Surfactin suppresses TPA-induced breast cancer cell invasion through the inhibition of MMP-9 expression

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Abstract. Metastasis is the main cause of cancer mortality. In this study, we investigated the effects of surfactin, a cyclic lipopeptide produced by *Bacillus subtilis*, on cancer metastasis *in vitro* and the underlying molecular mechanisms involved. Surfactin inhibited the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced invasion, migration and colony formation of human breast carcinoma cells. Western blot analysis, gelatin zymography and reverse transcription-PCR analysis revealed that matrix metalloproteinase-9 (MMP-9) expression and activation was significantly suppressed by surfactin in a dose-dependent manner. Surfactin attenuated TPA-induced nuclear translocation and activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Furthermore, surfactin strongly repressed the TPA-induced phosphorylation of Akt and extracellular signal-regulated kinase (ERK). Treatment with specific inhibitors of Akt and ERK suppressed MMP-9 expression and activation. These results suggest that the surfactin-mediated inhibition of breast cancer cell invasion and MMP-9 expression involves the suppression of the NF-κB, AP-1, phosphatidylinositol 3-kinase (PI-3K)/Akt and the ERK signaling pathways. Thus surfactin may have potential value in therapeutic strategies for the treatment of breast cancer metastasis.

Introduction

Breast cancer is one of the most common forms of malignancy in females worldwide, and the main cause of mortality from breast cancer is its metastasis from the primary tumor site (1,2). Metastasis is a multi-step process that involves invasion into the local area at the primary site, followed by intravasation of tumor cells which leads to general circulation, extravasation and colonization in distant organs (3,4). Despite successful treatment of the primary tumor, subsequent metastatic spread can still occur in other areas of the body through the bloodstream or lymphatic vessels. Thus, effective chemopreventive treatments for breast cancer metastasis may have a significant impact on breast cancer morbidity and mortality.

The metastatic process is initiated by invasion which involves changes in cell adhesion, proteolytic degradation of the surrounding tissue and migration of tumor cells through tissue (3). The extracellular matrix (ECM), a biochemical and mechanical barrier to cancer cell movement, is degraded by extracellular proteinases. Among these proteinases, the matrix metalloproteinases (MMPs) have been shown to play an essential role in tumor metastasis (5-7). MMPs are a family of zinc- and calcium-dependent endoproteinases that are divided into four subclasses based on their substrate; namely, collagenase, gelatinase, stromelysin and membrane-associated MMPs (6,8). MMPs are synthesized as pre-proenzymes and the secreted from cells as proenzymes. MMP-2 and MMP-9 are the key enzymes involved in the degradation of the main constituent of the basement membrane due to their ability to degrade type IV collagen which is a major component of the basement membrane; accordingly, they are essential to cancer invasion and metastasis (6,9). While MMP-2 is constitutively overexpressed in highly metastatic tumors, the enhanced expression of MMP-9 has been shown to be associated with the progression and invasion of tumors, and to strongly correlate with the malignant phenotype in various types of cancer. The expression of MMP-9 can be stimulated by various agents, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the inflammatory cytokine, TNF-α, or epidermal growth factor EGF (10-12). Consequently, inhibiting MMP-9 expression and/or its upstream regulatory pathways is critical to the treatment of malignant tumors, including breast cancer.

Surfactin, a biosurfactant produced by *Bacillus subtilis*, is a cyclic lipopeptide built from a heptapeptide and a β-hydroxy fatty acid with variable chain lengths of 13-15 carbon atoms (13). Biosurfactants have certain advantages over their chemical counterparts as they are biodegradable, less toxic and effective at extreme temperatures or pH values. As a result, they have the potential for application in various fields of industry including biomedicine (13). Surfactin has been shown to exert anticarcinogenic, antifungal, antibacterial and anti-inflammatory effects (13-17). However, the effects of surfactin on cancer invasion and metastasis remain unknown. In this study, we investigated effects of surfactin on the invasion, migration and

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the colony-forming ability of human breast carcinoma cells and the molecular mechanism underlying theses processes.

Materials and methods

Reagents. Surfactin (from *B. subtilis*), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other reagents not referred were purchased from Sigma-Aldrich (St. Louis, MO, USA). BD BioCoat™ Matrigel™ Invasion Chambers were obtained from BD Biosciences (San Jose, CA, USA). SB203580, SP600125 and LY294002 were purchased from A.G. Scientific (San Diego, CA, USA). The FuGene 6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN, USA). Antibodies against phosphorylated p38 (p-p38), phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated Akt (p-Akt), phosphorylated inhibitory protein κB (p-IκB)-α, MMP-2 and MMP-9 were purchased from Cell Signaling Technology (Beverly, MA, USA), and antibodies against ERK, JNK, p38, Akt, c-Jun, c-Fos, nuclear factor-κB (NF-κB), inhibitory protein κB-α (IκB-α) and histone H1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. The human breast cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI (Gibco BRL, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37˚C in a humidified incubator with 5% CO_2.

Determination of cell viability. The effect of surfactin on cell viability was determined using an established MTT assay. Briefly, cells ($5x10^4$ cells/24-well) were seeded in wells and incubated at 37˚C for 24 h to allow attachment. The attached cells were treated with TPA in the presence or absence of surfactin for 24 h at 37˚C. The cells were washed with phosphate-buffered saline (PBS), after which MTT (62.5 μg/ml) was added and the cells were incubated at 37˚C for 30 min. Following incubation, formazan crystals were dissolved with dimethylsulfoxide (150 μl/well) and detected at 570 nm using a microplate reader (Wallac 1420, PerkinElmer Life Sciences, Boston, MA, USA).

Wound-healing assay. For the cell migration assay, cells were seeded into a 24-well culture dish until 90% confluent. The cells were then maintained in serum-free medium for 12 h. The monolayers were carefully scratched using a 200-μl pipette tip. The cellular debris was subsequently removed by washing with PBS, and the cells were incubated in medium without serum. The migrated cells were fixed in cold 75% methanol for 30 min and washed three times with PBS. The cultures were photographed at 0 and 24 h to monitor the migration of cells into the wounded area, and the closure of the wounded area was calculated.

