

# Suppression of MMP-9 and FAK expression by pomolic acid via blocking of NF- $\kappa$ B/ERK/mTOR signaling pathways in growth factor-stimulated human breast cancer cells

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**Abstract.** The expression of matrix metalloproteinase-9 (MMP-9) and the phosphorylation of focal adhesion kinase (FAK) have been implicated in the invasion, metastasis and cell motility of cancer cells. It is considered that epidermal growth factor (EGF) may increase cell motility, an event involved in cancer cell invasion and metastasis. Pomolic acid (PA), an active triterpenoid from *Euscaphis japonica*, is known to inhibit the proliferation of a variety of cancer cells, but the effect of PA on the invasiveness of cancer cells is largely unknown. In this study, we first determined the molecular mechanism by which PA inhibits the migratory and invasive abilities of highly metastatic MDA-MB-231 cells. Transwell invasion, wound-healing assay and F-actin reorganization showed that PA significantly inhibits the EGF-induced invasion, migration and cell motility

by reducing expression of MMP-9 and FAK phosphorylation. In particular, PA potently suppressed the phosphorylation of nuclear factor (NF)- $\kappa$ B, extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway. Furthermore, PA treatment inhibited the DNA binding activity of NF- $\kappa$ B and activator protein (AP)-1, which is known to mediate the expression of EGFR and MMP-9. These results suggest that PA may be a potential therapeutic candidate for treatment of breast cancer metastasis.

## Introduction

Epidermal growth factor (EGF)-mediated activation of its receptor (EGFR) is a principal regulatory event in breast cancer growth and progression (1). Cell migration and invasion can be enhanced by numerous growth factors. High expression of EGFR is a major contributing factor to the development and progression of cancer, and is associated with a poor prognosis (2). Basal or triple-negative (ER-negative, PR-negative, and Her2/neu-negative) breast cancer cells, like MD Anderson-mammary breast cancer cell line-231 (MDA-MB-231), often express high levels of EGFR, which is associated with a loss of sensitivity to hormonal therapies and/or high probability of metastasis (3). The metastasis and invasion of cancer cells have been considered important therapeutic targets, because the inability to control metastasis and invasion remains the most formidable obstacle to successful treatment (4).

Cancer metastasis occurs by a complex series of events, which include detachment of cells from the primary tumor, migration, invasion of the cells into either blood or lymphatic vessels with the help of matrix metalloproteinases (MMPs), and finally, interaction with target organs such as the lungs, liver, and bones (5,6). Among the MMPs, MMP-9 is known to be involved in the degradation of type IV collagen, an important component of the extracellular matrix (ECM) (7). Focal adhesion kinase (FAK) is also a critical factor for cell motility and metastasis, and it is found in the cell membrane where the cytoskeleton interacts with the proteins of the ECM (8). Cancer cell invasion is regulated by growth factors that can rapidly activate cell surface receptors to induce actin polymerization

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**Abbreviations:** AP-1, activator protein-1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; F-actin, filamentous actin; FAK, focal adhesion kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDA-MB-231, MD Anderson-mammary breast cancer cell line-231; MMPs, matrix metalloproteinases; NF- $\kappa$ B, nuclear factor- $\kappa$ B; mTOR, mammalian target of rapamycin; p70S6K, 70 kDa ribosomal protein S6 kinase; PI3K/Akt, phosphatidylinositol 3-kinase/protein kinase B

**Key words:** pomolic acid, matrix metalloproteinase-9, focal adhesion kinase, actin filaments, epidermal growth factor, activator protein-1

and reorganization into actin-based extensions (9). Recent reports have suggested that MMP-9 and FAK are associated with the EGFR activation via EGF induction, and that they promote tumor cell motility and invasion (10). It has been shown that EGF-induced phosphorylation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), mammalian target of rapamycin (mTOR) pathway, leads to MMP-9 expression and FAK phosphorylation (5). Furthermore, the EGFR/PI3K/Akt signaling pathways regulate mTOR. The inhibition of the mTOR signaling pathway suppresses filamentous-actin (F-actin) reorganization, MMP expression, and FAK phosphorylation, which is a functional indicator of cell migration (11,12).

Triterpenoids (friedeline, glut-5-en-ol, pomolic acid and methylotundate) were isolated from *Euscaphis japonica* (Tunb.) Kuntz (*Staphyleaceae*) found in China, Japan, and Korea (13). Among them, pomolic acid (PA) was shown to inhibit the growth of leukemia cell lines with high efficacy (Fig. 1A) (14,15), and other investigations have demonstrated that PA mediates the apoptosis of human ovarian cancer cells through the mitochondrial-mediated intrinsic and death receptor-induced extrinsic pathways (16). PA has demonstrated anti-proliferative activity against human gastric adenocarcinoma, human uterine carcinoma, and murine melanoma (17). We previously demonstrated that PA inhibits invasion of breast cancer cells through the suppression of CXCR4 expression (18). However, the molecular mechanisms of the anti-metastatic potential of PA have not been fully elucidated in breast cancer cells. Therefore, we investigated the anti-metastasis mechanism by examining the effect of PA on EGF-induced breast cancer cells.

## Materials and methods

**Cell cultures and reagents.** MDA-MB-231 cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in a humidified incubator 5% CO<sub>2</sub> and 95% air. DMEM, RPMI-1640, FBS, antibiotics, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA). Pomolic acid (PA) was purified and received from Dr Ki Yong Lee, professor of the College of Pharmacy, Korea University (13). PA was dissolved in dimethylsulfoxide (DMSO) as a 10-mM stock solution and stored at 4°C. Further dilution was done in cell culture medium. Recombinant human EGF was purchased from R&D System Inc. (Minneapolis, MN, USA). Signal inhibitors were obtained from Calbiochem (Cambridge, MA, USA).

**Wound-healing and invasion assays.** Wound healing assay was performed in 6-well plates. When cells reached 80% confluence, synchronized cells were pretreated with PA (10 µM) for 1 h, followed by stimulated with EGF (20 ng/ml) for 24 h. A single wound was created in the center of the cell monolayers by gentle removal of the attached cells with a sterile plastic pipette tip. After 24 h of incubation, the cells that migrated into the wounded area or protruded from the border of the wound were visualized and photographed with

a microscope (Carl Zeiss, Sartrouville, France). *In vitro* invasion assay was done using Bio-Coat Matrigel invasion assay system (BD Biosciences, Lexington, KY, USA) according to the manufacturer's instructions (19). MDA-MB-231 cells were suspended in medium and seeded into the Matrigel-precoated Transwell chambers with polycarbonate membranes of 8 µm pore size. The lower chambers were filled with DMEM supplemented with EGF (20 ng/ml) in the absence or presence of PA (10 µM). The chamber was incubated at 37°C for 24 h. The cells from the upper surface of the filters were carefully removed while the cells on the lower surface of the filters were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Migrant cells were quantified by blind counting under a microscope (Carl Zeiss). The number of transmigrated cells were counted and averaged in five random areas.

