BIOCHEMICAL PHARMACOLOGY 73 (2007) 504–514

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Myricetin induces human osteoblast differentiation through bone morphogenetic protein-2/p38 mitogen-activated protein kinase pathway

Ya-Ling Hsu [a], *Jiunn-Kae Chang* [b], *Chu-Hung Tsai* [b], *Tzu-Tsung Chang Chien* [b], *Po-Lin Kuo* [b,*]

[a] *Department of Pharmacy, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan*
[b] *Cell Biology Laboratory, Department of Biotechnology, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan*

ARTICLE INFO

Article history:
Received 29 August 2006
Accepted 20 October 2006

Keywords:
Myricetin
Osteoblasts
Differentiation
Bone morphogenetic protein-2
p38

ABSTRACT

Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), a flavonoid compound, is present in vegetables and fruits. By means of alkaline phosphatase (ALP) activity, osteocalcin, and type I collagen enzyme-linked immunosorbent assay (ELISA), we have shown that myricetin exhibits a significant induction of differentiation in MG-63 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 myricetin stimulates osteoblast differentiation at various stages, from maturation to terminally differentiated osteoblasts. Induction of differentiation by myricetin is associated with increased bone morphogenetic protein-2 (BMP-2) production. The BMP-2 antagonist noggin blocked myricetin-mediated ALP activity and osteocalcin secretion enhancement, indicating that BMP-2 production is required in myricetin-mediated osteoblast maturation and differentiation. Induction of differentiation by myricetin is associated with increased activation of SMAD1/5/8 and p38 mitogen-activated protein kinases. Cotreatment of p38 inhibitor SB203580 inhibited myricetin-mediated ALP upregulation and osteocalcin production. In conclusion, myricetin increased BMP-2 synthesis, and subsequently activated SMAD1/5/8 and p38 MAPK, and this effect may contribute to its action on the induction of osteoblast maturation and differentiation, followed by an increase of bone mass.

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 include bisphosphonates, calcitonin, estrogen, Vitamin D analogues, and ipriflavone. These are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts [4,5]. However, the effect of these drugs in increasing or recovering bone mass is relatively small, certainly no more than 2% per

* *Corresponding author*. Tel.: +886 6 2664911x520; fax: +886 6 2662135.
E-mail address: kuopolin@seed.net.tw (P.-L. Kuo).

Abbreviations: MAPK, mitogen-activated protein kinase; phospho-p38, phosphorylated p38; BMP, bone morphogenetic protein; ELISA, enzyme-linked immunosorbent assay; PNPP, 4-nitrophenyl phosphate; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole

0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.10.020

year [4]. It is desirable, therefore, to have satisfactory bone-building (anabolic) agents such as teriparatide, that stimulate new bone formation and correct the imbalance of trabecular microarchitecture characteristic of established osteoporosis [1,6]. Since new bone formation is primarily a function of the osteoblasts, agents which regulate bone formation act either by increasing the proliferation of cells of the osteoblastic lineage, or inducing differentiation of the osteoblasts [1,7].

Bone morphogenetic proteins (BMP) form a unique group of proteins within the transforming growth factor beta (TGF-β) superfamily and have pivotal roles in the regulation of bone induction, maintenance and repair, as well as being important determinants of mammalian embryological development [8,9]. Fifteen bone morphogenetic proteins have presently been identified, and have been further divided into subfamilies according to their amino acid sequences [10,11]. Bone morphogenetic proteins induce osteoblast differentiation of various types of cells, including undifferentiated mesenchymal cells, bone marrow stromal cells, and preosteoblasts [12–14]. In the BMP subfamily, bone morphogenetic protein-2 (BMP-2) was the earliest BMP 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, both of which have subcategories. Various signaling pathways appear to be activated by the ligand binding to the receptors. BMPs stimulate that activation of SMAD proteins that accumulate in the nucleus, and control transcription of a large number of target genes. The SMAD1, SMAD5, and SMAD8 are recognized by BMP type I receptor [8,9]. p38, one subfamily of MAPK, is activated not only by MKKs, but also by the BMP-2 pathway through SMAD-dependent or independent pathways [16,17]. In some types of cells, BMP-2 activates MAP kinase kinases kinase (MAPKKK), including TAK1, and consequently the MAPKKK elicits MKK3 or MKK6 that directly phosphorylates and activates p38 kinase [18,19]. Many studies have reported that p38 activation is necessary for differentiation in osteoblasts [20,21].

Flavonoids are a broadly distributed class of plant pigments, universally present in vascular plants, and responsible for much of the coloring in nature [22]. Several flavonoids, such as daidzein, genistein, biochanin A, quercetin, glycitein, and kaempferol, have been reported to induce osteoblast differentiation [11,23–27]. These studies have provided evidence that the high dietary intake of flavonoids found in fruits and vegetables may be associated with a potential benefit in osteoporosis prevention. Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), a flavonoid compound, has been reported to possess antioxidative, antiproliferative, and anti-inflammatory effects [28–30]. Myricetin exhibits an anti-inflammatory effect through inhibiting IL-1 mRNA expression and NO production in LPS-treated RAW246.7 cells [29,30]. In an earlier report, we showed that myricetin is able to protect osteoblasts against apoptotic cell death induced by inflammatory cytokines [31]. In the present study, we have further examined the molecular mechanisms of myricetin on the differentiation of osteoblasts.

