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Simvastatin induces osteogenic differentiation of MSCs via Wnt/β-catenin pathway to promote fracture healing

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Abstract. – OBJECTIVE: This study was designed to investigate whether Simvastatin could facilitate osteogenic differentiation of rat marrow mesenchymal stem cells (MSCs) by modulating the Wnt/β-catenin pathway, thus promoting fracture healing.

MATERIALS AND METHODS: MSCs were isolated from rat bone marrow specimens and their purity was identified. The third generation of MSCs was cultured in osteoinduction medium containing simvastatin of gradient concentration, and the highest dose of simvastatin that did not cause cell proliferation was determined by the result of the CCK8 assay. The effects of simvastatin on osteogenic differentiation of MSCs were evaluated by ALP activity, Alizarin red staining, alkaline phosphatase staining and osteoblast-specific gene expression. Finally, Wnt pathway antagonist DKK1 and β-catenin disturbing agent were added to MSCs to detect the ALP activity, Alizarin red staining, alkaline phosphatase staining and osteoblast-specific genes of MSCs respectively, and to evaluate whether simvastatin promoted osteogenic differentiation of MSCs by activating Wnt/β-catenin pathway.

RESULTS: After osteoinduction, simvastatin of 0.3 nmol/L was found to be the highest dose that did not induce the proliferation of MSCs. After treated with 0.3 nmol/L simvastatin for 7 days, the ALP activity of cells and the number of cell calcified nodules significantly increased. Meanwhile, the expression of osteoblast-related genes, including ALP, Runx2, OCN, and OPN, were clearly up-regulated. However, when the MSCs were treated with DKK1 for 7 days, the ALP activity and the expression of osteoblast-related genes, including ALP, Runx2, OCN, and OPN, were found decreased. Simvastatin markedly up-regulated the expression of the β-catenin protein, while transfection of β-catenin shRNA inhibited the expression of osteoblast-related genes including ALP, Runx2, OCN, and OPN.

CONCLUSIONS: Simvastatin can promote the differentiation of rat MSCs into osteoblast-like cells, and its mechanism may be related to the Wnt/β-catenin pathway.

Key Words:
Simvastatin, Mesenchymal stem cells, Osteogenic differentiation, Wnt/β-catenin Pathway.

Introduction

Osteoblasts are differentiated from bone marrow mesenchymal stem cells, and their functions are mainly involved in bone formation and repair of bone injury. Mature osteoblasts can synthesize and secrete extracellular matrix proteins, including various collagenous and non-collagenous proteins. Meanwhile, they are able to form osteoid and initiate the process of matrix mineralization[1]. Wnt/β-catenin signaling pathways are expressed in mesenchymal stem cells and osteoblasts in many mammals[2]. There is increasing evidence that Wnt/β-catenin signaling pathway is involved in directional differentiation of MSCs, proliferation of osteoblast precursors and terminal differentiation, mineralization and apoptosis of osteoblasts, which is important for bone development and metabolic balance[3].

As 3-hydroxy-3-methylglutaric acid (HMG-CoA) reductase inhibitors, statins are able to inhibit cholesterol biosynthesis and reduce serum cholesterol concentrations and have been used in the treatment of cardiovascular diseases. In 1999, for the first time, Mundy et al[4] found that statins can improve mRNA level of bone morphogenetic protein-2 (BMP2) *in vitro* culture. Moreover, BMP2 is currently recognized as an osteoblast-promoting transforming factor[5].

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A growing number of studies have shown that statins have the potential to promote osteogenic differentiation. Some *in vitro* researches[6,7] have confirmed that simvastatin plays a key role in the proliferation and osteogenic differentiation of mesenchymal stem cells. Bone marrow mesenchymal stem cells (BMSCs) are easy to obtain and proliferate *in vitro*. Therefore, BNSCs have become the most widely used cells in the field of tissue engineering research[8-10].

At present, there are few studies on simvastatin involvement in Wnt/β-catenin signaling pathway to promote osteogenic differentiation of mesenchymal stem cells. In this work, the MSCs cultured *in vitro* were used as research objects to investigate whether simvastatin can promote the differentiation of MSCs into osteoblasts *via* Wnt/β-catenin signaling pathway, which may provide a theoretical basis for the pharmacological study of Simvastatin.