**Actin filament staining.** Serum-starved cells grown in 6-well-plates were pretreated with or without PA for 1 h, followed by stimulation with or without EGF (20 ng/ml) for 24 h. Cells were then fixed with 4% ice cold formaldehyde in PBS for 20 min at 4°C and washed with 0.2% Triton X-100 in PBS for 5 min. Cells were stained with FITC conjugated phalloidin (1 µg/ml, Sigma, MO, USA) for 30 min. Slides were mounted using ProLong® Gold antifade reagent (Molecular Probes® by Life Technologies™, CA, USA). Actin filaments (F-actin) were examined using a confocal microscope (Nikon, Tokyo, Japan).

**Protein isolation and immunoblot analysis.** Cytosolic and nuclei protein fractions were obtained as described (20). The protein concentration was determined with a Bio-Rad Bradford kit (Bio-Rad Laboratories, CA, USA). The samples were boiled for 5 min and equal volumes were loaded on a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, the proteins were transferred to a nitrocellulose membrane for 1 h at 4°C and blocked overnight with PBS-T [0.1% (v/v) Tween-20, 5% (w/v) powdered milk in 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4] at 4°C. Immune complexes were detected with a horseradish peroxidase (HRP)-conjugated secondary antibody and were visualized by an enhanced chemiluminescence (ECL) detection system (Bio-Rad Laboratories). The primary antibodies used in this study were: anti-MMP-9, anti-p-FAK, anti-FAK, anti-p-EGFR, anti-EGFR, anti-p-IKKα/β, anti-IKKα/β, anti-p-IκBα, anti-IκBα, anti-p-NF-κB p65, anti-NF-κB, anti-p-ERK1/2, anti-ERK1/2, anti-p-PI3K, anti-PI3K, anti-p-Akt, anti-Akt, anti-p-mTOR, anti-mTOR, anti-p-70S6K, anti-70S6K, anti-p-4E-BP1, anti-4E-BP1, anti-lamin B and anti-β-actin (all from Cell Signaling Technology, MA, USA). Lamin B and β-actin were used as a loading control. The luminescent signals were analyzed using an ImageQuant LAS 4000 Scanner of GE Healthcare (Piscataway, NJ, USA).

**Construction of human MMP-9 promoter, transient transfection and luciferase gene assays.** A 700-bp fragment at the 5'-flanking region of the human MMP-9 gene was amplified by PCR from human genomic DNA (6). Specific primers were designed to contain the appropriate restriction enzyme site: sense 5'-CGG GGT ACC TGC TAC TGT CCC CTT TAC TG-3' (*Kpn*I) and antisense 5'-CCC AGA TCT GTG AGG GCA GAG GTG TCT-3' (*Bgl*II). The amplified promoter DNA

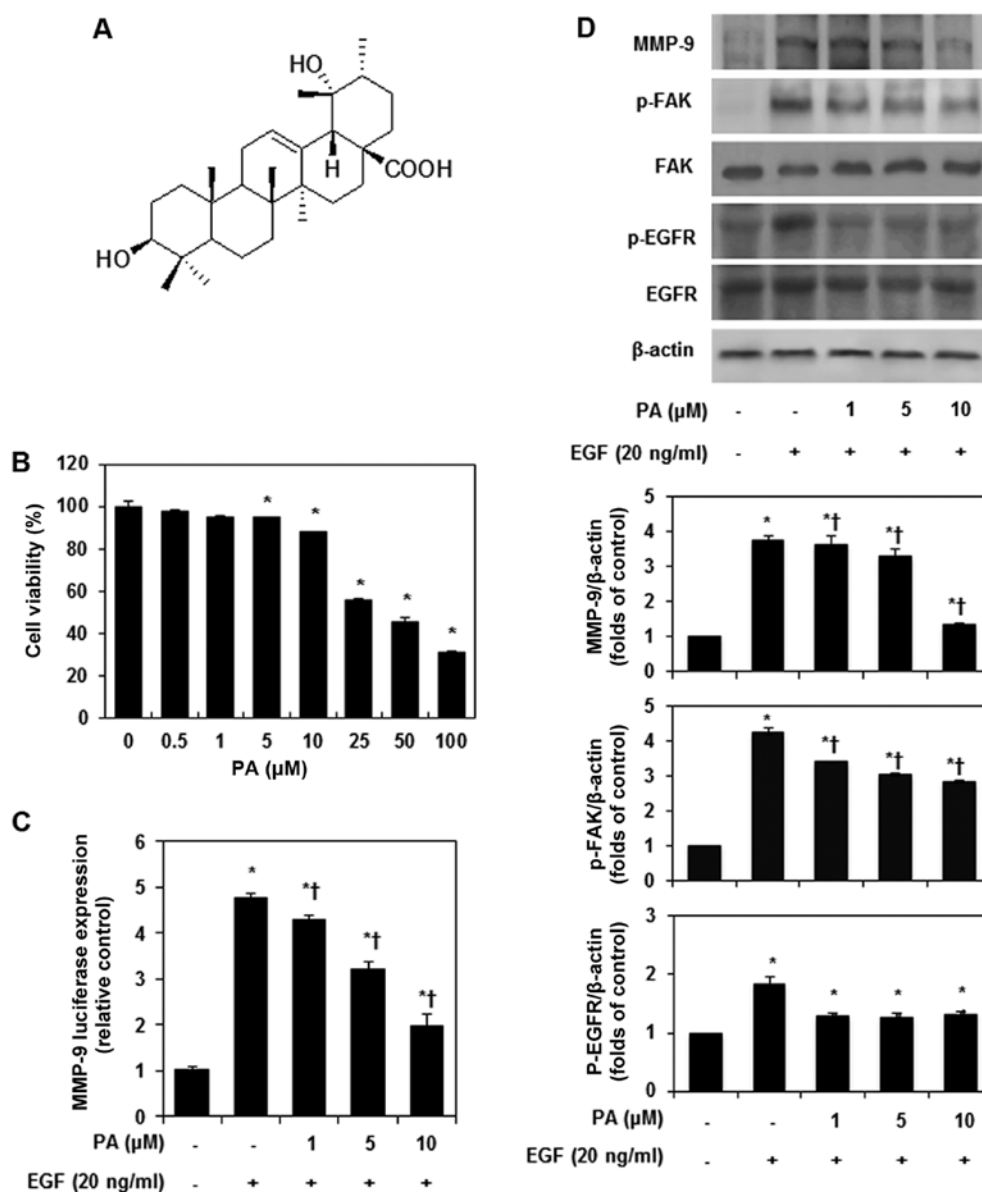


Figure 1. Inhibitory effect of PA on EGFR-mediated expression of MMP-9 and FAK phosphorylation in MDA-MB-231 cells. (A) The chemical structures of PA (C<sub>30</sub>H<sub>48</sub>O<sub>4</sub>). The molecular weight is 472.71. (B) Dose-dependent effect of PA on the viability of MDA-MB-231 cells. Cells were treated with various concentration of PA for 24 h. Viability was determined using the MTT assay. The percentage of cell survival was defined relative to the number of surviving untreated MDA-MB-231 cells. (C) Cells were transiently transfected with MMP-9 promoter reported construct. PA inhibits EGF-induced MMP-9 luciferase activity. (D) Cells were pretreated with PA for 1 h, followed by stimulation with EGF for 24 h. PA inhibited the EGF-induced MMP-9 expression, FAK and EGFR phosphorylation. β-actin was used to confirm equal sample loading. MMP-9, p-FAK and p-EGFR followed by densitometric analysis. The data are representative of three similar experiments and quantified as mean values ± SE. \*p<0.05 compared to normal control; †p<0.05 compared to EGF treatment.

was digested with *KpnI* and *BglII*, and then cloned upstream of the luciferase gene in pGL3 plasmid. The DNA sequence of the MMP-9 promoter was confirmed, and the resultant reporter plasmid was named pGL3-wtMMP-9. The AP-1, NF-κB, and SP-1 mutants from pGL3-wtMMP-9 were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, CA, USA); all the mutants were confirmed by DNA sequencing.