2. Materials and methods

2.1. Chemicals and reagents

Fetal bovine serum (FBS), minimal essential medium (MEM), penicillin G, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD, USA). BMP-2 ELISA kit, human BMP-2 protein, and noggin were purchased from R&D Systems

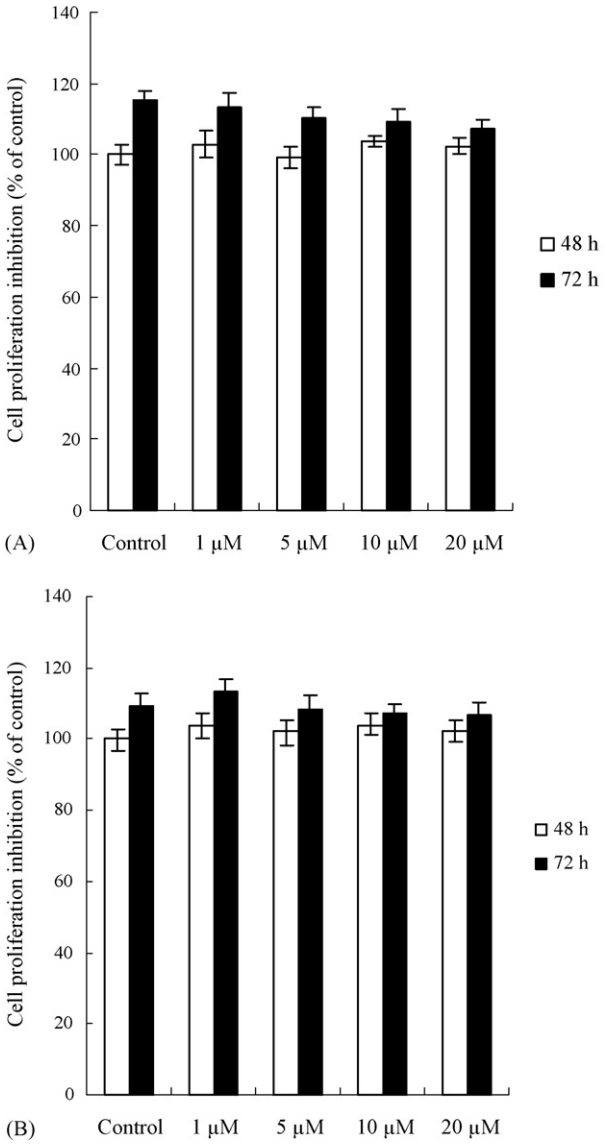

Fig. 1 – The effect of myricetin on the cell proliferation of MG-63 (A) and hFOB (B) cells. Adherent cells that proliferated in 96-well plates (8×10^3 cells/well) were incubated with different concentrations (μM) of myricetin for various time intervals. Cell proliferation was determined by XTT assay. Each value is the mean ± S.D. of three independent experiments. Standard deviations were less than 10%.

(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). The antibodies to p38 (5F11), SMDA1/5/8 (N18), phospho-p38 (3D7), and phospho-SMAD1/5/8 (41D10) were purchased from Cell Signaling Technology (Beverly, MA, USA). Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO, USA). The stock solution of myricetin 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 human osteoblastic cell lines were used: (1) the conditionally immortalized fetal osteoblastic cell line hFOB and (2) the human osteosarcoma cell line MG-63. 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

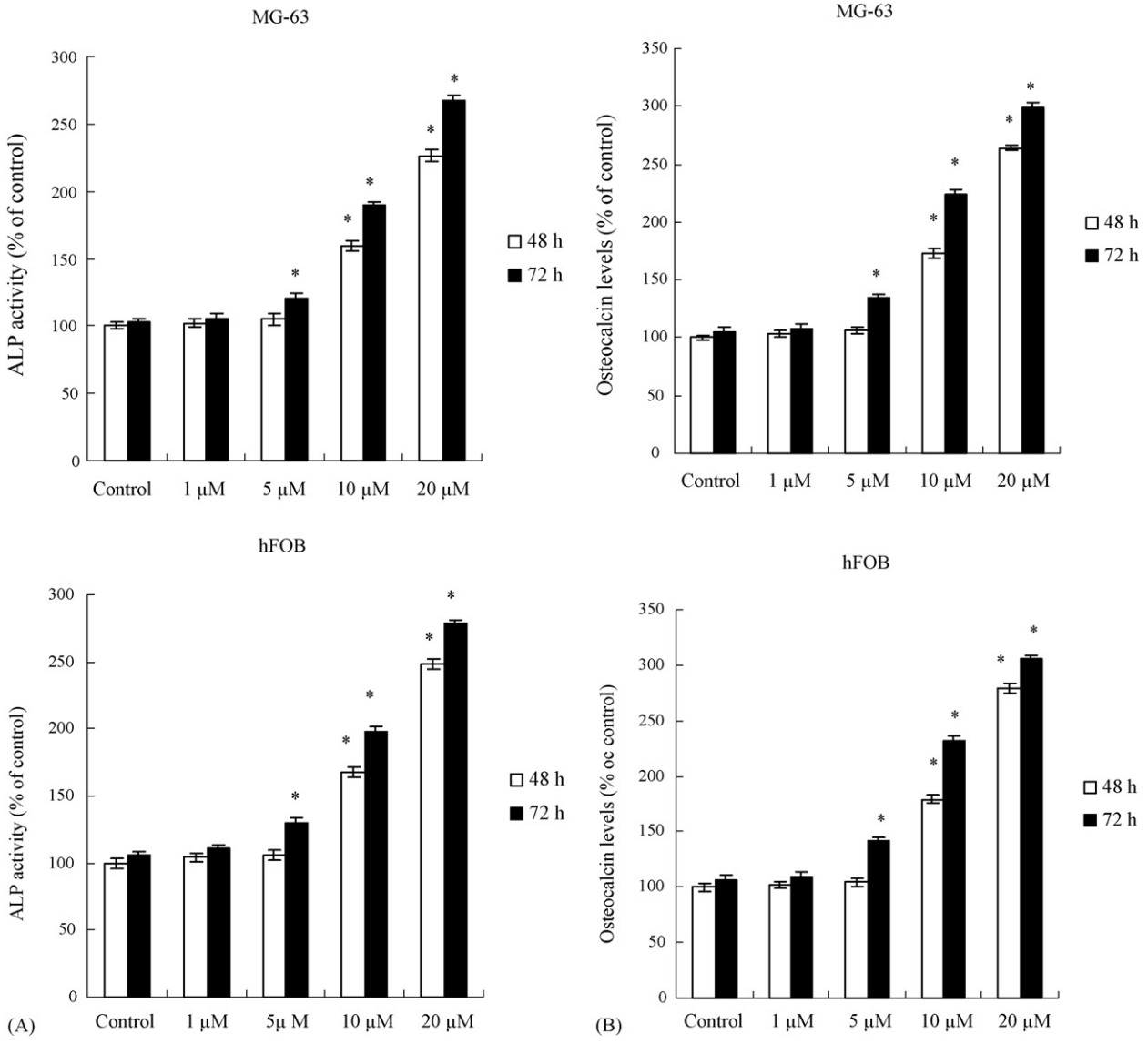

Fig. 2 – Myricetin increased the osteoblastic activity in MG-63 and hFOB cells. Myricetin increased ALP activity (A), osteocalcin production (B), type I collagen synthesis (C), and mineralization (D). ALP activity was assessed by the conversion of *p*-nitrophenyl phosphate in 0.1 M $NaHCO_3$–Na_2CO_3 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 ± S.D. of three independent experiments. The asterisk indicates a significant difference between control and myricetin-treated groups, as analyzed by Dunnett's test ($P < 0.05$).

