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Psoralen accelerates osteogenic differentiation of human bone marrow mesenchymal stem cells by activating the TGF-β/Smad3 pathway

YONGQUAN HUANG[1,2], LIU LIAO[2], HAITAO SU[1], XINLIN CHEN[3], TAO JIANG[1], JUN LIU[1] and QIUKE HOU[4]

[1]Department of Orthopaedics, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine,
Guangzhou, Guangdong 510006; [2]Graduate School and [3]Department of Preventive Medicine and Health Statistics,
Guangzhou University of Chinese Medicine; [4]Department of Gastroenterology, The First Affiliated Hospital of
Guangzhou University of Chinese Medicine, Guangzhou, Guangdong 510405, P.R. China

Abstract. Psoralen, one of the active ingredients in *Psoralea corylifolia*, has been previously reported to regulate the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). A previous study revealed that psoralen can regulate the expression levels of microRNA-488 and runt-related transcription factor 2 (Runx2) to promote the osteogenic differentiation of BMSCs. However, the underlying signalling pathway in this process remains to be fully elucidated. BMSCs have also been confirmed to play a key role in the occurrence and development of osteoporosis, and are expected to be potential seed cells in the treatment of osteoporosis. In order to explore the potential signalling pathways of psoralen acting on BMSCs, in the present study, human BMSCs (hBMSCs) were treated with different concentrations of psoralen (0.1, 1, 10 and 100 μmol/l) and the TGF-β receptor I (RI) inhibitor SB431542 (5 μmol/l) *in vitro* for 3, 7 or 14 days. Cell Counting Kit-8 and MTT assays were used to measure cell proliferation and cell viability of hBMSCs following psoralen administration. Alkaline phosphatase (ALP) activity and alizarin red S staining were used to assess the osteogenic differentiation ability of hBMSCs. Reverse transcription-quantitative PCR and western blotting were used to measure the expression of osteogenic differentiation-related genes [bone morphogenetic protein 4 (BMP4), osteopontin (OPN), Runx2 and Osterix] and proteins associated with the TGF-β/Smad3 pathway [TGF-β1, TGF-β RI, phosphorylated (p-)Smad and Smad3]. Psoralen was found to increase the proliferation and viability of hBMSCs. Although different concentrations of psoralen enhanced ALP activity and the calcified nodule content in hBMSCs, the enhancement effects were more potent at lower concentrations (0.1, 1 and 10 μmol/l). The expression of BMP4, OPN, Osterix, Runx2, TGF-β1, TGF-β RI and p-Smad3 was also promoted by psoralen at lower concentrations (0.1, 1 and 10 μmol/l). In addition, whilst SB431542 could inhibit calcium deposition and osteogenic differentiation-related gene expression in hBMSCs, psoralen effectively reversed the inhibitory effects of SB431542. In conclusion, psoralen accelerates the osteogenic differentiation of hBMSCs by activating the TGF-β/Smad3 pathway, which may be valuable for the future clinical treatment of osteoporosis.

Introduction

Osteoporosis is a common systemic skeletal bone disease that is mainly caused by age-related bone loss (1). For the treatment of osteoporosis, inhibition of bone resorption is an important strategy to enhance bone mineral density to slow the deterioration of the condition (2). However, the toxicity and side effects of a number of agents remain to be problematic. Although bisphosphonates confer clear analgesic effects, improve bone density and decrease the risk of fracture, they are associated with gastrointestinal reactions (2). In addition, teriparatide can increase the activity of osteoblasts, but is expensive and therefore yields poor patient compliance (2). At present, it is difficult to achieve satisfactory clinical outcomes following the treatment of osteoporosis (3). A novel strategy of promoting the differentiation of osteoblasts or the regeneration of bone tissues to slow the development of osteoporosis has been previously proposed by using bone marrow mesenchymal stem cells (BMSCs) (4,5). Mesenchymal stem cells are pluripotent stem cells derived from the mesoderm and are widely distributed in tissues, including adipose tissues,

Correspondence to: Professor Qiuke Hou, Department of Gastroenterology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, 12 Jichang Road, Guangzhou, Guangdong 510405, P.R. China
E-mail: houqiuke@gzucm.edu.cn

Professor Jun Liu, Department of Orthopaedics, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, 55 Inner Ring West Road, Guangzhou, Guangdong 510006, P.R. China
E-mail: liujun_tcm@163.com

periosteum and bone marrow (6). It has been previously reported that BMSCs can continuously promote the formation of new fibroblasts and bone, thereby serving a potentially beneficial role in the reconstruction of bone (6). Therefore, BMSCs are currently the subject of extensive osteoporosis research due to their diverse source as they are easily extracted from bone marrow, lack of immune rejection (7) and have no ethical issues when compared with embryonic stem cells (8).

As understanding on the pathophysiology of osteoporosis deepens in the field of Traditional Chinese Medicine (TCM), psoralen has been reported to exert an effect on the osteogenic differentiation of BMSCs (9). Psoralen occurs naturally in the seeds of the *Psoralea corylifolia* herb, which has been extensively applied for the clinical treatment of fractures, bone defects and osteoporosis as a herbal TCM (10). Mechanistically, a previous study revealed that psoralen can promote the differentiation of BMSCs into osteoblasts (11). Li *et al* (12) treated hFOB1.19 cells with different concentrations (0, 5, 10, 15 and 20 μM) of psoralen and found that psoralen stimulated the proliferation of hFOB1.19 cells in a dose-dependent manner. The results demonstrated that the exact mechanism may be related to the activation of the NF-κB signalling pathway (12). In addition, Tang *et al* (13) stimulated primary mouse calvarial osteoblasts with different doses of psoralen and found that psoralen promoted their differentiation into osteoblasts in a dose-dependent manner (13). Psoralen upregulated the expression of *Bmp2* and *Bmp4* genes, and activated the BMP reporter gene (12xSBE-OC-Luc) (13). This indicates that the promotion of osteoblast differentiation by psoralen may be related to BMP signalling (13). Despite the findings of previous studies, the exact underlying molecular mechanism and signalling pathway mediating this process remain to be fully elucidated.

