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Anti-metastatic effects of arctigenin are regulated by MAPK/AP-1 signaling in 4T-1 mouse breast cancer cells

MIN-GU LEE, KYU-SHIK LEE and KYUNG-SOO NAM

Department of Pharmacology and Intractable Disease Research Center, School of Medicine, Dongguk University, Gyeongju 38066, Republic of Korea

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Abstract. Arctigenin is a natural lignan that is found in burdock with anti-viral, -oxidative, -inflammatory and anti-tumor activities. In the current study, the effect of arctigenin on metastatic potential was examined in 4T-1 mouse triple-negative breast cancer cells. The results indicated that arctigenin inhibited cell motility and invasiveness, which was determined using wound healing and transwell invasion assays. Arctigenin suppressed matrix metalloprotease-9 (MMP-9) activity via gelatin zymography, and protein expression of cyclooxygenase-2 (COX-2) and MMP-3. Furthermore, arctigenin attenuated the mRNA expression of metastatic factors, including MMP-9, MMP-3 and COX-2. Based on these results, the effect of arctigenin on the mitogen-activated protein kinase (MAPK)/activating protein-1 (AP-1) signaling pathway was assessed in an attempt to identify the regulatory mechanism responsible for its anti-metastatic effects. Arctigenin was demonstrated to inhibit the phosphorylation of extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK), and the nuclear translocations of the AP-1 subunits, c-Jun and c-Fos. In summary, the present study demonstrated that in 4T-1 mouse triple-negative breast cancer cells the anti-metastatic effect of arctigenin is mediated by the inhibition of MMP-9 activity and by the inhibition of the metastasis-enhancing factors MMP-9, MMP-3 and COX-2, due to the suppression of the MAPK/AP-1 signaling pathway. The results of the current study demonstrated that arctigenin exhibits a potential for preventing cell migration and invasion in triple negative breast cancer.

Correspondence to: Professor Kyung-Soo Nam, Department of Pharmacology and Intractable Disease Research Center, School of Medicine, Dongguk University, 123 Dongdea-ro, Gyeongju 38066, Republic of Korea
E-mail: namks@dongguk.ac.kr

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Introduction

Arctigenin (ATG) is a bioactive natural lignan found in the seeds of *Arctium lappa* (Burdock) in the family Asteraceae (1). Burdock has long been used as a folk medicine to treat various infectious symptoms such as inflammation and sore throat (2), and several scientific studies have demonstrated that arctigenin has various physiological activities, which include anti-viral, -oxidative, -inflammatory, and anti-tumor effects (2-12). Furthermore, several recent investigations have reported that ATG exhibits anti-cancer activity in various human cancer cells, including those of breast, pancreatic, hepatic, and colon cancer (1,2,12-18).

Breast cancer is the one of the most common causes of female mortality worldwide (19). Breast cancer may be classified as progesterone receptor (PR) and estrogen receptor (ER) positive, HER2 (human epidermal growth factor 2) over-expressing, and triple-negative breast cancer (TNBC). In particular, TNBC cannot treated using a selective target therapy because it lacks HER2, ER, and PR and has a poor prognosis caused by its high metastatic potential (20-22).

Metastasis is a complex process that results in secondary tumor formation and is caused by the detachment, migration, invasion, and attachment of cells at secondary sites. This process requires the participations of many proteases to degrade extracellular matrix (ECM) and basement membrane (BM), and the matrix metalloproteinases (MMPs) are known play important roles in development, progression, and in the invasion and migration of breast cancers (23). MMPs are classified into 23 types of proteases [e.g., collagenase, stromelysin, gelatinase, and matrilysin) (24)], and MMP-9 (a gelatinase B type) is known to degrade ECM and BM by breaking down gelatin, and to be an important player during invasion and migration. Notably, MMP-9 has been reported to be an important predictor of cancer invasion, metastasis, prognosis, and angiogenesis in breast cancer (24-26). MMP-3 (a stromelysin-1 type) also plays an important role during metastasis and enhances metastasis by activating of MMPs (e.g., MMP-1, MMP-7, and MMP-9) and degrading collagen types II, IV, and IX, proteoglycans, laminin, fibronectin, gelatin, and elastin in ECM and BM (22,23,25,26). In addition, MMP-3 activates MMP-9 via the proteolytic removal of the pro-domain in pro-MMP-9 (27). Flores-Pliego *et al* showed increased MMP-3 secretion in placental leukocytes was closely linked

with MMP-9 secretion and that the activity of MMP-9 was diminished by treating cells with MMP-3 inhibitor (28), which adequately demonstrated MMP-9 and MMP-3 secretions and activities are closely linked.

Furthermore, cyclooxygenase-2 (COX-2) is another known metastasis-enhancing factor and catalyzes the synthesis of prostaglandin E_2 (PGE_2) from arachidonic acid, and thus, enhances metastasis and angiogenesis (23). In one notable study conducted in a COX-2-silenced MDA-MB-231 TNBC xenograft model tumor growth and metastasis to lung were found to be inhibited (29). In another, transfection of siCOX-2 into pancreatic cancer tumors significantly downregulated MMP-9 expression (30). Therefore, it appears the downregulations of MMP-9, MMP-3 and COX-2 are needed to prevent metastatic potential in breast cancer.

The mitogen-activated protein kinases (MAPKs) are typical serine/threonine protein kinases that participate in the regulations of many cellular processes (e.g., growth, proliferation, differentiation, migration, and death) (31-33), and several studies have revealed that MAPKs such as extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), and P38 play important roles during tumor development, progression, metastasis, invasion, and angiogenesis (34,35). MMPs and COX-2 contain promoter sites that bind to transcription factors, such as activating protein-1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and signal transducer and activator of transcription 3 (STAT3), and the activities of these transcription factors are regulated by MAPK, Akt, and STAT signaling pathways, respectively (36-40). AP-1 is formed by a homodimeric or heterodimeric interactions with Jun, Fos, or ATF subunits and the formed complexes bind to AP-1 binding sites on DNA (39-42). Jun/Fos heterodimers are more stable than other AP-1 complexes and have greater DNA binding activity (41,42). Therefore, we evaluated the effect of arctigenin on cancer metastatic potential and investigated whether MAPK/AP-1 signaling is involved in suppression of metastatic potential by arctigenin in 4T-1 mouse TNBC cells.

