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Osthole inhibits the invasive ability of human lung adenocarcinoma cells via suppression of NF-κB-mediated matrix metalloproteinase-9 expression

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ABSTRACT

The induction of matrix metalloproteinase (MMP)-9 is particularly important for the invasiveness of various cancer cells. Osthole, a natural coumarin derivative extracted from traditional Chinese medicines, is known to inhibit the proliferation of a variety of tumor cells, but the effect of osthole on the invasiveness of tumor cells is largely unknown. This study determines whether and by what mechanism osthole inhibits invasion in CL1-5 human lung adenocarcinoma cells. Herein, we found that osthole effectively inhibited the migratory and invasive abilities of CL1-5 cells. A zymographic assay showed that osthole inhibited the proteolytic activity of MMP-9 in CL1-5 cells. Inhibition of migration, invasion, and MMP2 and/or MMP-9 proteolytic activities was also observed in other lung adenocarcinoma cell lines (H1299 and A549). We further found that osthole inhibited MMP-9 expression at the messenger RNA and protein levels. Moreover, a chromatin immunoprecipitation assay showed that osthole inhibited the transcriptional activity of MMP-9 by suppressing the DNA binding activity of nuclear factor (NF)-κB in the MMP-9 promoter. Using reporter assays with point-mutated promoter constructs further confirmed that the inhibitory effect of osthole requires an NF-κB binding site on the MMP-9 promoter. Western blot and immunofluorescence assays demonstrated that osthole inhibited NF-κB activity by inhibiting IκB-α degradation and NF-κB p65 nuclear translocation. In conclusion, we demonstrated that osthole inhibits NF-κB-mediated MMP-9 expression, resulting in suppression of lung cancer cell invasion and migration, and osthole might be a potential agent for preventing the invasion and metastasis of lung cancer.

Introduction

Lung cancer is the leading cause of cancer death in industrialized countries. Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. People with pulmonary adenocarcinomas have a high mortality rate because of the local invasion and subsequent widespread metastasis that occur in late stage tumors leading to poor prognoses (Yoshida et al., 2000; Kerr, 2001). Despite advances in diagnosis and therapy, the overall 5-year survival rate in many countries is generally <15% (Erridge et al., 2007). Therefore, the development of novel therapies to treat patients with metastatic tumors is necessary to improve mortality rates.

Cancer cell metastasis involves multiple processes and various cytophysiological changes, including changes to the adhesion capabilities between cells and the extracellular matrix (ECM) and damage to intercellular interactions. Thus, degradation of the ECM and components of the basement membrane caused by proteinases, such as matrix metalloproteinases (MMPs), cathepsins, and the plasminogen activator (PA), play critical roles in tumor invasion and metastasis (Westermarck

Abbreviations: AP-1, activator protein 1; AP-1-mut, mutant AP-1; ChIP, chromatin immunoprecipitation; ECM, extracellular matrix; FACS, fluorescence-activated cell sorter; IκB, inhibitor of NF-κB; MMP, matrix metalloproteinase; mRNA, messenger RNA; NF-κB, nuclear factor-κB; NF-κB-mut, mutant NF-κB; NSCLC, non-small-cell lung cancer.

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and Kahari, 1999). MMPs are overexpressed in many types of human cancers (Cakarovski et al., 2004; Patel et al., 2007) including NSCLC (Passlick et al., 2000). Type-IV collagen is a major constituent of the basement membrane that separates the epithelial and stromal compartments. MMP-9 (a 92-kDa type-IV collagenase or gelatinase B) and MMP-2 (a 72-kDa type-IV collagenase or gelatinase A) play critical roles in the degradation of type-IV collagen. They are expressed by various malignant tumors and are closely related to the invasion and metastasis of cancer cells (Nelson et al., 2000; Bjorklund and Koivunen, 2005). The 5′ flanking region of the MMP-9 gene contains several functional regulatory motifs that binds well-characterized transcription factors, including nuclear factor-kappa B (NF-κB; −600), activator protein-1 (AP-1; −533 and −79), stimulatory protein-1 (SP-1; −558), and polyoma virus enhancer activator-3 (PEA-3; −540) (Sato and Seiki, 1993; Takahra et al., 2004). Through one or more of these binding sites on specific element-containing target genes, various chemical and physical stimulators regulate the expression of MMP-9. These stimulators include growth factors, cytokines, and oncogenes (eg, Ras) (Zeigler et al., 1999; Hozumi et al., 2001). An interaction with the tissue inhibitor of metalloproteinase-1 also regulates MMP-9 at the posttranslational level (Zhang et al., 2004b). Therefore, upstream molecules that regulate MMP-9 expression and enzymatic activity can potentially be used as targets for treating cancer metastasis.

It was recently demonstrated that certain foods (including many vegetables, fruits, and grains), and phytochemicals with diverse pharmacological efficacies, offer significant protection against various cancers (Huang et al., 2008; Shankar et al., 2008). There is increasing focus on providing a scientific basis for using these agents as a preventive strategy for people who have a high risk of cancer. The dried fruit of *Cnidium monnieri* (L.) Cusson, a well-known traditional Chinese medicine, has a variety of pharmacological, biological, and therapeutic uses. Osthole, an active constituent isolated from dried *C. monnieri* fruit, has many biological applications. It has been used for its anti-inflammatory (Liu et al., 2005), anti-osteoporosis (Zhang et al., 2007), antihepatitis (Okamoto et al., 2003), anti-allergenic (Matsuda et al., 2002), and estrogen-like (Kuo et al., 2005) effects. Additionally, accumulating evidence indicates that osthole possesses an antitumor effect by inhibiting tumor cell growth and inducing apoptosis (Kawaii et al., 2001; Okamoto et al., 2005; Riviere et al., 2006; Xu et al., 2011). However, besides breast cancer studies (Yang et al., 2010; Hung et al., 2011), few studies have investigated the anticancer migration and invasion effects of osthole. This study investigates the effects of osthole on cell migration and invasion in several highly invasive lung adenocarcinoma cells and examines the possible underlying mechanisms.

Materials and methods

Cell culture. Lung cancer cell lines (CL1-5, A549, and H1299) were grown in RPMI 1640 plus 10% fetal bovine serum (FBS; Invitrogen/Gibco) in a humidified atmosphere containing 5% CO_2 at 37 °C. CL1-5 cells were established in National Health Research Institute laboratory and displayed progressively increasing invasiveness (Chu et al., 1997). A549 and H1299 cells were obtained from the American Type Culture Collection (Manassas, VA).

Materials and reagents. Cell culture materials and FBS were obtained from Gibco-BRL (Gaithersburg, MD). An enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL). Osthole, propidium iodide (PI), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Antibodies, specifically Bax, Bcl-2, IκBα, NF-κB p65 (for Western blot analysis), c-Fos, and α-tubulin, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MMP-9, NF-κB p65 (for immunofluorescence), and β-actin antibodies were purchased from Epitomic (Burlington, CA). N-cadherin, E-cadherin, vimentin, snail, slug, foxc-2, and twist antibodies were purchased from Cell Signaling Technology (Danvers, MA). Unless otherwise specified, additional chemicals used in this study were purchased from Sigma.

Cell viability assay. CL1-5, A549, and H1299 cells were grown to 80% confluence, treated with osthole (0–60 μM) for 24 h, and then subjected to a cell viability assay (MTT assay). Data were collected from 3 replicates.

In vitro wound closure. CL1-5 cells (3×10^5 cells/well) or A549 and H1299 cells (4×10^5 cells/well) were plated in 6-well plates for 24 h. Cells were scratched with a pipette tip to wound them, incubated in an RPMI medium containing 0.5% FBS, and treated with or without osthole (0–60 μM) for 24 h. Cells were photographed using a phase-contrast microscope (×100), as described by Ho et al. (2011).