Matrigel invasion assay. A cell invasion assay was conducted using BioCoat Matrigel Invasion Chambers according to the manufacturer's instructions. Briefly, the Matrigel coating was re-hydrated in 0.5 ml DMEM for 30 min immediately before the experiments. Cells ($5x10^4$) suspended in 0.5 ml of serum-free medium were then added to the upper chamber of Matrigel-coated filter inserts. After treatment with surfactin for 1 h, 0.5 ml of serum-free medium containing TPA was added to the bottom well as a chemoattractant. The chambers were then incubated for 24 h. After incubation, cells on the upper side of the chamber were removed using cotton swabs. Cells that had migrated were then fixed and stained with 2% ethanol containing 0.2% crystal violet powder. Invading cells were enumerated under a light microscope using a x10 objecive lens.

Gelatin zymography assay. The activity of MMP-2 and MMP-9 in the conditioned medium was determined by gelatin zymography protease assays. Briefly, cells ($2x10^5$) were seeded in 6-well plates and allowed to grow to 80% confluency. The cells were then maintained in serum-free medium for 12 h prior to being treated with surfactin and TPA for 24 h. The conditioned medium was subsequently collected, cleared by centrifugation and mixed with 2X sodium dodecyl sulphate (SDS) sample buffer, followed by electrophoresis on polyacrylamide gels containing 0.1% (w/v) gelatin. Following electrophoresis, the gels were incubated in renaturing buffer (2.5% Triton X-100) with gentle agitation to remove the SDS and were then incubated in developing buffer (50 mM Tris-HCl buffer, pH 7.4, and 10 mM $CaCl_2$) overnight at 37˚C to allow digestion of the gelatin. Finally, the gels were stained with SimplyBlue SafeStain (Invitrogen Corp., Carlsbad, CA, USA) until clear bands indicative of gelatin digestion appeared.

Western blot analysis. Cells were harvested in ice-cold lysis buffer consisting of 1% Triton X-100, 1% deoxycholate and 0.1% sodium SDS. The protein content of the cell lysates was then determined using Bradford reagent (Bio-Rad, Hercules, CA, USA). Proteins in each sample were resolved by 12% SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and exposed to the appropriate antibodies. The proteins were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) using horseradish peroxidase-conjugated secondary antibodies. Images were acquired using an ImageQuant 350 analyzer (Amersham Biosciences).

Reverse transcription (RT)-PCR and real-time PCR. Total cellular RNA was isolated using RNA Spin Mini RNA isolation kits (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using Maxime RT PreMix (Intron Biotechnology, Seongnam, Korea) and anchored oligo-$(dT)_{15}$-primers. The amplification sequence protocol was as follows: 25 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min. The PCR products were subjected to 1.5% agarose gel electrophoresis and images were captured by an ImageQuant 350 analyzer (Amersham Biosciences). Real-time PCR was performed using the SYBR-Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a Chromo4 instrument (Bio-Rad). The relative amount of target mRNA was determined using the Ct method by normalizing target mRNA Ct values to those for GAPDH (ΔCt) (18). Real-time PCR was conducted by subjecting the samples to the following conditions: 95˚C for 5 min followed by 40 cycles of 95˚C for 30 sec, 55˚C for 20 sec and 72˚C for 30 sec. The following primers were used for

PCR: MMP-9 sense, 5'-TTCCCTGGAGACCTGAGAACC-3' and antisense, 5'-CGGCAAGTCTTCCGAGTAGTTT-3'; MMP-2 sense, 5'-GATGGCACCCATTTACACCT-3' and antisense, 5'-CACAGTCCGCCAAATGAA-3'; and GAPDH sense, 5'-AGGTGGTCTCCTCTGACTTC-3' and antisense, 5'-TACCAGGAAATGAGCTTGAC-3'.

Immunofluorescence confocal microscopy. MCF-7 cells were cultured directly on glass coverslips in 35 mm-diameter dishes. Cells were fixed with -20˚C methanol for 10 min. To investigate the cellular localization of NF-κB, we treated cells with a 1:100 dilution of polyclonal antibody against NF-κB for 24 h. The cells were then extensively washed with PBS, after which they were further incubated with a 1:1,000 dilution of secondary fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG antibody for 4 h at room temperature. Cell nuclei were stained with 1 μg/ml of 4',6-diamidino-2-phenylindole (DAPI), and then analyzed by confocal microscopy using an LSM 510 Meta microscope (Zeiss, Jena, Germany).

Chromatin immunoprecipitation (ChIP) assay. To detect the *in vivo* association of nuclear proteins with human MMP-9 promoter, ChIP analysis was conducted as described previously (19) with some modifications. Briefly, $2x10^7$ cells were incubated in culture medium containing 1% formaldehyde for 10 min at room temperature, after which the cross-linking reaction was quenched by adding glycine to a final concentration of 0.125 M. The isolated nuclei were then digested with 10 U of MNase at 37˚C for 15 min followed by sonication to produce chromatin of primarily mononucleosome size. Fragmented chromatin was then reacted with antibodies for 3 h at 4˚C. Protein-DNA complexes were recovered using protein A agarose beads, washed and eluted with elution buffer. Crosslinks were reversed at 65˚C in 0.25 M NaCl overnight and the DNA was then digested with proteinase K for 2 h at 50˚C. The immunoprecipitated DNAs were subsequently isolated and used for PCR. PCR primers specific for the MMP-9 promoter [including NF-κB/activator protein-1 (AP-1) cluster, GenBank accession no. AF538844] were as follows: sense, 5'-CACTTCAAAGTGGTAAGA-3' antisense, 5'-GAAAGTGATGGAAGACTCC-3'.

Transient transfection and dual luciferase assay. We used a dual-luciferase reporter assay system (Promega, Madison, WI, USA) to determine promoter activity. Briefly, cells were transfected with NF-κB luciferase reporter plasmid (20) or AP-1 luciferase reporter plasmid (Stratagene, Grand Island, NY, USA) using FuGENE-6 reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Renilla luciferase control plasmid pRL-CMV (Promega) was co-transfected as an internal control to evaluate transfection efficiency. Twenty four hours after transfection, the cells were incubated with the indicated reagents for 1 h and then treated with TPA for 24 h. Luciferase activity was assayed using the dual-luciferase assay kit (Promega) according to the manufacturer's instructions. Luminescence was measured with a GloMax™ 96 microplate luminometer (Promega).

