Comparison between 8-prenylnaringenin and naringenin concerning their activities on promotion of rat bone marrow stromal cells' osteogenic differentiation *in vitro*

L. G. Ming*, B. F. Ge*, M. G. Wang† and K. M. Chen*

*Institute of Orthopaedics, Lanzhou General Hospital, Lanzhou Command of CPLA, Lanzhou, China, and †School of Life Sciences and Engineering, Lanzhou University of Technique, Lanzhou, China

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Abstract

A number of recent studies have suggested that flavonols (a class of phytochemical with many biological activities), might exert protective effects against post-menopausal bone loss. In the present study, we compared naringenin (NG) and 8-prenylnaringenin (PNG), two major naturally occurring flavonols, on *in vitro* differentiation of osteoblasts and bone resorbing activity, of rat bone marrow stromal cells (BMSCs). Our results indicated that both compounds, at 10^{-6} M, enhanced BMSCs' differentiation. Then effects of the two compounds at 10^{-6} M on ALP activity, osteocalcin secretion and calcium deposition, were compared over a time course. Numbers and areas of colonies stained for ALP (CFU-F_{ALP}) expression, and mineralized bone nodules, were histochemically analysed after 12 days and 16 days osteogenic induction, respectively. Expression of *BMP-2*, *OPG*, *OSX*, *RUNX-2* genes and p38MAPK protein were examined using real-time PCR and western blotting, respectively. The data presented indicate that PNG, significantly enhanced the rat BMSCs' differentiation and mineralization through the BMP-2/p38MAPK/Runx2/Osterix signal pathway, greater than did NG. In conclusion, PNG has a more pronounced ability to enhance osteoblast differentiation and mineralization, than NG.

Introduction

During adulthood, bone is continuously remodelled and a balance between resorption of old bone by osteoclasts,

and formation of new bone by osteoblasts, is required for maintenance of skeletal integrity. Osteoblasts are of crucial importance in bone tissues as they are critical for bone formation and normal bone density. Cellular events involved in bone formation include chemotaxis, proliferation and differentiation of osteoblast precursors (1,2). Precursors of osteoblasts in bone marrow, bone marrow stromal cells (BMSCs) have been investigated *in vitro*, and have been induced into osteoblastic cell differentiation. In inducing medium, *Runt-related gene-2* (*Runx-2*), Osterix (*OSX*), BMP, OPG and p38 mitogen-activated protein kinase (p38MAPK) are important factors in this process; in particular, they have been shown to enhance osteoblast proliferation and differentiation (3–8). MAPKs have been implicated in many physiological processes, including cell proliferation, differentiation and apoptosis. Three major types of MAPKs in mammalian cells are the ERK1/2, JNK and p38 MAPKs (9,10). p38MAPK plays a critical role in the bone-related cell differentiation. We found that the activation of BMP-2/p38MAPK/Runx-2/Osterix pathway enhanced the osteoblasts differentiation. Several recent studies have suggested that flavonols, a class of phytochemical with many biological activities, might exert a protective effect on post-menopausal bone loss. A great deal of evidence indicates that isoflavonoids and flavonoids, mainly represented by genistein and daidzein, which significantly prevented bone loss in ovariectomized rats (12), have positive effects on both osteoblast (13,14) and osteoclast activity. Genistein and daidzein (so-called phyto-oestrogens) share some structural similarity with natural oestrogen, and their beneficial effects are attributable to their capacity to bind the oestrogen receptor; some residual groupings may play important roles in anti-osteoporosis activity. Structural differences in methoxylation and hydroxylation of genistein and daidzein may contribute to significant alteration in their biological effects

Correspondence: K. M. Chen, Institute of Orthopaedics, Lanzhou General Hospital, Lanzhou Command of CPLA, Lanzhou 730050, China. Tel.: (618) 8302 1944; Fax: (618) 8302 1087; E-mail: chkeming@yahoo.com.cn

(15). However, our studies have revealed that icariin and osthole are more potent than genistein in promoting osteoblast differentiation and mineralization; this is mainly due to existence of a prenyl group at C-8 of icariin and osthole (11,16). Naringenin (NG) and 8-prenylnaringenin (PNG) belonging to the flavonoids, have similar structures to icariin and osthole, as isolated from *Spica humuli lupuli*. PNG has a prenyl group at C-8, and NG has the same structure with PNG except that no prenyl group exists at C-8 (Fig. 6). More recent data have considered PNG to be a novel phytoestrogen (17,18), being one of the strongest plant-derived oestrogen receptor (ERs) ligands (19). However, little is known so far concerning its structural components which determine its bone metabolism activities. Taking into consideration that prenylation reactions are relatively common in plant secondary metabolism, activity of NG and PNG have been compared with regard to structure–oestrogenicity relationships, on BMSCs. In the prophase study, we found that NG and PNG at 10^{-6} M enhanced osteoblast differentiation and mineralization (20,21). In the experiments described below, PNG and NG were found to enhance osteogenic differentiation of rat BMSCs.

