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ORIGINAL PAPER

Amentoflavone enhances osteogenesis of human mesenchymal stem cells through JNK and p38 MAPK pathways

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Abstract Amentoflavone is a bioflavonoid found in a variety of traditional Chinese medicines such as Gingko and *Selaginella tamariscina*. It has been reported that amentoflavone has anti-inflammatory, antioxidant, antiviral and anticancer effects. However, the effect of amento-flavone on osteogenic differentiation of human mes-enchymal stem cells (hMSCs) has not been studied. In this study, we aim to explore the effect of amentoflavone on the proliferation and osteogenic differentiation of hMSCs. The results showed that amentoflavone significantly enhanced the proliferation, alkaline phosphatase (ALP) activity and mineralization in hMSCs. Western blot analysis revealed that the expression of runt-related transcription factor 2 and osterix proteins was upregulated in amentoflavone-treated hMSCs. Furthermore, we investigated the possible signaling pathways responsible for osteogenic differentiation of hMSCs by amentoflavone. We found that amentoflavone significantly increased the levels of phosphorylated JNK and p-p38. The amentoflavone-induced increases of ALP and mineralization were significantly diminished when the JNK and p38 MAPK pathways were blocked by selected inhibitors (SP600125, SB203580) in hMSCs. Furthermore, in vivo evidence indicated that amentoflavone protected against the dexamethasone-induced inhibition of osteoblast differentiation in tg(sp7:egfp) zebrafish larvae. Thus, we showed for the first time that amentoflavone improves the osteogenesis of hMSCs through the JNK and p38 MAPK pathway. Amentoflavone may be beneficial in treating bone-related disorders.

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Introduction

Osteoporosis, characterized by a high risk of fragility fractures and skeletal pain, is one of the most common bone disorders and results in low bone mineral density and the deterioration of bone microarchitecture [1]. What causes osteoporosis is the imbalance of function between osteoblasts and osteoclasts [2]. Drugs, such as bisphos-phonates, calcitonin and selective estrogen receptor mod-ulators, have been used in osteoporosis treatments to inhibit bone resorption. They are effective in stabilizing bone architecture, but they cannot increase bone formation and restore the bone structure and have obvious side effects [3–

5]. Thus, it is very important to develop anabolic agents to treat osteoporosis.

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into osteoblasts, adipocytes, chondrocytes, myoblasts, neurons, muscle cells and endothelial cells in response to different stimuli [6, 7]. The multipotent property of MSCs makes it possible for them to be used in cell therapy for musculoskeletal applications including bone tissue engineering and osteoporosis [8]. In recent years, MSCs showed promising results for healing bone-related diseases such as bone fracture and segmental bone defects [9–11]. In addition, the osteogenic differentiation potential of MSCs was weak in osteoporotic patients [12]. Therefore, it could be a potential therapy to enhance osteogenesis of MSCs for bone tissue engineering and osteoporotic patients [13].

In recent years, the zebrafish has become an ideal model for bone research. The cortical bone transgenic zebrafish line, tg(sp7:egfp), which labels osteoblast cells with green fluorescent protein, makes the skeleton structure visible during the development of the zebrafish embryo [14]. Fleming et al. successfully used glucocorticoid to establish an osteoporosis-like phenotype in zebrafish. This model has become popular in high-throughput screening for bone anabolic compounds and is used to study bone-related diseases [15–17].

Amentoflavone is a bioflavonoid found in a variety of traditional Chinese medicines such as Gingko and *Selaginella tamariscina*. It has been reported to have anti-inflammatory, antioxidant, antiviral and anticancer effects [18–21]. In addition, studies have reported that flavonoids have an anabolic effect on bone metabolism [22]. However, the effect of amentoflavone on the osteogenic differentiation of human mesenchymal stem cells (hMSCs) has not been reported.

In the present study, we investigated the effect of amentoflavone on the osteogenic differentiation of hMSCs. The results showed that amentoflavone could promote the osteogenic differentiation of hMSCs by activating the JNK and p38 MAPK pathways. In vivo results also proved that amentoflavone protected against the dexamethasone (Dex)-induced inhibition of osteoblast differentiation in tg(sp7:egfp) zebrafish larvae.

Materials and methods

Materials

Amentoflavone (>99 % purity) was purchased from Chengdu Best Reagent Co., Ltd. (Chengdu, China). Human fetal bone marrow-derived MSCs were offered from the Stem Cell Bank in the Prince of Wales Hospital (Hong Kong, China). Human ethics approval was obtained from the Joint CUHK-NTEC Clinical Research Ethics Committee of the Chinese University of Hong Kong (reference no. CRE-2011.383). Tg(*sp7:egfp*) zebrafish line was kindly provided by Dr. Chung-Der Hsiao's group from Chung Yuan Christian University, Taiwan. Minimum essential medium α (α-MEM), MSC qualified fetal bovine serum (FBS) and 0.25 % trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Paraformaldehyde was purchased from ChengDu Kelong Chemical Reagent Co. (Chengdu, China). Agarose was purchased from Biowest (Spain). Methyl thiazolyl tetrazolium (MTT), SP600125 and SB203580, NBT-BCIP solution, L-ascorbic acid 2-phosphate, β-glycerophosphate, Dex and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alkaline Phosphatase Assay Kits were purchased from the Beyotime Institute of Biotechnology. Anti-GAPDH, anti-runt-related transcription factor 2 (Runx2) and anti-osterix (Osx) antibodies were purchased from Abcam. Anti-p38, anti-phosphorylated-p38 (p-p38), anti-JNK and anti-phosphorylated-JNK (p-JNK) antibodies were purchased from Cell Signaling Technology.