Materials and methods

Materials. Arctigenin and bovine serum albumin (BSA) were bought from Santa Cruz Biotechnology (Dallas, TX, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Pierce™ BCA Protein Assay Kits were purchased from Thermo Scientific (Waltham, MA, USA). Dimethyl sulfate (DMSO) was obtained from Duksan Pure Chemicals (Ansan, Korea) and protease inhibitor cocktail and phosphatase inhibitor cocktail were from GenDEPOT (Barker, TX, USA). Dulbecco's modified Eagle's medium (DMEM), antimycotic/antibiotic solution and fetal bovine serum (FBS) were obtained from Welgene (Daegu, Korea), Tris-base and glycine were from BioShop Canada Inc. (Burlington, ON, Canada). Polyvinylidenefluoride (PVDF) membranes were purchased from Pall Life Sciences (Port Washington, NY, USA). Antibodies for ERK1/2 (cat. no. 4695), p-ERK1/2 (cat. no. 4370), JNK1/2 (cat. no. 9258), p-JNK1/2 (cat. no. 4668), P38 MAPK (cat. no. 8690), p-P38 MAPK (cat. no. 4511), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB; cat. no. 8242), p-NF-κB (cat. no. 3033), c-Jun (cat. no. 9265), c-Fos (cat. no. 2250), COX-2 (cat. no. 12282), GAPDH (cat. no. 5174), and histone H3 (cat. no. 14269) were obtained from Cell Signaling Technology (Beverly, MA, USA). β-actin (cat. no. sc-69879) was from Santa Cruz Biotechnology (Dallas, TX, USA); HRP-conjugated anti-rabbit IgG (cat. no. NCI1460KR) and -mouse IgG (cat. no. NCI1430OKR) from Thermo Scientific Fisher Scientific, Inc (Rockford, IL, USA); sodium dodecyl sulfate (SDS) from Amresco (VWR Life Science, Radnor, PA, USA), and 30% polyacrylamide solution from SERVA (Heidelberg, Germany). Collagen type I and matrigel were bought from Corning Life Sciences (Bedford, MA, USA), and hematoxylin and eosin were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture and viability assay. 4T-1 mouse TNBC cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and routinely cultured in DMEM containing 10% FBS and 1% antibiotic-antimycotic solution at 37˚C in a 5% CO_2 incubator. The effect of arctigenin on cell viability was evaluated using an MTT assay. Briefly, $2x10^3$ cells/well were seeded into 96-well plates incubated for 24 h at 37˚C, treated with 0, 25, 50, 100 or 200 μM arctigenin and cultured for an additional 24, 48 or 72 h. Cell viabilities were determined by measuring absorbances at 570 nm using a Spectramax M2*e* (Molecular Devices, Sunnyvale, CA, USA).

Wound healing assay. Cells were seeded in 6-well plates coated with collagen type I (Corning Life Sciences, Bedford, MA, USA), grown until confluent, and scratched with a blue tip to create wounds. They were then treated with culture media containing 0, 50, 100, 150 or 200 μM arctigenin. Optical microscopic images were captured from two different areas of each well at 0 and 48 h after wounding.

Transwell invasion assay. The effect of arctigenin on the invasiveness of 4T-1 mouse TNBC cells was determined using transwell chambers (Corning Life Sciences) inserts in 24-well plates. Lower faces of polycarbonate filters (transwell inserts) were coated with matrigel for 1 h at 37˚C, and then, $3x10^4$ cells were seeded into matrigel-coated transwell chambers and 750 μl of culture media was added to lower chambers. After 24 h, cells were treated with conditioned media containing 2 or 10% FBS and 0, 50, 100, 150 or 200 μM arctigenin and incubated at 37˚C in 5% CO_2 atmosphere for 24 h. Cells that migrated across membranes were then fixed and stained using hematoxylin and eosin (H&E) and photographed under an inverted microscope at x200.

Gelatin zymography. The effect of arctigenin on MMP-9 activity was evaluated by gelatin zymography. Briefly $2x10^5$ cells/well were seeded into 6-well plates and allowed to attach for 24 h. Cells were then serum-starved for 4 h and treated with serum-free media supplemented with various concentrations of arctigenin (0, 50, 100, 150 or 200 μM) for 24 h. Conditioned media were then transferred to new conical tubes and centrifuged to remove cell debris. The supernatants were objected by 8% SDS-polyacrylamide gel electrophoresis (PAGE) containing 0.1% (v/v) gelatin under non-reducing conditions. The gel was then washed with 2.5% Triton X-100 for 1 h at

room temperature to remove SDS and gelatinase reactions were performed in reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 0.04% NaN_3) at 37˚C for 24 h. The gel was then stained with Coomassie staining solution (0.05% Coomassie brilliant blue R, 45% methanol, and 10% acetic acid) and destained at room temperature. Densitometric analysis was performed using ImageJ.

RNA extraction, cDNA synthesis, and RT-qPCR. 4T-1 mouse TNBC cells ($2x10^5$ cells/well) were seeded into 6-well plates, allowed to attach for 24 h, and cultured in serum-free DMEM containing 0, 25, 50, 100, 150 or 200 μM arctigenin for an additional 24 h. The cells were then collected by trypsinization for RNA extraction, which was performed using the easy-BLUE™ Total RNA extraction kit (iNtRON Biotechnology, Inc., Sungnam, Korean). Extracted total RNA was quantified using a NanoDrop spectrophotometer (Schimazu Scientific Instruments, Kyoto, Japan), and cDNA was synthesized from 1 μg of total RNA in 1X Goscript reaction buffer containing 2 mM $MgCl_2$, 0.5 mM and Goscript™ Reverse Transcriptase (all from Promega, Madison, WI, USA). RT-q PCR was conducted using Q Green SYBR Green Master Mix Kits (Cellsafe, Suwon, Korea) using an Eco™ Real-Time PCR machine (Illumina, San Diego, CA, USA). cDNA amplification reactions were performed as follows: Pre-heating for 5 min at 95˚C, 45 cycles at 95˚C for 10 sec, 60˚C for 15 sec and 72˚C for 20 sec. Relative mRNA expressions were calculated automatically using the $2^{-\Delta\Delta Cq}$ method and Eco™ Software v3.1.7 (Illumina, Inc.) (43). The primer sequences used for RT-q PCR were as follows: MMP-9, forward, 5'-TGT CTGGAGATTCGACTTCA-3' and reverse, 5'-TGAGTTCCA GGGCACACCA-3'; MMP-3, forward, 5'-CTTTGAAGCATT TGGGTTTCTCTAC-3' and reverse, 5'-AGCTATTGCTCT TCAATATGTGGGT-3'; COX-2, forward, 5'-CCTGCTGCC CGACACCTTCA-3' and reverse, 5'-AGCAACCCGGCCAGC AATCT-3'; β-actin, forward, 5'-CATCCGTAAAGACCTCTA TGCCAAC and reverse, 5'-ATGGAGCCACCGATCCACA-3'.