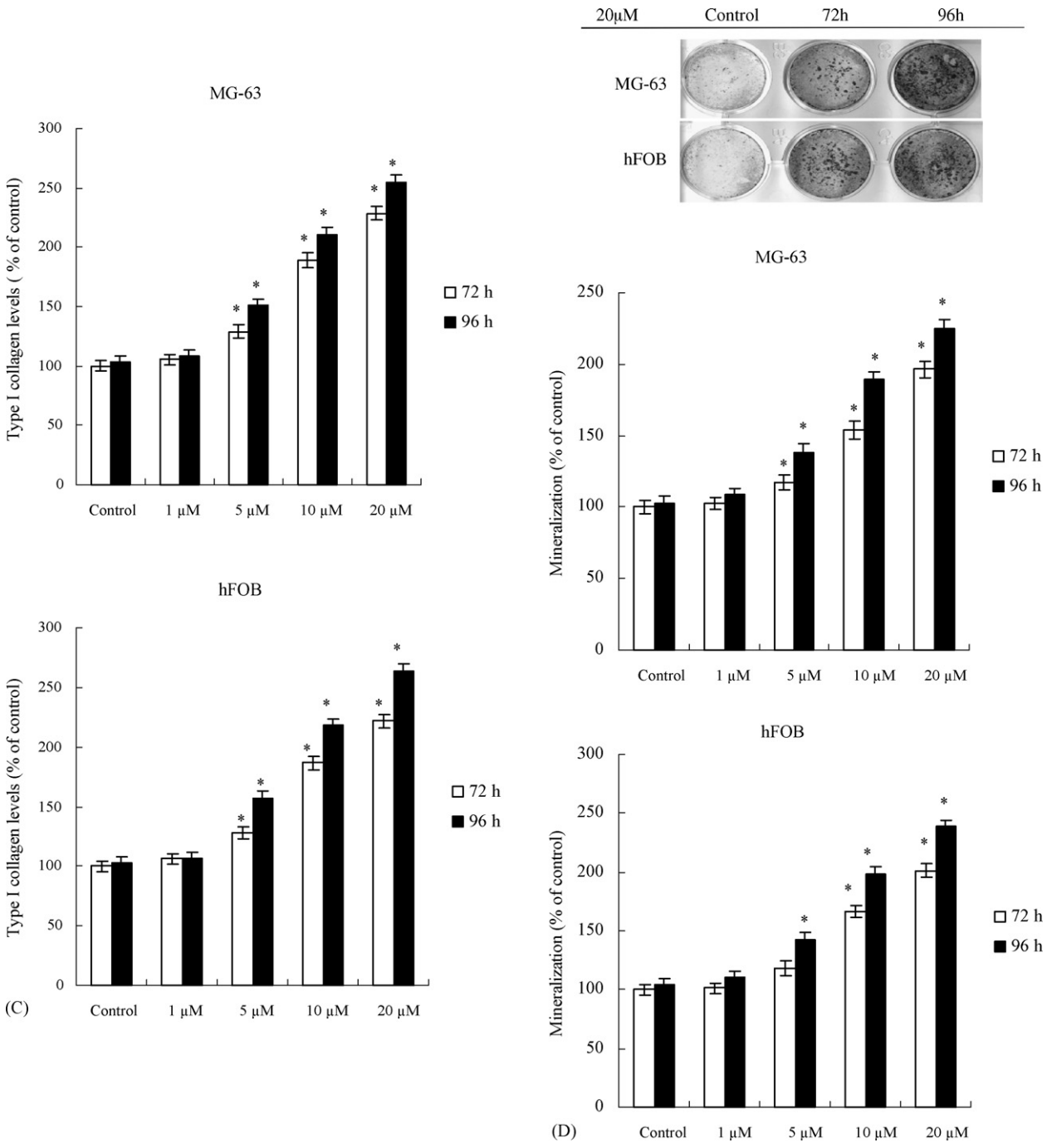

Fig. 2. (Continued).

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. The human osteosarcoma cell line (MG-63, CRL-1427) was cultured in minimal essential medium (MEM) (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml of penicillin G and 100 μg/ml of streptomycin).

2.3. *Cell proliferation assay (XTT)*

Inhibition of cell proliferation by myricetin 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 myricetin (0, 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 then added to each well. After 4 h of incubation, absorbance was measured on an ELISA reader