Cell invasion assay. The invasion assay was performed using 24-well transwell units (Costar, Cambridge, NY). Each well was coated with Matrigel matrix (25 mg/50 mL; BD Biosciences, Bedford, MA) to form a continuous thin layer. CL1-5 cells (1.5×10^5) or A549 and H1299 cells (2×10^5) were plated on the Matrigel surface with a serum-free medium and various concentrations (0–60 μM) of osthole. After a 24-h incubation, cells that had invaded the lower side of the membrane were stained with 4′,6-diamidino-2-phenylindole (DAPI). Stained cells in each well were photographed (at 40× magnification) and counted. Triplicate samples were conducted, and data are expressed as the average cell number of 10 fields.

Gelatin zymography. MMP-2 and MMP-9 activities in a conditional medium from lung adenocarcinoma cells (CL1-5, A549, and H1299) were measured using gelatin zymography protease assays, as described by Ho et al. (2011). An appropriate volume of collected media was subjected to electrophoresis on 8% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 and incubated in a reaction buffer (40 mM Tris–HCl at pH 8.0, 10 mM $CaCl_2$, and 0.01% NaN_3) for 12 h at 37 °C. The gel was then stained with Coomassie brilliant blue R-250.

Reverse-transcriptase polymerase chain reaction (RT-PCR). Messenger RNA (mRNA) was isolated and amplified as described by Chen et al. (2010). Primer sequences are shown as supplementary data (Supplementary Table 1).

Nuclear and cytosolic protein extraction. For nuclear and cytosolic protein extraction, protein extracts were prepared from 60-μM osthole-treated CL1-5 cells using an NE-PER Cytoplasmic and Nuclear Protein extraction kit (Pierce Biotechnology, Rockford, IL).

Western blot analysis. Protein lysates were prepared as described by Chen et al. (2010). Western blot analysis was performed with primary antibodies for MMP-9, IκBα, NF-κB p65, N-cadherin, E-cadherin, vimentin, snail, slug, foxc-2, twist, α-tubulin, or β-actin.

Flow cytometric analysis. CL1-5 cells were grown in RPMI supplemented with 10% FBS. After cells had grown to subconfluence, they were rendered quiescent and challenged with 10% FBS and vehicle or 60 μM osthole. After 24 h, they were harvested, washed twice with PBS/0.1% dextrose, and fixed in 70% ethanol at −20 °C. The nuclear DNA was stained with a reagent containing PI (50 mg mL^{-1}) and DNase-free RNase (2 U mL^{-1}) and measured using a fluorescence-activated cell sorter (FACS). The proportion of nuclei in each phase of the cell cycle was determined using WinMDI 2.9 DNA analysis software.

Construction of MMP-9 promoter/reporter plasmids. The MMP-9 promoter was provided by Prof Ko (Chung Shan Medical University, Taichung, Taiwan). The −720 bp to −11 bp fragment of the MMP-9

promoter was inserted into the pGL3-basic vector to generate the MMP-9 promoter/reporter plasmid. Mutations were introduced into the pGL3-MMP-9 promoter using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to instruction manual recommendations. Three complementary primers contained the mutated NF-κB (−600) sites, AP1-1 (−533), and AP1-2 (−79) sites of the human MMP-9 promoter (NF-κB-mut sense, 5′-GGGGGTTGCCCCAGTTTAATTCCCCAGCCTTG-3′; NF-κB-mut antisense, 5′-CAAGGCTGGGGAATTAAACTGGGGCAACCCCC-3′; AP1-1-mut sense, 5′-AAGCAGGGAGAGGAAGCTATGTCAAAGAAGGCTGTCAG-3′; AP1-1-mut antisense, 5′-CTGACAGCCTTCTTTGACATAGCTTCCTCTCCCTGCTT-3′; AP1-2-mut sense, 5′-CACACCCTGACCCCTATGTCAGCACTTGCCTG-3′; and AP1-2-mut antisense, 5′-CAGGCAAGTGCTGACATAGGGGTCAGGGTGTG-3′). The mutated sites were extended during temperature cycling by Pfu Ultra II DNA polymerase resulting in mutated plasmids. The products were then treated with Dpn I endonuclease, which selectively digests methylated (parental) and hemimethylated (semi-parental) plasmids. Mutation-containing plasmids were then transduced into *Escherichia coli* XL10-Gold. Finally, all mutants were confirmed using sequence analysis.

Luciferase assay. CL1-5 cells were seeded at 5×10^4/well in 6-well culture plates. They were cotransfected with 1 μg pGL3-basic (vector) or MMP-9 promoter constructs (wild type, NF-κB-mut, or AP-1-mut) and 0.5 μg pRL-TK Renilla control vector (Promega, Madison, WI) using PolyJet™ (SignaGen Laboratories, Ijamsville, MD), according to manufacturer instructions. After 6 h, a medium containing 10% FBS was added, and cells were incubated overnight. Cells were then treated with osthole (0–60 μM) for 24 h. Firefly and Renilla luciferase activities were measured using a dual-luciferase reporter assay (DLR) kit (Promega). Firefly luciferase activity was adjusted to Renilla luciferase activity to control for variations in cell viability and transfection efficiency.

Chromatin immunoprecipitation (ChIP) analysis. A ChIP analysis was performed as described by Chen et al. (2010). DNA immunoprecipitated with antibodies specific to c-Fos, NF-κB p65, or the control, rabbit immunoglobulin G (IgG), was purified and extracted using phenol-chloroform. Immunoprecipitated DNA was analyzed with a PCR or quantitative (q)PCR using specific primers which are described in the supplementary data (Supplementary Table 1).

Immunofluorescence. To evaluate NF-κB translocation, CL1-5 cells grown on glass chamber slides were treated with 60 μM osthole and then washed with phosphate-buffered saline (PBS) followed by fixation with 4% paraformaldehyde. Cells were permeabilized with PBS containing 1% Triton X-100, labeled with a primary antibody against NF-κB p65 (Epitomic, Burlingame, CA), followed by incubation with a rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology); nuclei were stained with DAPI, and images were acquired on a Zeiss Axioplan microscope.

Statistical analysis. Values are presented as the mean ± SE. Statistical analysis was performed using Statistical Package for Social Science software, vers. 16 (SPSS, Chicago, IL). Data were analyzed using Student's *t* test when 2 groups were compared. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to analyze 3 or more groups. Differences were considered significant at the 95% confidence interval ($p<0.05$).

Results

Osthole treatment inhibits lung adenocarcinoma cell migration and invasion

It was reported that a high concentration (up to 100 μM) and long-term treatment (48 h) of osthole (Fig. 1A) can inhibit the growth of lung adenocarcinoma A549 cells (Xu et al., 2011). To further investigate the pharmacological potential of osthole against lung cancer, we first examined the effect of osthole on cell migration and invasion in lung adenocarcinoma cells. To determine the role of osthole in cell migration and invasion, 3 highly invasive lung cancer cell lines, A549, H1299, and CL1-5, were treated with various concentrations of osthole for 24 h. Figs. 1B–D are photographs of A549, H1299, and CL1-5 cells migrating into scratch wounds, respectively. The number of cells migrating into the wound decreased in an osthole concentration-dependent manner (Figs. 1E–G). At 60 μM, osthole decreased the number of migrating cells from A549, H1299, and CL1-5 cells by 71%, 59%, and 61%, respectively.

This study then examined whether osthole inhibits invasion in lung cancer cells. Invasion assay results (Figs. 2A–F) show that osthole treatment significantly inhibits penetration of A549, H1299, and CL1-5 cells into the Matrigel-coated filter. After incubating cells with 60 μM osthole, the invasion inhibitory percentages in A549, H1299, and CL1-5 cells are 54%, 34%, and 58%, respectively (Figs. 2D–F). To exclude the possibility that decreased numbers of migrating and invading cells were a consequence of reduced proliferation, we performed viability assays using A549, H1299, and CL1-5 cells treated with the same osthole concentration that was used in the migration and invasion assays for 24 h. There was no significant difference between the treated cells and control cells (Figs. 3A–C).