Nuclear fractionation. 4T-1 mouse TNBC cells were seeded into 6-well plates at $2x10^5$ cells/well and allowed to attach for 24 h. The cells were then serum-starved for 4 h, treated with conditioned-media containing various concentration of arctigenin (0, 25, 50, 100, 150 or 200 μM) for 24 h, and washed twice with ice-cold phosphate buffered saline. Hypertonic buffer (20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$] containing protease inhibitor cocktail and phosphatase inhibitor cocktail (GenDEPOT, Barker, TX, USA) was then added to each well. The cells were detached with a rubber policeman (SPL Life Sciences, Pocheon, Korea), transferred to 1.5 ml microtubes, kept on ice for 15 min, treated with 10% NP-40 (final NP-40 concentration 0.125%) with vortex-mixing for 10 sec at the highest setting, and left on ice for 10 min. Cell mixtures were then centrifuged at 3,000 rpm for 10 min at 4˚C, supernatants (cytosolic fractions) were removed and pellets were lysed with Cell Extraction Buffer (Invitrogen, Carlsbad, CA, USA) containing phosphatase and protease inhibitor cocktail for 30 min on ice, lysates were centrifuged at 14,000 x g for 30 min at 4˚C, and supernatants (nuclear fractions) were collected. Cytosolic and nuclear fractions were stored at -80˚C until required.

Western blotting. After treating 4T-1 mouse TNBC cells for 24 h with various concentration of arctigenin (0, 25, 50, 100, 150 or 200 μM), they were lysed with RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 2 mM ethylenediaminetetraacetic acid] (Biosesang, Seongnam, Korea) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (GenDEPOT, Barker, TX, USA), and centrifuged at 13,000 rpm for 10 min at 4˚C. Supernatants (whole cell lysates) were transferred to microtubes and stored at -80˚C until required. Total protein in whole cell lysates was quantified using the BCA method, and same amounts of total protein in whole cell lysates were subjected to SDS-PAGE. After transferring proteins to PVDF membranes, membranes were blocked with 1% BSA in Tris-buffered saline (TBS)-Tween (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature, probed with primary antibodies diluted 1:3,000 with 1% bovine serum albumin in TBS-Tween solution overnight at 4˚C, washed three times in TBS-Tween, and reacted with secondary antibodies (dilution 1:5,000) for 1 h at room temperature in TBS-Tween. Target proteins were visualized using a homemade chemiluminescent substrate and photographed using a Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo).

Statistical analysis. One-way analysis of variance followed by the Tukey's *post hoc* test was used to determine the significances of differences. The analysis was performed using SPSS Ver. 20.0 software (SPSS, Inc., Chicago, IL, USA), and results are presented as means ± SDs. Statistical significance was accepted for P-values of <0.05.

Results

The effect of arctigenin on the cell viability, migration, and invasion of 4T-1 mouse TNBC cells. We firstly evaluated the effect of arctigenin on the viability of 4T-1 mouse TNBC cells using an MTT assay. As shown in Fig. 1A, arctigenin (100 and 200 μM at 48 and 72 h) slightly reduced cell viability. Furthermore, arctigenin inhibited cell migration and invasiveness as determined by the matrigel invasion and wound healing assays in a concentration-dependent manner, respectively. Lignans should enters into cells with simple diffusion or a low affinity transporter (44). Therefore, these results suggest arctigenin should inhibit invasion and migration by 4T-1 mouse TNBC cells and we postulated the effect was mediated via the regulation of signaling molecules by arctigenin entered into the cells with simple diffusion or a low affinity transporter.

Arctigenin inhibited MMP-9 activity and its gene expression in 4T-1 mouse TNBC cells. Due to its important role on metastasis in breast cancer and the associations between MMP-9 activity and cell migration and invasiveness, we evaluated the effects of arctigenin on MMP-9 activity and its mRNA level. Arctigenin was found to reduce both the protein and mRNA levels of MMP-9 dose-dependently (Fig. 2A and B), which suggested that the suppression of MMP-9 expression by arctigenin was responsible for its inhibition of cell migration and invasion.

