RESEARCH ARTICLE

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 (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 ELISA, we have shown that syringetin exhibits a significant induction of differentiation in MC3T3-E1 mouse calvaria osteoblasts and human fetal osteoblastic 1.19 cell line human osteoblasts. ALP 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 (ERK1/2). Cotreatment of ERK1/2 inhibitor 2′-amino-3′-methoxyflavone 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:
Bone morphogenetic protein-2 / Differentiation / Extracellular signal-regulated kinase1/2 / Osteoblasts / Syringetin

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

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Abbreviations: ALP, alkaline phosphatase; **BMP,** bone morphogenetic protein; **ERK,** extracellular signal-regulated kinase; **hFOB,** human fetal osteoblastic 1.19 cell line; **MAPK,** mitogen-activated protein kinase; **MEK,** mitogen-activated protein kinase kinase; **PD98059,** 2′-amino-3′-methoxyflavone

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 (BMPs) form a unique group of proteins within the transforming growth factor β superfamily. Fifteen BMPs have presently been identified and divided into subfamilies according to their amino acid sequences [8–11]. BMPs 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 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, such as SMADs, appear to be activated by the binding of BMPs and their 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].

Mitogen-activated protein kinases (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 the tyrosine residues of a conserved T-X-Y motif within the activation loop by a dual-specificity MAPK kinase (MKK or mitogen-activated protein kinase kinase (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 2′-amino-3′-methoxyflavone (PD98059) [18, 19]. The downstream factors of MEKs, extracellular signal-regulated kinase 1/2 (ERK1/2), show 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 human fetal osteoblastic 1.19 cell line (hFOB) human osteoblasts that are mediated through BMP/ERK1/2 pathway.

2 Materials and methods

2.1 Chemicals and reagents

Fetal bovine serum, α-minimal essential medium, 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, phosphorylated 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). 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 hFOB (CRL-11372), was maintained in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (GIBCO-BRL) containing 10% fetal bovine serum 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-minimal essential medium and antibiotics.

2.3 Cell proliferation assay (XTT)

Inhibition of cell proliferation by syringetin was measured by XTT assay (Roche Diagnostics). 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. In total, 50 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 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.

Alkaline phosphatase (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 with *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) 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, and 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 by following 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

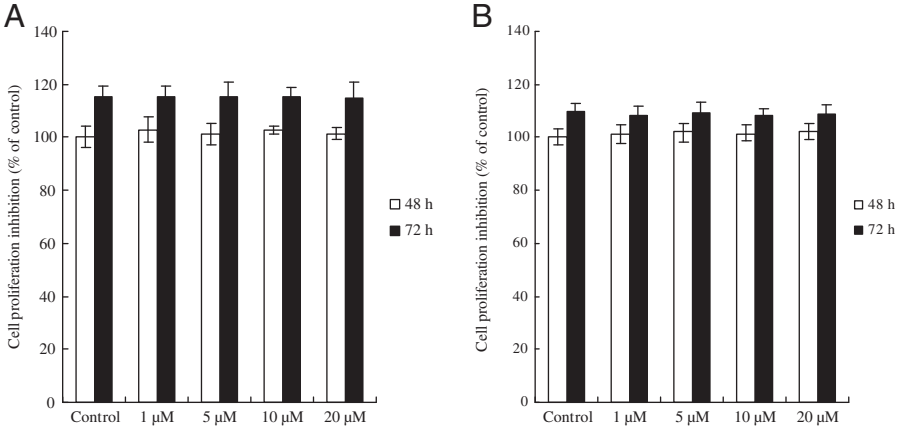

Figure 1. The effect of syringetin on the cell proliferation of MC3T3-E1 (A) and hFOB (B) cells. Adherent cells that proliferated in 96-well plates (8×10^3 cells/well) were incubated with different concentrations (μM) of syringetin for various time intervals. Cell proliferation was determined by XTT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