Materials and methods

Animals

Four male Wistar rats 4 weeks of age, weighing 80–100 g, were obtained from the Animal Breeding Center of Gansu College of Traditional Chinese Medicine (Lanzhou, China). Procedures for obtaining bone marrow samples for isolating bone marrow stromal cells, from rats, were carried out according to the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health.

Reagents

8-prenylnaringenin (purity $\geq 97\%$) was purchased from AXXORA, LLC (San Diego, CA, USA). NG (purity $>99\%$) was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Culture media (DMEM/F12) and foetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). Penicillin and streptomycin were obtained from Gibco BRL (Gaithersburg, MD, USA). The majority of drugs used were purchased from Sigma (Steinheim, Germany), including dexamethasone, β -glycerophosphate, ascorbic acid phosphate and 1-naphthyl phosphate sodium salt monohydrate. The alkaline phosphatase activity measurement kit was

purchased from Nanjing Jiancheng Company (Nanjing, China) and the calcium colorimetric assay kit was obtained from Biovision (San Francisco, CA, USA); the enzyme immunoassay ELISA kit for quantitative determination of osteocalcin was purchased from Immunodiagnostic Systems Ltd (Baldon, UK). All other chemicals were of analytical grade.

Cell culture

Primary culture of rBMSCs was established as described previously (22). Four adult male Wistar rats weighing ~80–100 g were sacrificed by dislocation of the cervical spine. Tibias and femurs were immediately dissected from attached muscles and tissues using aseptic techniques. Ends of bones were removed, and marrow plugs were flushed out by injection of DMEM/F12 medium, containing 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cell plugs were dispersed by repeated pipetting and cells were forcefully passed through a 19-gauge needle to obtain a single cell suspension; finally they were filtered through a 76 μ m stainless steel cell strainer. Suspensions were adjusted to 1×10^7 cells/ml and were applied to 6-well plates at 2 ml/well, 12-well plates at 1 ml/well and 24-well plates at 0.5 ml/well. Cells were then cultured at 37 °C in 5% humidified CO₂ atmosphere; media were replaced every 4 days.

Time course measurement of alkaline phosphatase activity and histochemical staining for osteoblasts

rBMSCs were plated in 12-well tissue culture plates. When cells were confluent, osteogenic medium (10^{-8} M dexamethasone, 10 mM β -glycerophosphate and 50 mg/ml ASAP) containing 10^{-6} M PNG or identical concentration of NG was changed. 10^{-6} M had previously been found to be optimal concentration for PNG, to improve osteogenic differentiation (20). ALP activity was measured after 4, 8, 12, and 16 days ($n = 3$ per time point). Equal volumes of vehicle (DMSO) were used as control. Cells were rinsed twice and sonicated for 15 s in 2 ml of 50 mM Tris–HCl, pH 7.2, containing 0.1% Triton X-100 and 2 mM MgCl₂. ALP activity was measured using a commercial kit, as instructed (Nanjing Jiancheng Bioengineering Ltd, Nanjing, China). A modified method of King (Powell and Smith 1954) was used in the kit and results were expressed as nmol phenol/15 min/mg protein. Protein concentrations were determined using a BCA protein assay kit. To further compare potency of PNG and NG to stimulate osteogenic differentiation, numbers of colonies positive for ALP (CFU-F_{ALP}) were also compared on day 12. Cells were

fixed in 3.7% formaldehyde and 90% ethanol solution for 5 min, washed then stained for 15–20 min at 37 °C in 20 ml Michalis buffer, pH 8.9, containing 10 mg 1-naphthyl sodium phosphate and 10 mg fast blue RR salt. Numbers and total areas of blue colonies were measured using image analysis with Image-Pro Plus 6.0 software (Media Cybernetics, Inc. Bethesda, MD, USA).

