

# Salidroside promotes the osteogenic and odontogenic differentiation of human dental pulp stem cells through the BMP signaling pathway

XIAOLING WEI<sup>1,2\*</sup>, JIAYANG LI<sup>1,2\*</sup>, HUI LIU<sup>1,2</sup>,  
CHENGUANG NIU<sup>2-4</sup> and DONG CHEN<sup>1,2</sup>

<sup>1</sup>Department of Endodontics; <sup>2</sup>Shanghai Key Laboratory of Craniomaxillofacial Development and Diseases, Shanghai Stomatological Hospital, Fudan University, Shanghai 200001; <sup>3</sup>Department of Endodontics, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine; <sup>4</sup>College of Stomatology, Shanghai Jiao Tong University, Shanghai 200011, P.R. China

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**Abstract.** Regenerative endodontics, as an alternative approach, aims to regenerate dental pulp-like tissues and is garnering the attention of clinical dentists. This is due to its reported biological benefits for dental therapeutics. Stem cells and their microenvironment serve an important role in the process of pulp regeneration. Regulation of the stem cell microenvironment and the directed differentiation of stem cells is becoming a topic of intensive research. Salidroside (SAL) is extracted from the root of *Rhodiola rosea* and it has been reported that SAL exerts antiaging, neuroprotective, hepatoprotective, cardioprotective and anticancer effects. However, the ability of SAL to regulate the osteo/odontogenic differentiation of hDPSCs remains to be elucidated. In the present study, the effect of SAL on the proliferation and osteogenic/odontogenic differentiation of human dental pulp stem cells (hDPSCs) was investigated. This was achieved by performing CCK-8 ARS staining assay, reverse transcription-quantitative PCR to detect mRNA of ALP, OSX, RUNX2, OCN, DSPP and BSP, western blotting to detect the protein of MAPK, Smad1/5/8, OSX, RUNX2, BSP and GAPDH and immunofluorescence assays to detect DSPP. The results indicated that SAL promoted the

cell viability and the osteogenic/odontogenic differentiation of hDPSCs whilst increasing the expression of genes associated with osteogenic/odontogenic differentiation by ARS staining assay. In addition, SAL promoted osteogenic and odontogenic differentiation by activating the phosphorylation of Smad1/5/8. Collectively, these findings suggest that SAL promoted the osteogenic and odontogenic differentiation of hDPSCs activating the BMP signaling pathway.

## Introduction

The typical treatment option for the irreversible inflammation and necrosis of the dental pulp tissue is root canal treatment (1). However, the development of regenerative medicine has enabled the potential application of regenerative endodontics as an alternative approach, which aims to regenerate dental pulp-like tissues (2,3). This method is increasingly attracting the attention of dentists and researchers due to the reported beneficial effects on both tooth function and preservation (2,3). However, identification of the conditions for optimal pulp tissue regeneration has not been fully realized (4). Stem cells and their microenvironment serve an important role in pulp regeneration (5). This microenvironment may include growth factors, scaffold materials and the removal of infection which can create optimal conditions for the tissue-induced induction of stem cells (6). During pulp regeneration, the microenvironment can regulate the directional differentiation of stem cells, which in turn affects downstream processes, including the generation of new blood vessels, neurons and dentin, in the regenerated pulp-like tissues (6). In the majority of studies that previously investigated dental pulp-like tissue regeneration, the regulation of transplanted human dental pulp stem cell (hDPSC) differentiation serves an important role (7,8). Regulation of the stem cell microenvironment and its influence of the direct differentiation of stem cells has been a subject of intense research. It has been previously reported that the osteogenic and odontogenic differentiation, vascularization and neurogenesis of stem cells can be regulated by drug-loaded scaffolding materials or small-molecular weight

*Correspondence to:* Professor Dong Chen, Department of Endodontics, Shanghai Stomatological Hospital, Fudan University, 356 Beijing Road, Shanghai 200001, P.R. China  
E-mail: chendong19690124@126.com

Dr Chenguang Niu, Department of Endodontics, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, P.R. China  
E-mail: niuchg09@alumni.sjtu.edu.cn

\*Contributed equally

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agents (2,9). These findings may provide useful clinical strategies for dental pulp-like tissue regeneration.

Significant progress has been made in the exploration of the therapeutic use of naturally-occurring products for the treatment of several diseases (10,11). In particular, new applications have emerged with the development of molecules, such as artemisinin, used in traditional Chinese medicine (10,11). Salidroside (SAL) is a compound that can be extracted from the root of *Rhodiola rosea* and is considered to be an important Chinese herbal medicine (12). It has been previously reported that SAL can exert anti-aging, neuroprotective, hepatoprotective, cardioprotective and anticancer effects (13-16). However, few studies have performed a therapeutic evaluation of SAL on hDPSCs. Therefore, the effects of SAL on the regulation of hDPSCs osteogenic and odontogenic (osteo/odontogenic) differentiation remain unclear. The aim of the present study was to explore the potential effects of SAL, a small-molecular weight compound derived from traditional Chinese medicine, on the osteo/odontogenic differentiation of hDPSCs *in vitro*. The effects of SAL on the viability and osteo/odontogenic differentiation of hDPSCs and whether these effects were mediated via the regulation of osteo/odontogenic differentiation-associated gene expression were examined. The role of the bone morphogenetic protein (BMP) pathway in osteo/odontogenic differentiation was also investigated.

