

Shikonin stimulates MC3T3-E1 cell proliferation and differentiation via the BMP-2/Smad5 signal transduction pathway

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Received August 12, 2015; Accepted May 10, 2016

DOI: 10.3892/mmr.2016.5363

Abstract. Shikonin, the predominant naphthoquinone pigment isolated from the Chinese plant *Lithospermum erythrorhizon*, is anti-inflammatory, antiviral and exerts anticancer effects, amongst other biological activities. However, it is unknown whether shikonin affects bone formation. In the present study, the role of shikonin on cell proliferation was assessed via MTT assay, and shikonin was identified to markedly promote cell growth in a time- and dose-dependent manner in the MC3T3-E1 cell line. In addition, flow cytometric analysis was performed to evaluate the effect of shikonin on the cell cycle, and it was observed that shikonin markedly increased the percentage of S-phase MC3T3-E1 cells to accelerate the G₁/S transition. To investigate the potential molecular mechanism by which shikonin enhances bone formation, the changes in bone morphogenetic protein-2 (BMP-2), SMAD family member 5 (Smad5), runt related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin (OC) expression levels induced by shikonin were investigated using western blot analysis and quantitative polymerase chain reaction. The results indicated that shikonin increased the BMP-2 and Smad5 mRNA levels, and upregulated Smad5 and Runx2 protein expression levels to promote osteoblast differentiation. Furthermore, ALP staining was performed, and revealed that shikonin enhanced ALP activity. These results indicate that shikonin promotes cell proliferation and differentiation of MC3T3-E1 cells via the BMP-2/Smad5 signaling pathway.

Introduction

Osteoporosis is a serious public health issue that is characterized by a reduction of bone mass, which is caused by an imbalance between bone resorption and bone formation (1). Osteoporosis affects numerous individuals throughout the world (2,3); however, currently there are no effective and economical therapeutic strategies to cure osteoporosis (4). Therefore, establishing a novel therapeutic agent for the prevention and treatment of osteoporosis is considered to be critical. Traditional Chinese medicine has become the focus of basic research and clinical studies due to reduced side-effects when compared with cytokine and hormone therapy (5).

Shikonin (5,8-dihydroxy-2-[(1S)-1-hydroxy-4-methyl pent-3-en-1-yl] naphthalene-1,4-dione), is a predominant type of naphthoquinone pigment that is extracted from the Chinese plant, *Lithospermum erythrorhizon* (6), with a molecular weight of 288 (Fig. 1). Shikonin performs many biological activities; it is an antioxidant, anti-inflammatory, antithrombotic, antiviral, and antimicrobial, it has anticancer properties and is associated with accelerated wound healing (7,8). Previous studies have demonstrated that shikonin inhibits cell growth, mediates cell apoptosis and alters the cell cycle in various types of tumor cell (9,10). However, whether shikonin exerts an effect on bone formation remains unknown. Therefore, the aim of the present study was to investigate the possible influence and associated mechanisms of shikonin on MC3T3-E1 cell proliferation and differentiation.

Many studies have demonstrated that bone morphogenetic proteins (BMPs) and transforming growth factor- β (TGF- β) are the most important cytokines affecting the proliferation, differentiation and function of osteoblasts (11,12). BMP-2, a member of the TGF- β superfamily, is a key signaling component in osteoblast proliferation and differentiation (13,14). SMAD family member 5 (Smad5) is a downstream transcription factor that is phosphorylated and activated by the receptors of BMP-2. Phosphorylated Smad5 forms a complex with Smad4 (co-Smad), and translocates into the nucleus to activate the transcription factor, runt related transcription factor 2 (Runx2) (15,16). The BMP-2/Smad5 signal transduction pathway is important in osteoblast proliferation and differentiation. In the present study, the function of shikonin on

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Key words: shikonin, osteoporosis, bone formation, MC3T3-E1 cells

biological behaviors of MC3T3-E1 cells, such as cell growth, cell division and ALP activity were assessed. In addition, the potent mechanism of shikonin-enhanced bone formation was investigated by examining the expression levels of BMP-2, Smad5, Runx2, alkaline phosphatase (ALP) and osteocalcin (OC) in the MC3T3-E1 cell line.