Cell proliferation assay

The hMSCs were cultured in 96-well flat-bottomed plates with 5×10^3 cells per well. After 24 h, amentoflavone was added to the culture medium at different concentrations (0.1–10 μM). Then, the cells were incubated for 1, 2, 3, 7 and 14 days. The effect of amentoflavone on hMSC cell proliferation was determined by MTT assay. After incubation, the hMSCs were treated with 10 μl MTT solution of 0.5 mg/ml at 37 °C for 4 h. Then, the medium was abandoned, and the dark blue formazan crystals were subsequently dissolved in 200 μl DMSO at 37 °C for shaking 10 min. The absorbance at 490 nm was measured with a microplate reader.

Cell culture and osteogenic differentiation of hMSCs

The hMSCs were kept in α-MEM supplemented with 10 % FBS and 1 % penicillin-streptomycin (Gibco) and cultured at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. After reaching 80 % confluence, the hMSCs were rinsed twice with phosphate-buffered saline (PBS), trypsinized with 0.25 % trypsin-EDTA for 2 min and then using serum-containing medium to end the trypsinization immediately. The mixed fluid was collected and centrifuged with 900*g* for 3 min. Next, leaving the supernatant gently, the precipitate was resuspended in 10 % serum-containing medium. Finally, the cells were subcultured 1:3. After reaching 80 % confluence, the cells were stimulated by osteogenic induction medium (OIM) consisting of 10 %

serum-containing medium with 100 nM Dex, 10 mM β-glycerophosphate and 0.05 mM L-ascorbic acid 2-phosphate. The medium was replaced every 3 days.
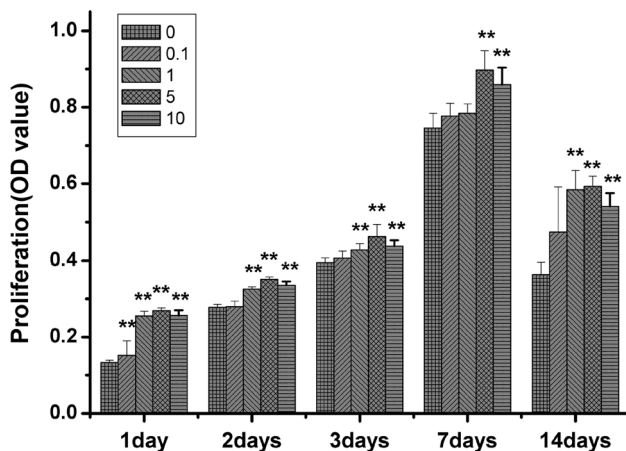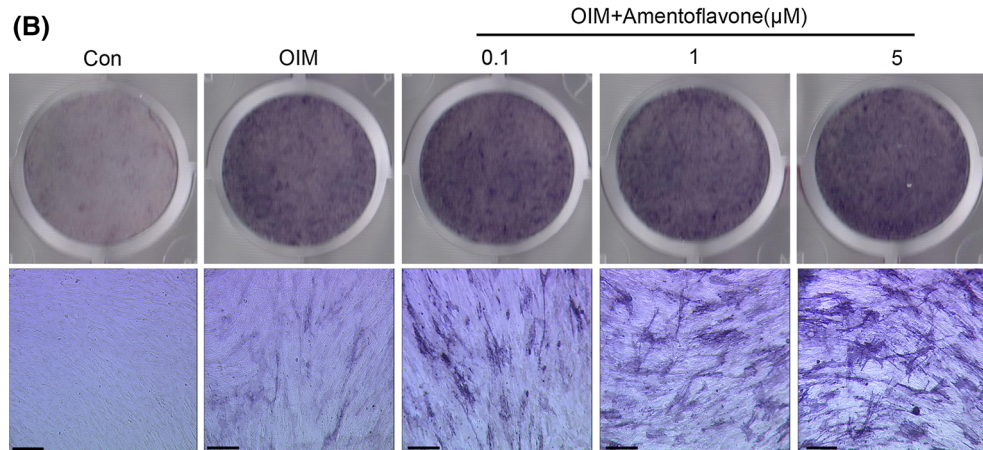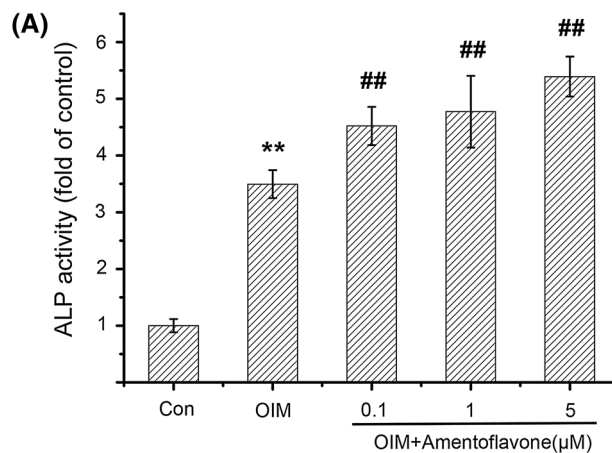

Fig. 1 The proliferation of hMSCs incubated with amentoflavone at concentrations of 0.1–10 μM for 1, 2, 3, 7 and 14 days. The MTT assay was designed to test the proliferation ($n = 6$). **$P < 0.01$ compared with Con

ALP activity assay

Alkaline phosphatase (ALP) activity was measured by ALP Assay Kits (Beyotime, China). After 7 days of osteogenic induction, the cells in 24-well flat-bottomed plates were washed lightly with PBS and lysed in 0.1 % (v/v) Triton X-100 in PBS for 30 min. The activity was then assessed under the protocol of ALP Assay Kits. The concentration of cell lysate protein was determined by using bicinchoninic acid (BCA) protein assay reagent (Beyotime, China). The absorbance was measured at 562 nm with a microplate reader. ALP activity was normalized to the total protein concentration.

