

Original Article

Naringin promotes osteogenic differentiation of bone marrow stromal cells by up-regulating Foxc2 expression via the IHH signaling pathway

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Abstract: Naringin is an active compound extracted from *Rhizoma Drynariae*, and studies have revealed that naringin can promote proliferation and osteogenic differentiation of bone marrow stromal cells (BMSCs). In this study, we explored whether naringin could promote osteogenic differentiation of BMSCs by upregulating Foxc2 expression via the Indian hedgehog (IHH) signaling pathway. BMSCs were cultured in basal medium, basal medium with naringin, osteogenic induction medium, osteogenic induction medium with naringin and osteogenic induction medium with naringin in the presence of the IHH inhibitor cyclopamine (CPE). We examined cell proliferation by using a WST-8 assay, and differentiation by Alizarin Red S staining (for mineralization) and alkaline phosphatase (ALP) activity. In addition, we detected core-binding factor $\alpha 1$ (Cbf $\alpha 1$), osteocalcin (OCN), bone sialoprotein (BSP), peroxisome proliferation-activated receptor gamma 2 (PPAR $\gamma 2$) and Foxc2 expression by using RT-PCR. We also determined Foxc2 and IHH protein levels by western blotting. Naringin increased the mineralization of BMSCs, as shown by Alizarin red S assays, and induced ALP activity. In addition, naringin significantly increased the mRNA levels of Foxc2, Cbf $\alpha 1$, OCN, and BSP, while decreasing PPAR $\gamma 2$ mRNA levels. Furthermore, the IHH inhibitor CPE inhibited the osteogenesis-potentiating effects of naringin. Naringin increased Foxc2 and stimulated the activation of IHH, as evidenced by increased expression of proteins that were inhibited by CPE. Our findings indicate that naringin promotes osteogenic differentiation of BMSCs by up-regulating Foxc2 expression via the IHH signaling pathway.

Keywords: Naringin, osteogenic differentiation, bone marrow stromal cells (BMSCs), forkhead box C2 (Foxc2), indian hedgehog (IHH), osteoporosis

Introduction

Osteoporosis develops as a result of an imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation [1, 2]. It is characterized by bone microarchitectural deterioration and loss of bone mass, with a resultant increase in bone fracture risk, posing a major public health challenge. An acute loss of bone mineral density (BMD) occurs in the perimenopausal period coinciding with a dramatic decline in serum estrogen in perimenopausal women. This and a subsequent more gradual and progressive decline in BMD help explain why the prevalence of primary osteoporosis peaks among postmenopausal women [3-5]. Currently, novel therapeutic options are being pursued to combat osteoporosis, aiming at inhibiting bone resorp-

tion by osteoclasts and/or increasing bone formation by osteoblasts [6]. Drugs currently in use for treating osteoporosis, including bisphosphonates, vitamin D analogues, estrogen, calcitonin, and ipriflavone, are all bone absorption-inhibitor drugs that rescue bone mass by inhibiting osteoclast activity. However, they only demonstrate modest effects in increasing or recovering bone mass, which is less than 2% per year [7]. Development of therapeutic agents with an anabolic effect on the osteoporotic bone could provide a new alternative to or complement these agents for treating osteoporosis [3].

BMSCs are noted for their capacity to differentiate into osteoblasts, chondrocytes, adipocytes, myocytes and fibroblasts. Of these, the

osteogenic and adipogenic lineages derived from BMSCs are inverse related, such that differentiation towards an osteoblast phenotype accompany with decreasing an adipocytic phenotype [8, 9]. An imbalance between osteoblasts and adipocytes is present in osteoporotic bones, especially in age-related osteoporotic bone cases that are accompanied by an increase in the number of adipocytes in the bone marrow [10]. Specific inhibition of bone marrow adipogenesis, and a concomitant enhancement of osteogenesis of BMSCs may provide a novel therapeutic approach for the treatment of osteogenic disorders, such as osteoporosis.

Naringin is an active compound extracted from the Chinese medicinal herb *Rhizoma Drynariae* [11]. Naringin possesses many pharmacological properties, such as the promotion of human mesenchymal stem cell differentiation [11, 12] and upregulation of bone morphogenetic protein (BMP) in osteoblasts. The latter involves, at least partially, phosphoinositide 3-kinase (PI3K), Akt, c-Fos/c-Jun and the AP-1-dependent signaling pathway [13]. However, the underlying signaling pathway(s) whereby naringin promotes proliferation and osteogenic differentiation of BMSCs remains uncertain.

Forkhead box C2/mesenchymal forkhead-1 (Foxc2/MFH-1) is a member of the family of winged helix/forkhead transcription factors [14]. Foxc2 is expressed during early skeletal cell condensation and in C1 mesodermal cells [15]. Foxc2 also stimulates osteoblast differentiation of mesenchymal cells and preosteoblasts by activating the canonical Wnt/ β -catenin signaling pathway [16]. Park et al. found that Foxc2 plays an important role in osteoblastogenesis by promoting osteoblast proliferation, survival and differentiation through up-regulation of integrin β 1 in response to bone formation stimuli [17]. Moreover, Foxc2 over-expression in BMSCs stimulates osteogenic differentiation and inhibits adipogenic differentiation [18].