Materials and Methods

Isolation and Culture of MSCs

In this experiment, 28 to 35-day-old rats weighed 80 to 100 grams were sacrificed by dislocations of cervical vertebrae and then immersed in 75% ethanol for 10 minutes. The bilateral femurs of rats were taken and placed in 75% ethanol for minutes. Then, bilateral femurs were placed in a sterile culture dish with buffer solution to remove the attached muscle from the bone surface. After aseptic washing, an injector with 10 mL Dulbecco's modified Eagle medium (DMEM) medium was inserted into bone marrow cavity and marrow was flushed out into a centrifuge tube. The marrow was repeatedly pipetted and centrifuged to aspirate and discard the supernatant, and added into DMEM containing 10% fetal bovine serum (FBS), followed by incubation at 37°C, 5% CO_2 saturated humidity incubator. After 24-hour cultivation, the medium was renewed for the first time and then changed every 48 hours. The cells were passaged at a ratio of 1:2 when they were grown to 80-90% confluence. Light microscope and inverted microscope were used to observe the cell growth, proliferation and changes of cell morphology. This study was approved by the Animal Ethics Committee of Shandong University Animal Center.

Medium components of MSCs were as follows. 50 mL fetal bovine serum and 5 μl penicillin-streptomycin were added to 450 mL DMEM medium in a super clean bench to prepare a complete BMSCs culture medium with 10% FBS and 1% penicillin-streptomycin.

Osteoinduction medium components of MSCs were complete culture medium of MSCs with 10 mmol/L β-glycerophosphate and 50 μg/mL ascorbic acid.

Flow Cytometry Identification of MSCs

When the MSCs of passage three reached 80% confluence, a total of 5×10^6 cells were trypsinized, and then centrifuged to aspirate and discard the supernatant. The cells were resuspended at a density of 3000-6000 cells/μL and labeled with CD29, CD90, CD34 and CD45 antibodies respectively. Subsequently, they were incubated for 25 min in the dark and fixed overnight in paraformaldehyde at 4°C. Expression of surface markers CD29, CD90, CD34, and CD45 were detected next day.

Construction of Lentivirus Vector and Cell Transfection

Lentivirus shRNA vector pLKO.1 and control shRNA targeting GFP were designed and synthesized by a reagent manufacturer. Well-growing MSCs at passage 3 were taken for lentivirus transfection. 2 days after transfection, expression of β-catenin was detected by Western blot to screen for shRNA vectors with high inhibition rate for subsequent experiments. The sequences of β-catenin shRNA and control shRNA were 5'-GGACAAGCCACAGGATTACAA-3' and 5-TTCTCCGAACGTGTCACGT-3' respectively.

Detection of MSCs Proliferation

MSCs treated with different concentration of simvastatin were cultured in 96-well plates for 24 hours. Then, the cells in each well with the addition of 10 μL of cell counting kit-8 (CCK8) were incubated in the dark at 37°C for 3 hours and detected optical density value (OD value) by microplate reader at 450 nm. There were 5 parallel holes in each group.

Isolation of RNA and qRT-PCR Detection

Total RNA from different groups of cells cultured for 7 days was isolated using TRIzol. cDNA was obtained by reverse transcription and amplified for fluorescence-based quantitative polymerase chain reaction (PCR) detection. The following osteoblast-related genes were examined: ALP, Bglap, OSX, and Runx2. Primer sequences were as follows.

ALP (F: 5'-AAGGCTTCTTCTTGCTGGTG-3', R: 5'-GCCTTACCCTCATGATGTCC-3'), Bglap (F: 5'-AGCAAAGGTGCAGCCTTTGT-3', R: 5'-GCGCCTGGTCTCTTCACT-3'), OSX (F: 5'-CCCTTCTCAAGCACCAATGG-3', R: 5'-AAGGGTGGGTAGTCATTTGCATA-3'), Runx2 (F: 5'-ACTTCCTGTGCTCCGTGCTG-3', R: 5'-TCGTTGAACCTGGCTACTTGG-3'), GAPDH (F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3').

Testing of ALP Activity

MSCs of different groups cultured for 7 days were collected and added with 400 mL 10 mmol/L Tris-HCL (PH7.5) containing 0.1% TritonX-100 in each well. They were broke up by freeze-thaw method and centrifuged at 4°C, 10000 r/min for 10 minutes to aspirate the supernatant. Then, ALP activity of the cells was tested using ALP assay kit.

Detection of Protein Expression by Western Blot

Each group of cells was lysed in lysis buffer and total protein was extracted. After determining the total protein concentration, samples containing 50 μg of total protein were loaded and separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane, which was blocked in blocking buffer at room temperature for 2 hours and incubated overnight at 4°C with specific primary antibodies. In the next day, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 2 hours. And total proteins were finally visualized by chemiluminescence.

Detection of Osteogenic Ability of MSCs by Alkaline Phosphatase Staining

The MSCs were subjected to the ALP staining on the 7th day after grouping. All staining steps were performed according to the manufacturer's recommendations. After adding incubation medium on cover slips of 6 well plates, the cells were incubated at 37°C for 15 minutes and then washed for 2 minutes. After that, the cells were counterstained by Hematoxylin, placed under running water for 2 minutes, air dried, and then observed under light microscope.