Therefore, the present study explores the effect of varying concentrations of psoralen on the osteogenic differentiation of human BMSCs (hBMSCs) and investigates the association between psoralen and the TGF-β/Smad3 pathway, with aims of providing a reference for the development of treatment strategies for osteoporosis.

Materials and methods

Cell culture. hBMSCs (cat. no. BNCC338194) were obtained from BeNa Culture Collection. hBMSCs were cultured in DMEM (Thermo Fisher Scientific, Inc.) with 100 mg/ml streptomycin and 100 U/ml penicillin (Wuhan Boster Biological Technology, Ltd.) and 10% FBS (Sigma-Aldrich; Merck KGaA). The cells were grown in an incubator at 37˚C and 5% CO_2. The medium was replaced every 3 days. The cells were digested using 0.05% EDTA and 0.25% trypsin (Sigma Aldrich; Merck KGaA) and passaged when they filled ~80% of the culture flask. hBMSCs at passage three were used in the present study.

Treatment of cells. The hBMSCs were seeded into a 96-well plate at a density of $1x10^4$ cells/well and cultured in complete medium. After 24 h the complete medium was replaced with serum-free medium and psoralen (purity >99%; cat. no. 110739-201416; China Food and Drug Administration) was added and dissolved in DMSO. The concentrations used were 0.1, 1, 10 and 100 μmol/l, with each concentration added to 6 replicate wells per experimental repeat. The 0 μmol/l psoralen condition was used as the negative control (NC) group. When SB431542 was required, 5 μmol of SB431542 and different concentrations of psoralen (0.1, 1, 10 and 100 μmol/l) were administered at the same time and co-cultured with hBMSCs at 37˚C for 3, 7 or 14 days.

Cell Counting Kit-8 (CCK-8) assay of cell proliferation. Third-passage hBMSCs were collected and seeded into 96-well plates at a density of $1x10^4$ cells/well and co-cultured with different concentrations (0.1, 1, 10 and 100 μmol/l) of psoralen. Following cell culture for 12, 24, 36, 48 and 72 h at 37˚C, 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added into each well. Cells were then incubated in the dark at 37˚C for 2 h. Absorbance values were detected at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). hBMSCs cultured with 0 μmol/l psoralen were used as a NC group. A total of 6 replicate wells were established for each group before the average values were calculated.

Cell viability analysis. Third-passage hBMSCs were seeded into 96-well plates at $1x10^4$ cells/well. Following cell adherence to the culture flask, the original medium (DMEM containing 10% FBS) was discarded and replaced with serum-free medium. Following 24 h of incubation at 37˚C, hBMSCs were co-cultured with psoralen (0.1, 1, 10 and 100 μmol/l) for 24, 48 and 72 h at 37˚C. A total of 20 μl 5 g/l MTT was added to each well and cells were incubated at 37˚C for 4 h. Subsequently, the medium was discarded, 150 μl of DMSO was added before shaking for 10 min and absorbance was measured using a microplate reader at 570 nm. A total of 6 replicate wells were established for each group and the average values were calculated.

Alkaline phosphatase (ALP) activity analysis. Third-passage hBMSCs were seeded into 48-well plates. Following cell adherence to the culture flask, the DMEM was discarded and hBMSCs were incubated with different concentrations (0.1, 1, 10 and 100 μmol/l) of psoralen and osteogenic differentiation medium (cat. no. RASMX-90021; Cyagen Biosciences, Inc.) for 7 days at 37˚C. An osteogenic differentiation inducer without any treatment was used as a NC group. The medium was changed every 2 days. Subsequently, the cells were rinsed three times with PBS and treated with 150 μl 0.05% Triton X-100 for 10 min at room temperature. An ALP kit (cat. no. AP0100; Sigma-Aldrich; Merck KGaA) was used to detect the ALP activity of hBMSCs according to the manufacturer's instructions. The wavelength was set to 520 nm and a total of 6 replicate wells were established in each group. The average value was calculated.

Calcium deposition detection. The Alizarin Red staining method (AR-S) for calcium nodules was used to evaluate the extent of bone formation in hBMSCs. Third-passage hBMSCs were inoculated into a 6-well plate with different concentrations of psoralen (0.1, 1, 10 and 100 μmol/l) and osteogenic differentiation medium. Six replicate wells were established for each concentration. An osteogenic differentiation inducer

without any treatment was used as a NC group. The medium was replaced every 2-3 days for 2 weeks at 37˚C. The cells were fixed with 4% formaldehyde for 15 min at room temperature, adjusted to pH 4.2 and stained with 0.1% Alizarin Red (Sigma-Aldrich; Merck KGaA). Cells were incubated overnight at room temperature and subsequently washed three times with PBS. Stained cells were photographed using a digital camera (Nikon Corporation) under a light microscope (magnification, x100). In order to detect calcium deposits, cells were then treated with 10% cetylpyridinium chloride (Sigma-Aldrich; Merck KGaA) in 10 mM sodium phosphate for 15 min at room temperature. A multifunctional microplate reader (Varioskan LUX; Thermo Fisher Scientific, Inc.) was used to measure the absorbance value at 560 nm in each well. The experiment was repeated three times and the average value was taken.

