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Involvement of the ERK Signaling Pathway in Fisetin Reduces Invasion and Migration in the Human Lung Cancer Cell Line A549

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This study is the first to investigate the antimetastatic effect of fisetin in human lung adenocarcinoma A549 cells. Fisetin exhibited an inhibitory effect on the abilities of adhesion, migration, and invasion via inhibiting the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and downregulating the expressions of matrix metalloproteinase-2 (MMP-2) and urokinase-type plasminogen activator (u-PA) at both the protein and mRNA levels in A549 cells. Next, fisetin significantly decreased the nuclear levels of nuclear factor kappa B (NF-κB), c-Fos, and c-Jun. Also, treating A549 cells with fisetin also leads to a concentration-dependent inhibition on the binding abilities of NF-κB and activator protein-1 (AP-1). Furthermore, reduction of ERK1/2 phosphorylation by ERK small interfering RNA (ERK siRNA) potentiated the effect of fisetin, supporting the inhibition of ERK1/2 being beneficial to antimetastasis. Finally, the transient transfection of ERK siRNA significantly downregulated the expressions of MMP-2 and u-PA concomitantly with a marked inhibition of cell invasion and migration. Taken together, these results implied a critical role for ERK1/2 inhibition in fisetin-reduced invasion and migration of A549 cells.

KEYWORDS: Fisetin; invasion; migration; ERK1/2; MMP-2; u-PA

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality in both men and women (*1*), and lung adenocarcinoma accounts for approximately 75−85% of all lung cancers (*2*). Lung adenocarcinoma commonly develops resistance to radiation and chemotherapy, and often presents at stages too late for surgical intervention. Most patients present with locally advanced (37%) or metastatic (38%) disease at diagnosis, and a large percentage of those diagnosed with early stage disease eventually experience recurrence of metastatic disease. About two-thirds of such recurrences are in distant organs such as the brain, contralateral lung, and bone as a result of the hematogenous spread of cancer cells (*3*). Thus, effective chemopreventive treatment for metastasis would have an important impact on lung cancer mortality rates.

Flavonoids are a broadly distributed class of plant pigments, universally present in vascular plants and responsible for much of the coloring in nature (*4*). Fisetin (3,3′,4′,7-tetra- hydroxyflavone) (**Figure 1A**) is a naturally occurring flavonoid commonly found in various vegetables and fruits such as onions, cucumbers, apples, persimmons, and strawberries (*5*). Dietary fisetin possesses antioxidative (*6*), anti-inflammatory (*7*), and antiproliferative activities in a wide variety of cancer cells including hepatoma (*8*), lung adenocarcinoma, and Burkitt lymphoma (*9*). Another report on human prostate cancer LNCaP cells indicated that fisetin exerted a chemopreventive effect through modulations in the cyclin-dependent kinase (cdk) network and inhibition of PI3K and Akt, resulting in the inhibition of cell growth (*10*). Although it was clear that fisetin may inhibit the growth of various cancers by inducing cancer cells toward apoptosis, the precise impact and related molecular mechanism of fisetin on migration and invasion were still unclear.

The metastatic process comprises multiple events involving cell invasion, migration, surface adhesion properties, and degradation of extracellular matrix (ECM). Proteolytic degradation of the ECM components is a central event of this process. Specifically, the ability to penetrate the basement membrane (BM) is related to an increased potential for metastasis. Matrix metalloproteinases (MMPs), a family of zinc-dependent neutral endopeptidases, have been associated with tumor cell invasion and migration because of their ability to hydrolyze various ECMs. Also, MMPs substantially contribute to other steps in the metastatic cascade, such as angiogenesis, differentiation, proliferation, and apoptosis (*11*). Two members of the MMP family, the 72 kDa

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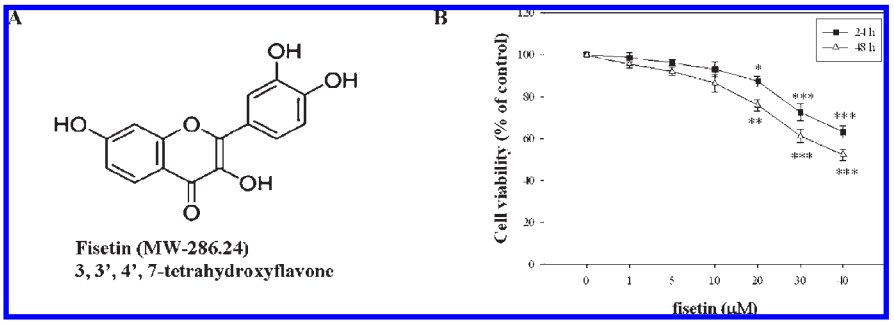

Figure 1. Effect of fisetin on the viability in A549 cells. (**A**) Chemical structures of fisetin. (**B**) Cells (3×10^4 cells/mL) were treated with various concentrations (0, 1, 5, 10, 20, 30, and 40 μM) of fisetin for 24 and 48 h. Cell viability was determined by the MTT assay. The cell viability was directly proportional to the production of formazan, which was measured spectrophotometrically at 563 nm. Values are expressed as the mean ± SD of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with that of the untreated control.

type IV collagenase (MMP-2) and 92 kDa type IV collagenase (MMP-9), were demonstrated to play a pivotal role in the degradation of ECM. A previous study has demonstated that the MMP-2 and MMP-9 activities are strongly expressed in metastatic lung adenocarcinoma cells (*12*). In addition, u-PA may initiate the activation of an enzymatic cascade and convert the zymogen plasminogen to plasmin (*13*). Meanwhile, activating these enzymes enables the degradation of the extracellular matrix (ECM) by tumor cells, allowing their access to the vasculature, migration, and invasion into the target organ and development of tumor metastasis (*14*). Also, the transcription of MMPs or u-PA gene is regulated by the upstream sequences, including motifs corresponding to the NF-κB and AP-1 binding sites (*15*−*18*). NF-κB is a multisubunit transcription factor involved in cellular responses to viral infection and inflammation. The active NF-κB consists of a dimer of an REL family/p65 subunit and a p50 or p52 subunit. NF-κB is maintained in the cytoplasm through interactions with an inhibitor of NF-κB (IκB), but upon dissociation, it moves into the nucleus and promotes cancer cell proliferation, angiogenesis, and metastasis. AP-1 is a nuclear transcription which is involved in cell proliferation, differentiation, apoptosis, and neoplastic transformation. AP-1 consists of homodimers and heterodimers of members from Fos (c-Fos, Fos B, Fra-1, and Fra-2) and Jun (c-Jun, Jun B, and Jun D) families (*19*, *20*). Activation of NF-κB and AP-1, downstream of the MAPK or PI3K-Akt pathway, are involved in many pathological processes, such as inflammation, cancer cell adhesion, invasion, migration, and angiogenesis (*21*−*23*). Therefore, MMPs, u-PA, and their regulatory pathways have been considered promising targets for anticancer drugs and chemopreventive agents. Our study aimed at examining the inhibitory effects and the related signaling pathways of fisetin on the invasion and migration of human lung adenocarcinoma A549 cells in vitro.