## Materials and methods

**Reagents and antibodies.** SAL was purchased from Chengdu Must Bio-Technology Co., Ltd. (cat. no. MUST-19021504) and was dissolved in dimethyl sulfoxide (stock concentration of 200 mM).  $\alpha$ -modification of Eagle's minimum essential medium ( $\alpha$ -MEM) and penicillin/streptomycin were purchased from Gibco; Thermo Fisher Scientific, Inc. Alizarin Red S (ARS), Triton X-100 and DAPI were obtained from Sigma-Aldrich; Merck KGaA. Primary antibodies against GAPDH (Cell Signaling Technology, Inc., cat. no. 5174T), Smad 1 (Cell Signaling Technology, Inc., cat. no. 6944T), phosphorylated (p-)Smad1/5/8 (Cell Signaling Technology, Inc., cat. no. 13820T), runt-related transcription factor 2 (RUNX2) (Cell Signaling Technology, Inc., cat. no. 12556S), bone sialoprotein (BSP) (Cell Signaling Technology, Inc., cat. no. 5468S), and osteocalcin (OCN) (Cell Signaling Technology, Inc., cat. no. 12888) were purchased from Cell Signaling Technology, Inc. Osterix (OSX) (Abcam, cat. no. ab209484) were purchased from Abcam. Alkaline Phosphatase (ALP) assay kit (cat. no. P0321S) was obtained from Beyotime Institute of Biotechnology.

**Isolation and culture of hDPSCs.** The present study was approved by The Shanghai Stomatological Hospital Ethics Association [approval no. SSDC-(2020)0006] and all patients or their parents provided written informed consent. The hDPSCs were isolated from completely healthy premolars, without caries or periodontitis, which were extracted during the course of orthodontic treatment. The donors were aged between 14 and 18 years ( $n=10$ , five male, five female, aged  $15.6 \pm 1.26$  years) and they had their teeth extracted in Shanghai Stomatological Hospital between August and September 2019. The pulp was gently separated

from the tooth and cultured with a modified tissue piece method in maintenance medium containing Dulbecco Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 units ml<sup>-1</sup> penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO<sub>2</sub> in an incubator. The medium was replaced every 3 days after cells grew out from the tissue pieces until cells reached 80-90% confluence. hDPSCs were digested by 0.05% trypsin-EDTA and then seeded into a new dish at 1:1. Flow cytometric analysis was used to determine the cell surface markers present on hDPSCs. The hDPSCs of each donor were isolated and cultured alone.

**Cell viability assay.** The effects of SAL on hDPSC proliferation were measured using a Cell Counting Kit-8 kit (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocols (17). In brief, hDPSCs were plated into 96-well plates at a density of  $5 \times 10^3$  cells/well and were incubated for 48 or 96 h at 37°C with serial dilutions of SAL (0, 10, 20, 50 and 100  $\mu$ M). Subsequently, 10  $\mu$ l CCK-8 solution was mixed with 90  $\mu$ l complete  $\alpha$ -MEM and added to each well. Following incubation for 2 h at 37°C, the optical density was measured using an ELX800 absorbance microplate reader (Bio-Tek Instruments, Inc.) at 450 nm (650 nm reference).

**ARS staining assay.** hDPSCs were first cultured in 12-well plates ( $4 \times 10^4$  cells per well). The wells were then divided into eight groups as follows at 37°C and 5% CO<sub>2</sub> in an incubator for 21 days: i) Growth medium group (GM); ii) osteo/odontogenic induction medium (OM) group; iii) OM + 1  $\mu$ M SAL group (1  $\mu$ M); iv) OM + 5  $\mu$ M SAL group (5  $\mu$ M); v) OM + 10  $\mu$ M SAL group (10  $\mu$ M); vi) OM + 20  $\mu$ M SAL group (20  $\mu$ M); vii) OM + 50  $\mu$ M SAL group (50  $\mu$ M); viii) and OM + 100  $\mu$ M SAL group (100  $\mu$ M).

The osteo/odontogenic medium was comprised of 100 nM dexamethasone, 50  $\mu$ M ascorbic acid and 10 mM  $\beta$ -glycerophosphate dissolved in  $\alpha$ -MEM (all from Sigma-Aldrich; Merck KGaA) (18). The growth medium was 10% FBS and 1% Penicillin-Streptomycin in  $\alpha$ -MEM. To assess osteogenic differentiation, the cells were stained with ARS (18). Briefly, hDPSCs were collected on day 21, fixed in 4% paraformaldehyde for 15 min and stained with 40 mM ARS (pH 4.9; Sigma-Aldrich; Merck KGaA) for 15 min, both at 25°C. The plates were observed under an inverted microscope (magnification, x200; Nikon Corporation). The darker the intensity of the red dots, the higher the number of calcium nodules and therefore the higher degree of differentiation.

**ALP staining assay.** hDPSCs were cultured in 12-well plates ( $4 \times 10^4$  cells per well). The wells were divided into three groups as follows: i) GM; ii) OM; and iii) 10  $\mu$ M SAL group. hDPSCs were then treated for 7 days at 37°C. Subsequently, they were fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were stained for ALP using the ALP assay kit (cat. no. P0321S; Beyotime Institute of Biotechnology) according to the manufacturer's protocols (19,20). The cells were subsequently washed three times with distilled water. ALP staining was observed under an inverted microscope (magnification, x200; Nikon Corporation). Increases in the

concentration of the insoluble nitroblue tetrazolium formazan was associated with a change in color from dark blue to blue/purple, which is in turn associated with higher ALP activity.

