Walpen S and Pfeilschifter J: Amplification of IL-1 beta-induced matrix metalloproteinase-9 expression by superoxide in rat glomerular mesangial cells is mediated by increased activities of NF-kappa B and activating protein-1 and involves activation of the mitogen-activated protein kinase pathways. J Immunol 165: 5788-5797, 2000.