This study then investigated the effects of osthole on cell cycle regulation. Results show that treatment of CL1-5 cells with osthole (60 μM) for 24 h does not increase the incidence of apoptosis. The lack of significant changes in the sub G1 population between control and osthole-treated CL1-5 cells provides evidence for this (Fig. 3D). The CL1-5 cell proliferation rate was also not affected by osthole because the number of cells in the S-phase did not change significantly after 24-h osthole treatment (Fig. 3D). Furthermore, after 24-h osthole treatment (60 μM), the Bcl-2/Bax ratio between the control and osthole-treated CL1-5 cells was not significantly different (Fig. 3E). Similar results for the Bcl-2/Bax ratio after osthole treatment were observed in H1299 and A549 cells (data not shown). According to these data, osthole significantly inhibits cancer cell invasion at a non-cytotoxic concentration (≤60 μM), indicating that osthole is an effective inhibitor of cell migration and invasion in lung adenocarcinoma cells. All subsequent experiments used this osthole concentration range.

Osthole reduces the expression and proteolytic activity of MMP-9

MMP-9 and MMP-2 are important ECM-degrading enzymes. Both enzymes are involved in cancer cell invasion and metastasis (Westermarck and Kahari, 1999). The cancer cell invasion inhibiting effects of osthole prompted us to examine the effect of osthole on MMP proteolytic activities and gene expressions. Fig. 4A shows that gelatin zymography tested the proteolytic activity of MMP-2 in conditioned media from A549, H1299, and CL1-5 cells. Osthole concentration-dependently inhibits the proteolytic activity of MMP-2 in A549 and H1299 cells, but not in CL1-5 cells. By contrast, the inhibitory effect of osthole on MMP-9 proteolytic activity is only observed in H1299 and CL1-5 cells (Fig. 4B). Additionally, MMP-9 mRNA and protein expressions in CL1-5 cells are significantly suppressed after 60 μM osthole treatment of different lengths (Figs. 4C and D). Maximal mRNA expression inhibition occurred 2 h after osthole treatment. This response continued until 10 h after treatment (Fig. 4C). By contrast, the maximal inhibitory effect of osthole on the MMP-9 protein level occurred 12 h after osthole treatment (Fig. 4D). These results indicate that osthole selectively suppresses MMP-9 expression at protein and mRNA levels, and subsequently inhibits the enzymatic activity of MMP-9 in CL1-5 cells.

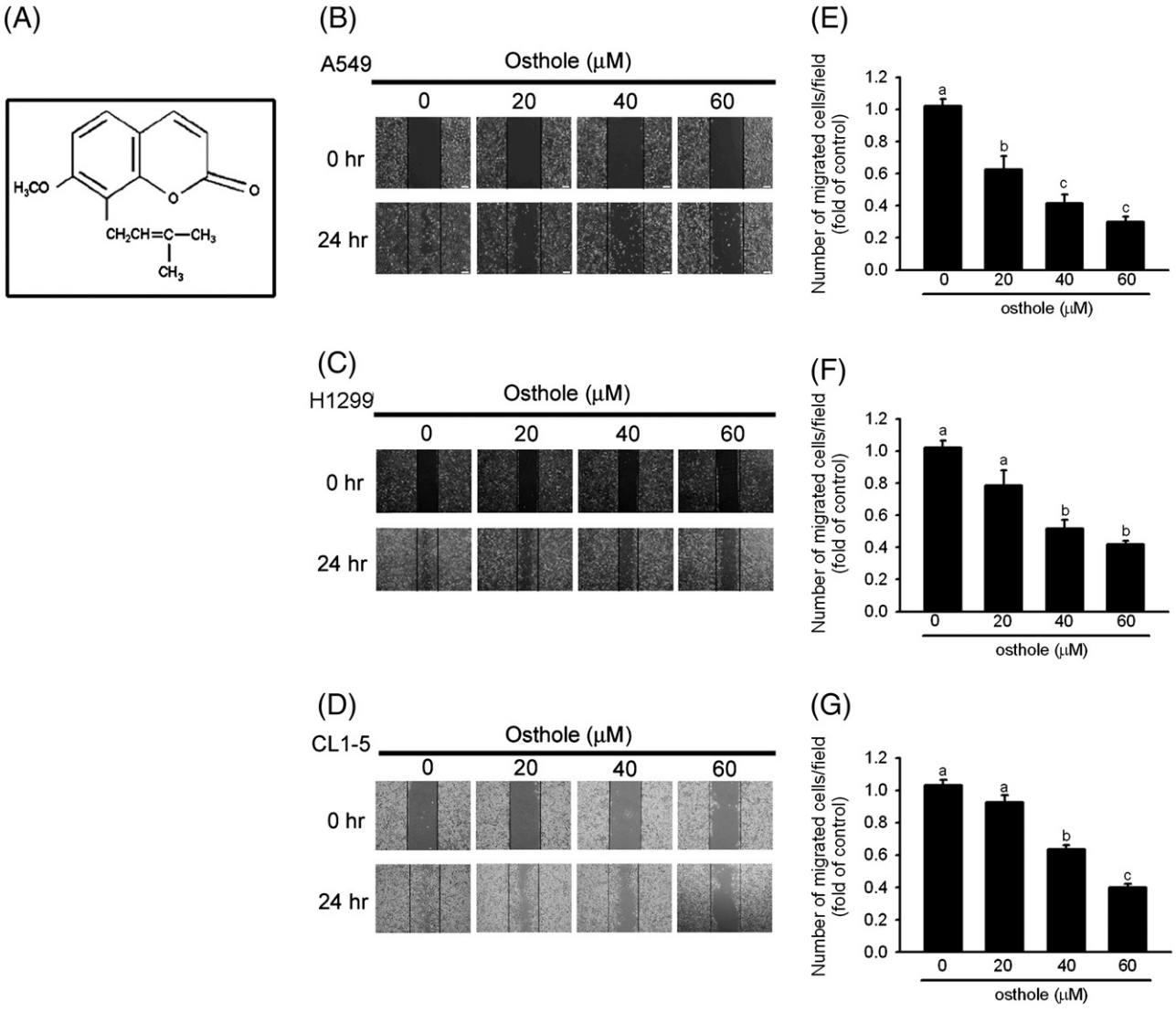

Fig. 1. Effects of osthole on in vitro wound closure in lung adenocarcinoma cells. (A) The chemical structure of osthole. A549 (B, E), H1299 (C, F), and CL1-5 (D, G) cells were wounded and then treated with the vehicle (DMSO) or osthole (0–60 μM) for 24 h in a serum-containing medium. At 0 h and 24 h, phase-contrast pictures of the wounds were taken at 3 different locations (B–D). Cells migrating into the wound area were counted using the dashed line as time zero. A quantitative assessment of the mean number of cells in the denuded zone is the mean ± SE ($n = 3$) (E–G). Results were analyzed using one-way ANOVA with Tukey's post hoc tests at 95% confidence intervals. Different letters represent different levels of significance.

Osthole inhibits the transcriptional activity of MMP-9 by suppressing NF-κB activity

Because osthole inhibits the mRNA expression of MMP-9, this study examines whether osthole affects the promoter activity of MMP-9. A luciferase reporter gene containing the MMP-9 promoter region (−720/−11) was transiently transfected into CL1-5 cells, and luciferase activity was determined. Fig. 5A shows that osthole suppresses the promoter activity of MMP-9 in a concentration-dependent manner, indicating that osthole inhibits MMP-9 expression at the transcriptional level. Results show that 60 μM osthole inhibits 57% of MMP-9 promoter activity. Studies indicate that NF-κB and AP-1 affect MMP-9 expression in various cancer cell lines (Noh et al., 2010; Park et al., 2010). Thus, reporter plasmids with single-site mutations in the NF-κB (−600 bp) or AP-1 (−533 bp and −79 bp) binding site of the MMP-9 promoter were constructed, and a luciferase assay analyzed promoter activity (Figs. 5B and C). Results show that 60 μM osthole only partially inhibits MMP9-NF-κB-mut promoter activity, with 29% inhibition (Fig. 5B). Whether the AP-1 binding site was mutated at −533 or −79 bp, osthole still exhibits similar inhibitory potential (60% or 58% inhibition) against the MMP9-AP-1-mut promoters and the wild promoter (Fig. 5C).