**RNA extraction and gene expression analysis using reverse transcription-quantitative PCR (RT-qPCR).** hDPSCs were cultured in six-well plates ( $8 \times 10^4$  cells per well). The wells were divided into three groups as follows: i) GM; ii) OM; and iii)  $10 \mu\text{M}$  SAL. hDPSCs were in turn treated for 3 and 7 days at  $37^\circ\text{C}$ . Total mRNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (21). Complementary DNA synthesis was performed using a FastQuant RT kit (Tiangen Biotech Co., Ltd. cat. no. KR106-03). qPCR was performed using the SYBR Green Premix (Tiangen Biotech Co., Ltd.; cat. no. FP207) according to the manufacturer's protocols under the following conditions: 15 min of denaturation at  $42^\circ\text{C}$  and 1 cycle of 1 min of denaturation at  $95^\circ\text{C}$  and 40 cycles each of 5 sec of denaturation at  $95^\circ\text{C}$  and 15 sec of amplification at  $60^\circ\text{C}$ . The following primer sequences were used as previously described: human actin (22) forward, 5'-CATGTACGTTGC TATCCAGGC-3' and reverse, 5'-CTCCTTAATGTCACG CACGAT-3'; human ALP (22) forward, 5'-CCTCCTCGG AAGACACTCTG-3' and reverse, 5'-GCAGTGAAGGGC TTCTTGTC-3'; human RUNX2 (22) forward, 5'-CCACTG AACCAAAAAGAAATCCC-3' and reverse, 5'-GAAAAC AACACATAGCCAAACGC-3'; human OCN (23) forward, 5'-GCGCTACCTGTATCAATGGC-3' and reverse, 5'-AAC TCTCACAGTCCGGATT-3'; human dentin sialophospho-protein (DSPP) (7) forward, 5'-CGACATAGGTCACAATGA GGATGTCG-3' and reverse, 5'-TTGCTTCCAGCTACTTGA GGTC-3'; human BSP (7) forward, 5'-CAGGCCACGATA TTATCTTTACA-3' and reverse, 5'-CTCCTCTTCTTCCTC CTCCTC-3' and human OSX (24) forward, 5'-CTGTGA AACCTCAAGTCCTATGGA-3' and reverse, 5'-GCTCTG CAGTCAAGGGAGATG-3'. The relative gene expression was calculated according to the comparative  $2^{-\Delta\Delta C_q}$  method and normalized to that of actin (25).

**Immunofluorescence analysis.** hDPSCs were cultured in 24-well plates ( $4 \times 10^4$  cells per well). The wells were divided into three groups as follows: i) GM; ii) OM; and iii)  $10 \mu\text{M}$  SAL. hDPSCs were treated for 3 days at  $37^\circ\text{C}$ . The cell samples were then fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Subsequently, the cells were permeabilized in the presence of PBS with 0.25% Triton X-100 for 15 min (26) and blocked with 5% non-fat milk diluted in TBS at room temperature for 1 h. The cells were incubated overnight at  $4^\circ\text{C}$  with anti-DSPP (1:200; cat. no. SC-73632; Santa Cruz Biotechnology, Inc.) in the presence of 2.5% BSA. The cells were subsequently washed three times in PBS with 0.1% Tween-20 (PBST) and stained for 1 h at room temperature with a goat anti-mouse FITC-conjugated secondary antibody (1:1,000; cat. no. F-2761; Thermo Fisher Scientific, Inc.) in 2.5% BSA. The samples were washed five times with PBST and the cells were counterstained with DAPI (1:10,000) at room temperature for 10 min. The cells were imaged using a fluorescence microscope (magnifications,  $\times 200$ ; Leica Microsystems, Inc.).

**Western blot analysis.** hDPSCs were cultured in six-well plates ( $8 \times 10^4$  cells per well). The wells were divided into three groups as follows: i) GM; ii) OM; and iii)  $10 \mu\text{M}$  SAL group. hDPSCs were treated for 3 days at  $37^\circ\text{C}$ . The cells were collected in RIPA lysis buffer (Thermo Fisher Scientific, Inc.). The protein concentration was determined by BCA method (Thermo Fisher Scientific, Inc., cat. no. 23225). The lysate proteins ( $30 \mu\text{g}$ ) were separated using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The PVDF membranes were blocked with 5% non-fat milk diluted in TBS at room temperature for 1 h and were incubated overnight at  $4^\circ\text{C}$  with a series of primary antibodies targeted at GAPDH, Smad 1, p-Smad1/5/8, RUNX2, BSP, OSX, and OCN (1:1,000 dilution). The following day, the PVDF membranes were incubated with HRP-conjugated secondary antibodies (Cell Signaling Technology, Inc. cat. nos. 7074 and 7076; 1:10,000 dilution) for 1-2 h at room temperature. A Western Chemiluminescent HRP Substrate system (EMD Millipore; cat. no. WBKLS0100) was used for visualizing the bands. The signals were captured with an Amersham Imager 600 monitoring system (GE Healthcare). ImageJ 1.8.0 (National Institutes of Health) was used to quantify the bands.