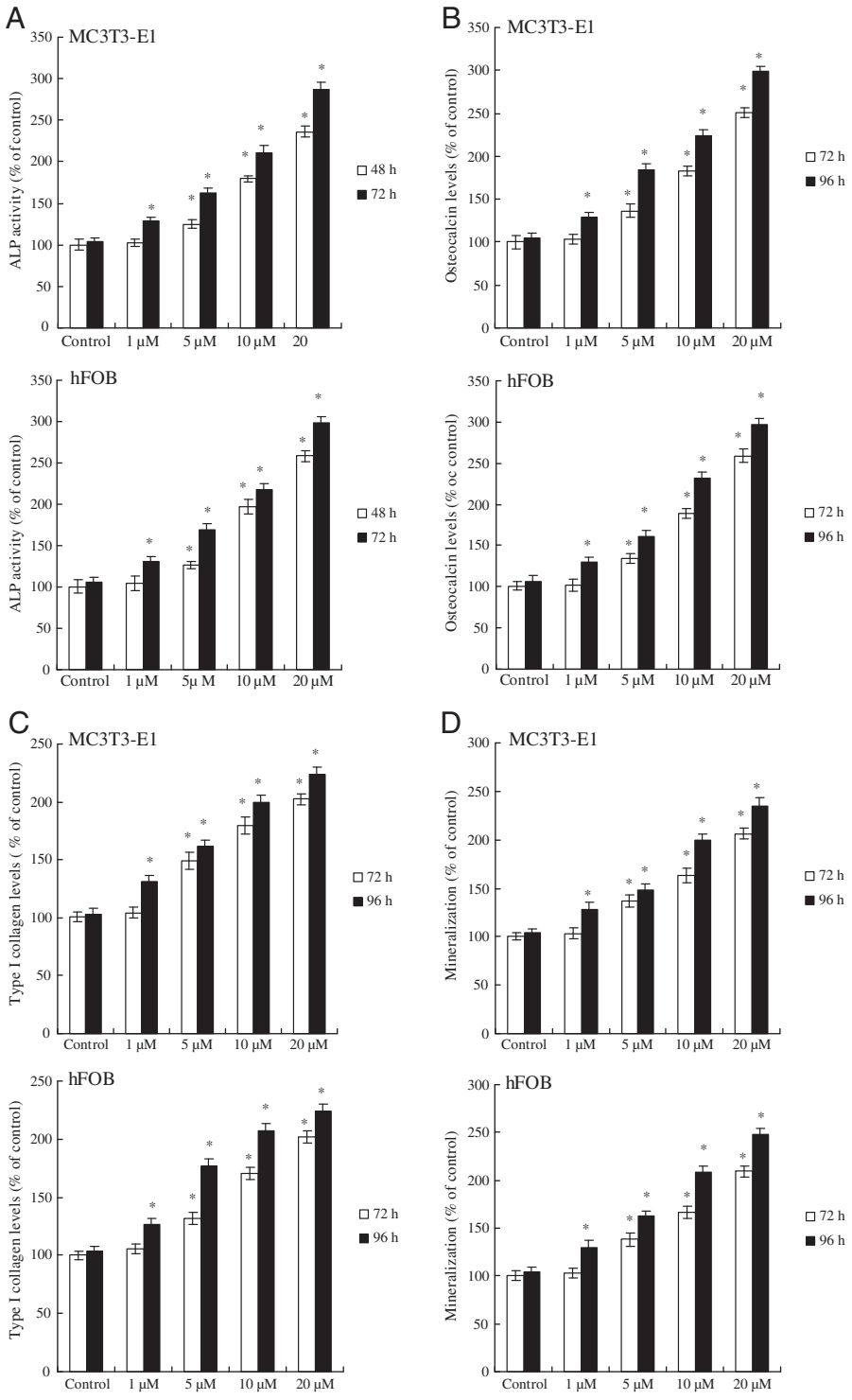

Figure 2. Syringetin increased the osteoblastic activity in MC3T3-E1 and hFOB cells. Syringetin increased ALP activity (A), osteocalcin production (B), type I collagen synthesis (C), and mineralization (D). ALP activity was assessed by the conversion of *p*-nitrophenyl phosphate in 0.1 M $NaHCO_3$–Na_2CO_3 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 ($p < 0.05$).