The Indian hedgehog (IHH) protein, a member of the hedgehog family, plays an important role in the regulation of tissue patterning, skeletogenesis, and cellular proliferation [19, 20]. Mice deficient in the IHH gene show impaired proliferation and maturation of chondrocytes, as well as failure of osteoblast development in

endochondral bones [21, 22]. Loss of IHH leads to diminished ossification of the secondary hard palate [23]. In addition, IHH regulates osteoblast differentiation of mesenchymal cells through Gli2-mediated up-regulation of Runx2 expression and function [24]. We hypothesized that naringin promotes the differentiation of the osteoblastic lineage of BMSCs by up-regulating Foxc2 expression via the IHH signaling pathway.

Materials and methods

Chemicals and reagents

Naringin (chemical purity $\geq 98.0\%$) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Shanghai, China). Culture flasks and plates were purchased from Corning (Lowell, MA, USA). The ALP colorimetric assay kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). The Bradford Protein Assay Kit was purchased from Generay Biotech. Co., Ltd (Shanghai, China). Alizarin Red S, cetylpyridinium chloride, β -glycerophosphate, ascorbic acid, and dexamethasone were all purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and culture of rat BMSCs

All animal protocols were in accord with the Guidelines of the Animal Care and Use Committee of Shantou University Medical College. BMSCs were isolated from 4- to 6-week-old female Sprague-Dawley rats (Experimental Animal Center, Shantou University Medical College, Shantou, China) as previously reported [25]. The femurs were dissected, the ends of the bones were cut, and the marrow was flushed out with 5 mL DMEM containing 10% (v/v) FBS by using a 10 mL syringe. A single bone marrow cell suspension was obtained by repeated aspirations and blowing of the dropper. Cells were seeded into a 60-mm Petri dish at a density of 1×10^6 cells/mL, and cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin (basal medium, as control), and cultured in a humidified incubator at 37°C, with 5% CO₂ and 95% air. Non-adherent cells were removed and the medium was refreshed three or four times a week. Cells at passages three to six were used for experiments.

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Table 1. PCR primer sequences for real-time PCR

Gene and GenBank accession No.	Forward primer sequence	Reverse primer sequence
OCN (NM_001137)	5'-TGAGGACCCCTCTCTGCTC-3'	5'-GGTAGCGCCGGAGTCTATTC-3'
BSP (NM_012587)	5'-GCCACACTCTCAGGGTAAC-3'	5'-CCTTGCCCTCTGCATCTCC-3'
Cbfa1 (NM_00127)	5'-AACCACAGAACCACAAGTGC-3'	5'-TCACTGACTCGGTTGGTCTC-3'
FoxC2 (NM_001101)	5'-AAGGTAGCTACTGGACGCTC-3'	5'-TCCTCCTTATCCTTGGGCAC-3'
PPAR γ 2 (NM_013124)	5'-ATCCCGTTCACAAGAGCTGA-3'	5'-GCAGGCTCTACTTTGATCGC-3'
β -Actin (NM_001307)	5'-ATCGTGGGCCGCCCTAGGCA-3'	5'-TGGCCTTAGGGTTCAGAGGGG-3'

CCK-8 assays

Cells were inoculated into 96 well plates, at a density of 1000 cells per well, and treated with basal medium containing 1, 10, 50, or 100 μ g/mL naringin, and cell proliferation was determined nine days post treatment using the CCK-8 assay according to the manufacturer's instructions (Xingzhi Biotechnology Co., Ltd, Guangdong, China). Briefly, cells were incubated basal medium containing 10% (v/v) CCK-8 for 1 hour. Absorbance was recorded at 450 nm using a microplate reader (Thermo Scientific, Shanghai, China). Proliferation of BMSCs was presented as the optical density (OD) value.

Osteogenic differentiation and cell treatments

For osteogenic differentiation, BMSCs were inoculated at approximately 1×10^4 cells/cm² into 12-well plates and induced in an osteogenic induction medium (OIM, 0.1 μ M dexamethasone, 50 μ M ascorbic acid, and 10 mM β -glycerophosphate) containing 0, 1, 10, or 50 μ g/mL naringin. BMSCs were grown under 5% CO₂ at 37°C in a humidified atmosphere. Osteogenic differentiation was evaluated by measuring alkaline phosphatase (ALP) activity and mineralization assays. Furthermore, BMSCs in OIM were treated with 50 μ g/mL naringin in the presence of the IHH inhibitor CPE (0.5 μ M).

ALP activity determination

Intracellular ALP assays were performed in quadruplicate on days 1, 3, 5, 7, and 9 post naringin treatment, as depicted previously [26]. Absorbance was measured at 520 nm using a microplate reader (Thermo Scientific, Shanghai, China). Total protein concentrations were determined by the Bradford method, and ALP activity was normalized against total protein.

Alizarin Red S staining

At day 21 post naringin treatment, cells were fixed with ice-cold 70% ethanol (v/v) for 10 min and then stained with 40 mM Alizarin Red S in deionized water (pH 4.2) for 10 min at room temperature. After rinses with distilled water and drying at room temperature, cells were digitally photographed under a microscope. Calcium deposits were evaluated using the cetylpyridinium chloride method. Stained cultures were photographed, followed by a quantitative analysis using 10% cetylpyridinium chloride. Alizarin Red S concentrations were calculated through comparison with an Alizarin Red S dye standard curve and presented as nmol/mL [27].