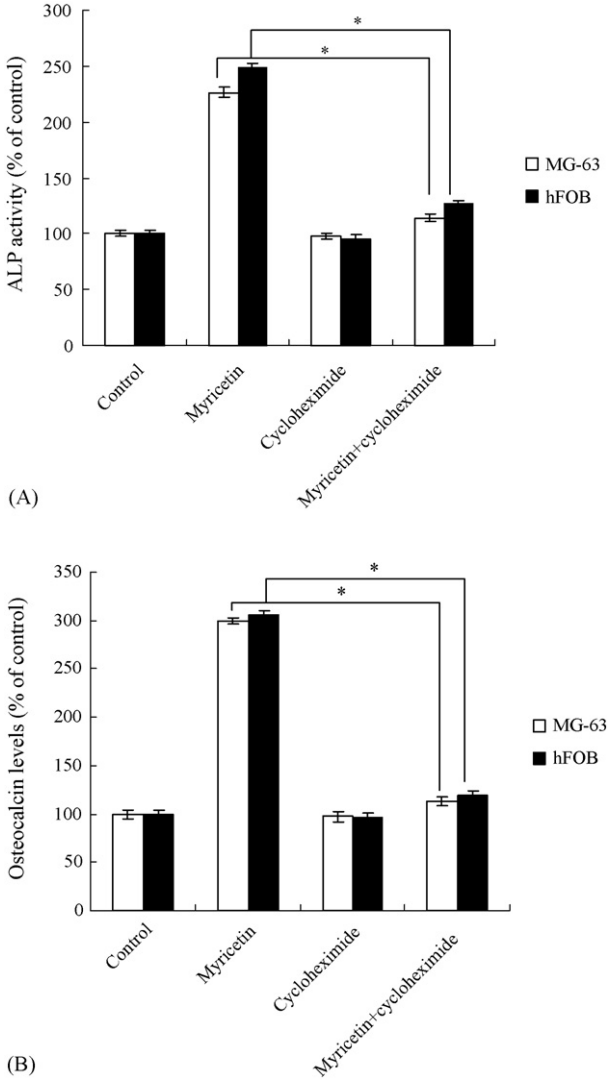

Fig. 3 – Inhibition of cycloheximide on myricetin-induced increase of ALP activity and osteocalcin production. (A) The effect of cycloheximide on myricetin-induced ALP activity. (B) The effect of cycloheximide on myricetin-mediated osteocalcin production. Cells were pretreated with or without 10 μg/ml cycloheximide for 24 h, then 20 μM myricetin 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 ± S.D. of three independent experiments. The asterisk indicates a significant difference between control and test groups, as analyzed by Dunnett's test ($P < 0.05$).

(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 [32].

2.6. Assaying the levels of type I procollagen

Cells were treated with various concentrations of myricetin 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 myricetin 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 [33].

2.8. Analysis for Western blotting

Cells treated with myricetin for the indicated times were lysed and the protein concentrations determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). For Western blotting, 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 °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, phosphorylated SMAD1/5/8, p38 and phosphorylated p38) for 2–15 h, followed by horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham, USA).

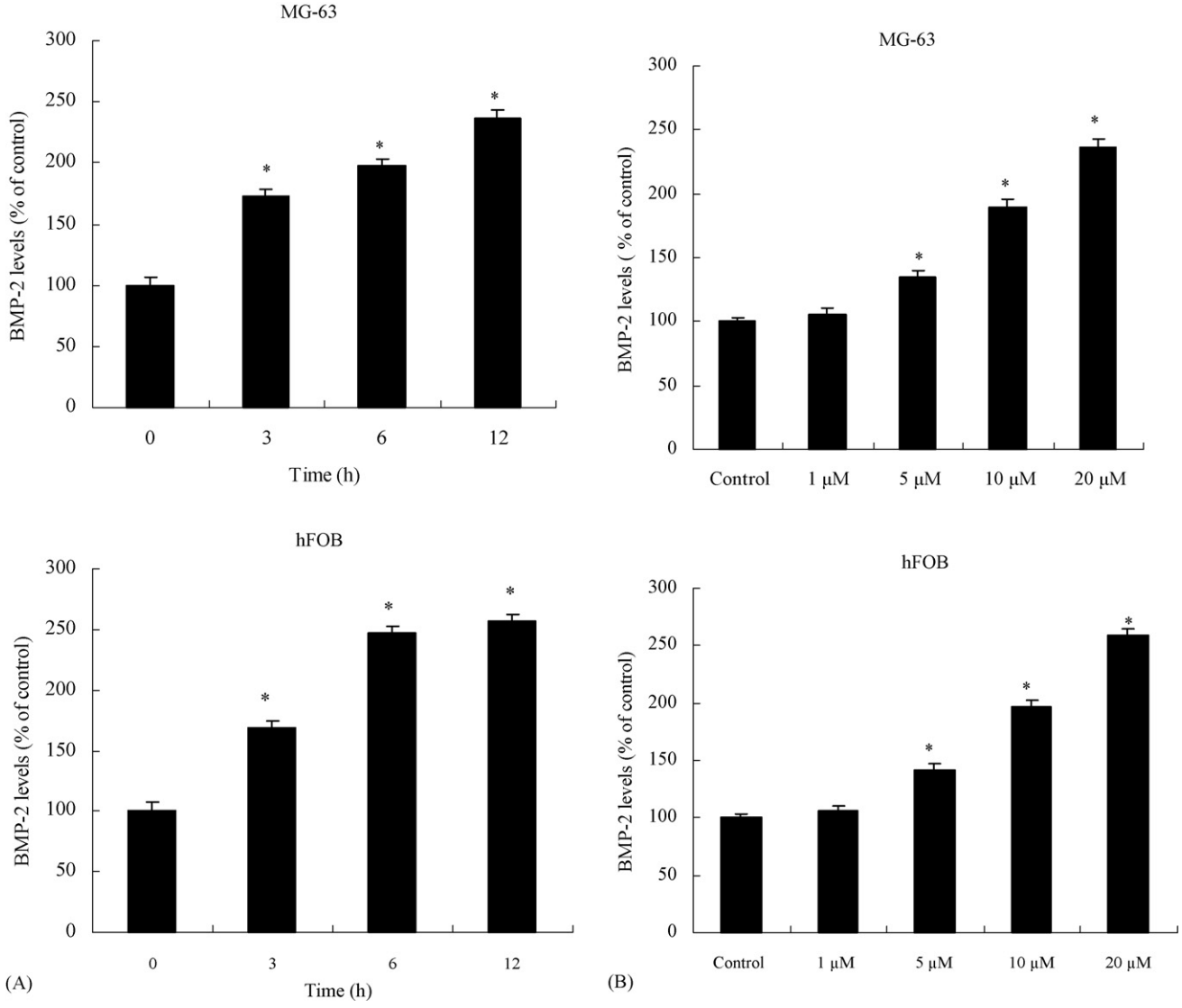

Fig. 4 – The role of BMP-2 in upregulation of osteoblastic activity by myricetin in MG-63 and hFOB cells. Myricetin increased the production of BMP-2 in a time-dependent (A) and dose-dependent manner (B). Noggin inhibited the induction of myricetin on ALP upregulation (C) and osteocalcin stimulation (D). For (A) and (B), cells were treated with 20 μM myricetin for the indicated times (0, 3, 6, and 12 h) at various concentrations (1, 5, 10, and 20 μM) of myricetin 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 myricetin 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 ± S.D. of three independent experiments. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett's test ($P < 0.05$).

