

## Naringin enhances osteogenic differentiation through the activation of ERK signaling in human bone marrow mesenchymal stem cells

Huichao Wang<sup>1</sup>, Chunbo Li<sup>2</sup>, Jianming Li<sup>1</sup>, Yingjie Zhu<sup>1</sup>, Yudong Jia<sup>1</sup>, Ying Zhang<sup>1</sup>, Xiaodong Zhang<sup>1</sup>, Wenlong Li<sup>3</sup>, Lei Cui<sup>4</sup>, Wuyin Li<sup>1</sup>, Youwen Liu<sup>1\*</sup>

<sup>1</sup> Luoyang Orthopedic Hospital of Henan Province, Orthopedic Institute of Henan Province, Luoyang 471002, China

<sup>2</sup> Shanghai First Maternity and Infant Hospital, Tongji University of Medicine, Shanghai, 200126, China

<sup>3</sup> Henan University of Traditional Chinese Medicine, Henan Province, Zhengzhou 450000, China

<sup>4</sup> Medical Science & Research Center, Beijing Shijitan Hospital Affiliated to Capital Medical University, 10 Tieyi Road, Beijing 100038, China

### ARTICLE INFO

#### Article type:

Original article

#### Article history:

Received: Mar 28, 2016

Accepted: Jan 12, 2017

#### Keywords:

Bone marrow-  
mesenchymal stem cells  
ERK signaling pathway  
Naringin  
Osteogenic differentiation

### ABSTRACT

**Objective(s):** Naringin has been reported to regulate bone metabolism. However, its effect on osteogenesis remains unclear. The aim was to investigate the effect of naringin on osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) through the activation of the ERK signaling pathway in osteogenic differentiation.

**Materials and Methods:** Annexin V-FITC assay and MTT assay were used to measure the effect of naringin on cytotoxicity and proliferation of hBMSCs, respectively. Alkaline phosphatase activity analysis, Alizarin Red S staining, Western blotting, and real-time PCR assay were used to evaluate both the potential effect of naringin on osteogenic differentiation and the role of ERK signaling pathway in osteogenic differentiation.

**Results:** Our results showed that naringin had no obvious toxicity on hBMSCs, and could significantly promote the proliferation of hBMSCs. Naringin also enhanced the osteogenic differentiation of hBMSCs and increased the protein and mRNA expression levels of osteogenic markers such as Runx-2, OXS, OCN, and Col1 in a dose-dependent manner. In addition, we found that the enhancing effect of naringin on osteogenic differentiation was related to the activation of phosphor-ERK, with an increase in duration of activity from 30 min to 120 min. More importantly, both the enhancing effect of naringin on osteogenic differentiation and the activity effect of naringin on ERK signaling pathway were reversed by U0126 addition.

**Conclusion:** Our findings demonstrated that naringin promoted proliferation and osteogenesis of hBMSCs by activating the ERK signaling pathway and it might be a potential therapeutic agent for treating or preventing osteoporosis.

#### ► Please cite this article as:

Wang H, Li CH, Li L, Zhu Y, Jia Y, Zhang Y, Zhang X, Li W, Cui L, Li W, Liu Y. Naringin enhances osteogenic differentiation through the activation of ERK signaling in human bone marrow mesenchymal stem cells. Iran J Basic Med Sci 2017; 20:408-414; 10.22038/IJBMS.2017.8582

### Introduction

Osteoporosis, characterized by low bone mass density and deterioration of bone microarchitecture is regarded as one of the challenging clinical entities in the elderly population (1-3). Bone mass homeostasis depends on mainly the balance between bone formation and bone resorption, which is tightly regulated by bone-forming osteoblasts and bone-resorbing osteoclasts (2). There is now a mounting body of scientific evidence to suggest that the impaired osteogenic differentiation of resident bone mesenchymal stem cell (BMSCs) may play a decisive role in mediating osteoporotic bone loss (4). In general, MSCs differentiate into osteoprogenitors, pre-osteoblasts, and subsequently mature osteoblasts,

which are accompanied by the mineralization of the extracellular matrix (ECM) in the form of hydroxyapatite (5). Recently, several researchers have demonstrated that patients with bone fracture treated with MSCs present promising results. A study found that directing MSCs to the bone surface could augment bone formation and increase bone mass (6). Thus, enhancing osteogenesis of MSCs is considered as a potential therapeutic strategy for osteoporosis.

