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## Salvianolic acid B promotes osteogenesis of human mesenchymal stem cells through activating ERK signaling pathway



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### ABSTRACT

Salvianolic acid B, a major bioactive component of Chinese medicine herb, *Salvia miltiorrhiza*, is widely used for treatment of cardiovascular diseases. Our recent studies have shown that Salvianolic acid B can prevent development of osteoporosis. However, the underlying mechanisms are still not clarified clearly. In the present study, we aim to investigate the effects of Salvianolic acid B on viability and osteogenic differentiation of human mesenchymal stem cells (hMSCs). The results showed Salvianolic acid B (Sal B) had no obvious toxic effects on hMSCs, whereas Sal B supplementation (5  $\mu$ M) increased the alkaline phosphatase activity, osteopontin, Runx2 and osterix expression in hMSCs. Under osteogenic induction condition, Sal B (5  $\mu$ M) significantly promoted mineralization; and when the extracellular-signal-regulated kinases signaling (ERK) pathway was blocked, the anabolic effects of Sal B were diminished, indicating that Sal B promoted osteogenesis of hMSCs through activating ERK signaling pathway. The current study confirms that Sal B promotes osteogenesis of hMSCs with no cytotoxicity, and it may be used as a potential therapeutic agent for the management of osteoporosis.

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### 1. Introduction

Osteoporosis is the most widespread bone disease, characterized by low bone mineral density and the deterioration of bone microarchitecture, leading to bone fragility fractures (Kanis et al., 2009; Raisz, 2005). Currently, the therapeutic agents of osteoporosis are mainly inhibitors of bone resorption including bisphosphonates, calcitonin and selective estrogen receptor modulators (Marie and Kassem, 2011; Sandhu and Hampson, 2011). Although these agents are effective in stabilizing bone mass, they do not increase bone formation. Mesenchymal stem cells (MSCs) are non-hematopoietic cells which can be easily isolated from bone

marrow and other tissues, such as adipose, umbilical cord and peripheral blood. MSCs have multi-potent capacity to differentiate into a variety of other cell types, including osteoblasts, adipocytes, chondrocytes, myoblasts and neurons (Jiang et al., 2002; Pittenger et al., 1999). MSCs have also been shown to be immunosuppressive and anti-inflammatory, as they do not express MHC-II, CD80, CD86 and CD40, and minimally express MHC-I on the cell surface (Deans and Moseley, 2000; Pittenger et al., 1999; Tse et al., 2000). In recent years, MSCs have been used to promote healing with promising results, such as bone fracture and segmental bone defect (Kumar et al., 2010; Shekkeris et al., 2012; Undale et al., 2011). Most recently, Guan et al. have found that directing MSCs to bone could augment bone formation and increase bone mass (Guan et al., 2012). In osteoporotic patients, studies have shown that the osteogenic differentiation potential of MSCs is reduced (Benisch et al., 2012; Dalle Carbonare et al., 2009; Rodriguez et al., 2000). Therefore, enhancing osteogenesis of MSCs is thought to be a useful therapeutic strategy for bone diseases such as osteoporosis (Pino et al., 2012).

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MSCs differentiation into mature functional osteoblasts is a complex process involving many transcriptional factors and signaling pathways. Runx2 (Runt-related transcription factor 2) is the central control gene of the osteoblast phenotype. Mice with homozygous mutation in Runx2 gene show a complete lack of ossification (Komori et al., 1997). The ERK signaling pathway has been intensively investigated in regulating MSCs differentiation. ERK1/2 is constantly activated during osteogenic differentiation, and the study by Jaiswal et al. has suggested that the commitment of hMSCs into osteogenic or adipogenic lineages is governed by activation or inhibition of ERK1/2, respectively (Jaiswal et al., 2000). ERKs activation can increase the phosphorylation and transcription potential of Runx2 (Park et al., 2010).

Salvianolic acid B (Sal B), a major bioactive component of traditional Chinese medicine, *Salvia miltiorrhiza*, is widely used for treatment of cardiovascular diseases (He et al., 2008; Joe et al., 2012). Studies have shown that Sal B exerts neuroprotective effects (Kim et al., 2011; Lee et al., 2013) and also could alleviate liver fibrosis (Wang et al., 2012). Recently we have investigated the role of Sal B on bone metabolism in glucocorticoid induced osteoporosis in rats and found that Sal B was able to prevent osteoporosis induced by glucocorticoid (Cui et al., 2012). However, the effect of Sal B on osteogenic differentiation of MSCs was not studied and the underlying mechanisms of Sal B on bone metabolism were still not clarified. In the present study, we have investigated the effect of Sal B on osteogenic differentiation in hMSCs. The results show that Sal B can promote the osteogenic differentiation of hMSCs by activating ERK signaling pathway, which may partially explain our previous finding that Sal B prevents osteoporosis induced by glucocorticoid.

## 2. Materials and methods

### 2.1. Reagents and cell culture

Sal B was purchased from Paipai Limited Technology Corporation (Guangzhou, China). Human fetal bone marrow-derived MSCs (hMSCs) were donated from the Stem Cell Bank in the Prince of Wales Hospital. 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). Informed written consent form was approved by the Clinical Research Ethics Committee and signed by donor before sample collection. The hMSCs were kept in Modified Eagle's Medium of Alpha (a-MEM) (Gibco) supplemented 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco).