Materials and methods

Materials and reagents. Purified shikonin (>98%) was purchased from the National Institute for the Control Pharmaceutical and Biological Products (Beijing, China). Shikonin was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C. The final concentrations of shikonin were 0 (control), 10, 50 and 100 ng/ml, and the final concentration of DMSO in the culture was <0.01%. α -Minimum Essential Medium (α -MEM), fetal bovine serum (FBS) and trypsin-EDTA were obtained from GE Healthcare Life Sciences (Hyclone; Logan, UT, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Rabbit anti-Smad5 (cat. no. ab13724), mouse anti-Runx2 (cat. no. ab76956) and mouse anti-GAPDH (cat. no. ab8245) monoclonal antibodies were purchased from Abcam (Cambridge, MA, USA). Invitrogen TRIzol reagent was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Primers were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Cell culture. The MC3T3-E1 cells were purchased from the Cell Center of the Chinese Academy of Medical Sciences (Shanghai, China) and cultured in α -MEM containing 10% FBS and 100 U/ml penicillin and 100 μ g/ml streptomycin in 5% CO₂ at 37°C. The medium was replaced every 3 days, and the cells were subcultured using 0.25% trypsin with 0.01% EDTA.

Cell proliferation assay. The effect of shikonin on cell proliferation was evaluated using the MTT assay. The cells were seeded in 96-well plates at a density of 1.0x10³ cells/well. Following incubation for 24 h at 37°C, the cells were treated with various final concentrations (0, 10, 50 and 100 ng/ml) of shikonin. Cells were treated with 20 μ l MTT (5 mg/ml) during the final 4 h of the culture and the optical density of the wells was measured at 490 nm using a microplate reader.

Cell cycle assay. MC3T3-E1 cells (1x10⁵ cells/ml) were plated in four tissue culture flasks. After 24 h, cells were treated with various concentrations of shikonin (0, 10, 50 and 100 ng/ml) for 48 h. Then, cells were harvested, fixed in 70% ethanol for 12 h, washed with phosphate-buffered saline (PBS) and stained in 5 mg/ml propidium iodide in PBS supplemented with RNase A (Roche Diagnostics, Indianapolis, IN, USA) for 30 min at room temperature. Data were analyzed using CellQuest v3.3 (BD Biosciences, San Jose, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). After 48 h of shikonin treatment, total RNA was extracted with TRIzol reagent. Then total RNA was used to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) with 5 μ g oligo (dT) primers per sample. qPCR was performed using SYBR

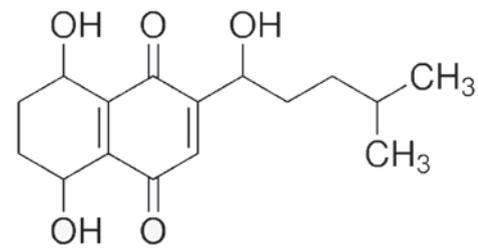


Figure 1. Chemical structure of shikonin.

Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a total volume of 20 μ l using a 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: 95°C for 30 sec, and 40 cycles of 95°C for 5 sec and 60°C for 30 sec. A dissociation step was performed to generate a melting curve to confirm the specificity of the amplification and GAPDH served as the reference gene. The relative levels of gene expression were represented as $\Delta\Delta Cq = Cq_{\text{gene}} - Cq_{\text{reference}}$, and the fold change of gene expression was calculated according to the $2^{-\Delta\Delta Cq}$ method (17). Experiments were repeated in triplicate. The primer sequences were as follows: Forward, 5'-GCTGGTCACAGATAAGGC CA-3' and reverse, 5'-TTTCTCGTTTGTGGAGCGGA-3' for BMP-2; forward, 5'-GTGAAGCGATTGTTGGGCTG-3' and reverse 5'-CAGGTGGCATATAGGCAGGG-3' for Smad5; forward, 5'-GCGCATTCCTCATCCAGTA-3' and reverse, 5'-AGTTCTGAAGCACCTGCCTG-3' for Runx2; forward, 5'-TGACCTTCTCCTCCATCC-3' and reverse, 5'-CTT CCTGGGAGTCTCCTCCT-3' for ALP; forward, 5'-TGC TTGTGACGAGCTATCAG-3' and reverse, 5'-GAGGACAGG GAGGATCAAGT-3' for OC; and forward, 5'-GTGAAGCAG GCATCTGAGGG-3' and reverse, 5'-GCCGTATTCATTGTC ATACCAGG-3' for GAPDH.

Western blot analysis. Total proteins from cell lines were harvested in lysis buffer (Thermo Fisher Scientific, Inc.) and quantified according to the Bradford method. Fifty micrograms of protein were separated by SDS-PAGE (12%) at a constant voltage (110V) for 2 h, and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked in 5% nonfat dry milk diluted with Tris-buffered saline Tween-20 [TBST; 20 mmol/l Tris-HCl, 150 mmol/l NaCl (PH 7.5) and 0.1% Tween 20] at room temperature for 1 h. Samples were incubated overnight at 4°C with monoclonal antibodies against Smad5 (1:1,000), Runx2 (1:1,000) and GAPDH (1:1,000) followed by incubation for 2 h with a goat-anti rabbit peroxidase-conjugated IgG (cat. no. ab6721; Abcam; 1:1,000) and anti-mouse horseradish peroxidase-conjugated IgG (cat. no. ab131368; Abcam; 1:1,000). The bound proteins were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and detected using a BioImaging System (UVP Inc., Upland, CA, USA). The relative protein levels were calculated based on GAPDH as the loading control.

ALP staining. To observe the influence of shikonin on osteoblast differentiation, staining of ALP (an early maker of osteoblast differentiation) was performed. Cells (2x10⁵ cells/well) were plated and cultured in 6-well plates for

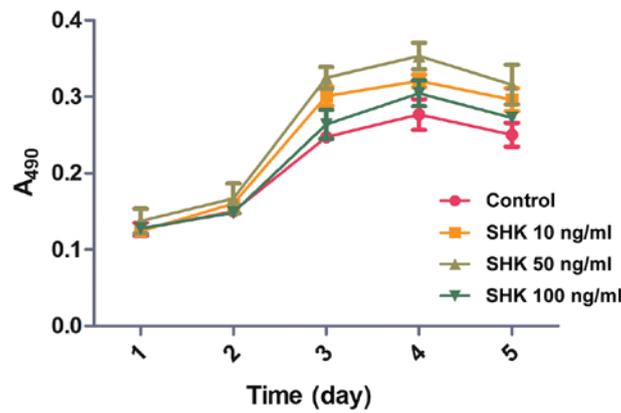


Figure 2. Influence of shikonin on MC3T3-E1 cell growth curves. Cells were exposed to 0 (control), 10, 50, 100 ng/ml SHK. The MTT assay indicates that shikonin may stimulate cell proliferation, particularly in the 50 ng/ml group on day 4. SHK, shikonin.