MATERIALS AND METHODS

Materials. Fisetin (>99% purity), dimethyl sulfoxide (DMSO), Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), gelatin, crystal violet, leupeptin, Nonidet P-40, deoxycholic acid, and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, MO, USA); the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Dulbecco's phosphate buffered solution (PBS), trypsin-EDTA, and powdered Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies, Inc. (Gibco/BRL, Gaithersburg, MD). Antibody against PKB/Akt, MAPK/ERK1/2, p38 MAPK and JNK/SAPK, proteins, and phosphorylated proteins were purchased from Cell Signaling Tech. (Beverly, MA, USA). Matrigel, MMP-2, MMP-9, u-PA, NF-κB (p65), c-Fos, c-Jun, β-actin, and C23 antibodies were from BD Transduction Laboratories (San Jose, CA, USA). ERK siRNA and control siRNA were purchased from Santa Cruz Biochnology, Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham GE Healthcare UK Ltd. (Buckinghamshire, England).

Cell Culture and Fisetin Treatment. A549, a human lung adenocarcinoma cell line, was obtained from BCRC (Food Industry Research and Development Institute in Hsin-Chu, Taiwan). Cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL of penicillin and 100 mg/mL streptomycin mixed antibiotics, and 1 mM sodium pyruvate. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2−95% air. For fisetin treatment, the stock solution of fisetin was dissolved in dimethyl sulfoxide (DMSO) and sterilized by filtration through 0.2 μm disk filters. Appropriate amounts of stock solution (1 mg/mL in DMSO) of fisetin were added into the cultured medium to achieve the indicated concentrations (final DMSO concentration was less than 0.2%) and then incubated with cells for the indicated time periods.

Analysis of Cell Viability by the MTT Assay. To evaluate the cytotoxicity of fisetin, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to determine cell viability. Briefly, cells were seeded at a density of 3×10^4 cells/mL in a 24-well plate for 24 h. Then, the cells were treated with fisetin at various concentrations (0, 1, 5, 10, 20, 30, and 40 μM) for various periods of time (24 and 48 h). Then, the medium was changed and incubated with MTT solution (5 mg/mL)/well for 4 h. The medium was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm.

Cell-Matrix Adhesion Assay. After a pretreatment with fisetin (0, 1, 5, and 10 μM) for 24 h, cells were seeded at a density of 5×10^4 cells/mL in a 24-well plate and coated with 150 μL type I collagen (10 μg/mL) and cultured for 30 min. Afterward, nonadherent cells were removed by PBS washes, and adherent cells were fixed in ethanol. After a staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton-100, and measured spectrophotometrically at 550 nm.

Wound-Healing Assay. To determine the cell motility, A549 cells (1×10^5cells /mL) were plated in 6-well culture plate and grown to 80−90% confluence. After aspirating the medium, the center of the cell monolayer was scraped with a sterile micropipet tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, then A549 cells were exposed to various concentrations of fisetin (0, 1, 5, and 10 μM). The wound closure was monitored and photographed at 0, 12, 24, 36, and 48 h by an Olympus CKX-41 inverted microscope and Olympus E410 camera. The migrated cells across the white lines were counted in five random fields from each triplicate treatment, and data are presented as the mean ± SD.

Boyden Chamber Invasion and Migration Assay. The ability of A549 cells to pass through Matrigel-coated filters was measured by the Boyden chamber invasion assay. Matrigel was diluted to 200 μg/mL with distilled water and applied to the top side of the 8-μm pore polycarbonate filter. Briefly, A549 cells were treated with various concentrations of fisetin. After 48 h, the cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10% FBS-medium was applied to the lower chamber as the chemoattractant, and then the cells were seeded on the upper chamber at a density of 1×10^5 cells/well in 50 μL of serum-free medium. The chamber was incubated for 8 h at 37 °C. At the end of incubation, the cells that invaded to the lower surface of the

membrane were fixed with methanol and stained with 5% Giemsa solution. The invasive cells on the lower surface of the membrane filter were counted with a light microscope.

To measure the ability of A549 cells on migration, cells were seeded into a Boyden chamber with 8 μm pore polycarbonate filters, which were not coated with Matrigel. The migration assay was measured as described in the invasion assay.

Analysis of MMP-2, MMP-,9 and u-PA Activity by Zymography. The activities of MMP-2 and MMP-9 were assayed by gelatin zymography. Briefly, conditioned media from cells cultured without serum for 24 h were collected. Samples were mixed with loading buffer and electrophoresed on 8% SDS−polyacrylamide gel containing 0.1% gelatin. Electrophoresis was performed at 140 and 110 V for 3 h. Gels were then washed twice in Zymography washing buffer (2.5% Triton X-100 in double-distilled H_2O) at room temperature to remove SDS, followed by incubation at 37 °C for 12−16 h in Zymography reaction buffer (40 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 0.02% NaN_3), stained with Coomassie blue R-250 (0.125% Comassie blue R-250, 0.1% amino black, 50% methanol, and 10% acetic acid) for 1 h, and destained with destaining solution (20% methanol, 10% acetic acid, and 70% double-distilled H_2O). Nonstaining bands representing the levels of the latent form of MMP-2 and MMP-9 were quantified by densitometer measurement using a digital imaging analysis system.

Visualization of u-PA activity was performed by casein-plasminogen zymography. Briefly, 2% casein and 20 μg/mL plasminogen were added to 8% SDS−polyacrylamide gel electrophoresis (PAGE) gel. Samples of about 20 μg of total protein were then loaded onto the gels. The u-PA activity of cells treated or untreated with fisetin was measured as described in the gelatin zymography section.

Isolation of Total RNA, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), and DNA Electrophoresis. Total RNA was isolated from human lung adenocarcinoma A549 using the total RNA Extraction Midiprep System (Viogene BioTek Corporation, Taiwan). Total RNA (2 μg) was transcribed to 20 μL of cDNA with 1 μL of dNTPs (2.5 mM), 1 μL of Oligo dT (10 pmol/μL), and 1 μL of RTase (200 U), 1 μL of RNase inhibitor, and 5× reaction buffer. The appropriate primers (sense of MMP-2, 5′-GGCCCTGTCACTCCTGAGAT-3′ nt 1337−1356; antisense of MMP-2, 5′-GGCATCCAGGTTATCGGGGA-3′, nt 2026−2007; sense of MMP-9, 5′-AGGCCTCTACAGAGTCTTTG-3′ nt 1201−1220; antisense of MMP-9, 5′-CAGTCCAACAAGAAAG-GACG-3′, nt 1702−1683; sense of u-PA, 5′-TTGCGGCCATCTACAG-GAG-3′, nt 654−672; antisense of u-PA 5′-ACTGGGGATCGTTA-TACATC-3′, nt 1205−1124; sense of GADPH, 5′-CGGAGTCAACG-GATTGGTGTT-3′, nt 94−126; antisense of 5′-AGCCTTCTCC ATGGTTGGTGAAGAC-3′, nt 399−375) were used for PCR amplifications. PCR was performed with Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) under the following conditions: 30 cycles of 94 °C for 1 min, 59 °C (MMP-2 and u-PA) or 60 °C (MMP-9 and GAPDH) for 1 min, and 72 °C for 1 min followed by 10 min at 72 °C.