### 2.2. Plasmid construction, transfection, lentivirus production and infection

The shRNA used for silencing ERK1 and ERK2 was designed as published (Hong et al., 2009). ERK1, ERK2 shRNA and scrambled shRNA sequence templates were inserted into pLL3.7 plasmid and pseudo-lentiviruses were produced by transfection of 293FT packaging cells (Invitrogen, USA) using the calcium phosphate method. For transduction,  $1 \times 10^5$  cells were seeded into 6-well plate and incubated with lentiviruses and 8  $\mu\text{g}/\text{mL}$  polybrene in the incubator for 24 h (Xu et al., 2012).

### 2.3. Phenotypic characterization of hMSCs

After reaching 80% confluence, the cells were rinsed twice with phosphate buffered saline (PBS) and treated with 0.05% trypsin-EDTA for 2 min. Then, serum-containing medium was immediately added to the culture to end trypsinization. Then, the fluid was collected and centrifuged ( $800 \times g$  for 5 min). After discarding the supernatant, the precipitate was resuspended in

staining buffer and incubated with fluorochrome-conjugated primary antibodies against CD34, CD44, CD45, CD73, CD90, CD105, or corresponding isotype control (BD Biosciences, USA) at 4°C for 30 min. The stained cells were immediately detected using Flow Cytometry (BD Biosciences, USA).

### 2.4. Cell viability assay

Samples ( $5 \times 10^3$  per well) were subcultured in a 96 flat-bottomed well plate. After 24 h of incubation, the medium was changed into Sal B containing media at different concentrations. Cells were incubated at 37°C for 1 and 3 days. The cell proliferation was determined using methyl thiazolyl tetrazolium (MTT) reduction assay. After incubation, cells were treated with the MTT solution (final concentration, 0.5 mg/ml) for 4 h at 37°C. The dark blue formazan crystals formed in intact cells were solubilized with 150  $\mu\text{L}$  DMSO and the plate was shaken for 10 min. The absorbance at 570 nm was measured with a microplate reader.

### 2.5. ALP activity assay

After MSCs were treated with or without OIM (osteogenic induced medium) and Sal B (5  $\mu\text{M}$ ) for 7 days, the plate was washed with PBS and the cells were lysed by lysis buffer consisting 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100. 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.

### 2.6. ALP staining

After MSCs were treated with or without OIM and Sal B for 7 days, the cells were washed with PBS twice and fixed with 70% ethanol for 10 min. The cells were equilibrated by ALP buffer (0.15 M NaCl, 0.15 M Tris-HCl, 1 mM  $\text{MgCl}_2$ , pH 9.0) twice, incubated with ALP substrate solution (5  $\mu\text{L}$  BCIP and 10  $\mu\text{L}$  NBT in 1 mL ALP buffer) at 37°C in dark for 60 min. Then the reaction was stopped by distilled water and the plate was dried before taking photo.

### 2.7. Mineralization assay

After 14 days of osteogenic induction, cells were fixed with 70% ethanol for 10 min. Then the fixed cells were stained with 0.5% alizarin red S (pH 4.1) for 10 min at room temperature and washed three times with deionized water. Orange red staining indicated the position and intensity of calcium deposits. The calcium deposition was extracted with 10% cetylpyridinium chloride (CPC, Sigma) and quantified by measuring the OD of the extract at 550 nm.

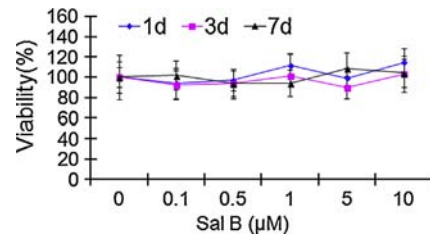
### 2.8. RNA extraction and real-time PCR

Total RNA was extracted from cultured cells with RNeasy Mini Kit (Qiagen, USA) and first-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. Real-time PCR was performed using the Step One Plus Real-Time PCR System (Applied Biosystems, USA). The reaction conditions consisted of 15  $\mu\text{L}$  reaction volumes with diluted cDNA template 3  $\mu\text{L}$ , 7.5  $\mu\text{L}$  SYBR-Green Master Mix (2 $\times$ ), 3.9  $\mu\text{L}$  PCR-Grade water and 0.3  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ). Amplification conditions were as follows: first at 95°C for 5 min, and then 40 cycles of 95°C for 15 s and 60°C for 60 s. Primer sequences were as follows: osteopontin (OPN) forward: 5'gtaccctgatgctacagcagc-3', reverse:

ttcataactgtccttcccac; Runx2 forward: 5'acttctgtgctcggtgct3', reverse: 5'gacggttatgtcaagtgaa3'; Osterix (Osx) forward: 5'ccagggaacactcctactcc3', reverse: 5'gccttgccatacaccttg3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5'ggcatggactgtggtcatgag3', reverse: 5'tgcaccaccaactgttagc3'. The relative quantification of gene expression was determined by calculating the values of  $2^{-\Delta\Delta CT}$ , with each sample being normalized to the expression level of GAPDH.

