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Diosmetin Induces Human Osteoblastic Differentiation Through the Protein Kinase C/p38 and Extracellular Signal-Regulated Kinase 1/2 Pathway

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ABSTRACT:

Introduction: The survival of osteoblasts is one of the determinants of the development of osteoporosis. This study is the first to investigate the osteoblastic differentiation induced by diosmetin, a flavonoid derivative, in osteoblastic cell lines MG-63, hFOB, and MC3T3-E1 and bone marrow stroma cell line M2-10B4.
Materials and Methods: Osteoblastic differentiation was determined by assaying alkaline phosphatase (ALP) activity and mineralization degree and measuring various osteoblast-related markers using ELISA. Expression and phosphorylation of Runt-related transcription factor 2 (Runx2), protein kinase Cδ (PKCδ), extracellular signal-regulated kinase (ERK), p38, and c-*jun*-N-terminal kinase (JNK) was assessed by immunoblot. Rac1 activity was determined by immunoprecipitation, and Runx2 activity was assessed by EMSA. Genetic inhibition was performed by small hairpin RNA plasmids or small interfering RNA (siRNA) transfection.
Results: Diosmetin exhibited an effect on osteoblastic maturation and differentiation by means of ALP activity, osteocalcin, osteopontin, and type I collagen production, as well as Runx2 upregulation. Induction of differentiation by diosmetin was associated with increased PKCδ phosphorylation and the activations of Rac1 and p38 and ERK1/2 kinases. Blocking PKCδ by siRNA inhibition significantly decreased osteoblastic differentiation by inhibiting Rac1 activation and subsequently attenuating the phosphorylation of p38 and ERK1/2. In addition, blocking p38 and ERK1/2 by siRNA transfection also suppressed diosmetin-induced cell differentiation.
Conclusions: In this study, we show that diosmetin induced osteoblastic differentiation through the PKCδ-Rac1-MEK3/6-p38 and PKCδ-Rac1-MEK1/2- ERK1/2-Runx2 pathways and that it is a promising agent for treating osteoporosis.
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Key words: diosmetin, differentiation, osteoblast, protein kinase C, mitogen-activated protein kinase

INTRODUCTION

THE HALLMARK OF OSTEOPOROSIS is a reduction in skeletal mass caused by an imbalance between bone resorption and bone formation, whereas bone homeostasis requires balanced interactions between the osteoblasts and osteoclasts.[1–3] Osteoporosis is estimated to affect >200 million people worldwide, causing morbidity and mortality in the aged population.[1,4] Drugs currently used to treat osteoporosis include bisphosphonates, calcitonin, estrogen, vitamin D analogs, and ipriflavone. These are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of the osteoclasts.[5] The effectiveness of these drugs in increasing or recovering bone mass, however, is relatively small, and certainly no more than 2% per year.[5] It is desirable, therefore, to have satisfactory bone-building (anabolic) agents, such as teriparatide, that would stimulate new bone formation and correct the trabecular microarchitecture imbalance characteristic of established osteoporosis.[2,6] Because new bone formation is primarily a function of the osteoblast, agents that regulate bone formation act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts.[2,7]

The mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases, are mediators of intracellular signals in response to various stimuli.[8,9] JNK (c-*jun* NH_2-terminal protein kinase), p38, and ERK1/2 (extracellular signal–regulated kinase) are the three main members of three different MAPK pathways that can be activated by growth factors, DNA damage, cytokines, oxidant stresses, UV light, anti-cancer drugs, and osmotic shock.[8–12] Evidence is accumulating to indicate that MAPK signaling induces osteoblastic differentiation. Activated ERK1/2 has been reported to be responsible for osteoblastic differentiation and skeletal development through Runx2 phosphorylation.[13,14] Similar to ERK1/2, activation of p38 has also

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been reported to be involved in osteoblastic differentiation by regulating the expression of osterix.[4,15,16] However, the precise mechanism of p38 and ERK1/2 MAPK-mediated osteoblastic differentiation still remains unknown.

Members of the protein kinase C (PKC) family are activated by various stimuli and involved in regulation of many cellular physiological functions, such as growth, cell death, and differentiation.[17,18] The novel PKC isoform, PKC delta (PKCδ), has been associated with osteoblastic differentiation induced by fibroblast growth factors and PTH.[19–21] Similar to other PKCs, activation of PKCδ induces its translocation from the cytoplasm to the cellular membrane and increases its serine/threonine kinase activity.[17] The activity of PKCδ is also regulated by tyrosine phosphorylation, dependent on phosphorylation sites and cell types.[22,23] Once activated, PKCδ can induce cell differentiation by activating several downstream signaling proteins, including all three MAPK cascades and Runx2.[19,20]

Flavonoids represent a large class of phenolic compounds present in fruit and vegetables. Flavonoids have been shown to influence a variety of biological functions, including induction of osteoblastic differentiation.[16,24,25] Previous studies have shown that diosmetin possesses CYP1A1 and CYP1B1 inhibition, as well as anti-*Helicobacter pylori*, anti-inflammatory, and anti-allergic effects.[26–28] The aim of this study was to evaluate the ability of diosmetin to induce osteoblastic differentiation. In this study, we showed that PKCδ-Rac1-MEK3/6-p38 and PKCδ-Rac1-MEK1/2-ERK1/2-Runx2 play important roles in diosmetin-mediated osteoblastic maturation and differentiation.

MATERIALS AND METHODS

Materials

FBS, MEM, αMEM, phenol-free DMEM/Ham's F12, penicillin G, streptomycin, and amphotericin B were obtained from GIBCO BRL (Gaithersburg, MD, USA). Dimethyl sulfoxide (DMSO) and trypsin-EDTA were purchased from Sigma Chemical (St Louis, MO, USA). XTT was obtained from Roche Diagnostics. PKCδ, ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, Rac1, MEK1/2, phospho-MEK1/2 antibodies and JNK and p38 activity assay kits were obtained from Cell Signaling Technology (Beverly, MA, USA). MEK3/6, phospho-MEK3/6, and Runx2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PKCδ siRNA plasmid, PAK agarose, ERK2, and p38 siRNA were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-tyrosine antibodies were purchased from Calbiochem (Cambridge, MA, USA). Lipofectamine 2000 reagent was obtained from Life Technologies (Rockville, MD, USA).

Cell cultures

The human osteoblast-like cell line MG-63 (CRL-1427), hFOB (CRL-11372), and mouse bone marrow stromal cell M2-10B4 (CRL-1972) were purchased from American Type Culture Collection (ATCC). MG-63 cells were cultured in MEM supplemented with 10% FBS and antibiotics (100 IU/ml of penicillin G and 100 μg/ml of streptomycin). M2-10B4 cells were maintained in RPMI 1640 with 10% FBS, supplemented with 1 mM sodium pyruvate and antibiotics. MC3T3-E1 mouse calvaria osteoblasts were kindly provided by Dr Renny T Franceschi (University of Michigan, Ann Arbor, MI, USA) and grown in αMEM and antibiotics. The conditionally immortalized human fetal osteoblastic cell line, hFOB, was maintained in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (GIBCO-BRL) 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 with hFOB cells were carried out at the permissive temperature of 33.5°C.

Cell proliferation assay (XTT)

Inhibition of cell proliferation by diosmetin was measured by XTT assay. Briefly, cells were plated in 96-well culture plates (8×10^3 cells/well). After 24 h of incubation, the cells were treated with vehicle (0.1% DMSO) or diosmetin (1, 5, 10, and 20 μM) for 48 h. Fifty microliters of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 μl of electron coupling reagent, was 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 activity

Cells were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured for 24 h. The agent to be tested was added to the wells, and incubation continued for 4 days. The cells were washed three times with physiological saline, and cellular protein concentration was determined by incubation for 1 h at 37°C in bicinchoninic acid (BCA) protein assay reagent containing 0.1% Triton X-100. The reaction was arrested by adding 1 M NaOH, and absorbance was measured at 560 nm.

Alkaline phosphatase (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 measured by incubation for 1 h at 37°C in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 0.1% Triton X-100, 2 mM $MgSO_4$, and 6 mM PNPP (4-nitrophenyl phosphate). The reaction was stopped by adding 1 M NaOH, and absorbance was measured at 405 nm. The percentages of changes of ALP activity with respect to the values found in the control were calculated according to this formula: M = value of absorbance at 405 nm/value of absorbance at 560 nm. Percentage of change = [(M of the test – M of the control)/M of the control] × 100.

Assaying the levels of osteocalcin and osteopontin

Osteocalcin and osteopontin kits were used to detect osteocalcin and osteopontin, respectively. Briefly, cells were treated with various concentrations of diosmetin for 5 days. The culture medium was collected and measured for osteocalcin and osteopontin. These samples were placed in 96-well microtiter plates coated with monoclonal detective an-

tibodies and incubated for 2 h at room temperature. After removing unbound material 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 proportionate 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 level compared with the untreated control.

Assaying the levels of type I procollagen

Cells were treated with various concentrations of diosmetin for 5 days. Type I procollagen assay, which measures the propeptide portion of the molecule and reflects the synthesis of the mature form of the protein, was carried out using Prolagen-C kit (for human cell lines) and Mouse IgG Anti-Type I Collagen ELISA Kit (for MC3T3-E1), in accordance with 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 of mineralization

The degree of mineralization was determined in the 12-well plates using Alizarin red S staining (Sigma Chemical) after 5 days of treatment. Briefly, cells were fixed with ice-cold 70% (vol/vol) ethanol for 1 h and stained with 40 mM Alizarin red S in deionized water (pH = 4.2) for 10 min at room temperature. After removing Alizarin red S solution by aspiration, cells were incubated in PBS for 15 min at room temperature on an orbital rotator and rinsed once with fresh PBS. The cells were subsequently destained for 15 min with 10% (wt/vol) cetylpyridinium chloride in 10 mM sodium phosphate (pH = 7.0). The extracted stain was transferred to a 96-well plate, and absorbance at 562 nm was measured using an ELISA reader (Multiskan EX; Labsystems). The concentration of Alizarin red S staining in the samples was determined by comparing the absorbance values with those obtained from Alizarin red S standards. Mineralization values were normalized to the relative number of viable cells, as determined directly in the 96-well plates using the XTT assay.[29]

EMSA

Nuclear extract was prepared, using the Nuclear Extract kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's specifications. EMSA was performed by using lightshift chemiluminescent EMSA kit (Pierce, Rockford, IL, USA), also following the manufacturer's protocol. Five micrograms of nuclear protein and 50 fmol 3′-biotin–labeled wildtype OSE2 (5′-CCCGTATTAACCACAATAAAACTCG-3′ for human; 5′-GATCCGCTGCAATCACCAACCACAGCA-3′ for mouse) or cold DNA probes (Geneka Biotechnology) were mixed in the binding reagent containing 1× binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol [pH 7.5], 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/ml poly [dI-dC], and 0.05% NP-40). After incubation for 30 min, the reaction mixture was subjected to gel electrophoresis on 8% native polyacrylamide gel and transferred to a nylon membrane. Biotin end-labeled DNA was detected using streptavidin–horseradish peroxidase conjugate and a chemiluminescence blotting detection system.[30,31]

Immunoprecipitation/immunoblot, and ERK1/2, p38, and Runx2 activity assays

Cells were treated with 20 μM diosmetin for the indicated times. For immunoblot, the cells were lysed on ice for 40 min in a solution containing 50 mM Tris, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EGTA, 12 mM β-glycerophosphate, 10 mM NaF, 16 μg/ml benzamidine hydrochloride, 10 μg/ml phenanthrolene, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsufonyl fluoride. Cell lysate was centrifuged at 14,000*g* for 15 min, and the supernatant fraction was collected for immunoblot. Equivalent amounts of protein were resolved by SDS-PAGE (10–12%) and transferred to PVDF membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1–16 h. The membrane was treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham) according to the manufacturer's instructions.

For analysis of Runx2 activity, the nuclear extracts were incubated with anti-Runx2 antibody, after which the immune complexes were precipitated with protein A-agarose beads (Sigma Chemical). Immunocomplexes were subjected to SDS-PAGE and immunoblot. The total amount of Runx2 was detected by anti-Runx2 antibody (Santa Cruz Biotechnology) and phosphorylated by a specific mouse anti-phosphotyrosine antibody (Oncogene, Boston, MA, USA), followed by horseradish peroxidase–conjugated IgG as the second antibody. ERK1/2 and p38 activities were determined using kits from Cell Signaling Technology according to the manufacturer's instructions.

PKC activity assay

PKC activity was assessed by PKC Kinase Activity Assay Kit according to the manufacturer's instructions (Assay DesignsMI). PKC activity kit is based on a solid-phase ELISA that uses a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate.

siRNA knockdown of ERK and p38 expression

Monolayer cells were transfected with PKCδ siRNA expression plasmid pKD-PKCδ-v3 or pKD-NegCon-v3 (Upstate Biotechnology), ON-TARGET smart pool siRNA, and ON-TARGET *plus* si*CONTROL* Nontargeting Pool (Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000 (Invitrogen). For p38 and ERK1/2 inhibition, cells were transfected with ON-TARGET smart pool MAPK1 (ERK2) and p38 siRNA duplexes or nonspecific control siRNA duplexes (Dharmacon). Immunoblot analyses

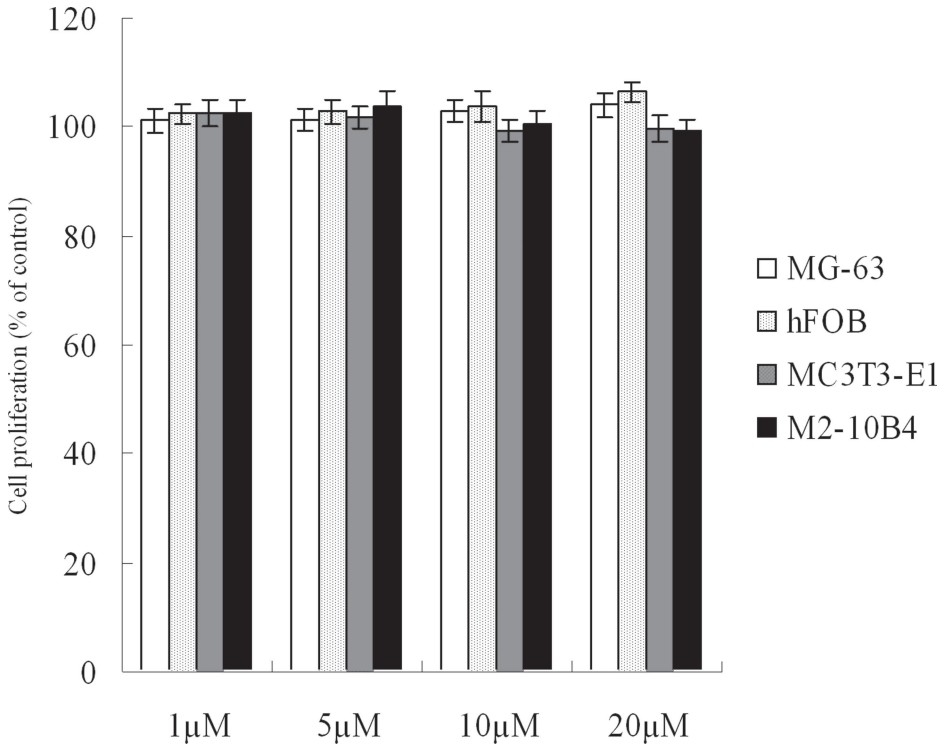

FIG. 1. The effect of diosmetin on the proliferation of MG-63, hFOB, MC3T3-E1, and M2-10B4 cells. Adherent cells that proliferated in 96-well plates (8×10^3 cells/well) were incubated with different concentrations (μM) of diosmetin for 48 h. Cell proliferation was determined by XTT assay. Each value is the mean ± SD of three independent experiments.

showed that expression of PKCδ, ERK1/2, and p38 remained low but detectable, whereas expression of β-actin was unaffected by siRNA treatment.