Osteogenesis is an intricate process, which is mediated by the action of several key transcriptional factors, including Runx-related transcription factor-2 (Runx-2) and osterix (OSX), and is usually accompanied by the increased expression of bone matrix proteins, such as alkaline phosphatase (ALP), type I collage

\*Corresponding author: Youwen Liu. Luoyang Orthopedic Hospital of Henan Province, Orthopedic Institute of Henan Province, Luoyang 471002, China. Tel/Fax: 00 86- 379 63546511; email: liuyouwen543@sina.com

(ColA1), and Osteocalcin (OCN) (4). ERK signaling pathway has been intensively investigated in regulating many cellular functions, such as meiosis, mitosis, apoptosis, and differentiation (7). Recently, it was reported that the activation or inhibition of ERK signaling pathway is involved in the commitment of MSCs into osteogenic lineages (8, 9). Several studies have found that ERK1/2, activated by mitogen-activated protein kinase 1/2 (MEK1/2) through phosphorylation at a threonine and an adjacent tyrosine residue, could strongly increase the Runx-2 protein level though an increase in acetylation and a decrease in ubiquitination (8, 10, 11). In addition, many studies have shown that traditional medicinal herbs such as Salvianolic acid B (12) and Chlormadinone acetate (13) could enhance the osteogenic differentiation of MSCs through the activation of ERK signaling pathway.

Naringin, a major traditional Chinese medicine with a long medicinal history, is the main effective component of rhizome dryariae (14). Rhizome dryariae, specifically naringin, is commonly used to manage orthopedic disorders (15). In addition, numerous studies revealed that naringin could enhance the proliferation and osteogenic differentiation of the MC3T3-E1 cell line (16). A study by Zhang *et al* also demonstrated that naringin had anti-osteoporotic and enhancing bone-unifying properties through the promotion of proliferation and osteogenic differentiation of human BMSCs (17). However, it remains unclear whether the effect of naringin for enhancing osteogenic differentiation is related to the activation of the ERK signaling pathway. Thus, the present study seeks to investigate the effects of naringin on the proliferation and osteogenic differentiation of hBMSCs by activating the ERK signaling pathway.

## Materials and Methods

### Chemical reagents

Naringin,  $\beta$ -glycerophosphate, L-ascorbic acid-2-phosphate, dimethyl Sulfoxide (DMSO), 1,25-dihydroxyvitamin D<sub>3</sub>, and Alizarin Red S were purchased from Sigma (St. Louis, MO, USA). Calcium Colorimetric Assay Kit and Alkaline Phosphatase Activity Colorimetric Assay Kit were obtained from Biovision, Inc. The anti-ERK antibody and anti-pERK antibody were purchased from Cell Signaling (Boston, MA, USA). U0126 was from Beyotime (Shanghai, China). All other chemicals were purchased from sigma (St. Louis, MO, USA).

### Cell Cultures and differentiation

Bone marrow aspirates were obtained from four healthy volunteers (A-D) (A, 27 years old; B, 29 years old; C, 34 years old; D, 47 years old) during the routine orthopedic surgical procedure in Shanghai 9th People's Hospital and hBMSCs were isolated and expanded using the previous method (18). All protocols of human tissue handling were approved by the Research Ethical

Committee of our Hospital. Briefly, the aspirates were immediately re-suspended in DMEM supplemented with 10% fetal bovine serum (FBS, Thermo scientific), 1% penicillin-streptomycin (Thermo scientific) (growth medium, GM), and plated at  $4 \times 10^4$  cells/cm<sup>2</sup> in 100 mm culture dishes (Falcon, USA) with the medium changed every three days at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. After the cells were cultured for 1 day, the growth medium was replaced with differentiation medium (DMEM with  $10^{-8}$  M dexamethasone, 50  $\mu$ g/ml ascorbic acid, and 5 mM glycerol phosphate), which was changed every 2 days during osteogenic differentiation. To evaluate the effect of naringin on osteogenesis, the cells were treated with osteogenic differentiation medium supplemented with various concentrations of naringin (0, 10, 50, and 100  $\mu$ g/ml). Three independent experiments were performed in quadruplicate.

### Cell viability

The hBMSCs from passage 3–5 were seeded in 6-well plates at a density of  $1 \times 10^4$  cells/well and cultured in growth or osteogenic medium supplemented with various concentrations of naringin at 0, 10, 50, and 100  $\mu$ g/ml. Then, cell viability analysis was performed at 1, 3, and 7 days based on a known method (19). The cells were washed twice in PBS and dissociated with 0.25% trypsin/EDTA, after which the cells were collected and stained using an Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol, and finally analyzed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

### MTT assay

The MTT assay was used to test the effect of naringin on cell proliferation based on an existing method (19). Briefly, hBMSCs at a density of  $5 \times 10^3$  cells/well were seeded in 96-well plates and cultured in growth or osteogenic medium supplemented with various concentrations of naringin (0, 10, 50, and 100  $\mu$ g/ml). Following that, the cells were cultured for 0, 1, 3, 7, and 14 days, respectively. At each time point, the cells were washed twice with PBS, and then 200  $\mu$ l DMEM supplemented with 20  $\mu$ l 5 mg/mL MTT (Amresco, USA) solution was added and incubated at 37°C for 4 hr. Subsequently, the medium was replaced with 200  $\mu$ l DMSO and vibrated for 10 min to dissolve the MTT formazan crystals. Finally, the absorbance was measured at 450 nm using an ELx Ultra Microplate Reader (BioTek, USA).