Detection of Mineralization of MSCs by Alizarin Red Staining

After the medium was discarded, MSCs cultured in medium with or without 0.3 nmol/L simvastatin to differentiate for 7 days were washed twice with phosphate buffered saline (PBS). 4% paraformaldehyde was added to fix cells for 15 min, and then the cells were washed with PBS again. Next, 1% alizarin red solution was added and cells were placed at room temperature for 5 min, then washed with PBS. Finally, mineralized nodules were observed under an inverted microscope.

Statistical Analysis

The experimental data were statistically analyzed by statistical product and service solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) and shown in $x \pm s$. The t-test was used for comparison of two groups, when $p < 0.05$, the difference was statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Results

Cultivation and Flow Cytometry Identification of MSCs

MSCs grew into a long fusiform shape with strong refraction on the 4th day when observed under an inverted microscope. After passage, the MSCs grew rapidly in the osteogenic induction medium (when the MSCs stopped growing, they differentiated and cell morphology changed) and reached about 80% confluence approximately 1 week later (with visible macroscopically calcified nodules) (Figure 1A). The result of flow cytometry showed that surface markers of MSCs at passage 3 were CD29-positive (95.5%), CD90-positive (97.2%), CD34-negative (4.3%) and CD45-negative (2.8%), which were consistent with the immunophenotypic characterization of bone marrow mesenchymal stem cells, rather than hematopoietic stem cells (Figure 1B).

Effect of Different Concentration of Simvastatin on the Activity and Osteogenesis Related Genes of MSCs

The result of the CCK-8 test showed that after addition of simvastatin with different concentrations, 0.3 nmol/L was found to be the highest dose that did not cause cell proliferation. When the concentration of simvastatin was further increased, the proliferation ability of MSCs showed