## 2.9. Western blot

Equal proteins were loaded onto 10% SDS-PAGE and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) for 75 min at 100 V. The membrane was blocked with 5% skim milk for 1 h at room temperature. After washing five times with TBS containing 0.1% Tween 20 (TBST), the membranes were incubated, respectively, with anti-ERK1/2 (BD, 1:2000), anti-p-ERK1/2 (BD, 1:1000), anti-OCN (abcam, 1:1000), anti-OPN (abcam, 1:1000), anti-Osx (abcam, 1:1000), anti-Runx2 (abcam, 1:1000), anti-BSP (abcam, 1:1000) or anti-GAPDH (Santa Cruz, 1:1000) antibodies at 4 °C overnight. After washing in TBST for three times (5 min for each time), the membrane was incubated with horseradish peroxidase-linked secondary antibodies (anti-mouse, anti-goat or anti-rabbit) for 1 h at room temperature. After washing five times with TBST, proteins were detected with the ECL (enhanced chemiluminescence) blotting reagents or ECL prime western blotting detection reagent (Amersham Biosciences) according to the manufacturer's instruction. The band intensity was quantified using Image J software.



**Fig. 1.** Viability of hMSCs treated by Sal B at different concentrations. The cells were incubated with Sal B (0.1–10 μM) for 1, 3 and 7 days, then MTT assay was performed to test the cell viability.

## 2.10. Statistical analysis

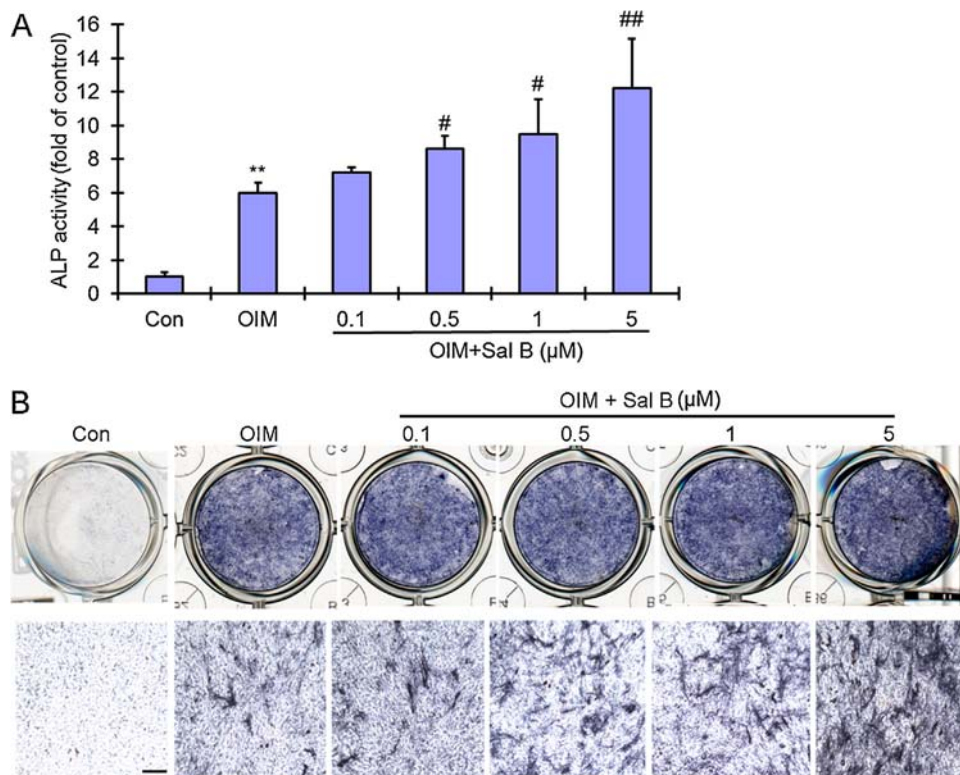
All data were presented as mean ± SD and statistical analysis was performed using one-way analysis of variance (one-way ANOVA). A value of  $P < 0.05$  was considered statistically significant.

## 3. Results

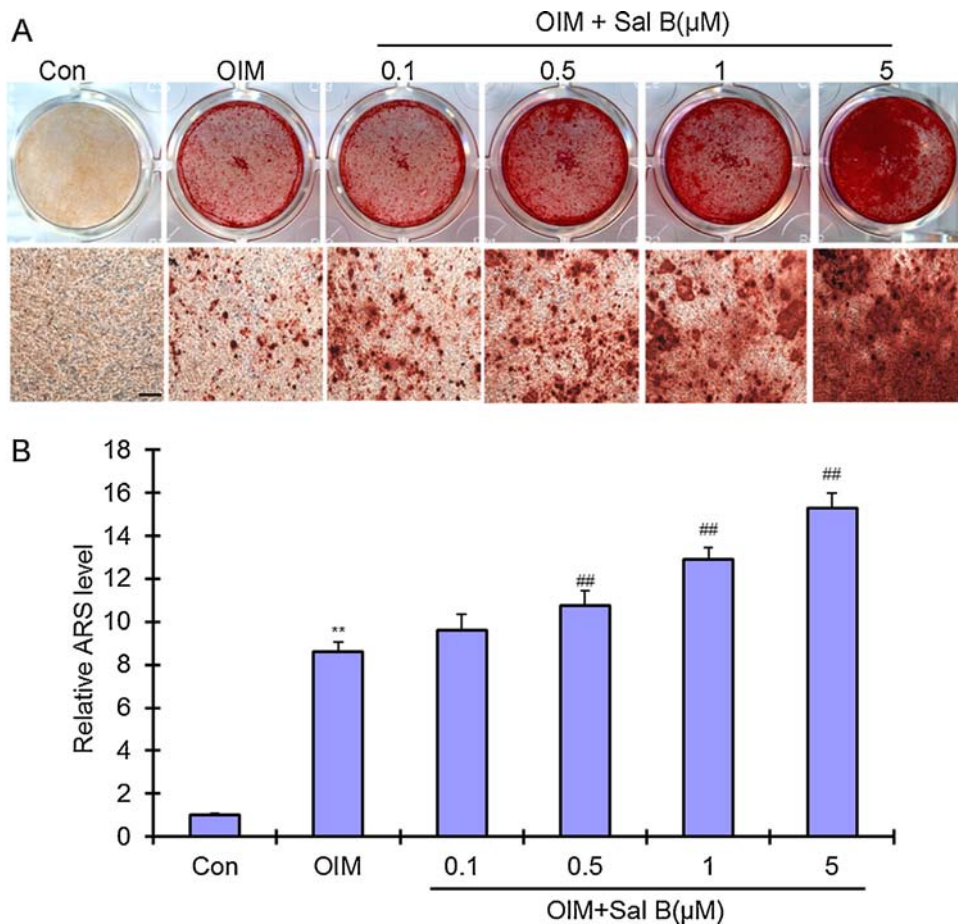
### 3.1. Phenotypic characterization of hMSCs