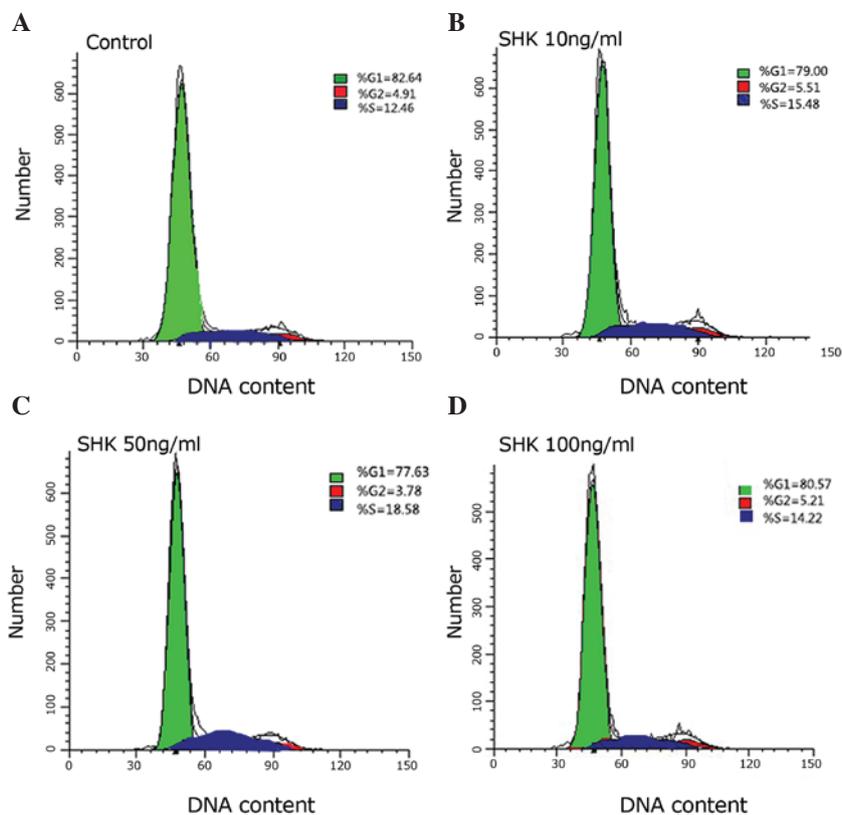


Figure 3. Effect of (A) 0, (B) 10, (C) 50 and (D) 100 ng/ml SHK on the cell cycle in MC3T3-E1 cells. Flow cytometry demonstrated that shikonin promotes cell cycle progression. SHK, shikonin; concent, concentration.

24 h at 37°C, and treated with 0 (control), 10, 50, 100 ng/ml shikonin. The medium was replaced every 3 days. A week later, cells were washed three times with PBS and fixed in 10% paraformaldehyde for 10 min at 25°C. The cells were stained using 300 µg/ml 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium buffer (Thermo Fisher Scientific, Inc.) for 20 min at 25°C. ALP-positive cells were stained blue/purple. The stained cells were visualized using a digital microscope (DP73; Olympus, Tokyo, Japan).

Statistical analysis. All statistical analysis were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla,

CA, USA). Data were presented as the mean ± standard error of the mean, and statistically analyzed using a two-tailed Student's *t* test and one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Shikonin stimulates cell proliferation. The effects of different concentrations of shikonin on the proliferation of MC3T3-E1, following 24, 48, 72, 96 and 120 h treatments, were examined by MTT (Fig. 2) to examine whether shikonin stimulates MC3T3-E1 cell proliferation *in vitro*. During the initial