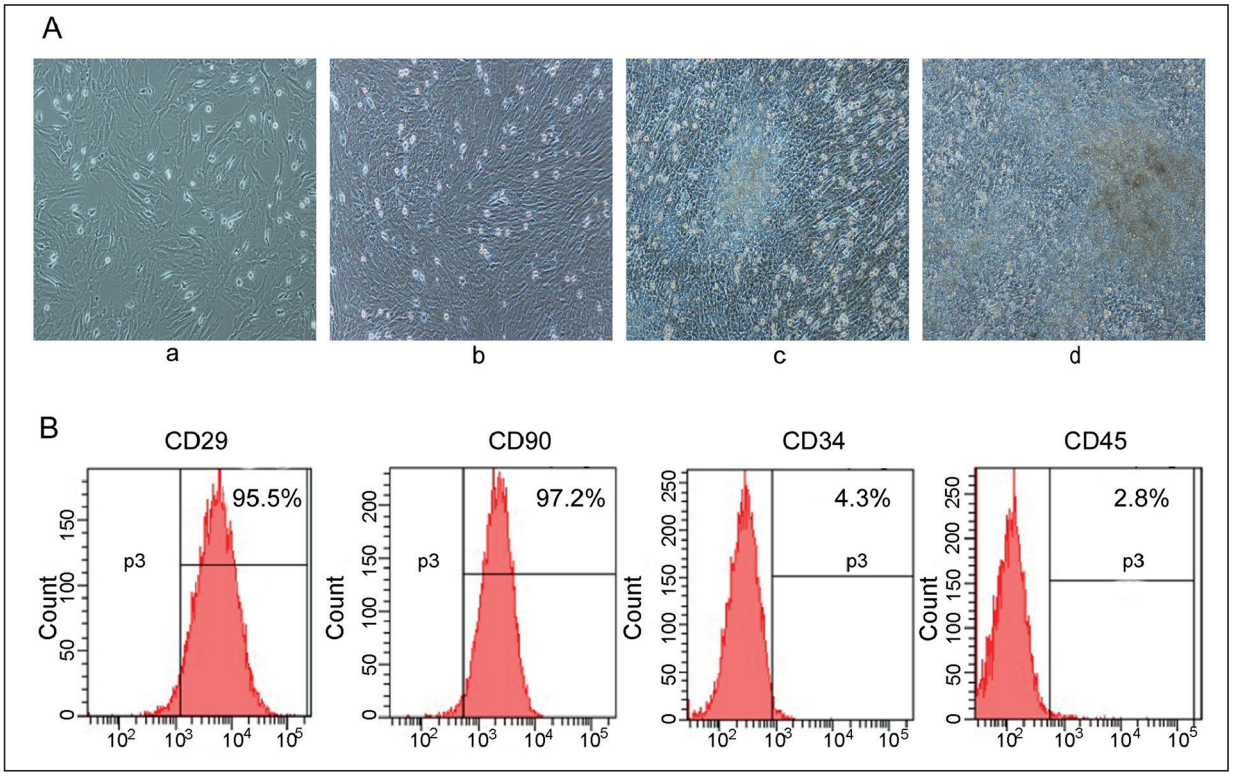

Figure 1. Phenotype identification of marrow mesenchymal stem cells. ***A,*** (a) Under normal circumstances, MSCs grew into a long fusiform shape on the 4th day; (b) The cell morphology of MSCs cultured in osteoinduction medium for 1 day; (c) The cell morphology of MSCs cultured in osteoinduction medium for 7 day; (d) The cell morphology of MSCs cultured in osteoinduction medium for 14 day. ***B,*** Specific surface antigen of MSCs were identified by flow cytometry, including CD29-positive (95.5%), CD90-positive (97.2%), CD34-negative (4.3%) and CD45-negative (2.8%).

an upward trend. The proliferation activities of MSCs in 1 nmol/L and 3 nmol/L simvastatin significantly increased ($p < 0.05$) (Figure 2A). Then, MSCs were cultured in a medium containing 0.3 nmol/L simvastatin for 7 days. As a result, no significant difference was found in the proliferation of MSCs compared with the control group. Therefore, the optimal concentration of simvastatin was 0.3 nmol/L. After MSCs were cultured for 7 days with different treatments, the results of qRT-PCR showed that the expression of ALP, Bglap, OSX and Runx2 in induction group with 0.3 nmol/L simvastatin was considerably higher than that in control group (Figure 2C, D, E, F).

Simvastatin Can Promote the Osteogenic Differentiation of MSCs

The ALP activity of the two groups of cells was detected on the 7th day after administration of simvastatin. The results showed that the ALP activity of MSCs in the group containing simvastatin was higher than that in the control group ($p < 0.05$) (Figure 3A). The results of Western blot showed that the expression of osteoporosis-related proteins (ALP, Runx2, OCN, OPN) in the simvastatin group was noticeably higher than that in control group, which was consistent with the results of qRT-PCR (Figure 3B). The results of ALP staining showed that compared with the control group, a significant red-brown color and more coarse granules in endochylema of MSCs were found in the simvastatin group, suggesting that alkaline phosphatase increased significantly (Figure 3C). Calcium nodules are important signs of osteoblast maturation. MSCs in the simvastatin group were added with simvastatin at a concentration of 0.3 nmol/L, while MSCs in the control group were treated without simvastatin. After osteogenic induction for 7 days, alizarin red staining kit was used to stain calcium nodules. The results showed that the quantity and volume of mineralization nodules in the control group were relatively small, and the number and volume of calcium nodules in the simvastatin group were significantly larger than those in the control group (Figure 3D). The above results showed that simvastatin alone could induce differentiation of MSCS into osteoblast.

DKK1 Can Inhibit the Effect of Simvastatin on Osteogenic Differentiation

It is well known that DKK1 is only expressed in osteoblast and osteocyte and a specific blocker of Wnt/β-catenin signaling pathways[11]. After addition of 0.5 μg/mL DKK1 one hour prior to 0.3 nmol/L simvastatin treatment, MSCs were induced to differentiate for 7 days, then the changes of ALP activity in cells were detected. The results showed that simvastatin could significantly improve the ALP activity of cells, which was consistent with the previous results. DKK1 alone did not affect ALP activity, while DKK1 reduced ALP activity in the simvastatin group (Figure 4A). Subsequently, Western blot results demonstrated that the expression of osteoblast-related proteins (ALP, Runx2, OCN, and OPN) could be reduced in the simvastatin group DDK1 (Figure 4B, C, D, E). Therefore, the effect of simvastatin