Statistical analysis. Each experiment was repeated at least three times and all results are expressed as the means ± SE. Statistical analysis was performed using SPSS software (version 18.0) to determine significant differences. We used either one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for comparison between three or more groups. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxicity of surfactin against human breast cancer cells. To verify the effects of surfactin on cell viability, non-aggressive MCF-7 and aggressive MDA-MB-231 human breast cancer cells were treated with surfactin in the absence or presence of TPA for 24 h. When compared with the untreated control cells, MCF-7 and MDA-MB-231 cells treated with surfactin at concentrations between 0 and 10 μM exhibited no cytotoxicity, regardless of whether they were treated with TPA or not (Fig. 1). Thus, this concentration range of surfactin was applied in all the subsequent experiments.

Surfactin inhibits TPA-induced migration of human breast cancer cells in vitro. In order to investigate the effects of surfactin on the invasive potency of breast cancer cells, we carried out a wound-healing assay in non-aggressive MCF-7 and aggressive MDA-MB-231 human breast cancer cells. When confluent monolayers of cells were treated with TPA for 24, the MCF-7 and MDA-MB-231 cells readily closed the gap over 24 h, whereas the untreated cells did not. In the wound-healing assay, 10 μM of surfactin caused a decrease in the number of cells migrating into the wound area (Fig. 2).

Surfactin inhibits TPA-induced invasion of human breast cancer cells in vitro. Considering that invasion across the basement membrane by cancer cells is a critical process in tumor metastasis, we used Transwell invasion assay to investigate the effects of surfactin on cancer cell invasion. When MCF-7 and MDA-MB-231 cells were treated with TPA, the cells were able to invade freely through the Matrigel. The numbers of MCF-7 and MDA-MB-231 cells that passed through Matrigel were remarkably decreased by treatment with surfactin (Fig. 3), and the inhibition rates were approximately 68 and 84%, respectively.

Surfactin suppresses TPA-induced colony formation of human breast cancer cells in vitro. Tumor metastasis is a multi-step and complex process that includes the proteolytic digestion of the ECM, cell migration to the circulation system, as well as colonization at metastatic sites. Therefore, we examined the effects of surfactin on clonogenicity with soft agar colony formation assays. As shown in Fig. 4, surfactin inhibited the anchorage-independent growth of MCF-7 and MDA-MB-231 cells and the inhibition rates were approximately 70 and 61%, respectively.

Surfactin suppresses TPA-induced MMP-9 expression and enzyme activity. The upregulation of MMP-9 has been reported to play an essential role in invasion and metastasis in breast cancer cells (6). Thus, we examined whether the inhibitory effect of surfactin against breast cancer cell invasion is associated with the regulation of MMP-9 expression and enzyme

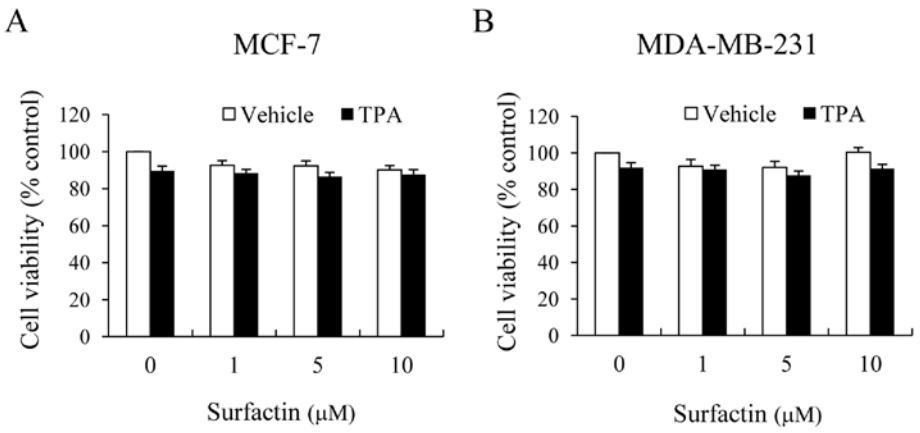

Figure 1. Effect of surfactin on breast cancer cell viability. (A) MCF-7 and (B) MDA-MB-231 cells were treated with the indicated concentrations of surfactin in the absence or presence of TPA (50 ng/ml) for 24 h. The cell viabilities were then determined by MTT assay.

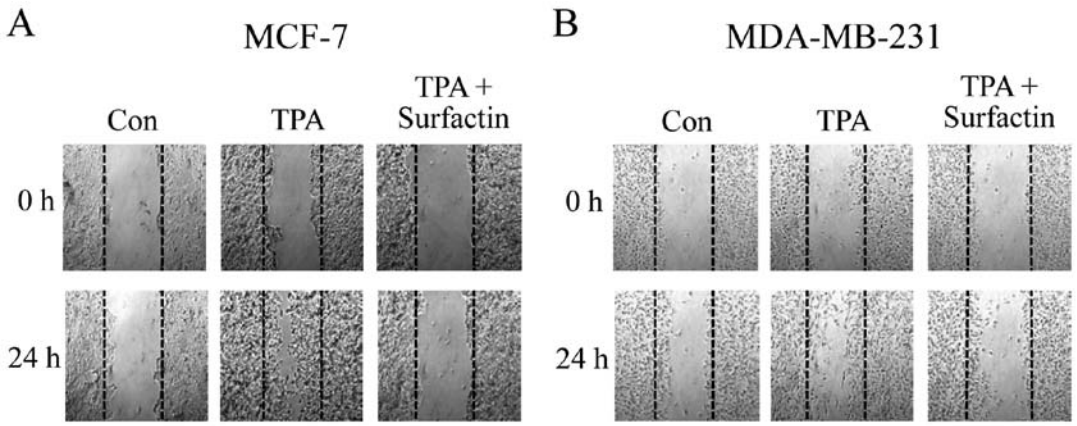

Figure 2. Effect of surfactin on breast cancer cell migration. (A) MCF-7 and (B) MDA-MB-231 cells were scratched with a pipette tip and then incubated with TPA (50 ng/ml) for 24 h in the absence or presence of surfactin (10 μM). Migrating cells were photographed under a phase contrast microscope.