### ALP staining and activity assay

hBMSCs were seeded in 6-well plates at a density of  $1 \times 10^4$  cells/well and cultured in DMEM with various concentrations of naringin, as described above. After the cells were cultured for 14 days, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 10 min. Then, the cells were equilibrated by ALP buffer

(0.15 M NaCl, 0.15 M Tris-HCl, 1 mM MgCl<sub>2</sub>, pH 9.0) twice, and incubated with ALP substrate solution (5 l BCIP 1 ml ALP buffer) at 37 °C in the dark for 60 min. Then the reaction was stopped by distilled water and the plate was visualized using a microscope. The ALP activity was determined using p-nitrophenylphosphate as the substrate. Absorbance at 405 nm was measured and the protein concentration of cell lysates was measured using the Bradford assay at 595 nm on a microplate spectrophotometer (Bio-Rad, USA). ALP activity was normalized according to the total protein concentration.

#### Alizarin Red S staining and quantification determination

To detect mineral accumulation in differentiation osteoblasts, the hBMSCs treated with osteogenic induction for 14 days were washed with PBS and fixed with 4% paraformaldehyde for 10 min. After washing with deionized water, the cells were stained with 0.1 % Alizarin Red solution (Sigma) at pH 4.2 for 30min at room temperature. Orange Red staining indicated the position and intensity of calcium deposits. For quantitation determination, 0.5 ml 100 mmol/l cetylpyridinium chlorides (CPC) solution (Sigma-Aldrich, MO, USA) was added to each well and shaken for 30 min. The mixed liquid was diluted with 100 mmol/l CPC solution to 1/20 of the original concentration and quantified by measuring the OD of the extract at 550 nm.

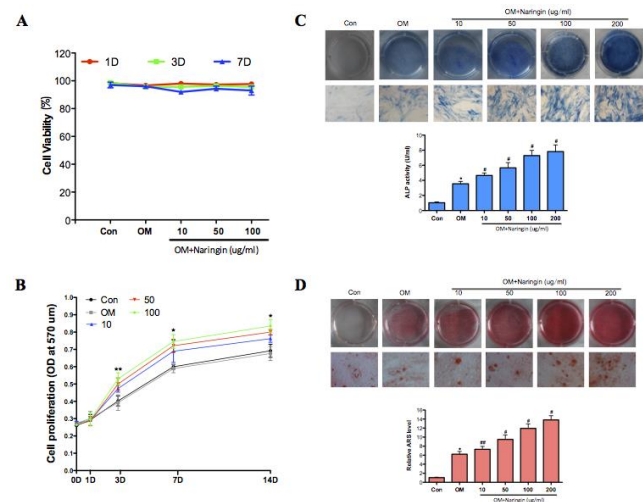
#### Quantitative real-time PCR

Total RNA of the hBMSCs was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA yield and purity were confirmed by the ratio of A(260)/A(280) using UV-spectroscopy. First-strand cDNA was synthesized from 1-3 µg of the extracted RNA samples using transcriptase reverse transcriptase (TaKaRa), real Time PCR was performed using quantitative real-time amplification system (Biorad, CFX Connect), and SYBR Green PCR MasterMix (Tiangen Biotech ) was used in each reaction. Expression of Runx-2, ALP, collagen type I (COL I), osteopontin (OPN), and OCN were evaluated. Amplification conditions were as follows first at 95°C for 15 min, and then 40 cycles of 95°C for 10 sec and 60° C for 40 sec. Primer sequences were as follows: Runx-2: forward 5'-GTCTTACCCTCCTACCTGA-3' and reverse 5'-TGCCTGGCTCTTCTTACTGA-3'; Col1: forward 5'-TGTTTCAGCTTTGTGGACCTC-3' and reverse 5'-CTTGGTCTCGTCACAGATCA-3', OSX: forward 5'-ACGTGGCTAAGAATGTCATC-3' and reverse 5'-CTGTTAGCGCATGTCCTTA-3'; OCN: forward 5'-CAAAGTGCAGCCTTTGTGTC-3' and reverse 5'-TCACAGTCCGGATTGAGCTCA-3'; α-tubulin: forward 5'-ACATCGCTCAGACACCATG-3' and reverse 5'-TGTTAGTTGAGGTCATGAAGGG-3', real expression levels for each gene of interest were calculated by

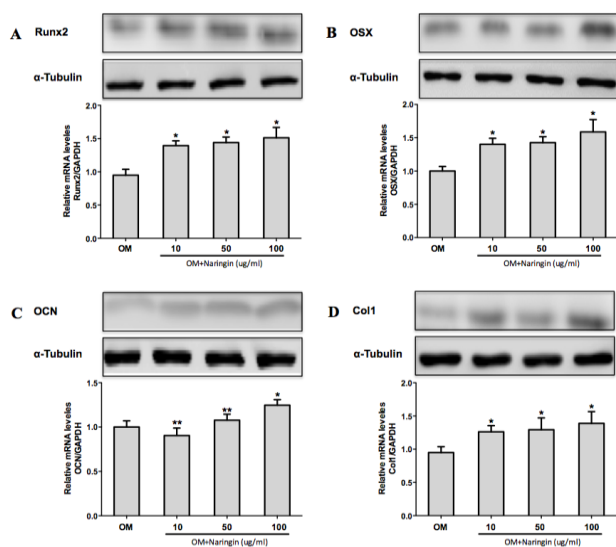
normalizing the quantified cDNA transcript level (cycle threshold) to that of the housekeeper gene α-tubulin.

