

Osthole-mediated cell differentiation through BMP-2/p38 and ERK1/2 pathway in human osteoblast cells

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*Abbreviations:* ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; phospho-p38, phosphorylated p38; phospho-ERK1/2, phosphorylated extracellular signal-regulated kinase; MEK1, mitogen-activated protein kinase kinase; BMP, Bone morphogenetic protein; ELISA, enzyme-linked immunosorbent assay; PNPP, 4-Nitrophenyl phosphate; PD98059, 2'-Amino-3'-methoxyflavone; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

## Abstract

The survival of osteoblast cells is one of the determinants of the development of osteoporosis in patients. Osthole is a coumarin derivative present in many medicinal plants. By means of alkaline phosphatase (ALP) activity, osteocalcin, osteopontin, and type I collagen ELISA assay, we have shown that osthole exhibits a significant induction of differentiation in two human osteoblast-like cell lines, MG-63 and hFOB. Induction of differentiation by osthole was associated with increased BMP-2 production, and the activations of SMAD1/5/8, and p38 and ERK1/2 kinases. Addition of purified BMP-2 protein did not increase the upregulation of ALP activity and osteocalcin by osthole, whereas the BMP-2 antagonist noggin blocked both osthole and BMP-2-mediated ALP activity enhancement, indicating that BMP-2 production is required in osthole-mediated osteoblast maturation. Pretreatment of osteoblast cells with noggin abrogated p38 activation, but only partially decreased ERK1/2 activation, suggesting that BMP-2 signaling is required in p38 activation, and is partially involved in ERK1/2 activation in osthole-treated osteoblast cells. Co-treatment of p38 inhibitor SB203580 or p38 siRNA expression inhibited osthole-mediated activation of ALP, but only slightly affected osteocalcin production. In contrast, the production of osteocalcin induced by osthole was inhibited by the MEK inhibitor PD98059, or by expression of an ERK2 siRNA. These data suggest

that BMP-2/p38 pathway links to the early phase, while ERK1/2 pathway is associated with the later phase in osthole-mediated differentiation of osteoblast cells. In this study, we demonstrate that osthole is a promising agent for treating osteoporosis.

## Introduction

Osteoporosis is a reduction in skeletal mass due to an imbalance between bone resorption and bone formation, whereas bone homeostasis requires balanced interactions between osteoblast and osteoclast (Manolagas and Jilka, 1995; Ducy et al., 2000; Teitelbaum, 2000). Current drugs used to treat osteoporosis include bisphosphonates, calcitonin, estrogen, vitamin D analogues and ipriflavone. These are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts (Rodan and Martin, 2000). The effect of these drugs in increasing or recovering bone mass is relatively small, certainly no more than 2% per year (Rodan and Martin, 2000). It is desirable, therefore, to have satisfactory bone-building (anabolic) agents, such as teriparatide, that would stimulate new bone formation and correct the imbalance of trabecular microarchitecture which is characteristic of established osteoporosis (Ducy et al., 2000; Berg et al., 2003). Since new bone formation is primarily a function of the osteoblast, agents which regulate bone formation act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts (Ducy et al., 2000; Lane and Kelman, 2003).

Bone morphogenetic proteins (BMPs) form a unique group of proteins within the transforming growth factor beta (TGF- $\beta$ ) superfamily and have pivotal roles in the

regulation of bone induction, maintenance and repair, as well as being important determinants of mammalian embryological development (Sykaras and Opperman, 2003; Nohe et al., 2004). Signaling by BMPs proteins is mediated through heterodimerization of types I and II serine/threonine kinase receptors, both of which have subcategories. Various signaling pathways appear to be activated by the ligand binding to the receptors. BMPs stimulate that activation of SMAD proteins that accumulate in the nucleus and control transcription of a large number of target genes. The SMAD1, SMAD5, and SMAD8 are recognized by BMP type I receptor (Sykaras and Opperman, 2003; Nohe et al., 2004). BMPs also activate extracellular signal regulated protein (ERK1/2), p38 and c-Jun amino terminal kinases (JNK) mitogen-activated proteins kinases (MAPKs), and this can proceed rapidly and independently of SMADs (Nohe et al., 2004; Gallea et al., 2001; Guicheux et al., 2003).

MAPKs, a family of serine/threonine kinases, are mediators of intracellular signals in response to various stimuli. MAPKs have demonstrated involvement in the regulation of many cellular physiological functions, including proliferation, differentiation, inflammation, and apoptosis (Chang and Karin, 2001). MAPK activation requires the phosphorylation of both the threonine and tyrosine residues of a conserved T-X-Y motif within the activation loop of the kinase by a dual-specificity

MAPK kinase (MKK or MEK) (Jadlowiec et al., 2004). p38, one subfamily of MAPK, is activated not only by MKKs, but also by the BMP-2 pathway through SMAD-dependent or independent pathways (Chang and Karin, 2001; Hu et al., 2003). In some types of cells, BMP-2 activates MAP kinase kinases kinase (MAPKKK), including TAK1, and consequently the MAPKKK elicits MKK3 or MKK6 that directly phosphorylates and activates p38 kinase (Goswami et al., 2001; Lai et al., 2002). Many studies have reported that p38 activation is necessary for differentiation in osteoblast cells (Jadlowiec et al., 2004; Guicheux et al., 2003; Hu et al., 2003). ERK1/2, another subfamily of MAPK, has demonstrated the dependence of mammalian cell differentiation on the ERK pathway (Rodriguez et al., 2004). Activation of MEK is necessary and sufficient for mesenchymal stem cells, and interference with its enzyme activity by the chemical inhibitor PD98059 blocks this differentiation (Jaiswal et al., 2000). Although p38 and ERK1/2 have been implicated in the regulation of osteoblast differentiation, the precise role of p38 and ERK1/2 kinase remains elusive.

Osthole, a coumarin derivative, has been extracted from many medicinal plants, such as *Cnidium monnieri* and *Angelica pubescens*, which are used in traditional Chinese medicine as tonics and aphrodisiacs (Ko et al., 1992). Previous studies have shown that osthole possesses anti-proliferation, vasorelaxation, anti-hepatitis,

anti-inflammatory, anti-aggregatory, and anti-allergic effects (Ko et al., 1992; Huang et al., 1996; Liu et al., 1998; Matsuda et al., 2002; Yang et al., 2003.) Osthole has been shown to exhibit estrogen-like effects, preventing post-menopausal osteoporosis in ovariectomized rats (Li et al., 2002). It has also been reported to stimulate cell proliferation of osteoblast-like cells (Meng et al., 2004). Here we report that osthole increases differentiation in human MG-63 and hFOB osteoblast. Furthermore, to establish the cell differentiating mechanism of osthole, we assayed BMP-2, SMADs, p38 and ERKs levels, which are strongly associated with the signaling pathways of cell differentiation. Furthermore, by focusing on osteoblast treated with osthole or BMP-2, we further characterize the relationship between BMP-2 and MAPKs.



## Methods

### Reagents and materials

Fetal bovine serum (FBS), minimal essential medium (MEM), penicillin G, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD, USA). Osthole (7-methoxy-8-isopentenoxycoumarin) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Dimethyl sulfoxide (DMSO) was supplied by Sigma Chemical Co. (St. Louis, MO, USA). BMP-2 ELISA kit, human BMP-2 protein, and noggin were obtained from R&D Systems (Minneapolis, MN, USA). Sodium 3'-[1-(phenylamino-carbonyl) -3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfoic acid hydrate (XTT) kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). PD98059 (2'-Amino-3'-methoxyflavone) and SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) were purchased from Calbiochem (Cambridge, MA, USA). The antibodies to p38, ERK1/2, SMAD1/5/8, phospho-p38, phospho-ERK1/2 and phospho-SMAD1/5/8, and p38 and ERK kinase assay kits were obtained from Cell Signaling Technology (Beverly, MA, USA). The osteocalcin ELISA kit was obtained from Biosource Technology (Nivelles, Belgium). The osteopontin ELISA kit was obtained from Assay Designs Inc. (Michigan, USA).