**Statistical analysis.** Data analysis was performed using the SPSS software (ver. 23.0; IBM Corp.). Experiments were repeated independently at least three times. The results were expressed as mean  $\pm$  standard deviation. Statistical analysis of the data was performed with one-way analysis of variance followed by the Scheffé's post hoc test for multiple comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**SAL promotes hDPSC viability and osteo/odontogenic differentiation.** To investigate the effects of SAL on the osteo/odontogenic differentiation of hDPSCs, CCK-8 assay was first used. Treatment with SAL for 48 or 96 h at concentrations  $< 100 \mu\text{M}$  did not induce cytotoxicity. SAL ( $10$  or  $20 \mu\text{M}$ ) significantly increased cell viability at 96 h time points of treatment compared with that in the control group (Fig. 1A and B).

ARS was subsequently used to detect the effect of SAL on the osteogenic differentiation of hDPSCs. SAL promoted the osteo/odontogenic differentiation of hDPSCs at  $100 \mu\text{M}$ ; Fig. 1C showed that differentiation showed by the calcium nodules was increased in all treatment groups vs. GM. SAL ( $10 \mu\text{M}$ ) markedly increased the osteo/odontogenic differentiation of hDPSCs (Fig. 1C). Based on this observation,  $10 \mu\text{M}$  SAL was selected for the following experiments.

**SAL increases the expression of genes associated with osteo/odontogenic differentiation.** ALP staining indicated that OM increased ALP activity compared with GM and SAL ( $10 \mu\text{M}$ ) markedly increased ALP activity in the hDPSCs compared with OM (Fig. 2A). Additionally, SAL further promoted the expression levels of ALP mRNA following incubation with the cells for 3 and 7 days compared with OM (Fig. 2B). The expression levels of OSX were also significantly increased in hDPSCs following treatment of the cells with



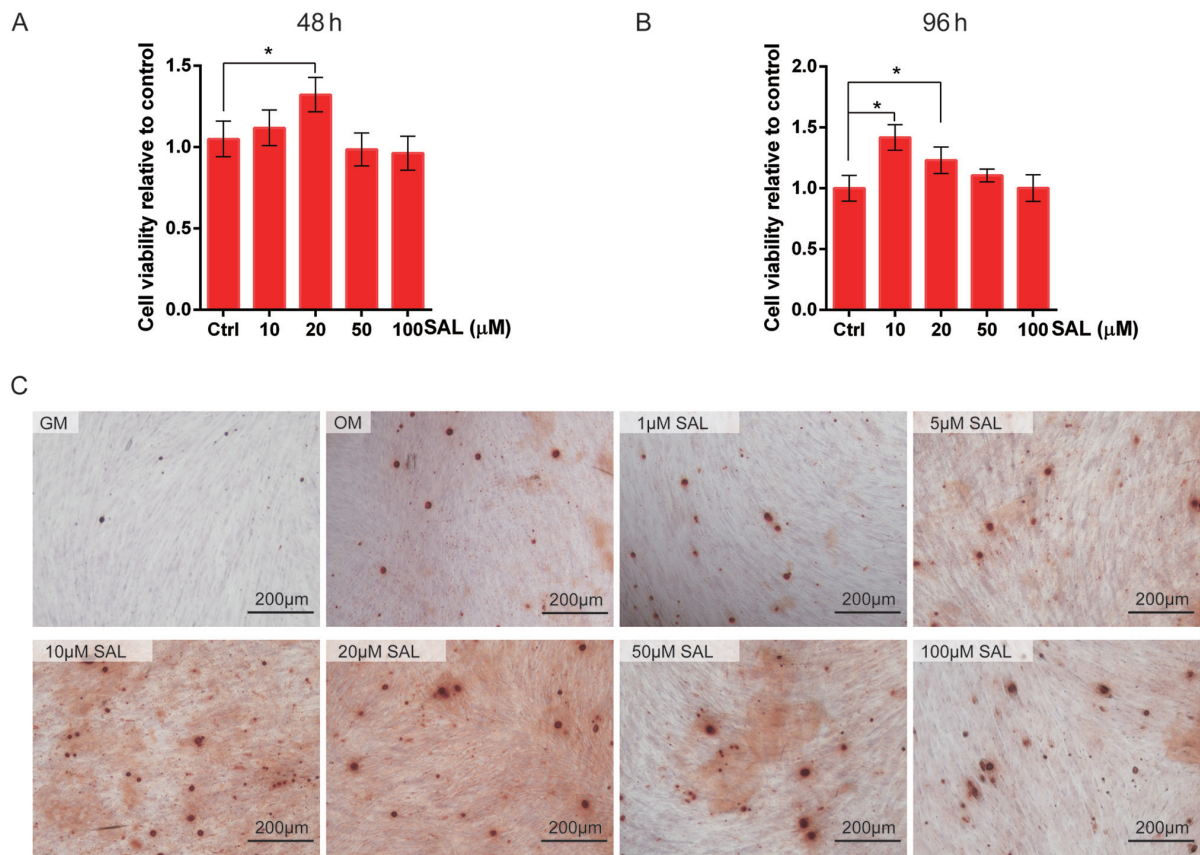


Figure 1. SAL promotes the proliferation and osteogenesis of hDPSCs. (A) Cell viability of hDPSCs were detected by Cell Counting Kit-8 following treatment with 10, 20, 50 and 100  $\mu$ M SAL for (A) 48 and (B) 96 h. (C) Alizarin red staining detected the osteogenesis of hDPSCs treated with 1, 5, 10, 20, 50 and 100  $\mu$ M SAL. Scale bars, 200  $\mu$ m. \* $P < 0.05$ . GM, growth medium; OM, osteogenic medium; SAL, salidroside; hDPSCs, human dental pulp stem cells.