Real-time quantitative PCR

Total cellular RNA was isolated from BMSCs using Trizol reagent (Invitrogen, CA, USA) Total RNA was then converted to cDNA synthesis using a PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). Real-Time quantitative PCR was then performed using the SYBR® PreMix Ex Taq™ (TaKaRa) on the CFX96™ Real-time PCR Detection System (Applied Biosystems). The PCR conditions and the sequences of the primers are listed in **Table 1** for the genes encoding the following proteins: rat ALP, BSP, Cbfa1, Foxc2, PPAR γ 2, and β -actin. Gene expression was calculated using the 2^{- $\Delta\Delta$ Ct} method, and β -actin was used as an internal control to normalize the mRNA levels. PCR was performed at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C, and 30 s at 56°C. At least three independent experiments for each reaction were performed in triplicate.

Western blotting assays

Cells were harvested by scraping in ice-cold RIPA lysis buffer (Boster, Wuhan, China). We-

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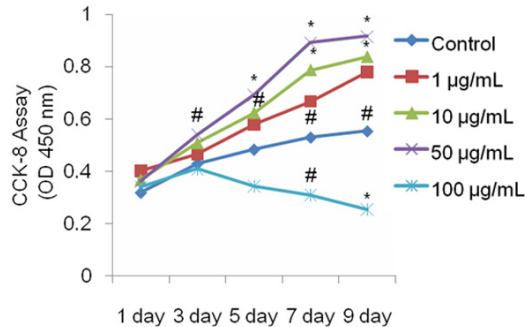


Figure 1. Naringin enhances the proliferation of BMSCs. Naringin promoted the proliferation of BMSCs in a dose-dependent manner. BMSCs were treated for 1-9 days, and the proliferation rate was assessed by CCK-8 assays. The proliferation of BMSCs was enhanced by naringin. Data is expressed as mean \pm SD and the experiments were done in quadruplicate ($n=4$). * $P<0.05$ versus controls at the same time point; # $P<0.05$ versus controls at the same time points.

stern blotting was performed as described previously [28] and blots were probed with anti-Foxc2 antibodies (Proteintech) and anti-IHH antibodies (Abcam, UK). Protein bands were detected using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL). The intensity of the bands was quantified by Quantity One Software v4.62 (Bio-Rad, Hercules, CA). As an internal control, GAPDH was used to normalize the protein levels.

Statistical analyses

Experiments were performed in triplicate and repeated more than three times independently. Data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL), and were presented as mean \pm standard deviation (SD). All data were analyzed for statistically significant differences by using Student's *t* test (two-tailed). In this study, a probability value (*P*) <0.05 was regarded as statistically significant.

Results

Naringin promotes the proliferation and mineralization of BMSCs

Primary BMSCs were isolated from the femora and tibia of rats and cultured under basal conditions. We first investigated the effects of naringin on proliferation of BMSCs in vitro. Our CCK-8 assays showed that 1, 10, and 50 $\mu\text{g}/\text{mL}$ naringin caused a dose and time-depen-

dent increase in the proliferation of BMSCs. At 50 $\mu\text{g}/\text{mL}$, naringin caused a statistically significant increase in the growth of BMSCs on days 5 to 9 post treatment, compared to controls ($P<0.01$ or 0.05). Naringin at a higher dose (100 $\mu\text{g}/\text{mL}$), by contrast, markedly depressed the proliferation of BMSCs (**Figure 1**). ALP assays revealed that 1 to 50 $\mu\text{g}/\text{mL}$ naringin caused a significant dose- and time-dependent increase in ALP activity ($P<0.01$) (**Figure 2A**). Furthermore, we found that naringin resulted in a statistically significant dose-dependent increase in mineralization, which peaked at 50 $\mu\text{g}/\text{mL}$ naringin (**Figure 2B and 2C**). The above findings indicated that 50 $\mu\text{g}/\text{mL}$ naringin was the optimal dose for potentiating osteogenic differentiation and was used for subsequent experiments.

Naringin enhances osteogenesis of BMSCs by up-regulating Foxc2 expression via the IHH signaling pathway

The IHH pathway has been shown to be intimately involved in the proliferation and osteogenic differentiation of BMSCs [29]. We investigated whether naringin promoted osteogenesis by up-regulating Foxc2 expression via the IHH signaling pathway. ALP assays showed that CPE significantly inhibited naringin-mediated increase in ALP activity (**Figure 3A**). We further investigated the effect of naringin on the expression of genes involved in osteogenesis of BMSCs. Real-time quantitative PCR assays showed that, compared to controls or BMSCs under OIM, 50 $\mu\text{g}/\text{mL}$ naringin treatment for 14 days caused a significant increase in the mRNA transcript levels for Cbfa1, OCN and BSP, and a noticeable reduction in the mRNA transcript levels for PPAR γ 2 (**Figure 3B**). However, CPE effectively abolished the effects of naringin on the mRNA transcript levels of the above genes. We treated BMSCs with IHH inhibitor CPE (0.5 μM), followed by 50 $\mu\text{g}/\text{mL}$ naringin for 21 days. Alizarin Red S assays showed that CPE markedly suppressed the naringin-mediated increase in calcium deposition (**Figure 3C and 3D**).

We then investigated whether naringin potentiated osteogenesis of BMSCs via the IHH signaling pathway by examining the protein levels of Foxc2 and IHH by immunoblotting assays. We found that Foxc2 expression was up-regulated, and IHH was activated in BMSCs under OIM.