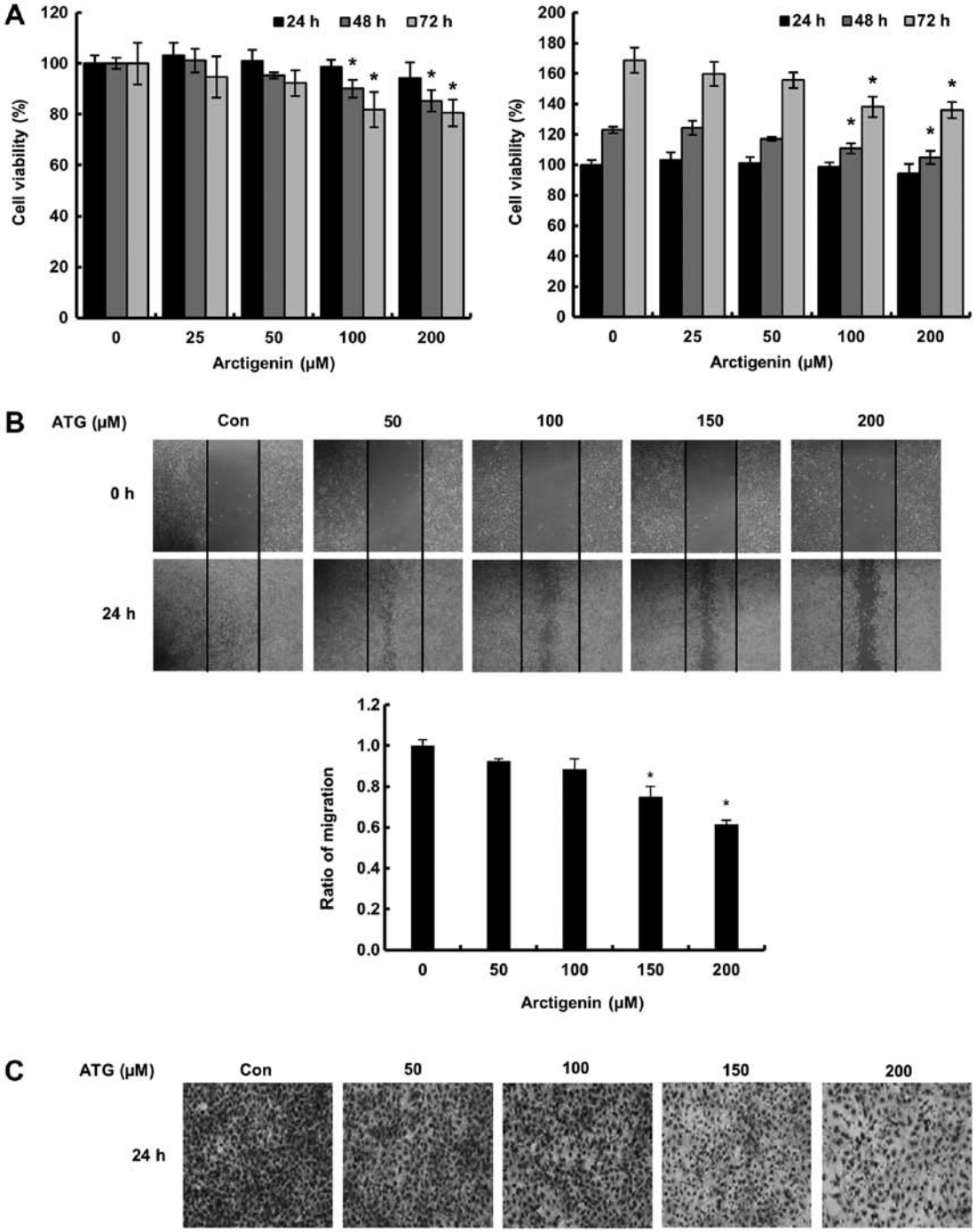

Figure 1. Effects of arctigenin on the viability and metastatic potential of 4T-1 mouse TNBC cells. (A) Arctigenin inhibited the viability of 4T-1 mouse TNBC cells. Cells were seeded into 96-well plates and treated with 25, 50, 100 or 200 μM of arctigenin and cell viabilities were measured at 24, 48 or 72 h. Results are presented as the means ± SD of three independent experiments. *P<0.05. (B) Arctigenin inhibited the motility of 4T-1 mouse TNBC cells. Changes in wound areas were observed using an optical microscope and photographed using a Nikon DS-U3 Digital Sight. Original magnification, x40. Cell migration was densitometrically analyzed and the results were expressed as the relative migration compared with 0 μM arctigenin. The migrations were measured at five points in each sample. Relative migrations are presented as the means ± SD. *P<0.05 compared with 0 μM arctigenin. (C) Arctigenin decreased invasiveness of 4T-1 mouse TNBC cells. Cells that invaded lower membrane surfaces in transwell chambers were observed using an optical microscope and photographed using a Nikon DS-U3 Digital Sight. Original magnification, x200. TNBC, triple-negative breast cancer; SD, standard deviation; ATG, arctigenin.

Arctigenin inhibited MMP-3 mRNA expression in 4T-1 TNBC cells. Active MMP-9 is produced by cleavage of the prodomain in pro-MMP-9 mediated by MMP-3 and MMP-9 and as mentioned above, its activity is also positively associated with MMP-3 activity. We found arctigenin at 150 or 200 μM suppressed MMP-3 transcription (Fig. 2C). Mehner *et al* showed metastatic potential and MMP-3 expression are associated in breast carcinoma (45), and Chu *et al* found breast cancer tumorigenesis and metastasis were prevented by MMP-3 knockdown by mir-519d (46). These reports indicate MMP-3 activity is closely linked with its gene expression. Therefore, our observation suggest arctigenin might inhibit MMP-9 activity by downregulating MMP-3 expression in 4T-1 mouse TNBC cells.

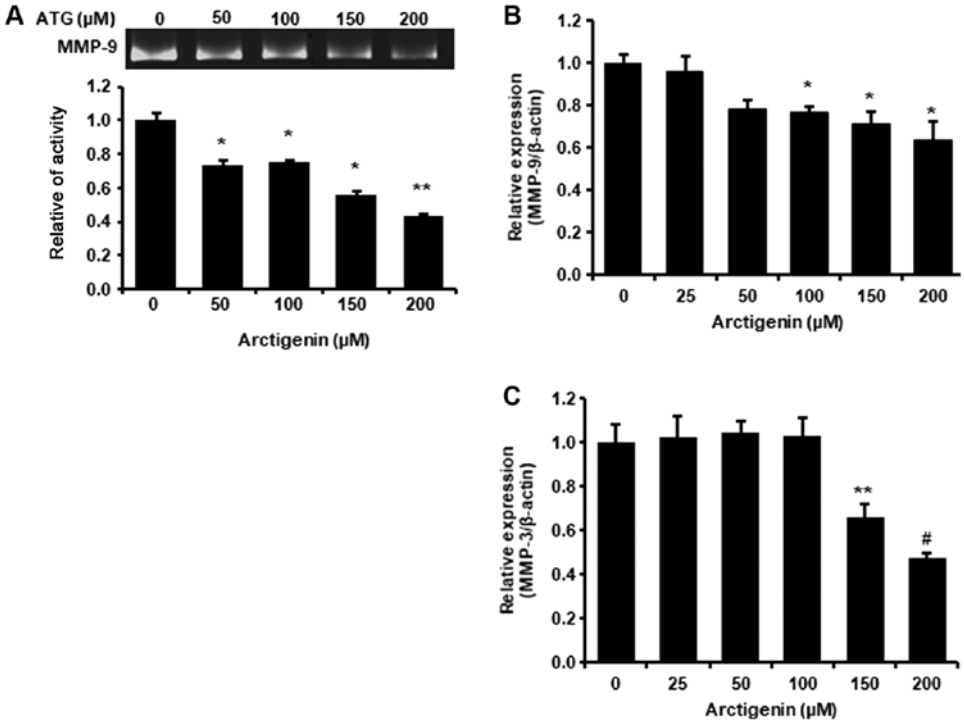

Figure 2. Effects of arctigenin on MMP-9 activity and MMP-9 and -3 mRNA expression in 4T-1 mouse TNBC cells. (A) Arctigenin dose-dependently reduced MMP-9 activity. Protein loading was normalized with respect to the protein concentration in whole cell lysate. Clear bands were analyzed using ImageJ ver. 1.6.0_20 (National Institutes of Health). The results are presented as the means ± SD of three independent experiments. *P<0.05 and **P<0.01, respectively compared with 0 μM arctigenin. (B) MMP-9 transcription was inhibited by arctigenin in 4T-1 mouse TNBC cells. Relative mRNA levels were determined by quantitative real-time RT-PCR. GAPDH was used as the internal control. Results are presented as the means ± SD of three independent experiments. (C) MMP-3 transcription was inhibited by arctigenin in 4T-1 mouse TNBC cells. Relative mRNA levels were determined by quantitative real-time RT-PCR. GAPDH was used as the internal control. Results are presented as the means ± SD of three independent experiments. *P<0.05, **P<0.01 and $^{\#}$P<0.0005, respectively, compared with 0 μM arctigenin. TNBC, triple-negative breast cancer; MMP, matrix metalloprotease-9; SD, standard deviation; ATG, arctigenin.