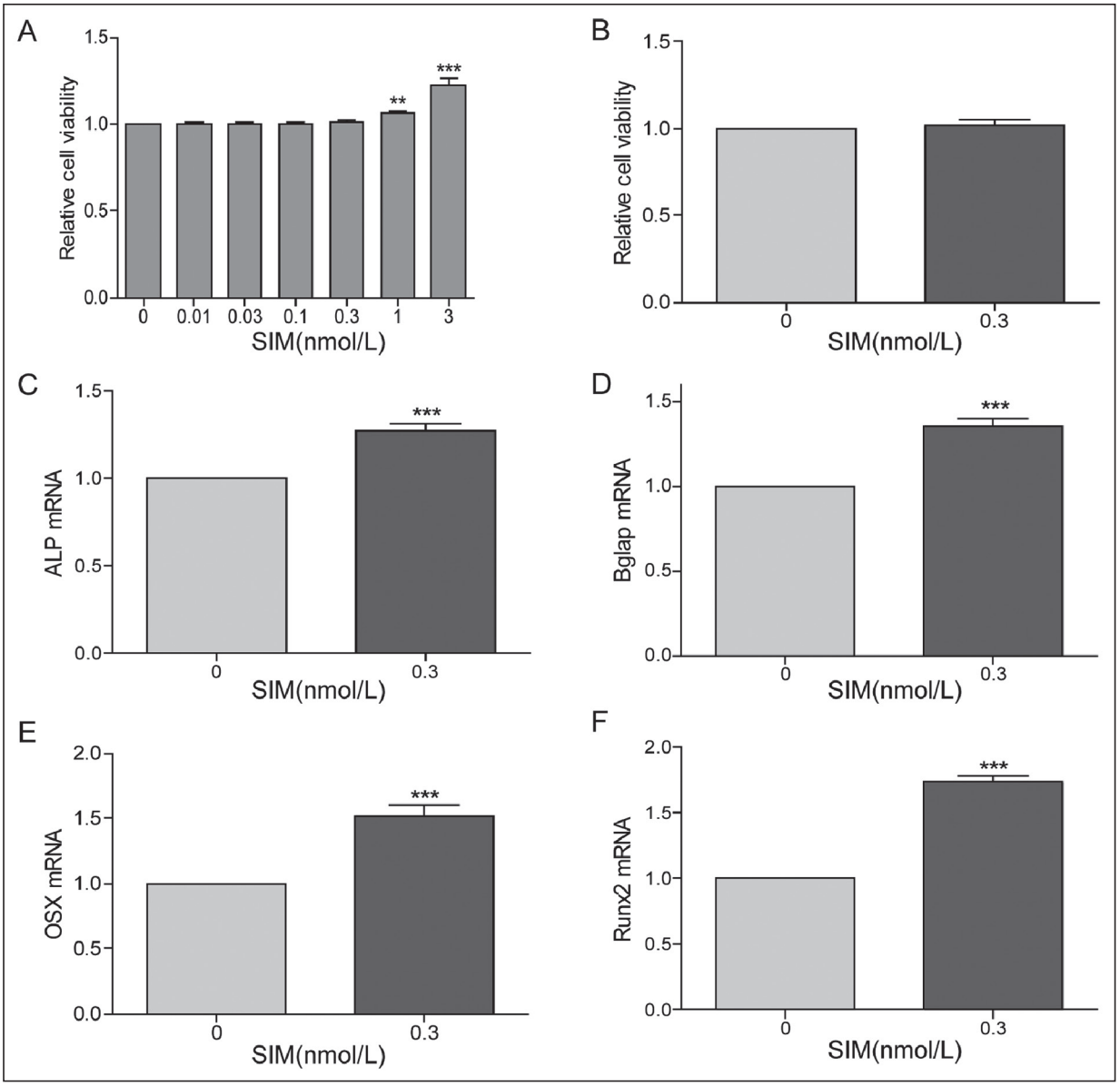

Figure 2. Effect of different concentration of simvastatin on the activity and osteogenesis related genes of MSCs. ***A,*** The effect of different concentration of simvastatin on activity of MSCs, 0.3 nmol/L was the highest dose of simvastatin that did not cause cell proliferation. ***B,*** After cultured for 7 days, the dose of 0.3 nmol/L did not cause changes of cell activity. ***C, D, E, F,*** After cultured in osteoinduction medium containing 0.3 nmol/L simvastatin for 7 days, the expression of ALP, Bglap, OSX and Runx2 increased significantly.