#### Western blotting analysis

Cells were washed three times with ice-cold PBS then lysed using cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2 g/l sodium azide, 1 g/l sodium dodecyl sulfate (SDS), 0.1 g/l aprotinin, 10 g/l NP-40, 5 g/l sodium deoxycholate, 0.1 g/l phenylmethylsulfonylfluoride, pH 8.0) on ice. After centrifugation at 12,000 rpm for 10 min at 4 °C, the protein concentrations were determined (BCA, Pierce). Equal amounts of protein lysates (20 µg) were electrophoresed in 10 % SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% skim milk for 1 hr at room temperature, the membranes were incubated, respectively, with anti-ERK1/2 (BD, 1:2000), anti-p-ERK1/2 (BD, 1:1000) or anti-GAPDH (Santa Cruz, 1:1000) antibodies at 4 °C overnight and then incubated with horseradish peroxidase-linked secondary antibodies (anti-mouse, anti-goat, or anti-rabbit) for 1 hr at room temperature. Signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).



**Figure 1.** Naringin enhances proliferative and osteogenic differentiation of hBMSCs without toxicity. (A): The viability of hBMSCs treated with growth or osteogenic medium supplemented with various concentrations of naringin (0, 10, 50, and 100 µg/ml) for 1, 3, and 7 days was measured by flowcytometer. (B): Effect of naringin on the proliferation of hBMSCs. Cells were treated with growth or osteogenic medium supplemented with various concentrations of naringin (0, 10, 50, and 100 µg/ml) for 1, 3, 7, 14, and 21 days were measured by MTT assay. \* $P < 0.05$  or \*\* $P > 0.05$  compared with Con. (C): ALP staining and activity of differentiating hBMSCs cultured in growth or osteogenic medium supplemented with various concentrations of naringin (0, 10, 50, and 100 µg/ml) for 14 days. # $P < 0.05$  compared with Con, \* $P < 0.05$  compared with Con. (D) Alizarin Red staining and quantitation of hBMSCs treated with various concentrations of naringin (0, 10, 50, and 100 µg/ml) for 14 days. # $P < 0.05$  or #  $P > 0.05$  compared with control, \* $P < 0.05$  or \*\* $P > 0.05$  compared with OM group. Con: cells treated with growth medium. OM: cell treated with osteogenic medium without naringin



**Figure 2.** Naringin enhances the protein and mRNA expression of osteoblastic markers in hBMSCs. Western blotting and real-time PCR evaluates the protein and mRNA expression of Runx-2 (A), Osx (B), Ocn (C) and Col1 (D) of hBMSCs treated with osteogenic medium supplemented with various concentrations of naringin (0, 10, 50, and 100 ug/ml) for 14 days \* $P < 0.05$  or \*\* $P > 0.05$  compared with the OM group. OM: cells treated with osteogenic medium without naringin

### Inhibition of ERK

U0126, a highly specific and potent ERK inhibitor (20), was used to examine the role of ERK signaling pathway in hBMSCs osteogenesis stimulated by naringin. The experimental specimens were treated with 10  $\mu$ M U0126. Then, 100 ug/ml naringin stimulation was performed. Control cells were cultured in the absence of U0126. The inhibitor was replaced every 3 days, along with the growth or osteogenic medium. At day 14, ALP staining and activity analysis were used to examine the osteogenic differentiation of the hBMSCs and Alizarin Red S staining was used to evaluate the formation of calcium deposits. Western blotting and real-time PCR were used to measure the proteins and genes expression of Runx-2 and OSX.

### Statistical analysis

The results were presented as mean  $\pm$  standard deviation (SD) from three individuals, and each of them was the mean of triplicate experiments. Statistical analysis was performed using the SPSS software (version 13.0). One-way analysis of variance was used to compare the differences between the experimental group, where a value of  $P < 0.05$  was considered to be statistically significant.

## Results

### Effect of naringin on viability and proliferation of hBMSCs

To explore whether the naringin treatment had toxicity on cells, the cell viability was measured. Our results showed that naringin exposure at various concentrations (10, 50, and 100 ug/ml) did not

influence the viability of hBMSCs cultured for 1, 3, and 7 days as compared to control (Figure 1A).

The MTT assay was used to compare the proliferation of hBMSCs, after being cultured in growth or osteogenic medium supplemented with different concentrations of naringin (0, 10, 50, and 100 ug/ml) for 1, 3, 7, and 14 days. Our findings showed that the cell numbers increased with increasing culture time, and naringin at various concentrations resulted in a high proliferation potential after 3 days of culture as compared to control (Figure 1B). These results indicated naringin seemed to be safe and effective for cell growth.