MMP-9 wild-type (pGL3-wtMMP-9), AP-1 site-mutated (pGL3-MMP-9-mtAP-1), NF-κB site-mutated (pGL3-MMP-9-mtNF-κB), and SP-1 site-mutated MMP-9 luciferase promoter constructs (pGL3-MMP-9-mtSP-1) were used in transient transfection assays. MDA-MB-231 cells were co-transfected with 1 μg of MMP-9 promoter-luciferase reporter constructs and 0.2 μg of the *Renilla* reporter plasmid

for 6 h using Lipofectamine reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. After transfection, MDA-MB-231 cells were pretreated with PA for 1 h and then stimulated with EGF for 24 h. Luciferase and *Renilla* activities were determined by following the manufacturer's protocol (Dual-Luciferase Reporter Assay system, Promega, WI, USA).

**Electrophoretic mobility shift analysis (EMSA) and super shift assay.** According to our previous studies, DIG Gel Shift kit (Roche, Mannheim, Germany) was performed to detect NF-κB p65, AP-1 and SP-1 DNA-binding activity, with the manufacturer's instructions (20,21). The binding activity of NF-κB p65, AP-1 and SP-1 in the EGF-induced MDA-MB-231 cell nuclear extract was confirmed by EMSA or super shift

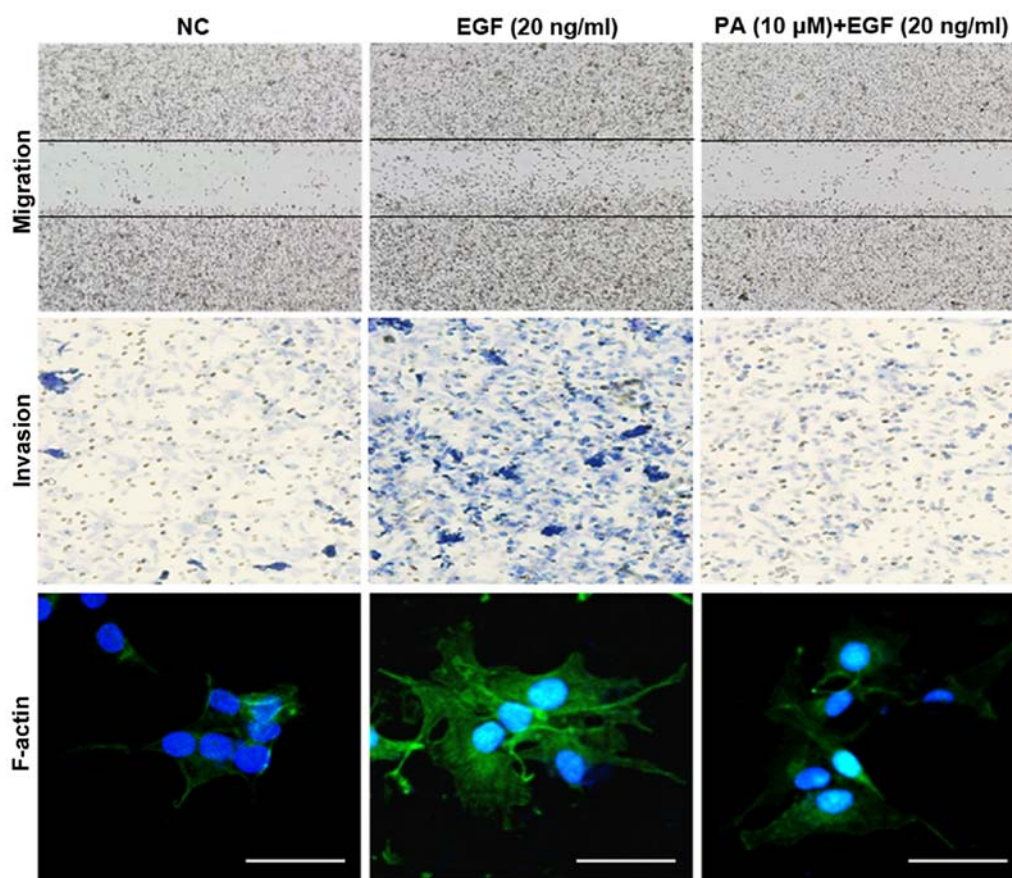


Figure 2. Inhibitory effect of PA on EGF-induced migration, invasion and cell motility in the MDA-MB-231 cells. Cell were pretreated with PA for 1 h, followed by stimulated with EGF for 24 h. Wound-healing assays (upper panel), invasion assays (middle panel) and F-actin staining (lower panel) were carried out using PA (10  $\mu$ M) in the presence of EGF (20 ng/ml) in MDA-MB-231 cells. Magnifications, x40 or x100. MDA-MB-231 cells were immunostained for F-actin (green) and nuclei (blue). Scale bar, 50  $\mu$ m.

assay with a DIG-labeled oligonucleotide (NF- $\kappa$ B, 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; AP-1, 5'-CGC TTG ATG AGT CAG CCG GAA-3'; SP-1, 5'-CTT GAA CCC CGC CCC TGT CTT-3') and NF- $\kappa$ B p65 antibody (Abcam, Cambridge, UK). EMSA was performed by incubating 10  $\mu$ g of the MDA-MB-231 cell nuclear extract in a 9- $\mu$ l binding reaction mixture containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 3 mM MgCl<sub>2</sub>, 0.05 mg/ml poly (dI-dC) and 10% (v/v) glycerol at 37°C for 10 min. The binding reaction mixture for the super shift assay containing 1  $\mu$ l of the non-diluted antibody of NF- $\kappa$ B p65 was added to 1  $\mu$ l of DIG-labeled double-stranded oligonucleotide and was incubated at 37°C for 20 min, followed by the addition of 1  $\mu$ l of the gel loading 10X buffer (250 mM Tris-HCl, pH 7.5, 40% glycerol) at room temperature. This mixture was then loaded on a pre-run 4% polyacrylamide gel and run at 10°C in 0.5X TBE buffer at 350 V until the bromophenol blue dye was three-fourth of the way down the gel. After electrophoresis, the DIG-labeled DNA was electroblotted on the positively-charged nylon membrane (Roche). The blotted nylon membrane was fixed by baking at 120°C for 15 min and the chemiluminescent detection was performed according to the manufacturer's instructions.