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 with 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, USA). For immunoblot, 50 μg of total cell lysates

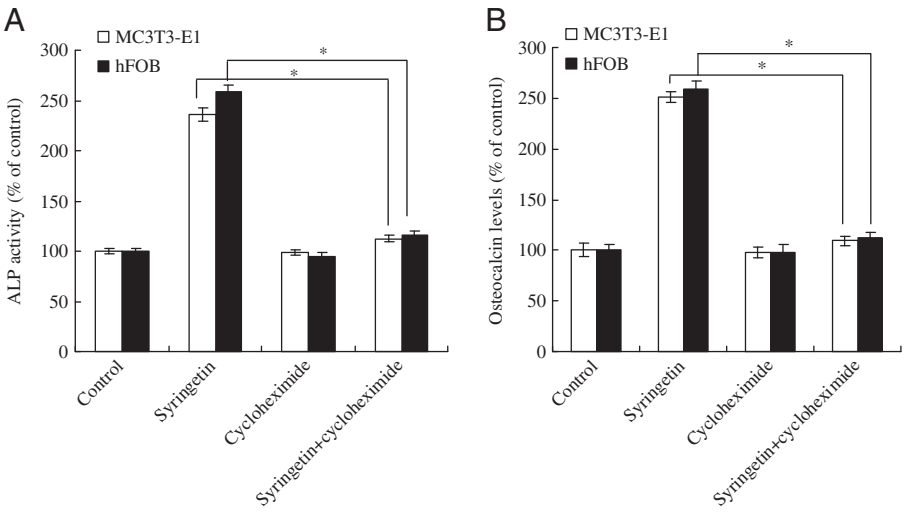

Figure 3. Inhibition of cycloheximide on syringetin-induced increase of ALP activity and osteocalcin production. (A) The effect of cycloheximide on syringetin-induced ALP activity. (B) The effect of cycloheximide on syringetin-mediated osteocalcin production. Cells were pretreated with or without 10 μg/mL cycloheximide for 24 h, and 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 in Section 2. 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 ($p < 0.05$).

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°C. 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, phosphorylated ERK, and phospho-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, Piscataway, NI, 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 (1–20 μM) after 48 and 72 h of treatment in MC3T3-E1 and hFOB cells (Figs. 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