Reverse transcription quantitative (RT-q)PCR. Third-passage hBMSCs were inoculated into a 6-well plate and co-cultured with osteogenic differentiation medium and different concentrations of psoralen (0.1, 1, 10 and 100 μmol/l) or 5 μmol SB431542 (cat. no. SF7890; Beyotime Institute of Biotechnology) for 72 h at 37˚C. Following 72 h of co-cultivation of hBMSCs with different concentrations of psoralen or SB431542, RT-qPCR was used to detect the expression of osteogenic differentiation-related genes of hBMSCs. Total RNA from the hBMSCs was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). NanoDrop2000 was used to measure RNA concentration and purity. PrimeScript™ RT Master Mix (cat. no. RR036A; Takara Bio, Inc.) was used for reverse transcription according to the manufacturer's instructions. The following protocol was used: 37˚C for 15 min (reverse transcription reaction) and 85˚C for 5 sec (reverse transcriptase inactivation reaction). The cDNA obtained by reverse transcription was amplified on StepOne Plus (Thermo Fisher Scientific, Inc.) using the SYBR™ Green PCR Premix kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The thermocycling conditions used were as follows: 50˚C for 2 min and 95˚C for 10 min; followed by 95˚C for 15 sec and 60˚C for 60 sec for 40 cycles; and final extension at 72˚C for 1 min. Relative mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (14). The ΔCqs values were obtained from Cq normalized to that of GAPDH. The primers were synthesized by Sangon Biotech Co., Ltd. and are displayed in Table I. The experiment was repeated three times and the average value was calculated.

Western blot (WB) analysis. After 7 days of treatment with different concentrations of psoralen or 5 μmol of SB431542, hBMSCs were lysed with RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors, and centrifuged at 14,000 x g at 4˚C for 20 min. The supernatant was the total protein and was used to determine the protein concentration using the BCA method. Subsequently, 40 μg of the extracted protein was added per lane and separated by SDS-PAGE on a 10% gel. Proteins were then transferred onto PVDF membranes and blocked with 5% non-fat milk in PBS with 0.1% Tween-20 at 4˚C overnight. The membranes were then probed using the following primary antibodies: Osteopontin (OPN; cat. no. bs-0019P; 1:1,000; BIOSS), bone morphogenic protein 4

Table I. Sequences of each primer used in the present study.

Gene name	Primer pairs
Runx2	F: 5'-TCTTAGAACAAATTCTGCCCTTT-3' R: 5'-TGCTTTGGTCTTGAAATCACA-3'
Osterix	F: 5'-AGAGATCTGAGCTGGGTAGAGG-3' R: 5'-AAGAGAGCCTGGCAAGAGG-3'
BMP4	F: 5'-CTCCAAGAATGGAGGCTGTAGGAA-3' R: 5'-CCTATGAGATGGAGCAGGCAAGA-3'
OPN	F: 5'-ATCTCCTAGCCCCACAGAAT-3' R: 5'-CATCAGACTGGTGAGAATCATC-3'
TGF-β1	F: 5'-CTGCTGACCCCCACTGATAC-3' R: 5'-AGCCCTGTATTCCGTCTCCT-3'
TGF-β RI	F: 5'-AAGATGACCGCT CTGACATCA-3' R: 5'-CTTATAGACCTCAGCAAAGCGAC-3'
GAPDH	F: 5'-ATTTGGTCGTATTGGGCG-3' R: 5'-TGGAAGATGGTGATGGGATT-3'

Runx2, runt-related transcription factor 2; BMP4, bone morphogenetic protein 4; OPN, osteopontin; RI, Receptor I; F, forward; R, reverse.

(BMP4; cat. no. GP20102; 1:1,000; GlpBio Technology), runt-related transcription factor 2 (Runx2; cat. no. ab114133; 1:2,000; Abcam), Osterix (cat. no. ab209484; 1:2,000; Abcam), TGF-β1 (cat. no. E1A0340C; 1:1,000; EnoGene Biotech Co., Ltd.), TGF-β Receptor I (RI; cat. no. E1A1126B-2; 1:1,000; EnoGene Biotech Co., Ltd.), Smad3 (cat. no. E1A0031B; 1:1,000; EnoGene Biotech Co., Ltd.), phosphorylated (p-) Smad3 (cat. no. E8ET1609-41; 1:1,000; EnoGene Biotech Co., Ltd.) and internal reference GAPDH (cat. no. ab8245; 1:20,000; Abcam) at room temperature for 2 h. Subsequently, the blot was incubated with the diluted secondary antibody (1:5,000; cat. no. ab205718; Abcam) and labelled with HRP at room temperature for 1 h, followed by a wash step with 0.1% TBST. Protein expression was detected using a SuperSignal™ West Femto Maximum Sensitivity Substrate kit (Roche Applied Science). The relative value of the target protein was calculated by comparing with the corresponding internal reference. Densitometry was conducted using the ImageJ software (v1.8.0; National Institutes of Health).