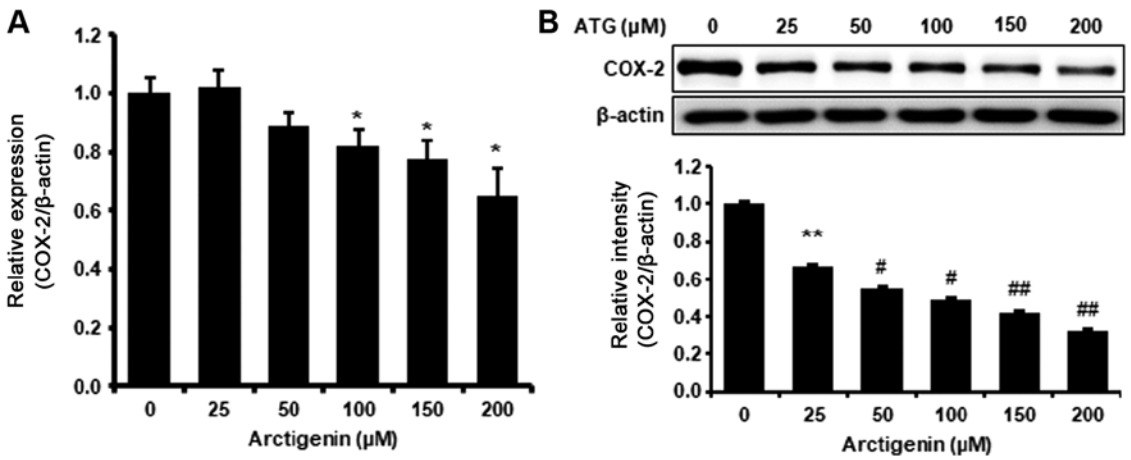

Figure 3. The effect of arctigenin on COX-2 mRNA expression in 4T-1 mouse TNBC cells. (A) COX-2 transcription reduced by arctigenin in 4T-1 mouse TNBC cells. Relative mRNA levels were determined using reverse transcription-quantitative PCR. GAPDH was used as the internal control. Results are presented as the means ± SD of three independent experiments. *P<0.05 compared with 0 μM arctigenin. (B) Arctigenin inhibited COX-2 protein expression in 4T-1 mouse TNBC cells. Each band was densitometrically analyzed using ImageJ ver. 1.6.0_20. β-actin was used as loading control. Results are presented as the means ± SD of three independent experiments. **P<0.01, $^{\#}$P<0.001 and $^{\#\#}$P<0.0005 respectively, compared with 0 μM arctigenin. COX-2, cyclooxygenase-2; TNBC, triple-negative breast cancer; SD, standard deviation.

Arctigenin inhibited COX-2 protein and mRNA levels in 4T-1 mouse TNBC cells. COX-2 is another important factor of metastasis in breast cancer and MMP-9 activity is positively associated with COX-2 expression. We found arctigenin dose-dependently downregulated COX-2 protein and mRNA levels (Fig. 3), which suggests that arctigenin might also reduce cancer cell growth and metastatic potential by inhibiting COX-2 expression in 4T-1 mouse TNBC cells.

Arctigenin inhibited nuclear c-Jun and c-Fos levels via the ERK1/2 and JNK1/2 signaling pathways. Because arctigenin appeared to inhibit 4T-1 TNBC cell migration and invasion

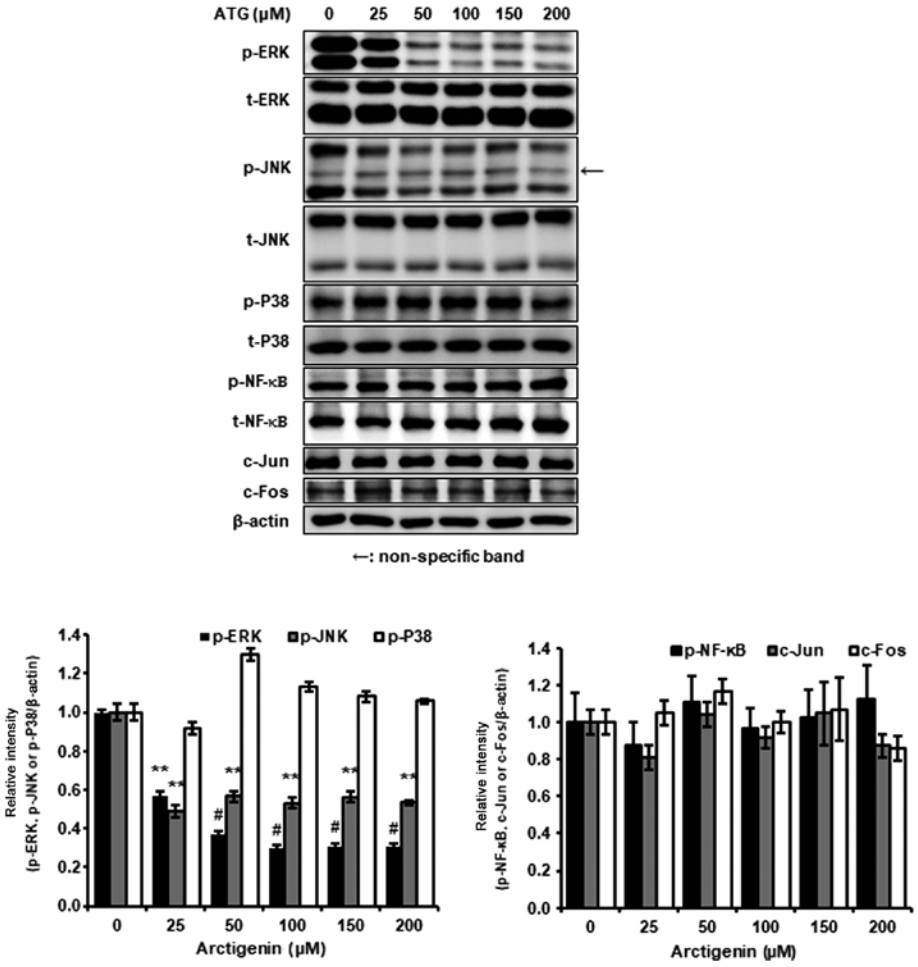

Figure 4. The effect of arctigenin on MAPK phosphorylations and on the protein expression of AP-1 subunits in 4T-1 mouse TNBC cells. Arctigenin was indicated to inhibit the phosphorylation of ERK1/2 and JNK1/2. Bands were densitometrically analyzed using ImageJ ver. 1.6.0_20. β-actin was used as loading control. Results are presented as the mean ± standard deviation of three independent experiments. **P<0.01 and $^{\#}$P<0.001, respectively, compared with 0 μM arctigenin. TNBC, triple-negative breast cancer; p, phosphorylated.

by suppressing MMP-9, MMP-3, and COX-2. Therefore, we investigated the effect of arctigenin on ERK1/2 and JNK1/2 signaling pathways, which are key regulators of the expressions of MMP-9, MMP-3, and COX-2. The results obtained showed that arctigenin inhibited the phosphorylations of ERK1/2 and JNK1/2 but not p38 MAPK (Fig. 4). Arctigenin did not change the whole cell expressions of c-Jun and c-Fos (AP-1 subunits), but attenuated their nuclear expressions and reduced AP-1 transcriptional activity (Fig. 5). Consequently, our results suggest that anti-metastatic activity of arctigenin is governed by reduction in nuclear c-Jun and c-Fos levels and associated inhibitions of the phosphorylations of ERK1/2 and JNK1/2.