Preparation of Whole-Cell Lysates and Nuclear Extracts. The cells were lysed with iced-cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl, pH 7.5), and then, the following were added: phenylmethylsulfonyl fluoride (10 mg/mL), leupeptin (17 mg/mL), and sodium orthovanadate (10 mg/mL). The samples were mixed for 30 min on ice and then centrifuged at 12000*g* for 10 min. Then, the supernatants were collected, denatured, and subjected to SDS−PAGE and Western blotting. Also, the nuclear pellet was resuspended in nuclear extract buffer (1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 420 mM NaCl). The nuclear suspension was incubated on ice for 20 min and then centrifuged at 14,000*g* for 5 min. The supernatant (corresponding to the soluble nuclear fraction) was saved, and then used for NF-κB, c-Fos, c-Jun, and AP-1 detection. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as a standard.

Western Blotting Analysis. To analyze the migration-related proteins, Western blotting was performed as follows. The denatured samples (50 μg purified protein) were resolved on 10−12% SDS−PAGE gels. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times for 10 min and incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4 °C. Subsequently, the membranes were washed with TBST and incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated goat antimouse or antirabbit IgG) for 1 h. After washing the membrane three times for 10 min in TBST, band detection was revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJFILM Las-3000 mini (Tokyo, Japan).

Small Interfering RNA for ERK. We assessed the effect of inducing RNA interference on ERK using Silencer siRNA. Control siRNA is a siRNA sequence that will not cause the specific degradation of any cellular message. A549 cells were transfected with 3 nmol of ERK-siRNA. ERK-siRNA is target specific 20−25 nt siRNAs designed to knock down gene expression. siRNA sequences were 5′-GACCGGAUGUUAAC-CUUUAUU (sense) and 5′-PUAAAGGUUAACAUCCGGUCUU (antisense) for ERK by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After siRNA transfection, we determined ERK, MMP-2, MMP-9, and u-PA contents in whole cell lysates by Western blotting using anti-ERK, anti-MMP-2, anti-MMP-9, and anti-u-PA, respectively, to confirm the silencing of these proteins. The expression of β-actin was unaffected by siRNA treatment.

Analysis of NF-κB and AP-1 Binding Activity by Electrophoretic Mobility Shift Assay. Cells (1×10^5/mL) were collected in PBS buffer (pH 7.4) and centrifuged at 2000*g* for 5 min at 4 °C. Cells were lysed with buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF (pH 7.9) containing 5% NP-40) for 10 min on ice, followed by vortexing to shear the cytoplasmic membranes. The lysates were centrifuged at 2000*g* for 10 min at 4 °C. The pellet containing the nuclei was extracted with high salt buffer B (20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, and 25% glycerol) for 15 min on ice. The lysates were clarified by centrifuging at 13000*g* for 10 min at 4 °C. The supernatant containing the nuclear proteins was collected and frozen at −80 °C until use. The protein content of nuclear fractions was determined with Bio-Rad protein assay. A 5 μg aliquot of nuclear proteins was mixed with either biotin-labeled NF-κB or AP-1 oligonucleotide probes for 15 min at room temperature. Oligonucleotides containing sense of NF-κB, 5′-AGTTGAGGGGACTTTCC-CAGGC-3′, antisense of NF-κB, 3′-TCAACTCCCCTGAAAGGG-TCCG-5′; sense of AP-1, 5′-CGCTTGATGACTCAGCCGGAA-3′, antisense of AP- 1, 3′-GCGAACTACTGAGTCGGCCTT. DNA probes were added to 10 μL binding reactions containing double-distilled H_2O, 5 μg of nuclear protein, 1 μL of poly (dI-dC), 1 μL of biotin-labeled double-stranded NF-κB or AP-1 oligonucleotides, and 2 μL of 10-fold binding buffer, placed into a microcentrifuge tube, and incubated for 15 min at room temperature. Specific competition binding assays were performed by adding 200-fold excess of unlabeled probe as a specific competitor. Following protein−DNA complex formation, samples were loaded on a 6% nondenaturing polyacrylamide gel in 0.5× TBE buffer and then transferred to positively charged nitrocellulose membranes (Millipore, Bedford, MA, USA) by a transfer blotting apparatus and cross-linked in a Stratagene UV cross-linker. Gel shifts were visualized with streptavidin-horseradish peroxidase followed by chemiluminescent detection.

Statistical Analysis. Data were expressed as means ± standard deviation of three independent experiments and analyzed by Student's *t*-test (Sigmaplot 2001). Significant differences were established at $p \leq 0.05$.

RESULTS

Cytotoxicity of Fisetin to A549 Cells. In this study, we first determined the cytotoxicity of fisetin on the prostate cancer cell line, A549. **Figure 1B** shows that the level of the cell viability effect of fisetin was assayed in a concentration- and time-dependent manner by the MTT assay. Compared to the 0 μM (DMSO was treated alone; data not shown), after 24 and 48 h treatment with fisetin at a concentration between 0 and 10 μM, the A459 cells were not significantly changed, indicating that fisetin was not toxic to A549 cells at these dosages. When cells were treated with 20−40 μM fisetin for 24 and 48 h, cell viability was significantly decreased. These results demonstrated that the treatment of