## **Cell cultures**

The human osteoblast-like cell line MG-63 (CRL-1427) was purchased from American Type Culture Collection (ATCC). Cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml of penicillin G and 100 µg/ml of streptomycin). The conditionally immortalized human fetal osteoblastic cell line (hFOB, CRL-11372), was maintained in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (GIBCO-BRL, Gaithersburg, MD, USA) containing 10% FBS supplemented with geneticin (300 µg/ml) and antibiotics at 33.5°C, the permissive temperature for the expression of the large T antigen. All experiments of hFOB cells were carried out at the permissive temperature of 33.5°C.

## **Cell proliferation assay (XTT)**

Inhibition of cell proliferation by osthole was measured by XTT assay. Briefly, cells were plated in 96 well culture plates ( $5 \times 10^3$  cells/well). After 24 h incubation, the cells were treated with osthole (0, 1, 5, 10, and 20 µM) for 48 h. Fifty µl of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 µl of electron coupling reagent, was then added to each well. After 4 h of incubation, absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

### **Alkaline phosphatase (ALP) activity**

Cells were seeded into 96 well plates at a density of  $5 \times 10^3$  cells/well and cultured for 24 h. The agent to be tested was added to the wells, and incubation continued for 2 days. The cells were then washed with three times with physiological saline, and cellular protein concentration was determined by incubation in BCA (bicinchoninic acid) protein assay reagent containing 0.1% Triton X-100 for 1 h at 37°C. The reaction was stopped by adding 1 M NaOH, and absorbance measured at 560 nm.

ALP activity in the cells was assayed after appropriate treatment periods by washing the cells three times with physiological saline. ALP activity in the cells was then measured by incubation for 1 h at 37°C in 0.1 M NaHCO<sub>3</sub>–Na<sub>2</sub>CO<sub>3</sub> buffer, pH 10, containing 0.1% Triton X-100, 2 mM MgSO<sub>4</sub>, and 6 mM PNPP (4-Nitrophenyl phosphate). The reaction was stopped by adding 1 M NaOH, and absorbance measured at 405 nm. The percentages of changes of ALP activity with respect to the value found in the control were calculated according to this formula:  $M = \text{value of absorbance at 405 nm} / \text{value of absorbance at 560 nm}$ . Percentage of change =  $[(M \text{ of the test} - M \text{ of the control}) / M \text{ of the control}] \times 100$  (Jia et al., 2003; Morais et al., 1998).

### **Assaying the levels of osteocalcin, osteopontin and BMP-2**

Osteocalcin, osteopontin, and BMP-2 ELISA kits were used to detect osteocalcin,

osteopontin, and BMP-2 levels, respectively. Briefly, cells were treated with various concentrations of osthole or BMP-2 for the indicated times. The culture medium was collected and measured for osteocalcin, osteopontin, and BMP-2 respectively. These samples were placed in 96 well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room temperature. After removing unbound material by washing with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution, with color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm. Results are presented as the percentage of change of the activity compared to the untreated control.

#### **Assaying the levels of type I procollagen**

Cells were treated with various concentrations of osthole or BMP-2 for 72 h. The type I procollagen assay, which measures the propeptide portion of the molecule, reflects the synthesis of the mature form of the protein, and was carried out using Prolagen-C kit as described in the manufacturer's protocol (Metra Biosystems, Mountainview, CA, USA). The type I procollagen levels obtained were normalized to total protein concentrations that were determined by BCA protein assay.

### **Analysis for Western blotting and in vitro kinase assay**

Cells treated with osthole or BMP-2 for the indicated times were lysed and the protein concentrations were determined by using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). For Western blotting, 50  $\mu$ g of total cell lysates were subjected to SDS-PAGE. The protein was transferred to PVDF membranes using transfer buffer (50 mM Tris, 190 mM glycine, and 10% methanol) at 100 V for 2 h. The membranes were incubated with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Tween 20, and 3% BSA) overnight at 4°C. After washing three times with washing buffer (blocking buffer without 3% BSA) for 10 min each, the blot was incubated with primary antibody (SMAD1/5/8, ERK1/2, p38, phospho-ERK, phospho-p38 and SMAD1/5/8) for 2-15 h, followed by horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham, USA).

The ERK1/2 and p38 MAPK activities were determined using in vitro kinase assay kits from Cell Signaling Technology (Beverly, MA) according to the manufacturer's instructions.

### **siRNA knockdown of ERK and p38 expression**

Osteoblast monolayers were transfected with SMARTpool™ MAPK1 (ERK2) and

p38 siRNA duplexes or non-specific control siRNA duplexes (Upstate Biotechnology Inc, NY). Briefly, osteoblast cells were plated in 6-well plates and transfected with 200 pmole/well of ERK2, p38 or control siRNA (at a final concentration of 100 nM) in Opti-MEM medium using LipofectAMINE reagent (Invitrogen, Co.). After 6 h transfection, the medium was replaced with complete growth medium, and the cells were allowed to recover for 24 h. Cells were then treated with or without osthole and BMP-2 for the indicated times, and cell lysates were analyzed by Western blot using anti-ERK or anti-p38 antibody. At these times, immunoblot analyses showed that expression of ERK1/2 and p38 remained low but detectable, whereas expression of  $\beta$ -actin was unaffected by siRNA treatment.

### **Statistical analysis**

Data were expressed as means  $\pm$  SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ( $p < 0.05$ ) between the means of control and test group were analyzed by Dunnett's test.

## **Results**

### **No effect of osthole on the proliferation of MG-63 and hFOB cells**

We first determined the effect of osthole on the cell proliferation of MG-63 and hFOB human osteoblast cells by XTT assay. As shown in Fig. 1, osthole did not exhibit significant effects on cell growth at the concentrations used (1-20  $\mu$ M) after 48 h of treatment in either cell line.

### **Effect of osthole on maturation and differentiation markers, ALP activity, collagen synthesis, osteopontin, and osteocalcin expression in MG-63 and hFOB cells**

The effects of osthole on the differentiation of osteoblasts were studied by determining ALP activity, collagen synthesis, osteopontin, and osteocalcin production in MG-63 and hFOB cells. The results showed that osthole increased ALP activity in a dose-dependent manner after 48 h of treatment in both cell lines (Fig. 2A).

The effect of osthole on the terminal differentiation of osteoblast cells was also assessed by determining the production of osteocalcin, osteopontin, and collagen synthesis. As shown in Fig. 2, B and C, treatment of MG-63 and hFOB cells with osthole increased the level of osteocalcin and osteopontin in a dose-dependent manner after 72 h of treatment. In addition, type I collagen protein levels were also enhanced in osthole-treated MG-63 and hFOB cells after 72 h of treatment (Fig 2D).

## **BMP-2 mediates osthole-induced maturation and differentiation in MG-63 and hFOB cells**

The upregulation of BMP-2 by 10  $\mu$ M osthole started to increase 3 h after treatment with osthole, and maximum expression was observed at 12 h (Fig. 3A). After 12 h of treatment, osthole increased production of BMP-2 in a dose-dependent manner (Fig. 3B). To further determine that BMP-2 may be involved in osthole-mediated osteoblast cell differentiation, we first determined the effect of purified BMP-2 on various cell differentiation markers. As shown in Fig. 3C, treatment of cells with purified BMP-2 protein resulted in a significant upregulation of ALP activity at 48 h of treatment. In addition, some bone matrix proteins, including osteocalcin, osteopontin, and type I collagen protein levels, all increased in BMP-2-treated MG-63 and hFOB cells after 72 h of treatment.