10  $\mu$ M SAL for 3 and 7 days compared with those in the OM group (Fig. 2C). Although SAL promoted the expression levels of RUNX2, OCN, DSPP and BSP mRNA following incubation with the cells for 3 and 7 days to some extent, there was no significant difference (Fig. 2).

*SAL promotes the expression levels of DSPP in hDPSCs.* DSPP is a key protein involved in the process of odontogenic differentiation (23). In the OM group, the expression of DSPP was higher compared with the GM group. SAL (10  $\mu$ M) effectively promoted the expression of DSPP in hDPSCs as detected by immunofluorescence analysis compared with the OM group (Fig. 3).

*SAL promotes osteo/odontogenic differentiation by activating the BMP pathway.* The effects of SAL on the expression of the osteo/odontogenic differentiation-associated proteins were detected in hDPSCs by western blot analysis. Western blot analysis indicated that SAL markedly increased the expression levels of the RUNX2 and BSP proteins compared with the OM group (Fig. 4A). To explore the specific mechanism underlying the involvement of SAL in the osteo/odontogenic differentiation of hDPSCs, the effects of SAL on the p38, JNK, ERK and Smad1/5/8 signaling pathways were examined. The results indicated that whilst SAL could significantly activate Smad1/5/8 signaling compared with that in the OM group, it had no effect on the p38, ERK or the JNK MAPK signaling pathways (Fig. 4B-C).

## Discussion

Dental pulp regeneration, compared with root canal therapy for the young permanent teeth with pulpitis, exhibits a number of methodological advantages as it restores the nutrition supply of tooth tissue and make the tooth harder to break (27,28). It would be of considerable benefit for the young permanent teeth with pulpitis to be able to regulate the directed differentiation of transplanted hDPSCs using different methods such as releasing cytokines or drugs by biomaterials (29). Small molecules derived from traditional Chinese medicine have been reported to confer specific biological activities that may resemble those of natural products (30). Previous studies have successively used molecules such as corylin and artemisinin for the regulation of stem cell differentiation (31,32). The aim of the present study was to explore whether SAL has the ability to affect the osteo/odontogenic differentiation of hDPSCs *in vitro*. The data demonstrated that SAL promoted the viability and osteo/odontogenic differentiation of hDPSCs.

In the present study, SAL exerted no cytotoxicity in the concentration range of 0-100  $\mu$ M, but could instead significantly increase the viability of hDPSCs at 10 and 20  $\mu$ M, which is consistent previous observations by Chen *et al* (13). However, further increases in SAL concentration to 50 and 100  $\mu$ M did not promote the proliferation of hDPSCs. This may be because with this increase, the toxicity of SAL increased correspondingly, counteracting its effect on cell viability. The possible