The surface antigens of hMSCs were detected by flow cytometry. The results showed that the cells were positive for CD90, CD44, CD73, and CD105 and negative for CD34, CD45 (Supplemental Fig. S1). The data showed that the cells expressed typical surface markers of MSCs and therefore were used for experiments described below.

Supplementary Fig. S1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2014.03.005>.



**Fig. 2.** Sal B increased ALP activity in hMSCs. (A) The ALP activity of hMSCs was measured after they were incubated with different concentrations of Sal B in OIM for 7 days. \*\* $p < 0.01$  compare with Con ( $\alpha$ -MEM); # $p < 0.05$ , ## $p < 0.01$  compared with OIM. (B) ALP in hMSCs was stained with BCIP/NBT kit after the cells were incubated with different concentrations of Sal B in OIM for 7 days. Scale bar = 200 μm.



**Fig. 3.** Sal B promoted mineralization of hMSCs. (A) hMSCs were treated with Sal B (0.1–5  $\mu$ M) in OIM for 14 days, then the mineralized nodules were stained by Alizarin Red S. Scale bar = 200  $\mu$ m. (B) The mineralization was quantified by extraction of Alizarin Red S dye with 10% CPC. \*\* $p$  < 0.01 compared with Con; ## $p$  < 0.01 compared with OIM.

### 3.2. Sal B had no effect on hMSCs viability

To investigate the effect of Sal B on cell viability, the MTT assay was performed. The result showed that Sal B did not influence cell viability when it was used at concentration between 0.1 and 10  $\mu$ M (Fig. 1).

### 3.3. Sal B increased ALP activity of hMSCs

ALP is a marker for the differentiation of osteoblast, so we measured the ALP activity of hMSCs after they were incubated with different concentrations of Sal B for 7 days. Our data showed that Sal B increased the ALP activity of hMSCs in a dose-dependent manner and the increase in the activity peaked at the concentration of 5  $\mu$ M (Fig. 2A). And similar result was observed by ALP staining (Fig. 2B).

### 3.4. Sal B promoted mineralization of hMSCs

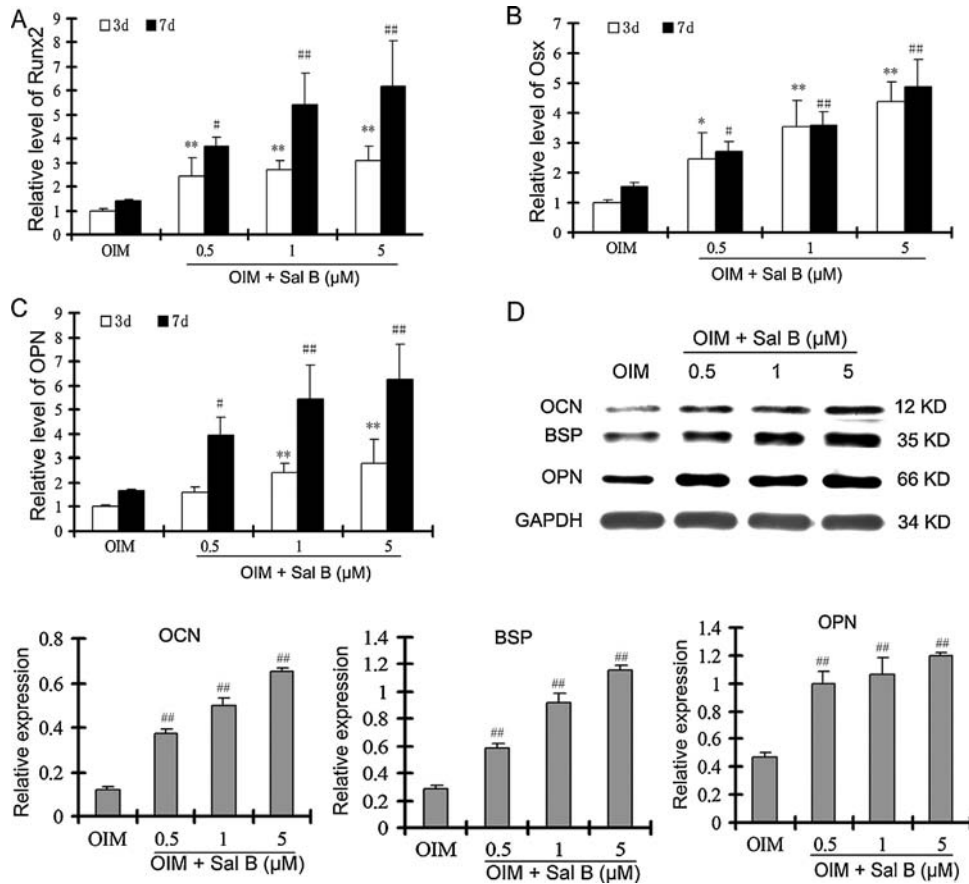
In addition, we examined the effect of Sal B on calcium mineralization of hMSCs. Alizarin Red S staining showed that there was no calcium nodules formation in absence of OIM at day 14; while in presence of OIM, Sal B increased mineralized nodule formation (Fig. 3A). Quantification of Alizarin Red S showed that Sal B markedly increased calcium deposition in a dose-dependent manner as compared with the control, and the maximal effect was observed at a concentration of 5  $\mu$ M (Fig. 3B).