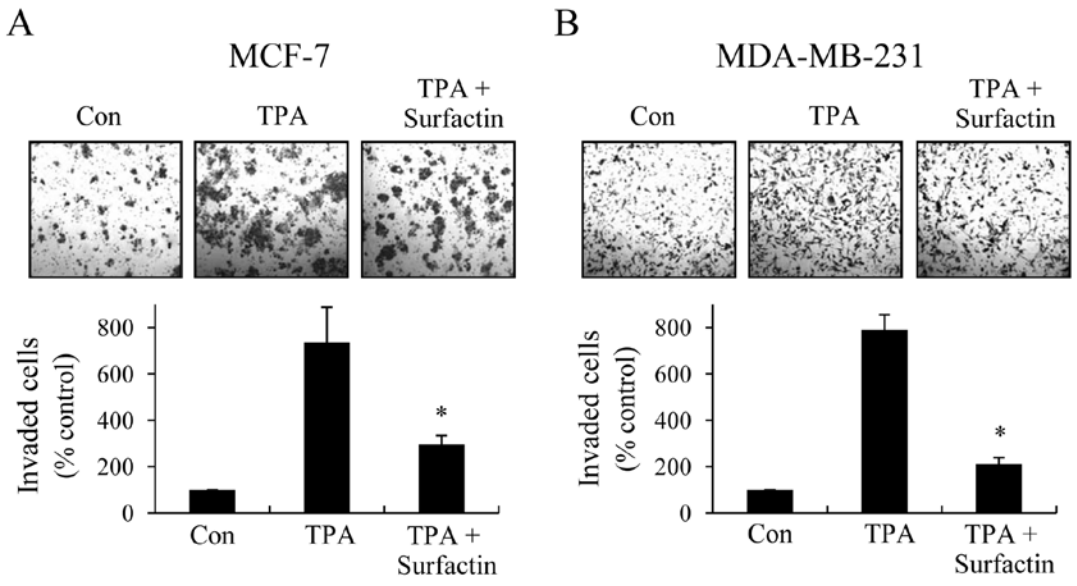

Figure 3. Effect of surfactin on breast cancer cell invasion. MCF-7 (A) and (B) MDA-MB-231 cells were incubated with TPA (50 ng/ml) for 24 h in the absence or presence of surfactin (10 μM), and the cells invading the bottom side of the filter were stained with crystal violet and counted under a microscope. The invaded cells were counted from three independent experiments (lower panel). $^{*}p<0.05$ vs. the TPA-treated group.

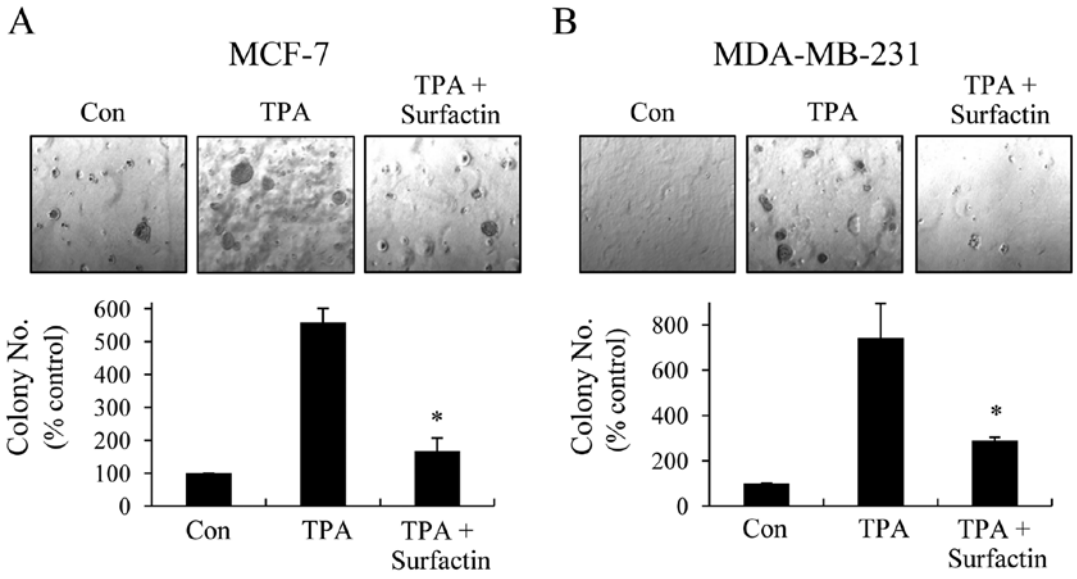

Figure 4. Inhibitory effect of surfactin on the colony-forming ability of human breast cancer cells. (A) MCF-7 and (B) MDA-MB-231 cells were cultured in soft agar gel with TPA (50 ng/ml) for two weeks in the absence or presence of surfactin (10 μM). The colonies were counted from three independent experiments (lower panel). *p<0.05 vs. the TPA-treated group.

activity by western blot analysis and gelatin zymography assay. To accomplish this, MCF-7 cells were treated with surfactin 1 h prior to the addition of TPA and then incubated for a further 24 h. The medium from the control cells contained weak proteolytic activity at 92 kDa corresponding to MMP-9 and high proteolytic activity at 72 kDa corresponding to MMP-2. Treatment with TPA for 24 h dramatically upregulated MMP-9 activity, while the activity of MMP-2 was not affected by TPA or surfactin. TPA-induced MMP-9 secretion (in conditioned medium) was also dramatically inhibited in the presence of surfactin in a dose-dependent manner (Fig. 5A and B). Furthermore, the treatment of breast cancer cells with surfactin decreased the TPA-stimulated intracellular expression of MMP-9 in a dose-dependent manner, while the level of MMP-2 was not affected by TPA or surfactin (Fig. 5A and B).

To determine whether the inhibition of MMP-9 expression by surfactin was due to a reduced level of transcription, we performed RT-PCR and real-time PCR. The treatment of breast cancer cells with surfactin significantly inhibited the levels of TPA-stimulated MMP-9 mRNA in a dose-dependent manner, whereas surfactin together with TPA had little effect on the MMP-2 mRNA levels (Fig. 5C and D). These results indicate that surfactin suppresses TPA-induced MMP-9 expression through the inhibition of its transcriptional activity. Surfactin had similar inhibitory effects on MMP-9 expression in MDA-MB-231 cells (data not shown).