### Naringin promoted osteogenic differentiation and mineral accumulation of hBMSCs

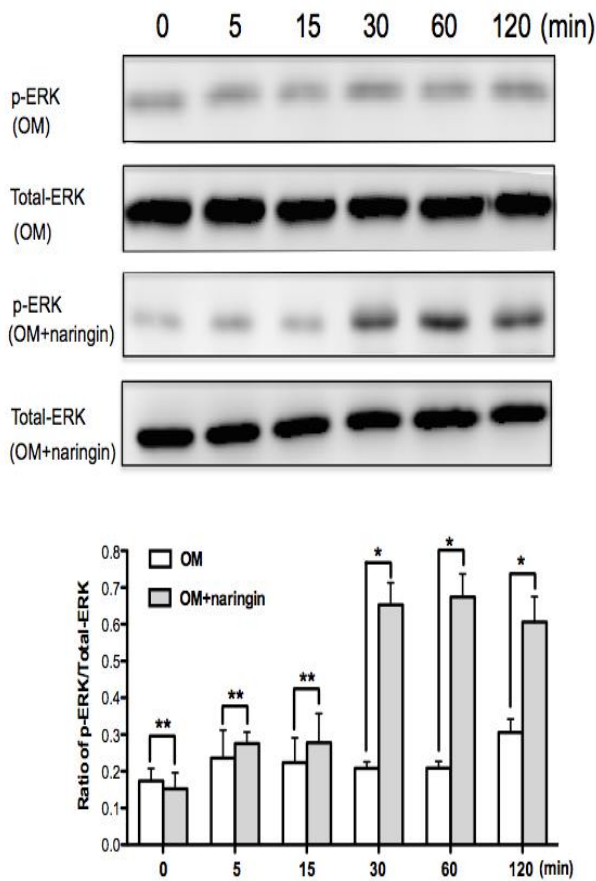
To examine whether naringin stimulated osteogenesis, ALP staining and activity analysis were carried out in the presence and absence of naringin after the cells were cultured for 14 days. ALP, a significant marker for the differentiation of osteoblast, was expressed at an early phase of differentiation and involved in the initiation of mineralization (6). Our results showed that naringin increased the ALP activity of hBMSCs in a dose-dependent manner, and the increase in the activity peaked at the concentration of 100 ug/ml (Figure 1C). Concurrently, naringin treatment increased the mineral accumulation and calcium deposition in a dose-dependent manner, which was confirmed by Alizarin red S staining (Figure 1D). Taken together, these results suggested that naringin promoted osteoblast differentiation and mineralization of hBMSCs.

### Naringin promoted osteogenic markers expression

To further investigate the effect of naringin on osteogenic differentiation in hBMSCs, Western blotting and real-time PCR were used to detect the expression of several osteogenic differentiation-related markers after the hBMSCs were treated with osteogenic medium supplemented with vehicle control or naringin for 14 days. Our results showed that naringin at various concentrations increased the expression level of Runx-2, OSX, and Col1 as compared to the vehicle control in osteogenesis induction condition (Figures 2A, B, and D). OCN, a major non-collagenous bone matrix synthesized protein, was secreted by normal maturing osteoblasts (6). Our findings showed that naringin at the concentration of 100 ug/ml increased the expression level of OCN, while there was no difference at the concentration of 10 or 50 ug/ml naringin as compared to control (Figure 2C). Taken together, these results supported that naringin promoted osteogenic differentiation of hBMSCs.

### Naringin activated the expression of the ERK signaling pathway components

To determine whether naringin-mediated osteogenesis was related to the ERK signaling pathway,



**Figure 3.** Naringin activates the ERK1/2 signaling pathway in hBMSCs. The phosphorylation of ERK and total-ERK in hBMSCs treated with naringin at 100 ug/ml for different time points (0-120 min) were evaluated by Western blotting (upper panel). The intensity of the protein bands was quantified and calculated as percentages of the control (lower panel). Values are expressed as the fold of increase to OM group. \* $P < 0.05$  or \*\* $P > 0.05$  compared with the OM group. OM: cells treated with osteogenic medium without naringin

We initially analyzed the effect of naringin (100 ug/ml) on the compounds of ERK signaling pathways by Western blotting. As shown in Figure 3, our results indicated that 100 ug/ml naringin induced the activation of phosphor-ERK1/2 from 30 min to 120 min and peaking at 60 min as compared to control. Therefore, these results suggested the hypothesis that naringin was sufficient to induce the activation of ERK1/2.