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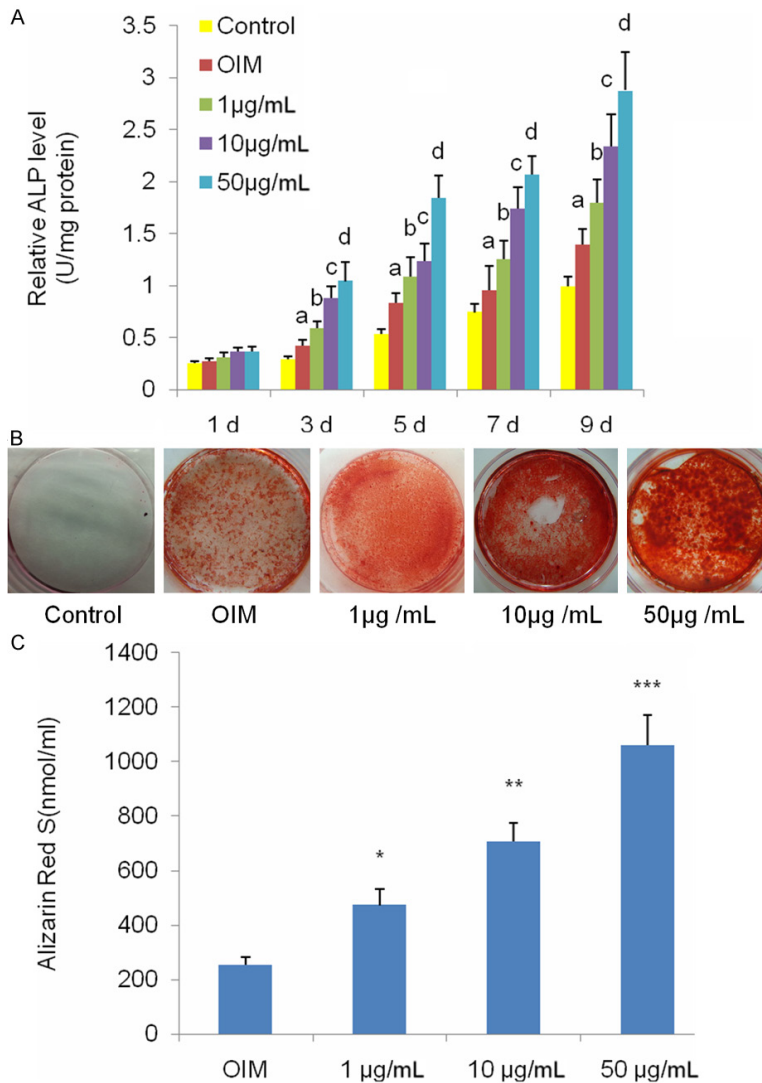


Figure 2. Naringin enhances osteogenic differentiation of BMSCs. A. Naringin enhanced ALP activities of BMSCs. ALP activities were measured by conventional methods and the data represent the mean \pm SD of quadruplicate samples ($n=4$). ^a $P<0.05$ versus the control group; ^b $P<0.01$ versus the OIM group; ^c $P<0.01$ versus the 1 $\mu\text{g}/\text{mL}$ group; ^d $P<0.01$ versus the 10 $\mu\text{g}/\text{mL}$ group. B. Naringin potentiated the mineralization of BMSCs under OIM. BMSCs underwent OIM or OIM with naringin at 1, 10, or 50 $\mu\text{g}/\text{mL}$ for 21 days. C. Mineralization of BMSCs was quantified by Alizarin Red S assays. Alizarin Red S showed that naringin enhanced the staining of BMSCs in a dose-dependent manner. Data represents mean \pm SD of experiments done in quintuplicate ($n=5$). * $P<0.01$ versus the OIM group; ** $P<0.01$ versus the 1 $\mu\text{g}/\text{mL}$ group; *** $P<0.01$ versus the 10 $\mu\text{g}/\text{mL}$ group.

Naringin further enhanced the expression of Foxc2 and the activation of IHH in BMSCs (Figure 4A-E). By contrast, CPE significantly attenuated the naringin-induced increase in Foxc2 expression and IHH activation (Figure 4A-E). These results suggest that naringin potentiated osteogenesis of BMSCs by increas-

ing the expression of Foxc2 via activation of the IHH signaling pathway.

Discussion

Osteoporosis poses an increasing public health challenge given the rapid aging of the population and the inadequacy of currently available therapies that are hampered by a myriad of side effects. An early study showed naringin, a polymethoxylated flavonoid, improves BMD in a rat model of osteoporosis [30]. Naringin was also found to possess estrogen-like activities and prevent loss of bone mass in ovariectomized mice and rats [31, 32]. Subsequent studies demonstrated that naringin stimulates osteogenic differentiation of bone marrow mesenchymal stem cells [12]. Consistently, in this study, we have found that naringin enhances osteogenesis of BMSCs in vitro. Fan et al. have recently found that naringin potentiates osteogenic differentiation by upregulating microRNA 20a and downregulating PPAR γ in BMSCs [33]. On the other hand, Wang et al. have demonstrated that naringin facilitates bone formation in ovariectomized mice by stimulating the canonical Wnt/ β -catenin signaling pathway by activated Akt and AMPK signaling [34]. Foxc2 also stimulates osteoblast differentiation of mesenchymal cells by activating the canonical Wnt/ β -catenin signaling pathway [16]. Here, we demonstrate that naringin increases mRNA transcript and protein levels of Foxc2 in BMSCs undergoing osteogenic differentiation. More importantly, inhibition of IHH signaling by CPE significantly attenuates naringin-induced increase in Foxc2, suggesting an intimate link between Foxc2 and