Statistical analysis

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

RESULTS

Diosmetin did not affect the proliferation of osteoblasts and bone marrow stromal cells

We first determined the effect of diosmetin on the proliferation of MG-63, hFOB, and MC3T3-E1 osteoblasts and bone marrow stromal cell M2-10B4 by XTT assay. As shown in Fig. 1, after 48 h of treatment in all cell lines, diosmetin did not exhibit significant effects on cell growth at the concentrations used (1–20 μM).

Effect of diosmetin on maturation and differentiation markers, ALP activity, collagen synthesis, osteopontin, osteocalcin expression, and mineralization in osteoblasts and bone marrow stromal cells

The effects of diosmetin on the differentiation of osteoblasts and bone marrow stromal cells were studied by determining ALP activity, collagen synthesis, osteopontin, osteocalcin production, and mineralization. The results showed that diosmetin increased ALP activity in a dose-dependent manner (4-day treatment) in all cell lines (Fig. 2A).

The effect of diosmetin on the terminal differentiation of osteoblasts and bone marrow stromal cells was also assessed by determining the production of osteocalcin, osteopontin, collagen synthesis, and the degree of mineralization. As shown in Figs. 2B and 2C, treatment of all cell lines with diosmetin increased the levels of osteocalcin and osteopontin in a dose-dependent manner after 5 days and in a time-dependent manner at a concentration of 20 μM. Type I collagen protein levels were also enhanced in diosmetin-treated MG-63, hFOB, and MC3T3-E1 cells in a dose-dependent manner after 5 days of treatment and a time-dependent manner at a concentration 20 μM (Fig. 2D). In contrast, M2-10B4 lacked type I collagen expression in both vehicle and diosmetin treatments (data not shown). In addition, treatment with diosmetin also increased the amount of osteoblastic mineralization in a dose- and time-dependent manner (Fig. 2E).

Diosmetin increased the expression and phosphorylation of Runx2 in MG-63, hFOB, and MC3T3-E1 cells

We next assessed the effect of diosmetin on the expression of osteoblast-specific transcription factor Runx2. As shown in Fig. 3A, the total amount of Runx2 was increased in diosmetin-treated MG-63, hFOB, and MC3T3-E1 cells. Furthermore, Runx2 protein levels in the nuclei were also enhanced after 24 h of diosmetin treatment. Runx2 activation was examined by immunoprecipitation, as previously described.[14] Our findings showed that Runx2 phosphorylation was dramatically increased by diosmetin (Fig. 3B).

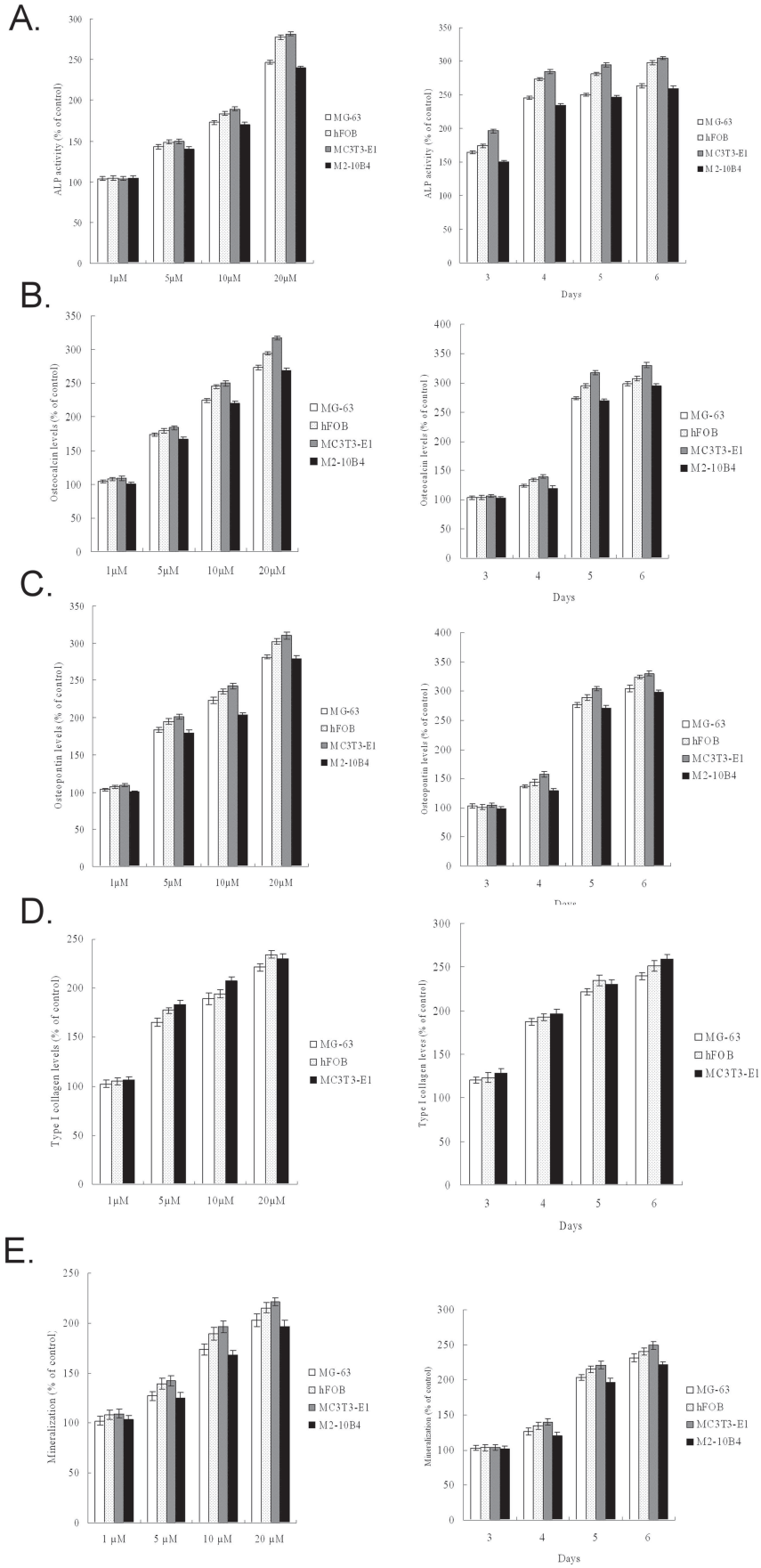

FIG. 2. Diosmetin increased the osteoblastic activity in MG-63, hFOB, MC3T3-E1, and M2-10B4 cells. Diosmetin increased ALP activity (A), osteocalcin production (B), osteopontin synthesis (C), type I collagen synthesis (D), and mineralization (E). Cells were treated with different concentrations (μM) of diosmetin for 4 (ALP assay) or 5 days (osteocalcin, osteopontin, type I collagen synthesis, and mineralization assay) or treated with 20 μM diosmetin for the indicated times. ALP activity was assessed by the conversion of *p*-nitrophenyl phosphate in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 2 mM $MgSO_4$ and 0.1% Triton. The amount of osteocalcin and osteopontin in culture medium was assessed by osteocalcin and osteopontin ELISA kit. Production of type I collagen was assayed by Prolagen-C and Mouse IgG Anti-Type I Collagen ELISA Kit, and the degree of mineralization was assayed by Alizarin red S staining. Each value is the mean ± SD of three independent experiments. Data were calculated as the percentage of control by the following formula: inhibition % = ODt/ODs × 100. ODt and ODs indicated, respectively, the optical density of the test substances and the solvent control at the same time.

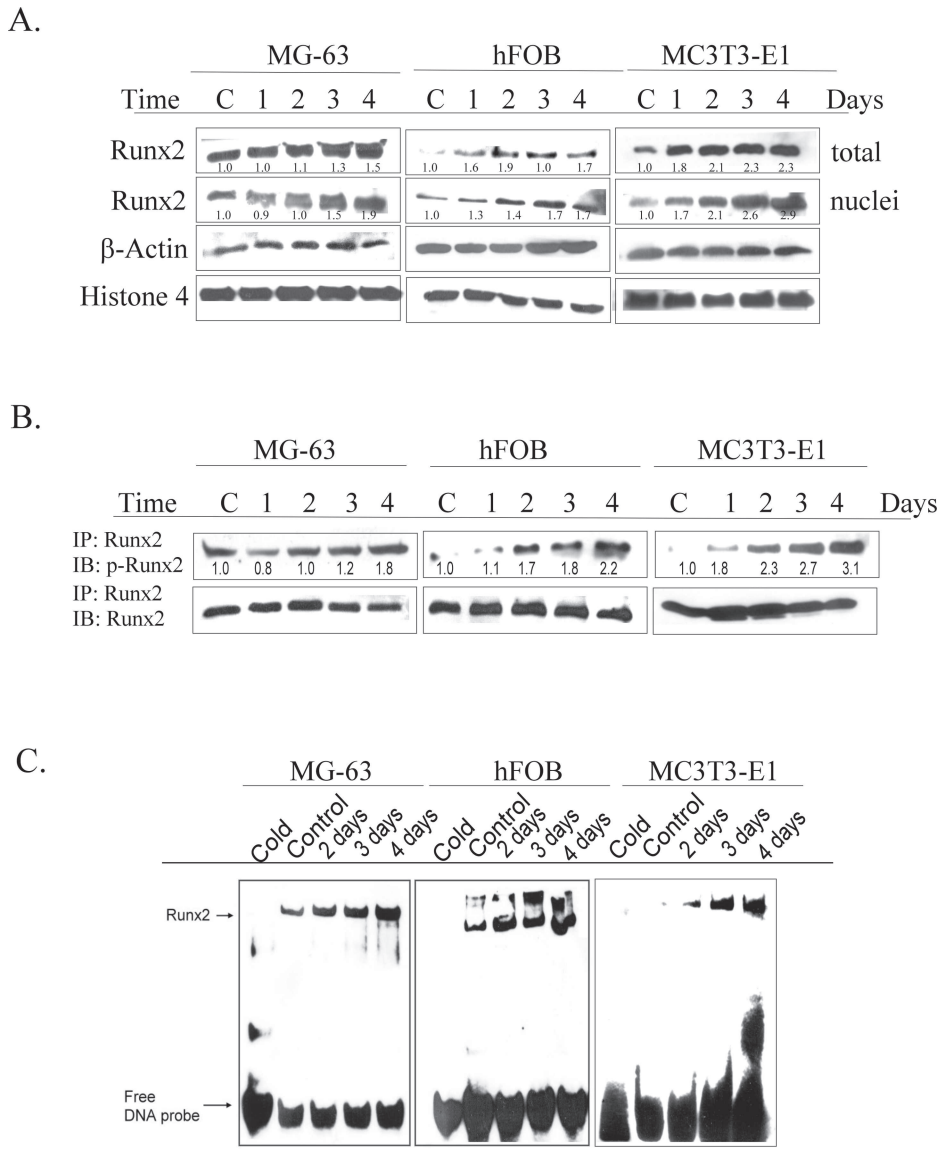

FIG. 3. The effect of diosmetin on Runx2 expression and activity in MG-63, hFOB, and MC3T3-E1 cells. (A) The amount of Runx2. (B) The phosphorylation of Runx2 in nuclei. (C) The DNA binding activity of Runx2 after diosmetin treatment. Extractions of cytoplasm and nuclei were separated from cell pellets by lysis buffer and centrifugation. Immunoblot analysis was used to assess protein expression. The activation of Runx2 was equal to the degree of phosphorylation of Runx2 immune complexes. The DNA binding activity of Runx2 was assessed by EMSA. Data shown are representative of three independent experiments.

Moreover, the DNA binding activity of Runx2 was also shown by EMSA in diosmetin-treated MG-63, hFOB, and MC3T3-E1 cells (Fig. 3C).

Effect of diosmetin on the MAPK pathway

Because MAPK has been shown to be involved in the phosphorylation of Runx2 and osteoblastic differentiation, we studied the role of MAPKs in diosmetin-treated cells. The results showed that exposure of all cell lines of osteoblasts to 20 μM diosmetin resulted in a sustained activation of p38 and ERK1/2. Activation (phosphorylation) of p38 and ERK1/2 was determined after 2 days of treatment and persisted for the duration of the experiment (Fig. 4A). On the other hand, the expression of p38 and ERK1/2 (unphosphorylated form) was not altered by diosmetin treatment. Also, diosmetin failed to affect JNK activation in MG-63, hFOB, or MC3T3-E1 cells at any of the examined points in time (Fig. 4A). Diosmetin-mediated activation of p38 and ERK1/2 was additionally confirmed by determining phosphorylation of their substrates (ATF-2 and Elk-1 for p38 and ERK1/2, respectively). As shown in Fig. 4B, in contrast with the control, phosphorylation of ATF-2 at Thr71 increased after a 2-day exposure of MG-63, hFOB, and MC3T3-E1 cells to 20 μM diosmetin (Fig. 4B). Similarly, phosphorylation of Elk-1 increased in diosmetin-treated MG-63, hFOB, and MC3T3-E1 cells, in contrast to the control (Fig. 4B).