**U0126 inhibited the effect of naringin for osteogenic differentiation**

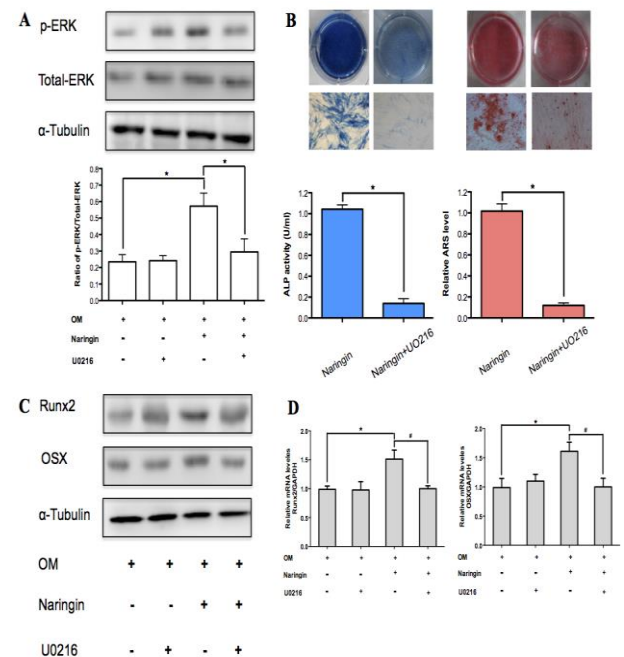
To demonstrate whether the activation of the ERK signaling pathway is required for the naringin-mediated osteogenic activity, U0126, an inhibitor of MEK1/2, was used to block the activation of p-ERK.

As shown in Figure 4A, the higher protein expression levels of p-ERK activated by naringin treatment were decreased in the presence of U0126, suggesting that U0126 completely inhibited the

naringin-induced activation of ERK1/2. Furthermore, our results indicated that pretreatment of U0126 effectively decreased the enhancing effect of naringin on ALP activity, which was shown by ALP staining. Concomitantly, Alizarin Red S staining revealed that U0126 treatment significantly inhibited the naringin-promoted calcium deposition (Figure 4B).

Then, we examined the effect of U0126 on the expression level of naringin-promoted osteogenic marker genes such as Runx-2 and OSX. Our findings demonstrated that the protein expression levels of Runx-2 and OSX elevated by naringin treatment were reduced in the presence of U0126 (Figure 3D). Moreover, the increased mRNA expression levels of Runx-2 and OSX due to naringin treatment were effectively attenuated by the addition of U0126 (Figure 4D).

These results supported the hypothesis that naringin stimulated osteoblast differentiation and mineralization of hBMSCs through activating the ERK signaling pathway.



**Figure 4.** U0126 blocked naringin-induced activation of ERK1/2 signaling pathway and naringin-induced enhancing effect of osteogenic differentiation. (A) U0126 blocked the protein expression of p-ERK and total-ERK, activated by naringin at 100 ug/ml in hBMSCs. (B) U0126 blocked the enhancing effect of naringin at 100ug/ml on osteogenic differentiation, which was confirmed by ALP staining and Alizarin Red staining. \* $P < 0.05$  compared with naringin alone. (C) U0126 reduced the protein expression of Runx-2 and OSX enhanced by naringin at 100 ug/ml in hBMSCs. (D) U0126 reduced the gene expression of Runx-2 and OSX enhanced by naringin treatment in hBMSCs. \* $P > 0.05$  or \*\* $P < 0.05$  compared with OM without naringin and U0126 treatment. # $P > 0.05$  or ## $P < 0.05$  compared with OM supplemented naringin treatment. OM: cells treated with osteogenic medium without naringin

## Discussion

Previous studies have demonstrated that the enhancing osteogenesis of MSCs to osteoblast is a potential therapeutic method for osteoporosis (4, 6). Many natural products capable of regulating osteogenic differentiation have been explored to treat osteoporosis (8, 11, 12). In the present study, we demonstrated that naringin could enhance osteogenic differentiation by up-regulating the expression level of osteogenic transcription factors and activating the ERK signaling pathway.

Naringin has been reported to have antioxidant, anti-inflammatory, and anti-apoptosis effect, which could exert its potential therapeutic benefits for a wide gamut of human disorders such as atherosclerosis, cardiovascular disorders, diabetes, and cancer (14). A study demonstrated that naringin at a dose of 0.5-5 mmol/l significantly suppressed iron-induced lipid peroxidation, protein oxidation and DNA damage (21). Liu *et al* showed that naringin at a dose of 50–200  $\mu$ M mediated its anti-inflammatory effect by inhibiting IL-8, MCP-1, and MIP-1 secretion and mRNA expression as well as by inhibiting the phosphorylation of ERK1/2, JUK, and p38 MAPK signaling pathway (22). Recently, a study revealed that naringin (10 nM to 1  $\mu$ M) increased cell proliferation and ALP activity in rat osteoblast-like UMR-106 cells, which presented a potential therapeutic effect for osteoporosis and bone loss (23). A study indicated that naringin at a dose of 2  $\mu$ g/ml could improve osteogenic proliferation and differentiation in MC3T3-E1 cells via up-regulation of Runx-2, Col1, and OCN protein expressions as well as modulation of BMP-2 (24). Recently, research showed that naringin at a dose of 1–100  $\mu$ g/mL also enhanced the proliferation and osteogenic differentiation of hBMSC (17). According to these published studies, we estimated that a high concentration of naringin is required to exert its anti-apoptosis and anti-inflammatory effects. However, a low concentration of naringin is enough to promote proliferation and induce differentiation of cells. A study demonstrated that naringin could increase the proliferation and ALP activity of human adipose-derived stem cells (hADSCs) in the range of 1–100  $\mu$ g/mL, while a dose of 200  $\mu$ g/ml naringin resulted in reduced cell numbers (25). Thus, in the present study, a naringin dose of 10–100  $\mu$ g/ml was utilized to evaluate the enhancing effect of naringin on osteogenic differentiation of hBMSCs.