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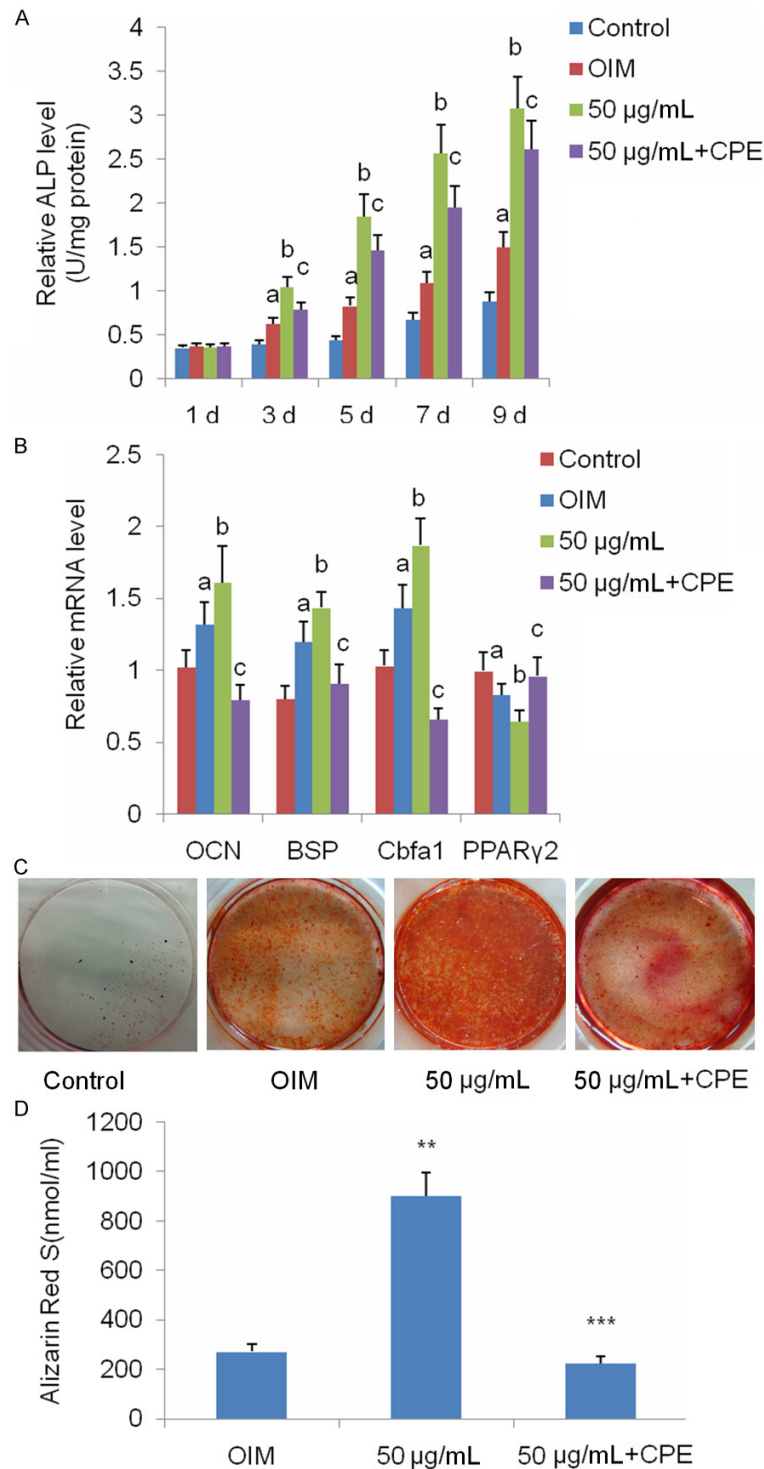


Figure 3. Naringin potentiates osteogenesis of BMSCs via the IHH signaling pathway. A. BMSCs were cultured in basal medium, OIM or OIM with 50 µg/mL naringin in the presence or absence of CPE for 1, 3, 5, 7, and 9 days, then subjected to intracellular ALP assays. Data represent the mean \pm SD of five independent experiments (n=5). ^a P <0.01 versus the control group; ^b P <0.01 versus the OIM group; ^c P <0.05 versus the 50 µg/mL group. B. Expression of genes involved in osteogenesis of BMSCs was quantified by real-time quantitative RT-PCR. BMSCs were grown in basal medium, OIM, or OIM with 50 µg/mL naringin in the absence or presence of CPE for 14 days. ^a P <0.05 versus

the control group; ^b P <0.05 versus the OIM group. ^c P <0.01 versus the 50 µg/mL group. Alizarin Red S assay of BMSCs cultured in basal medium, OIM or OIM with 50 µg/mL naringin, in the presence or absence of CPE, for 21 days. C. Alizarin Red S assays show that CPE inhibits naringin-potentiated mineralization of BMSCs. D. The amount of calcium deposit was quantified and statistically analyzed (n=5). ^{**} P <0.01 versus the OIM group; ^{***} P <0.01 versus the 50 µg/mL group.