ALP staining

After 7 days of osteogenic induction, the cells were rinsed twice with PBS and fixed with 500 μl 70 % ethanol at room temperature for 10 min. After aspirating the ethanol, the cells were equilibrated by 500 μl ALP buffer (0.15 M NaCl, 0.15 M Tris-HCl, 1 mM MgCl₂, pH9.0) twice for 10 min and stained with NBT/BCIP solution at 37 °C for about 20 min in dark. Upon the appearance of purple-blue

Fig. 2 Amentoflavone increased ALP activity of hMSCs. a The ALP activity was tested after hMSCs had been treated with different concentrations of amentoflavone in OIM for 7 days ($n = 4$). b ALP of hMSCs was stained with BCIP/NBT kit after culturing in the same conditions for 7 days. Scale bars represent 200 μm. **$P < 0.01$ compared with Con; ##$P < 0.01$ compared with OIM

precipitates, the reaction was stopped by distilled water. Photographs were taken of all samples.

Alizarin Red S staining and quantification

After 14 days of osteogenic induction, the cells in 24-well flat-bottomed plates were washed twice with PBS, fixed with 500 μl 70 % ethanol for 15 min and then incubated with 0.5 % Alizarin Red S (pH 4.1) for 5 min at 37 °C. The samples were then washed three times with deionized water to stop the reaction. After taking photos, the samples were extracted with 10 % cetylpyridinium chloride (CPC, Sigma) for 10 min, and the absorbance at 562 nm was measured with a microplate reader.

Western blot analysis

Western blot analysis was performed as previously described [23]. Briefly, equal proteins were fractionated by 10 % SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Then, the membrane was blocked with 5 % skim milk for 1 h at room temperature. Specific antibodies against Runx2, Osx, p38, p-p38, JNK, p-JNK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used. The band intensity was quantified by Quantity One software.

Zebrafish skeleton development assay

Zebrafish skeleton development assay was performed as previous described [17]. In brief, tg(sp7:egfp) embryos were raised in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$) at 28.5 °C for 2 days. The embryos were then randomly divided into five groups: vehicle control (0.1 % DMSO), 10 μM Dex, Dex + 0.1 μM amentoflavone, Dex + 1 μM amentoflavone and Dex + 5 μM amentoflavone. All embryos were kept in 24-well flat-bottomed plates with ten fish per well, and the medium was half changed every day. After 6 days, the fish were fixed in 4 % paraformaldehyde in PBS for 2 h and fixed with 1 % low melting agarose (LMA, Invitrogen, USA). Images were scanned using a confocal laser scanning microscope. The fluorescent area

Fig. 3 Amentoflavone promoted the mineralization of hMSCs. a The hMSCs were treated with amentoflavone in OIM for 14 days, and the mineralized nodules of hMSCs were stained by Alizarin Red S. b The mineralization, for three independent experiments, was quantified by extraction of Alizarin Red S dye with 10 % CPC. Scale bars represent 200 μm. **P < 0.01 compared with Con; ##P < 0.01 compared with OIM
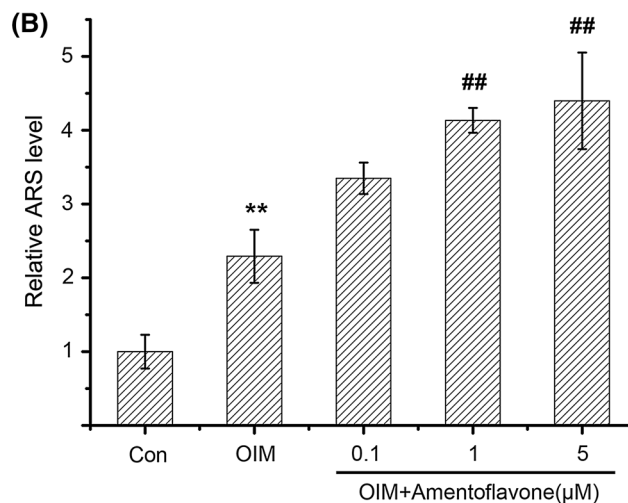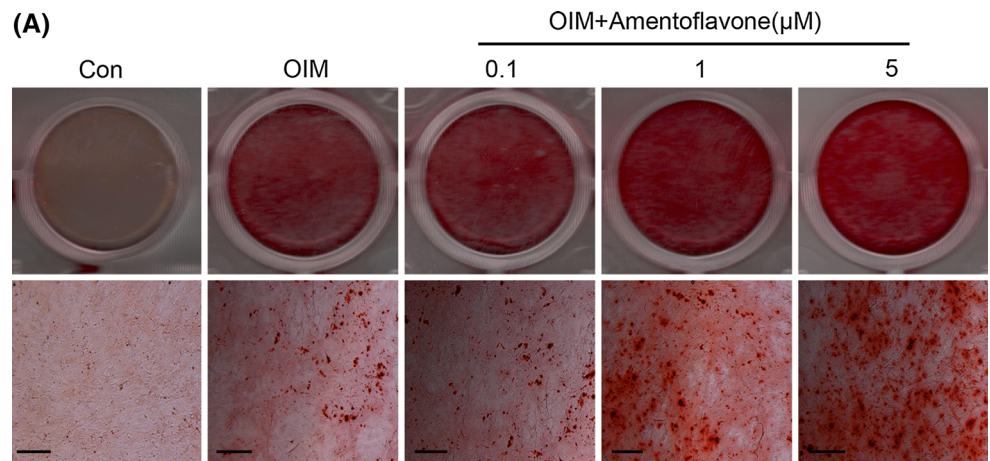
and integrated optical density (IOD) were calculated by Image Pro Plus 6.0.

Statistical analysis

All statistical data were presented as the mean ± SD. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Student-Newman-Keuls post hoc test for multiple-group comparison. Probability values of $P < 0.05$ were considered to represent statistical significance.