### 3.5. Sal B up-regulated osteogenesis-related markers in hMSCs

Furthermore, we detected the gene expression of OPN which is a marker of osteoblastic differentiation, and transcription factors Runx2 and Osx. The real time PCR result showed that OPN, Runx2 and Osx were all significantly up-regulated by Sal B in a dose-dependent manner (Fig. 4A–C). The result obtained from the western blot demonstrated that the protein levels of OCN, BSP and OPN were also significantly up-regulated by Sal B (Fig. 4D). Based on the above-mentioned results, we concluded that 5  $\mu$ M Sal B was the most suitable dosage that could be used to promote osteogenesis of hMSCs. So, we only used this concentration in the following experiments.

### 3.6. Sal B increased phosphorylation of ERK1/2 in hMSCs

It is well known that ERK signaling pathway is one of the most important signaling pathways that play an important role in regulating the osteogenic differentiation of MSCs. In order to check whether ERK signaling pathway was activated or not when hMSCs were treated with Sal B, we performed western blot to detect the level of phosphorylated ERK1/2 (p-ERK1/2) in hMSCs. The result showed that the level of p-ERK1/2 was significantly up-regulated in hMSCs when they were treated with OIM (Fig. 5A). Also, the protein level of Runx2 and Osx were also significantly increased during the process of osteogenic induction (Fig. 5A). In addition, our result demonstrated that Sal B significantly increased the level

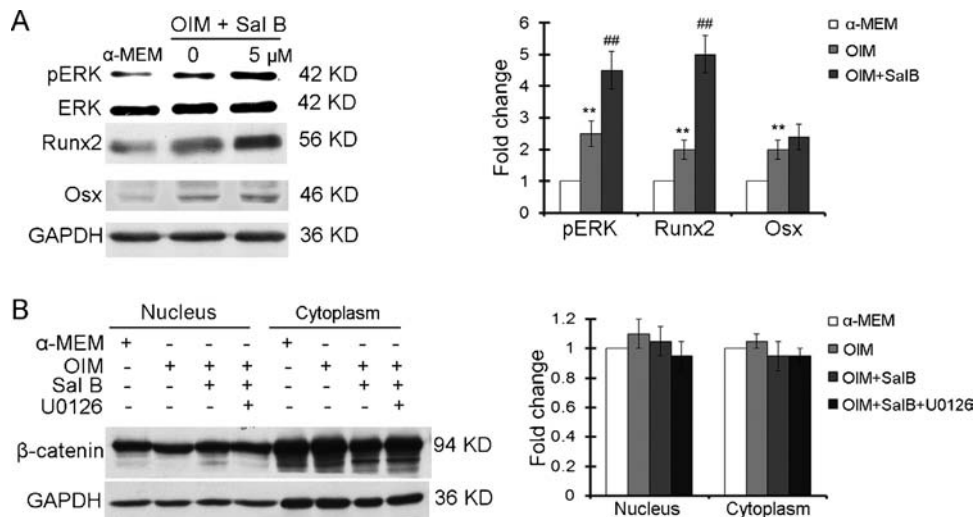


**Fig. 4.** The effect of Sal B on Runx2, Osx and OPN in hMSCs. (A–C) hMSCs were treated with Sal B (0.5 μM, 1 μM, and 5 μM) in OIM for 3 and 7 days, then gene expressions were detected by real time PCR. (D) hMSCs were treated with Sal B (0.5 μM, 1 μM, and 5 μM) in OIM for 7 days, the protein levels of OCN, BSP and OPN were detected by western blot with the indicated antibodies. GAPDH was used as loading control. The experiments were repeated three times. The intensity of the bands was quantified using Image J. \**p* < 0.05, \*\**p* < 0.01 compared with OIM.