A ChIP assay was then performed to investigate the effect of osthole on NF-κB and AP-1 DNA-binding activities and validate the transcription factor involvement in the transcriptional inhibitory effects of osthole on MMP-9. Fig. 5D shows that NF-κB binding to the MMP-9 promoter decreases in CL1-5 cells after 60 μM osthole treatment. However, the binding activity of AP-1 is unaffected by osthole in CL1-5 cells. A quantitative real-time PCR assay confirms that osthole significantly suppresses NF-κB binding to the MMP-9 promoter (Fig. 5E). These results indicate that the NF-κB transcription factor and NF-κB binding site in the MMP-9 promoter region might contribute to the inhibitory effect of osthole on MMP-9 transcription.

Osthole inhibits IκBα degradation and NF-κB nucleus translocation

NF-κB is sequestered in the cytoplasm by binding to the IκB family and is activated by IκBα phosphorylation and subsequent degradation in proteasomes, thus allowing NF-κB subunits, including p65 and p50, to enter the nucleus and activate target genes (Magnani et al.,

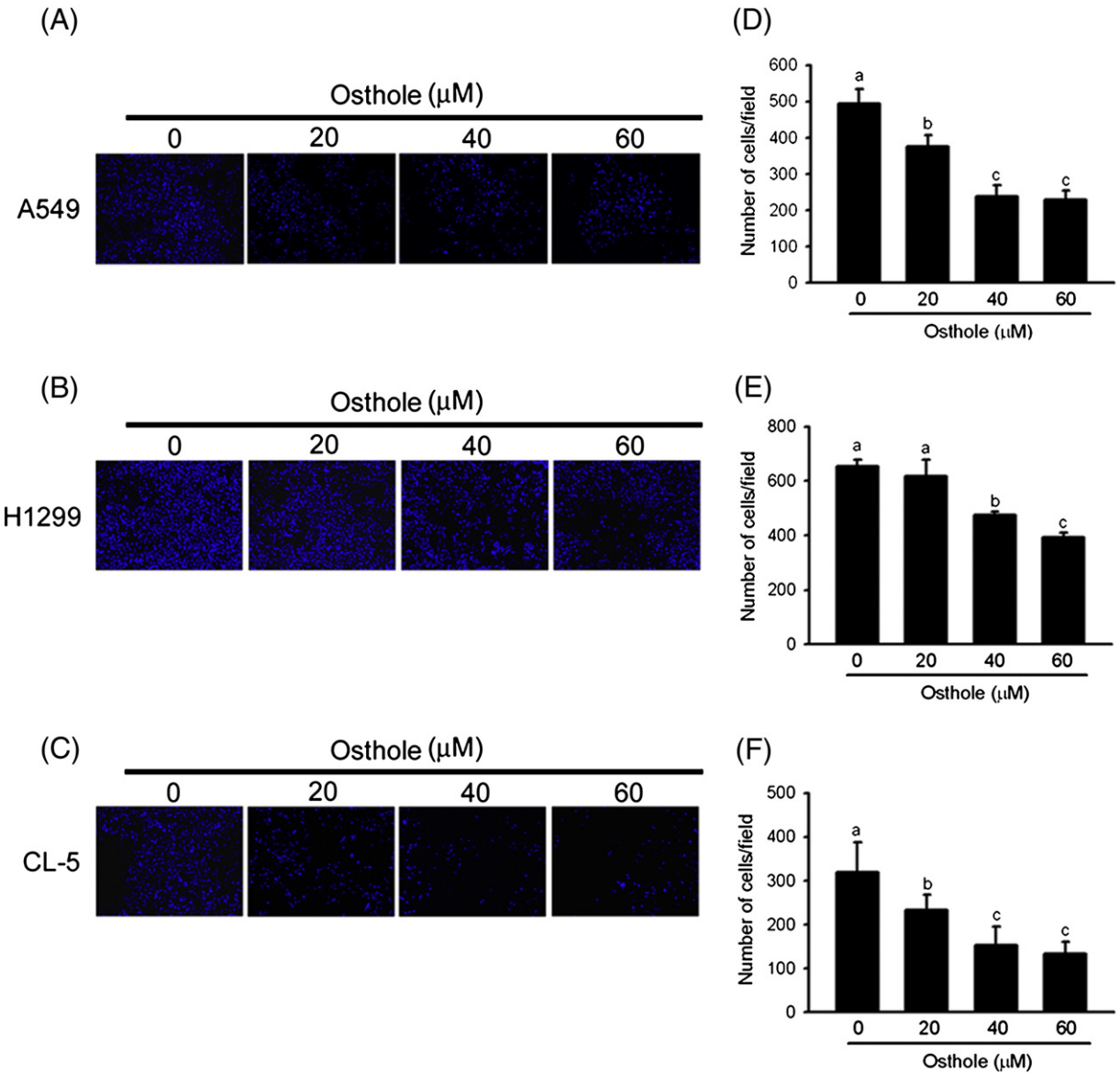

Fig. 2. Concentration-dependent inhibitory effects of osthole on A549 (A, D), H1299 (B, E), and CL1-5 (C, F) cell invasion. Photographs (40×) show DAPI-labeled cells invading through a Matrigel-coated membrane (A–C). Bar graphs (D–F) represent the invasive cells that were treated with various concentrations (0–60 μM) of osthole for 24 h. Values represent the mean ± SE ($n = 3$). Results were analyzed using one-way ANOVA with Tukey's post hoc tests at 95% confidence intervals and different letters represent different levels of significance.

2000). This study examines whether osthole affects the phosphorylation and degradation of IκBα and nucleus translocation of the NF-κB p65 subunit in CL1-5 cells. Compared to the vehicle-treated group, degradation of IκBα, and nuclear translocation of NF-κB p65 were attenuated after treating CL1-5 cells with 60 μM osthole for 30 min (Fig. 6A).

To further evaluate the effect of osthole on IκBα phosphorylation, we normalized the phosphorylation levels of IκBα to total IκBα levels at the same time after vehicle or osthole treatment. The differences in IκBα phosphorylation levels between vehicle and osthole treatment groups were then compared. IκBα phosphorylation is also suppressed significantly after treating CL1-5 cells with 60 μM osthole for 10–60 min (Fig. 6B). We further confirmed this result by using the proteasome inhibitor, MG132, to block the proteasome-mediated IκBα degradation. CL1-5 cells were preincubated with 15 μM MG132 for 30 min, and then treated with 60 μM osthole for 30 min. The inhibitory effect of osthole on IκBα phosphorylation was observed (Supplementary data 1). Immunofluorescence staining confirms the effect of osthole on the cellular distribution of NF-κB, and results show that osthole suppresses NF-κB p65 nuclear translocation (Fig. 6C). These results indicate that osthole inhibits NF-κB activation by suppressing the NF-κB nuclear translocation which inhibits phosphorylation and subsequent degradation of IκBα in CL1-5 cells.