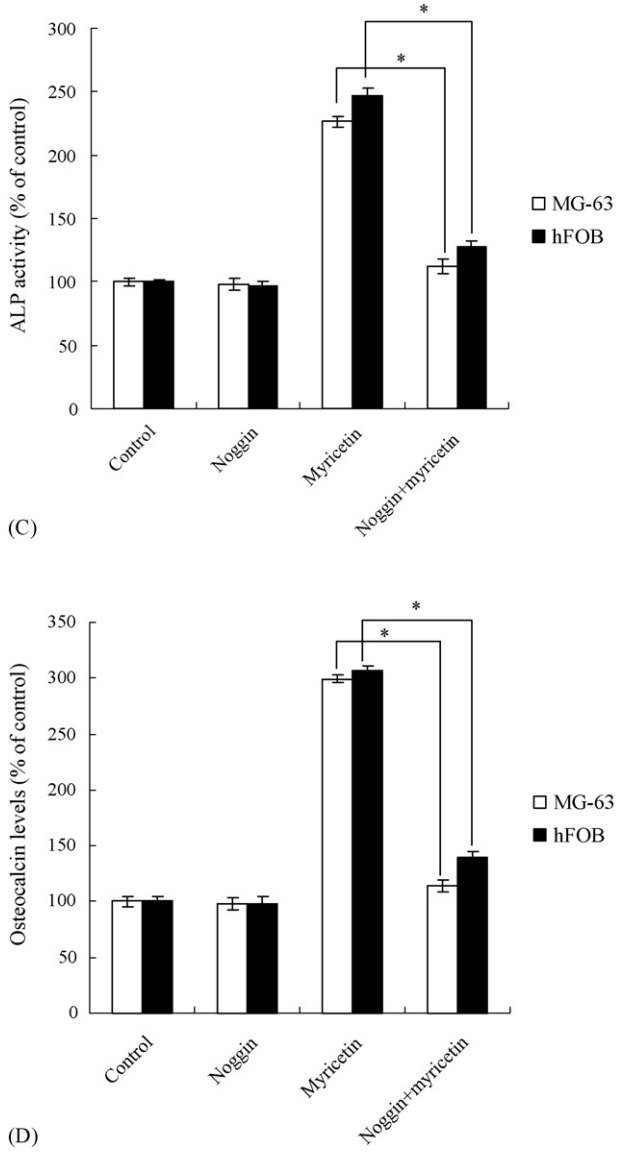

Fig. 4. (Continued).

2.9. Statistical analysis

Data were expressed as means ± S.D. 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. Myricetin had no effect on the proliferation of MG-63 and hFOB cells

We first determined the effect of myricetin on the cell proliferation of MG-63 and hFOB by XTT assay. Our results showed that myricetin did not exhibit significant effects on cell proliferation at the concentrations used (1–20 μM) after 48 and 72 h of treatment in MG-63 and hFOB cells (Fig. 1A and B).

3.2. Effect of myricetin on maturation and differentiation markers, ALP activity, osteocalcin expression, type I collagen synthesis, and mineralization in MG-63 and hFOB cells

The effect of myricetin on the maturation of osteoblasts was studied by determining ALP activity in MG-63 and hFOB cells. The results showed that myricetin increased ALP activity in MG-63 and hFOB cells in a dose-dependent manner after 48 and 72 h of treatment (Fig. 2A). The effect of myricetin 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 MG-63 and hFOB cells with myricetin increased the levels of osteocalcin protein in a dose-dependent manner after 48 and 72 h of treatment. In addition, type I collagen protein levels were also enhanced in myricetin-treated MG-63 and hFOB cells after 72 and 96 h of treatment (Fig. 2C). Treatment with myricetin for 72 and 96 h also increased the amount of osteoblasts mineralization in a dose-dependent manner (Fig. 2D).

3.3. Myricetin 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 myricetin on the increase of ALP activity and osteocalcin production was due to the enhancing of protein synthesis. The results showed that the myricetin-mediated increase of ALP activity and osteocalcin production is almost completely inhibited by a 24 h pretreatment of MG-63 and hFOB cells with protein synthesis inhibitor cycloheximide (10 μg/ml) (Fig. 3A and B).

3.4. BMP-2 mediates myricetin-induced maturation and differentiation in MG-63and hFOB cells

To confirm whether either transcriptional or translational levels of BMP-2 expression were influenced by the presence of myricetin, we examined the expression of the BMP-2 in the presence and absence of myricetin using bone morphogenetic proteins ELISA kits. The results indicated that myricetin caused a significant increase in BMP-2 protein levels in MG-63 and hFOB. The upregulation of BMP-2 by 20 μM myricetin protein started to increase 3 h after treatment with myricetin, and maximum expression was observed at 12 h (Fig. 4A). After 12 h of treatment, myricetin increased production of BMP-2 in a dose-dependent manner (Fig. 4B).

To further examine the role of BMP-2 in myricetin-induced cell differentiation, osteoblasts were pretreated with a BMP-2 inhibitor, 100 ng/ml noggin protein [34], for 1 h, then cotreated with 20 μM myricetin and the inhibitor for the indicated times. Addition of purified noggin protein did not change ALP activity and osteocalcin secretion, but abrogated myricetin-induced cell differentiation (Fig. 4C and D). Therefore, myricetin-induced

cell differentiation may operate by a BMP-2-dependent pathway.

3.5. Activations of SMAD1/5/8 and p38 in myricetin-treated MG-63 and hFOB cells

Ligation of BMP-2 to BMP receptor induces receptor heteromeric complexes and subsequently activates SMADs or MAPKs by phosphorylation. We first assessed activation (phosphorylation) of SMAD proteins in myricetin-treated MG-63 and hFOB cells. As shown in Fig. 5A, treatment with myricetin 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 myricetin, 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 p38 in myricetin-treated cells. The results showed that myricetin treatment increased the activation (phosphorylation) of p38 (Fig. 5A). p38 activation occurred later (9 h) than SMADs (3 h), indicating that p38 activation may be a downstream event of SMADs.