Results

The effect of amentoflavone on hMSC proliferation

In order to test the effect of amentoflavone on hMSC proliferation, the MTT assay was performed. The result showed that amentoflavone enhanced hMSC proliferation in a dose-

dependent manner, and the most effective dose was 5 μM (Fig. 1).

Amentoflavone increased the ALP activity of hMSCs

As ALP is a sign of osteogenic differentiation of hMSCs, we evaluated whether amentoflavone would increase the ALP activity. The hMSCs were treated with different concentrations of amentoflavone (0.1, 1, 5 μM) in OIM for 7 days. The ALP staining and quantitative data showed amentoflavone increased ALP activity in a dose-dependent manner, and the concentration of 5 μM was the peak (Fig. 2).

Amentoflavone increased the mineralization of hMSCs

Alizarin Red staining (ARS) was carried out to estimate whether hMSCs incubated with amentoflavone resulted
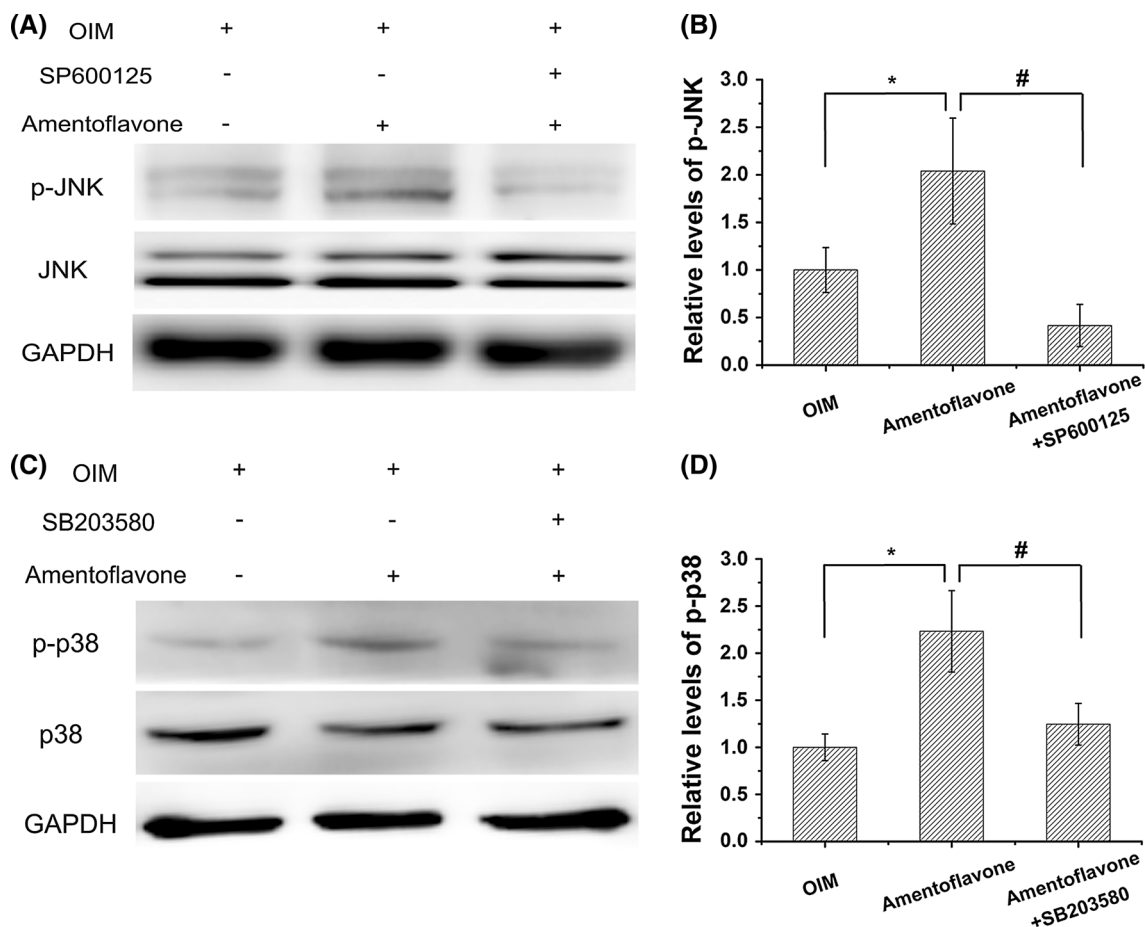

Fig. 4 Amentoflavone induced activation of the JNK and p38 MAPK signaling pathways. The hMSCs were pretreated with SP600125 or SB203580 for 1 h and incubated with amentoflavone (5 μM) in OIM for 3 days. The proteins were then separated by 10 % SDS-PAGE and analyzed by Western blot with specific antibodies. **a** The level of p-JNK was detected by Western blot. **b** Quantification of three independent Western blot experiments was shown with the mean level of p-JNK normalized to total JNK. *$P < 0.05$; #$P < 0.05$. **c** The level of p-p38 was detected by Western blot. **d** Quantification of three independent Western blot experiments was shown with the mean level of p-p38 normalized to total p38. *$P < 0.05$; #$P < 0.05$

in an increase of extracellular calcium deposition. The result showed that samples had no calcium deposition formation in the absence of OIM at 14 days, while in the presence of OIM and amentoflavone, they had remarkable calcium nodule formation (Fig. 3). Furthermore, quantification of calcium deposition showed that amentoflavone significantly promoted calcium deposition in a dose-dependent manner, and 5 μM was the best (Fig. 3).

Amentoflavone increased the phosphorylation of JNK and p38 in hMSCs

It is well known that the JNK and p38 MAPK pathways play an important role in regulating the osteogenesis of MSCs [24, 25]. In order to check whether amentoflavone stimulated the osteogenesis of hMSCs by activating the JNK and p38 MAPK pathways, we detected the levels of p-JNK and p-p38 in hMSCs through Western blot. The results showed that amentoflavone significantly increased

the levels of p-p38 and p-JNK, while SB203580 and SP600125 eliminated this effect of amentoflavone (Fig. 4).