Discussion

Approximately 70% of drugs used for treating cancer are derived from or based on natural products (Newman et al., 2002). Many phytochemicals are able to inhibit tumor metastasis or cell invasion through suppressing enzymatic activities or gene expressions of MMPs (Aggarwal and Shishodia, 2006). Osthole, an active constituent in *C. monnieri*, is extracted from many medicinal plants and herbs such as *C. monnieri*, *Angelica pubescens,* and some species of the Leguminosae and Compositae. Osthole has many applications because of its antihepatitis, anti-osteoporosis, anti-inflammatory, and anti-allerginic effects (Matsuda et al., 2002; Okamoto et al., 2003; Liu et al., 2005; Zhang et al., 2007). Furthermore, both in vitro and in vivo studies show that osthole also has an anticancer effect by inhibiting

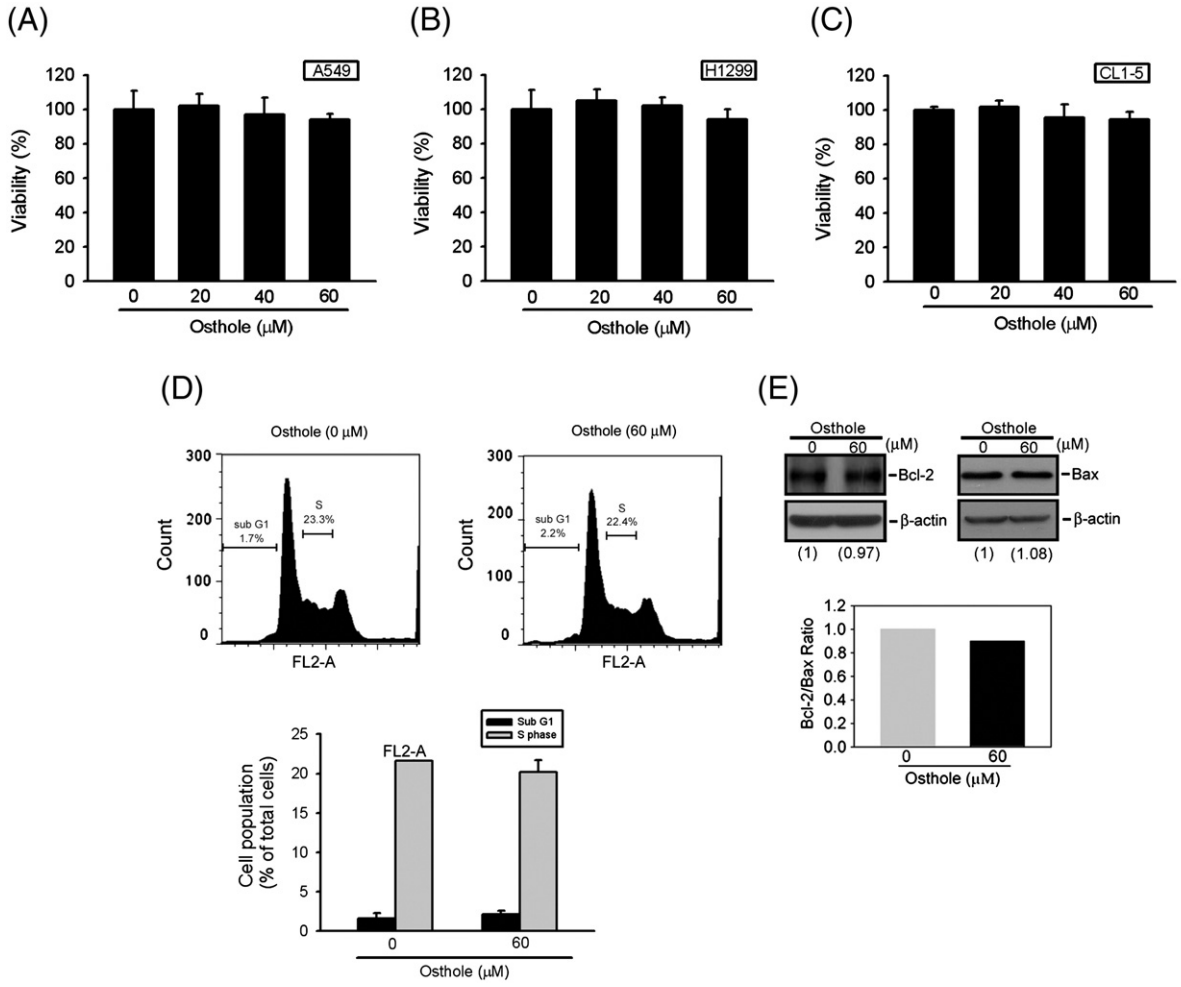

Fig. 3. Effects of osthole on the viability of A549 (A), H1299 (B), and CL1-5 (C) cells. Cells were incubated in a serum-containing medium with various concentrations (0–60 μM) of osthole for 24 h before being subjected to an MTT assay for cell viability. Cells in a serum-containing medium with 0.05% DMSO were used as the control. Values represent the mean ± SE ($n = 3$). CL1-5 cells were released from quiescence by incubation in a culture medium supplemented with 10% FBS and 0.05% DMSO or osthole (60 μM) in 0.05% DMSO for 24 h. Cells were harvested and cell cycle distribution in the sub-G1 and S-phases (D) and bcl-2/bax ratio (E) was determined by a FACS analysis and Western blot, respectively.

human cancer cell growth and inducing apoptosis (Kawaii et al., 2001; Okamoto et al., 2005; Riviere et al., 2006; Chou et al., 2007; Xu et al., 2011). To date, only a few studies describe the antimetastatic (inhibition of migration and invasion) effects of osthole on breast cancer (Yang et al., 2010; Hung et al., 2011). This study demonstrates for the first time, that osthole at noncytotoxic concentrations, (I) inhibits the migration and invasion of lung adenocarcinoma A549, H1299, and CL1-5 cells; (II) inhibits enzyme activities of MMP-2 and/or MMP-9 in A549, H1299, and CL1-5 cells; (III) inhibits mRNA expression, protein expression, and promoter activity of MMP-9 in CL1-5 cells; and (IV) inhibits NF-κB nuclear translocation by suppressing IκBα phosphorylation, degradation, and the binding activity of NF-κB to the MMP-9 promoter in CL1-5 cells.

Cancer cell metastasis involves tumor cell adhesion to the ECM, proteolytic cleavage or destruction of the ECM, and cell migration through resultant defects. This study shows that osthole (20–60 μM) decreases the invasion and migration of several highly invasive lung adenocarcinoma cells (Figs. 1 and 2). Previous study shows that a high concentration (50–150 μM) and long treatment (48 h) of osthole can induce lung cancer cell, A549, apoptosis or cell cycle arrest after 48 h of treatment. Even after treating cells with 50 μM of osthole for 48 h, cell cycle arrest was not significantly induced. (Xu et al., 2011). However, our study demonstrates that treatment of three different lung cancer cell lines (CL1-5, A549, and H1299) with lower concentrations of osthole (20–60 μM) for a shorter time (24 h) did not cause cell apoptosis nor proliferation inhibition (Fig. 3). These results suggest that osthole can inhibit lung cancer cell invasion at a non-cytotoxic concentration.

The critical step in invasion and metastasis is a breaking down of the basement membrane, which requires activation of proteolytic enzymes (Coussens and Werb, 1996) such as MMPs. MMPs are key to tumor angiogenesis and metastasis, and stimulating growth factor release from the ECM (Coussens and Werb, 1996). Several studies indicate that inhibiting MMP expression or enzyme activity are early targets for preventing cancer metastasis (Waas et al., 2003; Guruvayoorappan and Kuttan, 2008). MMP-2 and MMP-9 may participate in this initial step because they hydrolyze basal membrane type-IV collagen. Research associates these enzymes with the invasive metastatic potential of tumor cells (Zhang et al., 2004a). The zymographic data in this study indicate that osthole significantly inhibits MMP-2 and/or MMP-9 enzyme activities in A549, H1299, and CL1-5 cells (Figs. 4A and B). Osthole also significantly inhibits MMP-9 protein expression in CL1-5 cells (Fig. 4D). Previous studies show that regulation of MMP expression is primarily at the transcriptional, posttranscriptional (modulation of mRNA stability), or protein level, depending on different activators or inhibitors, and cell surface localization (Westermarck and Kahari, 1999; Rao, 2003). In this study, osthole inhibits promoter activity (Fig. 5A) and MMP-9 mRNA expression (Fig. 4C), indicating that osthole regulates the expression of MMP-9, at least partially, at a transcriptional level.