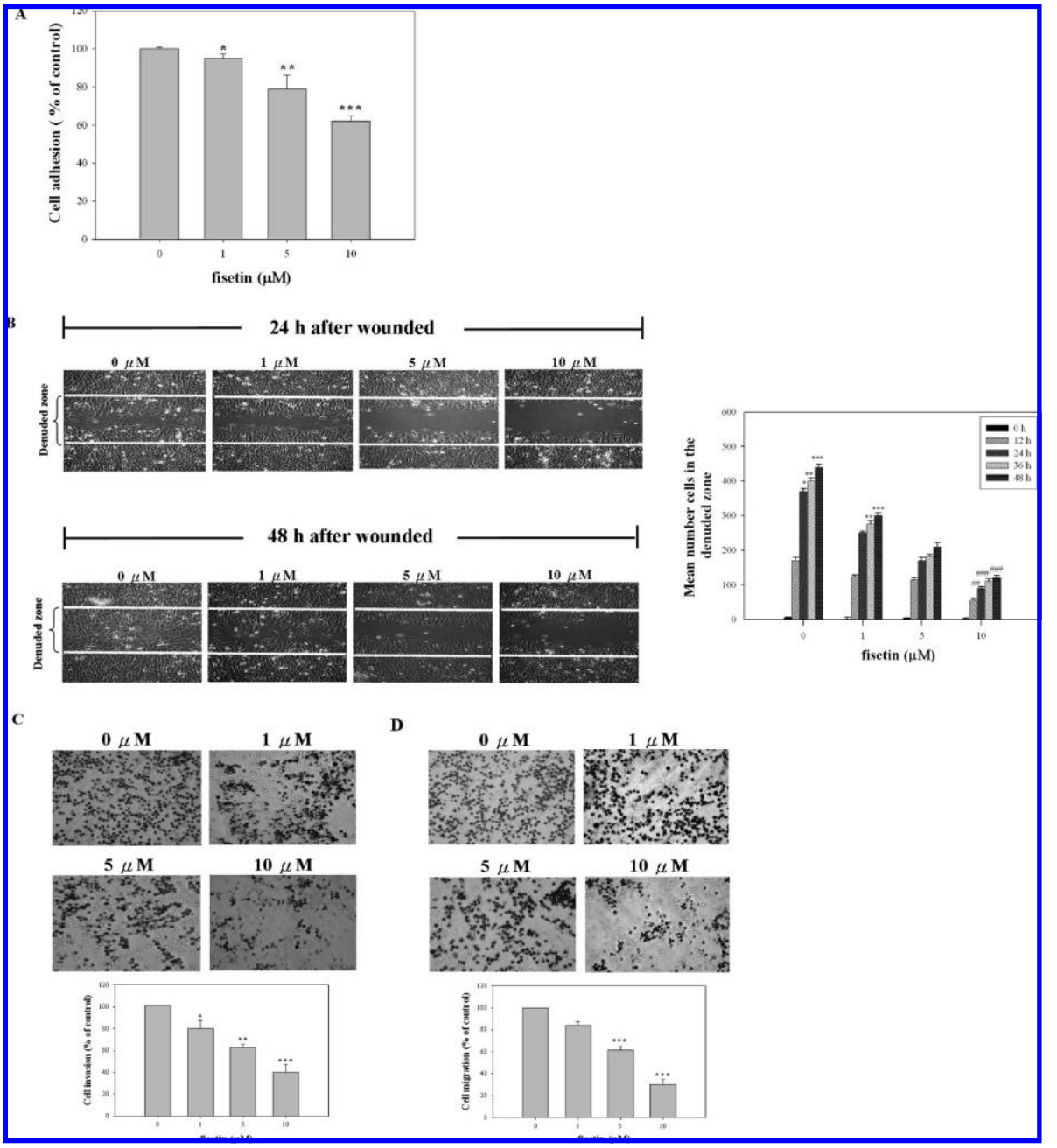

Figure 2. Effect of fisetin on the cell-matrix adhesion, invasion, and migration in A549 cells. (**A**) Cells were treated with various concentrations (0, 1, 5, and 10 μM) of fisetin for 24 h, and were then subjected to analyses for cell-matrix adhesion as described in Materials and Methods. (**B**) In the wound-healing assay, cell monolayers were scraped by a sterile micropipet tip, and the cells were treated with various concentrations of fisetin for 0, 12, 24, 36, and 48 h. The number of cells in the denuded zone was quantitated after the indicated times (0, 12, 24, 36, and 48 h) by inverted microscopy. White lines indicate the wound edge. Pictures were only presented for 24 and 48 h. (**C**) In the Boyden chamber invasion assay, cells were treated with various concentrations of fisetin for 48 h, then cell invasion was measured by the Boyden chamber for 8 h; polycarbonate filters (pore size, 8 μm) were precoated with matrigel. (**D**) In the Boyden chamber migration assay, cells were treated with various concentrations of fisetin for 48 h, and then cell migration was measured by the Boyden chamber for 6 h with polycarbonate filters. Values are expressed as the mean $\pm$ SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with that of the untreated control; ##$p < 0.01$ and ###$p < 0.001$ compared with the 0 h-treated time.

fisetin with concentrations higher than 10 μM for 24 and 48 h resulted in concentration- and time-dependent loss of cell viability in A549 cells, but concentrations lower than 10 μM for 24 and 48 h did not cause cytotoxicity. In the following experiments, these concentrations below 10 μM of fisetin were applied in all subsequent experiments.

Fisetin Inhibits the Adhesion, Invasion, and Migration in A549 Cells. In the cell-matrix adhesion assay, fisetin showed a concentration-dependent inhibitory effect on the cell adhesion ability of A549 cells. At 5 μM, cell adhesion was reduced to 81%, and at 10 μM, the cell adhesion was reduced to 62% (**Figure 2A**). In the wound healing assay, the result showed that 10 μM of fisetin exhibited the most inhibiting effect on cell motility after 48 h of incubation (**Figure 2B**). Also, compared with the untreated cells, fisetin (10 μM) for 48 h significantly inhibits the motility of A549 cells by 90%. With a cell invasion assay with Boyden chamber coated with Matrigel, it was shown that fisetin induced a concentration-dependent decrease in invasion with increasing

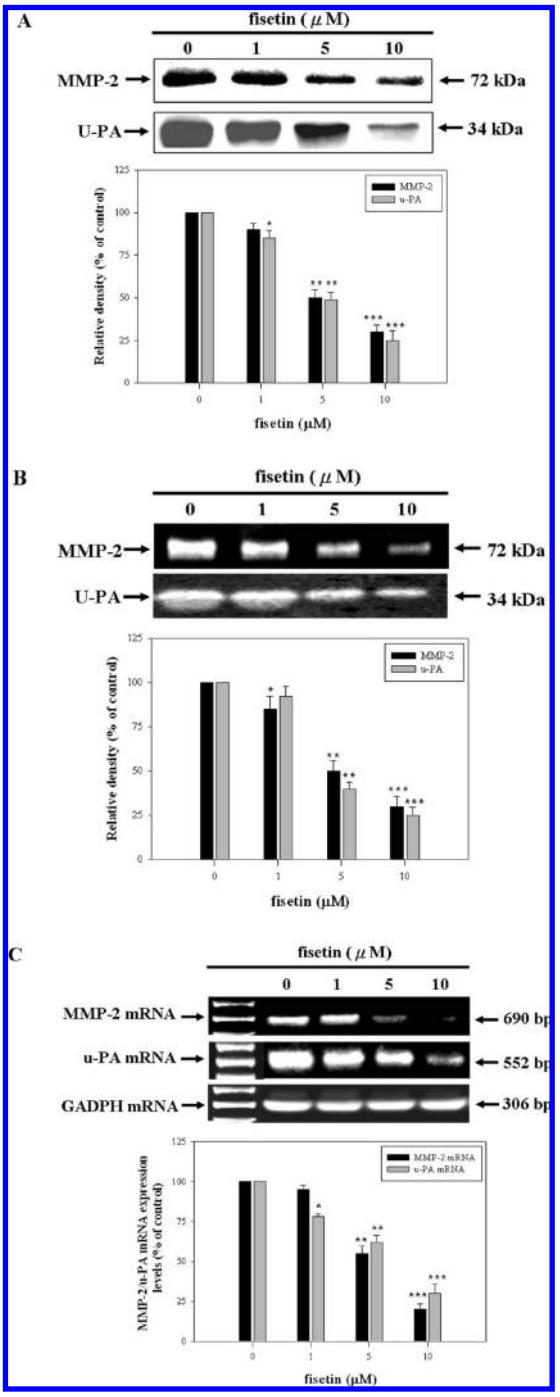

Figure 3. Effect of fisetin on MMP-2 and u-PA expressions in A549 cells. (**A**) Cells were treated with various concentrations (0, 1, 5, and 10 μM) of fisetin for 24 h. The protein levels of MMP-2 and u-PA from whole-cell lysates was analyzed by Western blotting. β-Actin was used as the loading control. It was determined that the protein expressions of MMP-2 and u-PA were subsequently quantified by densitometric analysis with that of the control being 1-fold. (**B**) The conditioned media were collected, and MMP-2 and u-PA activities were determined by gelatin or casein zymography. MMP-2 and u-PA activities were quantified by densitomeric analysis. (**C**) Cells were treated with various concentrations of fisetin for 24 h, and then, RNA samples were extracted and subjected to a semiquantitative RT-PCR for MMP-2 and u-PA with GADPH being an internal control. The PCR products were quantitated by densitometric analysis with that of the untreated group being 100%. Values are expressed as the mean $\pm$ SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with that of the untreated control.

concentrations of fisetin (**Figure 2C**). At 5 μM, the invasion was reduced to 63% and at 10 μM, the invasion was reduced to 41%. Subsequently, fisetin also induced a concentration-dependent decrease in migration with increasing concentrations of fisetin (**Figure 2D**). At 5 μM, the migration was reduced to 62%, and at 10 μM, the migration was reduced to 31%. The results demonstrated that fisetin significantly inhibited the invasion and migration of A549 cells.