The development of osteogenesis involved in several proteins and transcription factors and their coordinated function has been envisaged. ALP, a cell membrane-associated enzyme increases if there is active bone formation occurring, which is used as an indicator of early osteogenic differentiation (6). ALP activity is associated with matrix formation in osteoblasts formation prior to the initiation of mineralization (6). In the present study, our outcomes indicated that naringin treatment enhanced the

osteogenic differentiation, as measured by ALP staining and significantly increased the activity of ALP in hBMSCs. As expected, naringin enhanced calcium nodule formation, a functional marker of mineralization, as presented by Alizarin Red staining. Next, we evaluated the changes of osteogenesis-related genes in naringin-treated hBMSCs, such as Runx-2, OSX, OCN, and Col1. Runx-2, a pivotal osteogenic transcription regulator, plays a crucial role in the process of osteoblast maturation (26). OSX, as a downstream factor of Runx-2, is required for osteoblast differentiation in developing bones (27). OCN, characterized by mature cells of the osteoblastic lineage, is usually involved in controlling the mineralization process and appears at a late stage of osteogenic differentiation (28). Col1 is the most abundant protein in the bone matrix; it constitutes about 90% of the organic bone matrix and high levels of Col1 mRNA expression would be observed during proliferation (27). As expected, our findings indicated that naringin up-regulated the protein and mRNA expression levels of Runx-2, OSX, OCN, and Col1 in a concentration-dependent manner. These findings indicated that naringin could promote the osteogenic differentiation of hBMSCs by controlling these biosynthesis-related genes during osteogenic differentiation.

Previous studies have shown that ERK signaling pathway plays an important role in the regulation of osteogenesis by enhancing the osteogenic transcription regulators such as Runx-2 and OSX (28). Studies found that the activation of ERK signaling pathway involved in taurine (11), lactoferrin (8), and phosphatidylserine (29) promoted osteogenic differentiation. In the present study, we found that naringin significantly activated the phosphorylation of ERK1/2 in a dose-dependent manner. To further confirm our findings, we also checked whether blocking ERK1/2 signaling pathway could impair the effect of naringin on osteogenesis of hBMSCs. Our outcomes indicated that a higher phosphorylation level of ERK1/2 activated by naringin treatment could be blocked completely by the addition of U0126. In addition, U0126 also inhibited the ALP activity and reduced the mineralized nodule formation that was enhanced by naringin in hBMSCs. Moreover, the relatively higher expression levels of Runx-2 and OSX due to naringin treatment were reversed by U0126; indicating Runx-2 was a target of the ERK1/2 pathway. These results demonstrated that ERK signaling pathway was related with naringin-mediated regulation of osteogenic differentiation of hBMSCs.

## Conclusion

According to our findings, naringin promoted osteogenesis of hBMSCs through activating the ERK signaling pathway. This might be one of the mechanisms by which naringin increased bone mass. More studies were needed to explore other potential

mechanisms involved in the differentiation of naringin-treated hBMSC, which might help the application of naringin in osteoporotic patients.

### Acknowledgment

We appreciated all the help and suggestions from other members of the laboratory. Huichao Wang and Chunbo Li contributed equally to this study.

### Conflict of interest

None declared.