**MAPK inhibitor treatment and transient transfection with small interfering RNA.** MDA-MB-231 cells were pre-treated

with mitogen-activated protein kinase (MAPK) inhibitors such as ERK-specific inhibitor: PD98059 (20  $\mu$ M), JNK-specific inhibitor: SP600125 (40  $\mu$ M), p38-specific inhibitor: SB203580 (20  $\mu$ M), PKC-specific inhibitor: GO6978 (10  $\mu$ M), PI3K/Akt-specific inhibitor: LY294002 (50  $\mu$ M), mTOR-specific inhibitor: rapamycin (20 nM), and EGFR-specific inhibitor: AG1478 (2  $\mu$ M). After 30 min, the cells were treated and co-cultured with EGF for 24 h. Cell were transfected with negative control small interfering RNA (siRNA) or mTOR specific siRNA (Cell Signaling Technology) using RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions.

**Statistical analysis.** Statistical analysis was performed by Student's t-test and ANOVA using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) to study the relationship between the different variables. Values of  $p < 0.05$  were considered to indicate statistical significance.

## Results

**Effects of PA on the EGF-induced MMP-9 expression and FAK phosphorylation.** To investigate the cytotoxic effect of PA on the proliferation of MDA-MB-231 cells, we treated them with PA for 24 h and measured cell viability using the MTT assay. As shown in Fig. 1B, PA treatment for 24 h at

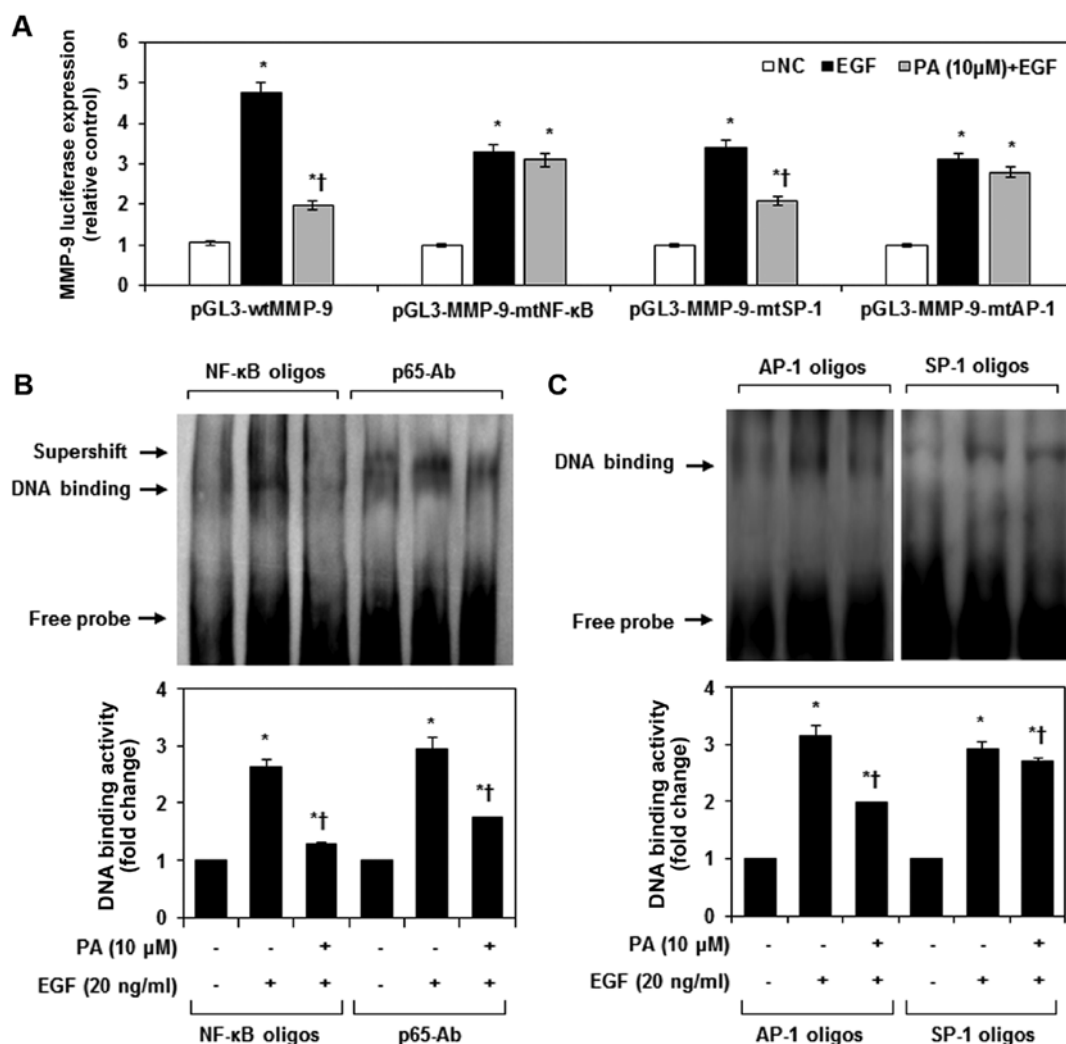


Figure 3. Effect of PA on EGF-induced MMP-9 transcriptional activity. (A) PA inhibits EGF-induced MMP-9 luciferase activity. Mutation of SP-1 binding sites have influence on the inhibitory effects of PA. Mutation of NF-κB and AP-1 binding sites diminish the inhibitory effects of PA, suggesting that PA suppresses the expression of MMP-9 through NF-κB and AP-1 binding sites. DNA and Ab-binding activity of NF-κB (B), and DNA binding activity of AP-1 and SP-1 (C) in nuclear extracts of the cells was measured by EMSA and supershift assay. NF-κB oligos and p65 Ab followed by densitometric analysis. The data are representative of three similar experiments and quantified as mean values  $\pm$  SE. \* $p < 0.05$  compared to normal control; † $p < 0.05$  compared to EGF treatment.

concentration ranging from 0.5 to 10  $\mu$ M had no significant cytotoxic effect on MDA-MB-231 cells. Thus, we used PA at a concentration of 1, 5 and 10  $\mu$ M for subsequent experiments. We investigated whether the PA treatment inhibited the MMP-9 transcriptional activity induced by EGF. To this end, MDA-MB-231 cells were transiently transfected with MMP-9 promoter reported construct. As shown Fig. 1C, PA inhibited EGF-stimulated MMP-9 transcriptional activity in a dose-dependent manner. Previous studies have shown that EGF induces significant MMP-9 expression and phosphorylation of FAK in MDA-MB-231 cells (7). Therefore, the inhibitory effects of PA on the activation of MMPs and FAK were analyzed in EGF-stimulated MDA-MB-231 cells. PA strongly inhibited EGF-induced MMP-9 expression and FAK phosphorylation in a dose-dependent manner (Fig. 1D). Also, PA significantly suppressed EGF-induced EGF receptor (EGFR) phosphorylation.