Osteocalcin secretion and mineralization assays for osteoblasts

Levels of osteocalcin (produced by osteoblasts and secreted into culture medium) in 0.5 ml of media, were collected on days 0–4, 4–8, 8–12 and 12–16 and were measured (ng/ml) using a Rat-Mid™ Osteocalcin EIA kit (Immunodiagnostic Systems Ltd, Fountain Hills, AZ, USA). Calcium deposition measurements were performed on days 4, 8, 12 and 16. Briefly, cultures were rinsed twice in PBS and decalcified for 24 h in 0.1 M HCl; calcium content in HCl supernatant samples were measured using a calcium colorimetric assay kit (Biovision) and results were expressed as mg/dish. Histochemical alizarin red staining of mineralized cell nodules was carried out on day 16. Briefly, cells were fixed in 3.7% formaldehyde for 10 min and stained in 0.1% alizarin red for 1 h at 37°C. Numbers of and total areas of red nodules were assessed using Image-Pro Plus 6.0 software.

Real-time PCR quantification of gene expression

Effects of PNG and NG on osteogenic gene expression were examined by quantitative RT-PCR. mRNA expression of growth factor BMP-2 and transcription factors OSX and RUNX-2 were examined after 0, 6, 12, 24, 48 and 72 h osteogenic induction of osteoblasts, with or without supplement of PNG and NG. Total RNA was extracted from cells using RNAiso Kit (Takara Biotechnology, Dalian, China). Single-stranded cDNA was

synthesized from 1 µg total RNA using a Primescript™ RT reagent kit (Takara Biotechnology). Real-time PCR was performed using 2 µl of cDNA product in a 25-µl reaction volume with 7300 Real Time PCR System (Applied Biosystems, Singapore). SYBR® Premix Ex Taq™ II (Takara Biotechnology), specific primers (see below) and 2 µl of cDNA were used in each PCR reaction (95 °C for 30 s, 40 cycles of denaturation at 94 °C for 5 s, and annealing and extension at 60 °C for 30 s). Sense and antisense primers were designed with Primer Express 5.0 based on published cDNA sequences. *GAPDH* was used as internal control gene. Primer sequences are listed in Table 1. All real-time PCR reactions were performed in triplicate, and results after calibration with *GAPDH* expression were calculated using the $\Delta\Delta CT$ method and are presented as fold increase, relative to non-stimulated control.

Western blot analysis

p38MAPK, a signal transduction protein, was examined by western blotting after 12, 24 and 36 h. Cells were washed twice in distilled water and total protein was collected by adding lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 100 mg/l PMSF; 1 mg/l Aprotinin; 1 ml 1% Tween-20; 1 g SDS; 0.5 g deoxysodium cholate; 100 ml demineralized water). Crude homogenates were centrifuged at 4 °C for 15 min at 12 000g and supernatants were collected. After total protein content quantification by BCA assay kit (Biosynthesis Biotechnology, Beijing, China), 50 µg total protein from each sample was separated using SDS-PAGE (12% gel) and was transferred to PVDF membranes. After incubation in blocking solution (2% non-fat milk) for 2 h at room temperature, membranes were incubated overnight at 4 °C with primary antibodies at 1:1000 dilution [mouse anti-p38MAPK (Abcam, Hong Kong)], and loading control antibody (mouse anti β -actin polyclonal

Table 1. Primer sequences RT-real time PCR

Gene	Gene Bank No.	Primer sequence	Product length/bp
BMP-2	NM-017178.1	Forward 5'-ACCGTGCTCAGCTTCCATCAC-3'	156
		Reverse 5'-TTCCTGCATTTGTTCCTCGAAA-3'	
OSX	NM-001037632.1	Forward 5'-GCCTACTTACCCGTCTGACTTT-3'	131
		Reverse 5'-GCCCACTATTGCCAACTGC-3'	
Runx-2	NM-053470.1	Forward 5'-GCACCCAGCCCATATAAGA-3'	165
		Reverse 5'-TTGGAGCAAGGAGAAGCC-3'	
OPG	NM-012870	Forward 5'-ATTTGCTTTCGGCATCAT -3'	123
		Reverse 5'-GCTCCCTCCTTTCATCAG-3'	
GAPDH	NM-017008.3	Forward 5'-GGCACAGTCAAGGCTGAGAATG-3'	143
		Reverse 5'-ATGGTGGTGAAGACGCCAGTA-3'	

antibody, 1:500) (Zhongshan Goldenbridge, Beijing, China). After three washes in TBS-Tween-20, membranes were incubated in 1:3000 dilution of second antibody for 2 h, and immunoreaction signals were detected by enhanced chemiluminescence reagent (Millipore Corp, Billerica, MA, USA) and were exposed on X-ray film (Kodak, Shanghai, China). Relative intensities were scanned using Image-Pro plus 6.0. Data (densities of product bands) were expressed as relative optical density units after being standardized against the β -actin band of each sample.