ALP activity is a phenotypic marker for the early and mature differentiations of osteoblast cells (Franceschi and Iyer, 1992), whereas osteocalcin production is a biologic marker for terminal differentiation (Jia et al., 2003). However, purified BMP-2 protein did not increase the activity and secretion of ALP and osteocalcin induced by osthole, supporting the hypothesis that BMP-2 may mediate osthole-induced mature differentiation (Fig. 3, D and E). To further examine the role of BMP-2 in cell differentiation by osthole, osteoblast cells were pretreated with a



BMP-2 inhibitor, 100 ng/ml noggin protein, for 1 h and then co-treated with 10  $\mu$ M osthole and the inhibitor for the indicated times. Noggin directly binds to BMP-2, thereby preventing its interaction with BMP receptor (Hallahan et al., 2003). Addition of purified noggin protein did not change ALP activity and osteocalcin secretion, but abrogated BMP-2 induced cell differentiation as predicted (Fig. 3, F and G). In contrast, noggin pre-treatment only decreased osthole-induced enhancement of ALP (83.5% and 80.4% inhibition for MG-63 and hFOB), but showed it had a lesser effect on osteocalcin production (45.1 % and 49.4 % inhibition for MG-63 and hFOB) than that on ALP activity induced by osthole. Therefore, osthole-induced cell differentiation may operate by BMP-2-dependent and independent pathways.

### **The activations of SMAD1/5/8, p38, ERK1/2 pathways in osthole-treated MG-63 and hFOB cells**

Ligation of BMP-2 to BMP receptor induces receptor heteromeric complexes and subsequently activates SMADs or MAPKs by phosphorylation. We first assessed activation (phosphorylation) of SMAD proteins in osthole-treated MG-63 and hFOB cells. As shown in Fig. 4A, treatment with osthole did not affect on the expression levels of unphosphorylated SMAD1/5/8, but it did increase the amount of phospho-SMAD1/5/8 after a 3 h exposure of osteoblast cells to osthole, with a progressive increase for up to 12 h. The activation of SMADs closely matched the

appearance of BMP-2.

We also investigated the implication of MAPKs in osthole-treated cells. The result showed that osthole treatment increased that activation (phosphorylation) of p38 as well ERK1/2 (Fig. 4B). p38 activation occurred later (6 h) than SMADs (3 h), indicating that p38 activation may be a downstream event of SMADs. In contrast, ERK1/2 activation was observed at 3 h and persisted for the duration of the osthole treatment experiment.

The osthole-mediated effect on the activity of MAPKs was further confirmed by determining phosphorylation of one of its substrates (ATF-2 and Elk-1 for p38 and ERK1/2, respectively.). As shown in Fig. 4C, in comparison with the control, the activity of p38 (phospho-ATF-2, Thr71) increased after a 6 h exposure of MG-63 and hFOB cells to osthole, then maintained similar levels up to 12 h of treatment (Fig. 4C). The activity of ERK1/2 (phospho-Elk-1, Ser383) increased at 3 h, and sustained activation for the duration of the osthole treatment experiment, which was consistent with the phosphorylated activation of ERK1/2.

To further confirm whether BMP-2 was involved in osthole-mediated on SMADs, p38, and ERK1/2 activation, we also assessed the effect of purified BMP-2. As shown in Fig. 4D, Purified BMP-2 increased the phosphorylation of SMAD1/5/8 without affecting on the levels of unphospho-SMAD1/5/8. The activation pattern of p38 by

purified BMP-2 treatment, including p38 activation and specific substrate (ATF-2) phosphorylation, was similar to those of osthole-treated osteoblasts (Fig. 4, E and F). In contrast, although purified BMP-2 increased activation and activity of ERK1/2, the duration of ERK1/2 activation, was shorter (3-6 h) than that of osthole-treated osteoblasts (3-24 h).

### **BMP-2 signaling is required in p38 activation, but only partially involved in ERK1/2 activation by osthole treatment**

To determine the role of BMP-2 on the activation of p38 and ERK1/2 in osthole treated osteoblastic cells, we tested the effect of noggin on the activation of SMAD1/5/8, p38, and ERK1/2 by osthole and BMP-2. Results showed that noggin pretreatment abrogated the activation of SMAD1/5/8, p38, and ERK1/2 induced by purified BMP-2 (Fig. 5A). In contrast, noggin pretreatment inhibited the activation of SMAD1/5/8 and p38, but only had a lesser effect the activation of ERK1/2 induced by osthole (Fig. 5B). Thus, BMP-2 signaling is both necessary and sufficient to mediate the activation of SMAD1/5/8 and p38, and is partially involved in the activation of ERK1/2 in both MG-63 and hFOB cells.

### **p38 and ERK1/2 are necessary for osthole-mediated osteoblast differentiation in MG-63 and hFOB**

As p38 and ERK1/2 activation was observed with BMP-mediated differentiation in

osthole treated osteoblastic cells, we next assessed the role of these by using inhibitor specific to p38 and ERK1/2. MG-63 and hFOB cells were pretreated for 1 h with MEK1 inhibitor (an upstream kinase in the ERK1/2 signaling pathway), PD98059, or a potent specific inhibitor for p38, SB203580. Subsequently, the inhibitor-treated cells were exposed to osthole or BMP-2, and ALP activity and osteocalcin secretion then determined. As shown in Fig. 6A, the osthole and BMP-2-mediated p38 and ERK1/2 activation was effectively inhibited by 20  $\mu$ M SB203580 and 20  $\mu$ M PD98059 respectively (Fig. 6A). SB203580 decreased the ALP activity at 48 h in both osthole and BMP-2 treated osteoblast (88.7 % and 79.1 % inhibition in osthole-treated MG-63 and hFOB; 85.7 % and 78.5 % inhibition in BMP-2-treated MG-63 and hFOB), but slightly inhibited the accumulation of osteocalcin induced by osthole or BMP-2 at 72 h (30.56 % and 27.0 % inhibition in osthole-treated MG-63 and hFOB; 25.9 % and 24.5 % in BMP-2-treated MG-63 and hFOB) (Fig. 6, B and C). In contrast, PD98059 pretreatment completely inhibited osteocalcin production and slightly decreased the upregulation of ALP activity in osthole-treated MG-63 and hFOB cells (33.4 % and 24.5 % inhibition for ALP; 85.8 % and 78.3 % inhibition for osteocalcin in osthole-treated MG-63 and hFOB). However, PD98059 only exhibited less inhibition on BMP-2-induced ALP activity and osteocalcin production (33.8 % and 26.9 % inhibition for ALP; 28.5 % and 28.0 % inhibition for osteocalcin in BMP-2-treated

MG-63 and hFOB) (Fig. 6, B and C).

### **Genetic inhibition of p38 and ERK1/2 by siRNA blocks osthole-induced cell differentiation in MG-63 and hFOB cells**

We further confirmed the role of BMP-2, p38 and ERK1/2 in osthole-mediated cell differentiation by siRNA-based inhibition. To do so, MG-63 and hFOB cells were transfected with a pool of siRNAs target p38 or ERK2, after which the cells were exposed to osthole or BMP-2. The effect of p38 and ERK2 genetic inhibition on osthole and BMP-2-induced ALP activity and osteocalcin production was examined. Transfection of MG-63 and hFOB cells with p38 and ERK2 siRNA reduced basal levels of p38 and ERK1/2 and blocked osthole and BMP-2-mediated p38 and ERK1/2 activation (Fig. 7A). Specific knock down p38 expression by p38 siRNA inhibited ALP upregulation, but had a lesser effect on osteocalcin production in both osthole and BMP-2 treated cells (77.5 % and 72.0 % inhibition for ALP; 23.7 % and 26.5 % inhibition for osteocalcin in osthole-treated MG-63 and hFOB; 73.2 % and 73.8 % inhibition for ALP; 22.7 % and 25.5 % inhibition for osteocalcin in BMP-2-treated MG-63 and hFOB) (Fig. 7, B and C). In contrast, selective genetic inhibition of ERK blocked osthole-mediated osteocalcin production, but only somewhat influenced the increase of ALP activity induced by osthole and BMP-2, and osteocalcin production induced by BMP-2 in both cell lines (39.7 % and 34.7 % inhibition for ALP; 81.35 %

and 75.8 % inhibition for osteocalcin in osthole-treated MG-63 and hFOB; 37.6 % and 36.0 % inhibition for ALP; 18.4 % and 19.1 % inhibition for osteocalcin in BMP-2-treated MG-63 and hFOB) (Fig. 7, D and E). These findings coincided with results in the study using chemical inhibitors.