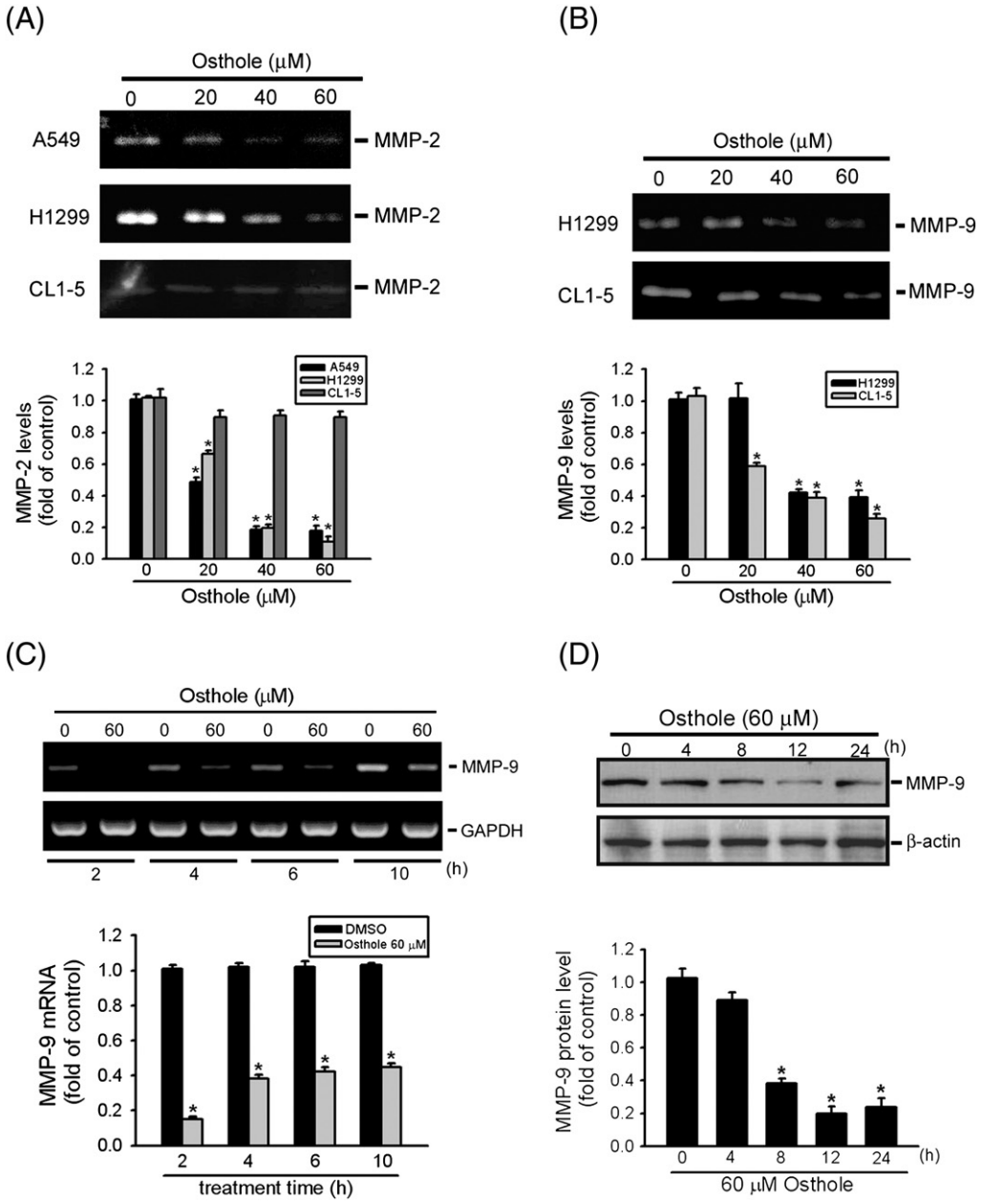

Fig. 4. Effects of osthole on the proteolytic activity and expression of MMP-2 and MMP-9. A549, H1299, and CL1-5 cells were treated with osthole (0–60 μM) for 24 h and then subjected to gelatin zymography to analyze the activities of MMP-2 (A) and MMP-9 (B). (C) CL1-5 cells were treated with 60 μM osthole for different periods (0–10 h) and then subjected to an RT-PCR. GAPDH was used as an internal control. (A–C) (upper panel) represents zymography (A, B) or PCR gel (C). (A–C) (lower panel) is the densitometric analysis of zymography or PCR gel (inversed image used) measuring the band intensities of MMP-2 (A) and MMP −9 (B, C). Values represent the mean ± SE of 3 independent experiments. * $p<0.05$, compared to the vehicle group. (D) CL1-5 cells were treated with 60 μM osthole at different times (0–24 h) and then subjected to Western blot analysis. Quantitative MMP-9 protein level results were adjusted to the β-actin protein level. Values represent the mean ± SE of 3 independent experiments. * $p<0.05$, compared to the vehicle group.

Transcription of the *MMP-9* gene is regulated by the upstream promoter sequence, including AP-1, NF-κB, stimulatory protein (SP)-1, and polyoma virus enhancer activator (PEA)-binding sites (Sato and Seiki, 1993; Takahra et al., 2004). The AP-1 and NF-κB elements of the MMP-9 promoter are centrally involved in the induction of the *MMP-9* gene which is associated with the invasion of tumor cells. For instance, Cheng et al. (2006) indicate that NF-κB modulates radiation-enhanced MMP-9 activity in HepG2 cells. Huang et al. (2005) show that suppression of NF-κB and AP-1 DNA-binding activities mediates the inhibitory effect of carnosol on melanoma cell migration and invasion by reducing MMP-9 expression. Chung et al. (2004) find that caffeic acid and caffeic acid phenyl ester inhibit the transcriptional activity of MMP-9 in PMA-induced HepG2 cells by blocking NF-κB activation. Based on this research, we examine MMP-9 promoter activity using mutated reporter plasmids. When AP-1-binding sites are mutated, the inhibitory effect of osthole on MMP-9 promoter activity does not significantly change (Fig. 5C). Surprisingly, the inhibitory effect of osthole on MMP-9 promoter activity is only partially reversed when the NF-κB binding site is mutated (Fig. 5B). Results from the ChIP assay show that AP-1 and NF-κB are constitutively bound to the *MMP-9* gene promoter. However, osthole only inhibits the DNA-binding ability of NF-κB but not that of AP-1 (Figs. 5D and E). Together, these data suggest that in addition to NF-κB, other transcription factors such as SP-1 and PEA-3, which are located in the MMP-9 promoter (Sato and Seiki, 1993; Takahra et al., 2004), might also contribute to the inhibitory effect of osthole on MMP-9 transcription. For instance, Huang et al. (2007) indicate that the suppression of NF-κB and SP-1 DNA-binding activities mediates the inhibitory effect of lycopene on SK-Hep-1 cell invasion by reducing