Statistical analysis

Statistical analyses were carried out using SPSS 16.0 software (SPSS China, Shanghai, China). Data for MTT assays were from six parallel experiments and others from triplicate experiments. All data shown are mean $\pm$ SD and significance levels were determined by ANOVA. Multiple comparisons were carried out using the Tukey method. Values of $P < 0.05$ was taken to be significant.

Results

To compare osteogenic activity of PNG and NG, ALP activity was measured first over a time course (Fig. 1a). At all points analysed, PNG treatment always produced higher ALP activity compared to controls ($P < 0.01$) and compared to the NG group ($P < 0.01$). NG group specimens also had higher values controls at all time points, but lower than those of the PNG group; ALP activity was significantly higher after 8 days. Histochemical staining for ALP on day 12 (Fig. 1b) revealed that the PNG group had significantly higher numbers of, and larger areas of CFU-F_{ALP} colonies than the NG group; also, the NG group produced slightly higher numbers and areas of CFU-F_{ALP} colonies (Fig. 1c,d).

Osteocalcin is a non-collagen protein marker of bone formation. During early stages of osteogenic culture (days 0–4), there were no differences in osteocalcin concentration, between the three groups ($P > 0.05$). On the following days, however, differences of increase in

Figure 1. Time course induction of ALP activity in rBMSCs over 16 days osteogenic culture (a). ALP stained CFU-F_{ALP} colonies formed by rBMSCs after 12 days of osteogenic induction culture (b). Naringenin (NG) or 8-prenylnaringenin (PNG) was supplemented at 10^{-6} M from the beginning of induction. Numbers (c) and total areas (d) of CFU-F_{ALP} colonies were measured using Olympus SP 1000 Image Analysis. Results are mean $\pm$ SD of triplicate cultures. # $P < 0.05$, ## $P < 0.01$ versus control; & $P < 0.05$, && $P < 0.01$ versus NG group.

osteocalcin secretion became readily observable (Fig. 2a). The NG-treated group produced 1.5-fold higher osteocalcin secretion than the control group during days 4–8 and over days 8–12, but was significantly lower than that of the PNG-treated group. The PNG-treated group had 3-fold higher osteocalcin secretion than the control group over days 4–12. By days 12–16, osteocalcin secretion declined in all the three groups but differences still existed between NG group control and the PNG group remained higher than the other two groups ($P < 0.05$).

Calcium deposition level is a direct indicator of mineralization. There were no differences in this amongst the PNG, NG and control groups on day 4 of observation. Beginning at day 8, however, calcium content increased steadily, the PNG group being always higher than NG group and control ($P < 0.01$, Fig. 2b), particularly between days 12 and 16. Mineralized nodule formation assay on day 16 revealed a similar tendency to calcium deposition (Fig. 3a). There were far more and larger areas of mineralized nodules in the PNG group than in the NG group ($P < 0.01$), and the NG group was significantly higher than control in numbers and areas of mineralized nodules ($P < 0.01$) (Fig. 3b,c). The PNG-treated group was 2.5 times higher than the control group and nearly double that of the NG group.

Real-time PCR analysis revealed that BMP-2 mRNA expression increased at 6 h and had peaked by 12 h of culture, then declined gradually (Fig. 4a). Consistent

with data from osteoblast differentiation and osteogenesis assays, the PNG group induced highest BMP-2 mRNA expression from 6 to 72 h, and was significantly higher than the NG and control groups from 12 to 48 h ($P < 0.01$). The NG group result was significantly higher than that of the control at 12 and 72 h ($P < 0.01$). OPG also increased during cell differentiation, PNG-treated group inducing highest OPG mRNA expression from 6 to 72 h; this was significantly higher than the NG and control groups from 24 to 48 h ($P < 0.01$). All groups displayed the same pattern, peaking at 24 h, NG-treated group being higher than the control group, but lower than the PNG-treated group (Fig. 4b).