Fisetin Inhibits the Expressions of MMP-2 and u-PA in A549 Cells. Since extracellular matrix degradation is crucial to cellular invasion and migration, indicating the inevitable involvement of matrix-degrading proteinases, the effects of fisetin on MMPs and u-PA activities were investigated by gelatin and casein zymography. **Figure 3A** shows that fisetin led to a significant reduction of MMP-2 and u-PA at the protein level in a concentration-dependent manner. Gelatin and casein zymography was also carried out to assess the activities of MMP-2 and u-PA in cells treated with fisetin. **Figure 3B** shows that fisetin inhibited the activities of MMP-2 and u-PA in a concentration-dependent manner. Quantification analysis indicated that MMP-2 activity was reduced by 15%, 50%, and 70% while u-PA by 8%, 60%, and 75% when cells were treated with 1, 5, and 10 μM of fisetin, respectively. Moreover, fisetin also exerted inhibitive effects on the MMP-2 and u-PA at the mRNA level in a concentration-dependent manner compared with the control group (**Figure 3C**). The data suggest that fisetin might regulate MMP-2 and u-PA expressions at the transcriptional level. However, the impact of fisetin on the protein and mRNA levels of MMP-9 was inconclusive because an extremely low level of MMP-9 was expressed in A549 cells, even in the absence of fisetin (data not shown). These results suggested that the antimetastatic effect of fisetin was related to the inhibition of the enzymatically degradative processes of tumor metastasis.

Fisetin Inhibits the Phosphorylation of ERK in A549 Cells. As we have shown that treatment of fisetin to A549 cells inhibited cell invasion, migration, and activities of MMP-2 and u-PA in the media, the underlying mechanisms were further investigated by studying the effect of fisetin on the constitutive activation status of MAPKs and PI3K/Akt. Hence, the phosphorylation of MAPKs, Akt, and the protein level of PI3K in A549 cells that were treated with various concentrations of fisetin for 6 h. **Figure 4A** shows that fisetin significantly suppressed the activation of ERK1 and ERK2, as shown by decreasing the phosphorylation of ERK1 and ERK2. In contrast, fisetin did not significantly affect phospho-JNK1/2, phospho-p38, and Akt activities. Moreover, no significant change in the total amount of ERK1/2, JNK1/2, p38, and Akt proteins was observed (data not shown).

To further investigate whether the inhibition of fisetin was mainly occurring through inhibition of the ERK1/2 signaling pathway, A549 cells were transiently transfected with ERK siRNA. Subsequently, the siRNA-transfected cells were exposed to the presence or absence of fisetin (1 μM) for 6 h. Then, the phosphorylation of ERK1 and ERK2 was determined by Western blot assay. Results have shown that a sole treatment of fisetin (1 μM) or ERK siRNA, respectively, reduced the expressions of ERK1 and ERK2 by 18% and 16% or 50% and 60% and that the combination treatment (ERK siRNA + 1 μM fisetin) could reduce phosphorylated ERK1 and ERK2 proteins even more dramatically, by 90% and 87% (**Figure 4B**). Indeed, **Figure 4A** and **B** shows that fisetin could inhibit the phosphorylation of ERK1/2, and the involvement of the MAPK pathway was further supported by the use of the ERK siRNA in our experimental model. It showed that treating with ERK siRNA could inhibit MMP-2 or u-PA expression, as well as reduce cell invasion and migration.

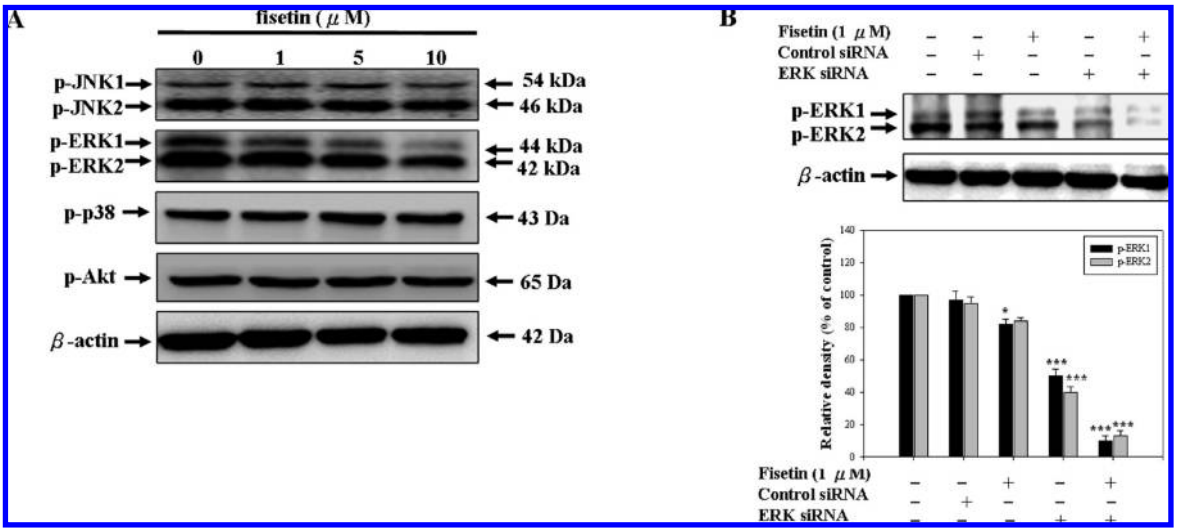

Figure 4. An inhibitory effect of fisetin on the phosphorylation of ERK1/2. (**A**) Cells were treated with various concentrations (0, 1, 5, and 10 μM) of fisetin for 6 h, and then cell lysates were subjected to SDS−PAGE followed by Western blotting and Immuno-probing with antiphospho-JNK1/2, antiphospho-ERK1/2, antiphospho-p38, and antiphospho-Akt antibodies. β-Actin was used as a loading control. The relative density of phosphorylated forms of JNK, ERK, p38, and Akt were normalized to total values of JNK, ERK, p38, and Akt, which were determined by densitometric analysis. Results from three repeated and separated experiments were similar. (**B**) For the determination of the effects of ERK siRNA and fisetin on the phosphorylation of ERK1/2, cells were plated in culture dishes and pretreated with ERK siRNA for 12 h and then incubated in the presence or absence of fisetin (1 μM) for 6 h. Afterward, the cell lysates were subjected to Western blotting to analyze the phosphorylation of ERK1/2. Values are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ and ***$p < 0.001$ compared with that of the untreated control.