Discussion

Although various studies have reported arctigenin has anti-cancer effects on some types of cancer cells, comparatively little is known of its effects on metastasis (2,13). In this study, we evaluated the effect of arctigenin on metastatic potential in 4T-1 mouse TNBC cells. We found arctigenin suppressed the migration and invasiveness of these cells (Fig. 1B and C) but did not significantly decrease cell viability in 24 h (Fig. 1A). These results suggest arctigenin has therapeutic potential for preventing the invasion and migration that are important roles in metastasis in triple-negative breast cancer.

Cell migration and invasiveness are closely associated with the activity of MMP-9 in breast cancer and MMP-9 is activated by proteolytic cleavage of the prodomain in pro-MMP-9 by MMP-3. Furthermore, MMP-9 gene expression is known to be closely associated with that of MMP-3 (26,28). It has also been well-established that the activity and expression of MMP-9 importantly contribute to breast cancer metastasis. Several authors have demonstrated that reductions in MMP-9 activity and expression in breast cancer cells are associated with reduced metastatic potential (22,47,48). In the present study, arctigenin decreased MMP-9 activity and suppressed its gene expression (Fig. 2A and B) and also downregulated MMP-3 mRNA expression (Fig. 2C). Furthermore, arctigenin also inhibited COX-2 at the protein and mRNA levels (Fig. 3), and as mentioned above, COX-2 also affects metastatic potential and MMP-9 activity and expression in cancer cells (29,30). Therefore, our results indicate arctigenin inhibits the migration and invasion of 4T-1 mouse TNBC cells by suppressing the activity and mRNA levels of MMP-9, transcription of MMP-3, and the protein and mRNA expression of COX-2.

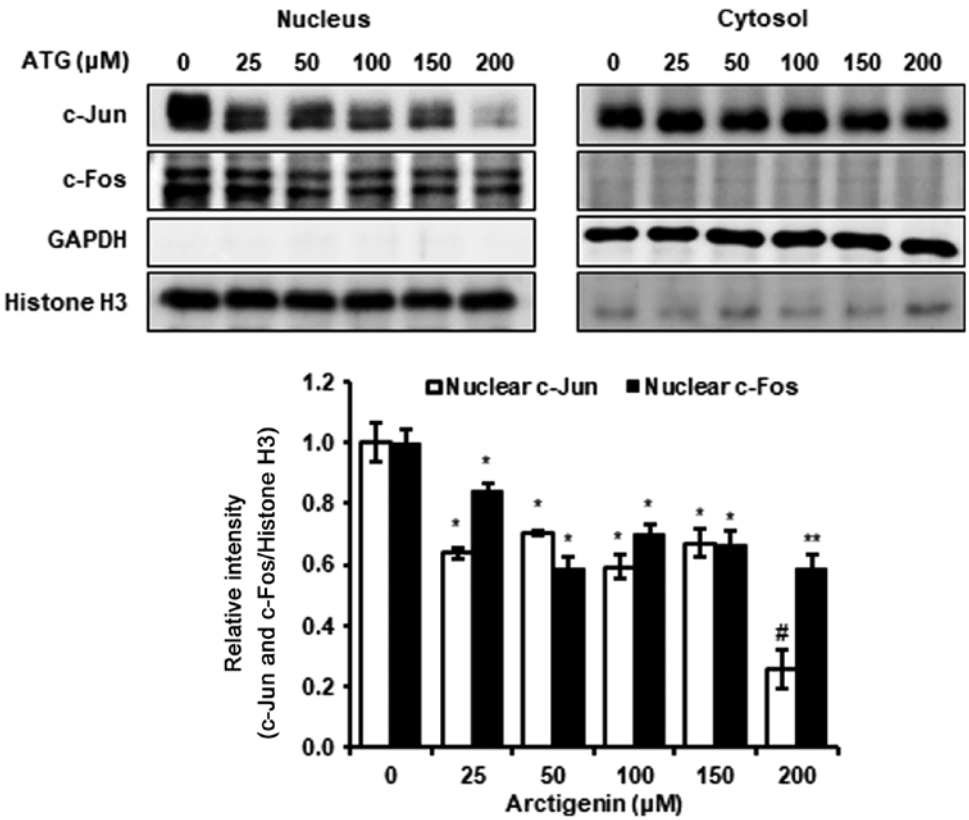

Figure 5. The effect of arctigenin on nuclear c-Jun and c-Fos levels in 4T-1 mouse TNBC cells. Arctigenin suppressed the nuclear level of c-Jun and c-Fos in 4T-1 mouse TNBC cells. Bands were densitometrically analyzed using ImageJ ver. 1.6.0_20. GAPDH and histone H3 was used as loading control for cytosolic and nuclear extracts, respectively. Results are presented as the means ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and $^{\#}$P<0.001, respectively, compared with the 0 μM arctigenin. TNBC, triple-negative breast cancer.

The MAPK/AP-1 signaling pathway plays an important role in the regulations of various metastasis-associated gene expressions. AP-1 is a transcription factor regulated by MAPKs, such as ERK1/2, JNK1/2, and p38 MAPK, and the transcriptional activity of AP-1 is determined by the nuclear levels of AP-1 subunits, which are in turn, governed by the regulation of MAPK phosphorylation. In the present study, we found that arctigenin suppressed the gene expressions of MMP-9, MMP-3 and COX-2 and the phosphorylations of ERK1/2 and JNK1/2, which were associated with reductions in the nuclear levels of c-Jun and c-Fos (AP-1 subunits) (Figs. 2B and C and 3-5). Furthermore, down-regulations of the gene expressions of MMP-9, MMP-3, and COX-2 corresponded to diminished phosphorylations of ERK1/2 and JNK1/2 and decreased nuclear c-Jun and c-Fos. However, p38 MAPK phosphorylation did not affected by arctigenin (Fig. 4). The promotor sites on MMP-9, MMP-3 and COX-2 genes contain AP-1 binding site, and thus, their gene expressions are closely linked with the nuclear levels of c-Jun and c-Fos (36,37,41,42,49,50). Consequently, our results suggest that the inhibitions of MMP-9, MMP-3, and COX-2 by arctigenin are mediated via partial suppression of MAPK/AP-1 signaling pathway. Also, these investigations implies that the inhibitory effects of arctigenin are not associated with the direct inhibition of protein kinase C.