SB203580 and SP600125 eliminated the increasing of ALP activity induced by amentoflavone

SB203580 and SP600125 are selective inhibitors of the p38 and JNK pathway, respectively [25]. ALP staining showed SB203580 and SP600125 strongly inhibited ALP activity increased by amentoflavone (Fig. 5). This was demonstrated by the ALP activity assay (Fig. 5).

SB203580 and SP600125 reduced the extracellular calcium deposition induced by amentoflavone

As expected, SB203580 and SP600125 also significantly reduced the calcium deposition stimulated by amentoflavone (Fig. 6). Quantification of calcium nodules also proved this (Fig. 6).
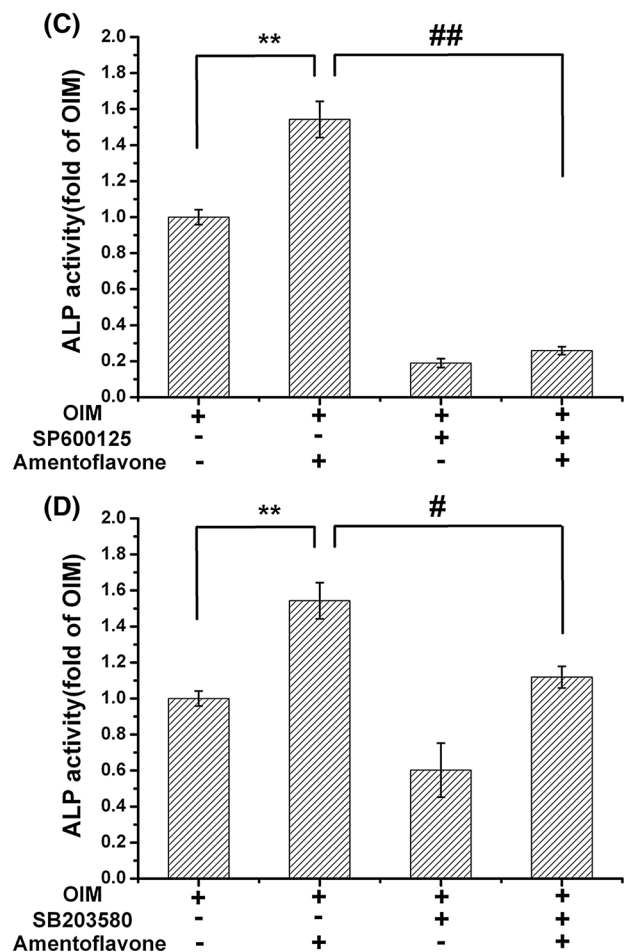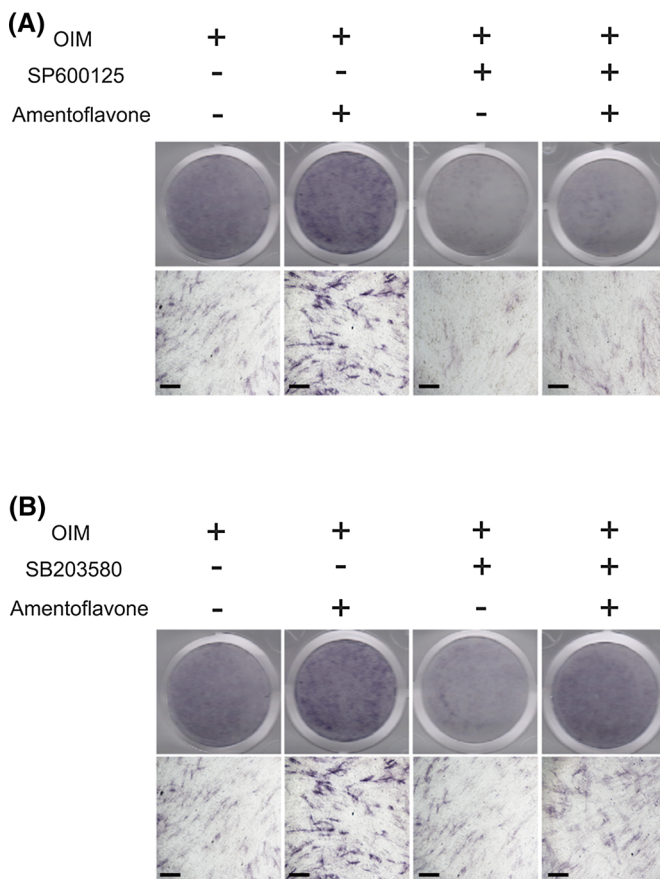