Statistical analysis. SPSS 21.0 software (IBM Corp.) was used for statistical analysis. Metrological data was first evaluated for normal distribution and data conforming to a normal distribution were expressed as mean ± standard deviation. Mixed design ANOVA with Bonferroni post hoc test was used to compare differences between groups with multiple factors. One-way ANOVA with Tukey's post hoc test was used to compare differences among groups with a single factor. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of psoralen on cell proliferation and viability. Results from the CCK-8 assay demonstrated that elevated cell proliferation was exhibited by hBMSCs treated with psoralen
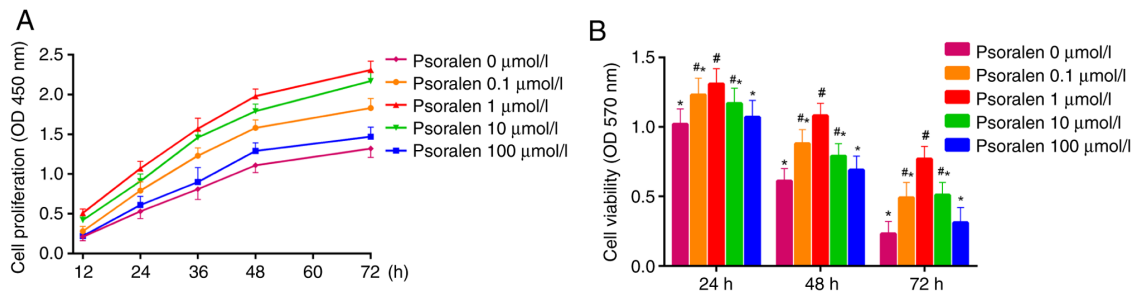
Figure 1. Effect of psoralen on cell activity. (A) Cell Counting Kit-8 assay demonstrated elevated cell viability in hBMSCs treated with psoralen at concentrations of 0.1, 1 and 10 μmol/l. (B) MTT assay revealed that with no psoralen treatment (0 μmol/l group), the cell viability of hBMSCs gradually decreased after 24, 48 and 72 h. In contrast, low concentrations of psoralen (0.1, 1 and 10 μmol/l) maintained the viability of hBMSCs following treatment for 24, 48 and 72 h. #$P<0.05$ vs. 0 μmol/l group. *$P<0.05$ vs. 1 μmol/l group. hBMSC, human bone marrow mesenchymal stem cells; OD, optical density.
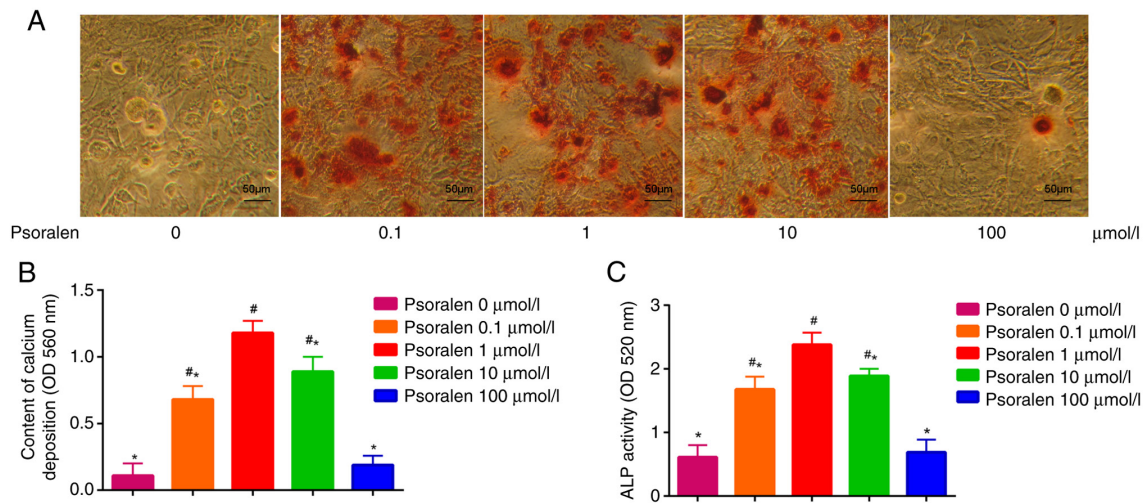

Figure 2. Effect of psoralen on the osteogenic differentiation of hBMSCs. (A) AR-S revealed that low concentrations (0.1, 1 and 10 μmol/l) of psoralen can markedly promote the formation of calcium nodules in hBMSCs (magnification, x100). (B) Comparison of calcium deposition in different groups. (C) ALP activity was significantly enhanced by psoralen at low concentrations (0.1, 1 and 10 μmol/l). #$P<0.05$ vs. 0 μmol/l group. *$P<0.05$ vs. 1 μmol/l group. hBMSC, human bone marrow mesenchymal stem cells; ALP, alkaline phosphatase; AR-S, alizarin red S; OD, optical density.

at concentrations of 0.1, 1 and 10 μmol/l, with 1 μmol/l demonstrating the highest elevation in proliferative activity compared with that in the NC group. There was no significant difference in cell proliferation between concentrations of 0 and 100 μmol/l, although cell proliferation was higher at 100 μmol/l (Fig. 1A). MTT assay revealed that at 24, 48 and 72 h after treatment with psoralen, cell viability of hBMSCs were increased at concentrations of 0.1, 1 and 10 μmol/l compared with that in the NC group (Fig. 1B). The optimal concentration was found to be 1 μmol/l. Similarly, there was no significant difference in cell viability between 0 and 100 μmol/l. (Fig. 1B).