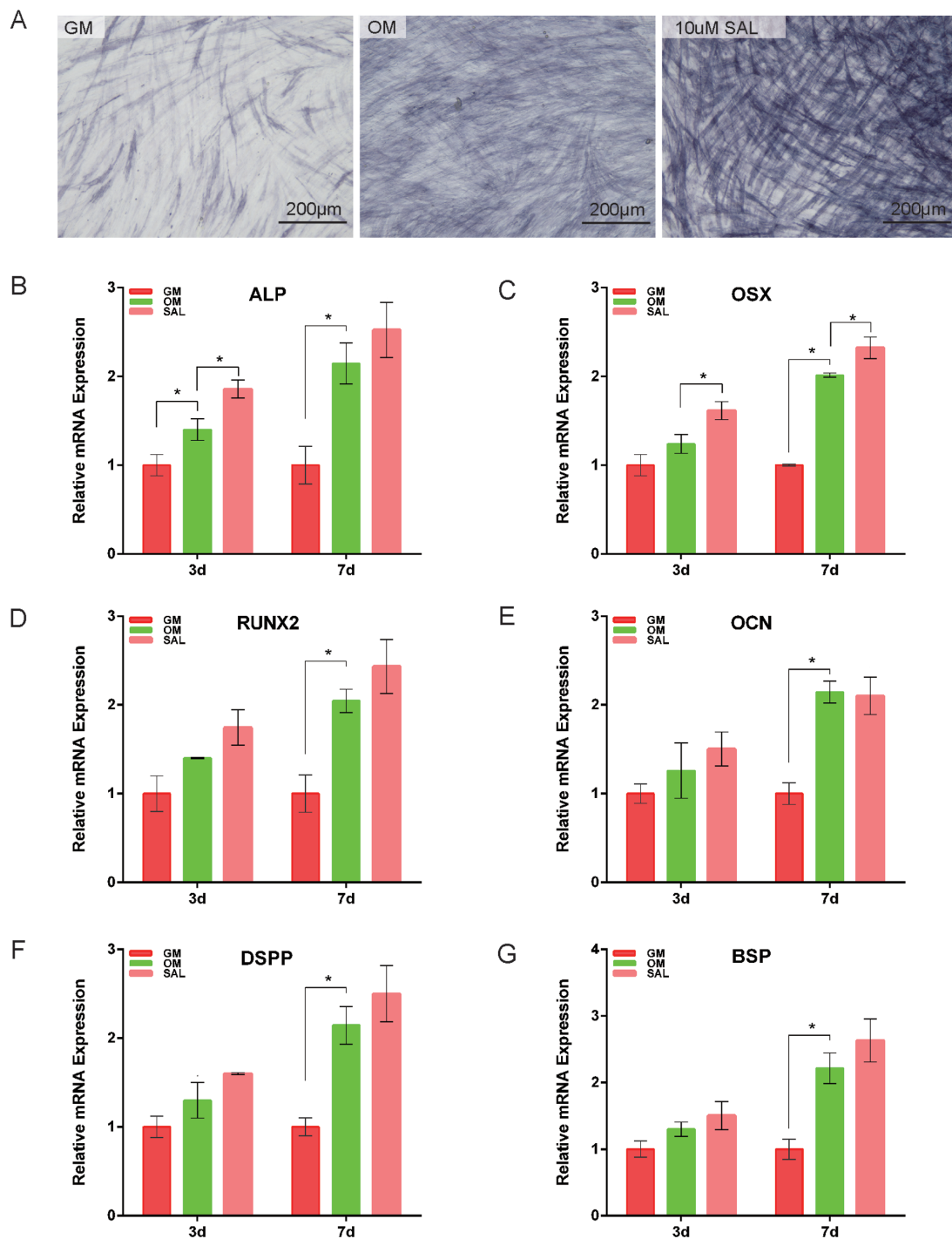


Figure 2. SAL promotes the expression of genes associated with osteogenesis. (A) Alkaline phosphatase staining was used to measure the degree of osteogenesis in hDPSCs treated with 10  $\mu$ M SAL. The mRNA expression of (B) ALP, (C) OSX, (D) RUNX2, (E) OCN, (F) DSPP and (G) BSP were detected after treatment with 10  $\mu$ M SAL for 3 and 7 days. The expression of OSX, RUNX2, OCN, DSPP and BSP in hDPSCs after treatment for 3 and 7 days with 10  $\mu$ M SAL was significantly increased. Scale bars, 200  $\mu$ m. \*P<0.05. GM, growth medium; OM, osteogenic medium; SAL, salidroside; RUNX2, runt-related transcription factor 2; BSP, bone sialoprotein; OCN, osteocalcin; OSX, osterix; ALP, Alkaline Phosphatase; DSPP, dentin sialophosphoprotein; hDPSCs, human dental pulp stem cells.

regulatory effect of SAL on the osteo/odontogenic differentiation of hDPSCs was then detected by ARS. The results of the ARS assay demonstrated that SAL exerted a promoting effect on the osteo/odontogenic differentiation of hDPSCs, which was the most prominent at 10  $\mu$ M. The biological effects of SAL on promoting osteogenesis have also been reported in other cells. Zhang *et al* (33) previously demonstrated that SAL treatment counteracted H<sub>2</sub>O<sub>2</sub>-induced osteogenic inhibition by increasing

ALP activity and mineralization in the MC3T3-E1 osteoblastic cell line. It has also been shown that SAL promotes osteoblastic differentiation in the murine pluripotent mesenchymal cell line C3H10T1/2 (13). A previous study reported that SAL promoted human periodontal ligament cell proliferation and osteocalcin secretion via the ERK1/2 and PI3K/AKT signaling pathways (16). These previous studies aforementioned at least in part support the findings of the present study.