To determine the role of BMP-2 on the activation of SMAD1/5/8 and p38 in myricetin treated osteoblasts, we tested the effect of noggin on the activation of SMAD1/5/8 and p38 by myricetin. Results showed that noggin pretreatment abrogated the activation of SMAD1/5/8 and p38 induced by myricetin (Fig. 5B and C). Thus, BMP-2 signaling is necessary and sufficient to mediate the activation of SMAD1/5/8 and p38 in myricetin-treated MG-63 and hFOB cells.

3.6. p38 is necessary for myricetin-mediated osteoblast maturation and differentiation in MG-63 and hFOB cells

As p38 activation was observed with myricetin treated osteoblasts, we next assessed the role of p38 by using an inhibitor specific to p38. MG-63 and hFOB cells were pretreated

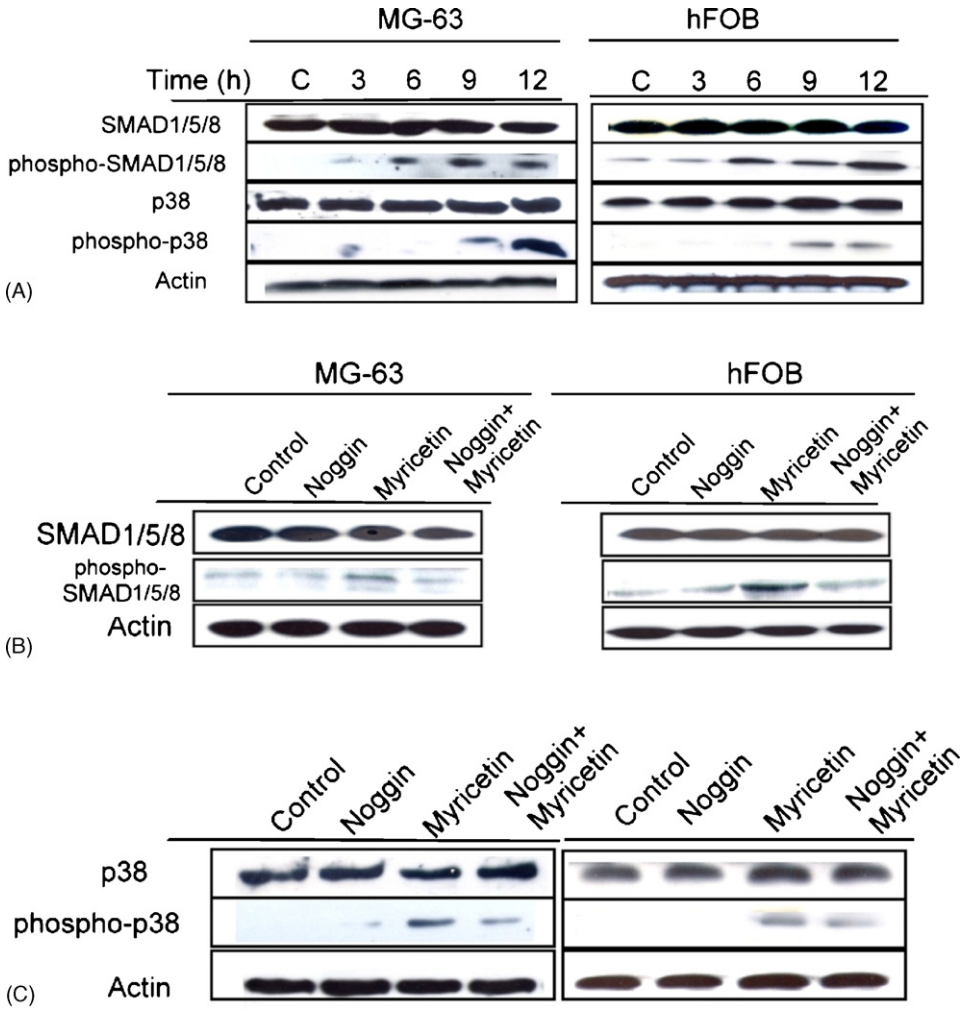

Fig. 5 – The activation of SMADs, p38 in myricetin-treated MG-63 and hFOB cells. Myricetin increased SMAD1/5/8 and p38 activation (A). Noggin decreased the effect of myricetin on the activation of SMAD1/5/8 (B) and p38 (C). For (A), unphospho- and phospho-SMAD and p38, cells were treated with 20 μM myricetin for the indicated times; the levels of SMAD1/5/8, p38, and their phosphorylated proteins were determined by Western blotting analysis. For (B) and (C), cells were incubated for 1 h in the presence or absence of noggin, and then 20 μM myricetin was added and incubated for 9 h. The levels of various proteins were determined by Western blotting analysis. Data shown are representative of three independent experiments.

for 1 h with a potent, specific inhibitor for p38, SB203580. The inhibitor-treated cells were then exposed to myricetin, and ALP activity and osteocalcin secretion then determined. As shown in Fig. 6A, the myricetin-mediated p38 activation was effectively inhibited by 20 μM SB203580 (Fig. 6A). SB203580 not only decreased the ALP activity of myricetin of 48 h, but also inhibited the accumulation of osteocalcin induced by myricetin 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 [11,35–37]. Our results indicate that the presence of myricetin 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 myricetin 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 myricetin treatment. In addition, bone formation, as measured by mineralization, was also increased in MG-63 and hFOB cells treated with myricetin. Furthermore, the inhibitory protein synthesis effect of cycloheximide on the myricetin-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 myricetin-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 [38]. 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 [11,33,39]. Our study indicates that the production of BMP-2 increases in myricetin-treated MG-63 and hFOB cells. Also, phosphorylations of SMAD1/5/8 are simultaneously enhanced in myricetin-treated osteoblasts. Indeed, bone morphogenetic proteins antagonist noggin not only blocked myricetin-mediated SMAD1/5/8 activation, but also exhibited a similar effect against myricetin-mediated cell differentiation (ALP upregulation and osteocalcin production). These results support the hypothesis that the BMP-2 signaling system plays an important role in myricetin-mediated cell maturation and differentiation in osteoblasts.