Effect of psoralen on the osteogenic differentiation of hBMSCs. The effects of different concentrations of psoralen on the osteogenic differentiation of hBMSCs were evaluated by measuring ALP activity and AR-S staining of calcium deposition. The results demonstrated that low concentrations of psoralen (0.1, 1 and 10 μmol/l) significantly promoted the formation of calcified nodules and enhanced ALP activity in hBMSCs compared with those in the NC cell group. However, there was no significant difference in these two parameters tested between the high concentration (100 μmol/l) and NC groups (0 μmol/l). The most potent effect on the osteogenic

differentiation of hBMSCs was found at 1 μmol/l psoralen (Fig. 2).

Effects of psoralen on the expression of genes associated with osteogenic differentiation and the TGF-β/Smad3 pathway of hBMSCs. The results of qPCR and western blot analysis revealed that low concentrations of psoralen promoted the expression of genes associated with osteogenic differentiation. Among these, at concentrations of 0.1, 1 and 10 μmol/l, psoralen significantly promoted the expression of Osterix and BMP4 compared with those in the 0 μmol/l NC group, whilst at concentrations of 1, 10 and 100 μmol/l, psoralen significantly promoted the expression of OPN compared with those in the 0 μmol/l NC group. In terms of Runx2, only 0.1 and 1 μmol/l of psoralen significantly promoted expression of Runx2 compared with that in the 0 μmol/l NC group (Fig. 3A and B). In all experimental conditions, 1 μmol/l was the optimal concentration. In addition, low concentrations of psoralen (1 and 10 μmol/l) promoted the expression of TGF-β1 and TGF-β RI (Fig. 3C and D), whilst all concentrations of psoralen (0.1, 1, 10 and 100 μmol/l) promoted the levels of p-Smad3 when compared with those in the 0 μmol/l NC group (Fig. 3C). In addition, the p-Smad3/Smad3 ratio was
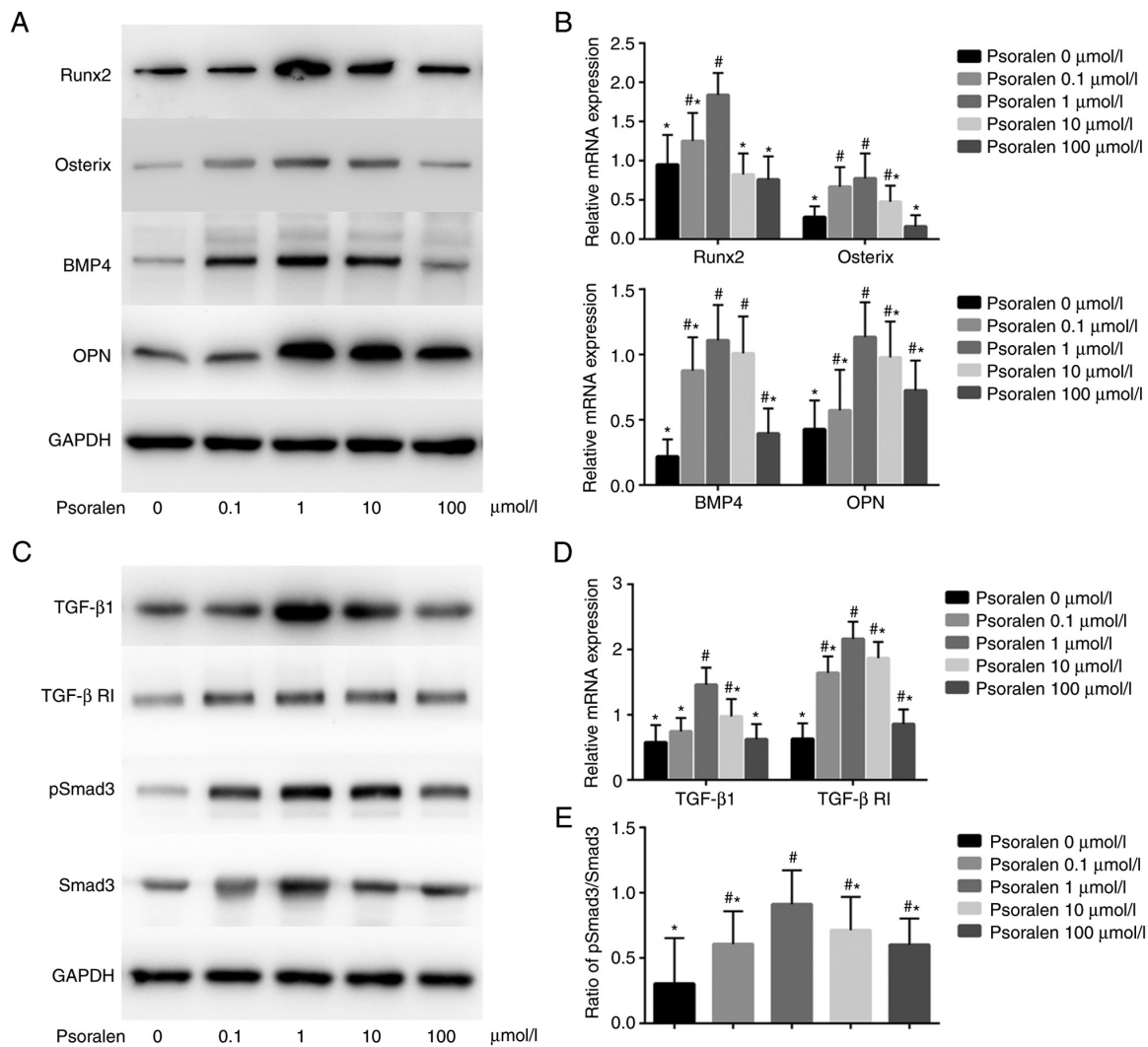
Figure 3. Effect of psoralen on the expression of genes associated with osteogenic differentiation and the TGF-β/Smad3 pathway of hBMSCs. Osteogenic differentiation-related gene and protein expression analysis using (A) western blotting and (B) RT-qPCR. (C) TGF-β/Smad3 pathway activity and expression analysis using western blotting. (D) The expression of TGF-β1 and TGF-β RI using RT-qPCR. (E) Comparison of the p-Smad3/Smad3 ratio. $^{\#}P<0.05$ vs. 0 μmol/l group. $^{*}P<0.05$ vs. 1 μmol/l group. hBMSC, human bone marrow mesenchymal stem cells; RT-qPCR, reverse transcription-quantitative PCR; RI, Receptor I; p-, phosphorylated; Runx2, runt-related transcription factor 2; BMP4, bone morphogenetic protein 4; OPN, osteopontin.