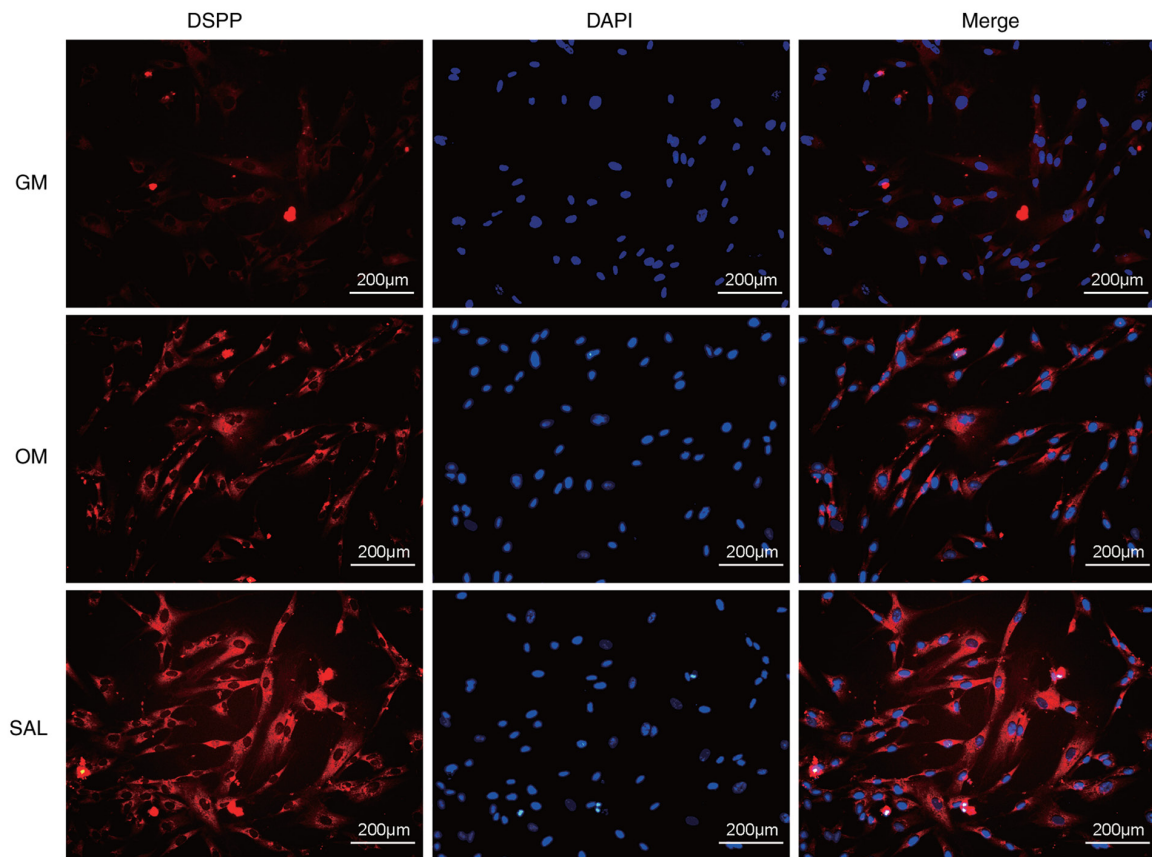


Figure 3. SAL promotes the expression of DSPP in hDPSCs as detected by immunofluorescence. In total, 10  $\mu$ M SAL can effectively promote the expression of DSPP in hDPSCs. Scale bars, 200  $\mu$ m. GM, growth medium; OM, osteogenic medium; SAL, salidroside; DSPP, dentin sialophosphoprotein; hDPSCs, human dental pulp stem cells.

ALP is a key marker of osteoblastic cells and serves a central role in the regulation of early osteogenesis, where it promotes cell maturation and calcification (24,34). To evaluate the effects of SAL, the expression levels of ALP were assessed by ALP staining and RT-qPCR. The results of ALP staining and gene expression analysis demonstrated that 10  $\mu$ M SAL exerted a significant promoting effect on early hDPSC osteogenesis compared with that in the OM groups. OSX is a zinc finger-containing transcription factor that is expressed in osteoblasts and may serve an important role in the osteoblast differentiation pathway by controlling the expression of specific genes such as OCN and collagen (35). A number of transcription factors are specific to regulating osteoblast differentiation and bone formation. RUNX2 is a multifunctional transcription factor that can regulate the differentiation of osteoblasts into mesenchymal stem cells and induces the differentiation of mesenchymal stem cells into osteoblasts (36). Furthermore, RUNX2 is necessary for tooth formation and is intimately involved in the development of calcified tooth tissues (35). Miyazaki *et al* (37) reported that RUNX2 is expressed in preodontoblasts, where its expression level is decreased during odontoblast differentiation. In the present study, the results suggested that RUNX2 was highly expressed during the early stages of hDPSC osteoblast/odontoblastic differentiation following SAL treatment compared with the OM group. RUNX2 and OCN are two extensively studied osteoblast-specific transcription factors that are also key markers of osteoblastic cells (38). RUNX2 is typically

expressed during early osteoblast differentiation and is indispensable for the differentiation and appropriate functioning of osteoblasts (38,39). OCN is considered to be a marker of mature osteocytes that is also expressed in odontoblasts (40). OCN has been reported to regulate the growth rate and direction of hydroxyapatite crystals during bone development (39).

During dentin formation, DSPP is considered the marker of dentin differentiation (41). DSPP is only expressed in odontoblasts and is regulated by RUNX2 to promote differentiation into odontoblasts (42). By contrast, to these observations, BSP comprises the majority of non-collagenous extracellular matrix in mineralized tissues and is one of the osteoblast-specific markers regulated by RUNX2 (43). In the present study, the expression levels of DSPP and BSP were significantly increased following SAL treatment. This indicates that SAL may promote osteoblastic/odontoblastic cell differentiation and mineralization by regulating DSPP and BSP gene expression in hDPSCs. BSP functions as a hydroxyapatite nucleator and it is possible that the bony spicules observed in the frontal and parietal bones may be formed as a result of BSP upregulation (44). In the present study, SAL (10  $\mu$ M) caused a significant upregulation in the mRNA expression levels of OSX, RUNX2, DSPP and BSP on either day 3 or day 7. These findings in the present study suggest that SAL may serve an important role in promoting the osteogenic and odontoblast differentiation of hDPSCs.

Subsequently, the present study explored the signaling pathways that were affected by SAL treatment with regards