*Effects of PA on the EGF-induced migration and invasion.* The regulatory proteins of ECM, such as MMPs and FAK,

are important pharmaceutical targets for the prevention of tumor invasion and metastasis (22). Since the most important characteristic of metastasis is the migratory and invasive ability of tumor cells, we next examined the effect of PA on the EGF-induced migration and invasion of the MDA-MB-231 cells using wound-healing and Transwell invasion assays. As shown in Fig. 2, 10  $\mu$ M of PA significantly inhibited EGF-induced cell migration and invasion. Tumor metastasis is associated with the polymerization of F-actin (23). Thus, to investigate the inhibitory effect of PA on EGF-induced cell motility, F-actin staining was performed. F-actin was randomly distributed across cells in the absence of EGF. After stimulation with EGF for 24 h, however, F-actin was condensed at the leading edge within structures resembling fans, an effect not seen in non-treated cells. PA markedly reduced EGF-stimulated F-actin reorganization at the leading edge. These results indicate that PA has an inhibitory effect on cell motility through the prevention of F-actin reorganization in MDA-MB-231 cells. Taken together, these results suggest that PA has a strong inhibitory effect on EGF-induced metastasis,

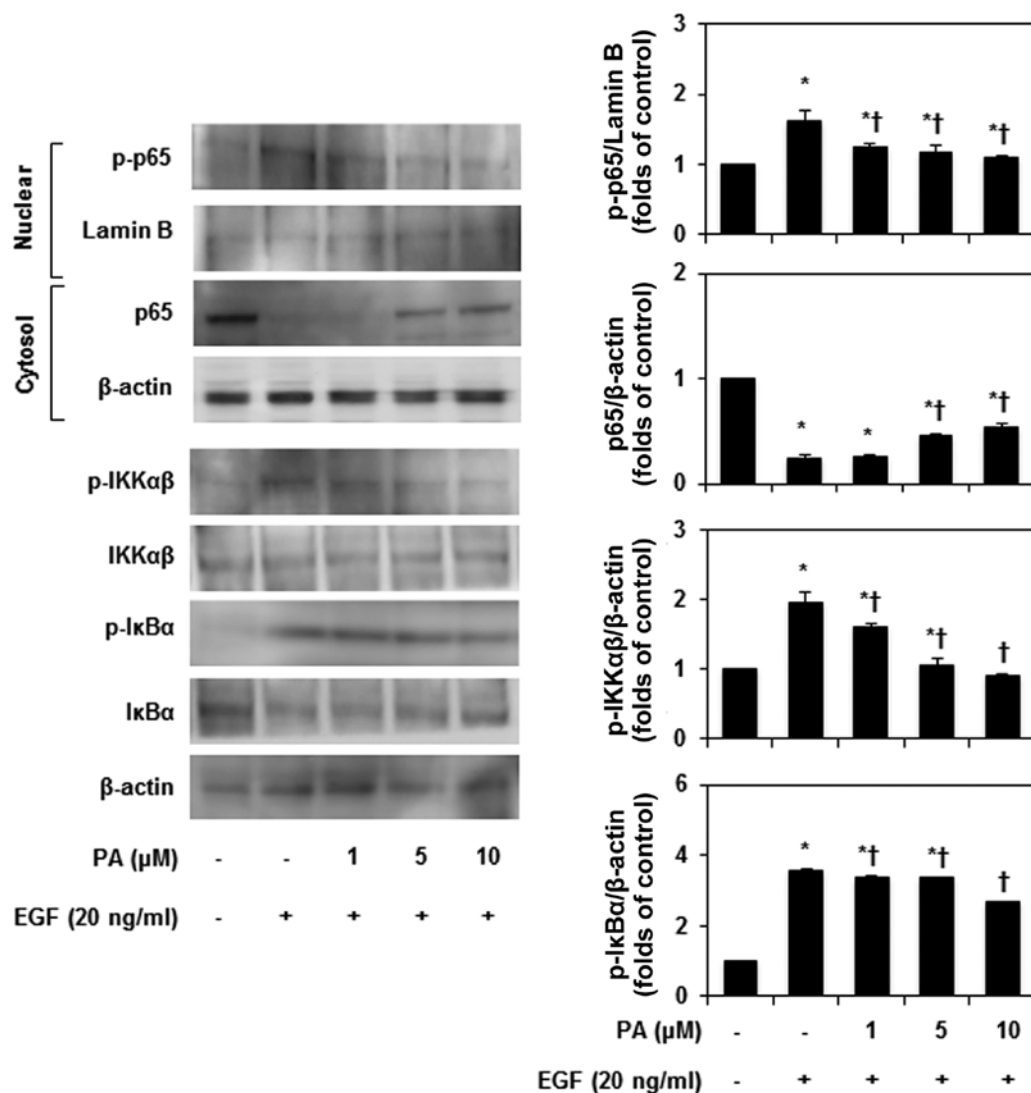


Figure 4. PA suppresses the EGF-induced NF- $\kappa$ B signaling pathway. MDA-MB-231 cells were pretreated with PA for 1 h, followed by stimulation with EGF (20 ng/ml) for 30 min. Nuclear extracts prepared from cells treated with EGF were analyzed for translocation of p65 and cytosol fraction of IKK $\alpha$  $\beta$  and I $\kappa$ B $\alpha$  by immunoblot analysis.  $\beta$ -actin and lamin B were used to confirm equal sample loading. p-p65, p65, p-IKK $\alpha$  $\beta$  and p-I $\kappa$ B $\alpha$  followed by densitometric analysis. The data are representative of three similar experiments and quantified as mean values  $\pm$  SE. \* $p$ <0.05 compared to normal control, † $p$ <0.05 compared to EGF treatment.

and that F-actin reorganization can be associated with inhibition of MMP-9 expression and FAK phosphorylation in MDA-MB-231 cells.

*PA suppresses the EGF-induced MMP-9 expression via inhibition of NF- $\kappa$ B and AP-1.* The promoter of MMP-9 contains cis-acting regulatory elements for transcription factors including an NF- $\kappa$ B site (located at -600 bp), an SP-1 site (located at -558 bp), and two AP-1 sites (located at -79 and -533 bp) (24). To test which of these transcription factors may regulate the MMP-9 gene in MDA-MB-231 cells, cells were transiently transfected with a reporter gene, either the wild-type (wt) MMP-9 promoter or the promoter with mutations in the NF- $\kappa$ B site, the AP-1 sites and the SP-1 site. As shown in Fig. 3A, wtMMP-9 luciferase activity was activated  $\leq$ 5-fold in cells treated with EGF. PA inhibited the EGF-induced wtMMP-9 luciferase activity, suggesting that PA may inhibit MMP-9 expression at the transcriptional level. Transfection

of MDA-MB-231 cells with MMP-9-mtAP-1 promoter and MMP-9-mtAP-1 promoter, which have mutated NF- $\kappa$ B and AP-1 binding sites, neutralized the ability of PA to prevent EGF-induced MMP-9 activation. We further evaluated the effects of PA on NF- $\kappa$ B DNA binding activity when cells were treated with EGF. NF- $\kappa$ B-DNA and NF- $\kappa$ B-Ab complexes were strongly upregulated in EGF-stimulated MDA-MB-231 cells, an effect that was significantly suppressed by treatment with PA (Fig. 3B). Also, PA significantly inhibited EGF-induced AP-1 DNA binding activity in MDA-MB-231 cells, but had no effect on SP-1 DNA binding activity (Fig. 3C).