## Discussion

Osthole is a coumarin derivative present in medicinal plants, such as *Cnidium monnieri* and *Angelica pubescens*, used in traditional Chinese medicine for the treatment of various orthopaedic diseases (Ko et al., 1992; Teng et al., 1994). In this study, we determined that osthole induced maturation and differentiation in two human osteoblast-like cell lines, MG-63 and hFOB, without exhibiting a significant effect on cell growth. Treatment of MG-63 and hFOB cells with osthole not only increased ALP activity (a marker of maturation and mineralization), but also enhanced osteocalcin secretion (a marker of terminal differentiation) in osteoblast cells. These results indicate that osthole stimulated maturation and differentiation of osteoblast cells could be affected at various levels, from early to terminal stages of the cell differentiation process.

BMPs play an important role in the process of bone formation and remodeling (Sykaras et al., 2003). It has been well documented that stimulation of osteoblast cell differentiation is characterized mainly by increased expression of ALP, type I collagen, and osteocalcin (Xiao et al., 2004). The action of BMPs is mediated by heterotetrameric serine/threonine kinase receptors and the downstream transcription factors SMAD1/5/8. After these transcription factors are phosphorylated on serine residues, they form a complex with a common mediator, SMAD4, and the complex is

translocated into the nucleus to activate the transcription of a specific gene (Sykaras et al., 2003; Nohe et al., 2004). Our study indicates that the production of BMP-2 increases in osthole-treated MG-63 and hFOB cells. Treatment of cells with purified BMP-2 not only increased ALP activity, but also enhanced the expression of bone matrix proteins (osteocalcin, osteopontin production, and Type I collagen level). Also, phosphorylations of SMAD1/5/8 are simultaneously enhanced in osthole or BMP-2-treated osteoblast cells. Furthermore, addition of purified BMP-2 did not increase osthole's effect on cell maturation, whereas BMP-2 antagonist noggin inhibited osthole's effect on cell maturation. Thus, our results have demonstrated that the BMP-2 signaling system plays an important role in osthole-mediated cell maturation in osteoblast cells.

BMP-2 has been shown to activate not only SMAD signaling but also to elicit p38 kinase pathway, which is involved in osteoblast differentiation (Nohe et al., 2004). Previous studies have reported the direct correlation between SMADs and p38 kinase signaling (Noth et al., 2003). Similarly, it has been shown that p38 pathway, by interacting with SMADs signaling, is involved in BMP-2 induced bone matrix gene expression and ALP activity in various osteoblast cell lines (Hu et al., 2003; Lai and Cheng, 2002). Our study observed an increase in p38 activity following BMP-2 production and SMAD1/5/8 phosphorylation, and suppression of BMP-2 signaling by



co-treating noggin abrogated SMAD1/5/8 and p38 activation in osthole-treated cells. Purified BMP-2 also increased p38 activation and activity, and the activation pattern was similar to that of osthole-treated MG-63 and hFOB cells. In addition, inhibition of p38 activity by SB203580 and siRNA-based inhibition decreased the effects of BMP-2 and osthole on osteoblastic maturation (ALP activity), but only slightly affected on later phase differentiation (osteocalcin production). These data therefore suggest that activation of p38 plays an important role in osthole and BMP-2 activity in osteoblast cells, and particularly on osteoblast cell maturation.

ERK1/2 is also important in osteoblast cell proliferation and differentiation (Jaiswal et al., 2000; Rodriguez et al., 2004). A number of studies have reported that ERK is an important mediator of BMP-2-induced osteoblast differentiation, and that inhibition of ERK1/2 results in the suppression of differentiation markers (Jaiswal et al., 2000; Cortizo et al., 2003). ERK1/2 has also been determined to induce osteoblast differentiation through BMPs-independent pathway in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated primary human osteoblasts (Chae et al., 2002). It suggests that sustained ERK activation operated independent on BMP also can be a critical determinant in the promotion of osteoblast differentiation (Chae et al., 2002). In our study, the activation of ERK1/2 and production of BMP-2 was simultaneous, and co-treatment of osteoblast cells with noggin and osthole only partially blocked the activation of ERK1/2. In addition, the

duration of ERK1/2 by purified BMP-2 was shorter than that of osthole-treated osteoblasts. These data indicate that the activation of ERK1/2 by osthole may be through BMP-2-dependent and independent pathways. Furthermore, since MEK inhibitor PD98059 and ERK2 siRNA-based inhibition abrogated osthole-induced osteocalcin secretion with little effect on ALP activity and BMP-2-induced osteocalcin production in osteoblast cells, this suggests that ERK1/2 activation is involved primarily in regulation of late stage cell differentiation induced by osthole, but not BMP-2.

The roles of ERK1/2 and p38 MAPKs in the phases of osteoblast differentiating process are disputable. Suzuki et al. reported that ERKs play an essential role in cell replication, whereas p38 is involved in the regulation of ALP expression during osteoblast cell differentiation in MC3T3-E1 cells (Suzuki et al., 1999). A study by Lai and Cheng showed BMP-2 up-regulation of type I collagen, fibronectin, osteopontin, osteocalcin, and alkaline phosphatase activity, whereas ERK mediated BMP-2 stimulation of fibronectin and osteopontin. Thus, ERK and p38 differentially mediate TGF-beta and BMP-2 functions in osteoblasts (Lai and Cheng, 2002). In contrast, one study of C2C12 indicates that p38 MAPK is required for BMP-2-induced expression of ALP and osteocalcin, and ERKs are necessary only for osteocalcin expression (Gallea et al., 2001). Our results show that p38 is particularly involved in the early

phase (ALP activity) in osthole and BMP-2- treated osteoblasts. In contrast, ERK1/2 participates mainly in the later phase (osteocalcin secretion) of osteoblast differentiation induced by osthole. Interestingly, ERK2 knockdown by siRNA caused less inhibition of BMP-2-mediated osteocalcin production than that of osthole-treated osteoblasts, suggesting that the duration of ERKs' activation may also be involved in osteocalcin production in response to various stimulators. However, the actual mechanism by which osthole operates the two different MAPK signals to regulate different phases of osteoblast differentiation requires further investigation.

Taken together, these observations indicate that osthole induces osteoblast maturation and differentiation in MG-63 and hFBO cells. Osthole's effect on cell maturation is strongly associated with BMP-2 production followed by SMAD1/5/8 and p38 activation. In addition, osthole also induces osteoblast differentiation by ERK1/2 activation via BMP-2-dependent and independent pathways. It would, therefore, suggest that osthole may be beneficial in stimulating the osteoblastic activity resulting in bone formation.

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## Figure Legends

**Fig. 1.** Osthole did not increase the proliferation of MG-63 and hFOB cells. Adherent cells that proliferated in 96-well plates ( $10^4$  cells/well) were incubated with different concentrations ( $\mu\text{M}$ ) of osthole for 48 h. Cell proliferation was determined by XTT assay. Each value is the mean  $\pm$  SD of three independent experiments.

**Fig. 2.** Osthole induced the differentiation of MG-63 and hFOB cells. (A) Osthole increased the activity of ALP at 48 h. (B) Osthole enhanced the production of osteocalcin at 72 h. (C) Osthole enhanced the production of osteopontin at 72 h. (D) Osthole enhanced the type I collagen synthesis at 72 h. For (A), cells were treated with various concentrations of osthole for 48 h. ALP activity was assessed by the conversion of PNPP in 0.1 M  $\text{NaHCO}_3$ - $\text{Na}_2\text{CO}_3$  buffer, pH 10, containing 2mM  $\text{MgSO}_4$  and 0.1 % Triton. For (B) and (C), cells were treated with various concentrations of osthole for 72 h. The amount of osteocalcin and osteopontin in culture medium was assessed by osteocalcin and osteopontin ELISA kit. For (D), the levels of Type I collagen were assessed by Prolagen-C kit. Each value is the mean  $\pm$  SD of three independent experiments. The asterisk indicates a significant difference between control and osthole-treated groups, as analyzed by Dunnett's test ( $p < 0.05$ ).