Role of p38 and ERK1/2 in diosmetin-mediated Runx2 activation and osteoblastic activation

To understand the upstream of Runx2, we assessed the roles of p38 and ERK1/2 on the activation of Runx2 by siRNA-based gene knockdown. To do so, MG-63 and hFOB cells were transfected with a pool of siRNAs targeting p38 and ERK2, after which the cells were exposed to 20 μM diosmetin. The effect of p38 and ERK2 genetic inhibi-

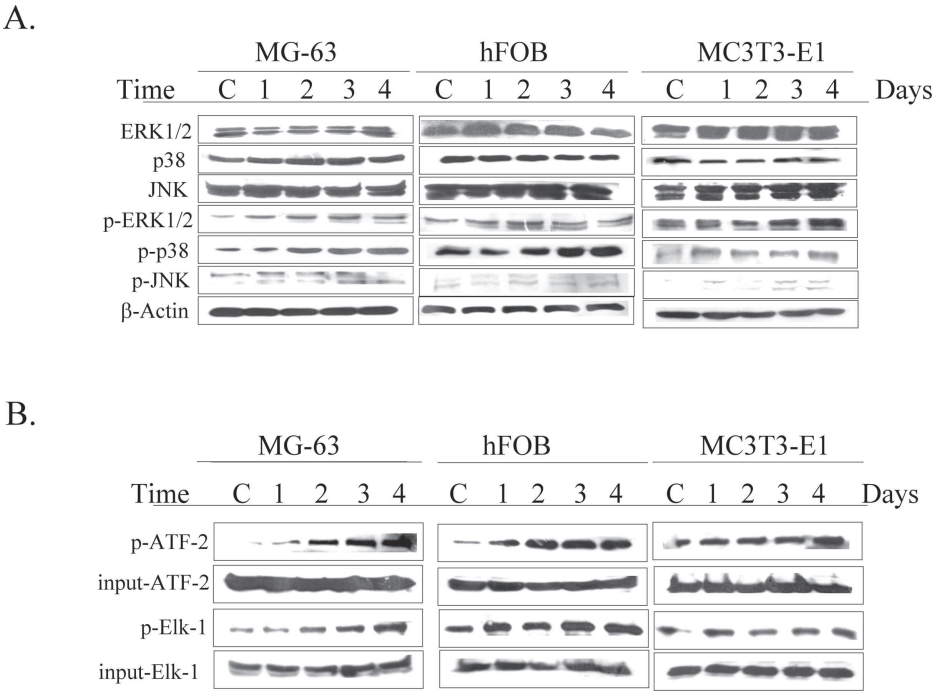

FIG. 4. Diosmetin increased the activation of p38 and ERK1/2. (A) The effect of diosmetin on the activation of MAPKs. (B) Diosmetin increased the activity of p38 and ERK1/2. Cells were treated with 20 μM diosmetin for the indicated times, and levels of JNK, p38, ERK1/2, and their phosphorylated proteins were determined by immunoblot analysis. p38 and ERK1/2 activity was assessed by p38 and ERK1/2 in vitro kinase assay kit. Data shown are representative of three independent experiments.

tion on diosmetin-induced Runx2 phosphorylation, 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 (Fig. 5A). As shown in Fig. 5B, selective genetic inhibition of ERK1/2 abrogated phosphorylation of Runx2. However, the p38-selective inhibition had no effect on Runx2 phosphorylation. To avoid the off-target effect of siRNA, we also assessed the role of p38 and ERK1/2 by using ON-TARGET smart pool p38 and ERK2 siRNA, which decrease nonspecific effects by chemical modification and pooling.[32,33] Transfection of MG-63, hFOB, and MC3T3-E1 cells with ON-TARGET smart pool p38 and ERK2 siRNA reduced basal levels of p38 and ERK1/2 (Fig. 5A). Similarly, inhibition of ERK2, but not p38, also decreased diosmetin-mediated Runx2 phosphorylation (Fig. 5B).

Specific knockdown p38 expression by p38 siRNA or ON-TARGET smart pool p38 siRNA not only inhibited ALP upregulation but also decreased osteocalcin production in diosmetin-treated cells (Figs. 5C and 5D). Similarly, blockade of ERK1/2 by ERK2 siRNA or ON-TARGET smart pool ERK2 siRNA also decreased the upregulation of ALP and osteocalcin by diosmetin (Figs. 5E and 5F). These results suggest that p38 and ERK1/2 may play key roles in diosmetin-mediated osteoblastic differentiation.

Diosmetin activates PKCδ, MEKs, and Rac1 in MG-63, hFOB, and MC3T3-E1 cell lines

Because it has been shown that PKCδ is involved in the activation of MAPK signaling, we assessed the status of PKCδ cascade signaling after diosmetin treatment. As shown in Fig. 6A, treatment of MG-63, hFOB, and MC3T3-E1 cells with diosmetin resulted in an increase of PKCδ phosphorylation (Fig. 6A). In addition, PKCδ activity was also increased by diosmetin in MG-63, hFOB, and MC3T3-E1 cell lines (Fig. 6B).

Next, we studied whether Rac1 and MEKs, the intermediate mediators of MAPK, are involved in diosmetin-mediated p38 and ERK1/2 activation. As shown in Fig. 6C, exposure of MG-63, hFOB, and MC3T3-E1 cells to diosmetin increased the phosphorylation of MEK1/2 and MEK3/6. Furthermore, treatment of cells with diosmetin dramatically increased Rac1/PAK binding activity, indicating diosmetin-induced Rac1 activation (Fig. 6D).

Role of PKCδ on diosmetin-mediated MAPK activation and cell differentiation

To confirm the central role of PKCδ as a key upstream of diosmetin-mediated p38 and ERK1/2 activation, we transfected MG-63 and hFOB cells with pKD-PKCδ-v3 plasmid, which constitutively expresses shRNAs targeting PKCδ. As shown in Fig. 7A, PKCδ siRNA reduced PKCδ expression ~85%, in comparison with control siRNA. Selective genetic inhibition of PKCδ abrogated the activity of Rac1 in diosmetin-treated cells (Fig. 7B). In addition, PKCδ inhibition also decreased phosphorylation of p38, ERK1/2, and Runx2 (Fig. 7C). To avoid the off-target effect of siRNA, we also assessed the role of PKCδ by using ON-TARGET smart pool PKCδ siRNA. Transfection of MG-63, hFOB, and MC3T3-E1 cells with ON-TARGET smart pool PKCδ siRNA reduced basal levels of PKCδ (Fig. 7A). Similarly, inhibition of PKCδ by ON-TARGET smart pool PKCδ siRNA also decreased diosmetin-mediated phosphorylation of p38, ERK1/2, and Runx2 (Figs. 7B and 7C). Furthermore, the upregulation and phosphorylation of Runx2 induced by diosmetin was attenuated by pretreatment with PKCδ specific inhibitor, rotterlin (20 μM; Fig. 7C).

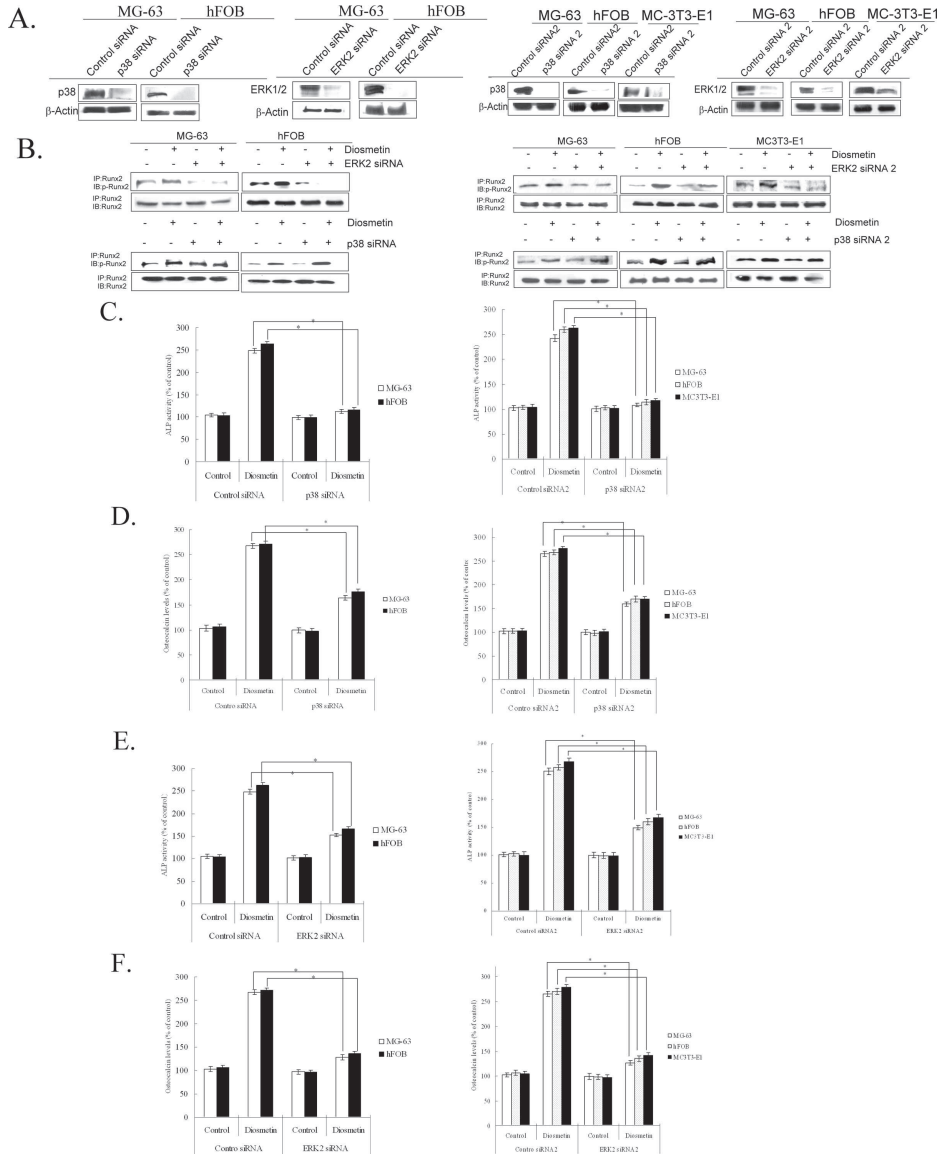

FIG. 5. The role of p38 and ERK1/2 on the upregulation of diosmetin on osteoblastic maturation and differentiation in MG-63, hFOB, and MC3T3-E1 cells. (A) The inhibition effect of p38 and ERK2 siRNA and ON-TARGET smart pool siRNA (siRNA2). (B) ERK2 inhibition decreased diosmetin-mediated Runx2 activation. ALP activity (C) and osteocalcin production (D) were inhibited by p38 siRNA and ON-TARGET smart pool p38 siRNA(siRNA2) transfection. ALP activity (E) and osteocalcin production (F) was inhibited by ERK2 siRNA and ON-TARGET smart pool ERK2 siRNA(siRNA2) transfection. Cells were transfected with control oligonucleotide, p38, or ERK2 siRNA or ON-TARGET smart pool p38 and ERK2 siRNA by lipofectamine 2000 agents and treated with diosmetin for the indicated times (4 days for ALP assay and 5 days for osteocalcin assay). Data shown are representative of three independent experiments. ALP activity (4-day treatment) and osteocalcin production (5-day treatment) were measured as described in Fig. 2. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($p < 0.05$).

Similar to PKCδ inhibition, knockdown Rac1 by dominant negative decreased diosmetin-induced p38 and ERK1/2 activation, suggesting that PKCδ is a potential upstream activator of Rac1-p38 and Rac1-ERK1/2 signaling (Figs. 7D and 7E). In addition, specific knockdown PKCδ expression by PKCδ siRNA and ON-TARGET smart pool PKCδ siRNA not only inhibited ALP upregulation, but also decreased osteocalcin production in diosmetin-treated MG-63, hFOB and MC3T3-E1 cells (Figs. 7F and 7G). These results suggest that PKCδ may play a key role in diosmetin-mediated p38 and ERK1/2 activation and osteoblastic differentiation.

DISCUSSION

Recent studies have shown that citrus juice possesses potential benefits for supplementing bone strength.[34] In addition, data have increasingly shown that several varieties of flavonoids, such as daidzein, genistein, apigenin, quercetin, myricetin, and kaempferol, protect against bone loss.[16,24,25,35] Diosmetin is a bioflavone derivative present in a variety of citrus fruits.[28] In this study, we determined that diosmetin induced maturation and differentiation in human osteoblast-like MG-63 and hFOB cells and mouse osteoblast MC3T3-E1, as well as in bone marrow stromal M2-10B4 cells, without exhibiting a significant effect on cell growth. Treatment of MG-63, hFOB, MC3T3-E1, and M2-10B4 cells with diosmetin not only increased ALP activity (a marker of maturation and mineralization), but also enhanced osteocalcin secretion, type I collagen synthesis, osteopontin production, and mineralization (markers of terminal differentiation) in osteoblasts. These results indicate that diosmetin-stimulated maturation and differentiation of osteoblasts could be affected at various levels, from early to terminal stages, of the cell differentiation process.