significantly increased after treatment with psoralen compared with that in the 0 μmol/l group, with 1 μmol/l resulting in the largest increase (Fig. 3E).

Effect of the TGF-β RI inhibitor SB431542 on osteogenic differentiation of hBMSCs. As indicated by the aforementioned results, 1 μmol/l psoralen demonstrated the optimal effect on the osteogenic differentiation of hBMSCs and the components of the TGF-β/Smad3 pathway. Therefore, 1 μmol/l psoralen was selected for subsequent experiments. AR-S results revealed that psoralen promoted calcium deposition in hBMSCs compared with all the other groups. By contrast, SB431542 significantly inhibited the calcium deposition of hBMSCs compared with the control group, whilst psoralen markedly reversed this inhibitory effect (Fig. 4A and B). There was no statistically significant difference between the control group and the psoralen + SB431542 group.

Effect of SB431542 on the expression of osteogenic differentiation-related genes. The results of the western blot analysis

and RT-qPCR revealed that SB431542 significantly inhibited the expression of osteogenic differentiation-related genes compared with the control group, whilst psoralen effectively reversed the inhibitory effect of SB431542 on the osteogenic differentiation of hBMSCs (Fig. 5A and B). There was no statistically significant difference between those in the NC and the psoralen + SB431542 group. This suggest that the mechanism underlying psoralen treatment in the promotion of osteogenic differentiation in hBMSCs is closely associated with the TGF-β/Smad3 pathway.

Discussion

Previous studies have revealed that psoralen has a relaxant effect on smooth muscle, in addition to possessing the ability to stimulate bone formation and induce osteoblast differentiation without affecting cell proliferation (12,13,15). BMSCs are abundant in bone tissues and have the potential for multidirectional differentiation (4,5,16). In certain conditions, BMSCs can differentiate into osteoblasts with the increased
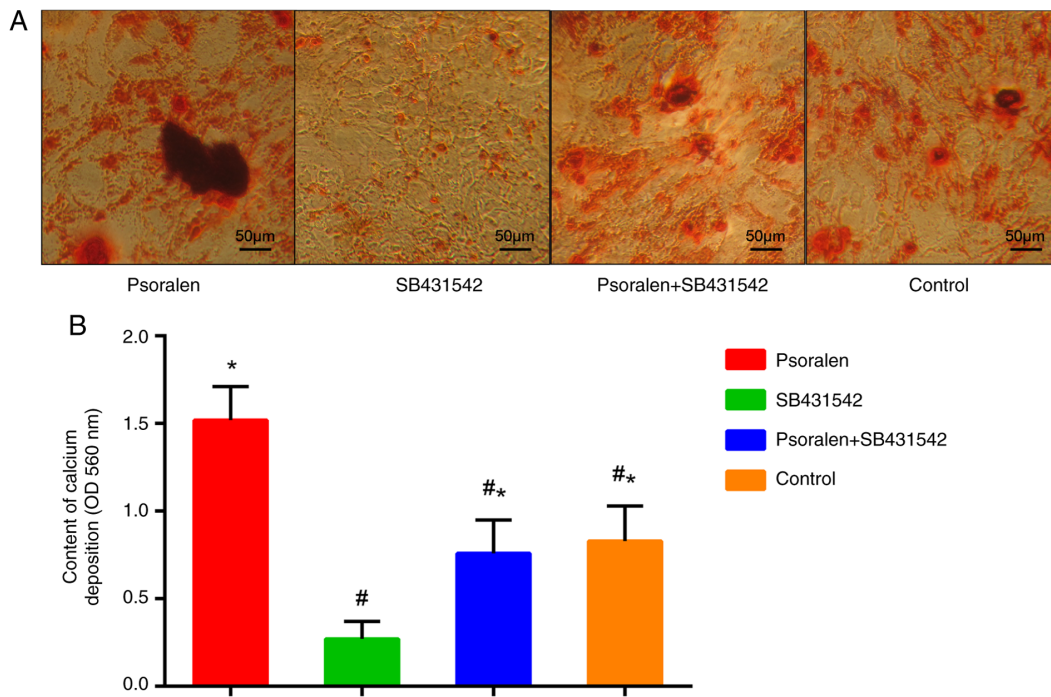
Figure 4. Effect of SB431542 on the osteogenic differentiation of human bone marrow mesenchymal stem cells. (A) Alizarin red S staining revealed formation of calcified nodules in the different groups (magnification, x100). (B) Comparison of calcium deposition among the different groups. #P<0.05 vs. psoralen group. *P<0.05 vs. SB431542 group. OD, optical density; NS, not significant.
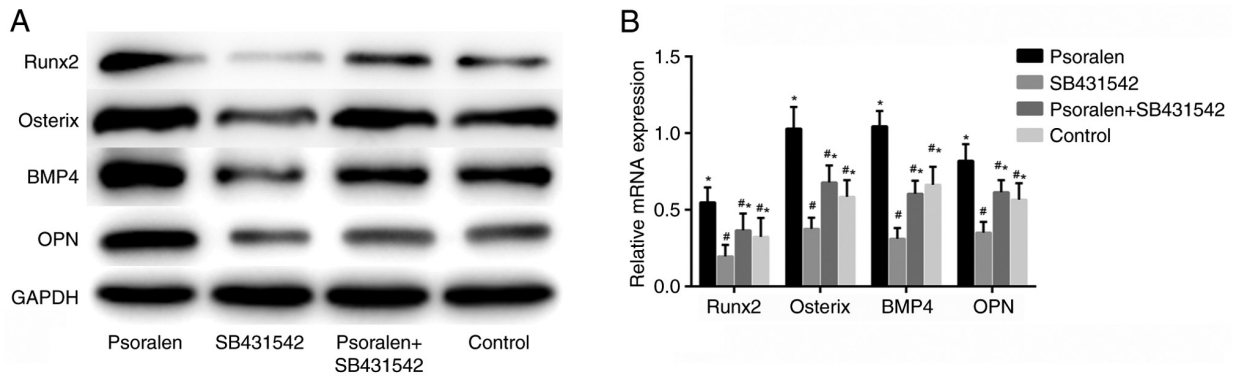