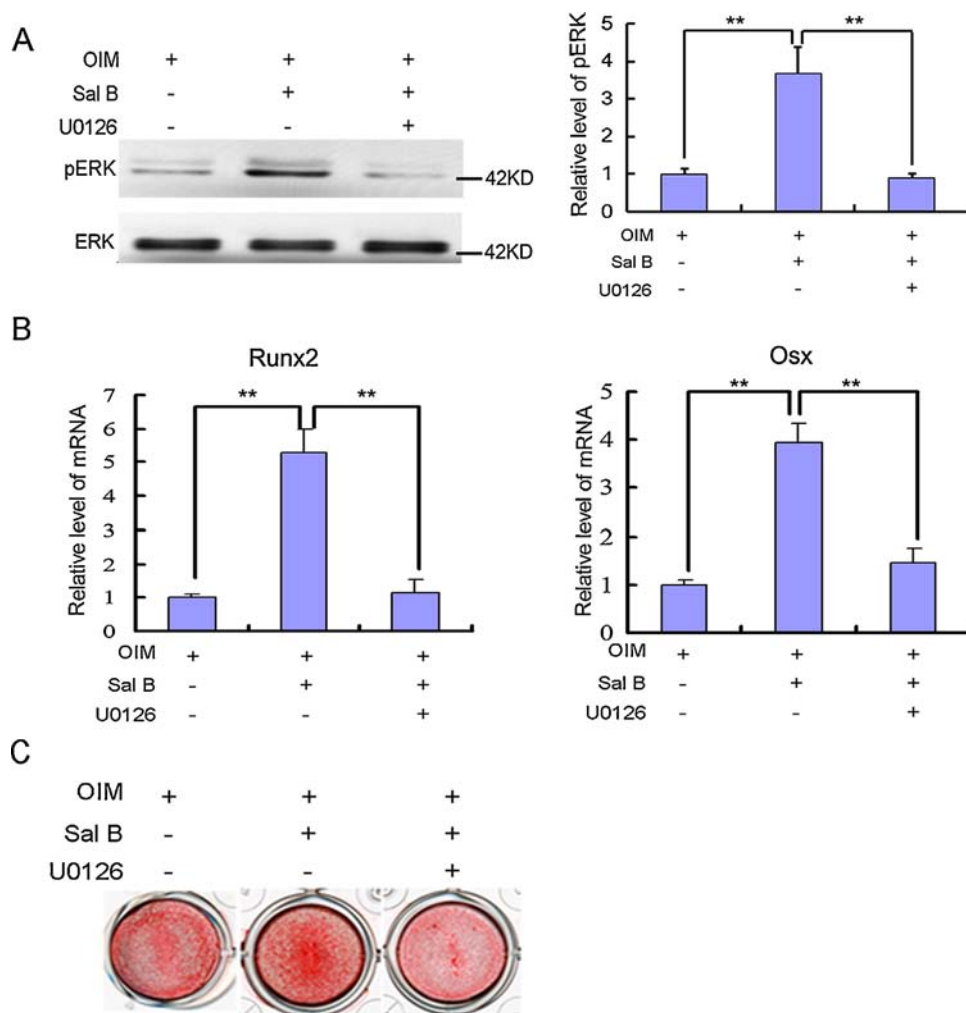
of p-ERK1/2 and Runx2 at the concentration of 5 μM (Fig. 5A). However, the levels of β-catenin which is another important regulator of osteogenesis were not significantly changed in both cytoplasmic and nuclear fractions (Fig. 5B).

3.7. U0126 inhibited the effect of Sal B on osteogenesis in hMSCs

Our data so far have suggested the possible involvement of ERK signaling pathway in Sal B-promoted osteogenesis in hMSCs. In



**Fig. 5.** The effect of Sal B on levels of Runx2, Osx, β-catenin and phosphorylated ERK1/2 in hMSCs. (A) The hMSCs were treated with Sal B in OIM for 48 h, then total proteins were extracted and separated by 10% SDS-PAGE, and detected with the indicated antibodies. GAPDH was used as loading control. (B) The hMSCs were treated with Sal B in OIM for 48 h, then the nuclear and cytoplasmic proteins were extracted and separated by 10% SDS-PAGE. The protein level of β-catenin was detected with indicated antibody. GAPDH was used as loading control. The experiments were repeated three times. The intensity of the bands was quantified using Image J. \*\**p* < 0.01 compared with α-MEM; ##*p* < 0.01 compared with OIM.



**Fig. 6.** U0126 inhibited the effect of Sal B on the osteogenesis in hMSCs. (A) The activation of pERK by Sal B was inhibited by U0126. hMSCs were pretreated with U0126 for 1 h, and incubated with Sal B and OIM for 24 h. Then proteins were separated by 10% SDS-PAGE and analyzed by western blot with specific antibodies. The experiments were repeated three times. The intensity of the bands was quantified using Image J.  $^{**}p < 0.01$ . (B) hMSCs were pretreated with U0126 for 1 h, and incubated with 5  $\mu$ M Sal B in OIM for 7 days. Then total RNAs were extracted, and the expression levels of *Osx* and *Runx2* were detected by real time PCR ( $n=3$ ).  $^{**}p < 0.01$ . (C) hMSCs were pretreated with U0126 for 1 h, and incubated with Sal B in OIM for 14 days. Then the mineralized nodules were stained by Alizarin Red S.

order to further prove the involvement of ERK signaling pathway during this process, we used U0126 (an inhibitor of MEK1/2) to block the activation of pERK. As shown in Fig. 6A, U0126 at dose of 20  $\mu$ M completely inhibited Sal B-induced phosphorylation of ERK1/2, suggesting that U0126 completely abolished the Sal B-induced activation of ERK1/2. Furthermore, the real time PCR data showed that the increases of *Runx2* and *Osx* by Sal B were significantly suppressed by U0126 at day 7 of osteogenic induction (Fig. 6B). Finally, the Alizarin Red S staining showed that treatment of hMSCs with 20  $\mu$ M U0126 significantly inhibited the Sal B-promoted calcium deposition (Fig. 6C).