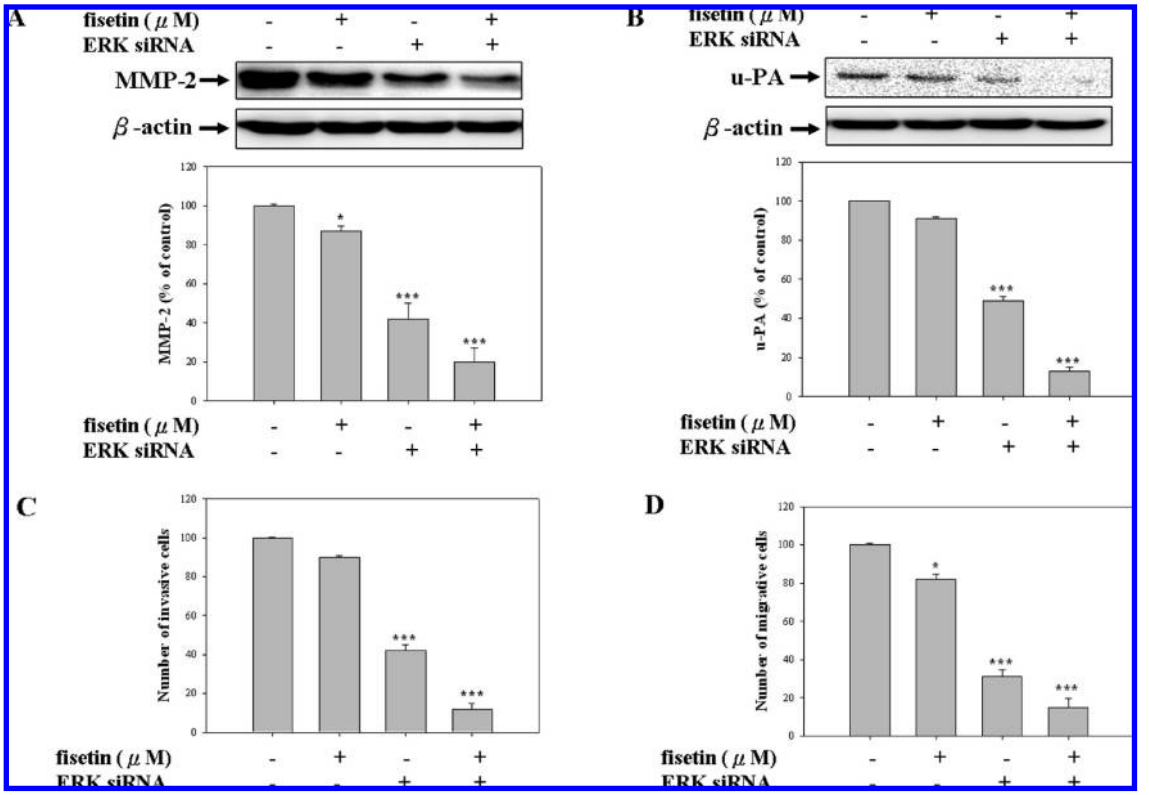

Figure 5. Effect of reducing ERK siRNA on fisetin-mediated protein expressions of MMP-2 and u-PA, and cell invasion and migration inhibition. For the determination of the effects of ERK siRNA and fisetin on the protein expressions of MMP-2 (**A**), u-PA (**B**), cell invasion (**C**), and cell migration (**D**), cells were plated in culture dishes and pretreated with ERK siRNA for 12 h and then incubated in the presence or absence of fisetin (1 μM) for 24 or 48 h. Afterward, the cell lysates were subjected to Western blotting to analyze the protein expressions of MMP-2 and u-PA. Also, cells were subjected to analyses for invasion and migration as described in Materials and Methods. Values are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ and ***$p < 0.001$ compared with that of the untreated control.

Fisetin Inhibits the Protein Expressions of MMP-2 and u-PA, and Cell Invasion and Migration via an Inactivation of the ERK Signaling Pathway. A549 cells were pretreated with ERK siRNA and then incubated with or without fisetin (1 μM) for 24 or 48 h.