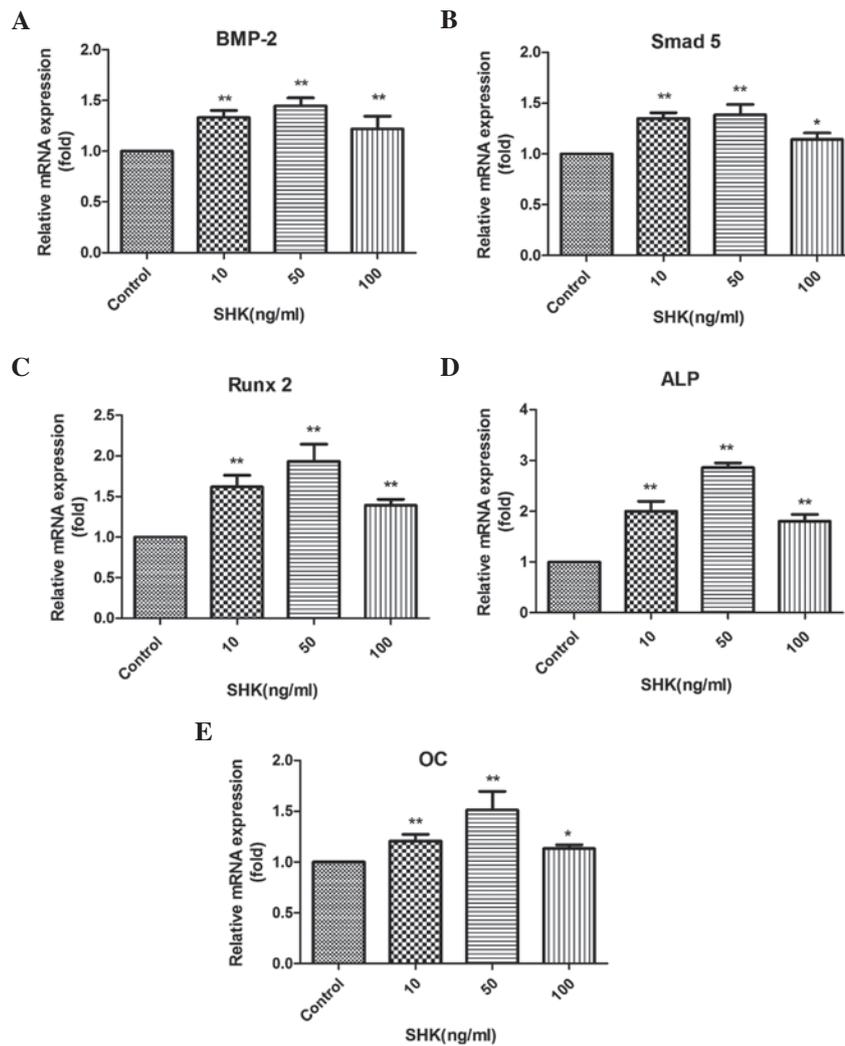


Figure 4. Effect of SHK on (A) BMP-2, (B) Smad5, (C) Runx2, (D) ALP and (E) OC mRNA expression levels in the MC3T3-E1 cells. Each experiment was performed in triplicate and data are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. control. SHK, shikonin; BMP-2, bone morphogenic protein-2; Smad5, SMAD family member 5; Runx2, runt related transcription factor 2; ALP, alkaline phosphatase; OC, osteocalcin.

2 days, no statistically significant differences in MC3T3-E1 cell viability were observed between the groups. However, compared with the control and the 100 ng/ml shikonin group, a marginally greater quantity of cells were observed in the 10 and 50 ng/ml shikonin groups on day 3 and 4. On day 4, the speed of cell proliferation peaked in the 50 ng/ml shikonin group and declined thereafter. These results demonstrated that shikonin treatment promotes MC3T3-E1 cell proliferation.

Shikonin stimulates cell division. Subsequently, cell cycle analysis was performed to assess the effect of shikonin on MC3T3-E1 cell cycle progression. As shown in Fig. 3, MC3T3-E1 cells treated with 10, 50 and 100 ng/ml shikonin exhibited increased percentages of S-phase cells, particularly in the 50 ng/ml shikonin groups. These data indicate that certain concentrations of shikonin accelerate cell cycle progression.

Effects of shikonin on BMP-2, SMAD5, Runx2, ALP and OC mRNA expression levels. Total mRNA was extracted after MC3T3-E1 cells were treated with 0, 10, 50 or 100 ng/ml shikonin for 48 h, and the mRNA expression levels of BMP-2,

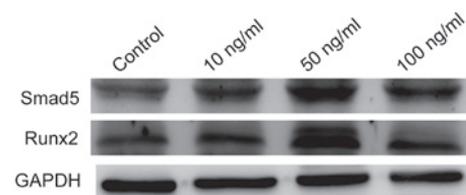


Figure 5. Effect of shikonin on Smad5 and Runx2 protein expression in MC3T3-E1 cells. The cells were treated with or without shikonin for 48 h. Western blot analysis demonstrated that different concentrations of shikonin (10, 50 and 100 ng/ml) markedly increased Smad5 and Runx2 expression compared with the control, particularly in the 50 ng/ml group. Cells treated with 0 ng/ml shikonin served as the controls. Smad5, SMAD family member 5; Runx2, runt related transcription factor 2.