ERK1/2 and p38, members of MAPKs, have been shown

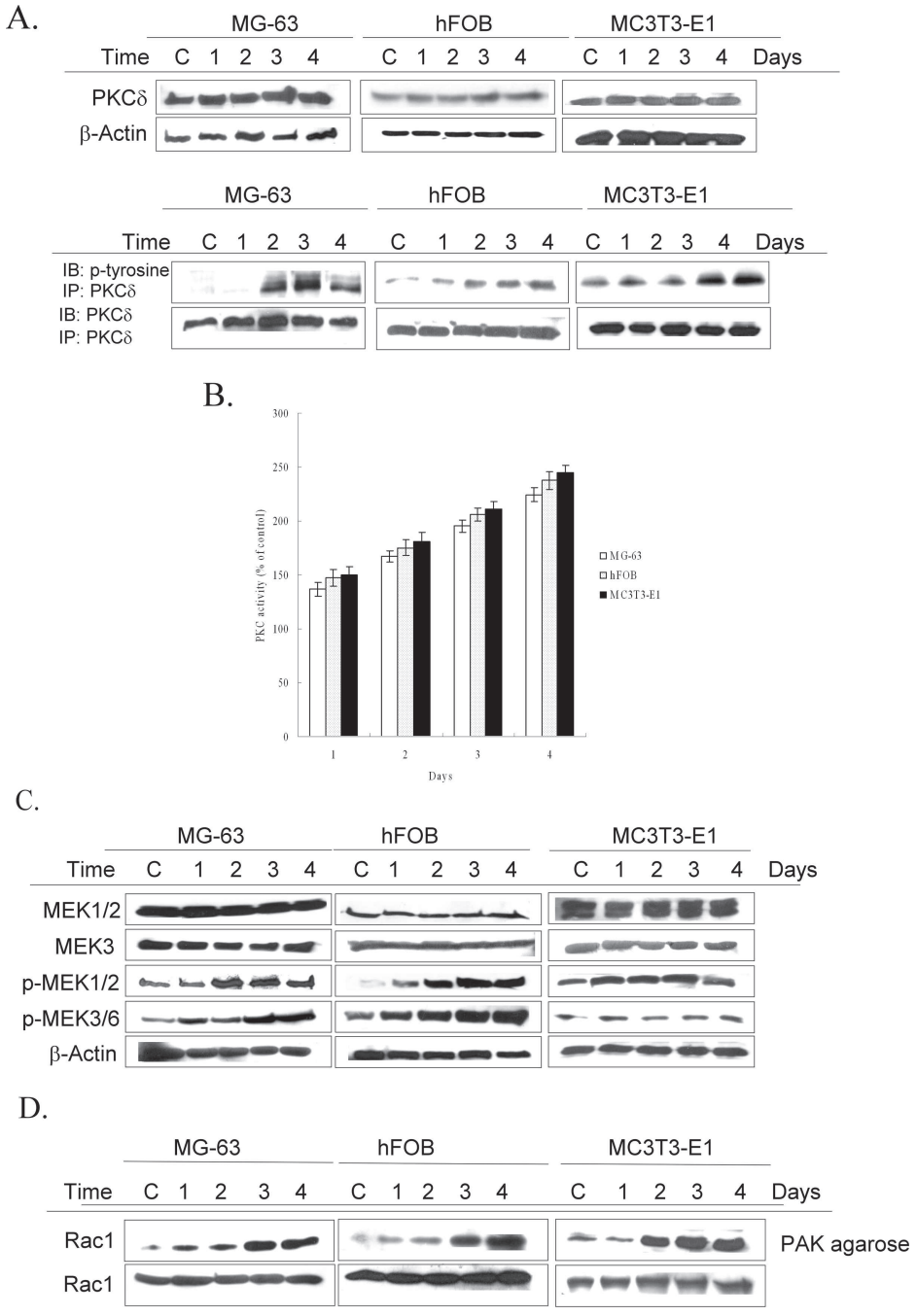

FIG. 6. The effect of diosmetin on activation of the PKC signaling pathway. Diosmetin increased PKCδ phosphorylation (A) and activity (B). The activation of MEKs (C) and Rac1 (D). Cells were treated with 20 μM diosmetin for the indicated times, and the expression and amount of proteins were assessed by immunoblot. PKCδ activity was determined by the PKCdelta kinase kit. The activation of Rac1 was detected by immunoblot analysis using anti-Rac antibody after incubation with PAK-conjugated agarose. Data shown are representative of three independent experiments.

to be important mediators of mammalian cell differentiation. A number of studies have reported that ERK is an important mediator of BMP-2–induced osteoblastic differentiation and that inhibition of ERK1/2 results in the suppression of differentiation markers.[36,37] ERK1/2 has also been determined to induce osteoblastic differentiation through a BMP-independent pathway in $1,25(OH)_2D_3$-treated primary human osteoblasts.[38] Previous studies have also shown that p38 activation plays a positive role in differentiation of various cells, including bone marrow osteoprogenitor cells, primary calvarial osteoblasts, and osteoblast-like cells.[17,38,39] Wang et al.[4] reported that p38 is involved in the regulation of osterix expression during osteoblastic differentiation induced by BMPs. In our study, we found that treatment of MG-63, hFOB, and MC3T3-E1 cell lines with diosmetin resulted in increases of MEK1/2 and MEK3/6 activation, followed by accumulation of ERK1/2 and p38 phosphorylation. Furthermore, selective knockdown ERK2 and p38 expression by siRNA-based inhibition approach also decreased the effects of diosmetin on ALP regulation and osteocalcin production, suggesting that the cooperation of ERK1/2 with p38 plays a crucial role in diosmetin-mediated cell maturation and differentiation in osteoblasts.

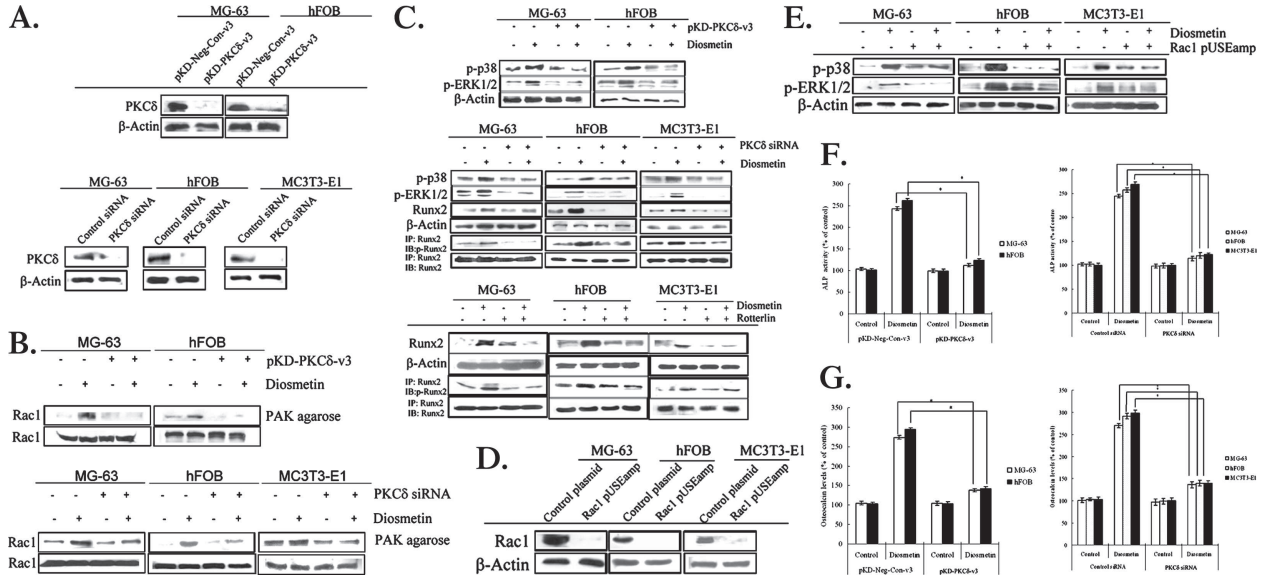

FIG. 7. The role of PKCδ on diosmetin-mediated osteoblastic differentiation. (A) The inhibition effect of PKCδ siRNA and ON-TARGET smart pool PKCδ siRNA (siRNA2). PKCδ inhibition decreased diosmetin-mediated Rac1 activation (B), p38, ERK1/2, and Runx2 phosphorylation (C). (D) The inhibition effect of Rac pUSEamp plasmid. Rac1 inhibition decreased diosmetin-mediated p38 and ERK1/2 activation (E), ALP upregulation (F), and osteocalcin production (G). Cells were transfected with control plasmid, pKD-PKCδ-v3, or Rac1 pUSEamp plasmid by Lipofectamine 2000 agents and treated with diosmetin for the indicated times (4 days for ALP assay, 5 days for osteocalcin assay, 2 days for Rac1, p38, and ERK1/2 activation). The expression and amount of proteins was assessed by immunoblot. ALP activity and osteocalcin production were measured as described in Fig. 2. Data shown are representative of three independent experiments. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($p < 0.05$).

Runx2 (Runt-related transcription factor 2) is a key transcription factor that plays an essential role in osteoblastic differentiation. During osteoblastic differentiation, Runx2 directly regulates bone marker genes, including osteocalcin, osteopontin, and type I collagen, by means of its specific DNA binding element, OSE2.[15,40] In Runx2-knockout animals, osteoblastic differentiation is impaired, and bone formation is absent.[40] Runx2 transcriptional activity is regulated at multiple levels by various kinds of intracellular signaling, such as growth factors, hormones, transcription factors, and cell–matrix interactions.[40] The ERK pathway has been shown to phosphorylate Runx2 on residues, which strongly correlates with enhanced Runx2 transactivation in osteoblastic differentiation and skeletal development.[14,41,42] Our study observed an increase in Runx2 expression and phosphorylation after ERK1/2 activation, whereas suppression of ERK1/2 signaling by siRNA abrogated Runx2 upregulation and phosphorylation. These data therefore suggest that activation of ERK1/2 acts as an upstream activator of osteoblast-specific Runx2 transcription factor in diosmetin-mediated cell differentiation.

Recent studies have shown that PKCδ signaling pathways regulate various types of cell differentiation. The differentiation of keratinocyte and tracheobronchial epithelial cells is increased by differentiating agents through a PKCδ/Ras/MEKK1/MEK3/7/AP-1 cascade.[21,22,43] Insulin-like growth factor-I (IGF-1) stimulated proliferation and fusion of the cells in C2C12 myoblasts by increasing selective translocation of PKCδ to the nucleus, and inhibition of PKCδ by the specific chemical inhibitor rotterlin completely aborted the differentiation effect of IGF-1.[44] All-trans retinoic acid (ATRA)– and phorbol 12-myristate 13-acetate (PMA) –induced monocytic differentiation is accompanied by enhancing phosphorylation of PKCδ, and ectopic expression of a constitutively active form of PKCδ directly increases leukemic cell differentiation.[45] Tu et al.[46] also have reported that Wnt3a signals through the $G\alpha_{q/11}$ subunits of G proteins to activate phosphatidylinositol signaling and PKCδ, resulting in the promotion of bone formation. PKCδ activation occurs through Fzd receptor–dependent mechanisms that require association with disheveled 2 and then translocate to the plasma membrane.[46] Both tyrosine phosphorylation and translocation are involved in regulating PKCδ activity. Phosphorylation of PKCδ at tyrosines 311, 332, and 532 respond to H_2O_2, and tyrosines 311 and 332 are phosphorylated in response to ceramide.[22] In this study, we showed that treatment of MG-63, hFOB, and MC3T3-E1 cell lines with diosmetin resulted in the increase of PKCδ tyrosine phosphorylation and activity. Moreover, selectively knockdown PKCδ expression by siRNA-based inhibition approach also decreased the effects of diosmetin on the induction of osteoblastic maturation and differentiation, suggesting that the cooperation of PKCδ plays a crucial role in diosmetin-induced cell differentiation.

PKCδ can be involved in p38 and ERK activation at several levels of the classical MAP kinase cascade, including activation by a direct interaction with MEKs or by Rac1.[47] Rac1, belong to the Rho family GTPases, is involved in activation of all three MAPKs. Rac1 stimulates

serine/threonine kinase PAK1, followed by activation of the downstream kinase MAPK/ERK kinase (MEK), which in turn phosphorylates p38 and ERKs.[48,49] We found that the treatment of MG-63, hFOB, and MC3T3-E1 cell lines with diosmetin resulted in increased Rac1 activity and subsequently triggered phosphorylation of MEK1/2 and MEK3/6, which lie upstream of ERK1/2 and p38, respectively. Inhibition of PKCδ effectively inhibited ERK1/2 and p38 MAPK signaling as well as Runx2 phosphorylation. In addition, inhibition of PKCδ decreased diosmetin-mediated Rac1 activation, whereas blockade of Rac1 attenuated p38 and ERK1/2 phosphorylation. These results suggest that activation PKCδ acts as the upstream activator of PKCδ-Rac1-MEK3/6-p38 and PKCδ-Rac1-MEK1/2-ERK1/2-Runx2 signaling in response to diosmetin.

The model of PKCδ signals downstream of Rac1 remains unknown. PKCδ activates Rac1 through proline-rich tyrosine kinase 2 (PYK-2) in angiotensin II–stimulated cells.[50] PKCδ that activate Rac1 by dissociation with SEK-1/MKK-4 and then activate Rac and SEK-1/MKK-4 have also been reported.[51] However, whether diosmetin-mediated PKCδ activation alters GTPase-activating protein (GAP)/guanine nucleotide exchange factors (GEFs) or other kinases, activities to regulate Rac require further study in the future.

In conclusion, this study showed that (1) diosmetin induces osteoblastic maturation and differentiation, (2) increased differentiation of all osteoblastic cell lines after exposure to diosmetin is associated with p38 and ERK1/2 activation, (3) diosmetin-induced cell differentiation is mediated by activation of ERK1/2, which phosphorylates Runx2, and (4) diosmetin increases the activity and phosphorylation of PKCδ, which activates p38 and ERK1/2 by increasing the activation of Rac1 and phosphorylation of MEK1/2 and MEK3/6. These findings suggest that diosmetin may be beneficial in stimulating osteoblastic activity, resulting in bone formation.

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Syringetin, a flavonoid derivative in grape and wine, induces human osteoblast differentiation through bone morphogenetic protein-2/extracellular signal-regulated kinase 1/2 pathway

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Syringetin, a flavonoid derivative in grape and wine, induces human osteoblast differentiation through bone morphogenetic protein-2/extracellular signal-regulated kinase 1/2 pathway

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; phospho-ERK1/2, phosphorylated extracellular signal-regulated kinase; BMP, Bone morphogenetic protein; ELISA, enzyme-linked immunosorbent assay; PNPP, 4-Nitrophenyl phosphate; PD98059, 2′-Amino-3′-methoxyflavone.

Abstract

Syringetin (3,5,7,4'-Tetrahydroxy-3',5'dimethoxyflavone), a flavonoid derivative, is present in grape and wine. By means of alkaline phosphatase (ALP) activity, osteocalcin, and type I collagen enzyme-linked immunosorbent assay (ELISA), we have shown that syringetin exhibits a significant induction of differentiation in MC3T3-E1 mouse calvaria osteoblasts and hFOB human osteoblasts. Alkaline phosphatase and osteocalcin are phenotypic markers for early-stage differentiated osteoblasts and terminally differentiated osteoblasts, respectively. Our results indicate that syringetin stimulates osteoblast differentiation at various stages, from maturation to terminally differentiated osteoblasts. Induction of differentiation by syringetin is associated with increased bone morphogenetic protein-2 (BMP-2) production. The BMP-2 antagonist noggin blocked syringetin-mediated ALP activity and osteocalcin secretion enhancement, indicating that BMP-2 production is required in syringetin-mediated osteoblast maturation and differentiation. Induction of differentiation by syringetin is associated with increased activation of SMAD1/5/8 and extracellular signal-regulated kinase 1/2 (ERK 1/2). Cotreatment of ERK1/2 inhibitor PD98059 inhibited syringetin-mediated ALP upregulation and osteocalcin production. In conclusion, syringetin increased BMP-2 synthesis, and subsequently activated SMAD1/5/8 and ERK1/2, and this effect may contribute to its action on the induction of osteoblast maturation and differentiation, followed by an increase of bone mass.