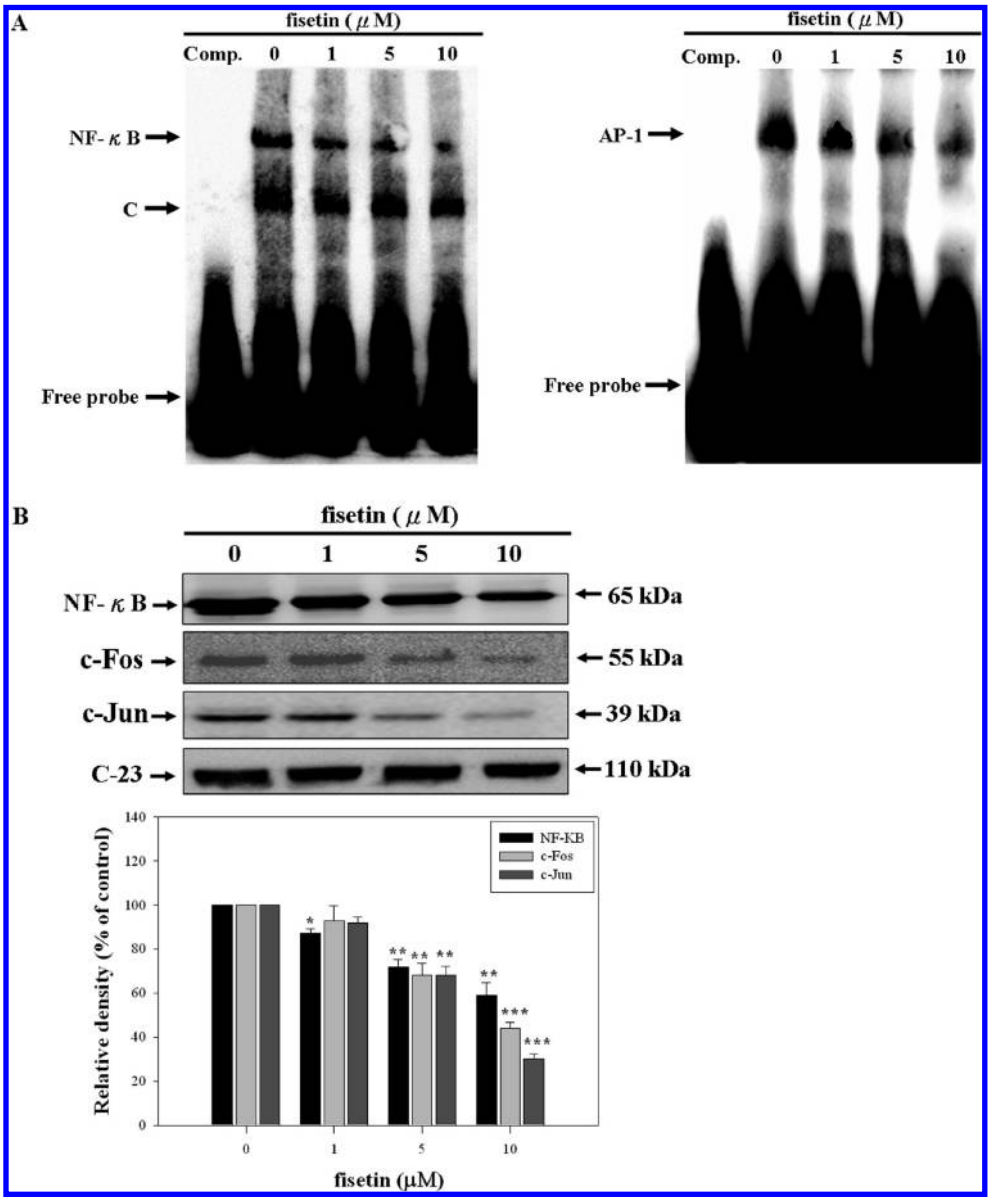

Figure 6. Effect of fisetin on the DNA binding activities of NF-κB and AP-1 in A549 cells. Cells were treated with various concentrations (0, 1, 5, and 10 μM) of fisetin for 12 h, and then nuclear extracts were prepared and analyzed for (**A**) NF-κB and AP-1 DNA binding activity using biotin-labeled consensus NF-κB and AP-1 specific oligonucleotide. Then the EMSA assay was performed as described in Materials and Methods. Lane 1: nuclear extracts incubated with 100-fold excess unlabeled consensus oligonucleotide (comp.) to confirm the binding specificity. C is a constitutive complex. Excess free probe is indicated at the bottom. Results from three repeated and separated experiments were similar. (**B**) Nuclear extracts were also analyzed by Western blotting with anti-NF-κB (p65), c-Fos, and c-Jun antibodies. C23 was a nucleus protein loading control. Determined protein expressions of NF-κB, c-Fos, and c-Jun were subsequently quantified by densitometric analysis with that of the control being 1-fold. The densitometric results are expressed as the mean $\pm$ SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with that of the untreated control.

Then, the protein expressions of MMP-2 and u-PA, and the cell invasion and migration were determined by the Western blotting assay and the Boyden chamber assay. As shown in **Figure 5A** and **B**, a sole treatment of fisetin (1 μM) or ERK siRNA, respectively, reduced the expressions of MMP-2 or u-PA by 13% and 56% or 9% and 52%, and the combination treatment (ERK siRNA + 1 μM fisetin) could reduce the expressions of MMP-2 or u-PA approximately 77% or 87% compared with that in the control. Also, the invasion and migration assay revealed a sole treatment with fisetin or ERK siRNA, respectively, decreased the invasion and migration by 10% and 58% or 17% and 68%, and the combination treatment (ERK siRNA + 1 μM fisetin) could further reduce the invasion and migration by 88% or 84% (**Figure 5C** and **5D**). Reduced of MMP-2 or u-PA expressions by ERK siRNA-based inhibition increased fisetin-inhibited invasion and migration. These data clearly indicate that the down-regulation of MMP-2 or u-PA might play a key role in fisetin-inhibited invasion and migration.

Fisetin Inhibits the DNA Binding Activities of NF-κB, c-Fos, and c-Jun in A549 Cells. NF-κB and AP-1 family of transcriptional factors have been known to translocate to the nucleus and regulate the expression of multiple genes involved in MMPs or u-PA secretion. To clarify the involvement of NF-κB and AP-1 proteins in the mechanism of fisetin's action, the effect of fisetin on the DNA binding activities of NF-κB and AP-1 in A549 cells was investigated by EMSA. **Figure 6A** shows that A549 cells were treated with 0−10 μM of fisetin for 12 h and that fisetin inhibited NF-κB and AP-1 transcriptional activities in a concentration-dependent manner.

Especially, the binding activities of NF-κB and AP-1 were inhibited by treating with 10 μM fisetin. Furthermore, the expressions of NF-κB, c-Fos, and c-Jun in nuclear extracts were analyzed by Western blotting. The results showed that the nuclear levels of NF-κB, c-Fos, and c-Jun were gradually diminished at concentrations of 1, 5, and 10 μM fisetin compared to the 0 μM after treatment for 12 h (**Figure 6B**).

DISCUSSION

This study is the first to show that fisetin can inhibit the invasion and migration in human lung adenocarcinoma A549 cells in vitro. We found that fisetin can suppress cancer cell invasion and migration possibly through inactivation of the ERK signaling pathway, exert inhibitory effects on NF-κB, c-Fos, and c-Jun transcriptional factors, inhibit NF-kB and AP-1 DNA binding activities, reduce the levels of MMP-2 and u-PA, and then have an antimetastatic effect. Our results strengthen the potential of fisetin as a new strategy for anticancer therapy and provide possible mechanisms responsible for their antimetastatic effect.

In recent years, attention has been drawn to the physiological relevance of MMPs and u-PA markers related to the metastatic ability and malignancy of tumor cells (*12*, *24*, *25*). Thus, many studies have shown that proteinases related to the degradation of the matrix are required for tumor cell metastasis and that heightened production of MMPs and u-PA correlates with the invasion, migration, and angiogenesis of the tumors (*26*). Also, to further explore the exact mechanism of fisetin-induced inhibition on the invasion and migration, we performed a set of experiments, including zymography, Western blotting, and RT-PCR, to detect MMP-2, MMP-9, and u-PA at the enzyme activities, protein, and mRNA levels. We demonstrated here that the expressions of MMP-2 and u-PA were reduced by fisetin treatment. These results suggested that the antimetastastic effect of fisetin was associated with the inhibition of the enzymatically degradative processes of tumor metastasis.

Several studies on different cell types have indicated that mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38MAPK (*27*) mediate signals from cell membrane receptors triggered by growth factors, cytokines, and cell-matrix interactions. Since MAPKs seem to play a central role in regulating the expressions of MMPs or u-PA (*16*, *28*), inhibition of the MAPK pathway might have the potential to prevent angiogenesis, proliferation, invasion, and migration for a wide range of tumors. ERK signaling is the major pathway in lung cancer cells and plays a variety of physiologic roles, including cell growth, cell cycle regulation, migration, and invasion (*29*, *30*). Recent studies have indicated that fisetin clearly decreased MMP-2 and u-PA expressions through inactivating the ERK signaling pathway, and such an inhibitory effect on proteinase expression may contribute to the capability of fisetin to inhibit cell invasion and migration. Moreover, treatment with fisetin significantly decreased the number of invasive and migrative cells in ERK siRNA-transfected cells. This may be due to fisetin treatment having decreased the level of ERK activity under these conditions. Thus, the ERK siRNA gene silencing result further confirms that the ERK signaling pathway is indeed the target of fisetin treatment.

In this study, the downstream activating enzymes of MMP-2 and u-PA were involved in invasion and migration. The transcription of MMP-2 and u-PA genes is regulated by upstream regulatory sequences, including the NF-κB and AP-1 binding sites (*15*−*17*, *29*). Therefore, this study provides insight into how fisetin suppresses the ERK1/2 signaling pathway and reduces NF-kB and AP-1 transcriptional activities in A549 cells. Indeed, one or more of these binding sites have been implicated in mediating the effects of a diverse set of agents. Here, we also found the treatment of A549 cells with fisetin results in an inhibition of NF-kB and AP-1 DNA binding activities, which was accompanied by the inhibition of nuclear translocation of these factors.

Finally, the involvement of the ERK signaling pathway in cell metastasis was further supported by experiments with ERK siRNA, showing that treatment with ERK siRNA to A549 cells inhibited cell invasion and migration. In conclusion, these results imply the therapeutic potential of fisetin for controlling tumor metastasis on the basis of the observation of its inhibitory effect on migration and invasion of A549 cells. This study suggested that fisetin may serve as an efficient antimetastastic drug in cancer treatment.

ABBREVIATIONS USED

ECM, extracellular matrix; BM, basement membrane; MMPs, matrix metalloproteinases; u-PA, urokinase-type plasminogen activator; MAPK, mitogen-activated protein kinase; ERK, extracellular signaling-regulating kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; PI3K, phosphoinositide 3-kinase; NF-κB, nuclear factor kappa B; AP-1, activator protein-1; IκB, inhibitor of NF-κB.