**Fig. 3.** The role of BMP-2 in osthole-mediated cell differentiation in MG-63 and hFOB cells. Osthole increased the production of BMP-2 in a time-dependent (A) and

dose-dependent manner (B). The treatment of purified BMP-2 increased ALP activity, osteocalcin, osteopontin, and type I collagen production in MG-63 and hFOB cells (C). Addition of purified BMP-2 protein did not increase the effect of osthole on ALP activity (D) or osteocalcin upregulation (E). The effect of BMP-2 antagonist noggin on osthole-induced ALP activity (F) and osteocalcin production (G). For (A) and (B), cells were treated with various concentrations of osthole for the indicated times. The production of BMP-2 in culture medium was assessed by BMP-2 ELISA kit. For ALP activity and osteocalcin, cells were treated with or without 10  $\mu$ M osthole, 100 ng/ml BMP-2 or osthole plus BMP-2 for 48 h and 72 h, respectively. For blocking assay, cells were pretreated with or without noggin for 1 h, and then added 10  $\mu$ M osthole or 100 ng/ml BMP-2 for the indicated times. ALP activity and osteocalcin levels were determined as described above. Each value is the mean  $\pm$  SD of three independent experiments. The asterisk indicates a significant difference between control and test groups, as analyzed by Dunnett's test ( $p < 0.05$ ).

**Fig. 4.** The activation of SMADs, p38, and ERKs in osthole and BMP-2-treated MG-63 and hFOB cells. Osthole increased the SMAD1/5/8 (A), p38 and ERKs activation (B) as well as their activity determined by *in vitro* kinase assay (C). BMP-2 increased the SMAD1/5/8 (D), p38 and ERKs activation (E) as well as their activity (F). For unphospho- and phospho-SMAD and MAPKs, cells were treated with 10  $\mu$ M

osthole or 100 ng/ml BMP-2 for the indicated times; the levels of SMAD 1/5/8, p38, ERK1/2, and their phosphorylated form proteins were determined by Western blotting analysis. For (C) and (F), p38 and ERK1/2 kinase activity was determined using a p38 and ERKs activity kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer's instructions. Data shown are representative of three independent experiments.

**Fig. 5.** The relation of p38, ERK1/2, and BMP-2 in osthole-treated MG-63 and hFOB cells. (A) Noggin inhibited the effects of BMP-2 on the activation of SMADs, p38, and ERKs. (B) Noggin inhibited the effects of osthole on the activation of SMADs, p38, and ERKs. Cells were incubated for 1 h in the presence or absence of noggin, then 10  $\mu$ M osthole or 100 ng/ml BMP-2 was added and incubated for specific times. The activation of p38 and ERK1/2 were measured as described in the legend to Fig. 4. Data shown are representative of three independent experiments.

**Fig. 6.** The effect of SB203580 and PD98059 in osthole-treated MG-63 and hFOB cells. (A) SB203580 and PD98059 inhibited osthole and BMP-2-induced p38 and ERK1/2 activation. The effects of SB203580 and PD98059 on osthole and BMP-2-induced ALP activity (B) and osteocalcin production (C). Cells were incubated for 1 h in the presence or absence of SB203590 or PD98059, then 10  $\mu$ M osthole or 100 ng/ml BMP-2 was added and incubated for specific times. The

activation of p38 and ERK1/2 was measured as described in the legend to Figure 4. Data shown are representative of three independent experiments. The asterisk indicates a significant difference between control and test groups, as analyzed by Dunnett's test ( $p < 0.05$ ).

**Fig. 7.** The effects of genetic suppression of p38 and ERK1/2 on osthole and BMP-2-induced cell differentiation. (A) p38 and ERK2 siRNA inhibited expression of p38 and ERK1/2 in osthole and BMP-2 treated cells. ALP activity (B, D) and osteocalcin production (C, E) was inhibited by p38 siRNA and ERK2 siRNA expression. Cells were transfected with control oligonucleotide, p38 or ERK2 siRNA by lipofectamine 2000 agents, then treated with osthole or BMP-2 for the indicated times. The activation of p38 and ERK1/2 was measured as described in the legend to Figure 4. Data shown are representative of three independent experiments. The asterisk indicates a significant difference between control and test groups, as analyzed by Dunnett's test ( $p < 0.05$ ). siRNA(-), the control transfection.

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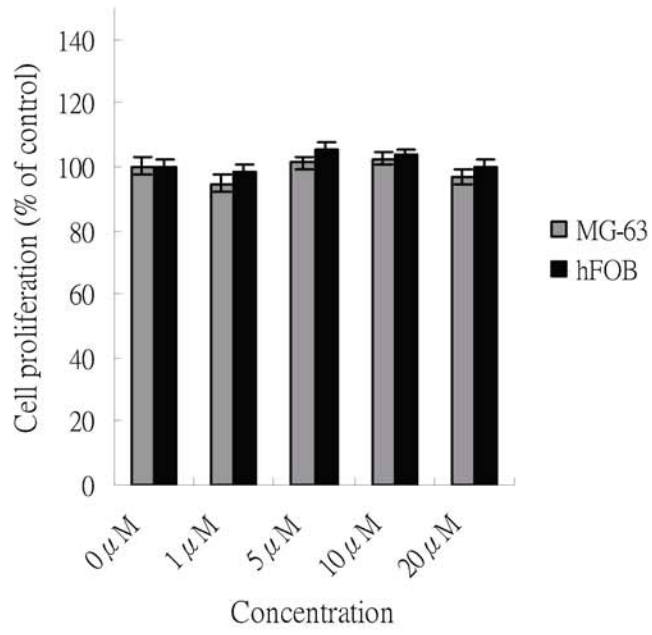
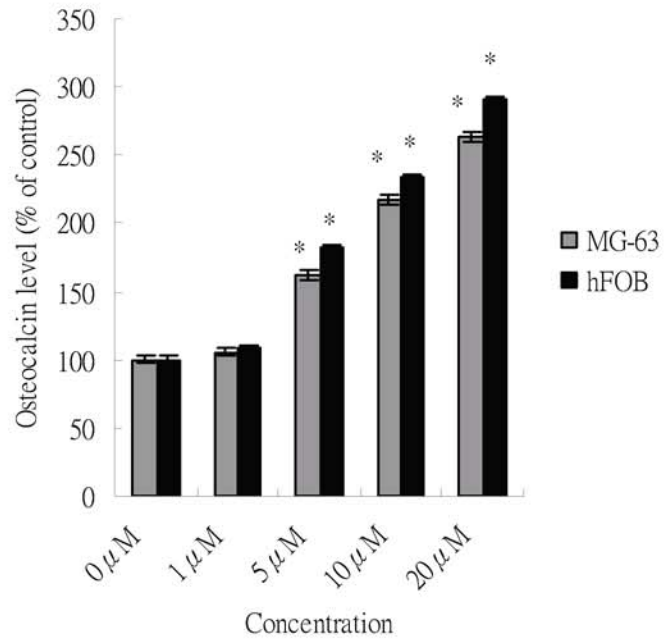
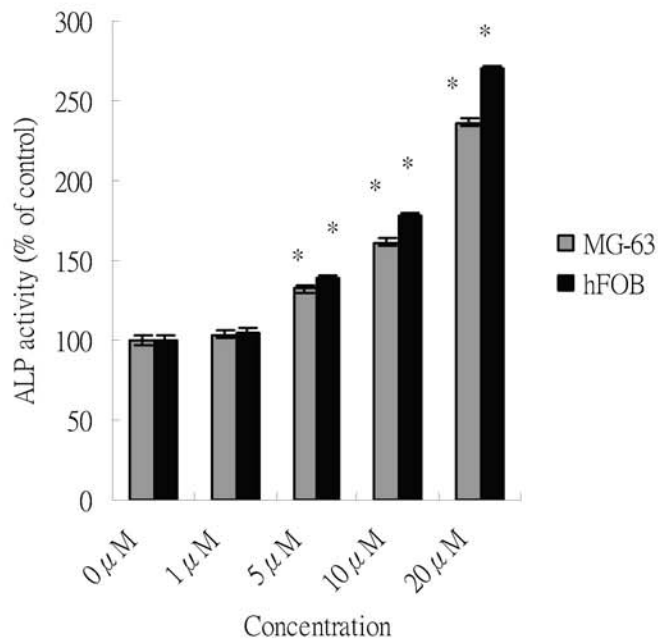
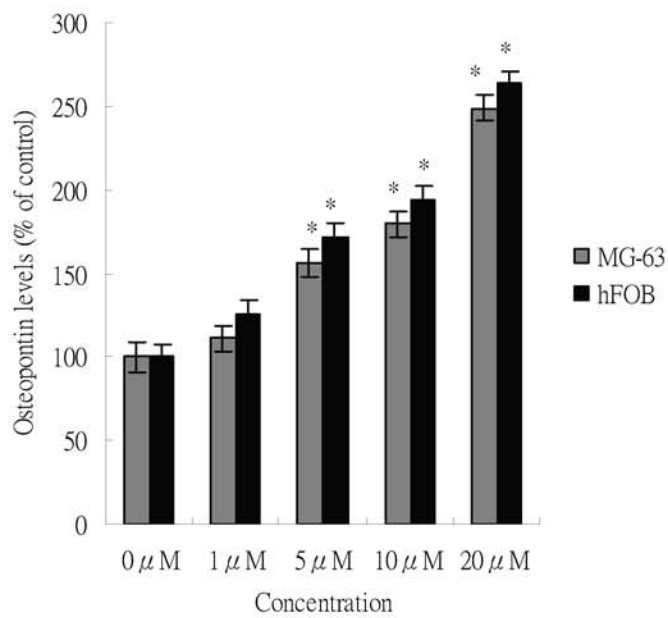