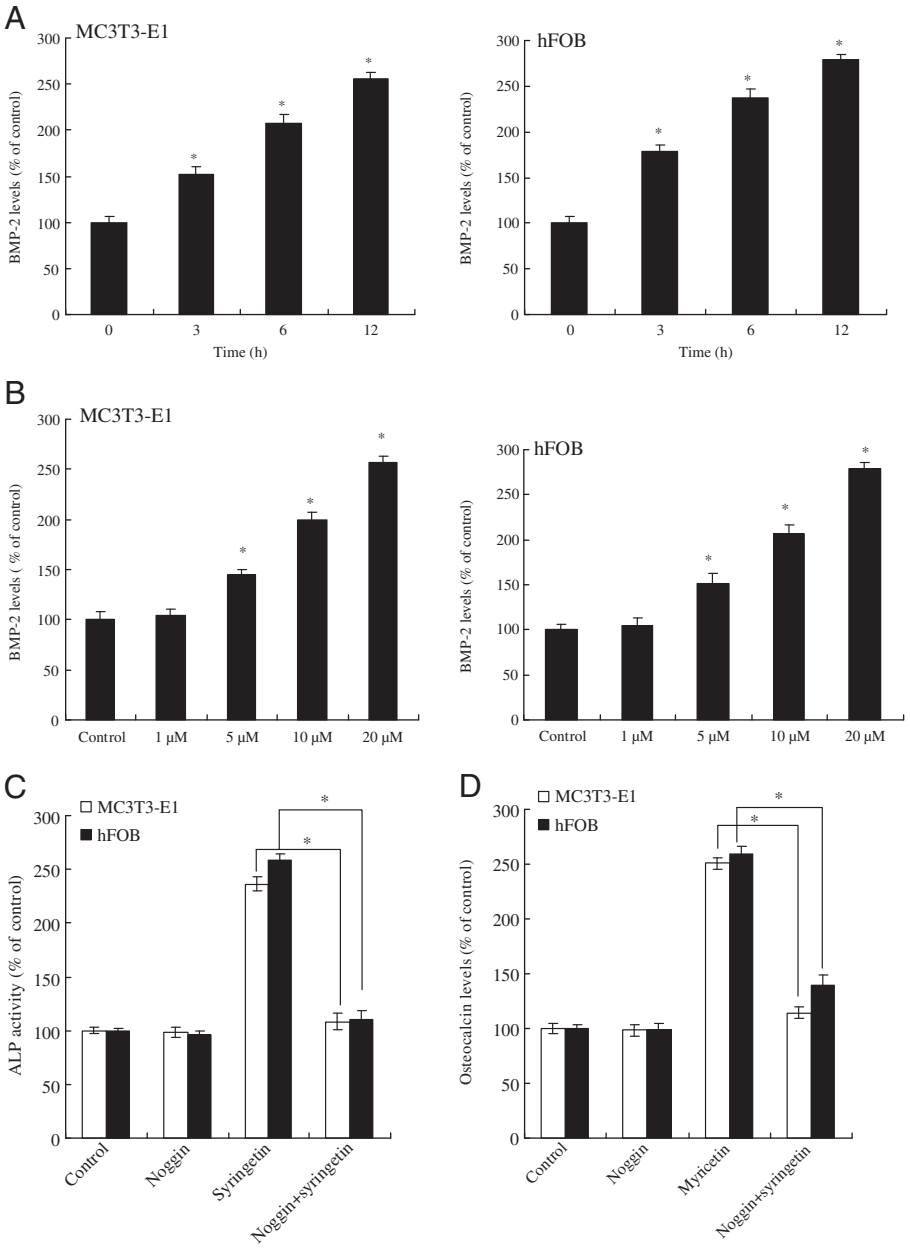

Figure 4. The role of BMP-2 in upregulation of osteoblastic activity by syringetin in MC3T3-E1 and hFOB cells. Syringetin increased the production of BMP-2 in a time-dependent (A) and dose-dependent manner (B). Noggin inhibited the induction of syringetin on ALP upregulation (C) and osteocalcin stimulation (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 ($p < 0.05$).

enhancement 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) (Figs. 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 BMPs 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 (Figs. 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 (Figs. 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.

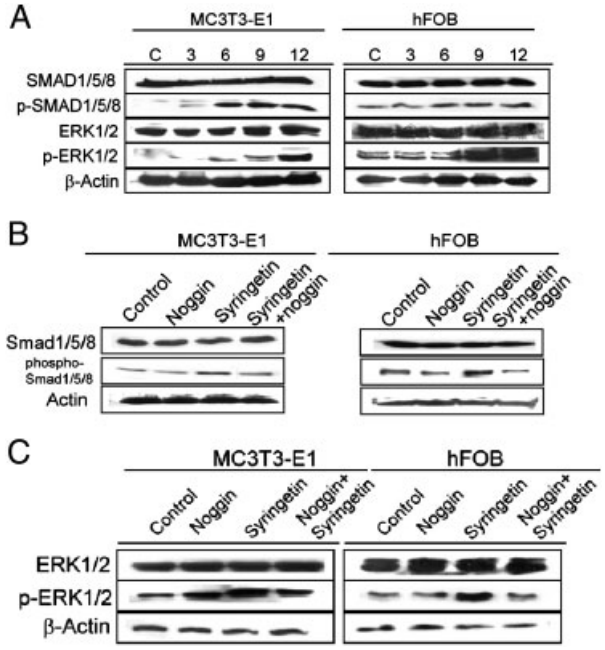

Figure 5. The activation of SMADs, ERK1/2 in syringetin-treated MC3T3-E1 and hFOB cells. Syringetin increased SMAD1/5/8 and ERK1/2 activation (A). Noggin decreased the effect of syringetin on the activation of SMAD1/5/8 (B) and ERK1/2 (C). For (A), unphospho- and phospho-SMAD and ERK1/2, cells were treated 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.