Runx-2 and Osterix (OSX) are two critical transcription factors concerned in osteoblast differentiation and bone formation (20). Expression level of Runx-2 mRNA increased at 6 h, peaked at 24 h, then declined (Fig. 4c). The PNG group had significantly higher expression of it the control at all time points ($P < 0.01$) and also higher than the NG group at 24 h ($P < 0.01$), to a lesser extent at 6, 12, 48 and 72 h ($P < 0.05$). The NG group was also higher than the control from 6 to 72 h, but to a lesser extent ($P < 0.05$), except at 12 and 24 h ($P < 0.01$). OSX mRNA expression produced a similar pattern (Fig. 4d). The three groups peaked at 24 h, PNG group being in the region of 2-fold higher than the control ($P < 0.01$) and 1.5-fold higher than the NG group ($P < 0.05$) at peak time points.

Secretion of p38MAPK was assessed by western blot analysis from cultured primary rBMSCs. Under reducing conditions, polyclonal anti-p38MAPK antibody was used in western blot. β -actin was analysed in all samples as an internal protein. As assessed by densitometry (IOD), PNG at 10^{-6} M induced p38MAPK increase to over 80% compared to controls, and over 40% compared to the NG group at 12, 24, 36 h respectively. However, in the NG group p38MAPK expression increased at 12 and 24 h only (Fig. 5).

Figure 2. Time course changes in osteocalcin concentration (a) in conditioned osteogenic culture medium and calcium deposition (b) of rBMSCs. Naringenin (NG) or 8-prenylnaringenin (PNG) was supplemented at 10^{-6} M. Results are the mean $\pm$ SD of triplicate cultures. $^{\#}P < 0.05$, $^{##}P < 0.01$ versus control; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus NG group.

Discussion

As we know, the p38MAPK signalling pathway acts in a crucial signal transduction cascade contributing to neuronal differentiation, adipogenesis and chondrogenesis (23). Previous reports have indicated that p38MAPK activation induces OPG expression in human osteoblastic cell lines and p38MAPK signalling pathways are also involved in transmission of BMP signals (24). The BMP pathway serves as the central axis of the cascade, with both ERK and p38 pathways converging at Runx2 to exert positive or negative roles on osteogenesis of rMSCs and independent studies have demonstrated that icariin promotes osteogenesis *via* BMP- and Runx2-

Figure 3. Mineralized nodules of rBMSCs after 16 days osteogenic induction culture as demonstrated by alizarin red staining (a). Naringenin (NG) or 8-prenylnaringenin (PNG) was supplemented at 10^{-6} M from beginning of induction. Numbers (b) and total areas (c) of mineralized nodules were measured using Olympus SP 1000 Image Analysis. Results are mean $\pm$ SD of triplicate cultures. # P < 0.05, ## P < 0.01 versus control; & P < 0.05, && P < 0.01 versus NG group.

Figure 4. Time course changes in mRNA expression of (a) BMP-2, (b) OPG, (c) Runx-2 and (d) OSX in rBMSCs over 72 h osteogenic induction culture. Naringenin (NG) or 8-prenylnaringenin (PNG) was supplemented at 10^{-6} M. Results are expressed as mean $\pm$ SD fold increase relative to non-supplemented control (n = 3 culture assays). # P < 0.05, ## P < 0.01 versus control; & P < 0.05, && P < 0.01 versus NG group.

dependent or MAPK-dependent pathways (25,26). We also have found phytoestrogen such as osthonol could activate the BMP-2/p38MAPK/Runx-2/Osterix pathway and

enhance osteoblast differentiation (11). NG and PNG also are phytoestrogens, which is why they were studied in this work.

Figure 5. Time course changes in protein expression of p38MAPK in rBMSCs over 36 h osteogenic induction culture. Naringenin (NG) or 8-prenylnaringenin (PNG) was supplemented at 10^{-6} M. Results expressed as mean $\pm$ SD fold increase relative to non-supplemented control ($n = 3$ culture assays). # $P < 0.05$, ## $P < 0.01$ versus control; & $P < 0.05$, && $P < 0.01$ versus NG group.

The current investigation has compared the relative ability of two flavonoid compounds, NG and PNG, to enhance differentiation and mineralization of rBMSCs *in vitro*. Here, they have been found to have a greater ability to improve rBMSCs osteogenic differentiation through the BMP-2/p38MAPK/Runx2/Osterix signal pathway. Osteogenic function was assessed by ALP activity, osteocalcin secretion, calcium deposition and number and area of mineralized bone nodules. The current data have shown that NG and PNG had roles in rBMSCs osteogenic differentiation, and that PNG was more potent than NG in this process.