Figure 1.

A.



C.



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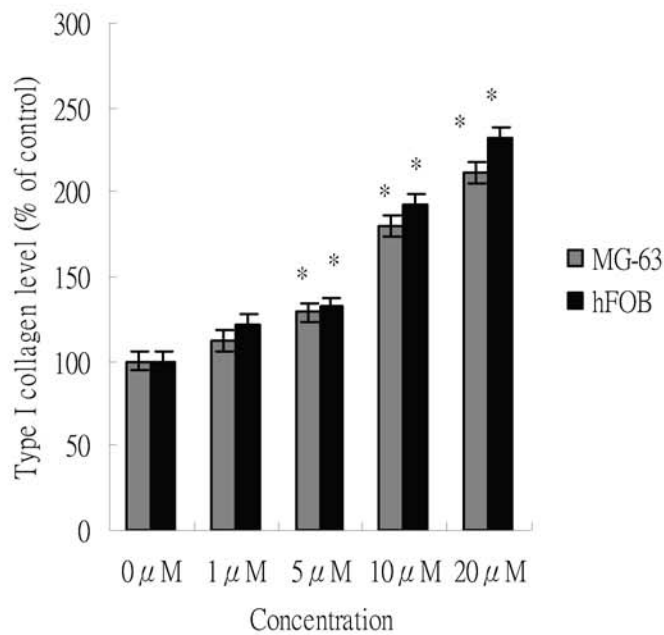
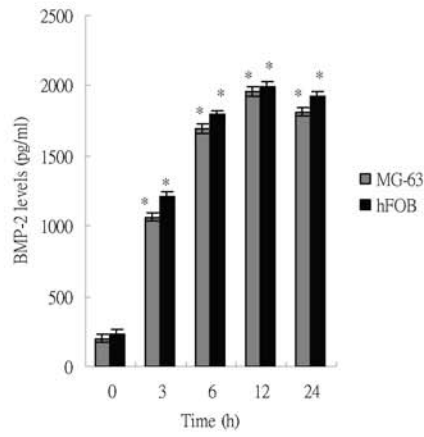
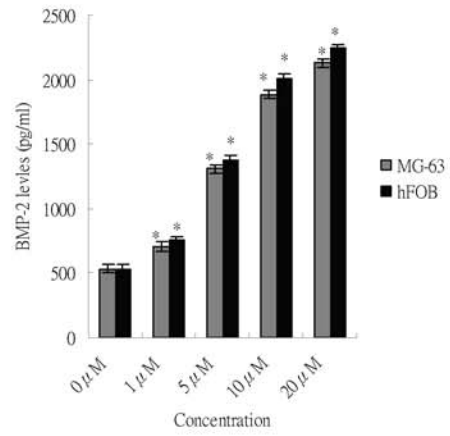


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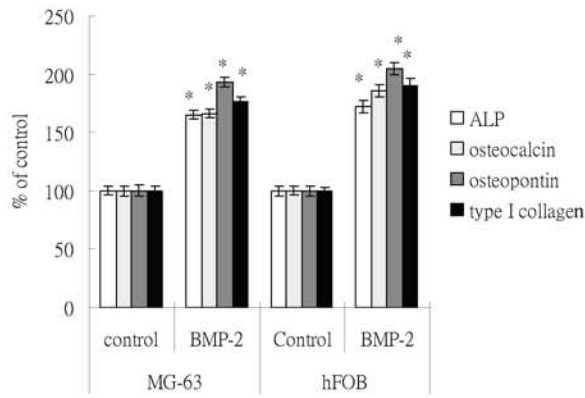
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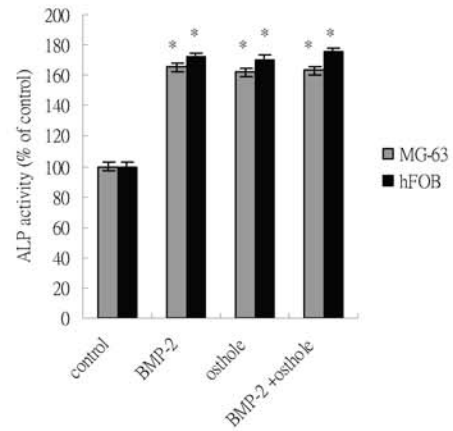
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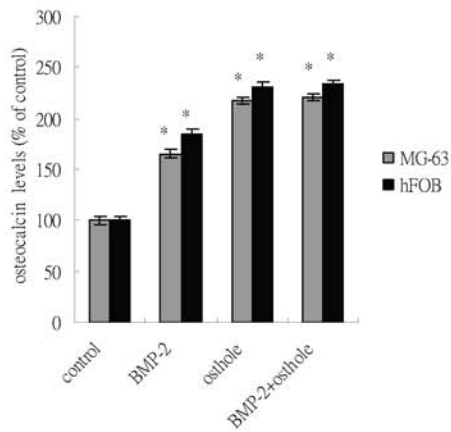
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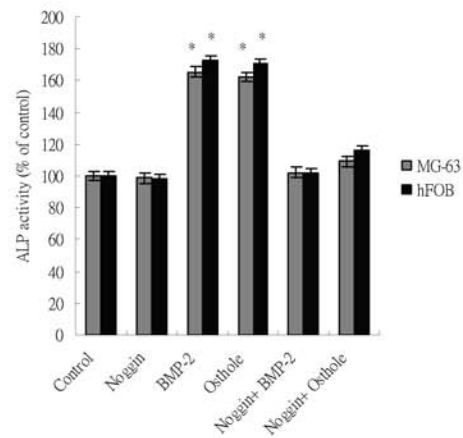
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F.