To confirm the involvement of MMP-9 in breast cancer cell invasion, we examined the effects of MMP-9 siRNA via a Matrigel invasion assay. MMP-9 siRNA reduced the expression of MMP-9 and the knockdown of MMP-9 markedly decreased the invasion of MCF-7 cells (Fig. 5E and F).

Surfactin does not affect the expression of tissue inhibitors of metalloproteinases (TIMPs). Since the physiological activity of MMP-9 is closely related to that of TIMPs, specific endogenous inhibitors of MMP-9 (21), we investigated the potential effects of surfactin on TIMP-1 and TIMP-2 expression. As shown in Fig. 5G, the expression levels of TIMP-1 and TIMP-2 were not altered by surfactin. When the MCF-7 cells were co-treated with surfactin and TPA, TIMP-1 and TIMP-2 were slightly upregulated by TPA, although their expression level was not altered by surfactin (Fig. 5H). Similar effects of surfactin on TIMP-1 and TIMP-2 expression were observed in the MDA-MB-231 cells (data not shown).

Surfactin inhibits MMP-9 activity through the suppression of NF-κB and AP-1 activity. We further investigated the mechanism of MMP-9 transcriptional regulation by surfactin. MMP-9 expression is known to be regulated by the interaction of transcription factors, such as NF-κB and AP-1 with binding elements in the MMP-9 gene promoter (22,23). Therefore, we examined the effect of surfactin on NF-κB and AP-1 activity in TPA-stimulated breast cancer cells. MCF-7 cells were transiently transfected with NF-κB-Luc reporter or AP-1-Luc reporter plasmid, and the reporter activities were found to be regulated by surfactin. The TPA-induced increases in NF-κB and AP-1 reporter activities were suppressed by surfactin in a dose-dependent manner (Fig. 6A and B).

The MMP-9 promoter contains *cis*-acting regulatory elements for transcription factors, including two Ap-1 sites (-79 and -533) and one NF-κB site (-600); therefore, we used a ChIP assay to determine the effects of surfactin on the binding activities of NF-κB and AP-1 with the MMP-9 promoter. Chromatin was extracted and immunoprecipitated using anti-NF-κB or AP-1 antibodies, and the MMP-9 promoter region (NF-κB/AP-1 cluster -739 to -358) was amplified by PCR (Fig. 6C) and real-time PCR (Fig. 6D). The *in vivo* binding of NF-κB and AP-1 to the MMP-9 promoter increased in response to TPA; however, the TPA-induced NF-κB and AP-1 binding activities were significantly inhibited by surfactin.

To elucidate whether surfactin can affect the nuclear translocation of NF-κB and AP-1 transcription factors, MCF-7 cells