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 were 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 (Figs. 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

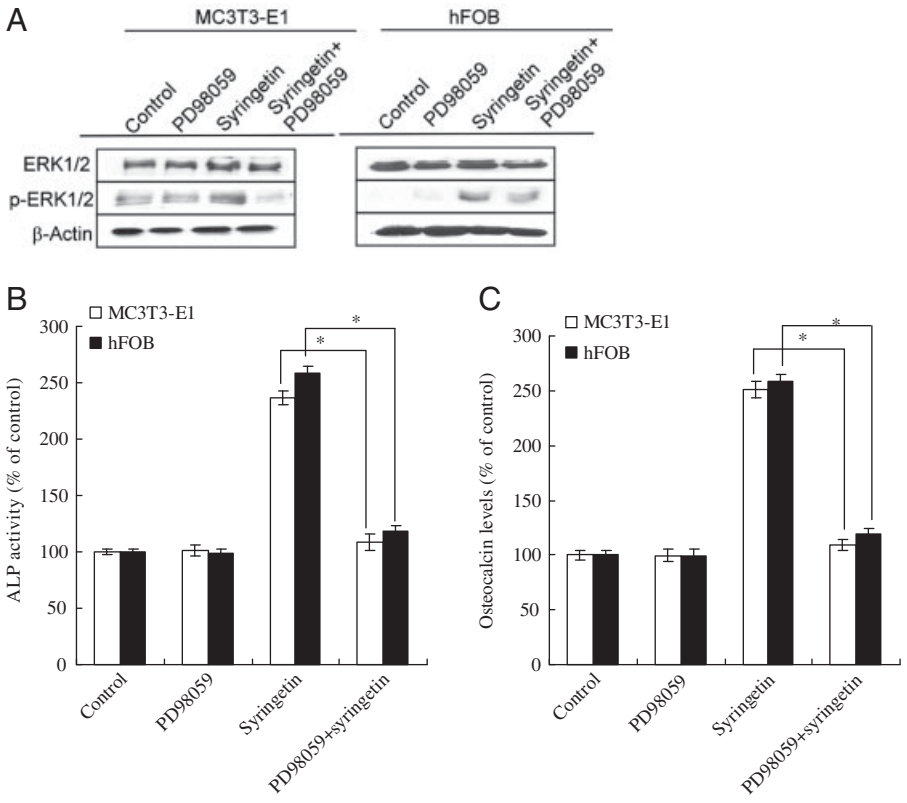

Figure 6. The role of ERK1/2 on the upregulation of syringetin on osteoblastic activity in MC3T3-E1 and hFOB cells. (A) PD98059 inhibited syringetin-induced ERK1/2 activation. Effect of PD98059 on syringetin-induced ALP activity (B) and osteocalcin production (C). Cells were incubated for 1 h in the 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 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 ($p < 0.05$).

osteoblasts 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 [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, BMPs 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.

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The authors have declared no conflict of interest.

5 References

[1] Ducy, P., Schinke, T., Karsenty, G., The osteoblast: A sophisticated fibroblast under central surveillance. *Science* 2000, *289*, 1501–1504.

[2] Manolagas, S.-C., Jilka, R.-L., Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N. Engl. J. Med.* 1995, *332*, 305–311.

[3] Teitelbaum, S.-L., Bone resorption by osteoclasts. *Science* 2000, *289*, 1504–1508.

[4] Rodan, G.-A., Martin, T.-J., Therapeutic approaches to bone diseases. *Science* 2000, *289*, 1508–1514.

[5] Schelonka, E.-P., Usher, A., Ipriflavone and osteoporosis. *J. Am. Med. Assoc.* 2001, *286*, 1836–1837.

[6] Berg, C., Neumeyer, K., Kirkpatrick, P., Teriparatide. *Nat. Rev. Drug Discov.* 2003, *2*, 257–258.

[7] Lane, N.-E., Kelman, A., A review of anabolic therapies for osteoporosis. *Arthritis Res. Ther.* 2003, *5*, 214–222.

[8] Sykaras, N., Opperman, L.-A., Bone morphogenetic proteins (BMPs): how do they function and what can they offer the clinician? *J. Oral Sci.* 2003, *45*, 57–73.

[9] Nohe, A., Keating, E., Knaus, P., Petersen, N.-O., Signal transduction of bone morphogenetic protein receptors. *Cell Signal* 2004, *16*, 291–299.