Our findings suggest arctigenin reduces the metastatic potential of 4T-1 mouse TNBC cells by reducing cell motility, invasiveness and MMP-9 activity and that these effects are linked with suppression of the gene expressions of MMP-9, MMP-3, and COX-2 via the partial suppression of MAPK/AP-1 signaling pathway. Taken together, our observations suggest arctigenin be considered a potential means of preventing metastatic potential in triple negative breast cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MGL, KSL and KSN designed the experiments. MGL performed experiments. MGL, KSL and KSN analyzed the data. MGL and KSL wrote the manuscript. KSN reviewed the manuscript. All authors confirmed 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. He Y, Fan Q, Cai T, Huang W, Xie X, Wen Y and Shi Z: Molecular mechanisms of the action of arctigenin in cancer. Biomed Pharmacother 108: 403-407, 2018.
2. Maxwell T, Chun SY, Lee KS, Kim S and Nam KS: The anti-metastatic effects of the phytoestrogen arctigenin on human breast cancer cell lines regardless of the status of ER expression. Int J Oncol 50: 727-735, 2017.
3. Chen J, Li W, Jin E, He Q, Yan W, Yang H, Gong S, Guo Y, Fu S, Chen X, *et al*: The antiviral activity of arctigenin in traditional Chinese medicine on porcine circovirus type 2. Res Vet Sci 106: 159-164, 2016.
4. Shen YF, Liu L, Chen WC, Hu Y, Zhu B and Wang GX: Evaluation on the antiviral activity of arctigenin against spring viraemia of carp virus. Aquaculture 483: 252-262, 2018.
5. Hayashi K, Narutaki K, Nagaoka Y, Hayashi T and Uesato S: Therapeutic effect of arctiin and arctigenin in immunocompetent and immunocompromised mice infected with influenza A virus. Biol Pharm Bull 33: 1199-1205, 2010.
6. Chang CZ, Wu SC, Chang CM, Lin CL and Kwan AL: Arctigenin, a potent ingredient of arctium Lappa L., induces endothelial nitric oxide synthase and attenuates subarachnoid hemorrhage-induced vasospasm through PI3K/Akt pathway in a rat model. Biomed Res Int 2015: 490209, 2015.
7. Wu RM, Sun YY, Zhou TT, Zhu ZY, Zhuang JJ, Tang X, Chen J, Hu LH and Shen X: Arctigenin enhances swimming endurance of sedentary rats partially by regulation of antioxidant pathways. Acta Pharmacol Sin 35: 1274-1284, 2014.
8. Jeong YH, Park JS, Kim DH and Kim HS: Arctigenin increases hemeoxygenase-1 gene expression by modulating PI3K/AKT signaling pathway in rat primary astrocytes. Biomol Ther (Seoul) 22: 497-502, 2014.
9. Gao Q, Yang M and Zuo Z: Overview of the anti-inflammatory effects, pharmacokinetic properties and clinical efficacies of arctigenin and arctiin from *Arctium lappa* L. Acta Pharmacol Sin 39: 787-801, 2018.
10. Zhao F, Wang L and Liu K: In vitro anti-inflammatory effects of arctigenin, a lignan from *Arctium lappa* L., through inhibition on NOS pathway. J Ethnopharmacol 122: 457-462, 2009.
11. Hyam SR, Lee IA, Gu W, Kim KA, Jeong JJ, Jang SE, Han MJ and Kim DH: Arctigenin ameliorates inflammation in vitro and in vivo by inhibiting the PI3K/AKT pathway and polarizing M1 macrophages to M2-like macrophages. Eur J Pharmacol 708: 21-29, 2013.
12. Maxwell T, Lee KS, Kim S and Nam KS: Arctigenin inhibits the activation of the mTOR pathway, resulting in autophagic cell death and decreased ER expression in ER-positive human breast cancer cells. Int J Oncol 52: 1339-1349, 2018.
13. Lou CH, Zhu Z, Zhao Y, Zhu R and Zhao H: Arctigenin, a lignan from *Arctium lappa* L., inhibits metastasis of human breast cancer cells through the downregulation of MMP-2/-9 and heparanase in MDA-MB-231 cells. Oncol Rep 37: 179-184, 2017.
14. Feng T, Cao W, Shen W, Zhang L, Gu X, Guo Y, Tsai HI, Liu X, Li J, Zhang J, *et al*: Arctigenin inhibits STAT3 and exhibits anticancer potential in human triple-negative breast cancer therapy. Oncotarget 8: 329-344, 2017.
15. Hsieh CJ, Kuo PL, Hsu YC, Huang YF, Tsai EM and Hsu YL: Arctigenin, a dietary phytoestrogen, induces apoptosis of estrogen receptor-negative breast cancer cells through the ROS/p38 MAPK pathway and epigenetic regulation. Free Radical Bio Med 67: 159-170, 2014.
16. Awale S, Lu J, Kalauni SK, Kurashima Y, Tezuka Y, Kadota S and Esumi H: Identification of arctigenin as an antitumor agent having the ability to eliminate the tolerance of cancer cells to nutrient starvation. Cancer Res 66: 1751-1757, 2006.
17. Sun Y, Tan YJ, Lu ZZ, Li BB, Sun CH, Li T, Zhao LL, Liu Z, Zhang GM, Yao JC and Li J: Arctigenin inhibits liver cancer tumorigenesis by inhibiting gankyrin expression via C/EBP and PPARα. Front Pharmacol 9: 268, 2018.
18. Li QC, Liang Y, Tian Y and Hu GR: Arctigenin induces apoptosis in colon cancer cells through ROS/p38MAPK pathway. J Buon 21: 87-94, 2016.
19. Tungsukruthai S, Petpiroon N and Chanvorachote P: Molecular mechanisms of breast cancer metastasis and potential anti-metastatic compounds. Anticancer Res 38: 2607-2618, 2018.
20. Campone M, Valo I, Jezequel P, Moreau M, Boissard A, Campion L, Loussouarn D, Verriele V, Coqueret O and Guette C: Prediction of recurrence and survival for triple-negative breast cancer (TNBC) by a protein signature in tissue samples. Mol Cell Proteomics 14: 2936-2946, 2015.
21. Gautam P, Karhinen L, Szwajda A, Jha SK, Yadav B, Aittokallio T and Wennerberg K: Identification of selective cytotoxic and synthetic lethal drug responses in triple negative breast cancer cells. Mol Cancer 15: 34, 2016.
22. Mehner C, Hockla A, Miller E, Ran S, Radisky DC and Radisky ES: Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget 5: 2736-2749, 2014.
23. Lee KS, Shin JS and Nam KS: Effect of proton beam irradiation on the regulation of metastasis-enhancing factors in MCF-7 human breast cancer cells. J Korean Phys Soc 63: 1373-1378, 2013.
24. Duffy MJ, McGowan PM and Gallagher WM: Cancer invasion and metastasis: Changing views. J Pathol 214: 283-293, 2008.
25. Brinckerhoff CE and Matrisian LM: Timeline-Matrix metalloproteinases: A tail of a frog that became a prince. Nat Rev Mol Cell Bio 3: 207-214, 2002.
26. Ye S, Eriksson P, Hamsten A, Kurkinen M, Humphries SE and Henney AM: Progression of coronary atherosclerosis is associated with a common genetic variant of the human stromelysin-1 promoter which results in reduced gene expression. J Biol Chem 271: 13055-13060, 1996.
27. Steenport M, Khan KM, Du B, Barnhard SE, Dannenberg AJ and Falcone DJ: Matrix metalloproteinase (MMP)-1 and MMP-3 induce macrophage MMP-9: Evidence for the Role of TNF-alpha and Cyclooxygenase-2. J Immunol 183: 8119-8127, 2009.
28. Flores-Pliego A, Espejel-Nuñez A, Castillo-Castrejon M, Meraz-Cruz N, Beltran-Montoya J, Zaga-Clavellina V, Nava-Salazar S, Sanchez-Martinez M, Vadillo-Ortega F and Estrada-Gutierrez G: Matrix metalloproteinase-3 (MMP-3) is an endogenous activator of the MMP-9 secreted by placental leukocytes: Implication in human labor. PLoS One 10: e0145366, 2015.
29. Stasinopoulos I, O'Brien DR, Wildes F, Glunde K and Bhujvalla ZM: Silencing of cyclooxygenase-2 inhibits metastasis and delays tumor onset of poorly differentiated metastatic breast cancer cells. Mol Cancer Res 5: 435-442, 2007.
30. Bu X, Zhao C and Dai X: Involvement of COX-2/PGE2 pathway in the upregulation of MMP-9 expression in pancreatic cancer. Gastroenterol Res Prac 2011: 214269, 2011.
31. Dhillon AS, Hagan S, Rath O and Kolch W: MAP kinase signalling pathways in cancer. Oncogene 26: 3279-3290, 2007.
32. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K and Cobb MH: Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev 22: 153-183, 2001.
33. Low HB and Zhang Y: Regulatory roles of MAPK phosphatases in cancer. Immune Netw 16: 85-98, 2016.
34. Reddy KB, Nabha SM and Atanaskova N: Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev 22: 395-403, 2003.
35. Yang M and Huang CZ: Mitogen-activated protein kinase signaling pathway and invasion and metastasis of gastric cancer. World J Gastroenterol 21: 11673-11679, 2015.
36. Benbow U and Brinckerhoff CE: The AP-1 site and MMP gene regulation: What is all the fuss about? Matrix Biol 15: 519-526, 1997.
37. Mishra M, Flaga J and Kowluru RA: Molecular mechanism of transcriptional regulation of matrix metalloproteinase-9 in diabetic retinopathy. J Cell Physiol 231: 1709-1718, 2016.
38. Huang F, Cao J, Liu Q, Zou Y, Li H and Yin T: MAPK/ERK signal pathway involved expression of COX-2 and VEGF by IL-1β induced in human endometriosis stromal cells in vitro. Int J Clin Exp Pathol 6: 2129-2136, 2013.