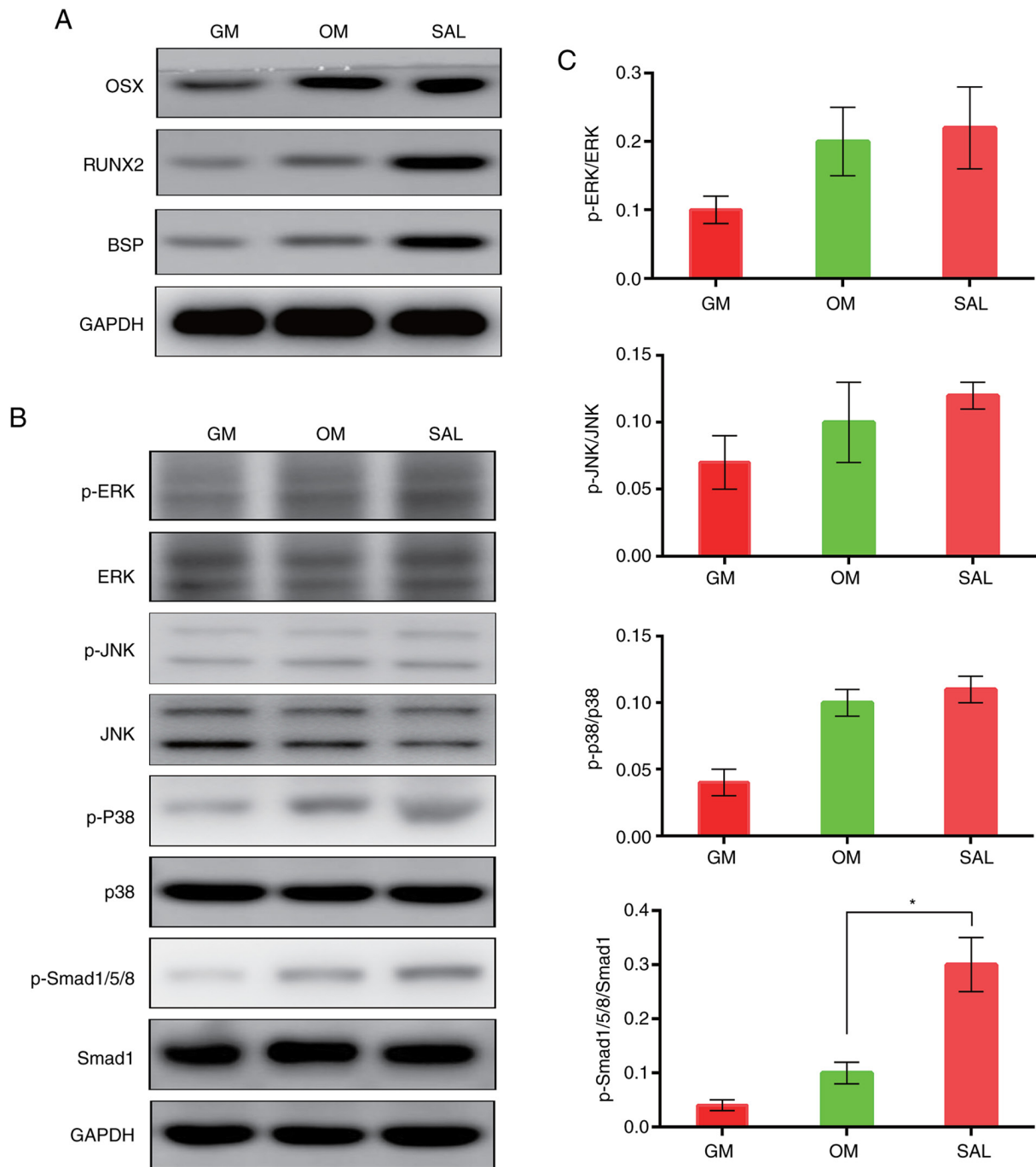


Figure 4. SAL promotes osteogenesis by activating the BMP signaling pathway. (A) The protein expression of OSX, RUNX2 and BSP in hDPSCs after treatment with 10  $\mu$ M SAL were detected by western blotting. (B) Western blotting was used to measure the phosphorylation of ERK, JNK, p38 MAPK and Smad1/5/8 in hDPSCs after treatment with 10  $\mu$ M SAL, (C) which was quantified against their corresponding total protein (\* $P=0.003$ ). GM, growth medium; OM, osteogenic medium; SAL, salidroside; RUNX2, runt-related transcription factor 2; BSP, bone sialoprotein; OSX, osterix; hDPSCs, human dental pulp stem cells.

to the osteogenic differentiation of hDPSCs. It was found that SAL exerted its effects on the hDPSCs mainly by activating Smad1/5/8, instead of by activating the MAPK signaling pathway. The BMP pathway is one of the main signaling cascades that stimulate the osteogenic and odontoblastic differentiation of hDPSCs (45). The phosphorylation of Smad1/5/8 is an important starting point for the activation of the BMP signaling pathway (46). Phosphorylation of Smad1/5/8 promotes the translocation of the downstream target Smad4 to the nucleus, where it activates intranuclear signaling transduction (47). SAL caused a significant increase in the

phosphorylation levels of Smad1/5/8, which largely indicated that it may regulate the differentiation of hDPSCs through the BMP signaling pathway. These findings may provide novel preliminary evidence of the involvement of BMP signaling in the SAL-induced osteo/odontogenic differentiation of hDPSCs. However, they also demonstrated that SAL did not affect the osteo/odontogenic differentiation of hDPSCs via the MAPK signaling pathway.

It should be noted here that other pathways may also be involved in the regulation of hDPSCs by SAL. In addition, the present study lacks *in vivo* experiments and the mechanism of

SAL action requires further investigation. Animal models will be constructed in subsequent studies to assess the *in vivo* effects of SAL and to provide potential clinical applications of this compound for the osteo/odontogenic differentiation of hDPSCs.

In conclusion, the present study demonstrated that SAL promoted the viability and osteo/odontogenic differentiation of hDPSCs, where it also increased the expression of osteo/odontogenic differentiation-associated genes. This process was mediated by activation of the BMP pathway.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XW, JL, HL and CN performed experiments. JL, CN and DC wrote and revised the manuscript. CN and DC conceived and designed the study. DC and CN confirm the authenticity of all raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by The Shanghai Stomatological Hospital Ethics Association [approval no. SSDC-(2020)0006] and all patients or their parents provided written informed consent.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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