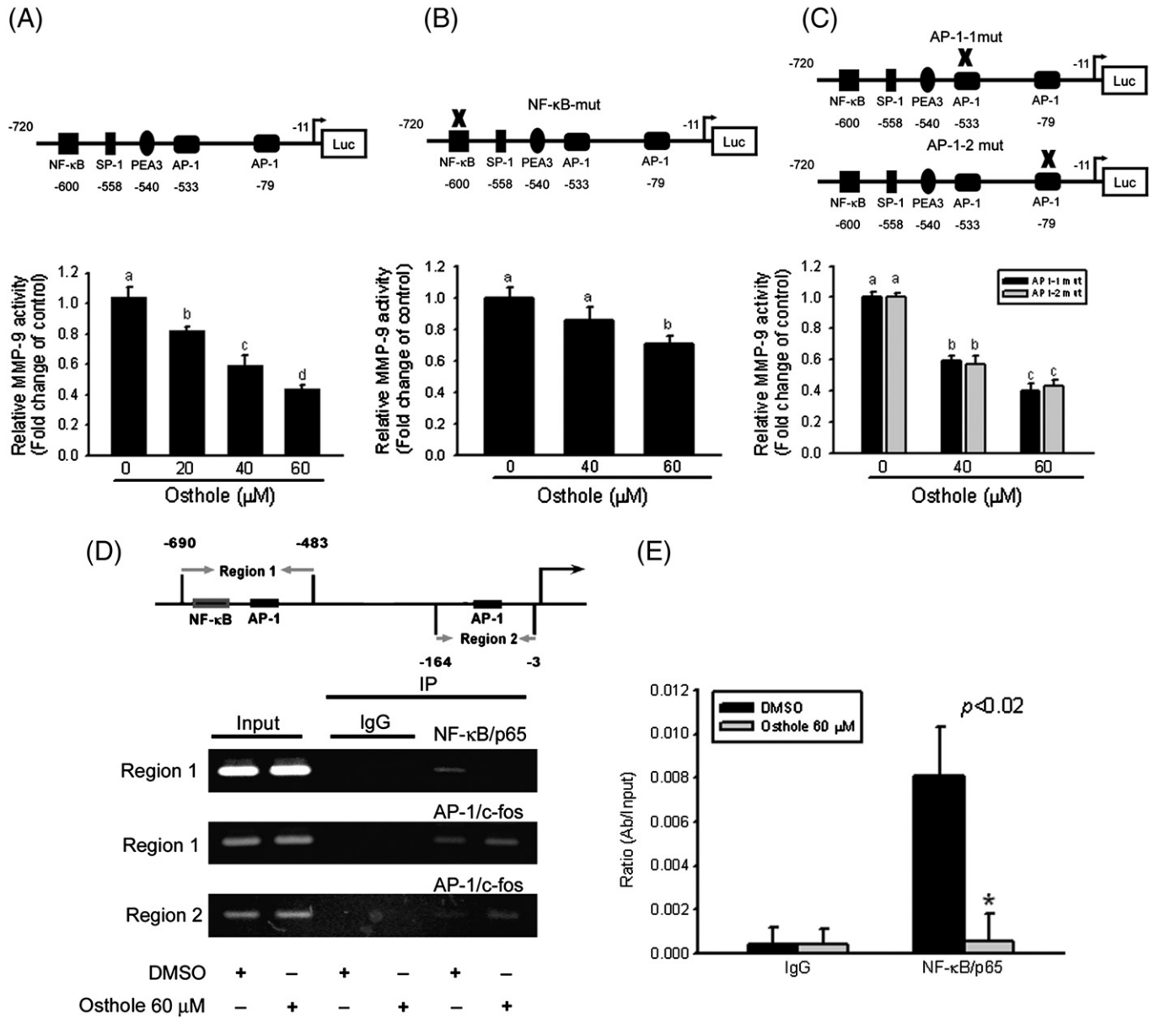

Fig. 5. Osthole inhibits the transcriptional activity of MMP-9 by suppressing the DNA-binding activity of NF-κB on the MMP-9 promoter. (A–C) Upper: schematic of the promoter region of the human MMP-9 gene and the utilized mutant constructs. (A–C) Lower: CL1-5 cells were co-transfected with pGL3-basic (vector) or MMP-9 promoter constructs (wild type, NF-κB-mut, or AP-1-mut) and pRL-TK Renilla control vector. Transfected cells were treated with the indicated concentrations of osthole for 24 h. Promoter activities were calculated as the firefly: renilla luciferase activity ratios and normalized to the vehicle control (0.05% DMSO). Each value represents the mean ± SE of 3 independent experiments. Results were analyzed using one-way ANOVA with Tukey's post hoc tests at 95% confidence intervals. Different letters represent different levels of significance. (D) ChIP analysis of the association of transcription factors NF-κB and AP-1 with the MMP-9 promoter region in CL1-5 cells. Upper: schematic illustration of PCR-amplified fragments of the MMP-9 promoter. Lower: ChIP assays were conducted on CL1-5 cells using p65 and c-fos antibodies to screen NF-κB and AP-1-bound MMP-9 promoter regions for PCR amplification (E). The effect of osthole on the DNA-binding activity of NF-κB was confirmed by a ChIP-qPCR assay. IgG was used as a negative control. Values represent the mean ± SE of 3 independent experiments. * $p<0.05$, compared to the vehicle control group.

MMP-9 expression. Further investigation is required to confirm the role of other transcription factors in the inhibitory effect of osthole on MMP-9 transcription.

NF-κB is central in promoting cancer cell motility and invasion (Baldwin, 2001). Cancer progression in many cancers, including lung cancer, is correlated with a significant elevation of NF-κB activation (McNulty et al., 2004; Tang et al., 2006). Activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκBα and the phosphorylation of p65, which leads to translocation of NF-κB to the nucleus, where it binds to specific response elements in the DNA (Magnani et al., 2000). In this study, osthole inhibits phosphorylation and the subsequent degradation of IκBα (Figs. 6A and B), indicating that the suppression of IκBα degradation by osthole might be because of the inhibition of IκBα phosphorylation. However, further investigation is required to confirm the effects of osthole on other factors which are important for the transcriptional activation of NF-κB, including IκBα ubiquitination and p65 serine 536 residue phosphorylation.

In addition to degradation of the ECM by MMPs, the epithelial to mesenchymal transition (EMT) is another vital step in cancer invasion. The EMT involves repression of epithelial-specific adhesion molecules, such as E-cadherin, with concomitant expression of proteins, such as N-cadherin and vimentin (Shih and Yang, 2011). This study shows that osthole decreases the protein level of the mesenchymal marker, vimentin and increases the epithelial marker, E-cadherin, in a concentration-dependent manner (Supplementary