Fig. 5 SB203580 and SP600125 inhibited the increasing of ALP activity induced by amentoflavone. **a, b** The hMSCs were pretreated with SP600125 or SB203580 for 1 h and incubated with amentoflavone (5 μM) in OIM for 7 days. Then, ALP was stained with BCIP/ NBT. **c, d** The ALP activity was measured by ALP Assay Kits. The experiments were repeated three times. *Scale bars* represent 200 μm. **P < 0.01$; $^{#}P < 0.05$; $^{##}P < 0.01$
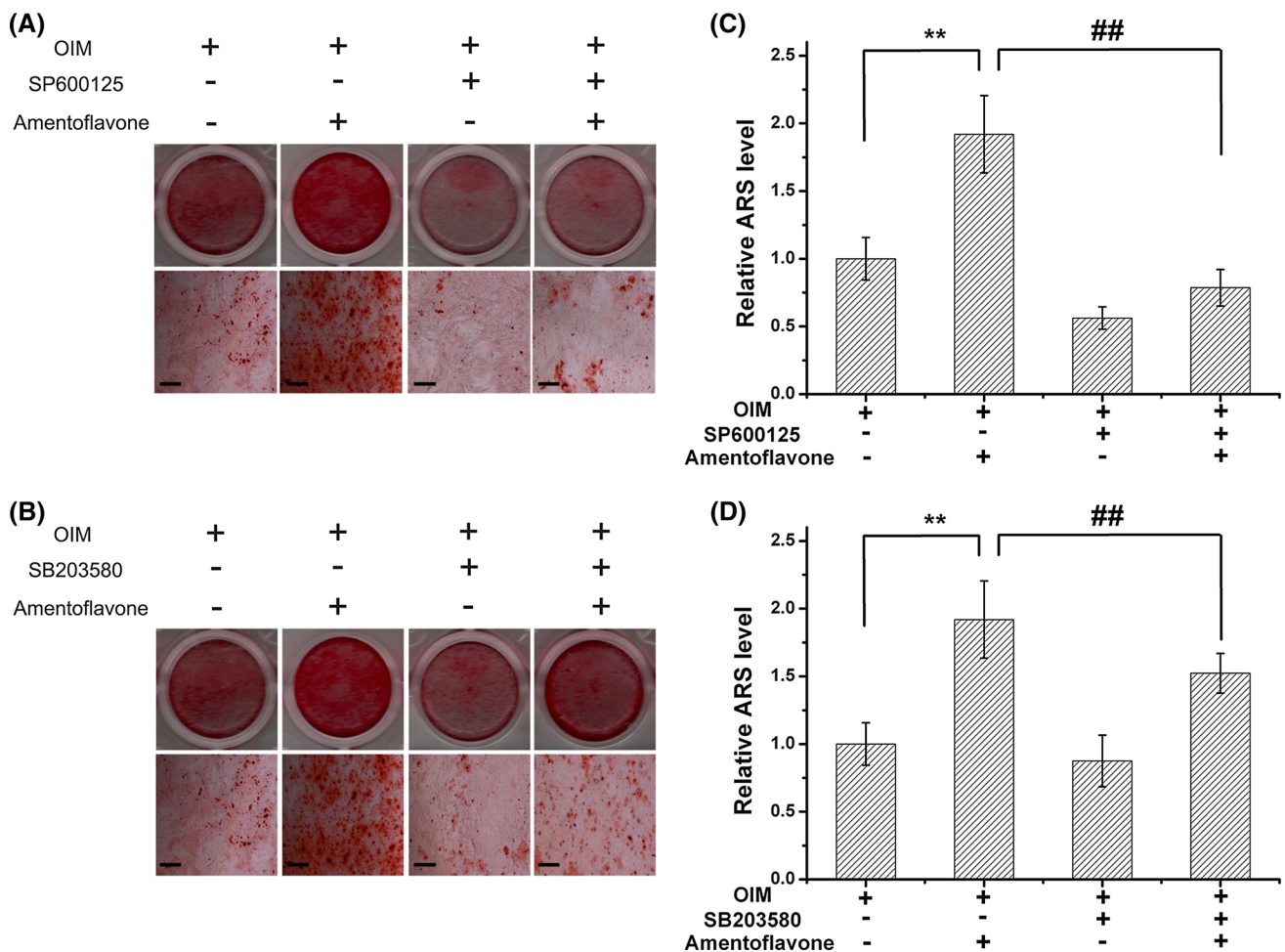
Fig. 6 SB203580 and SP600125 inhibited the effect of amentoflavone on mineralized nodules. **a**, **b** The hMSCs were pretreated with SP600125 or SB203580 for 1 h and incubated with amentoflavone (5 μM) in OIM for 14 days. Then, Alizarin Red S stained the mineralized nodules. **c**, **d** The mineralization was quantified by extraction of Alizarin Red S dye with 10 % CPC. *Scale bars* represent 200 μm. The experiments were repeated three times. **P < 0.01; ##P < 0.01

SB203580 and SP600125 abolished Runx2 and Osx protein expression induced by amentoflavone

We performed Western blot analysis to test Runx2 and Osx protein expression in cells treated with SB203580 and SP600125. The results showed amentoflavone increased the expression of Runx2 and Osx proteins. At the same time, SP600125 and SB203580 blocked the effect of amentoflavone on the expression of Runx2 and Osx proteins (Fig. 7).

Amentoflavone protected against the Dex-induced inhibition of osteoblast differentiation in tg(sp7:egfp) zebrafish larvae

We further examined the effect of amentoflavone on bone formation in tg(sp7:egfp) zebrafish larvae. The results showed that Dex exhibited a reduction in the fluorescence area and IOD. Amentoflavone alleviated the inhibition of Dex in the fluorescence area and IOD in a dose-dependent manner in tg(sp7:egfp) zebrafish larvae (Fig. 8).

Discussion

MSCs are multipotent cells with the capacity to differentiate into osteoblasts, adipocytes and chondrocytes [6, 7]. MSC differentiation into osteoblasts is an important part of the bone growth and remodeling process [9, 11]. Recently, hMSCs have be used extensively as a source of osteogenic cells for bone tissue engineering [10, 11]. Thus, it is important to promote osteogenic differentiation of hMSCs.