*Effects of PA on EGF-induced NF- $\kappa$ B signal pathway.* NF- $\kappa$ B regulates many responses of cancer cells involved in proliferation, apoptosis, metastasis, and angiogenesis (25). EGF-induced cellular migration corresponds to an increase in MMP-9 expression, which targets NF- $\kappa$ B activation and is correlated with NF- $\kappa$ B binding activity. Therefore, we

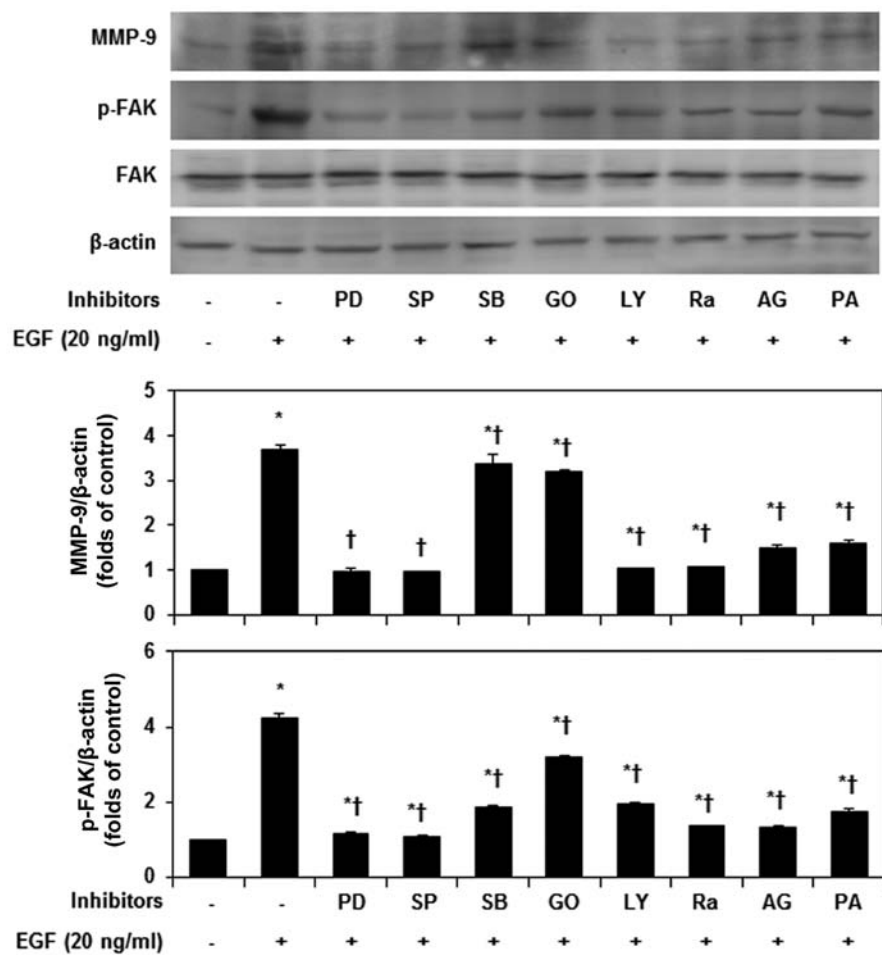


Figure 5. Role of MAPKs in EGF-induced expression of MMP-9 and FAK phosphorylation. MDA-MB-231 cells were pretreated with various kinase inhibitors (PD, PD98059; SP, SP600125; SB, SB203580; GO, GO6978; LY, LY294002; Ra, rapamycin; AG, AG1478) or with PA for 1 h and the exposed to EGF (20 ng/ml) for 24 h. MMP-9 and p-FAK followed by densitometric analysis. β-actin was used to confirm equal sample loading. The data are representative of three similar experiments and quantified as mean values ± SE. \*p<0.05 compared to normal control; †p<0.05 compared to EGF treatment.

examined the effects of PA on the NF-κB signaling pathway in MDA-MB-231 cells treated with EGF. As shown in Fig. 4, following EGF administration, expression of nuclear NF-κB and cytosolic phosphorylated IKKα/β and IκBα increased when MDA-MB-231 cells were treated with EGF. In addition, PA significantly reduced IKKα/β and IκBα phosphorylation in EGF-treated MDA-MB-231 cells. EGF stimulated nuclear translocation of p65, and PA inhibited the p65 translocation in a dose-dependent manner. These results confirmed that expression of the NF-κB DNA binding activity, decreased as a result of PA. It is likely that PA inhibited MMP-9 expression by decreasing the MMP-9 gene promoter binding activity of the NF-κB and AP-1 transcription factor. These results indicate that treatment with PA abrogated the effect of EGF on the expression levels of genes, relevant to breast cancer metastasis through NF-κB signaling.

**Effects of PA on EGF-induced MAPKs and PI3K/Akt/mTOR signal pathways.** MAPKs and PI3K/Akt/mTOR pathways play important roles in the migration and invasion of cancer cells through the regulation of MMP-9 and FAK (26,27). We therefore aimed to identify the precise MAPKs and PI3K/Akt/mTOR pathway involved in PA inhibition of

EGF-induced MMP-9 expression and FAK phosphorylation, the MDA-MB-231 cells were exposed to various kinase inhibitors. PD98059, SP600125, LY294002, rapamycin, AG1478 and PA most strongly inhibited MMP-9 expression and FAK phosphorylation, in EGF-stimulated MDA-MB-231 cells (Fig. 5). As shown in Fig. 6A, PA inhibited the EGF-induced phosphorylation of ERK, but had no effect on JNK and p38 phosphorylation (data not shown). In addition, PA significantly suppressed EGF-induced PI3K/Akt phosphorylation. These results indicate that inhibition of ERK and PI3K/Akt phosphorylation would be an important way to block the invasion of MDA-MB-231 cells. Additionally, EGF-induced MMP-9 expression and FAK phosphorylation seem to be involved in the PI3K and NF-κB signaling pathways in MDA-MB-231 cells. Akt-dependent regulation of NF-κB is controlled by the mTOR signaling pathway (28). Therefore, the effects of PA on EGF-induced mTOR phosphorylation were investigated in this study. PA significantly inhibited EGF-induced mTOR phosphorylation in MDA-MB-231 cells (Fig. 6B), and inhibited the phosphorylation of p70S6K and 4E-BP1, which are downstream effector molecules of mTOR. Subsequently, to confirm the ERK-MAPK and mTOR-dependent MMP-9 expression and FAK phosphorylation, MDA-MB-231 cells