[10] Groeneveld, E.-H., Burger, E.-H., Bone morphogenetic proteins in human bone regeneration. *Eur. J. Endocrinol.* 2000, *142*, 9–21.

[11] Jia, T.-L., Wang, H.-Z., Xie, L.-P., Wang, X.-Y., Zhang, R.-Q., Daidzein enhances osteoblast growth that may be mediated by increased bone morphogenetic protein (BMP) production. *Biochem. Pharmacol.* 2003, *65*, 709–715.

[12] Friedman, M.-S., Long, M.-W., Hankenson, K.-D., Osteogenic differentiation of human mesenchymal stem cells is regulated by bone morphogenetic protein-6. J. *Cell Biochem.* 2006, *98*, 538–554.

[13] Huang, W., Carlsen, B., Wulur, I., Rudkin, G., Ishida, K., Wu, B., Yamaguchi, D. T., Miller, T. A., BMP-2 exerts differential effects on differentiation of rabbit bone marrow stromal cells grown in two-dimensional and three-dimensional systems and is required for in vitro bone formation in a PLGA scaffold. *Exp. Cell Res.* 2004, *299*, 325–334.

[14] Lecanda, F., Avioli, L.-V., Cheng, S.-L., Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *J. Cell Biochem.* 1997, *67*, 386–396.

[15] Takuwa, Y., Ohse, C., Wang, E.-A., Wozney, J.-M., Yamashita, K., Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1. *Biochem. Biophys. Res. Commun.* 1991, *174*, 96–101.

[16] Chang, L., Karin, M., Mammalian MAP kinase signalling cascades. *Nature* 2001, *410*, 37–40.

[17] Jadlowiec, J., Koch, H., Zhang, X., Campbell, P.-G., Seyedain, M., Sfeir, C., Phosphophoryn regulates the gene expression and differentiation of NIH3T3, MC3T3-E1, and human mesenchymal stem cells via the integrin/MAPK signaling pathway. *J. Biol. Chem.* 2004, *279*, 53323–53330.

[18] Jaiswal, R.-K., Jaiswal, N., Bruder, S.-P., Mbalaviele, G., Marshak, D.-R., Pittenger, M.-F., Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J. Biol. Chem.* 2000, *275*, 9645–9652.

[19] Salasznyk, R.-M., Klees, R.-F., Hughlock, M.-K., Plopper, G.-E., ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin. *Cell Commun. Adhes.* 2004, *11*, 137–153.

[20] Rodriguez, J.-P., Rios, S., Fernandez, M., Santibanez, J.-F., Differential activation of ERK1,2 MAP kinase signaling pathway in mesenchymal stem cell from control and osteoporotic postmenopausal women. *J. Cell Biochem.* 2004, *92*, 745–754.

[21] Ge, C., Xiao, G., Jiang, D., Franceschi, R.-T., Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J. Cell Biol.* 2007, *176*, 709–718.

[22] Scalbert, A., Williamson, G., Dietary intake and bioavailability of polyphenols. *J. Nutr.* 2000, *130*, 2073S–2085S.

[23] De Wilde, A ., Lieberherr, M ., Colin, C ., Pointillart, A., A low dose of daidzein acts as an ERbeta-selective agonist in trabecular osteoblasts of young female piglets. *J. Cell Physiol.* 2004, *200*, 253–262.

[24] Morris, C., Thorpe, J., Ambrosio, L., Santin, M., The soybean isoflavone genistein induces differentiation of MG63 human osteosarcoma osteoblasts. *J. Nutr.* 2006, *136*, 1166–1170.

[25] Prouillet, C., Maziere, J.-C., Maziere, C., Wattel, A., Brazier, M., Kamel, S., Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MC3T3-E1 human osteoblasts through ERK and estrogen receptor pathway. *Biochem. Pharmacol.* 2004, *67*, 1307–1313.

[26] Wu, J.-B., Fong, Y.-C., Tsai, H.-Y., Chen, Y.-F., Tsuzuki, M., Tang, C.- H., Naringin-induced bone morphogenetic protein-2 expression via PI3K, Akt, c-Fos/c-Jun and AP-1 pathway in osteoblasts. *Eur. J. Pharmacol.* 2008, *588*, 333–341.