### References

- Makras P, Delaroudis S, Anastasilakis AD. Novel therapies for osteoporosis. *Metabolism* 2015; 64:1199-1214.
- Drake MT, Clarke BL, Lewiecki EM. The Pathophysiology and Treatment of Osteoporosis. *Clin Ther* 2015; 37:1837-1850.
- Lim SY, Bolster MB. Current approaches to osteoporosis treatment. *Curr Opin Rheumatol* 2015; 27:216-224.
- James AW. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica* 2013; 2013:684736.
- Bidwell JP, Alvarez MB, Hood M, Jr., Childress P. Functional impairment of bone formation in the pathogenesis of osteoporosis: the bone marrow regenerative competence. *Curr Osteoporos Rep* 2013; 11:117-125.
- Guan M, Yao W, Liu R, Lam KS, Nolte J, Jia J, et al. Directing mesenchymal stem cells to bone to augment bone formation and increase bone mass. *Nat Med* 2012; 18:456-462.
- McArthur GA. Combination Therapies to Inhibit the RAF/MEK/ERK Pathway in Melanoma: We are not Done Yet. *Front Oncol* 2015; 5:161.
- Ying X, Cheng S, Wang W, Lin Z, Chen Q, Zhang W, et al. Effect of lactoferrin on osteogenic differentiation of human adipose stem cells. *Int Orthop* 2012; 36:647-653.
- Gonzalez-Andrades MG, I, Alaminos M. Advances in the Field of Tissue Engineering and Regenerative Medicine: State of the Art and Regulatory Issues. *J Biomater Tissue Eng* 2013; 3:245-260.
- Li Y, Wang J, Chen G, Feng S, Wang P, Zhu X, et al. Quercetin promotes the osteogenic differentiation of rat mesenchymal stem cells via mitogen-activated protein kinase signaling. *Exp Ther Med* 2015; 9:2072-2080.
- Zhou C, Zhang X, Xu L, Wu T, Cui L, Xu D. Taurine promotes human mesenchymal stem cells to differentiate into osteoblast through the ERK pathway. *Amino Acids* 2014; 46:1673-1680.
- Xu D, Xu L, Zhou C, Lee WY, Wu T, Cui L, et al. Salvianolic acid B promotes osteogenesis of human mesenchymal stem cells through activating ERK signaling pathway. *Int J Biochem Cell Biol* 2014; 51:1-9.
- Kim JM, Lee JE, Ryu SH, Suh PG. Chlormadinone acetate promotes osteoblast differentiation of human mesenchymal stem cells through the ERK signaling pathway. *Euro J Pharmacol* 2014; 726:1-8.
- Bharti S, Rani N, Krishnamurthy B, Arya DS. Preclinical evidence for the pharmacological actions of naringin: a review. *Planta Med* 2014; 80:437-451.
- Li F, Sun X, Ma J, Ma X, Zhao B, Zhang Y, et al. Naringin prevents ovariectomy-induced osteoporosis and promotes osteoclasts apoptosis through the mitochondria-mediated apoptosis pathway. *Biochem Biophys Res Commun* 2014; 452:629-635.
- Li N, Xu Z, Wooley PH, Zhang J, Yang SY. Therapeutic potentials of naringin on polymethylmethacrylate induced osteoclastogenesis and osteolysis, *in vitro* and *in vivo* assessments. *Drug Des Devel Ther* 2014; 8:1-11.
- Zhang P, Dai KR, Yan SG, Yan WQ, Zhang C, Chen DQ, et al. Effects of naringin on the proliferation and osteogenic differentiation of human bone mesenchymal stem cell. *Euro J Pharmacol* 2009; 607:1-5.
- Baharara J, Amini E, Kerachian MA, Soltani M. The osteogenic differentiation stimulating activity of Sea cucumber methanolic crude extraction on rat bone marrow mesenchymal stem cells. *Iran J Basic Med Sci* 2014; 17:626-631.
- Fang B, Wan YZ, Tang TT, Gao C, Dai KR. Proliferation and osteoblastic differentiation of human bone marrow stromal cells on hydroxyapatite/bacterial cellulose nanocomposite scaffolds. *Tissue Eng Part A* 2009; 15:1091-1098.
- Hou X, Shen Y, Zhang C, Zhang L, Qin Y, Yu Y, et al. A specific oligodeoxynucleotide promotes the differentiation of osteoblasts via ERK and p38 MAPK pathways. *Int J Mol Sci* 2012; 13:7902-7914.
- Jagetia GC, Reddy TK, Venkatesha VA, Kedlaya R. Influence of naringin on ferric iron induced oxidative damage *in vitro*. *Clin Chim Acta* 2004; 347:189-197.
- Liu Y, Su WW, Wang S, Li PB. Naringin inhibits chemokine production in an LPS-induced RAW 264.7 macrophage cell line. *Mol Med Rep* 2012; 6:1343-1350.
- Pang WY, Wang XL, Mok SK, Lai WP, Chow HK, Leung PC, et al. 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.
- Li L, Zeng Z, Cai G. Comparison of neoeriocitrin and naringin on proliferation and osteogenic differentiation in MC3T3-E1. *Phytomedicine* 2011; 18:985-989.
- Liu M, Li Y, Yang ST. Effects of naringin on the proliferation and osteogenic differentiation of human amniotic fluid-derived stem cells. *J Tissue Eng Regen Med* 2014; doi: 10.1002/term.1911.
- Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997; 89:755-764.
- Potier E, Noailly J, Ito K. Directing Bone marrow-derived stromal cell function with mechanics. *J Biomech* 2010; 43:807-817.
- Miraoui H, Oudina K, Petite H, Tanimoto Y, Moriyama K, Marie PJ. Fibroblast growth factor receptor 2 promotes osteogenic differentiation in mesenchymal cells via ERK1/2 and protein kinase C signaling. *J Biol Chem* 2009; 284:4897-4904.
- Xu C, Zheng Z, Fang L, Zhao N, Lin Z, Liang T, et al. Phosphatidylserine enhances osteogenic differentiation in human mesenchymal stem cells via ERK signal pathways. *Mater Sci Eng C Mater Biol Appl* 2013; 33:1783-1788.