39. Karin M: The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270: 16483-16486, 1995.
40. Weekes D, Kashima TG, Zandueta C, Perurena N, Thomas DP, Sunters A, Vuillier C, Bozec A, El-Emir E, Miletich I, *et al*: Regulation of osteosarcoma cell lung metastasis by the c-Fos/AP-1 target FGFR1. Oncogene 35: 2852-2861, 2016.
41. Zhang Y, Pu X, Shi M, Chen L, Qian L, Song Y, Yuan G, Zhang H, Yu M, Hu M, *et al*: c-Jun, a crucial molecule in metastasis of breast cancer and potential target for biotherapy. Oncol Rep 18: 1207-1212, 2007.
42. Malnou CE, Brockly F, Favard C, Moquet-Torcy G, Piechaczyk M and Jariel-Encontre I: Heterodimerization with different Jun proteins controls c-Fos intranuclear dynamics and distribution. J Biol Chem 285: 6552-6562, 2010.
43. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
44. During A, Debouche C, Raas T and Larondelle Y: Among plant lignans, pinoresinol has the strongest antiinflammatory properties in human intestinal Caco-2 cells. J Nutr 142: 1798-1805, 2012.
45. Mehner C, Miller E, Nassar A, Bamlet WR, Radisky ES and Radisky DC: Tumor cell expression of MMP3 as a prognostic factor for poor survival in pancreatic, pulmonary, and mammary carcinoma. Genes Cancer 6: 480-489, 2015.
46. Chu C, Liu X, Bai X, Zhao T, Wang M, Xu R, Li M, Hu Y, Li W, Yang L, *et al*: MiR-519d suppresses breast cancer tumorigenesis and metastasis via targeting MMP3. Int J Biol Sci 14: 228-236, 2018.
47. Nalla AK, Gorantla B, Gondi CS, Lakka SS and Rao JS: Targeting MMP-9, uPAR, and cathepsin B inhibits invasion, migration and activates apoptosis in prostate cancer cells. Cancer Gene Ther 17: 599-613, 2010.
48. Xu DM, Mckee CM, Cao YH, Ding YC, Kessler BM and Muschel RJ: Matrix metalloproteinase-9 regulates tumor cell invasion through cleavage of protease nexin-1. Cancer Res 70: 6988-6998, 2010.
49. Park CH, Lee MJ, Ahn J, Kim S, Kim HH, Kim KH, Eun HC and Chung JH: Heat shock-induced matrix metalloproteinase (MMP)-1 and MMP-3 are mediated through ERK and JNK activation and via an autocrine interleukin-6 loop. J Invest Dermatol 123: 1012-1019, 2004.
50. Hannemann N, Jordan J, Paul S, Reid S, Baenkler HW, Sonnewald S, Bäuerle T, Vera J, Schett G and Bozec A: The AP-1 Transcription factor c-Jun promotes arthritis by regulating cyclooxygenase-2 and arginase-1 expression in macrophages. J Immunol 198: 3605-3614, 2017.