Smad5, Runx2, ALP and OC were detected by RT-qPCR. The BMP-2, Smad5, Runx2, ALP and OC expression level in the cells treated with 10, 50 and 100 ng/ml shikonin increased significantly compared with the untreated control cells ($P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively) (Fig. 4). In addition, the expression levels of BMP-2, Smad5, Runx2, ALP and OC were increased to the highest levels in

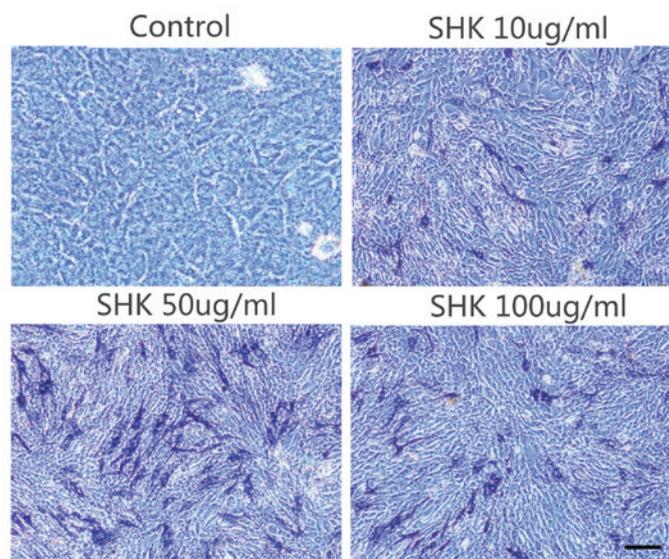


Figure 6. Effect of SHK on ALP activity in MC3T3-E1 cells. The MC3T3-E1 cells were treated with or without SHK for 7 days. The ALP activity was examined by ALP staining. ALP-positive cells were stained blue/purple. Treatment with 10, 50, 100 ng/ml SHK enhanced the ALP activity, markedly more so than that of the control, particularly in the 50 ng/ml group. Cells treated with 0 ng/ml SHK served as controls. Scale bar, 200 μ m. SHK, shikonin; ALP, alkaline phosphatase.

the 50 ng/ml shikonin group. This demonstrated that shikonin promotes osteoblast differentiation via its effect on BMP-2, SMAD5, Runx2, ALP and OC expression levels.

Effects of shikonin on Smad5 and Runx2 protein expression levels. To further investigate the mechanism by which shikonin stimulates osteoblast differentiation, western blotting was performed to examine the shikonin-induced changes in Smad5 and Runx2 protein expression (Fig. 5). Different concentrations of shikonin (10, 50 and 100 ng/ml) markedly increased Smad5 and Runx2 protein expression levels in the MC3T3-E1 cells compared with the control cells, particularly in the 50 ng/ml group. These findings revealed that shikonin regulates the expression levels of Smad5 and Runx2 proteins, which influences osteoblastic differentiation.

Effects of shikonin on ALP activity. The ALP activity in the MC3T3-E1 cells was examined by ALP histochemical staining 7 days after treatments with 0, 10, 50 or 100 ng/ml shikonin. The results demonstrate that treatment with different concentrations of shikonin elicits significantly greater ALP activity when compared with the control group (Fig. 6), particularly in the 50 ng/ml group. These results indicate that shikonin enhanced the activity of ALP in MC3T3-E1 cells.