Simultaneously, structure analysis indicated that the only difference between NG and PNG is the existence of a prenyl group at the C-8 of PNG. An interesting finding was that PNG had greater ability than NG to improve rBMSC osteogenic differentiation and maturation. Thus, we supposed that the NG parent nucleus would be required for their activity, and that the prenyl at C-8 could enhance the function of the NG. Recently, several studies have demonstrated that the prenyl group at C-8 may be highly related to osteogenic activity. Zhang *et al.* compared genistein derivatives (including 6-prenylgenistein, 8-prenylgenistein and 6, 8-prenylgenistein) in their effects on osteoblastic proliferation, differentiation and mineralization in UMR 106 cells, and found prenylation at C-8, but not at C-6 (15,19).

Bone-preserving actions of NG and PNG have been investigated over the last few years; however, knowledge concerning its activity mechanism was, until now, limited. It has been reported that phyto-oestrogen flavonoids induce osteogenic differentiation in an oestrogen receptor-dependent manner (17, 25, 27). In the present experiment, PNG was found to have higher ability than

Figure 6. Chemical structures of naringenin (a) and 8-prenylnaringenin (b).

NG to improve rBMSCs osteogenic differentiation, mineralization and expression of osteogenesis-related genes. The present data have also shown that NG and PNG significantly stimulated mRNA expression level of BMP-2, Runx2 and Osterix, and also enhanced p38MAPK signal transduction protein expression, thus indicating that NG and PNG improve rBMSCs osteogenic differentiation by activation of the BMP2/p38MAPK/Runx2/Osterix pathway.

In conclusion, our *in vitro* studies have demonstrated that NG and PNG both improve rBMSC osteogenic differentiation, but PNG has stronger ability than NG to enhance osteoblast differentiation. However, whether the NG or PNG flavonoids were more efficient in effecting osteoblasts and osteoclasts preserving bone mass and preventing bone loss induced by oestrogen deficiency, needs to be further investigated and studied *in vivo* in animal experiments.

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Conflict of interest

None.

Statement

All authors agreed to publish this article in Cell Proliferation.

References

- Mundy GR (1996) Regulation of bone formation by bone morphogenetic proteins and other growth factors. *Clin. Orthop.* **324**, 24–28.
- Jia TL, Wang HZ, Xie LP, Wang XY, Zhang RQ (2003) Daidzein enhance osteoblast growth that may be mediated by increased bone morphogenetic protein (BMP) production. *Biochem. Pharmacol.* **65**, 709–715.