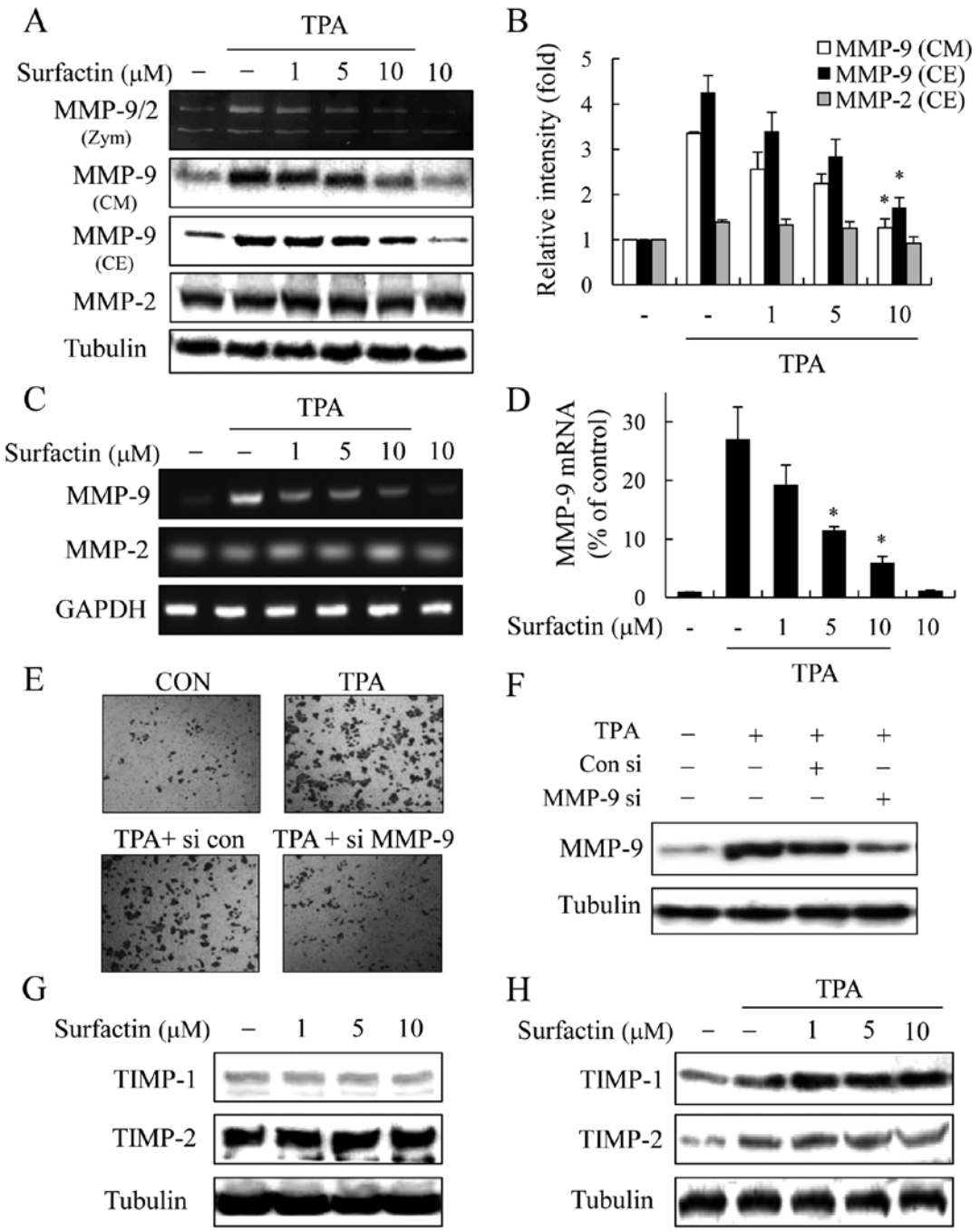

Figure 5. Effect of surfactin on TPA-induced expression and activation of MMP-9. (A) MCF-7 cells were treated with surfactin for 1 h followed by TPA (50 ng/ml) treatment for 24 h. Culture supernatant was analyzed by gelatin zymography (Zym) and western blot analysis with anti-MMP-9 antibody (CM, conditioned medium), while the cellular extract (CE) was analyzed by western blot analysis with anti-MMP-9 or -MMP-2 antibodies. (B) Relative intensity (MMP/tubulin) of data from western blot analysis was transformed into a histogram. (Cand D) MCF-7 cells were treated with surfactin followed by TPA (50 ng/ml) treatment for 6 h. MMP-9 and MMP-2 mRNA levels were analyzed by RT-PCR (C) and real-time RT-PCR (D). (E) MCF-7 cells were transfected with control siRNA (si con) or MMP-9 siRNA (si MMP-9). At 48 h after transfection, cells were seeded onto the upper chamber of Matrigel-coated filter inserts and treated with TPA (50 ng/ml) for 24 h. The cells invading the bottom of the filter were then stained with crystal violet and counted under a microscope. (F) MMP-9 silencing was confirmed by western blot analysis with anti-MMP-9 antibody. (G) MCF-7 cells were treated with surfactin for 24 h, after which the TIMP-1 and TIMP-2 protein levels were evaluated by western blot analysis. (H) Cells were treated with surfactin for 1 h and incubated with TPA (50 ng/ml) for 24 h. TIMP-1 and TIMP-2 protein levels were evaluated by western blot analysis. $^{*}p<0.05$ and vs. the TPA-treated group.

were treated with various concentrations of surfactin in the presence of TPA for 1 h, and nuclear extracts were prepared and examined by western blot analysis. While TPA induced the nuclear translocation of AP-1 (c-Jun and c-Fos) and NF-κB p65, surfactin inhibited the nuclear translocation of AP-1 and NF-κB in a dose-dependent manner (Fig. 6E). The effect of surfactin on the nuclear translocation of NF-κB p65 was confirmed by immunofluorescence confocal microscopy (Fig. 6F).

In unstimulated cells, NF-κB is present in the cytosol bound to IκB. In response to stimulation, IκBs are rapidly phosphorylated by IκB kinases (IKKs) and then ubiquitinated and degraded by the 26S proteasome complex. The free NF-κB