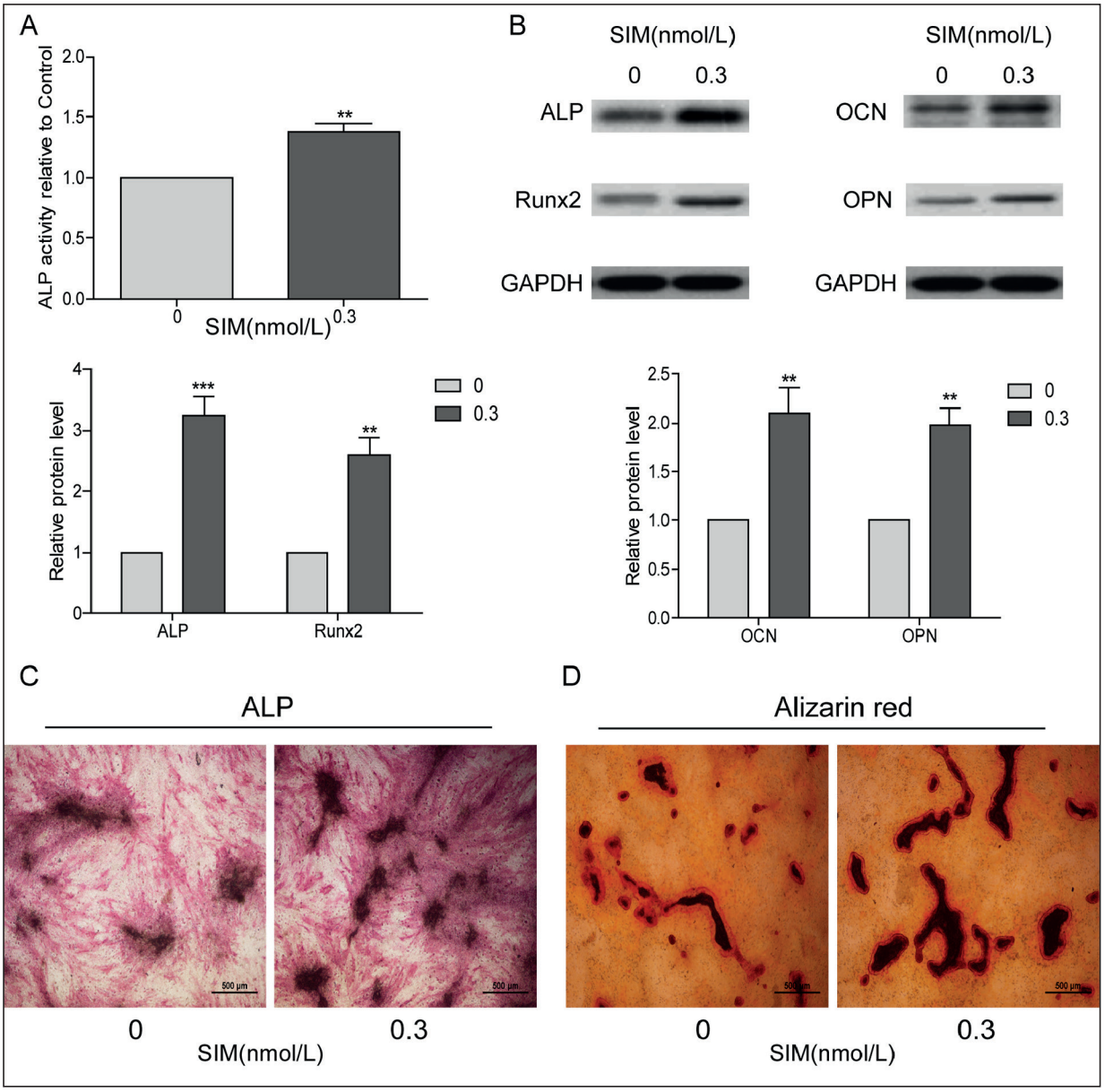

Figure 3. Simvastatin can promote the osteogenic differentiation of MSCs. After cultured in osteoinduction medium containing 0.3 nmol/L simvastatin for 7 days. ***A,*** The changes of ALP activity in cells. ***B,*** The expression of proteins (ALP, Runx2, OCN and OPN) in cells increased significantly. ***C,*** Alkaline phosphatase increased significantly after ALP staining on cells. ***D,*** Mineralized nodules increased significantly after alizarin red staining on cells.

on the osteogenic differentiation of MSCS could be inhibited by DKK1.

Inhibition of Osteogenic Differentiation of MSCs Induced by Simvastatin After Interfering with β-Catenin

Next, we transfected β-catenin shRNA and control shRNA into different groups of MSCs. Then, 0.3 nmol/L simvastatin were added to MSCs transfected with β-catenin shRNA and control shRNA while the control group was without simvastatin. The level of β-catenin in the cells was examined after the cells were transfected for 2 days. The β-catenin shRNA vector with high inhibition rate was screened by Western blot for subsequent experiments. In addition, simvastatin significantly up-regulated the expression of the β-catenin protein (Figure 5A). Subsequently, changes in the levels of osteoblast-related proteins (Runx2, OCN, and OPN) in the cells were exam-