The osteogenic differentiation of MSCs is characterized by cell proliferation, differentiation and mineralization. First, we studied the effect of amentoflavone on the proliferation of hMSCs. Our results showed that
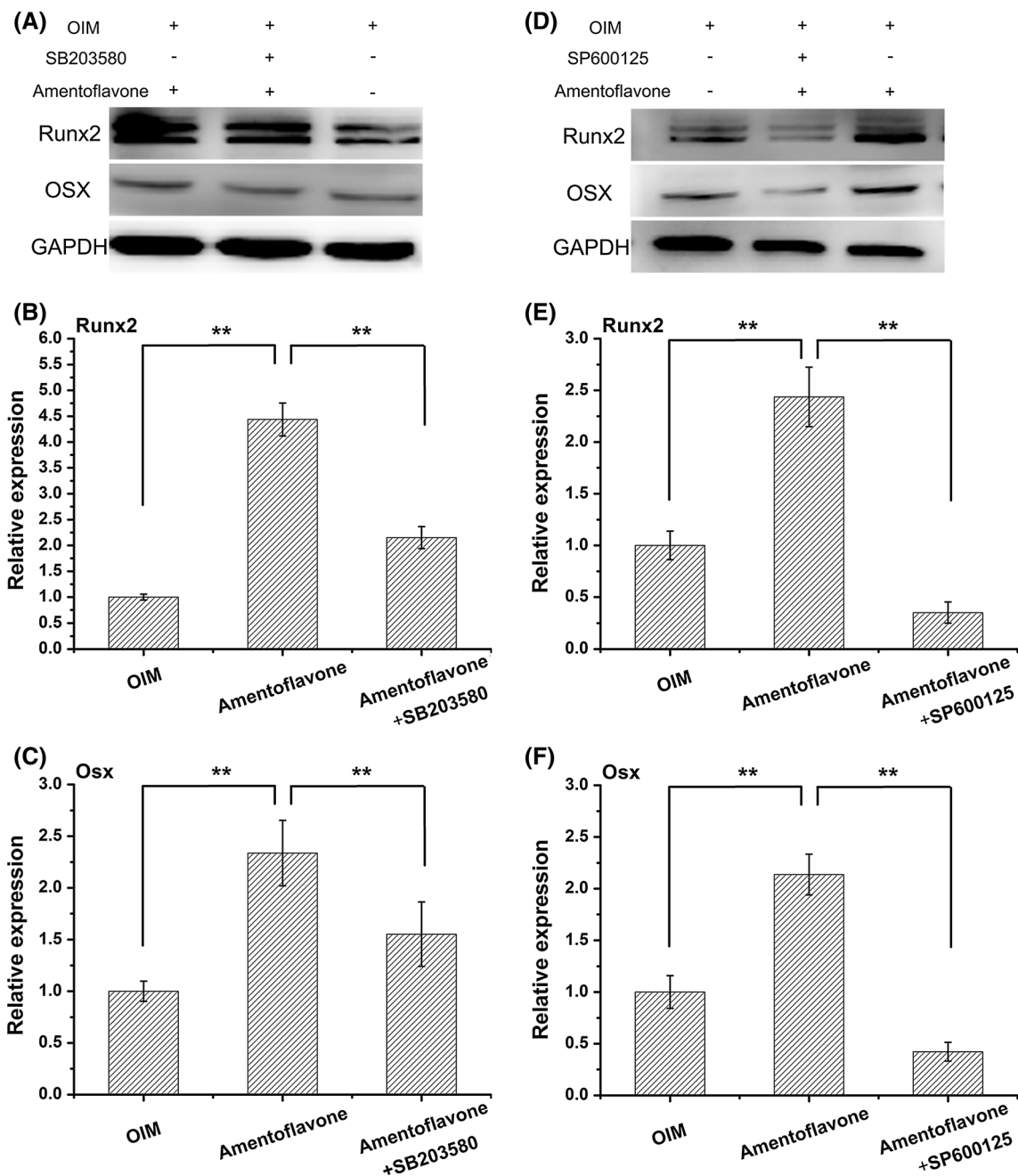
Fig. 7 SP600125 and SB203580 inhibited the effect of amentoflavone on the expression of Runx2 and Osx protein in hMSCs. The hMSCs were pretreated with SB203580 (**a–c**) or SP600125 (**d–f**) for 1 h and then incubated in OIM with amentoflavone (5 μM) for 7 days. The total proteins were extracted for Western blot to analyze the expression level of Runx2 and Osx. GAPDH was used as loading control. The experiments were repeated three times. **$**P < 0.01$

amentoflavone promoted the proliferation of hMSCs in a dose-dependent manner, suggesting that amentoflavone is advantageous to osteogenic differentiation of hMSCs in a wide range of concentrations.