Figure 5. Effect of SB431542 on the expression of osteogenic differentiation-related genes in human bone marrow mesenchymal stem cells. (A) The expression of BMP4, OPN, Runx2 and Osterix in hBMSCs was measured using western blot analysis. (B) The results of reverse transcription-quantitative PCR were consistent with the western blot analysis. #P<0.05 vs. psoralen group. *P<0.05 vs. SB431542 group. BMP4, bone morphogenetic protein 4; OPN, osteopontin; Runx2, runt-related transcription factor 2; NS, not significant.

expression of osteogenic genes, including Runx2, Osterix and ALP (17,18). In addition, alleviation of osteoporosis and the regeneration of osteoblasts have been previously shown to be closely associated with the number of BMSCs in the bone marrow and their osteogenic differentiation ability (4-6,19).

In a previous study, psoralen was revealed to promote the osteogenic differentiation of BMSCs via the miR-488/Runx2 pathway (11), however, the underlying molecular mechanism and signal pathway remained to be fully verified. In the present study, CCK-8 and MTT assays were used to measure cell viability and cell proliferation. These results demonstrated that psoralen promotes the proliferation of hBMSCs and maintains cell viability in a concentration-dependent manner. However, at the highest concentration tested (100 μmol/l), the result was not statistically significant when compared with 0.1 μmol/l,

with the overall optimal concentration demonstrated to be 1 μmol/l.

ALP is an important marker of the osteogenic activity of hBMSCs and is a non-specific phosphomonoesterase that is used to determine the early osteogenic differentiation ability of hBMSCs (20). In addition, ALP serves an important role in hBMSC calcium deposition (21). AR-S is a method that is commonly used for examining hBMSC calcium deposition and for determining differentiation into advanced osteoblasts (22). Results from the present study revealed that low concentrations of psoralen, particularly at 1 μmol/l, possessed the ability to potently promote ALP activity and calcified nodule formation in hBMSCs, suggesting that psoralen effectively promotes the osteogenic differentiation of hBMSCs. Huang *et al* (23) demonstrated that combination therapy with BMP-2 and
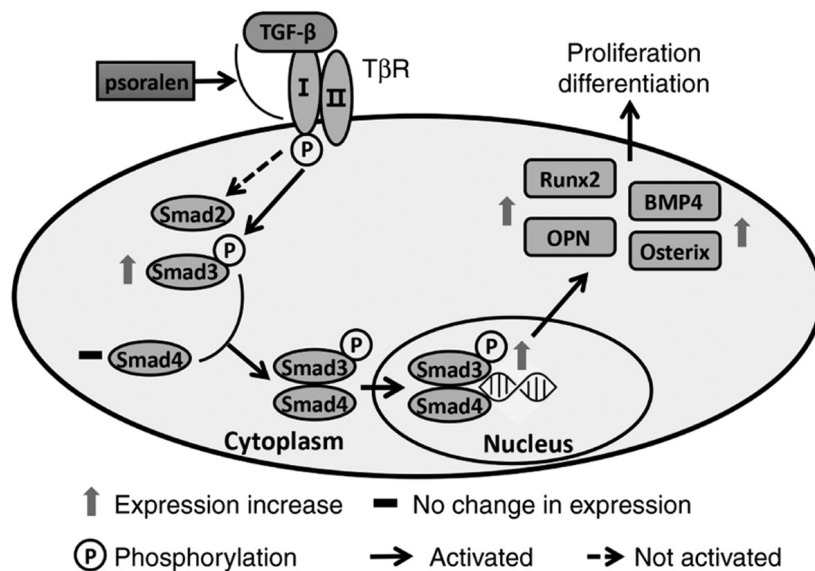
Figure 6. Proposed model of psoralen-induced osteogenic proliferation and differentiation through the TGF-β/Smad3 pathway in human bone marrow mesenchymal stem cells. BMP4, bone morphogenetic protein 4; OPN, osteopontin; Runx2, runt-related transcription factor 2; TβR, TGF-β receptor; p, phosphorylated.

psoralen enhanced fracture healing in ovariectomized mice through increased bone-specific ALP levels and decreased C-terminal telopeptide of type-1 collagen (23). Since hBMSCs are a type of adult stem cell with the potential for self-renewal and multi-directional differentiation (24,25), it is hypothesised that specific concentrations of psoralen can result in the following: i) Effective promotion of the differentiation of hBMSCs into osteoblasts; ii) an increase the activity of ALP; and iii) promotion of the formation of calcium mineralization nodules.