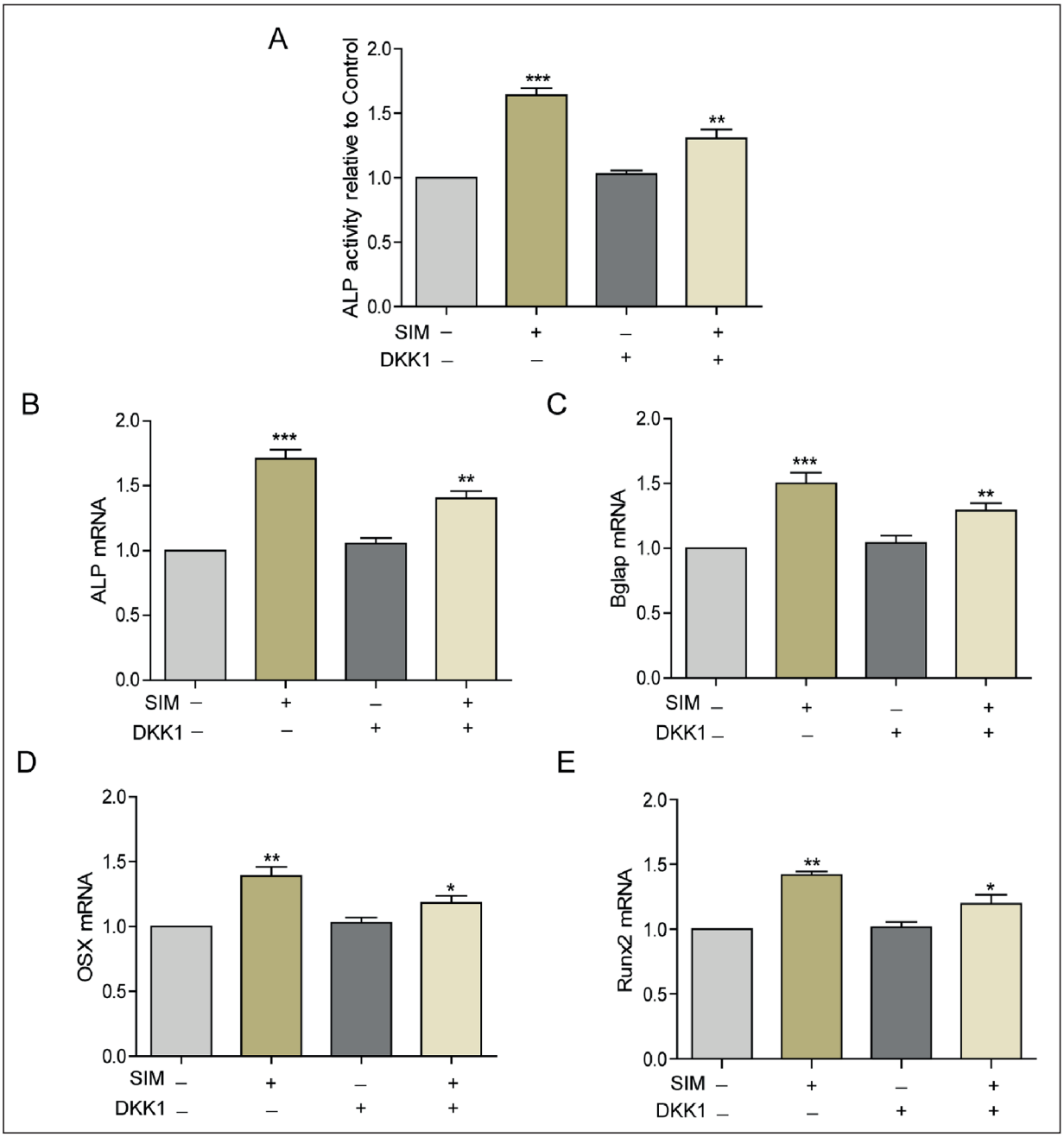

Figure 4. DKK1 can inhibit the effect of simvastatin on osteogenic differentiation. After addition of 0.5 μg/mL DKK1 1 h prior to 0.3 nmol/L simvastatin treatment, MSCs were induced to differentiate for 7 days. ***A,*** The changes of ALP activity in cells. ***B, C, D,*** The changes of gene expression of ALP, Bglap, OSX and Runx2 in cells.

ined after induction of osteogenic differentiation for 7 days. The results showed that the down-regulation of β-catenin significantly inhibited the expression of osteoblast-specific factor Runx2, OCN and OPN mRNA (Figure 5B), indicating that Wnt/β-catenin pathway played a key role in regulating the osteogenic differentiation induced by simvastatin.