- 3 Michitaka N, Eri O, Akira Y, Hiromi H (2004) Runx-2 is not essential for the vitamin D-regulated expression of RANKL and osteoprotegerin in osteoblastic cells. *J. BBRC* **324**, 655–660.
- 4 Ulsamer A, Ortuon MJ, Ruiz S (2008) BMP-2 induces Osterix expression through up-regulation of Dlx5 and its phosphorylation by p38. *J. Biol. Chem.* **283**, 3816–3826.
- 5 Liu T, Gao Y, Sakamoto K (2007) BMP-2 promotes differentiation of osteoblasts and chondroblasts in Runx2-deficient cell lines. *J. Cell. Physiol.* **211**, 728–735.
- 6 Nohe A, Keating E, Knaus P (2004) Signal transduction of bone morphogenetic protein receptors. *J. Cell Signal.* **16**, 291–299.
- 7 Jeon EJ, Lee KY, Choi NS (2006) Bone morphogenetic protein-2 stimulates Runx2 acetylation. *J. Biol. Chem.* **281**, 16502–16511.
- 8 Ichida F, Nishimura R, Hata K, Matsubara T, Ikeda F, Hisada K, *et al.* (2004) Reciprocal roles of Msx2 in regulation of osteoblast and adipocyte differentiation. *J. Biol. Chem.* **279**, 34015–34022.
- 9 Kong AN, Yu R, Chen C, Mandelkar S, Primiano T (2000) Signal transduction events elicited by natural products: role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. *Arch. Pharm. Res.* **23**, 1–16.
- 10 Seger R, Krebs EG (1995) The MAPK signaling cascade. *FASEB J.* **9**, 726–735.
- 11 Ming LG, Zhou J, Cheng GZ, Ma HP, Chen KM (2011) Osthol, a coumarin isolated from common cnidium fruit, enhances the differentiation and maturation of osteoblasts in vitro. *Pharmacology* **88**, 33–43.
- 12 Sugimoto E, Yamaguchi M (2000) Stimulatory effect of daidzein in osteoblastic MC3T3-E3 cells. *Biochem. Pharmacol.* **59**, 471–475.
- 13 Sugimoto E, Yamaguchi M (2000) Anabolic effect of genistein in osteoblastic MC3T3-E3 cells. *Int. J. Mol. Med.* **5**, 515–520.
- 14 Ma HP, Ming LG, Ge BF *et al.* (2011) Icaritin is more potent than genistein in promoting osteoblast differentiation and mineralization in vitro. *J. Cell. Biochem.* **112**, 916–923.
- 15 Sehmisch S, Hammer F, Christoffel J, Seidlova-Wuttke D, Tezval M, Wuttke W, *et al.* (2008) Comparison of the phytochemicals genistein, resveratrol and 8-prenylnaringenin as agents for preventing osteoporosis. *Planta Med.* **74**, 794–801.
- 16 Hsieh TP, Sheu SY, Sun JS, Chen MH, Liu MH. (2010) Icaritin isolated from *Epimedium pubescens* regulates osteoblasts anabolism through BMP-2, SMAD4, and Cbfa1 expression. *Phytomedicine* **17**, 414–423.
- 17 Hsieh TP, Sheu SY, Sun JS, Chen MH. (2011) Icaritin inhibits osteoclast differentiation and bone resorption by suppression of MAPKs/NF-kappaB regulated HIF-1alpha and PGE(2) synthesis. *Phytomedicine* **18**, 176–185.
- 18 Wada Y, Kataoka H, Yokose S, Ishizuya T, Miyazono K, Gao YH *et al.* (1998) Changes in osteoblast phenotype during differentiation of enzymatically isolated rat calvaria cells. *Bone* **22**, 479–485.
- 19 Zhang Y, Li XL, Yao XS, Wong MS. (2008) Osteogenic activities of genistein derivatives were influenced by the presence of prenyl group at ring A. *Arch. Pharm. Res.* **31**, 1534–1539.
- 20 Ming LG, Chen KM, Ge BF, Ma HP, Zhai YK (2011) Investigated the effect of 8-Prenylnaringenin on bone marrow stromal cells differentiation in vitro. *Chin. Pharmacol. Bull.* **27**, 421–427.
- 21 Ming LG, Chen KM, Ge BF, Ma HP, Zhai YK (2010) Effects of 8-Prenylnaringenin on osteoblasts differentiate and biomineralization in vitro. *Progr. Biochem. Biophys.* **38**, 1–8.
- 22 Nian H, Ma MH, Nian SS, Xu LL (2009) Antiosteoporotic activity of icaritin in ovariectomized rats. *Phytomedicine* **16**, 320–326.
- 23 Hamamura K, Goldring MB, Yokota H (2009) Involvement of p38 MAPK in regulation of MMP13 mRNA in chondrocytes in response to surviving stress to endoplasmic reticulum. *Arch. Oral Biol.* **54**, 279–286.
- 24 Pantouli E, Boehm MM, Koka S (2005) Inflammatory cytokines activate p38MAPK to induce osteoprotegerin synthesis by MG-63 cells. *Biochem. Biophys. Res. Commun.* **329**, 224–229.
- 25 Zhao J, Ohba S, Shinkai M, Chung UI *et al.* (2008) Icaritin induces osteogenic differentiation in vitro in a BMP- and Runx2-dependent manner. *Biochem. Biophys. Res. Commun.* **369**, 444–448.
- 26 Hsieh T, Sheu S, Sun J, Chen M (2011) Icaritin inhibits osteoclast differentiation and bone resorption by suppression of MAPKs/NF-kappaB regulated HIF-1[alpha] and PGE2 synthesis. *Phytomedicine* **18**, 176–185.
- 27 Mok SK, Chen WF, Lai WP, Leung PC, Wang XL, Yao XS, Wong MS (2010) Icaritin protects against bone loss induced by oestrogen deficiency and activates oestrogen receptor-dependent osteoblastic functions in UMR 106 cells. *Br. J. Pharmacol.* **159**, 939–949.