IHH signaling in naringin-potentiated osteogenic differentiation of BMSCs. The mechanisms whereby naringin promotes osteogenesis of BMSCs have hitherto remained largely undefined. Our study provides the first direct piece of experimental evidence that naringin-potentiated osteogenesis may involve Foxc2 and IHH signaling.

Osteoblast commitment and differentiation are controlled by complex activities involving signal transduction and transcriptional regulation of gene expression [35]. Cbfa1 (Runx2) is considered as an early marker of osteogenic differentiation, whereas OCN and BSP are mid and late markers [36, 37]. In the present study, we found that naringin markedly increased the mRNA transcript levels of Cbfa1. Furthermore, we found that inhibition of IHH signaling by CPE suppressed Cbfa1 expression. This is consistent with findings by Shimoyama et al., who found that IHH regulates osteoblast differentiation of mesenchymal cells by upregulating Runx2 via Gli2 [24]. PPARγ2 is a critical regulator of the balance of differentiation between adipogenesis and osteogenesis [38]. We

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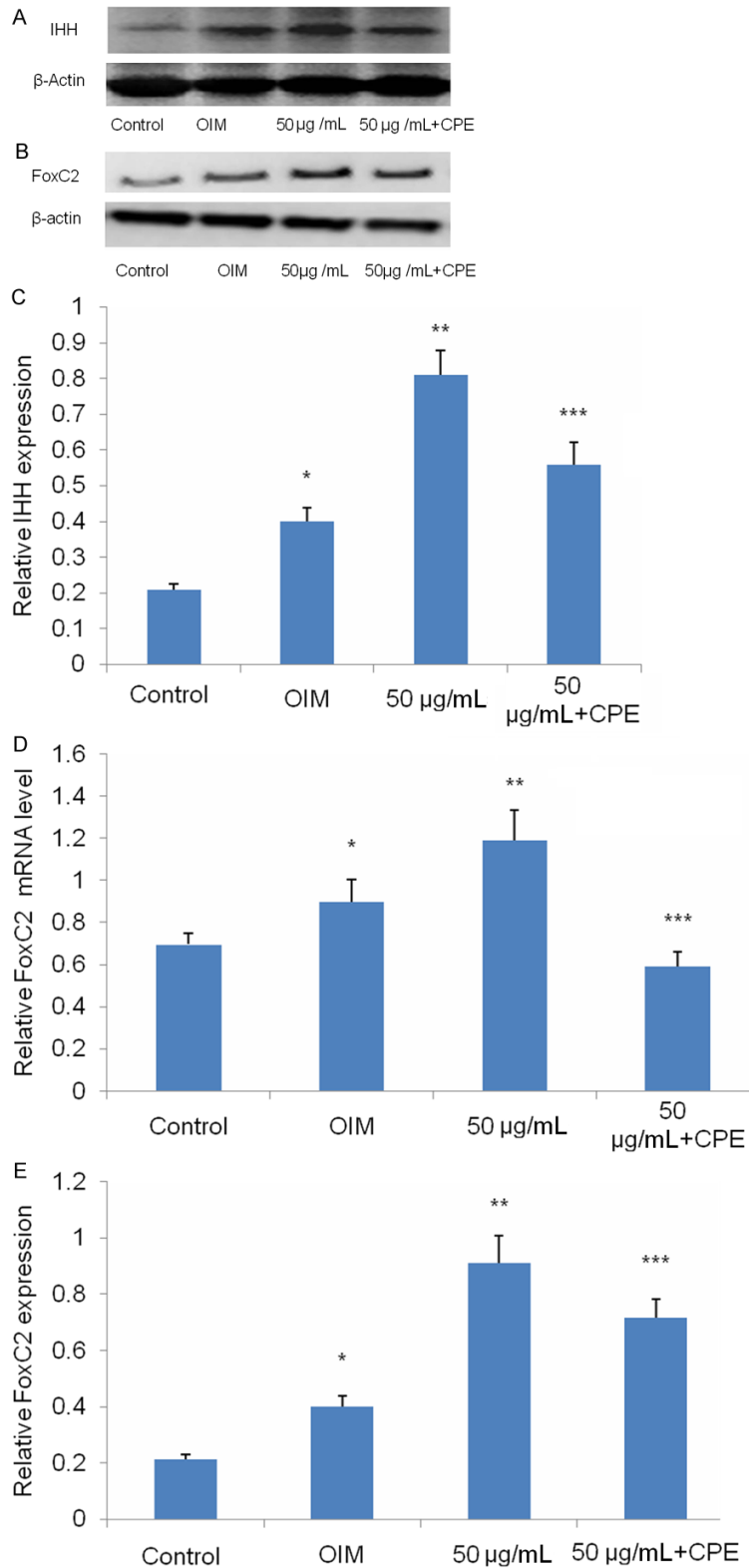


Figure 4. Naringin potentiates osteogenesis of BMSCs through up-regulating Foxc2 expression via the IHH signaling pathway. BMSCs were cultured in basal medium, OIM, OIM with 50 µg/mL naringin, and OIM with 50 µg/mL naringin in the absence or presence of 0.5 µM CPE for 14 days. A, B. Western blotting analysis of Foxc2 and IHH. C. Relative expression of Foxc2 in BM-