To explore the underlying mechanism of psoralen in promoting osteogenic differentiation of hBMSCs further, RT-qPCR and western blot analysis were performed to probe the expression of genes associated with osteogenic differentiation, namely BMP4, OPN, Runx2 and Osterix. BMP4 belongs to the TGF-β family that has been reported to induce cartilage and bone formation and regulates mesoderm induction, tooth development, limb formation and bone fracture repair (26). OPN is also known as secreted phosphoprotein 1, bone sialoprotein 1 and nephropontin (27). OPN binds tightly to hydroxyapatite and appears to form an integral part of the mineralized matrix, which is important for cell-matrix interactions (27). Runx2 is a specific transcription factor that is expressed in osteoblasts and is closely associated with the early proliferation of osteoblasts (28). Osterix is another key transcription factor, which is located downstream of Runx2 (29). Nakashima et al (29) demonstrated that the expression of osterix could not be detected in mice carrying a mutated version of Runx2; while the expression of Runx2 could be detected in osterix knockout mice (29). Subsequently, Nishio et al (30) further demonstrated that osterix was located downstream of the Runx2 gene in mesenchymal cells (30). It was revealed that overexpression of Runx2 significantly increased the promoter activity of osterix. This up-regulation was abrogated when the Runx2 responsive element on the osterix promoter was mutated. It is only expressed in the bone tissue and plays a key role in the late differentiation and maturation of osteoblasts (31). In the present study, low-dose psoralen (1 μmol/l) effectively increased the expression of hBMSC osteogenic markers BMP4, OPN, Runx2 and Osterix. Therefore, specific concentrations of psoralen can activate the differentiation of hBMSCs into osteoblasts.

Signal transduction by the TGF-β superfamily of proteins serves an important role in the regulation of cell proliferation, differentiation and development in many biological systems (32). Signal transduction starts with the ligand-induced oligomerization of serine/threonine receptor kinases and the phosphorylation of cytoplasmic signal transduction molecules Smad2 and Smad3 (33). The carboxyl terminus of Smad proteins is phosphorylated by an activated receptor, causing it to bind to the common signal transduction factor Smad4 and transporting it into the nucleus (34). Activated Smad proteins regulate various biological processes by binding to transcription factors, such as BMP, leading to the transcriptional regulation of cell states (35). Once bound to the corresponding receptors, transforming growth factors, activin, TGF-β and BMP will cause Smad to be phosphorylated, thereby activating the TGF-β/Smad signalling pathway (36). In the present study, psoralen (1 μmol/l) significantly promoted the activation of the TGF-β/Smad3 signalling pathway and mediated the strongest effect on the promotion of osteoblast differentiation. It was therefore hypothesised that psoralen promotes the binding of TGF-β to the TGF-β RI, which subsequently stimulates RI-mediated Smad3 signaling. Thus, Smad3 is activated in the nucleus which mediates the transcription of specific genes and induces the differentiation of hBMSCs into osteoblasts (Fig. 6). This then strengthens the activity of osteoblasts and promotes the mineralization of the extracellular matrix.

To test this hypothesis, 5 mol/l TGF-β RI inhibitor SB431542 (37) was applied to the hBMSCs. The results of the present study demonstrated that SB431542 effectively inhibited the formation of calcified nodules in hBMSCs and

the expression of genes associated with osteogenic differentiation. Furthermore, psoralen reversed the inhibitory effect of SB431542 on the osteogenic differentiation of hBMSCs. This further suggests that psoralen can promote the differentiation of hBMSCs into osteoblasts by activating the TGF-β/Smad3 pathway. Combining this with findings from previous studies (11), this indicate that psoralen regulates the expression of Runx2 through the TGF-β/Smad3 pathway, thereby regulating the osteogenic differentiation of BMSCs.

In conclusion, the present study suggest that psoralen promotes hBMSC cell proliferation, maintain cell viability and increase the expression of genes associated with osteogenic differentiation in hBMSCs, which may be closely associated with the activation of the TGF-β/Smad3 pathway. However, the present study has some limitations, such as the blocking of TGF-β RI alone and not the Smad pathway, which may lead to insufficient results. A combination of blocking both TGF-β RI and Smad would more accurately prove that psoralen promotes the differentiation of hBMSCs into osteoblasts through the TGF-β/Smad3 pathway. In addition, it remains unclear how psoralen activates the binding of TGF-β to the receptor, therefore the precise molecular mechanism underlying this phenomenon remains to be fully elucidated and is therefore a matter for future research.

Acknowledgements

Not applicable.

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

QH, YH and JL conceived and designed the experiments; YH, LL and TJ performed the experiments; HS and XC contributed to the statistical analysis of the data. YH and QH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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