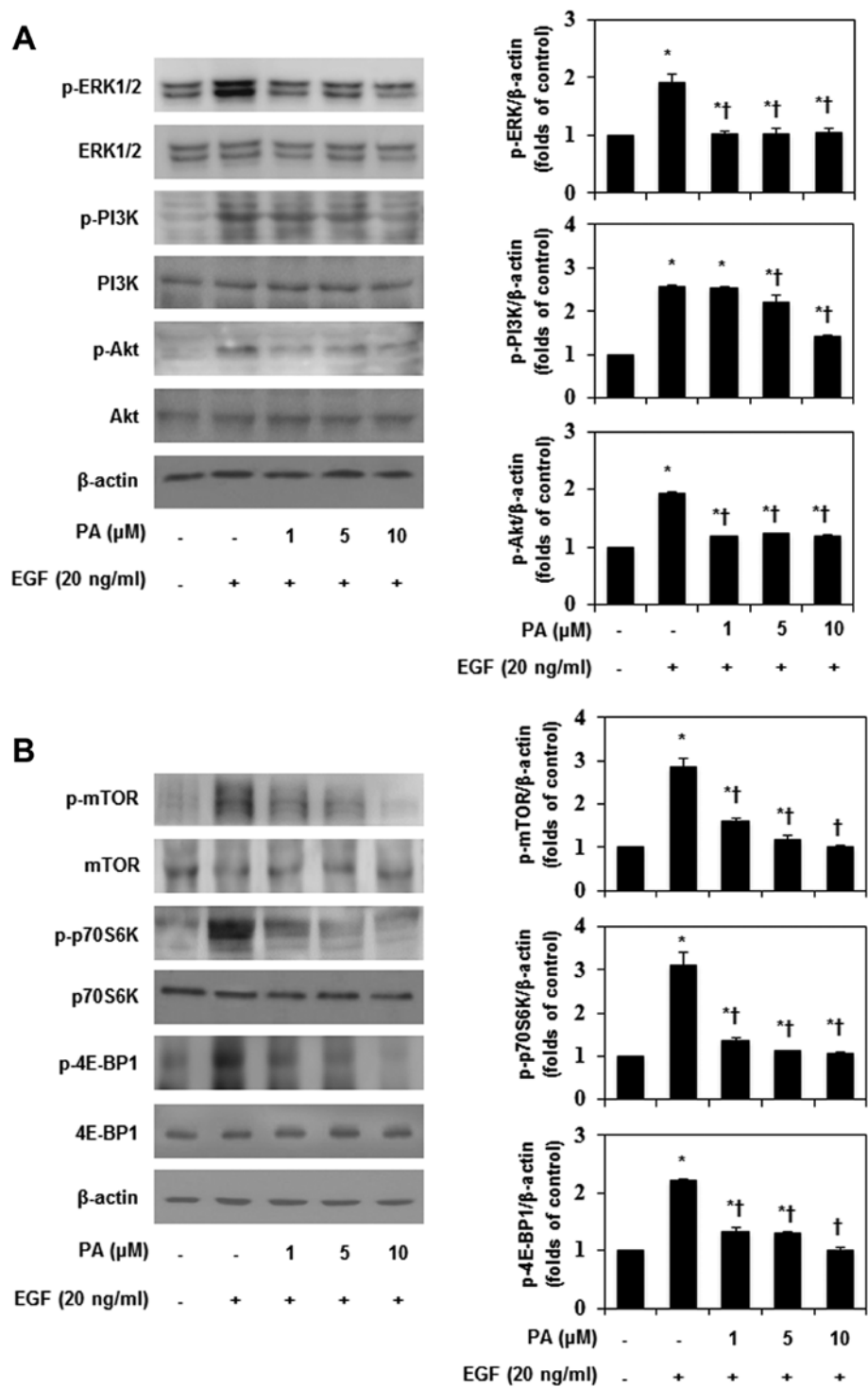


Figure 6. Effect of PA on the EGF-induced phosphorylation of ERK and PI3K/Akt (A), and mTOR (B) signal pathways. MDA-MB-231 cells were pretreated with PA for 1 h, followed by stimulated with EGF (20 ng/ml) for 30 min. p-ERK1/2, p-PI3K, p-Akt, p-mTOR, p-p70S6K, and p-4E-BP1 followed by densitometric analysis.  $\beta$ -actin was used to confirm equal sample loading. The data are representative of three similar experiments and quantified as mean values  $\pm$  SE. \* $p$ <0.05 compared to normal control; † $p$ <0.05 compared to EGF treatment.

were pretreated with mTOR siRNA. After transfection with mTOR siRNA for 24 h, the expression of mTOR declined in EGF-stimulated MDA-MB-231 cells (Fig. 7A). However, non-siRNA and transfection with control siRNA did not have any effects on mTOR accumulation. Furthermore, PA and mTOR siRNA most strongly suppressed MMP-9 expression and FAK phosphorylation in EGF-stimulated MDA-MB-231

cells. To further investigate the role of mTOR in EGF-induced phosphorylation of ERK-MAPK and translocation of p65, we examined the effects of mTOR siRNA and rapamycin on EGF-induced ERK-MAPK phosphorylation and p65 translocation by immunoblotting. As shown in Fig. 7B, EGF-induced phosphorylation of ERK-MAPK and p65 were inhibited by mTOR siRNA, rapamycin and PA. These results indicated