As ALP activity is an early osteogenic marker and plays an important role in bone formation [26], we investigated the role of amentoflavone on ALP activity to evaluate the effect of amentoflavone on osteogenic differentiation of
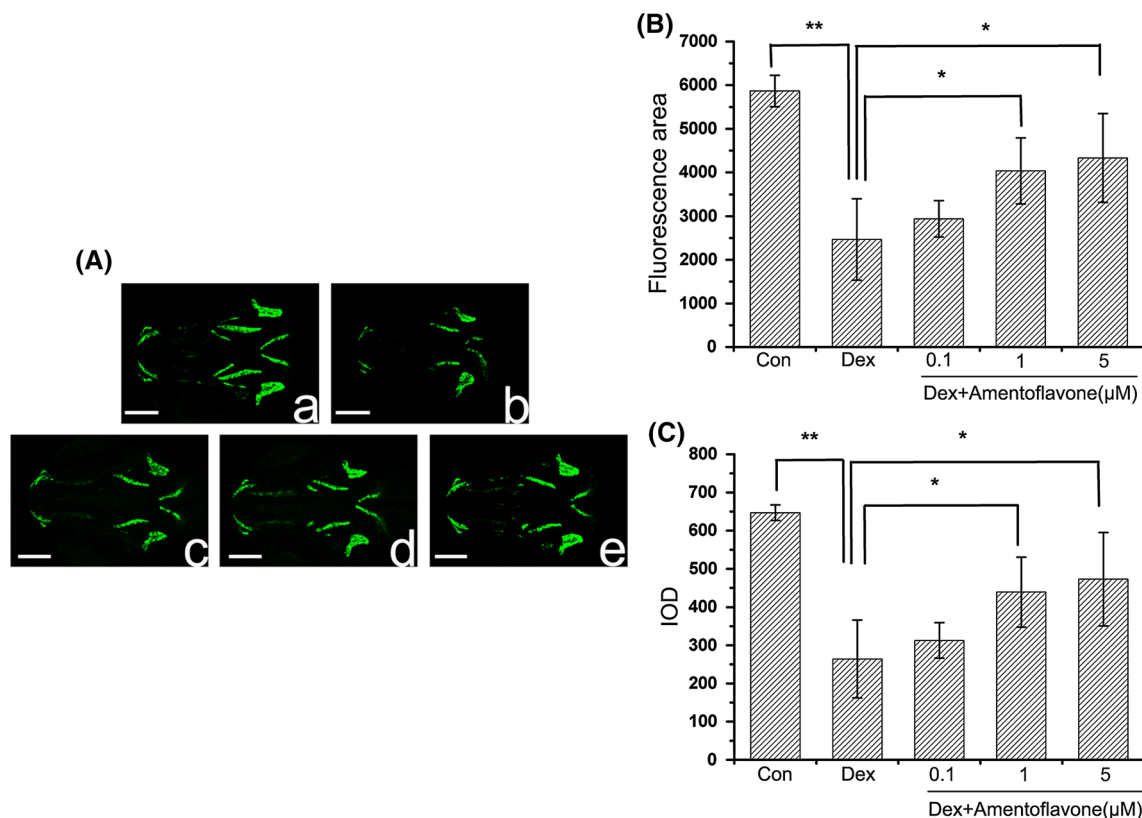
Fig. 8 The protection of amentoflavone against dexamethasone (Dex)-induced inhibition of osteoblast differentiation in tg(sp7:egfp) zebrafish larvae. **a** Fluorescence imaging of zebrafish larvae fixed in turn with 4 % paraformaldehyde and 1 % low-melting-point agarose. *a* Control; *b* Dex; *c* Dex + 0.1 μM amentoflavone; *d* Dex + 1 μM amentoflavone; *e* Dex + 5 μM amentoflavone. **b**, **c** The fluorescent area and integrated optical density (IOD) calculations of mean pixel number ($n = 10$). *Scale bars* represent 200 μm. *$P < 0.05$; **$P < 0.01$

Fig. 9 Schematic diagram showing the possible mechanisms by which amentoflavone enhances the osteogenesis of hMSCs

Amentoflavone
p38   JNK
Runx2
Osx
osteogenesis
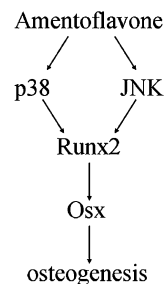
hMSCs. Our results indicated that amentoflavone significantly increased the activity of ALP. As expected, amentoflavone also strengthened mineralized nodule formation, a marker for a later stage of osteogenic differentiation [27]. These results revealed that amentoflavone promoted osteogenic differentiation of hMSCs.

Runx2 plays a pivotal role in the regulation of osteogenic differentiation. Osx, a zinc finger-containing transcription factor required for osteoblastic differentiation, acts as a downstream factor of Runx2 [28–30]. In this research, we found that amentoflavone significantly increased the expression levels of Runx2 and Osx. The

results suggested that amentoflavone might enhance the osteogenic differentiation of hMSCs via mediating the transcription factors Runx2 and Osx.

JNK and p38 MAPK signaling pathways play a vital role in the regulation of osteogenesis [24, 25]. The activation of JNK and p38 MAPK signaling pathways has been shown to be involved in both genistein and myocilin-promoted osteogenic differentiation [25, 31]. To explore whether JNK and p38 MAPK signaling pathways were also involved in the amentoflavone-mediated osteogenic differentiation in hMSC, we incubated cells with amentoflavone and OIM. We found amentoflavone significantly increased the levels of p-JNK and p-p38 in a dose-dependent manner. To further confirm our findings, we used inhibitors of p38 MAPK (SB203580) or JNK (SP600125) to check whether the stimulation of amentoflavone on osteogenic differentiation in hMSCs was abolished. The results showed that SB203580 or SP600125 significantly reduced the ALP activity and mineralized nodule formation induced by amentoflavone in the process of osteogenic differentiation. At the same time, SB203580 or SP600125 also efficiently decreased the expression of Runx2 and Osx

protein. These data clearly demonstrated that the JNK and p38 MAPK signaling pathways played a crucial role during amentoflavone-induced osteogenesis in hMSCs (Fig. 9).

The zebrafish is an ideal model for bone research because of the rapid generation time, prolific fecundity, external development, optically transparent embryos, and genomic conservation between zebrafish and humans [32–35]. In particular, zebrafish has great similarity to humans in terms of bone development [36–38]. Osx (sp7) is a transcription factor expressed in osteoblasts and a marker for studying osteoblasts. In transgenic zebrafish tg(sp7:egfp), the sp7 gene is expressed accompanying GFP expression during osteoblast differentiation, and GFP-positive osteoblasts are visible in zebrafish. Therefore, tg(sp7:egfp) provides an excellent tool for investigating bone formation directly [15–17]. We found that Dex inhibited the bone formation, while amentoflavone alleviated the inhibition of Dex on osteoblast differentiation in tg(sp7:egfp) zebrafish larvae, suggesting amentoflavone protected against the Dex-induced inhibition of osteoblast differentiation.

In conclusion, this study demonstrated that amento-flavone stimulated the osteogenesis of hMSCs at least partially by activating the JNK and p38 MAPK signaling pathways. More importantly, amentoflavone protected against the Dex-induced inhibition of osteoblast differentiation in larval zebrafish. These results suggest that amentoflavone may be useful for treating bone-related disorders.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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