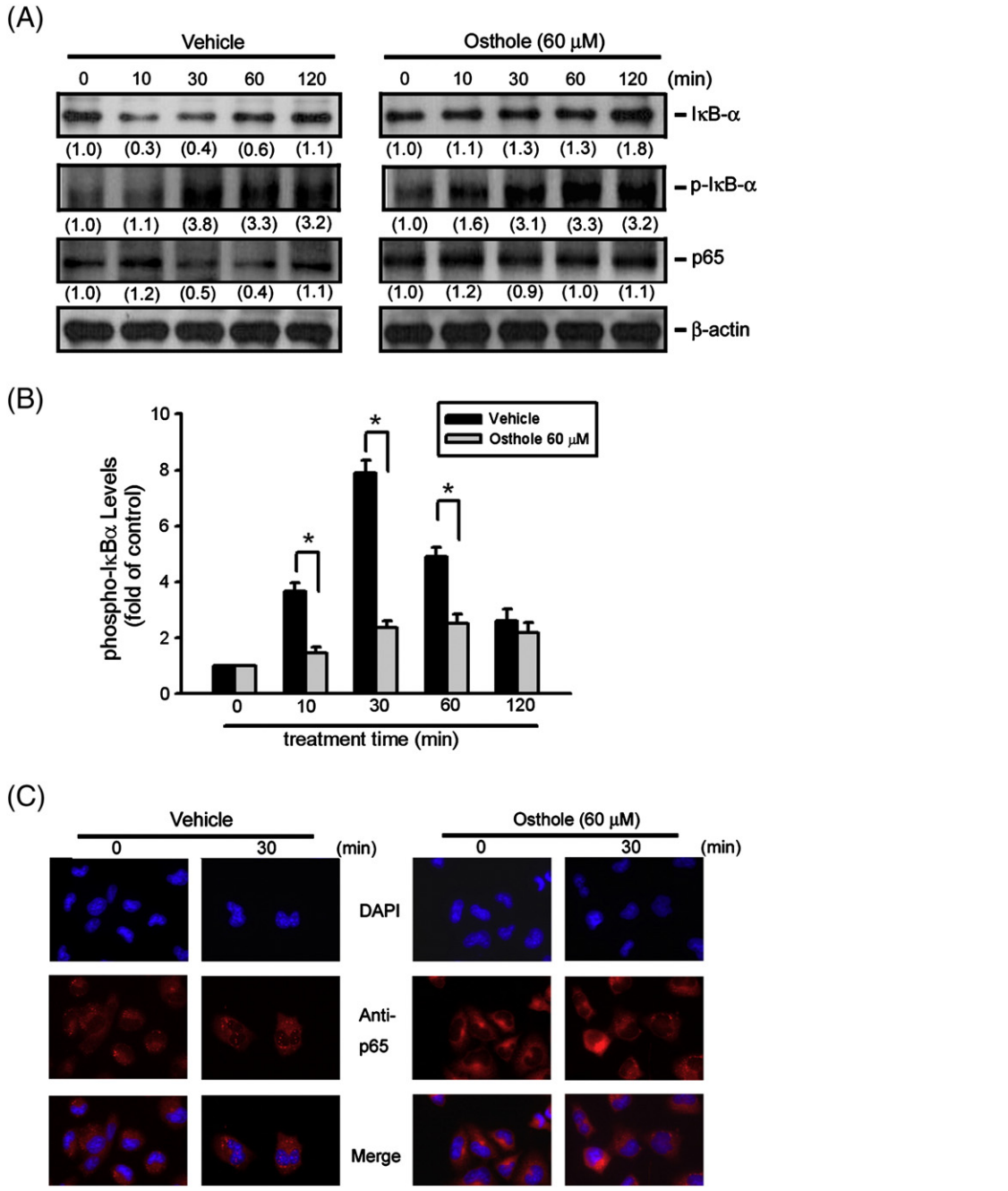

Fig. 6. Osthole inhibits IκBα phosphorylation, degradation, and NF-κB p65 nuclear translocation. (A) CL1-5 cells were pretreated with 60 μM osthole (right panel) or 0.05% DMSO (left panel) at different times (0–120 min). Cells were harvested and fractionated into cytoplasmic and nuclear fractions. Lysates from the cytoplasm were then subjected to Western blot analysis with p65, IκBα, and p-IκBα antibodies. The analysis was repeated 3 times and β-actin was used as an internal control. Protein levels were quantified by densitometric analyses. Results from a representative experiment are shown. (B) After adjusting the expression levels of IκBα and p-IκBα using internal control (β-actin), the phosphorylation levels of IκBα were normalized to total IκBα levels at the same time after vehicle or osthole treatment. Values represent the mean ± SE of 3 independent experiments. * $p<0.05$, compared to the vehicle control group. (C) The effect of osthole on p65 nuclear translocation was confirmed by immunofluorescence. Original magnification, 400×.

data 2). This suggests that the EMT might be involved in the osthole-mediated invasive inhibition of CL1-5 cells. Several transcription factors are also implicated in the transcriptional repression of E-cadherin, including snail, slug, foxc-2, and twist (Shih and Yang, 2011). This study evaluated these transcription factors and found that osthole significantly inhibits expression of slug in CL1-5 cells (Supplementary data 2). A complementary DNA micro-array analysis of a panel of lung adenocarcinoma cell lines (CL1-0, CL1-1, and CL1-5) shows that slug is overexpressed in invasive cell lines. Additionally, an analysis of tumor specimens from NSCLC patients demonstrates that slug mRNA in NSCLC tumor specimens is significantly associated with an increased rate of cancer recurrence and decreased survival (Shih and Yang, 2011). Therefore, slug may be important in the carcinogenesis and progression of lung cancer, and osthole might inhibit invasion, at least partially, through the slug-mediated E-cadherin pathway.

In conclusion, we demonstrate that osthole effectively inhibits lung cancer cell invasion; therefore, osthole could be an anti-invasive bioactive ingredient in *C. monnieri*. Fig. 7 shows the proposed mechanisms that osthole inhibits invasion by CL1-5 cells. The anti-invasive effects of osthole on CL1-5 cells might occur by inhibiting the degradation of IκBα protein expression to reduce NF-κB translocation and NF-κB DNA-binding activities, leading to the downregulation of

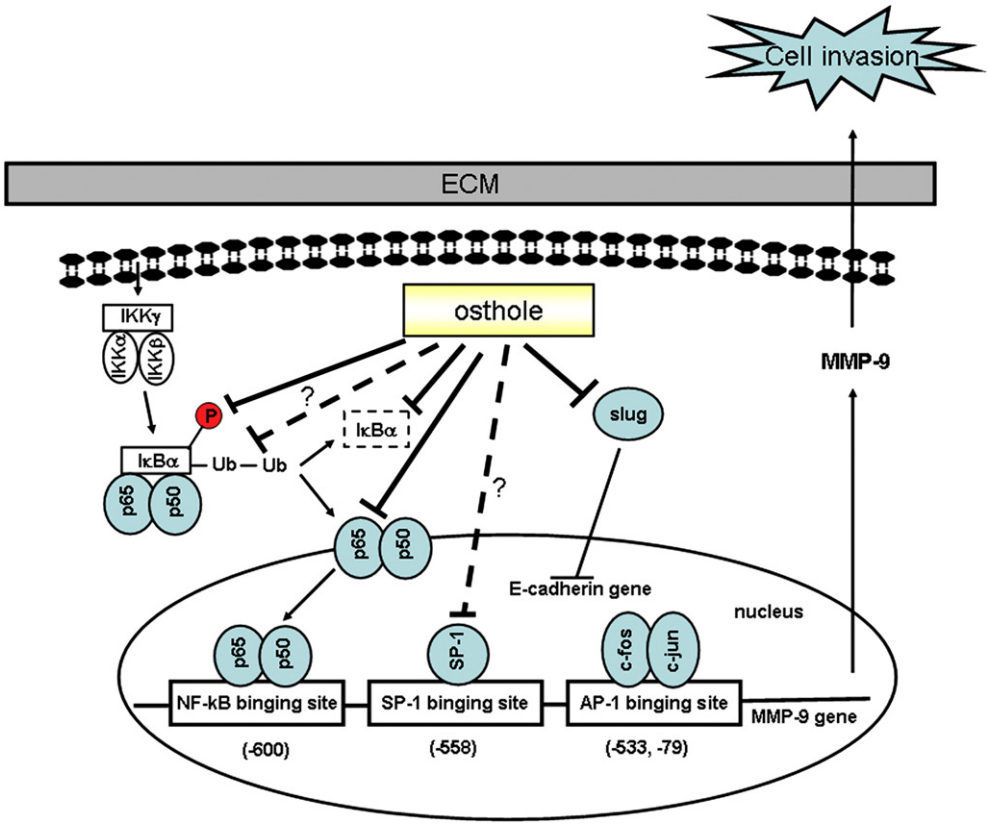

Fig. 7. Proposed signal transduction pathways by which osthole inhibits invasion of CL1-5 cells. Bold solid lines indicate pathways inhibited by osthole. Bold dashed lines indicate hypothetical pathways which might be inhibited by osthole.

MMP-9 expression. Osthole might also inhibit the slug-E-cadherin pathway to suppress cancer cell invasion. Previous research indicates that slug also mediates MMP-9 expression in oral cancer (Joseph et al., 2009). However, the interaction between slug and MMP-9 in the osthole-mediated inhibition of the invasive ability of CL1-5 cells should be further investigated. With clarification of signal transduction mediators and transcriptional factors involved in the anti-invasive process of osthole on human lung cancer cell lines, it might be possible to develop specific mediators to inhibit undesired cell invasion. Osthole should be further tested using an in vivo model to clarify if it is effective in preventing lung cancer invasion or metastasis.

Conflict of interest statement

None declared.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2012.03.020.

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