Keywords: Syringetin/ osteoblasts/ differentiation/ BMP-2/ ERK1/2

1. 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 osteoblasts and osteoclasts [1-3]. Current drugs used to treat osteoporosis are the bone resorption inhibitors which could inhibit the activities of osteoclasts, including bisphosphonates, calcitonin, estrogen, vitamin D analogues, and ipriflavone [4, 5]. However, the effect of these drugs in recovering bone mass is relatively small, certainly no more than 2% per year [4]. It is desirable, therefore, to have satisfactory bone-building (anabolic) agents, such as teriparatide. By increasing the osteoblastic lineage proliferation or inducing the differentiation of the osteoblasts, these agents stimulate new bone formation and correct the imbalanced trabecular microarchitecture characteristic of established osteoporosis [1, 6-7].

Bone morphogenetic proteins (BMP) form a unique group of proteins within the transforming growth factor beta (TGF-β) superfamily. Fifteen bone morphogenetic proteins have presently been identified and divided into subfamilies according to their amino acid sequences [8-11]. Bone morphogenetic proteins could induce osteoblast differentiation of various types of cells, including undifferentiated mesenchymal cells, bone marrow stromal cells, and preosteoblasts which have pivotal roles in the regulation of bone induction, maintenance and repair [12-14]. BMPs are also important

in the determinants of mammalian embryological development. The bone morphogenetic protein-2 (BMP-2) was detected in condensing prechondrocytic mesenchyme of developing limb buds, and has demonstrated a strong osteo-inductive capacity *in vivo* and *in vitro* [11, 15]. Signaling by BMP proteins is mediated through heterodimerization of types I and II serine/theronine kinase receptors. Various downstream factors, like SMADs, appear to be activated by the binding of BMPs and its receptors. After the stimulation and activation of BMP type I receptor, the SMAD1, SMAD5, and SMAD8 accumulated in the nucleus and controlled the transcription of a large number of target genes [8, 9].

MAPKs, another family of serine/threonine kinases, are mediators of intracellular signals in response to various stimuli and showed the involvement in the regulation of many cellular physiological functions, including proliferation, differentiation, inflammation, and apoptosis [16]. The activation of MAPK requires the phosphorylation of both the threonine and tyrosine residues of a conserved T-X-Y motif within the activation loop by a dual-specificity MAPK kinase (MKK or MEK) [17]. Previous research showed that activation of MEK is necessary and sufficient for the differentiation of mesenchymal stem cells and blocked by the chemical inhibitor PD98059 [18, 19]. And the downstream factors of MEKs, ERK1/2, shows the dependence of mammalian cell differentiation on the ERK pathway [20, 21].

Flavonoids represent a large class of phenolic compounds present in fruit and vegetables [22]. Several flavonoids have been shown to influence osteoblastic differentiation, including daidzein, genistein, quercetin, kaempferol, naringin, myricetin and diosmetin [23-28]. Syringetin (3,5,7,4'-Tetrahydroxy-3',5'dimethoxyflavone), a flavonoid derivative, is present in grape and wine [29-31]. The syringetin content in red grapes is 3.22% [30]. Syringetin-3-O-glycoside is the major form presented in red grapes, but syringetin-3-glycosides coexist with corresponding free aglycones released by hydrolysis in wine [32]. In this study, we first report on the effects and molecular mechanisms of action of syringetin in MC3T3-E1 mouse calvaria osteoblasts and hFOB human osteoblasts that are mediated through bone morphogenetic protein-2/extracellular signal-regulated kinase 1/2 pathway.

2. Materials and methods

2.1. Chemicals and reagents

Fetal bovine serum (FBS), α-Minimal Essential Medium (α-MEM), penicillin G, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD, USA). BMP-2 ELISA kit and noggin were purchased 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 purchased from Roche Diagnostics GmbH

(Mannheim, Germany). The osteocalcin ELISA kit was supplied by Biosource Technology (Nivelles, Belgium). PD98059 (2′-Amino-3′-methoxyflavone) was purchased from Calbiochem (Cambridge, MA, USA). The antibodies to ERK1/2, SMDA1/5/8, phospho-ERK1/2, and phospho-SMAD1/5/8 were obtained from Cell Signaling Technology (Beverly, MA, USA). Syringetin (3,5,7,4'-Tetrahydroxy-3',5'dimethoxyflavone) was purchased from Extrasynthese (Genay, France). Dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical (St. Louis, MO, USA). The stock solution of syringetin was prepared at a concentration of 8000 µM of DMSO. It was then stored at –20°C until use. For all experiments, the final concentrations of the test compound were prepared by diluting the stock with medium. Control cultures received the carrier solvent (0.05% DMSO).

2.2. Cell cultures

Two osteoblastic cell lines were used: (1) the conditionally immortalized fetal osteoblastic cell line hFOB, and (2) the mouse calvaria osteoblastic cell line MC3T3-E1. 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. hFOB cells proliferate at 33.5°C, (the permissive

temperature at which the temperature-sensitive mutant SV 40 large T antigen is active), and differentiate at 39.5 °C (the restrictive temperature, when the SV 40 large T antigen is inactive). All experiments on hFOB cells were carried out at the permissive temperature of 33.5°C. MC3T3-E1 mouse calvaria osteoblasts were kindly provided by Dr. Renny T Franceschi (University of Michigan, Ann Arbor, MI, USA), and grown in α-MEM and antibiotics.

2.3. Cell proliferation assay (XTT)

Inhibition of cell proliferation by syringetin was measured by XTT assay (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells were plated in 96 well culture plates (8×10^3 cells/well). After 24 h incubation, the cells were treated with vehicle (0.05% DMSO) or syringetin (1, 5, 10, and 20 µM) for 48 and 72 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.

2.4. Alkaline phosphatase (ALP) activity

Cells were seeded into 96 well plates at a density of 5×10^3 cells/well and cultured for 24 h. The agent to be tested was added to the wells, and incubation continued for 2 and 3 days. The cells were then washed 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, then measuring cellular activity by incubation for 1 h at 37°C in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 0.1% Triton X-100, 2 mM $MgSO_4$, and 6 mM *p*-nitrophenyl phosphate. The reaction was stopped by adding 1 M NaOH. Absorbance was measured at 405 nm and compared to *p*-nitrophenol standards.

2.5. Analysis of mineralization

The degree of mineralization was determined in the 12-well plates using Alizarin Red S staining (Sigma Chemical, St. Louis, MO, USA) after 72 and 96 h treatment. Briefly, cells were fixed with ice-cold 70% (v/v) ethanol for 1 h and then stained with 40 mM Alizarin Red S in deionized water (pH=4.2) for 10 min at room temperature. After removing Alizarin Red S solution by aspiration, cells were incubated in PBS for 15 min at room temperature on an orbital rotator, then rinsed once with fresh PBS. The cells were subsequently destained for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH=7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using an ELISA reader

(Multiskan EX, Labsystems). The concentration of Alizarin Red S staining in the samples was determined by comparing the absorbance values with those obtained from Alizarin Red S standards. The mineralization values were normalized to the relative number of viable cells, as determined directly in the 96-well plates using the XTT assay [33].

2.6. Assaying the levels of type I procollagen

Cells were treated with various concentrations of syringetin for 72 and 96 h. The type I procollagen assay, which measures the propeptide portion of the molecule and reflects the synthesis of the mature form of the protein, 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, as determined by BCA protein assay.

2.7. Assaying the levels of osteocalcin and BMP-2

Osteocalcin and BMP-2 ELISA kits were used to detect osteocalcin and BMP-2 levels, respectively. Briefly, cells were treated with various concentrations of syringetin for the indicated times. The culture medium was then collected and measured for osteocalcin and BMP-2. 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 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 [34].

2.8. Analysis for immunoblot

Cells treated with syringetin for the indicated times were lysed and the protein concentrations determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). For immunoblot, 50 μg of total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. The protein was transferred to polyvinylidene difluoride membranes using transfer buffer (50 mM Tris, 190 mM glycin, 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% bovine serum albumin) overnight at 4□. After washing three times with washing buffer (blocking buffer without 3% bovine serum albumin) for 10 min each, the blot was incubated with primary antibody (SMAD1/5/8, ERK1/2, phospho-ERK, and phospho-SMAD1/5/8) for 2 to 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).

2.9. Statistical analysis

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

3. Results

3.1. Syringetin had no effect on the proliferation of MC3T3-E1 and hFOB cells

We first determined the effect of syringetin on the cell proliferation of MC3T3-E1 and hFOB by XTT assay. Our results showed that syringetin did not exhibit significant effects on cell proliferation at the concentrations used (1 to 20 μM) after 48 and 72 h of treatment in MC3T3-E1 and hFOB cells (Fig. 1A and B).

3.2. Effect of syringetin on maturation and differentiation markers, ALP activity, osteocalcin expression, type I collagen synthesis, and mineralization in MC3T3-E1 and hFOB cells

The effect of syringetin on the maturation of osteoblasts was studied by determining ALP activity in MC3T3-E1 and hFOB cells. The results showed that syringetin increased ALP activity in MC3T3-E1 and hFOB cells in a dose-dependent manner after 48 and 72 h of treatment (Fig. 2A). The effect of syringetin on the terminal differentiation of osteoblasts was also assessed by determining the production of

osteocalcin, type I collagen protein, and the degree of mineralization. As shown in Fig. 2B, treatment of MC3T3-E1 and hFOB cells with syringetin increased the levels of osteocalcin protein in a dose-dependent manner after 72 and 96 h of treatment. In addition, type I collagen protein levels were also enhanced in syringetin-treated MC3T3-E1 and hFOB cells after 72 and 96 h of treatment (Fig. 2C). Treatment with syringetin for 72 and 96 h also increased the amount of osteoblasts mineralization in a dose-dependent manner (Fig. 2D).

3.3. Syringetin increases ALP activity and osteocalcin through de novo protein synthesis

ALP activity is a phenotypic marker for the early and mature differentiations of osteoblasts, whereas osteocalcin secretion is another biologic marker for terminal differentiation. Next, we assessed whether the effect of syringetin on the increase of ALP activity and osteocalcin production was due to the enhancing of protein synthesis. The results showed that the syringetin-mediated increase of ALP activity and osteocalcin production is almost completely inhibited by a 24 h pretreatment of MC3T3-E1 and hFOB cells with protein synthesis inhibitor cycloheximide (10 μg/ml) (Fig. 3A and B).

3.4. BMP-2 mediates syringetin-induced maturation and differentiation in MC3T3-E1and hFOB cells

To confirm whether either transcriptional or translational levels of BMP-2 expression were influenced by the presence of syringetin, we examined the expression of the BMP-2 in the presence and absence of syringetin using bone morphogenetic proteins ELISA kits. The results indicated that syringetin caused a significant increase in BMP-2 protein levels in MC3T3-E1 and hFOB. The upregulation of BMP-2 protein by 20 μM syringetin started to increase 3 h after treatment with syringetin, and maximum expression was observed at 12 h (Fig. 4A). After 12 h of treatment, syringetin increased production of BMP-2 in a dose-dependent manner (Fig. 4B).

To further examine the role of BMP-2 in syringetin-induced cell differentiation, osteoblasts were pretreated with a BMP-2 inhibitor, 100 ng/ml noggin protein [35], for 1 h, then cotreated with 20 μM syringetin and the inhibitor for the indicated times. Addition of purified noggin protein did not change ALP activity and osteocalcin secretion, but abrogated syringetin-induced cell differentiation (Fig. 4C and D). Therefore, syringetin-induced cell differentiation may operate by a BMP-2-dependent pathway.

3.5. Activations of SMAD1/5/8 and ERK1/2 in syringetin-treated MC3T3-E1 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 syringetin-treated MC3T3-E1 and hFOB cells. As shown in Fig. 5A, treatment with syringetin did not affect the expression levels of unphosphorylated SMAD1/5/8, but did increase the amount of phospho-SMAD1/5/8 after a 3 h exposure of osteoblasts to syringetin, with a progressive increase for up to 12 h. The activation of SMADs closely matched the appearance of BMP-2. We also investigated the role of ERK1/2 in syringetin-treated cells. The results showed that syringetin treatment increased the activation (phosphorylation) of ERK1/2 (Fig. 5A). ERK1/2 activation occurred later (6 h) than SMADs (3 h), indicating that ERK1/2 activation may be a downstream event of SMADs.

To determine the role of BMP-2 on the activation of SMAD1/5/8 and ERK1/2 in syringetin treated osteoblasts, we tested the effect of noggin on the activation of SMAD1/5/8 and ERK1/2 by syringetin. Results showed that noggin pretreatment abrogated the activation of SMAD1/5/8 and ERK1/2 induced by syringetin (Fig. 5B and C). Thus, BMP-2 signaling is necessary and sufficient to mediate the activation of

SMAD1/5/8 and ERK1/2 in syringetin-treated MC3T3-E1 and hFOB cells.

3.6. ERK1/2 is necessary for syringetin-mediated osteoblast maturation and differentiation in MC3T3-E1 and hFOB cells

As ERK1/2 activation was observed with syringetin treated osteoblasts, we next assessed the role of ERK1/2 by using an inhibitor specific to ERK1/2. MC3T3-E1 and hFOB cells were pretreated for 1 h with a potent, specific inhibitor for ERK1/2, PD98059. The inhibitor-treated cells were then exposed to syringetin, and ALP activity and osteocalcin secretion then determined. As shown in Fig. 6A, the syringetin-mediated ERK1/2 activation was effectively inhibited by 20 μM PD98059 (Fig. 6A). PD98059 not only decreased the ALP activity of syringetin of 48 h, but also inhibited the accumulation of osteocalcin induced by syringetin at 72 h (Fig. 6B and C).

4. Discussion

During differentiation *in vitro*, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of ALP, secretion of osteocalcin, and finally, mineralization of bone nodules [11, 36-38]. Our results indicate that the presence of syringetin causes a significant increase in ALP activity, osteocalcin production, type I collagen synthesis and mineralization. As the appearance of ALP activity is an early phenotypic marker for mature osteoblasts, our

results suggest that the presence of syringetin stimulates an early stage of osteoblast differentiation. The production of osteocalcin and type I collagen, both phenotypic markers for the later stage of osteoblast differentiation, was increased by syringetin treatment. In addition, bone formation, as measured by mineralization, was also increased in MC3T3-E1 and hFOB cells treated with syringetin. Furthermore, the inhibitory protein synthesis effect of cycloheximide on the syringetin-induced increase in ALP activity and octeocalcin production strongly suggests that *de novo* protein synthesis is essential for this response. In summary, these results indicate that syringetin-stimulated maturation and differentiation of osteoblasts could be affected at various levels, from early to terminal stages of the cell differentiation process.