### 3.8. Silencing ERK1/2 weakened the effect of Sal B on osteogenesis in hMSCs

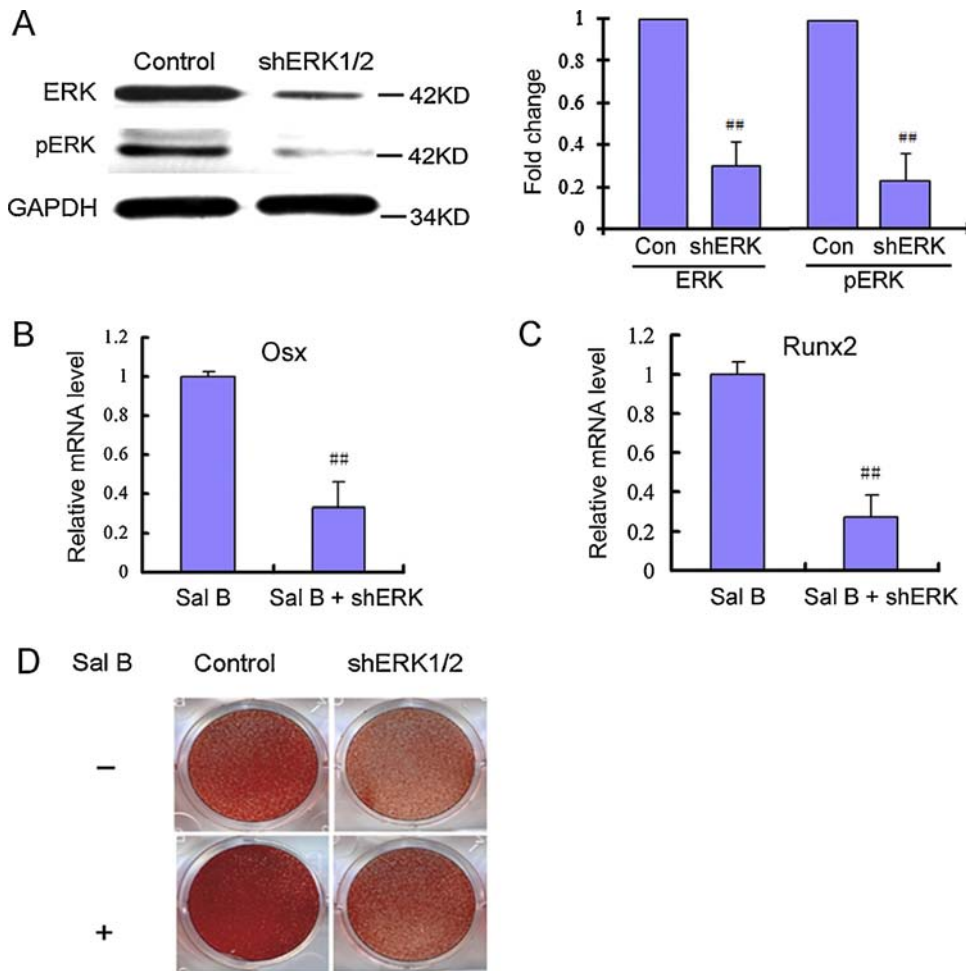
As the effect of U0126 on osteogenesis was not very consistent (our unpublished data), we constructed shRNAs which target ERK1 and ERK2 to further confirm our finding that Sal B promoted the osteogenesis of hMSCs by activating the ERK signaling pathway. Western blot was performed to detect the levels of ERK1/2 and pERK1/2 when the hMSCs were infected with lentiviruses carrying shRNAs targeting ERK1 and ERK2. The result showed that the shRNAs targeting the sequence of ERK1/2 significantly inhibited

ERK1/2 expression, and the total ERK1/2 was knocked down nearly 77%, the relative level of pERK1/2 in hMSCs was even less (Fig. 7A). Furthermore, the real time PCR data showed that the increases of *Runx2* and *Osx* by Sal B were significantly suppressed by shERK1/2 at day 7 of osteogenic induction (Fig. 7B and C). The Alizarin Red S staining showed that the effect of Sal B on the osteogenesis of hMSCs was significantly inhibited by silencing ERK1/2 (Fig. 7D). Taken together, we finally draw the conclusion that Sal B promoted osteogenic differentiation of hMSCs by activating the ERK signaling pathway.

## 4. Discussion

Our previous study has found that Sal B is able to prevent osteoporosis induced by glucocorticoid (Cui et al., 2012). The results from current study showed that Sal B did not affect the viability of hMSCs over a wide range of concentrations, indicating that Sal B had no cytotoxicity to hMSCs. Most importantly, we found that Sal B could promote osteogenesis of hMSCs through enhancing ERK1/2 signaling pathway.