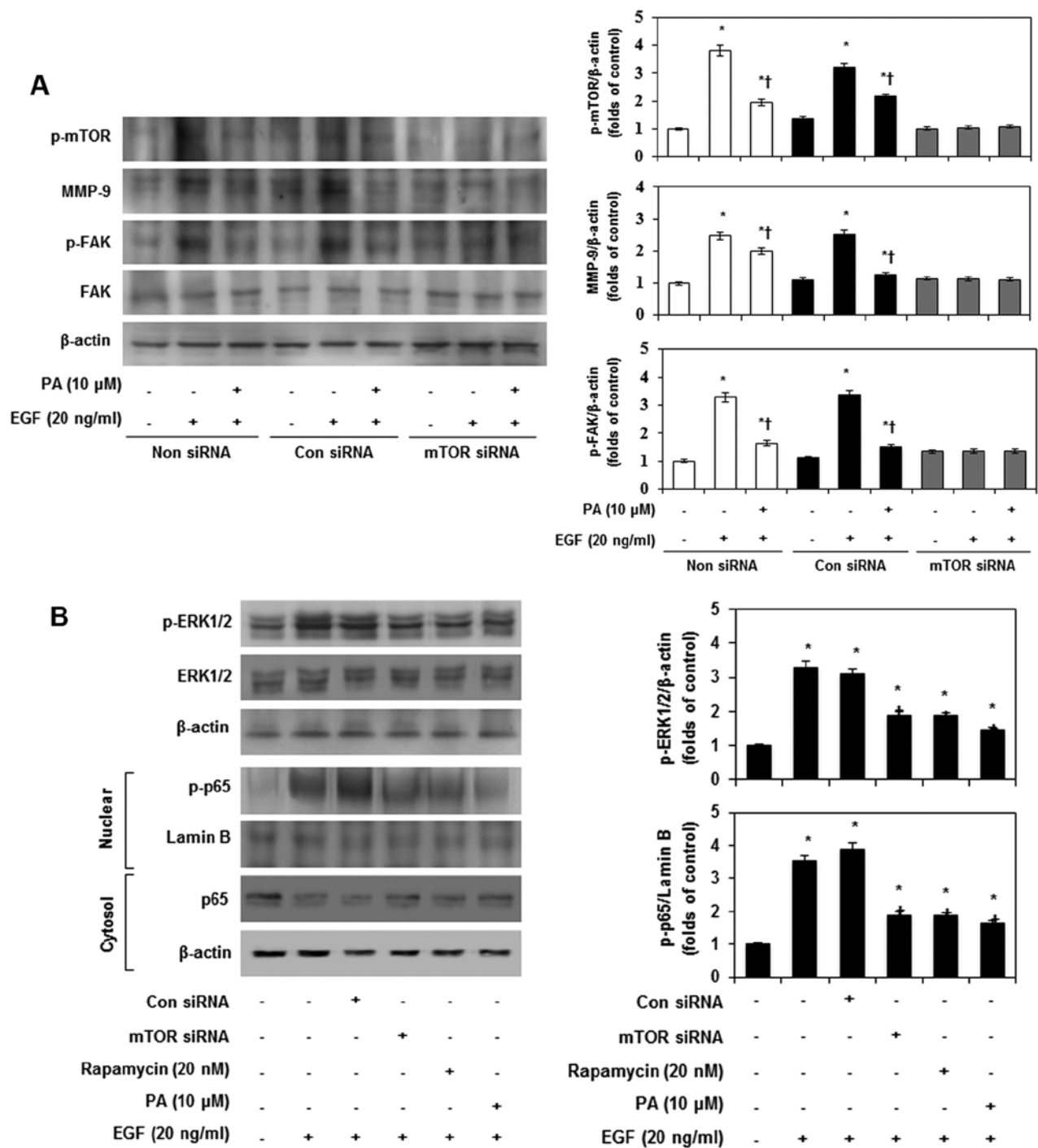


Figure 7. Role of mTOR in EGF-induced phosphorylation of ERK-MAPK and translocation of p65. (A) MDA-MB-231 cells were transfected with control or specific mTOR siRNA and then treated with EGF (20 ng/ml) for 1 h or 24 h. mTOR siRNA and PA inhibited EGF-induced expression of MMP-9 and FAK phosphorylation. (B) Cell were pretreated with PA for 1 h, followed by stimulation with EGF for 30 min. mTOR and PA contributes to EGF-induced ERK phosphorylation and p65 translocation. p-mTOR, MMP-9, p-FAK, p-ERK1/2 and p-p65 followed by densitometric analysis.  $\beta$ -actin and lamin B was used to confirm equal sample loading. The data are representative of three similar experiments and quantified as mean values  $\pm$  SE. \* $p < 0.05$  compared to normal control; † $p < 0.05$  compared to EGF treatment.

that mTOR partially contributes to EGF-induced MMP-9 expression, phosphorylation of FAK by ERK-MAPK, and p65 activation in MDA-MB-231 cells.

Discussion

Cancer invasion and metastasis are multistep and complex processes that involve changes in cell adhesion, migration,

and invasion through the ECM (29). Aberrations in EGFR expression and downstream signaling pathways contribute to the progression, invasion, and maintenance of the malignant phenotype of many human cancers, including breast cancer (2). In recent years, considerable enthusiasm was generated for EGFR inhibitors as targeted agents for cancer treatment. Evidence supporting the important role of MMP-9 in the invasive potential of malignancy *in vitro* and *in vivo* has been

reported (27). A downstream intracellular signaling effector, FAK is mainly located at the focal adhesion sites of migrating cells, and upon phosphorylation, FAK promotes cell adhesion (30). EGF stimulates the upregulation of MMP-9 and FAK signaling pathways in breast cancer cells (31).

Pomolic acid (PA), isolated from *Euscaphis japonica*, has been shown to have anticancer activities such as inhibiting the growth of leukemia cell lines and mediating the apoptosis of human ovarian cancer cells. PA has also been reported to demonstrate anti-proliferative activity against various cancer cell lines (17). However, the molecular mechanisms of the anti-metastatic potential of PA are not fully understood in breast cancer cells.

In this study, PA effectively suppressed EGF-induced MDA-MB-231 cell migration, invasion and cell motility through inhibition of the EGFR-mediated ERK/PI3K/Akt/mTOR signaling pathway, abating the expression of NF- $\kappa$ B and AP-1 and reducing the expression of MMP-9 and the phosphorylation of FAK resulting an anti-metastatic effect.

Lamellipodia and invadopodia formation are caused by the polymerization of F-actin through the regulation of cytoskeletal reorganization (23), a process that is stimulated by EGF at the leading edge of cell. We also found that PA inhibited EGF-induced cell motility and invasion in MDA-MB-231 cells.

EGF controls MMP-9 expression and FAK phosphorylation using various transcription factors such as AP-1, SP-1, and NF- $\kappa$ B through MAPK, PI3K/Akt, and mTOR signaling pathways (4,7,32). It has been reported that NF- $\kappa$ B, AP-1, and SP-1 regulate cancer proliferation and metastasis, and that they are also important transcriptional elements of MMP-9 (32). In agreement with these reports, our results showed that PA markedly suppresses the EGF-induced nuclear translocation of NF- $\kappa$ B and AP-1 DNA binding activity, whereas it did not affect the EGF-induced binding activity of SP-1.

The mitogenic effect of EGF is initiated by its interaction with overexpressed EGFR and is transmitted by the activation of the MAPKs, PI3K/Akt, and mTOR pathways (11). As several studies also indicated, the MAPKs and PI3K/Akt pathways play crucial roles in regulating the MMP-9 expression and FAK phosphorylation, and the suppression of MAPKs has the potential to prevent invasion and metastasis in various tumors (5). Our results showed that PA significantly inhibited EGF-induced ERK-MAPK and PI3K/Akt phosphorylation in MDA-MB-231 cells. The mitogenic effect of EGF is mediated via activation of PI3K/Akt and IKK-dependent activation of NF- $\kappa$ B (3). In this study, PA inhibited the activation of PI3K and Akt which led to inhibition of the downstream effector, NF- $\kappa$ B.

mTOR is regulated by the ERK-MAPK and PI3K/Akt pathways in various cancer cells. The inhibition of mTOR suppressed cell motility, which is independent of cell type and stimuli (33). Also, mTOR regulates the phosphorylation of 70S6K and 4E-BP1, which inhibit cell proliferation and growth by blocking the translation of mRNA. In addition, activated EGFR can regulate MMP-9 expression and cell proliferation through EGF binding. It has been reported that MMP-9 expression is needed to regulate the EGF-mediated activation of the PI3K/Akt/mTOR and MAPK pathways by phosphorylating protein translational regulators, including 70S6K and 4E-BP1

(34). Additionally, previous studies have demonstrated that the mTOR inhibitor also inhibited EGF-induced actin reorganization and cell migration (35). In accordance with these results, our results showed that PA suppresses the EGF-induced phosphorylation of mTOR/p70S6K/4E-BP1 in MDA-MB-231 cells. Also, we found that PD98059, SP600125, LY294002, rapamycin, AG1478, and PA specifically inhibited expression of MMP-9 and phosphorylation of FAK. Furthermore, mTOR siRNA transfection inhibited EGF-induced MMP-9 expression and FAK phosphorylation, and the suppressive effect of PA on the expression of MMP-9 and phosphorylation of FAK also decreased in mTOR-knockdown cells. These results suggest that PA can inhibit EGF-induced expression of MMP-9 and phosphorylation of FAK in MDA-MB-231 cells by inhibiting PI3K/Akt/mTOR signaling pathways, leading to activation of NF- $\kappa$ B and ERK/MAPK signaling pathways. Our findings demonstrated for the first time that PA inhibits the migration, invasion, and cell motility of highly metastatic MDA-MB-231 breast cancer cells by downregulating MMP-9 expression and FAK phosphorylation via the inhibition of EGFR-mediated NF- $\kappa$ B/ERK/mTOR signaling pathways. Based on these results, we suggest that PA is a potent candidate for a therapeutic anticancer agent and may be used for the prevention and treatment of breast cancer.

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