Bone morphogenetic proteins play an important role in the process of bone formation and remodeling [8]. It has been well documented that stimulation of osteoblast differentiation is characterized mainly by increased expression of ALP, type I collagen, and osteocalcin [39]. 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 [8, 9]. Several natural or chemical compounds have been reported to induce osteoblast

differentiation by induction of BMP and/or SMAD signaling, such as daidzein, osthole, and fraxetin [11, 34, 40]. Our study indicates that the production of BMP-2 increases in syringetin-treated MC3T3-E1 and hFOB cells. Also, phosphorylations of SMAD1/5/8 are simultaneously enhanced in syringetin-treated osteoblasts. Indeed, bone morphogenetic proteins antagonist noggin not only blocked syringetin-mediated SMAD1/5/8 activation, but also exhibited a similar effect against syringetin-mediated cell differentiation (ALP upregulation and osteocalcin production). These results support the hypothesis that the BMP-2 signaling system plays an important role in syringetin-mediated cell maturation and differentiation in osteoblasts.

ERK1/2 is also important in osteoblast cell proliferation and differentiation [18, 20]. 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 [18, 41]. Our study observed an increase in ERK1/2 activity after BMP-2 production and SMAD1/5/8 phosphorylation, and suppression of BMP-2 signaling by cotreating noggin abrogated ERK1/2 activation in syringetin-treated cells. In addition, inhibition of ERK1/2 activity by specific inhibitor PD98059 decreased the effects of syringetin on osteoblastic maturation and differentiation. These data suggest that activation of ERK1/2 plays an important role on the cell differentiation of syringetin activity in osteoblasts.

In summary, our study has clearly demonstrated that syringetin stimulates osteoblast differentiation at various stages in MC3T3-E1 and hFOB cells. Syringetin's effect on cell maturation and differentiation is strongly associated with BMP-2/SMAD1/5/8/ERK1/2 signaling pathway. This therefore suggests that syringetin may be beneficial in stimulating the osteoblastic activity resulting in bone formation.

Acknowledgment

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Figure legends

Figure 1. The effect of syringetin on the cell proliferation of MC3T3-E1 (A) and hFOB (B) cells. Adherent cells that proliferated in 96-well plates (8×10^3 cells/well) were incubated with different concentrations (μM) of syringetin for various time intervals. Cell proliferation was determined by XTT assay. Each value is the mean ± SD of three independent experiments. Standard deviations were less than 10%.

Figure 2. Syringetin increased the osteoblastic activity in MC3T3-E1 and hFOB cells. Syringetin increased ALP activity (A), osteocalcin production (B), type I collagen synthesis (C), and mineralization (D). ALP activity was assessed by the conversion of *p*-nitrophenyl phosphate in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 2 mM $MgSO_4$ and 0.1 % Triton. The amount of osteocalcin in culture medium was assessed by osteocalcin ELISA kit. The production of type I collagen was assayed by Prolagen-C immunoassay. The degree of mineralization was assayed by Alizarin Red S staining. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between control and syringetin-treated groups, as analyzed by Dunnett's test ($P < 0.05$).

Figure 3. Inhibition of cycloheximide on syringetin-induced increase of ALP activity and osteocalcin production. (A) The effect of cycloheximide on syringetin-induced ALP activity. (B) The effect of cycloheximide on

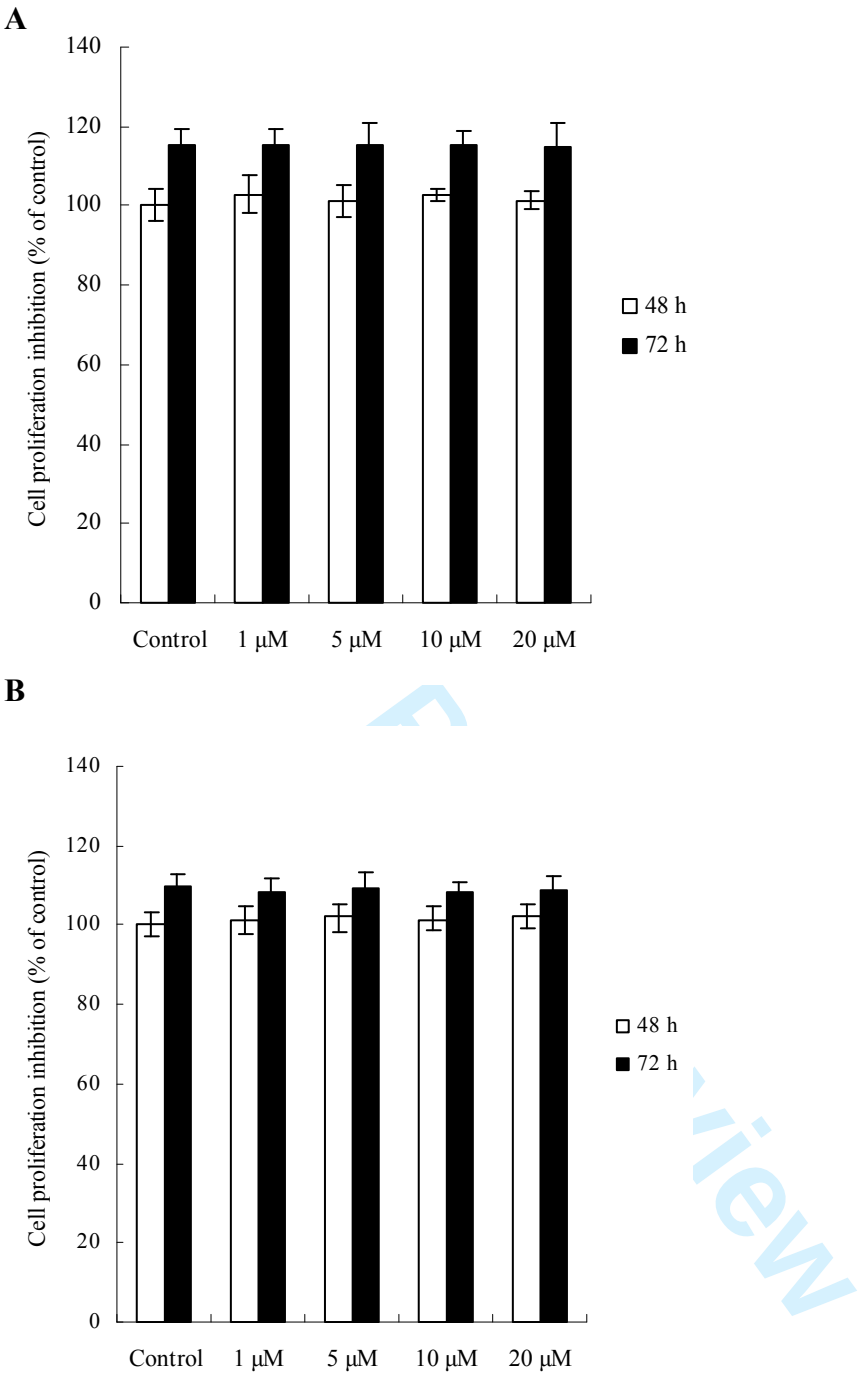
syringetin-mediated osteocalcin production. Cells were pretreated with or without 10 μg/ml cycloheximide for 24 h, then 20 μM syringetin was added for 48 h (for ALP) and 72 h (for osteocalcin). ALP activity and osteocalcin levels were determined as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between control and test groups, as analyzed by Dunnett's test ($P < 0.05$).

Figure 4. The role of BMP-2 in upregulation of osteoblastic activity by syringetin in MC3T3-E1 and hFOB cells. Syringetin increased the production of BMP-2 in a time-dependent (A) and dose-dependent manner (B). Noggin inhibited the induction of syringetin on ALP upregulation (C) and osteocalcin stimulation (D). For (A) and (B), cells were treated with 20 μM syringetin for the indicated times (0, 3, 6, and 12 h) at various concentrations (1, 5, 10, and 20 μM) of syringetin for 12 h. The production of BMP-2 in culture medium was assessed by BMP-2 ELISA kit. For (C) and (D), cells were pretreated with or without noggin for 1 h, and then 20 μM syringetin was added for the indicated times. ALP activity (48 h treatment) and osteocalcin levels (72 h treatment) were determined as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($P < 0.05$).

Figure 5. The activation of SMADs, ERK1/2 in syringetin-treated MC3T3-E1 and

hFOB cells. Syringetin increased SMAD1/5/8 and ERK1/2 activation (A). Noggin decreased the effect of syringetin on the activation of SMAD1/5/8 (B) and ERK1/2 (C). For (A), unphospho- and phospho-SMAD and ERK1/2, cells were treated with 20 μM syringetin for the indicated times; the levels of SMAD1/5/8, ERK1/2, and their phosphorylated proteins were determined by immunoblot analysis. For (B) and (C), cells were incubated for 1 h in the presence or absence of noggin, and then 20 μM syringetin was added and incubated for 9 h. The levels of various proteins were determined by immunoblot analysis. Data shown are representative of three independent experiments.

Figure 6. The role of ERK1/2 on the upregulation of syringetin on osteoblastic activity in MC3T3-E1 and hFOB cells. (A) PD98059 inhibited syringetin-induced ERK1/2 activation. Effect of PD98059 on syringetin-induced ALP activity (B) and osteocalcin production (C). Cells were incubated for 1 h in the presence or absence of PD98059, and then 20 μM syringetin was added and incubated for the specified times (48 h for ALP, 72 h for osteocalcin assay). The ERK1/2 activation was measured as described in the legend to Fig. 2. Data shown are representative of three independent experiments. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($P < 0.05$).


A
Cell proliferation inhibition (% of control)
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Control
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B
Cell proliferation inhibition (% of control)
140
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100
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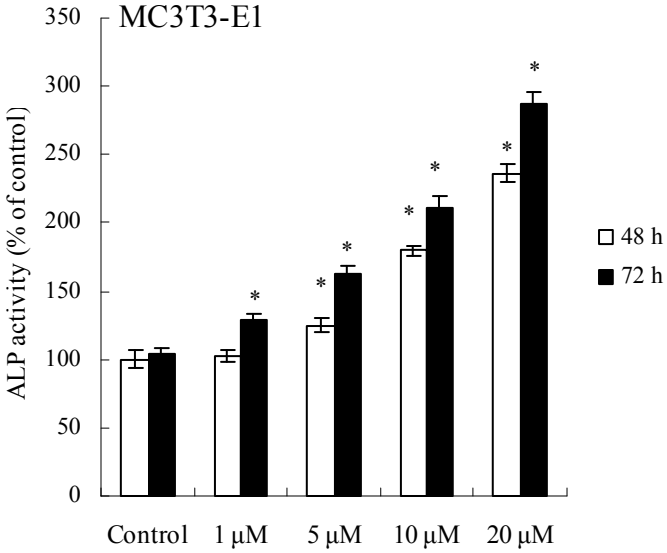
60
40
20
0
Control
1 μM
5 μM
10 μM
20 μM
48 h
72 h

Fig. 1.

A


MC3T3-E1
ALP activity (% of control)
350
300
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
48 h
72 h

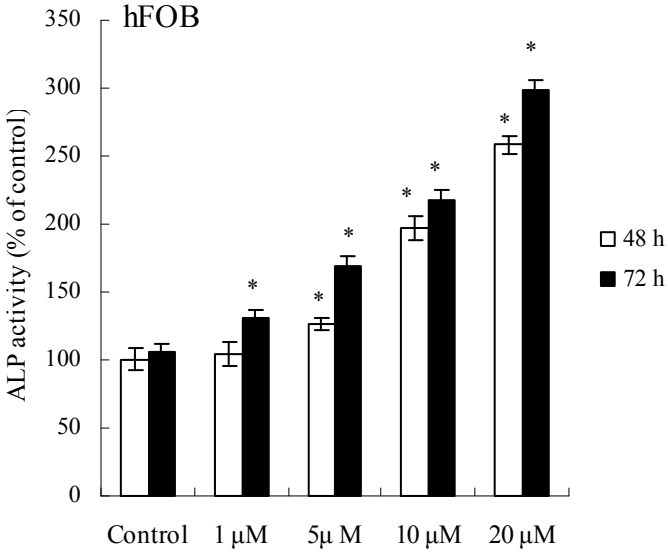
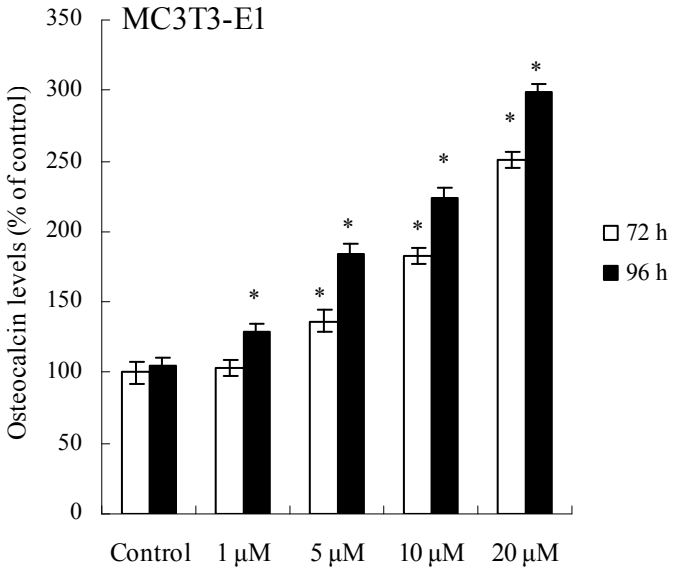

hFOB
ALP activity (% of control)
350
300
250
200
150
100
50
0
Control
1 μM
5μ M
10 μM
20 μM
48 h
72 h

Fig. 2.

B


MC3T3-E1
Osteocalcin levels (% of control)
350
300
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
72 h
96 h

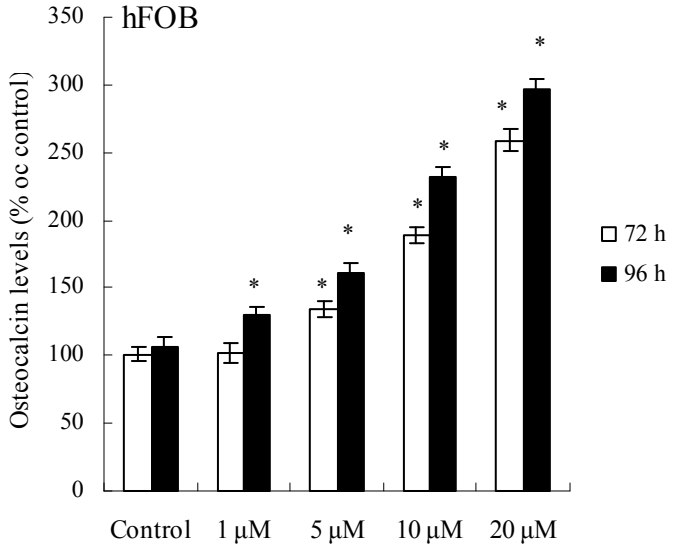

hFOB
Osteocalcin levels (% oc control)
350
300
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
72 h
96 h

Fig. 2.