In order to evaluate the effect of Sal B on osteoblastic differentiation of hMSCs, we first investigated the role of Sal B on ALP activity, an early marker of osteoblastic differentiation (Orimo, 2010; Orimo



**Fig. 7.** Silencing ERK1/2 ameliorated the effect of Sal B on osteogenesis in hMSCs. (A) hMSCs were transduced with shERK1 and shERK2 or control (scrambled shRNA). Then the cells were incubated with 5  $\mu$ M Sal B in OIM for 2 days. The total proteins were extracted from hMSCs transduced with shERK or control, and separated by 10% SDS-PAGE and analyzed by western blot with specific antibodies. GAPDH was used as loading control. The intensity of the bands was quantified using Image J.  $^{**}p < 0.01$ . (B) hMSCs were transduced with shERK1 and shERK2 or control (scrambled shRNA). Then the cells were incubated with 5  $\mu$ M Sal B in OIM for 7 days. Then total RNAs were extracted, and the expression levels of *Osx* and *Runx2* were detected by real time PCR ( $n = 3$ ).  $^{**}p < 0.01$ . (C) hMSCs were transduced with shERK1 and shERK2 or control (scrambled shRNA). Then the cells were incubated with 5  $\mu$ M Sal B or PBS in OIM for 14 days. The mineralized nodules of hMSCs were stained by Alizarin Red S after 14 days of osteogenic induction.

and Shimada, 2008). ALP hydrolyzes pyrophosphate to generate phosphate which reacts with calcium to form hydroxyapatite to promote mineralization, suggesting that ALP plays an important role in bone formation (Harrison et al., 1995; Orimo, 2010). Our result indicated that Sal B significantly increased the activity of ALP in hMSCs. As expected, Sal B also enhanced calcium nodule formation, a functional marker of mineralization (Gordon et al., 2007). These results showed that Sal B could promote the osteogenic differentiation of hMSCs.

Next, we checked the changes of some osteogenesis-related genes in Sal B treated hMSCs, such as *Runx2*, *Osx*, *OPN* and *OCN*. The runt family transcription factor *Runx2* is a pivotal transcription regulator and plays a crucial role in osteoblast differentiation (Ducy et al., 1997; Komori et al., 1997). Studies have revealed that both intramembranous and endochondral ossification are completely blocked in *Runx2* null mice and overexpression of *Runx2* could induce the expression of osteoblast phenotypic genes (Komori et al., 1997; Zhao et al., 2005). *Osx* is a zinc finger-containing transcription factor, and is required for osteoblastic differentiation (Kim et al., 2006; Nakashima et al., 2002). It has been shown that there are no endochondral or intramembranous bones in *Osx* null mice (Miraoui et al., 2009). *Osx* is not expressed in *Runx2* null mice but *Runx2* is normally expressed in *Osx* null mice, indicating that *Osx* acts as a

downstream factor of *Runx2* (Miraoui et al., 2009). *OPN* is a prominent bone matrix protein produced by osteoblastic cells and *Osx* also regulates the expression of *OPN* (Tu et al., 2006; Yao et al., 1994). In this study, we found that Sal B significantly increased the expression levels of *OPN*, *Runx2* and *OCN*. The results suggested that Sal B enhanced osteogenic differentiation of hMSCs through mediating transcription factor *Runx2*.

Studies show that ERK1/2 signaling pathway plays an important role in the regulation of osteogenesis, and ERK1/2 is constantly activated during osteogenic differentiation (Jaiswal et al., 2000; Lai et al., 2001). For example, the activation of ERK1/2 signaling pathway has been shown to be involved in both sodium butyrate and ugonin K promoted-osteogenic differentiation (Chen et al., 2007; Lee et al., 2011). To explore whether ERK1/2 signaling pathway was also involved in the Sal B-mediated osteogenic induction in hMSCs, the cells were incubated with Sal B and OIM, and we found Sal B significantly increased the phosphorylation of ERK1/2 in a dose-dependent manner. However, Lu et al. showed that Sal B inhibited the phosphorylation of ERK1/2 in rat MSCs (Lu et al., 2010). The discrepancy may be due to differences in cell types and culture condition. In addition, to further confirm our finding, we also checked whether blocking ERK1/2 signaling pathway could impair the effect of Sal B on osteogenesis of hMSCs. The cells were treated with

U1206 which could efficiently block ERK1/2 phosphorylation in hMSCs. We found that this compound significantly inhibited the effect of Sal B on mineralized nodule formation in hMSCs. Furthermore, we also used shRNAs targeting ERK1/2 to silence the endogenous expression of ERK1/2. The hMSCs were infected with pseudo-lentivirus carrying shERK1/2, and the result showed that total levels ERK1/2 were significantly knocked down by shERK1/2. The Alizarin Red S staining showed that silencing ERK1/2 significantly suppressed the effect of Sal B on osteogenesis of hMSCs. These data clearly demonstrated that ERK1/2 played a crucial role during Sal B induced osteogenesis in hMSCs.

The activation of ERK1/2 increases the phosphorylation and transcriptional activity of Runx2, suggesting Runx2 is a target of ERK1/2 pathway (Ge et al., 2007; Xiao et al., 2000). In the present study, we proved that Sal B promoted the phosphorylation of ERK1/2, which in turn up-regulated the expression of transcription factors Runx2, resulting in enhanced osteogenesis in hMSCs.

In conclusion, we have demonstrated that Sal B regulates osteogenesis of hMSCs. Sal B promotes osteogenesis at least partially through activating ERK1/2 signaling pathway. This may represent one of the mechanisms by which Sal B prevents osteoporosis.

#### Author's contribution

The authors, Daohua Xu, Liao Cui and Tie Wu conceived and designed the experiments. Daohua Xu, Liangliang Xu and Wayne YW Lee performed the experiments. Daohua Xu and Chenhui Zhou analyzed the data. Daohua Xu, Liao Cui and Gang Li wrote the paper.

#### Conflict of interest

No conflict of interest to declare.

#### Acknowledgments

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