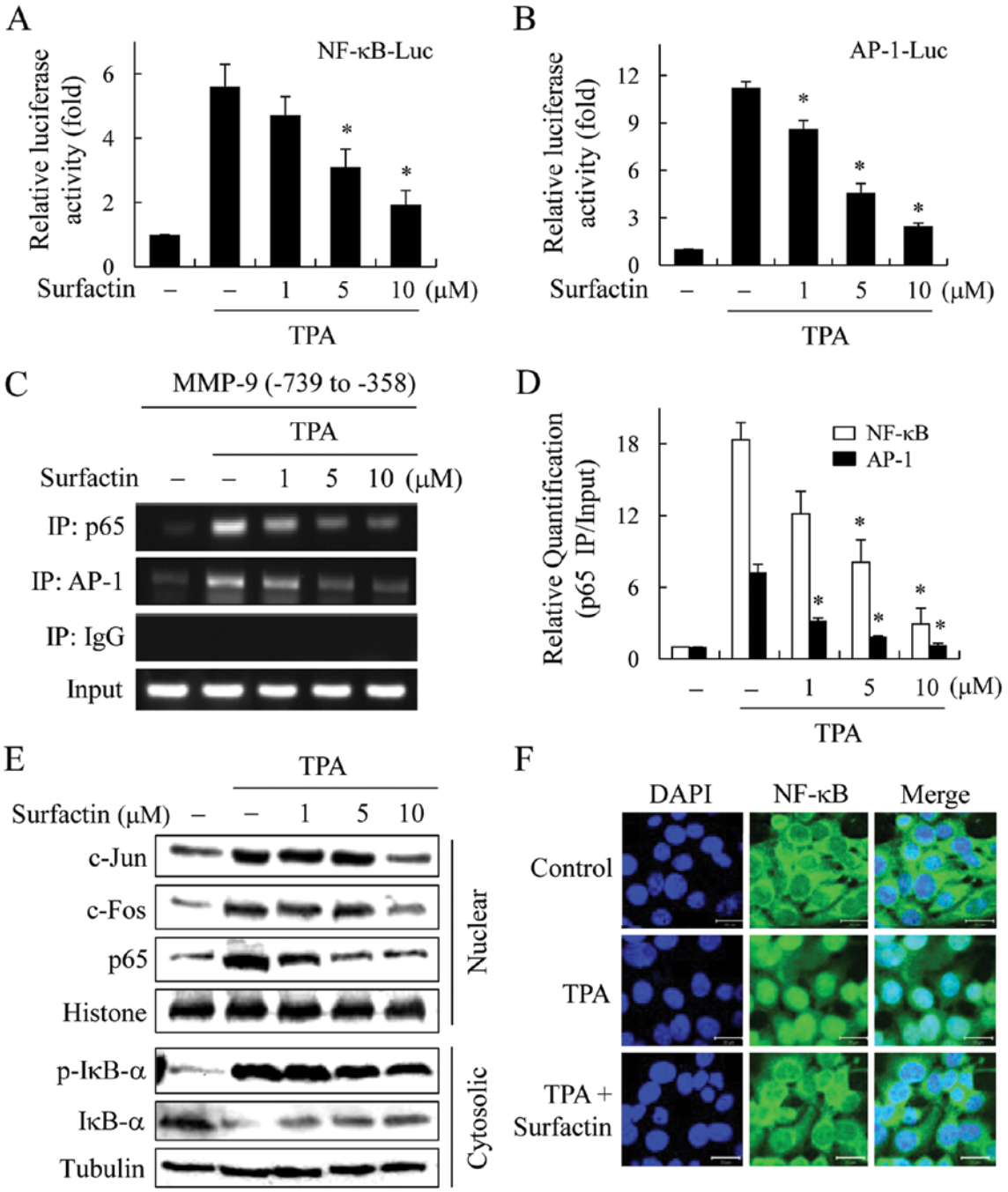

Figure 6. Inhibitory effects of surfactin on TPA-induced activation of NF-κB and AP-1. (A,B) MCF-7 cells were transfected with NF-κB-luciferase reporter or AP-1-luciferase reporter. Equal amounts of cell extract were assayed for dual-luciferase activity. (C and D) Cells were incubated with surfactin for 1 h and then incubated with TPA (50 ng/ml) for 4 h. A ChIP assay was performed with anti-NF-κB p65 or anti-AP-1 antibodies. The precipitated MMP-9 promoter region (-739 to -358 bp) was amplified by PCR (C) or real-time PCR (D). (E) Cells were treated with surfactin (10 μM) for 1 h, followed by TPA (50 ng/ml) treatment for 1 h. The nuclear (Nuc) and cytosolic (Cyt) extracts were prepared and analyzed by western blot analysis. (F) Cells were treated as described above and nuclear translocation of NF-κB was assessed by confocal microscopy as described in Materials and methods. *p<0.05 vs. the TPA-treated group.

dimers translocate to the nucleus, bind to the κB motif of the target genes and stimulate their transcription. Since IκB phosphorylation and degradation is the predominant pathway for NF-κB activation, we determined the levels of IκB-α and the phosphorylation of IκB-α proteins in cytosolic extract. Whereas the phosphorylation and degradation of IκB-α were stimulated by TPA, surfactin suppressed these effects (Fig. 6E). Surfactin also had similar inhibitory effects on NF-κB and AP-1 activities in the MDA-MB-231 cells (data not shown). These results indicate that surfactin inhibits TPA-stimulated AP-1 and NF-κB activities in breast cancer cells.

Surfactin inhibits MMP-9 activity through phosphatidylinositol 3-kinase (PI-3K)/Akt and ERK signaling pathways. MMP-9 gene expression may be activated by a number of signal transduction pathways, including those involving PI-3K/Akt and mitogen-activated protein kinases (MAPKs) such as ERK, c-Jun N-terminal kinase (JNK) and p38, which are also upstream

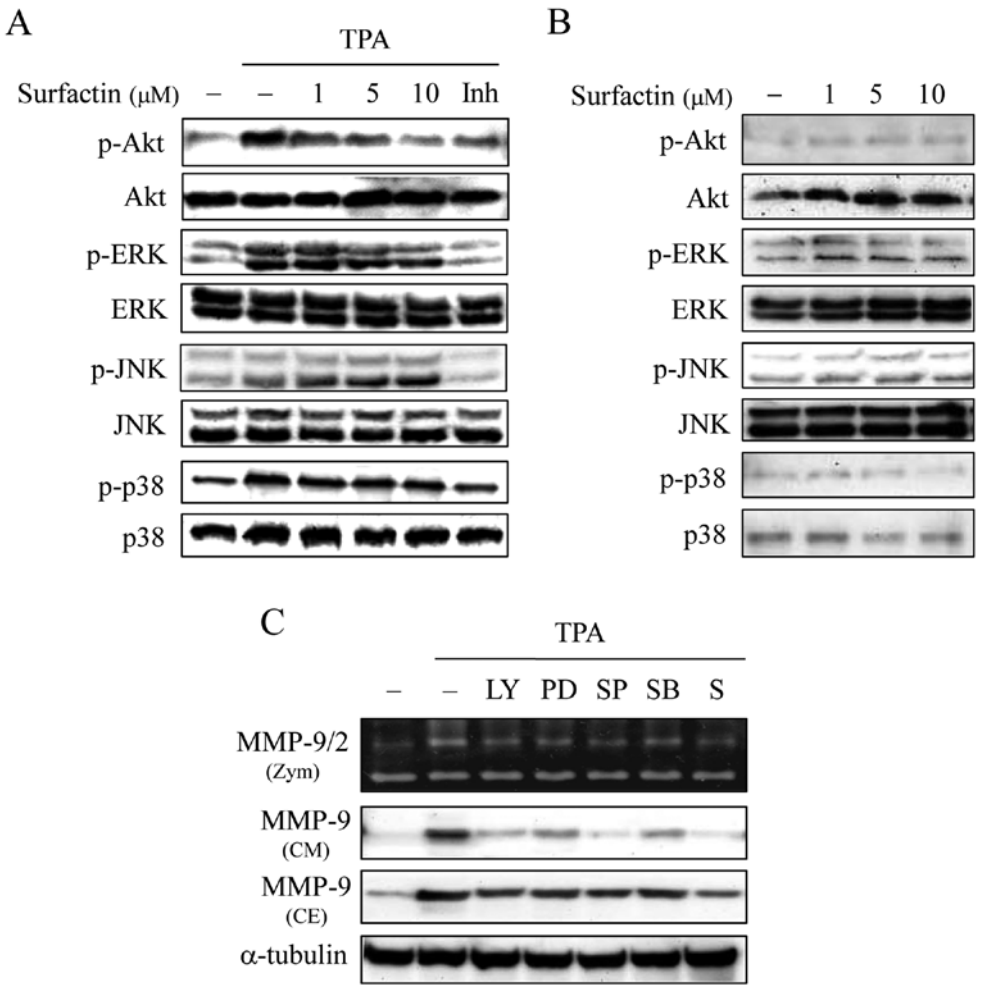

Figure 7. Effect of surfactin on TPA-induced activation of PI-3K/Akt and ERK. (A and B) MCF-7 cells were treated with the indicated concentrations of surfactin or specific kinase inhibitors (Inh; 10 μM, respectively) in the (A) presence or (B) absence of TPA (50 ng/ml) for 1 h. Equal amounts of cell extracts were analyzed by western blot analysis. (C) MCF-7 cells were treated with TPA (50 ng/ml) for 24 h in the presence of LY294002 (10 μM), PD98059 (10 μM), SP600125 (10 μM), SB203580 (10 μM) or surfactin (S; 10 μM). Subsequently, MMP-9 activity and intracellular protein expression were analyzed by gelatin zymography (Zym) and western blot analysis (CM, conditioned medium).

modulators of AP-1 or NF-κB (23-26). To evaluate the effect of surfactin on these signaling pathways, we monitored the activities of these kinases by examining their phosphorylated form. While TPA increased the level of PI-3K/Akt, ERK, JNK and p38 MAPK phosphorylation, surfactin specifically inhibited the TPA-induced phosphorylation of PI-3K/Akt and ERK in a dose-dependent manner, but not that of JNK and p38 (Fig. 7A). The phosphorylation of theses kinases was not affected by surfactin alone (Fig. 7B). To confirm the signal transduction pathways involved in TPA-stimulated MMP-9 expression, the effects of specific kinase inhibitors on the expression of MMP-9 in MCF-7 cells were analyzed by gelatin zymography and western blot analysis. The TPA-induced MMP-9 expression and secretion were inhibited by inhibitors of PI-3K/Akt (LY294002), ERK (PD98059), JNK (SP600125) and p38 (SB203580) (Fig. 7C). Surfactin had similar inhibitory effects on PI-3K/Akt and ERK activities in MDA-MB-231 cells (data not shown).