C

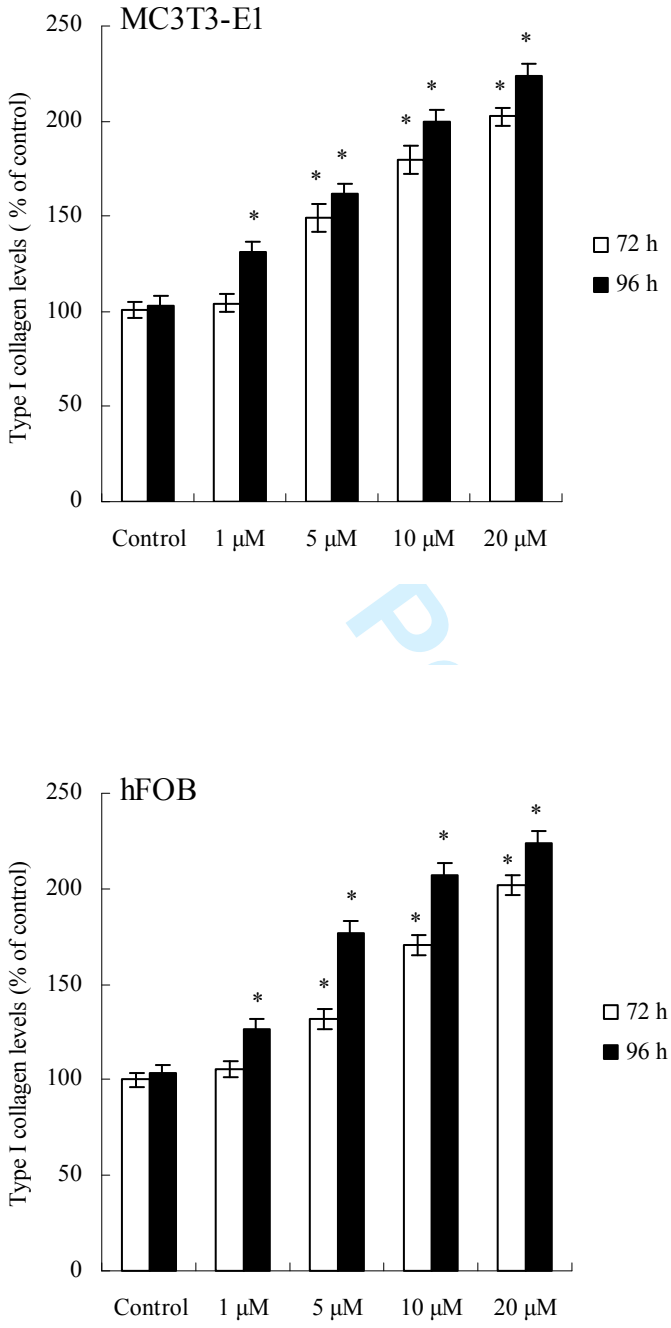

MC3T3-E1
Type I collagen levels (% of control)
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
72 h
96 h
hFOB
Type I collagen levels (% of control)
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
72 h
96 h

Fig. 2.

D

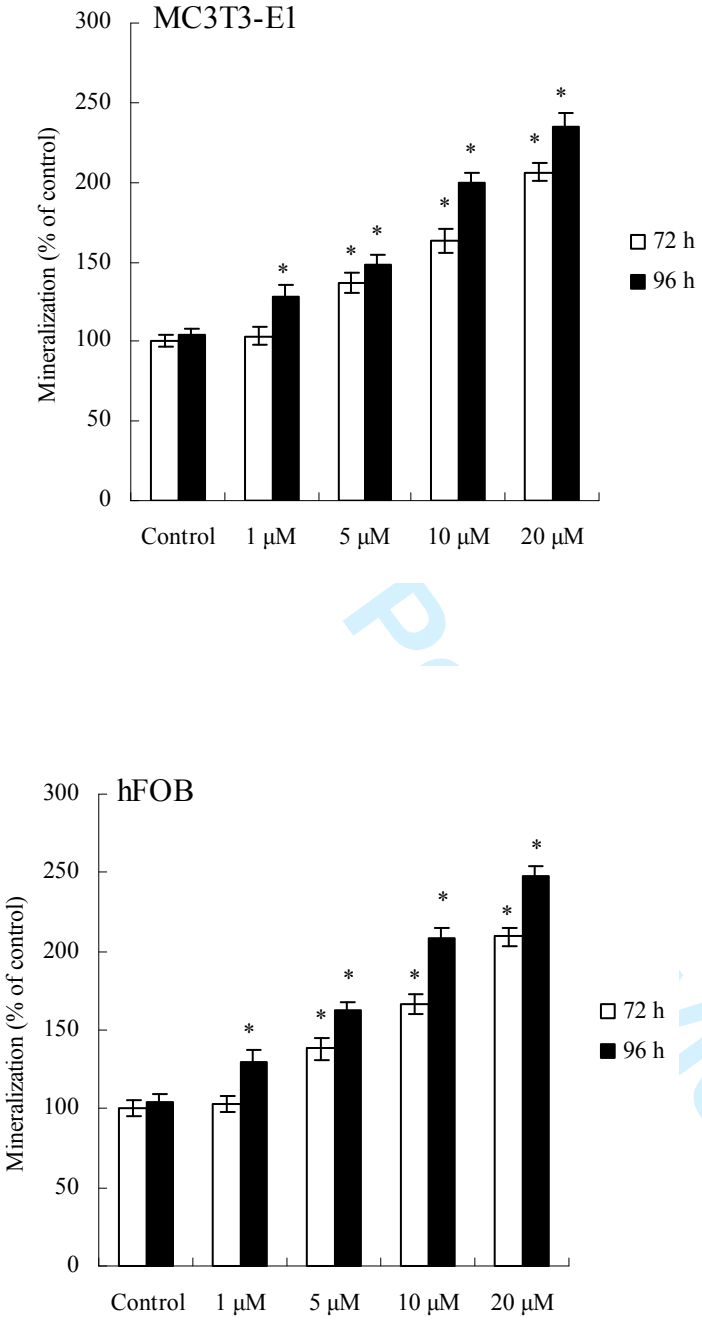
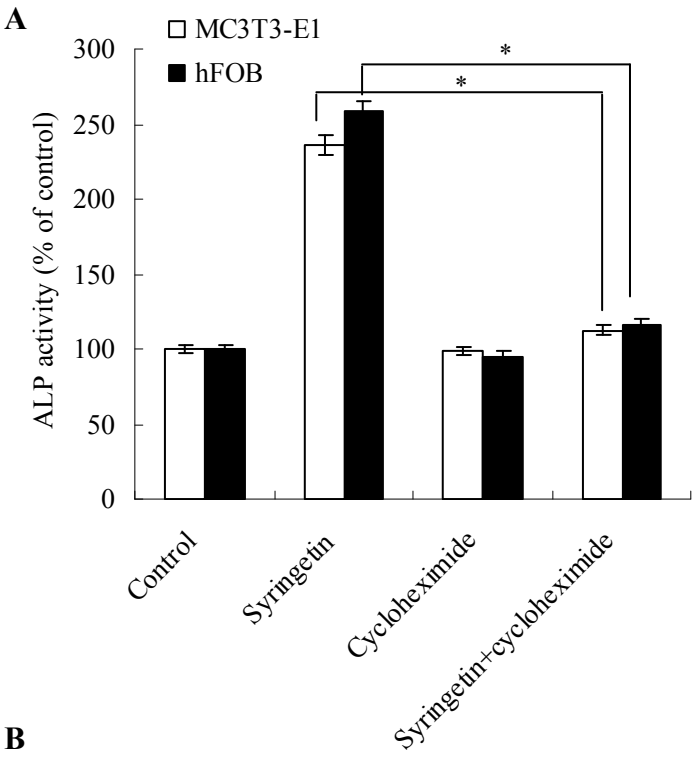

MC3T3-E1
Mineralization (% of control)
300
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
72 h
96 h
hFOB
Mineralization (% of control)
300
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
72 h
96 h

Fig. 2.


A
MC3T3-E1
hFOB
ALP activity (% of control)
300
250
200
150
100
50
0
*
*
Control
Syringetin
Cycloheximide
Syringetin+cycloheximide

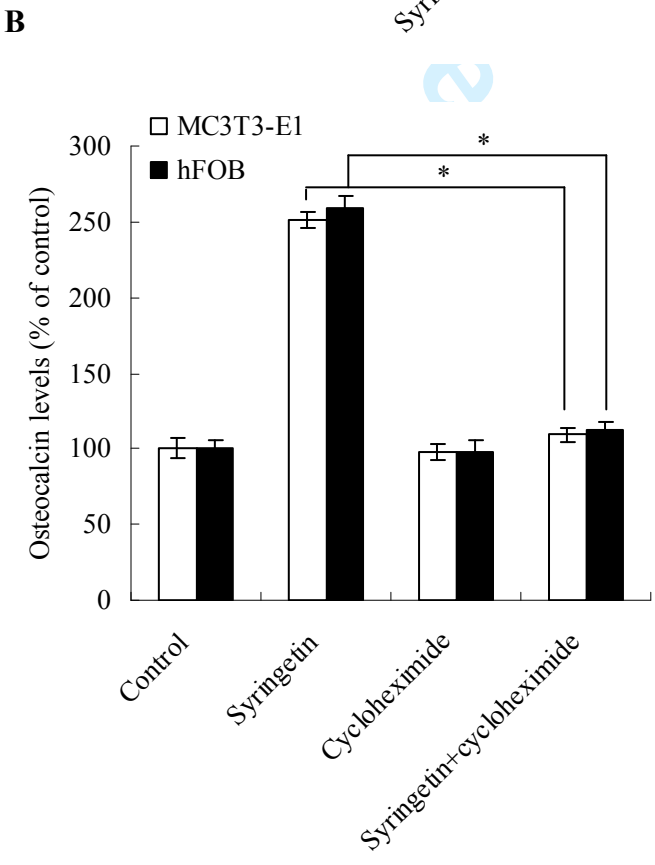
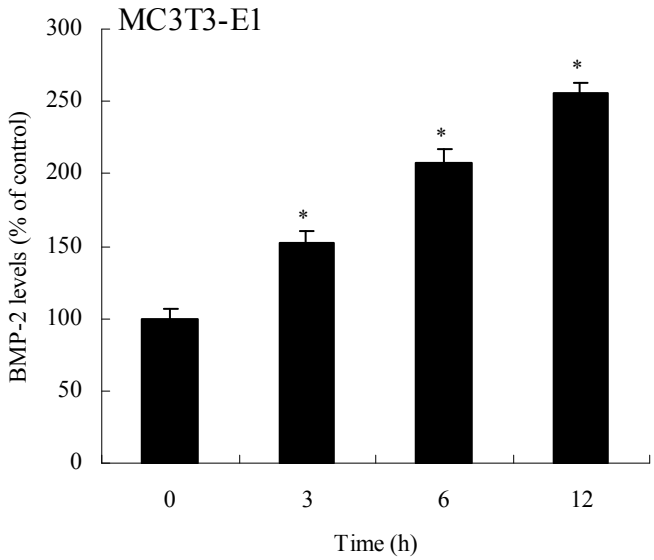

B
MC3T3-E1
hFOB
Osteocalcin levels (% of control)
300
250
200
150
100
50
0
*
*
Control
Syringetin
Cycloheximide
Syringetin+cycloheximide

Fig. 3.

A


MC3T3-E1
BMP-2 levels (% of control)
300
250
200
150
100
50
0
0
3
6
12
Time (h)
*
*
*
*

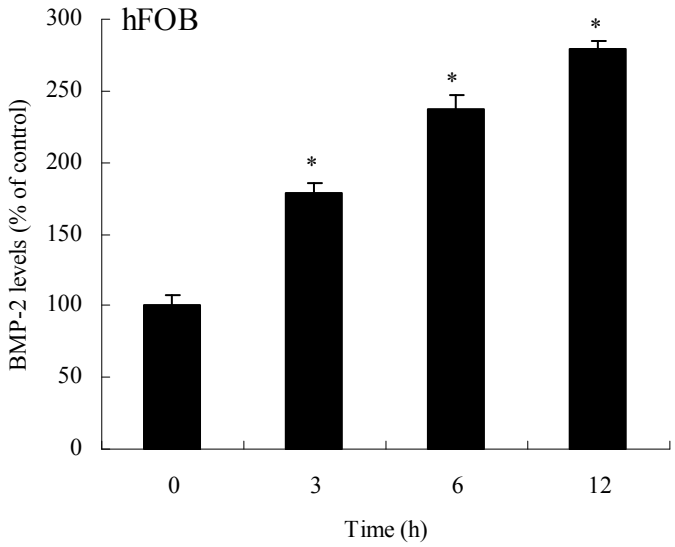
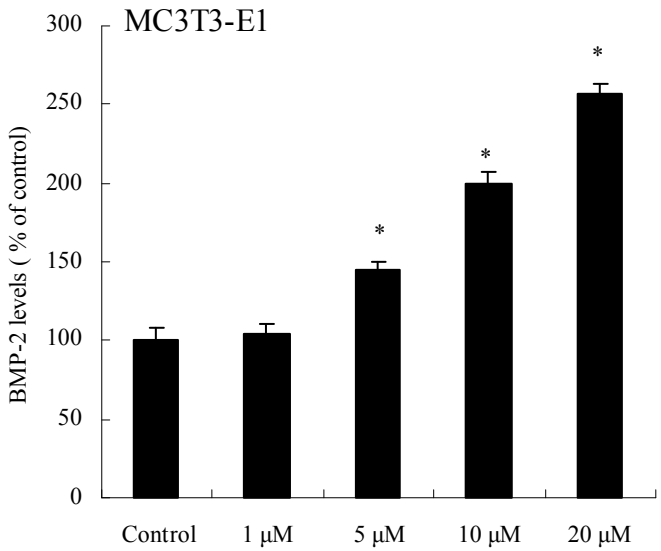

hFOB
BMP-2 levels (% of control)
300
250
200
150
100
50
0
0
3
6
12
Time (h)
*
*
*
*

Fig. 4.