LITERATURE CITED

(1) Greenlee, R. T.; Hill-Harmon, M. B.; Murray, T.; Thun, M. Cancer statistics. *CA Cancer J. Clin.* **2001**, *51*, 15–36.

(2) Shivapurkar, N.; Reddy, J.; Chaudhary, P. M.; Gazdar, A. F. Apoptosis and lung cancer: A review. *J. Cell Biochem.* **2003**, *88*, 885–898.

(3) Jemal, A.; Murray, T.; Ward, E.; Samuels, A.; Tiwari, R. C.; Ghafoor, A.; Feuer, E. J.; Thun, M. J. Cancer statistics, 2005. *CA Cancer J. Clin.* **2005**, *55*, 10−30.

(4) Cody, V. Crystal and molecular structures of flavonoids. *Prog. Clin. Biol. Res.* **1998**, *280*, 29–44.

(5) Arai, Y.; Watanabe, S.; Kimira, M.; Shimoi, K.; Mochizuki, R.; Kinae, N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *J. Nutr.* **2000**, *130*, 2243–2250.

(6) Noroozi, M.; Angerson, W. J.; Lean, M. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am. J. Clin. Nutr.* **1998**, *67*, 1210–1218.

(7) Higa, S.; Hirano, T.; Kotani, M.; Matsumoto, M.; Fujita, A.; Suemura, M.; Kawase, I.; Tanaka, T. Fisetin, a flavonol, inhibits TH2-type cytokine production by activated human basophils. *J. Allergy Clin. Immunol.* **2003**, *111*, 1299–1306.

(8) Chen, Y. C.; Shen, S. C.; Lee, W. R.; Lin, H.; Ko, C. H.; Shih, C. M.; Yang, L. L. Wogonin and fisetin induction of apoptosis through activation of caspase-3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SKHEP-1. *Arch. Toxicol.* **2002**, *76*, 351–359.

(9) Sung, B.; Pandey, M. K.; Aggarwal, B. B. Fisetin, an inhibitor of cyclin-dependent kinase 6, down-regulates nuclear factor-kappaB-regulated cell proliferation, antiapoptotic and metastatic gene products through the suppression of TAK-1 and receptor-interacting protein-regulated IkappaB alpha kinase activation. *Mol. Pharmacol.* **2007**, *71*, 1703–1714.

(10) Khan, N.; Afaq, F.; Syed, D. N.; Mukhtar, H. Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells. *Carcinogenesis* **2008**, *29*, 1049–1056.

(11) Chambers, A. F.; Matrisian, L. M. Changing views of the role of matrix metalloproteinases in metastasis. *J. Natl. Cancer Inst.* **1997**, *89*, 1260–1270.

(12) Shih, Y. W.; Chen, P. S.; Wu, C. H.; Jeng, Y. F.; Wang, C. J. α-Chaconine-reduced metastasis involves a PI3K/Akt signaling

pathway with downregulation of NF-κB in human lung adenocarcinoma A549 cells. *J. Agric. Food Chem.* **2007**, *55*, 11035–11043.
(13) Duffy, M. J.; Duggan, C. The urokinase plasminogen activator system: a rich source of tumour markers for the individualized management of patients with cancer. *Clin. Biochem.* **2004**, *37*, 541–548.
(14) Itoh, Y; Nagase, H. Matrix metalloproteinases in cancer. *Essays Biochem.* **2002**, *38*, 21–36.
(15) Nagase, H.; Woessner, J. F.Jr Matrix metalloproteinases. *J. Biol. Chem.* **1999**, *274*, 21491–21494.
(16) Westermarck, J.; Kahari, V. M. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J.* **1999**, *13*, 781–792.
(17) Sliva, D. Signaling pathways responsible for cancer cell invasion as targets for cancer therapy. *Curr. Cancer Drug Targets* **2004**, *4*, 327–336.
(18) Sato, H.; Seiki, M. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* **1993**, *8*, 395–405.
(19) Karin, M.; Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu. Rev. Immunol.* **2000**, *18*, 621–663.
(20) Lee, S. O.; Jeong, Y. J.; Im, H. G..; Kim, C. H.; Chang, Y. C.; Lee, I. S. Silibinin suppresses PMA-induced MMP-9 expression by blocking the AP-1 activation via MAPK signaling pathways in MCF-7 human breast carcinoma cells. *Biochem. Biophys. Res. Commun.* **2007**, *354*, 165–171.
(21) Chen, F.; Castranova, V.; Shi, X. New insights into the role of nuclear factor-kappaB in cell growth regulation. *Am. J. Pathol.* **2001**, *159*, 387–397.
(22) Takada, Y.; Singh, S.; Aggarwal, B. B. Identification of a p65 peptide that selectively inhibits NF-kappa B activation induced by various inflammatory stimuli and its role in down-regulation of NF-kappa B-mediated gene expression and up-regulation of apoptosis. *J. Biol. Chem.* **2004**, *279*, 15096–15104.
(23) Bahassi el, M.; Karyala, S.; Tomlinson, C. R.; Sartorm, M. A.; Medvedovic, M.; Hennigan, R. F. Critical regulation of genes for tumor cell migration by AP-1. *Clin. Exp. Metastasis* **2004**, *21*, 293–304.
(24) Chen, P. N.; Hsieh, Y. S.; Chiou, H. L.; Chu, S. C. Silibinin inhibits cell invasion through inactivation of both PI3K-Akt and MAPK signaling pathways. *Chem.-Biol. Interact.* **2005**, *156*, 141–150.
(25) Turner, N. A.; Aley, P. K.; Hall, K. T.; Warburton, P.; Galloway, S.; Midgley, L.; O'regan, D. J.; Wood, I. C.; Ball, S. G..; Porter, K. E. Simvastatin inhibits TNFalpha-induced invasion of human cardiac myofibroblasts via both MMP-9-dependent and -independent mechanisms. *J. Mol. Cell Cardiol.* **2007**, *43*, 168–176.
(26) Mackay, A. R.; Corbitt, R. H.; Hartzler, J. L.; Thorgeirsson, U. P. Basement membrane type IV collagen degradation: evidence for the involvement of a proteolytic cascade independent of metalloproteinases. *Cancer Res.* **1990**, *50*, 5997–6001.
(27) Robinson, M. J.; Cobb, M. H. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* **1997**, *9*, 180–186.
(28) Aguirre Ghiso, J. A.; Alonso, D. F.; Farias, E. F.; Gomez, D. E.; de Kier Joffe, E. B. Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur. J. Biochem.* **1999**, *263*, 295–304.
(29) Rothhammer, T.; Hahne, J. C.; Florin, A.; Poser, I.; Soncin, F.; Wernert, N.; Bosserhoff, A. K. The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma. *Cell. Mol. Life Sci.* **2004**, *61*, 118–128.
(30) Shih, Y. W.; Wu, P. F.; Lee, Y. C.; Shi, M. D.; Chiang, T. A. Myricetin suppresses invasion and migration of human lung adenocarcinoma A549 cells: possible mediation by blocking the ERK signaling pathway. *J. Agric. Food Chem.* **2009**, *57*, 3490–3499.

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