BMP-2 has been shown to activate not only SMAD signaling but also to elicit p38 kinase pathway, which is involved in

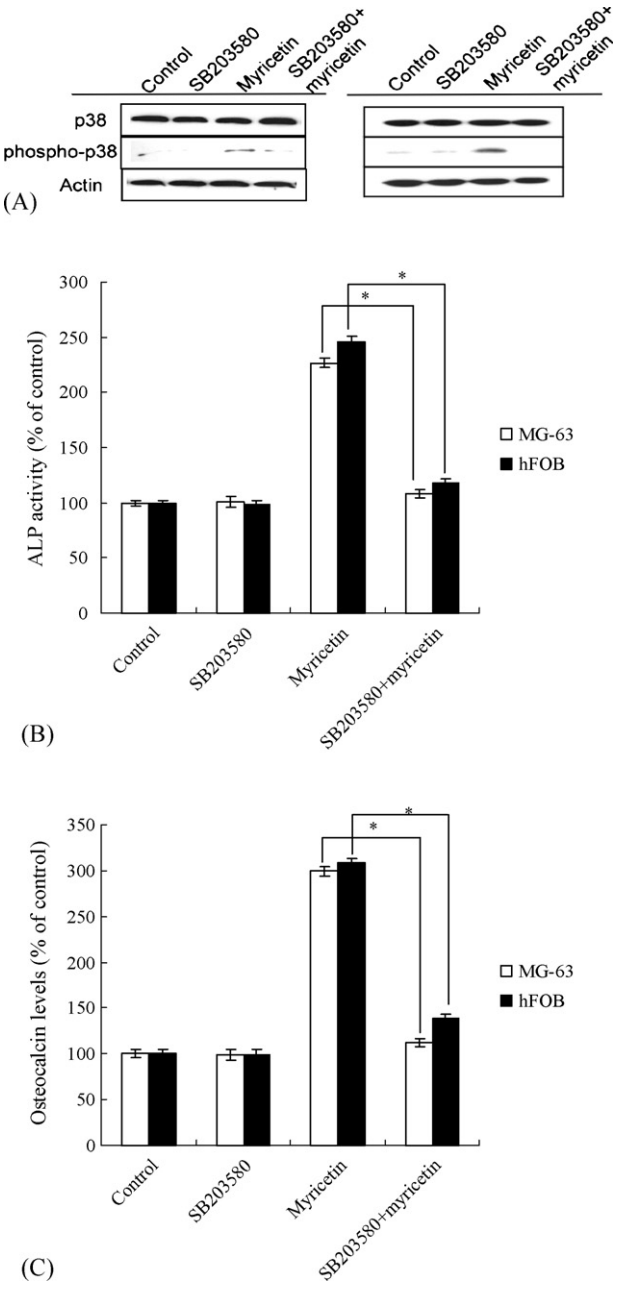

Fig. 6 – The role of p38 on the upregulation of myricetin on osteoblastic activity in MG-63 and hFOB cells. (A) SB203580 inhibited myricetin-induced p38 activation. Effect of SB203580 on myricetin-induced ALP activity (B) and osteocalcin production (C). Cells were incubated for 1 h in the presence or absence of SB203580, and then 20 μM myricetin was added and incubated for the specified times (48 h for ALP, 72 h for osteocalcin assay). The p38 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 ($P < 0.05$).

osteoblast differentiation [9]. Previous studies have reported the direct correlation between SMADs and p38 kinase signaling [9]. Similarly, it has been shown that p38 pathway, by interacting with SMADs signaling, is involved in BMP-2 induced bone matrix gene expression and ALP activity in various osteoblastic cells [20]. Our study observed an increase in p38 activity after BMP-2 production and SMAD1/5/8 phosphorylation, and suppression of BMP-2 signaling by cotreating noggin abrogated p38 activation in myricetin-treated cells. In addition, inhibition of p38 activity by specific inhibitor SB203580 decreased the effects of myricetin on osteoblastic maturation and differentiation. These data suggest that activation of p38 plays an important role on the cell differentiation of myricetin activity in osteoblasts.

A growing number of studies have shown that BMP plays a critical and controversial role in cancer progression. Several studies have reported that BMP-2 expression stimulates tumor growth, angiogenesis, and invasion [40,41]. In contrast, BMP-2 has also been found to inhibit growth and induce apoptosis in human myeloma cells [42]. Moreover, stimulation of BMP-2 by retinoids not only causes direct cell death in retinoid-sensitive cancer cells, but is also sufficient to induce apoptosis in surrounding retinoid-resistant cancer cells through a paracrine effect [43]. Although it has been implied that myricetin possesses anticancer activity [44], the actual effect of myricetin on the cancer, particularly in cancer cells localized in the bone, requires further investigation.

Increasing data show that several varieties of flavonoids, such as daidzein, genistein, quercetin, and kaempferol, are protective agents against bone loss [11,23,25,27]. Quercetin and kaempferol have been found to that increase osteoblast activity by extracellular regulated kinase and estrogen receptor pathway [27]. Daidzein enhances osteoblast growth that is mediated by increased BMP-2 production [11]. Genistein induced osteoblast differentiation may be partly involved in estrogen action [23]. Our observations indicate that myricetin stimulates osteoblast differentiation at various stages in MG-63 and hFOB cells. Myricetin's effect on cell maturation and differentiation is strongly associated with BMP-2/SMAD1/5/8/p38 signaling pathway. This therefore suggests that myricetin may be beneficial in stimulating the osteoblastic activity resulting in bone formation.

Acknowledgment

This study was supported by a research grant from the National Science Council of Taiwan (NSC95-2320-B-041-009).