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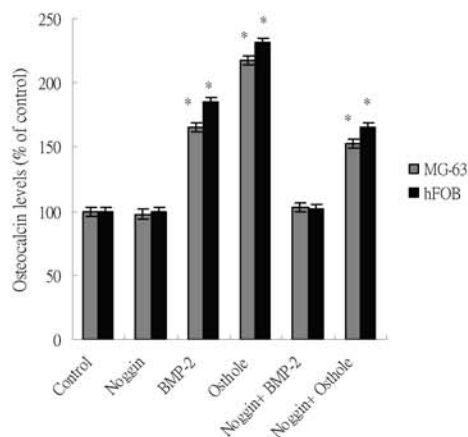
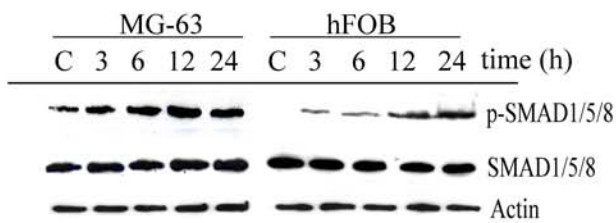


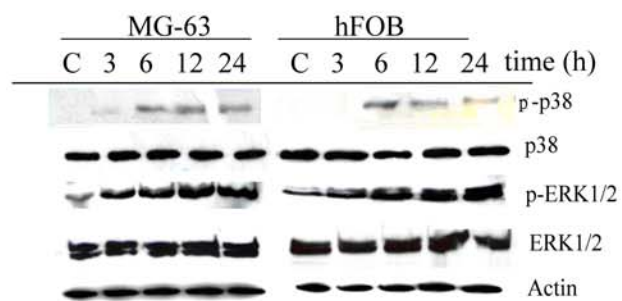
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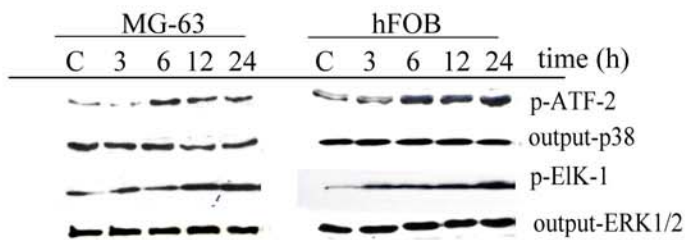
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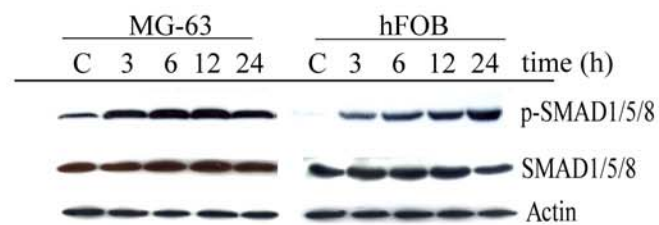
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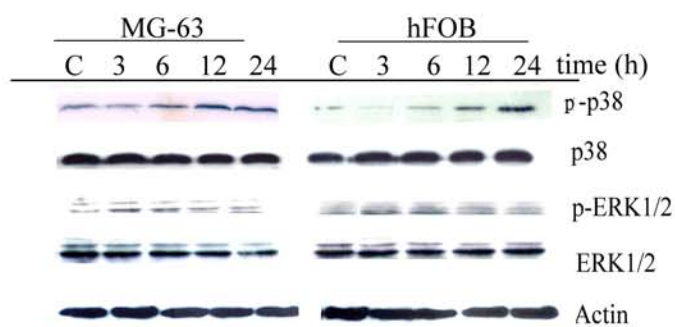
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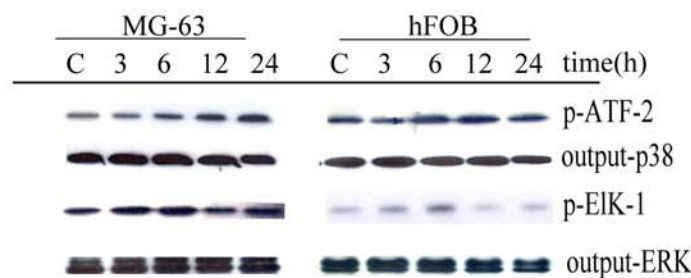
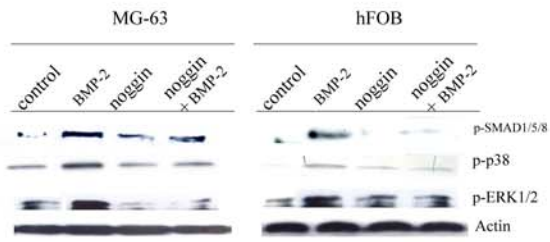


Figure 4.

A.



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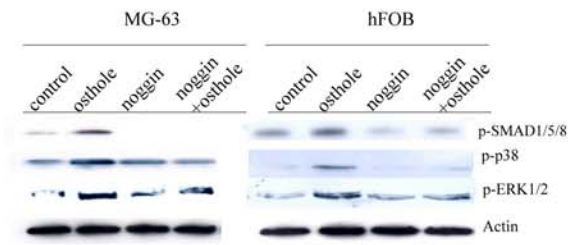
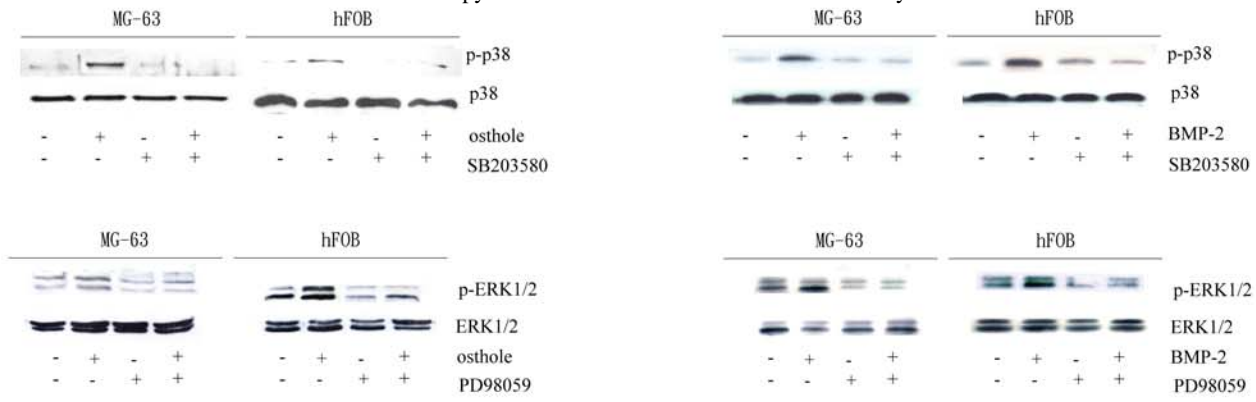
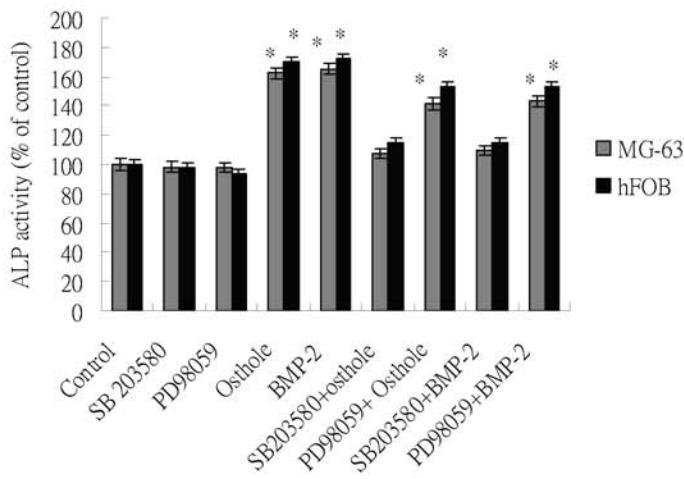


Figure 5.