SCs was checked using real time PCR. D, E. Densitometric quantification of activated Foxc2 and IHH was performed. C. * $P < 0.05$ versus the control group; ** $P < 0.01$ versus the OIM group; *** $P < 0.01$ versus the 50 µg/mL group. D. * $P < 0.05$ versus the control group; ** $P < 0.01$ versus the OIM group; *** $P < 0.05$ versus the 50 µg/mL group.

found that naringin-potentiated osteogenic differentiation of BMSCs is associated with marked downregulation of PPAR γ 2 in BMSCs, which is in accordance with a recent study by Fan et al., who demonstrated that naringin-potentiated osteogenic differentiation is associated with downregulation of PPAR γ in BMSCs [33]. These data indicate that naringin-potentiated osteogenic differentiation is associated with changes in a myriad of osteogenesis-associated genes. Similar to our findings, Foxc2 activation has been associated with stimulating the expression of osteogenesis-associated genes and inhibiting the expression of PPAR γ 2 in BMSCs [18]. These results suggest that naringin may exert its effect on osteogenesis-associated genes by indirectly regulating Foxc2 expression.

The IHH signaling pathway is important for osteogenesis [24], and IHH inhibitor CPE significantly inhibits osteogenic differentiation of BMSCs [39]. In the present study, we show that CPE attenuates the naringin-induced increase in osteogenic differentiation-associated genes, including genes encoding OCN, BSP and Foxc2. This indicates that the effects of naringin on osteogenesis-associated genes

could occur through activation of the IHH signaling pathway.

In conclusion, our study demonstrates that naringin promotes osteogenic differentiation of BMSCs in vitro by activating osteogenesis-associated genes, and is partly mediated by up-regulating Foxc2 expression via the IHH signaling pathway. Our findings shed light on the mechanisms whereby naringin potentiates osteogenesis of BMSCs. These findings indicate that naringin could be of value for treating osteogenic diseases such as osteoporosis.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

- [1] Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J Clin Invest* 2005; 115: 3318-3325.
- [2] Zimmermann EA, Schaible E, Bale H, Barth HD, Tang SY, Reichert P, Busse B, Alliston T, Ager JW 3rd and Ritchie RO. Age-related changes in the plasticity and toughness of human cortical bone at multiple length scales. *Proc Natl Acad Sci U S A* 2011; 108: 14416-14421.
- [3] Mulder JE, Kolatkar NS and LeBoff MS. Drug insight: Existing and emerging therapies for osteoporosis. *Nat Clin Pract Endocrinol Metab* 2006; 2: 670-680.
- [4] Deal C. Potential new drug targets for osteoporosis. *Nat Clin Pract Rheumatol* 2009; 5: 20-27.
- [5] Sowers MR, Zheng H, Jannausch ML, McConnell D, Nan B, Harlow S and Randolph JF Jr. Amount of bone loss in relation to time around the final menstrual period and follicle-stimulating hormone staging of the transition to menopause. *J Clin Endocrinol Metab* 2010; 95: 2155-2162.
- [6] Peng S, Zhang G, He Y, Wang X, Leung P, Leung K and Qin L. Epimedium-derived flavonoids promote osteoblastogenesis and suppress adipogenesis in bone marrow stromal cells while exerting an anabolic effect on osteoporotic bone. *Bone* 2009; 45: 534-544.
- [7] Rodan GA and Martin TJ. Therapeutic approaches to bone diseases. *Science* 2000; 289: 1508-1514.
- [8] Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997; 276: 71-74.
- [9] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; 284: 143-147.
- [10] Burkhardt R, Kettner G, Bohm W, Schmidmeier M, Schlag R, Frisch B, Mallmann B, Eisenmenger W and Gilg T. Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. *Bone* 1987; 8: 157-164.
- [11] Ramesh N, Viswanathan MB, Saraswathy A, Balakrishna K, Brindha P and Lakshmanaperumalsamy P. Phytochemical and antimicrobial studies on *Drynaria quercifolia*. *Fitoterapia* 2001; 72: 934-936.
- [12] Zhang P, Dai KR, Yan SG, Yan WQ, Zhang C, Chen DQ, Xu B and Xu ZW. Effects of naringin on the proliferation and osteogenic differentiation of human bone mesenchymal stem cell. *Eur J Pharmacol* 2009; 607: 1-5.
- [13] Wu JB, Fong YC, Tsai HY, Chen YF, Tsuzuki M and Tang CH. 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.