[27] Hsu, Y.-L., Chang, J.-K., Tsai, C.-H., Chien, T.-T., Kuo, P.-L., Myricetin induces human osteoblast differentiation through bone morphogenetic protein-2/p38 mitogen-activated protein kinase pathway. *Biochem. Pharmacol.* 2007, *73*, 504–514.

[28] Hsu, Y.-L., Kuo, P.-L., Diosmetin induces human osteoblastic differentiation through the protein kinase C/p38 and extracellular signal-regulated kinase 1/2 pathway. *J. Bone Miner. Res.* 2008, *23*, 949–960.

[29] Wang, H., Race, E.-J., Shrikhande, A.-J., Anthocyanin transformation in Cabernet Sauvignon wine during aging. *J. Agric. Food Chem.* 2003, *51*, 7989–7994.

[30] Mattivi, F., Guzzon, R., Vrhovsek, U., Stefanini, M., Velasco, R., Metabolite profiling of grape: flavonols and anthocyanins. *J. Agric. Food Chem.* 2006, *54*, 7692–7702.

[31] Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E., Hermosín-Gutiérrez, I., Flavonol profiles of *Vitis vinifera* red grapes and their single-cultivar wines. *J. Agric. Food Chem.* 2007, *55*, 992–1002.

[32] Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E., Gómez, M.-V., Velders, A.-H., Hermosín-Gutiérrez, I., Flavonol 3-O-glycosides series of *Vitis vinifera* Cv. Petit Verdot red wine grapes. *J. Agric. Food Chem.* 2009, *57*, 209–219.

[33] Reinholz, G.-G., Getz, B., Pederson, L., Sanders, E.-S., Subramaniam, M., Ingle, J.-N., Spelsberg, T.-C., Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. *Cancer Res.* 2000, *60*, 6001–6007.

[34] Kuo, P.-L., Hsu, Y.-L., Chang, C.-H., Chang, J.-K., Osthole-mediated cell differentiation through bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase 1/2 pathway in human osteoblast cells. *J. Pharmacol. Exp. Ther.* 2005, *314*, 1290–1299.

[35] Zhu, W., Kim, J., Cheng, C., Rawlins, B. A., Boachie-Adjei, O., Crystal, R.-G., Hidaka, C., Noggin regulation of bone morphogenetic protein (BMP) 2/7 heterodimer activity *in vitro*. *Bone* 2006, *39*, 61–71.

[36] Aubin, J.-E., Liu, F., Malaval, L., Gupta, A.-K., Osteoblast and chondroblast differentiation. *Bone* 1995, *17*, 77S–83S.

[37] Qu, Q., Perala-Heape, M., Kapanen, A., Dahllund, J., Salo, J., Vaananen, H.-K., Harkonen, P., Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. *Bone* 1998, *22*, 201–209.

[38] Wada, Y., Kataoka, H., Yokose, S., Ishizuya, T., Miyazono, K., Gao, Y.-H., Shibasaki, Y., Yamaguchi, A., Changes in osteoblast phenotype during differentiation of enzymatically isolated rat calvaria cells. *Bone* 1998, *22*, 479–485.

[39] Xiao, Y., Haase, H., Young, W.-G., Bartold, P.-M., Development and transplantation of a mineralized matrix formed by osteoblasts in vitro for bone regeneration. *Cell Transplant.* 2004, *13*, 15–25.

[40] Kuo, P.-L., Huang, Y.-T., Chang, C.-H., Chang, J.-K., Bone morphogenetic protein-2 and -4 (BMP-2 and -4) mediates fraxetin-induced maturation and differentiation in human osteoblast-like cell lines. *Biol. Pharm. Bull.* 2006, *29*, 119–124.

[41] Cortizo, A.-M., Lettieri, M.-G., Barrio, D.-A., Mercer, N., Etcheverry, S.-B., McCarthy, A.-D., Advanced glycation end-products (AGEs) induce concerted changes in the osteoblastic expression of their receptor RAGE and in the activation of extracellular signal-regulated kinases (ERK). *Mol. Cell Biochem.* 2003, *250*, 1–10.