REFERENCES

[1] Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 2000;289:1501–4.
[2] Manolagas SC, Jilka RL. Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. N Engl J Med 1995;332:305–11.
[3] Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504–8.
[4] Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. Science 2000;289:1508–14.
[5] Schelonka EP, Usher A. Ipriflavone and osteoporosis. J Am Med Assoc 2001;286:1836–7.
[6] Berg C, Neumeyer K, Kirkpatrick P. Teriparatide. Nat Rev Drug Discov 2003;2:257–8.
[7] Lane NE, Kelman A. A review of anabolic therapies for osteoporosis. Arthritis Res Ther 2003;5:214–22.
[8] Sykaras N, Opperman LA. 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 NO. Signal transduction of bone morphogenetic protein receptors. Cell Signal 2004;16:291–9.
[10] Groeneveld EH, Burger EH. Bone morphogenetic proteins in human bone regeneration. Eur J Endocrinol 2000;142:9–21.
[11] Jia TL, Wang HZ, Xie LP, Wang XY, Zhang RQ. Daidzein enhances osteoblast growth that may be mediated by increased bone morphogenetic protein (BMP) production. Biochem Pharmacol 2003;65:709–15.
[12] Friedman MS, Long MW, Hankenson KD. Osteogenic differentiation of human mesenchymal stem cells is regulated by bone morphogenetic protein-6. J Cell Biochem 2006;98:538–54.
[13] Huang W, Carlsen B, Wulur I, Rudkin G, Ishida K, Wu B, et al. 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;299:325–34.
[14] Lecanda F, Avioli LV, Cheng SL. 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;67:386–96.
[15] Takuwa Y, Ohse C, Wang EA, Wozney JM, 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] Hu Y, Chan E, Li B. Activation of p38 mitogen-activated protein kinase is required for osteoblast differentiation. Endocrinology 2003;144:2068–74.
[18] Goswami M, Uzgare AR, Sater AK. Regulation of MAP kinase by the BMP-4/TAK1 pathway in Xenopus ectoderm. Dev Biol 2001;236:259–70.
[19] Lai CF, Cheng SL. Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. J Biol Chem 2002;277:15514–22.
[20] Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G, Caverzasio J. Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. J Bone Miner Res 2003;18:2060–8.
[21] Jadlowiec J, Koch H, Zhang X, Campbell PG, Seyedain PM, 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;279:53323–30.
[22] Cody V. Crystal and molecular structures of flavonoids. Prog Clin Biol Res 1988;280:29–44.
[23] Morris C, Thorpe J, Ambrosio L, Santin M. The soybean isoflavone genistein induces differentiation of MG63 human osteosarcoma osteoblasts. J Nutr 2006;136:1166–70.
[24] Lee KH, Choi EM. Biochanin A stimulates osteoblastic differentiation and inhibits hydrogen peroxide-induced

production of inflammatory mediators in MC3T3-E1 cells. Biol Pharm Bull 2005;28:1948–53.
[25] Notoya M, Tsukamoto Y, Nishimura H, Woo JT, Nagai K, Lee IS, et al. Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts in vitro. Eur J Pharmacol 2004;485:89–96.
[26] Yoshida H, Teramoto T, Ikeda K, Yamori Y. Glycitein effect on suppressing the proliferation and stimulating the differentiation of osteoblastic MC3T3-E1 cells. Biosci Biotechnol Biochem 2001;65:1211–3.
[27] Prouillet C, Maziere JC, Maziere C, Wattel A, Brazier M, Kamel S. Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. Biochem Pharmacol 2004;67: 1307–13.
[28] Yanez J, Vicente V, Alcaraz M, Castillo J, Benavente-Garcia O, Canteras M, et al. Cytotoxicity and antiproliferative activities of several phenolic compounds against three melanocytes cell lines: relationship between structure and activity. Nutr Cancer 2004;49:191–9.
[29] Blonska M, Czuba ZP, Krol W. Effect of flavone derivatives on interleukin-1beta (IL-1beta) mRNA expression and IL-1beta protein synthesis in stimulated RAW 264.7 macrophages. Scand J Immunol 2003;57:162–6.
[30] Wang J, Mazza G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-gamma-activated RAW 264.7 macrophages. J Agric Food Chem 2002;50:850–7.
[31] Kuo PL. Myricetin inhibits the induction of anti-Fas IgM, tumor necrosis factor-α and interleukin-1β mediated apoptosis by Fas pathway inhibition in human osteoblastic cell line MG-63. Life Sci 2005;77(23):2964–76.
[32] Reinholz GG, Getz B, Pederson L, Sanders ES, Subramaniam M, Ingle JN, et al. Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. Cancer Res 2000;60:6001–7.
[33] Kuo PL, Hsu YL, Chang CH, Chang JK. 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;314:1290–9.
[34] Zhu W, Kim J, Cheng C, Rawlins BA, Boachie-Adjei O, Crystal RG, et al. Noggin regulation of bone morphogenetic protein (BMP) 2/7 heterodimer activity in vitro. Bone 2006;39:61–71.
[35] Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. Bone 1995;17:77S–83S.
[36] Qu Q, Perala-Heape M, Kapanen A, Dahllund J, Salo J, Vaananen HK, et al. Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 1998;22:201–9.
[37] Wada Y, Kataoka H, Yokose S, Ishizuya T, Miyazono K, Gao YH, et al. Changes in osteoblast phenotype during differentiation of enzymatically isolated rat calvaria cells. Bone 1998;22:479–85.
[38] Xiao Y, Haase H, Young WG, Bartold PM. Development and transplantation of a mineralized matrix formed by osteoblasts in vitro for bone regeneration. Cell Transplant 2004;13:15–25.
[39] Kuo PL, Huang YT, Chang CH, Chang JK. 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;29:119–24.
[40] Langenfeld EM, Kong Y, Langenfeld J. Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5. Oncogene 2006;25:685–92.
[41] Raida M, Clement JH, Leek RD, Ameri K, Bicknell R, Niederwieser D, et al. Bone morphogenetic protein 2 (BMP-2) and induction of tumor angiogenesis. J Cancer Res Clin Oncol 2005;131:741–50.
[42] Kawamura C, Kizaki M, Yamato K, Uchida H, Fukuchi Y, Hattori Y, et al. Bone morphogenetic protein-2 induces apoptosis in human myeloma cells with modulation of STAT3. Blood 2000;96:2005–11.
[43] Hallahan AR, Pritchard JI, Chandraratna RA, Ellenbogen RG, Geyer JR, Overland RP, et al. BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. Nat Med 2003;9:1033–8.
[44] Lu J, Papp LV, Fang J, Rodriguez-Nieto S, Zhivotovsky B, Holmgren A. Inhibition of mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity. Cancer Res 2006;66: 4410–8.