Discussion

In the present study, the osteoprotective effects of shikonin and its potential mechanism in MC3T3-E1 cells were examined. The results clearly demonstrated that treatment with 10, 50 and 100 ng/ml of shikonin, particularly 50 μ g/ml shikonin, enhances cell viability, stimulates cell cycle progression, resulting in a greater number of cells in the S-phase, and promotes ALP activity in MC3T3-E1 cells. Additionally, shikonin upregulated the expression levels of BMP-2, Smad5, Runx2, ALP and OC, indicating that the BMP-2/Smad5 signal

transduction pathway may be involved in shikonin-induced cell proliferation and differentiation.

Osteoporosis, a progressive disorder of aging bones, is widely recognized as a major public health issue (18). Bone is a dynamic tissue, which is mediated by the balance between osteoblastic bone formation and osteoclastic bone resorption (19). Osteoblasts, osteoclasts, and osteocytes are important in bone generation, maintenance and remodeling (20). Multiple factors, which cause the imbalance of osteoblasts and osteoclasts at the bone remodeling process, result in the loss of bone mass (21). Hence, therapeutic agents that increase the activity of osteoblasts may be administered to treat osteoporosis.

Due to fewer associated side-effects, Chinese herbs require investigation to identify more effective therapeutic agents that promote osteoblast proliferation and differentiation. Shikonin has attracted increasing attention, as it exhibits numerous biological activities, such as anti-inflammatory, antiviral and anti-cancer actions (22-24). Hence, the effects of shikonin on MC3T3-E1 cells were evaluated in the current study.

The results of the study confirm that shikonin promotes the proliferation of MC3T3-E1 cells in a dose- and time-dependent manner. The rate of cell proliferation peaked in response to 50 ng/ml shikonin on day 4 and decreased thereafter. Furthermore, the percentage of S-phase cells in the 50 ng/ml shikonin group was the greatest, which suggests active DNA synthesis and cell proliferation. Therefore, shikonin may lead to osteogenesis by stimulating osteoblast proliferation.

Numerous studies have demonstrated that TGF- β and BMPs are the most important cytokines affecting the proliferation, differentiation and function of osteoblasts (11,12). BMP-2 is a member of the TGF- β superfamily. Various studies have demonstrated that BMP-2 is a key signaling component in the regulation of bone induction, repair and maintenance (25-27). Smad5 is the intracellular mediator of BMP-2 and may be phosphorylated by heterotetrameric serine/threonine kinase receptors of BMP-2 (28). After

forming a complex with Smad4, phosphorylated Smad5 entered into the nucleus, activating the transcription factors of Runx2 (15,16). Our results indicated that the levels BMP-2, Smad5, Runx2, ALP and OC expression increased in shikonin-treated MC3T3-E1 cells, particularly in the 50 ng/ml group.

ALP is an early marker of osteoblast differentiation, thus, the effects of shikonin on ALP activity were detected by ALP staining. The results demonstrated that treatment with shikonin enhanced ALP activity, particularly in the 50 ng/ml group, suggesting that shikonin promotes osteoblast differentiation.

Shikonin, an active ingredient isolated from the Chinese plant, *Lithospermum erythrorhizon*, is widely administered as a traditional Chinese medicine to treat certain diseases, such as wet typhus, purpura, eczema and erysipelas. The present study demonstrates that shikonin stimulates MC3T3-E1 cell proliferation and differentiation via the BMP-2/Smad5 signaling pathway.

In conclusion, in addition to the anti-inflammatory, anti-viral and anti-cancer effects of shikonin, the present study is the first, to the best of our knowledge, to demonstrate that shikonin stimulates osteoblast proliferation and differentiation. Therefore, shikonin may present as a novel and potent candidate for the management of osteoporosis. However, further investigations are required to reveal the mechanism by which shikonin acts to promote bone formation.

Acknowledgements

This study was supported by grants from the National Nature Science Foundation of China (grant nos. 81370981 and 31201053) and the Outstanding Scientific Fund of Shengjing Hospital.

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