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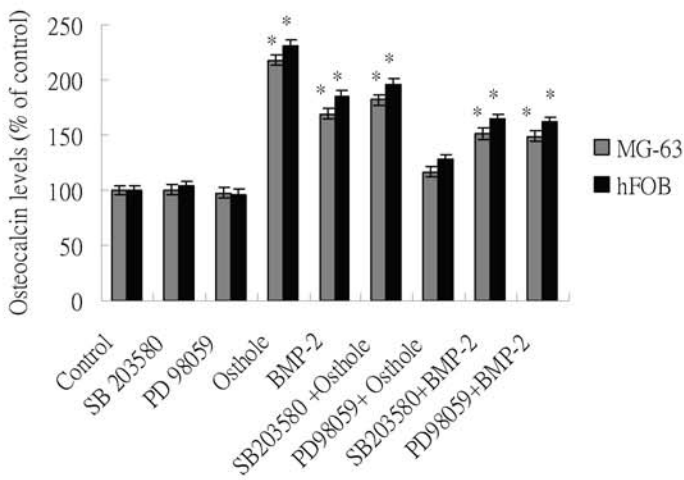
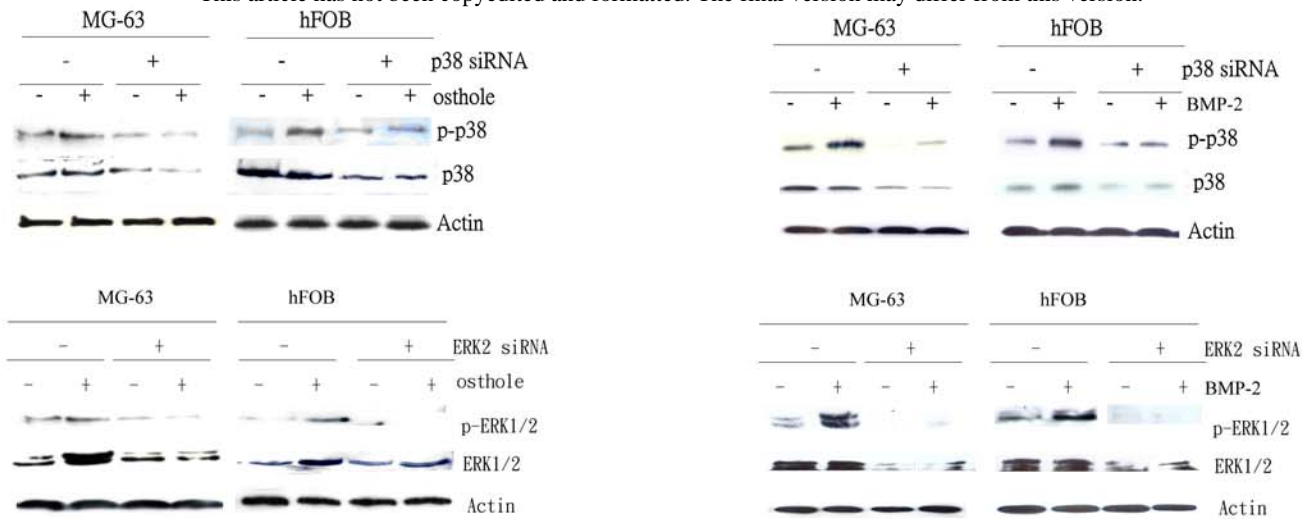


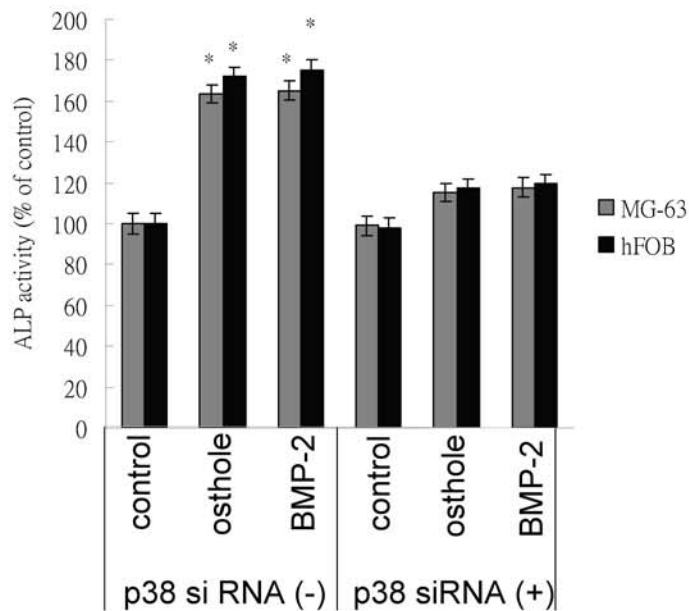
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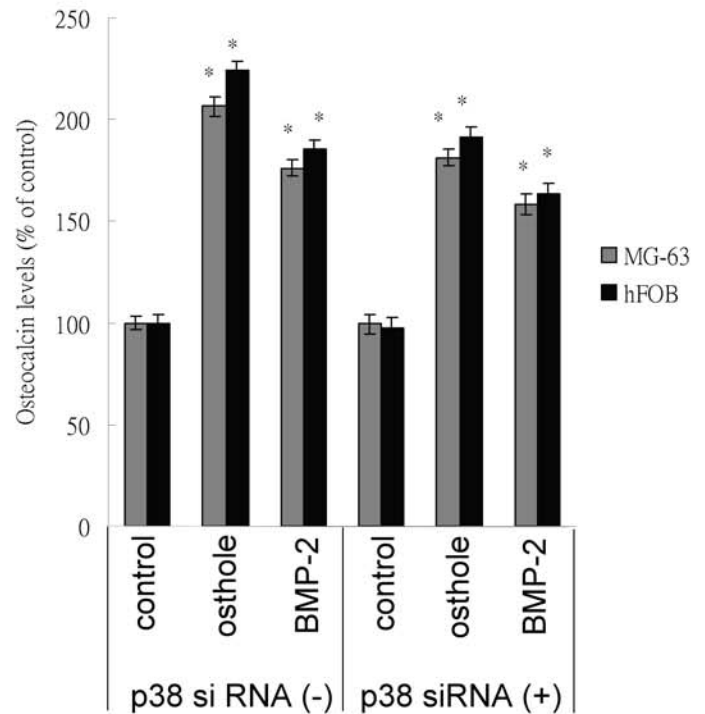
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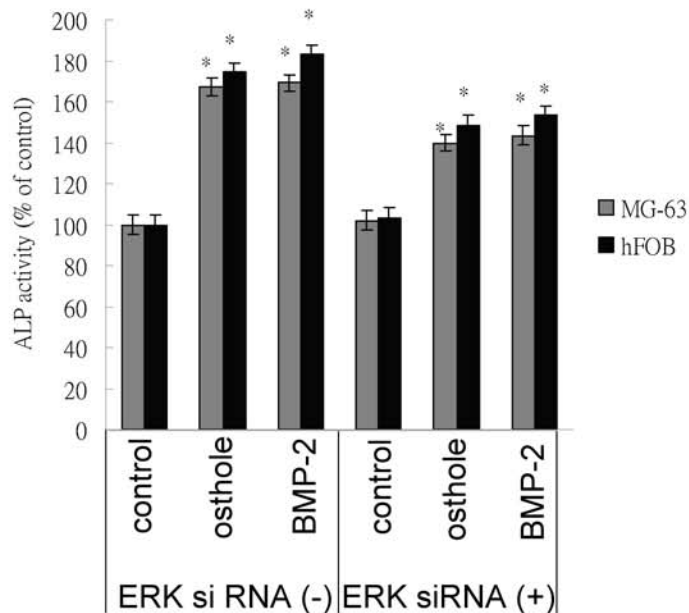
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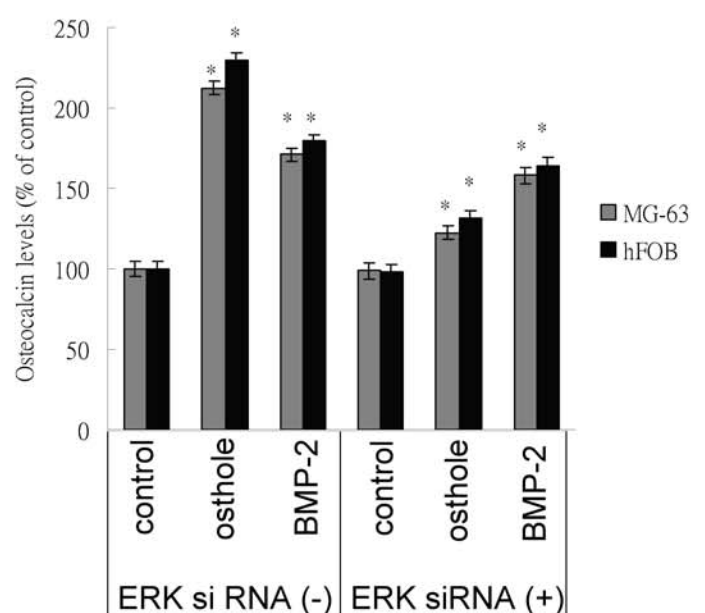


Figure 7.