B


MC3T3-E1
BMP-2 levels (% of control)
300
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
*

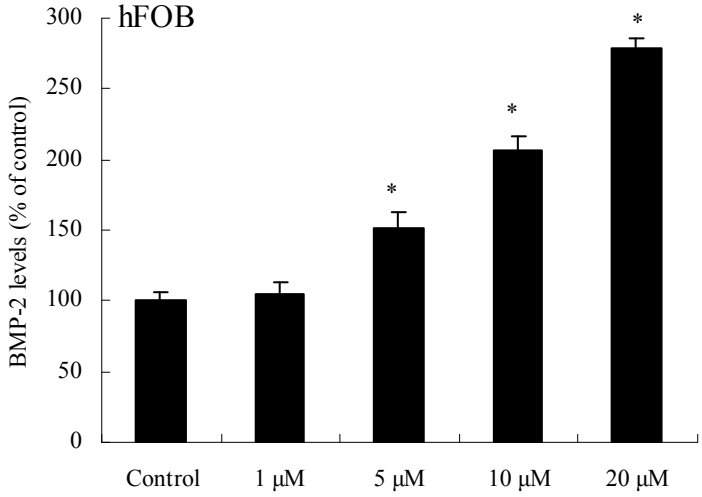

hFOB
BMP-2 levels (% of control)
300
250
200
150
100
50
0
Control
1 μM
5 μM
10 μM
20 μM
*

Fig. 4.

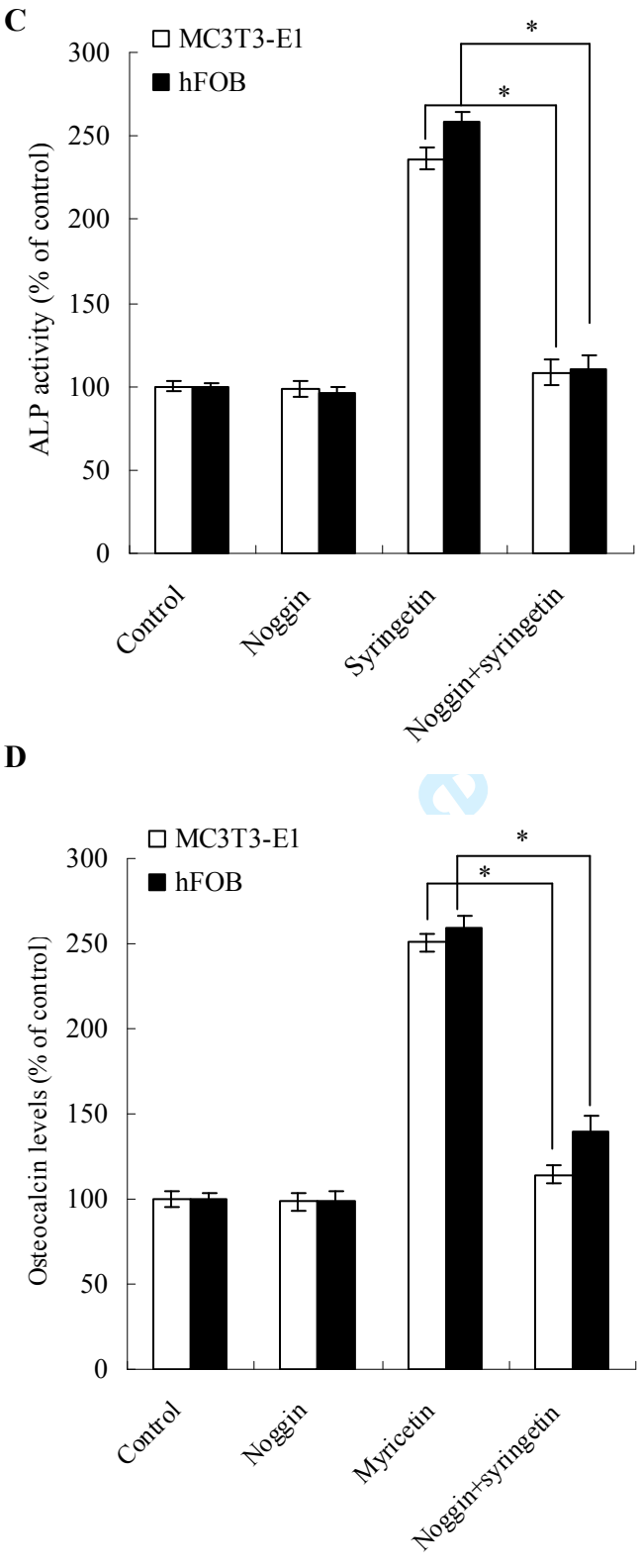

C
MC3T3-E1
hFOB
ALP activity (% of control)
300
250
200
150
100
50
0
*
*
Control
Noggin
Syringetin
Noggin+syringetin
D
MC3T3-E1
hFOB
Osteocalcin levels (% of control)
300
250
200
150
100
50
0
*
*
Control
Noggin
Myricetin
Noggin+syringetin

Fig. 4.

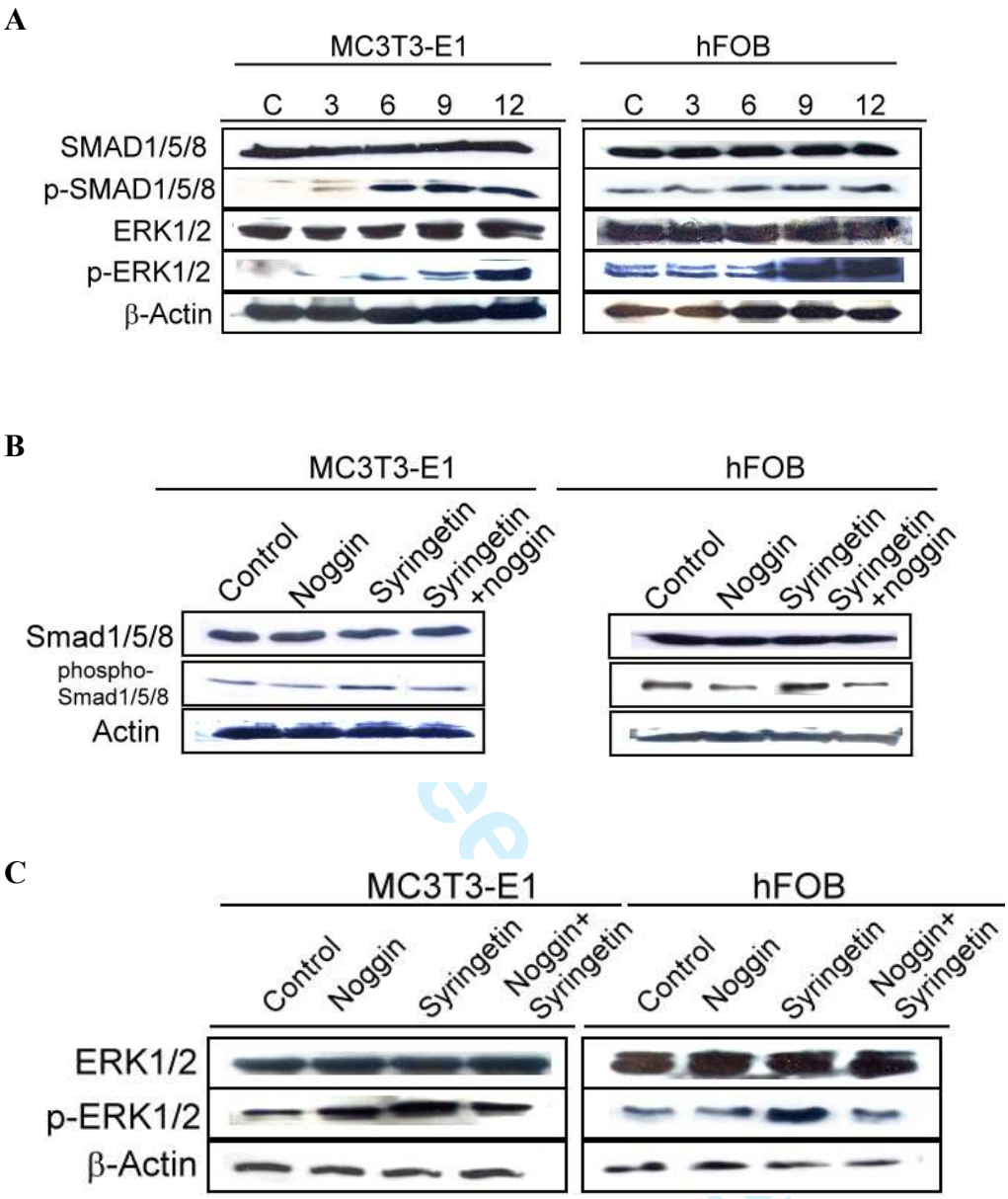

A
MC3T3-E1
hFOB
C 3 6 9 12
C 3 6 9 12
SMAD1/5/8
p-SMAD1/5/8
ERK1/2
p-ERK1/2
β-Actin
B
MC3T3-E1
hFOB
Control
Noggin
Syringetin
Syringetin +noggin
Smad1/5/8
phospho-Smad1/5/8
Actin
C
MC3T3-E1
hFOB
Control
Noggin
Syringetin
Noggin+ Syringetin
ERK1/2
p-ERK1/2
β-Actin

Fig. 5.

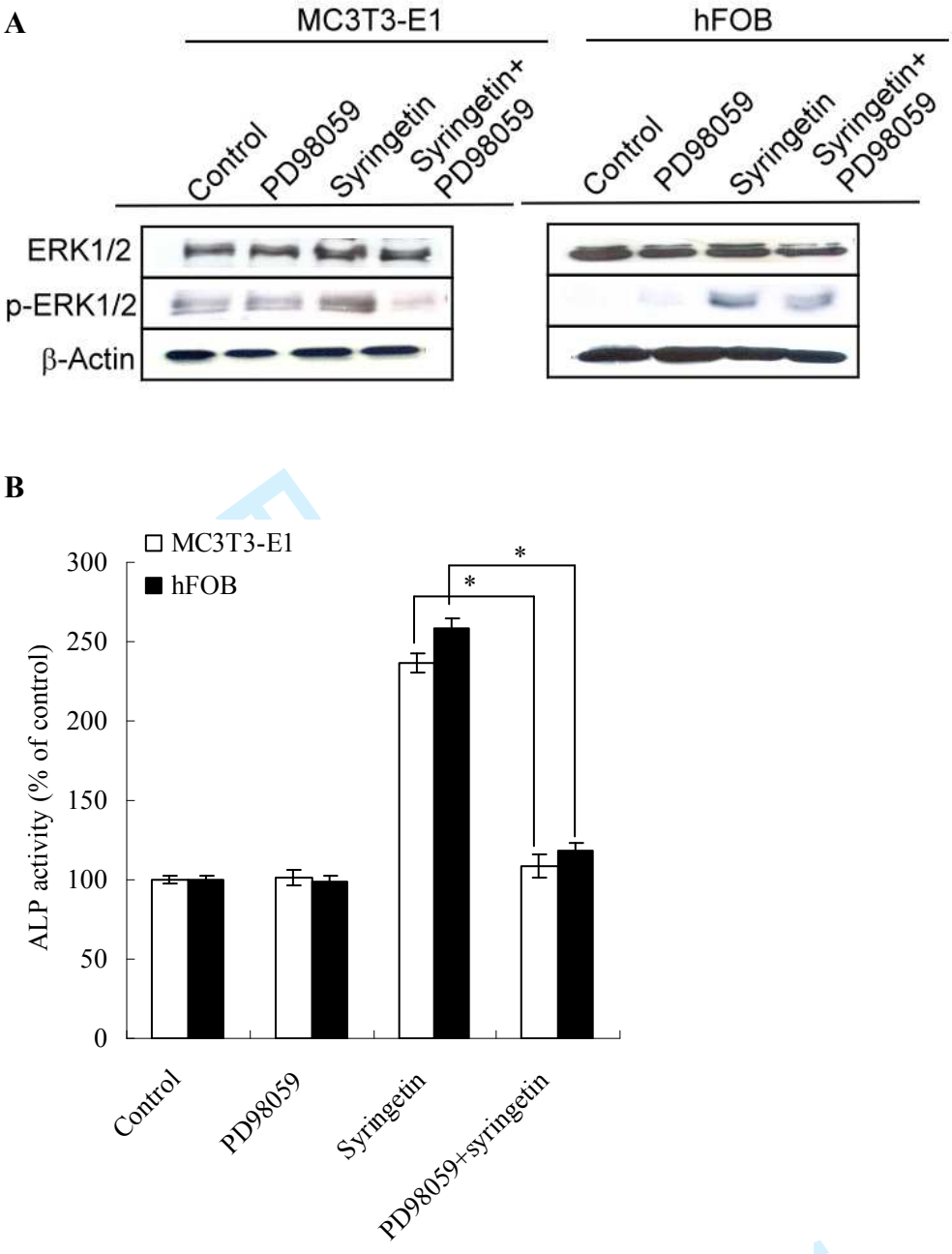

A
MC3T3-E1
hFOB
Control
PD98059
Syringetin
Syringetin+ PD98059
ERK1/2
p-ERK1/2
β-Actin
B
MC3T3-E1
hFOB
ALP activity (% of control)
300
250
200
150
100
50
0
*
*
Control
PD98059
Syringetin
PD98059+syringetin

Fig. 6.

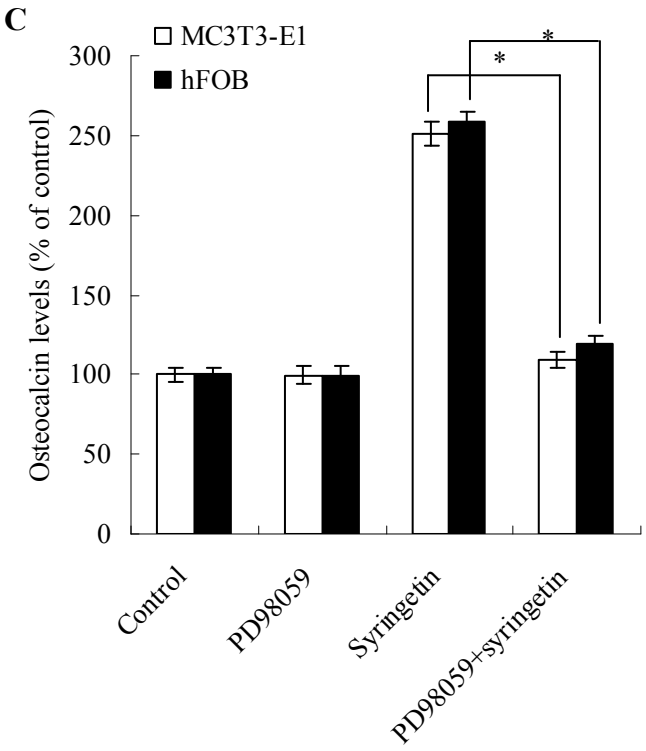


Fig. 6.