Discussion

Over the past few years, attention has been focused on the anticancer properties of natural products have received a great deal of attention and been shown to play an important role in the prevention of disease (27,28). Surfactin from *B. subtilis* has a wide range of pharmacological effects, including the inhibition of cell proliferation, cell cycle progression and apoptosis in various cancer cell lines. However, the effects of surfactin on cancer invasion and metastasis and the mechanisms underlying its action have not yet been elucidated. In this study, surfactin was found to suppress the invasion, migration and colony-forming ability *in vitro* of non-aggressive and aggressive breast cancer cells. Surfactin (15-80 μM) has been reported to exert antiproliferative and apoptotic effects in human colon carcinoma cells and MCF-7 human breast cancer cells (14,15). On the other hand, surfactin has been reported to cause neither mortality nor adverse side-effects at 2,500 mg/kg in a murine model of acute oral administration (29). Surfactin did not exert cytotoxicity in this study; surfactin had no cytotoxic effect at the concentrations of 10 μM or less on both MCF-7 and MDA-MB-231 cells. These results suggest that the anti-metastatic effects of surfactin are not due to its direct cytotoxicity against breast cancer cells.

The release of MMP-9 has been implicated as an important intermediary of tumor metastasis and certain studies have

reported the anti-metastatic effects of natural products associated with MMP-9 activity in various carcinoma cells (27,28). We found that the knockdown of MMP-9 significantly decreased the TPA-induced invasion of breast cancer cells. These results are consistent with those of other studies (30) and indicate that MMP-9 is critical to the invasion of breast cancer cells. Our results demonstrated that surfactin suppressed the enzymatic activity, secretion and expression of MMP-9 at the transcriptional levels in TPA-stimulated breast cancer cells, and the levels of TIMPs were not affected by surfactin. These results suggest that surfactin suppresses the invasion and metastasis of breast cancer cells through the inhibition of MMP-9 expression, but not through the induction of endogenous inhibitors of MMP-9. Accordingly, the inhibition of the expression and enzymatic activity of MMP-9 by surfactin would be an effective therapeutic approach for the treatment of breast carcinoma.

The results presented in this study also revealed that the pharmacological actions of surfactin are associated with the prevention of NF-κB and AP-1 activation. The MMP-9 promoter region contains multiple DNA binding sites for transcription factors, such as AP-1 (-533 and -79) and NF-κB (-600). The activation of these transcription factors plays a pivotal role in metastasis due to their ability to induce the transcription of metastasis-related genes, including MMP-9 (31,32). It currently is well known that MMP-9 expression is regulated by NF-κB and AP-1 and our results confirmed that the TPA-induced expression of MMP-9 was inhibited by inhibitors of NF-κB and AP-1 (data not shown). The present study demonstrates that surfactin inhibits the nuclear translocation and activation of NF-κB and AP-1. These results suggest that surfactin suppresses MMP-9 expression through the inhibition of NF-κB and AP-1 in breast cancer cells.

In this study, we also investigated the further upstream signaling pathways of TPA-induced MMP-9 expression. PI-3K/Akt and MAPKs have been known to be involved in the expression of a number of genes, including MMP-9. Thus, we examined whether surfactin regulates the activity of theses kinases and found that surfactin significantly inhibited PI-3K/Akt and ERK activation in TPA-stimulated breast cancer cells, while it did not have any influence on the phosphorylation of JNK and p38. Inhibitors of PI-3K/Akt, ERK, JNK and p38 kinases attenuated the expression and activity of MMP-9, which indicates that TPA-induced MMP-9 expression is mediated by these kinases; however, surfactin regulates PI-3K/Akt and ERK but not JNK and p38. Accordingly, our results suggest that surfactin suppresses TPA-induced MMP-9 expression via the inhibition of PI-3K/Akt and ERK in breast cancer cells. Cao *et al* reported that ERK was activated by 30 μM of surfactin (33). On the contrary, Kim *et al* reported that 30 μM of surfactin inhibits ERK (15), which is consistent with our results; we found that TPA-stimulated ERK activity was inhibited by 10 μM of surfactin and surfactin alone did not activate ERK. Although ERK activity may depend on the circumstances, it is evident that 10 μM of surfactin does not activate ERK but inhibits TPA-stimulated ERK in MCF-7 and MDA-MB-231 cells.

Surfactin is a lipopeptide produced by *B. subtilis* (13), which is a Gram-positive bacterium used in the Japanese delicacy, natto, as well as in the similar Korean food, fermented soybean paste. Fermented soybean paste has previously been reported to have antitumorigenic effects on breast, colon and stomach cancers (34-38). Epidemiological data have also shown a positive association between the high intake of fermented soybean paste and a low risk of prostate cancer (39). Additionally, Jung *et al* reported that extracts from fermented soybean paste had anti-metastatic effects against colon cancer (36). These results are consistent with those of the present study; therefore, it is reasonable to infer that the anti-metastatic effects of fermented soybean paste may be due to surfactin derived from *B. subtilis*.

In conclusion, surfactin is a potent inhibitor of TPA-induced MMP-9 expression and significantly blocks the NF-κB, AP-1, PI-3K/Akt and ERK signaling pathways. To our knowledge, this is the first study to demonstrate that surfactin suppresses cancer cell invasion *in vitro* by inhibiting MMP-9 expression. Thus, surfactin may be a potent candidate for preventing breast tumor invasion and metastasis *in vivo*.

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