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- [14] Carlsson P and Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. *Dev Biol* 2002; 250: 1-23.
- [15] Nifuji A, Miura N, Kato N, Kellermann O and Noda M. Bone morphogenetic protein regulation of forkhead/winged helix transcription factor Foxc2 (Mfh1) in a murine mesodermal cell line C1 and in skeletal precursor cells. *J Bone Miner Res* 2001; 16: 1765-1771.
- [16] Kim SH, Cho KW, Choi HS, Park SJ, Rhee Y, Jung HS and Lim SK. The forkhead transcription factor Foxc2 stimulates osteoblast differentiation. *Biochem Biophys Res Commun* 2009; 386: 532-536.
- [17] Park SJ, Gadi J, Cho KW, Kim KJ, Kim SH, Jung HS and Lim SK. The forkhead transcription factor Foxc2 promotes osteoblastogenesis via up-regulation of integrin beta1 expression. *Bone* 2011; 49: 428-438.
- [18] You W, Fan L, Duan D, Tian L, Dang X, Wang C and Wang K. Foxc2 over-expression in bone marrow mesenchymal stem cells stimulates osteogenic differentiation and inhibits adipogenic differentiation. *Mol Cell Biochem* 2014; 386: 125-134.
- [19] Ingham PW. Transducing Hedgehog: the story so far. *EMBO J* 1998; 17: 3505-3511.
- [20] Yamaguchi A, Komori T and Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr Rev* 2000; 21: 393-411.
- [21] St-Jacques B, Hammerschmidt M and McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 1999; 13: 2072-2086.
- [22] Hilton MJ, Tu X, Cook J, Hu H and Long F. Ihh controls cartilage development by antagonizing Gli3, but requires additional effectors to regulate osteoblast and vascular development. *Development* 2005; 132: 4339-4351.
- [23] Levi B, James AW, Nelson ER, Brugmann SA, Sorkin M, Manu A and Longaker MT. Role of Indian hedgehog signaling in palatal osteogenesis. *Plast Reconstr Surg* 2011; 127: 1182-1190.
- [24] Shimoyama A, Wada M, Ikeda F, Hata K, Matsubara T, Nifuji A, Noda M, Amano K, Yamaguchi A, Nishimura R and Yoneda T. Ihh/Gli2 signaling promotes osteoblast differentiation by regulating Runx2 expression and function. *Mol Biol Cell* 2007; 18: 2411-2418.
- [25] Lennon DP and Caplan AL. Isolation of rat marrow-derived mesenchymal stem cells. *Exp Hematol* 2006; 34: 1606-1607.
- [26] Li SH, Guo DZ, Li B, Yin HB, Li JK, Xiang JM and Deng GZ. The stimulatory effect of insulin-like growth factor-1 on the proliferation, differentiation, and mineralisation of osteoblastic cells from Holstein cattle. *Vet J* 2009; 179: 430-436.
- [27] Zou L, Zou X, Chen L, Li H, Mygind T, Kassem M and Burger C. Effect of hyaluronan on osteogenic differentiation of porcine bone marrow stromal cells in vitro. *J Orthop Res* 2008; 26: 713-720.
- [28] Allen JL, Cooke ME and Alliston T. ECM stiffness primes the TGFbeta pathway to promote chondrocyte differentiation. *Mol Biol Cell* 2012; 23: 3731-3742.
- [29] Steinert AF, Weissenberger M, Kunz M, Gilbert F, Ghivizzani SC, Gobel S, Jakob F, Noth U and Rudert M. Indian hedgehog gene transfer is a chondrogenic inducer of human mesenchymal stem cells. *Arthritis Res Ther* 2012; 14: R168.
- [30] Wei M, Yang Z, Li P, Zhang Y and Sse WC. Anti-osteoporosis activity of naringin in the retinoic acid-induced osteoporosis model. *Am J Chin Med* 2007; 35: 663-667.
- [31] Pang WY, Wang XL, Mok SK, Lai WP, Chow HK, Leung PC, Yao XS and Wong MS. Naringin improves bone properties in ovariectomized mice and exerts oestrogen-like activities in rat osteoblast-like (UMR-106) cells. *Br J Pharmacol* 2010; 159: 1693-1703.
- [32] Li N, Jiang Y, Wooley PH, Xu Z and Yang SY. Naringin promotes osteoblast differentiation and effectively reverses ovariectomy-associated osteoporosis. *J Orthop Sci* 2013; 18: 478-485.
- [33] Fan J, Li J and Fan Q. Naringin promotes differentiation of bone marrow stem cells into osteoblasts by upregulating the expression levels of microRNA-20a and downregulating the expression levels of PPARgamma. *Mol Med Rep* 2015; 12: 4759-4765.
- [34] Wang D, Ma W, Wang F, Dong J, Wang D, Sun B and Wang B. Stimulation of Wnt/beta-Catenin Signaling to Improve Bone Development by Naringin via Interacting with AMPK and Akt. *Cell Physiol Biochem* 2015; 36: 1563-1576.
- [35] Huang W, Yang S, Shao J and Li YP. Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front Biosci* 2007; 12: 3068-3092.
- [36] Komori T. Regulation of osteoblast differentiation by Runx2. *Adv Exp Med Biol* 2010; 658: 43-49.
- [37] Lee JH, Park JH, El-Fiqi A, Kim JH, Yun YR, Jang JH, Han CM, Lee EJ and Kim HW. Biointerface control of electrospun fiber scaffolds for bone regeneration: engineered protein link to mineralized surface. *Acta Biomater* 2014; 10: 2750-2761.
- [38] Watt J and Schlezinger JJ. Structurally-diverse, PPARgamma-activating environmental toxica-

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nts induce adipogenesis and suppress osteogenesis in bone marrow mesenchymal stromal cells. *Toxicology* 2015; 331: 66-77.

[39] Salem O, Wang HT, Alaseem AM, Ciobanu O, Hadjab I, Gawri R, Antoniou J and Mwale F.

Naproxen affects osteogenesis of human mesenchymal stem cells via regulation of Indian hedgehog signaling molecules. *Arthritis Res Ther* 2014; 16: R152.