Discussion

The differentiation ability of MSCs is a key step in the process of bone construction. Under specific inductive factors, MSC crossed a number of signal transduction pathways, experienced 5 stages, which are osteoprogenitor, preosteoblast, transitory osteoblast, secretory osteoblast and osteocytic osteoblast,

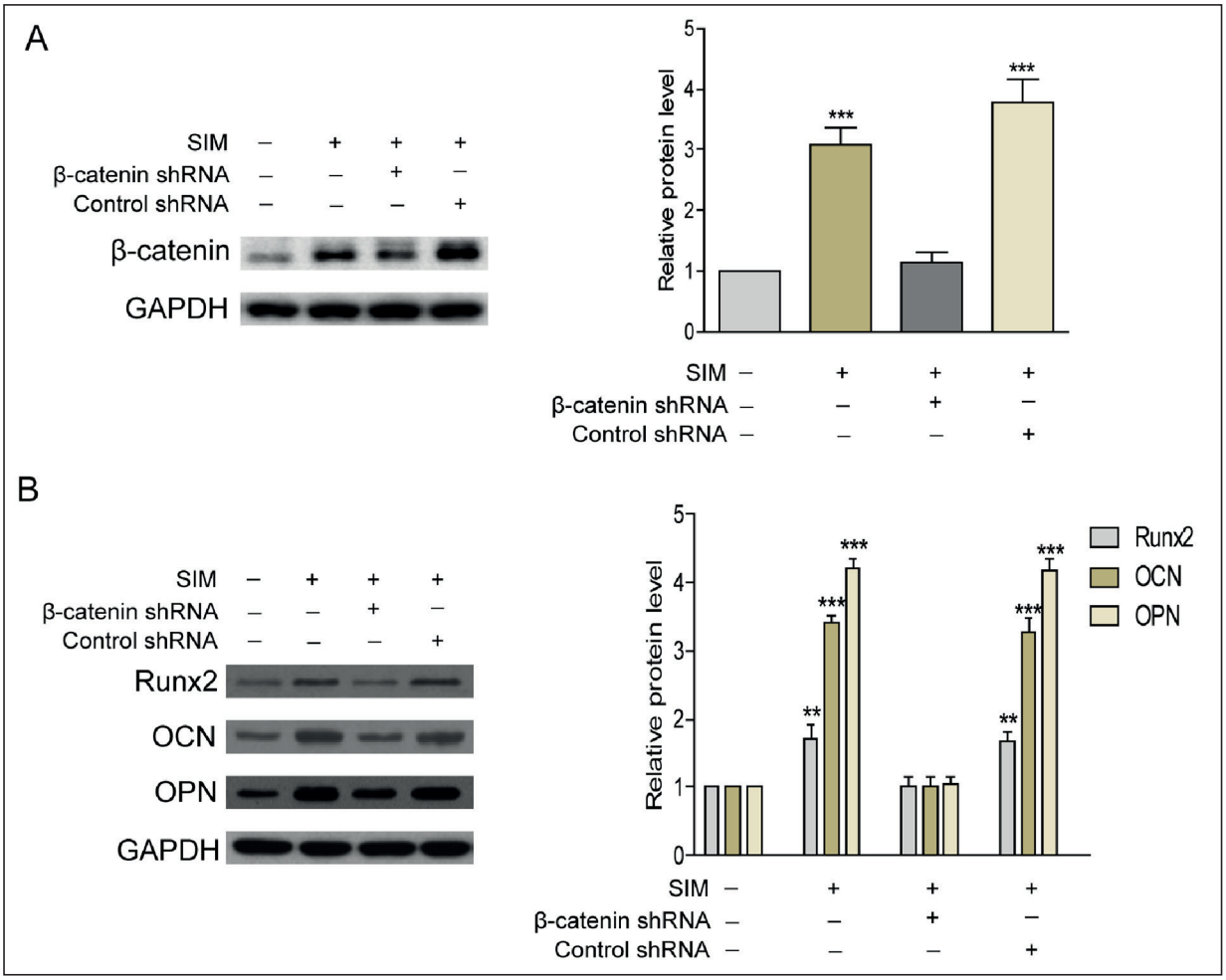

Figure 5. Inhibition of osteogenic differentiation of MSCs induced by Simvastatin after interfering with β-catenin. 0.3 nmol/L simvastatin were added into MSCs after transfected with β-catenin shRNA and control shRNA for 24 hours. ***A,*** The content changes of β-catenin in cells after transfection for 2 days. ***B,*** Changes of protein (Runx2, OCN and OPN) in cells after induction to differentiate for 7 days.

and eventually differentiated into osteocyte[12]. The classical Wnt signaling pathway plays a vital role in the osteogenic differentiation of bone marrow mesenchymal stem cells. Wnt is an important pathway that can induce cell proliferation and tumorigenesis. The activation of Wnt/β-catenin signaling pathway can promote the survival and spontaneous fusion of embryonic stem cells[13]. There is literature[14] demonstrating that activating Wnt/β-catenin signaling pathway can facilitate the proliferation of osteoblast and improve cell viability. However, the effect of simvastatin in regulating Wnt/β-catenin signaling in the proliferation and apoptosis of MSCs remains unclear.

Studies in recent years found that statins can not only promote bone formation, but also inhibit bone resorption, which plays an important role in the development of bone tissue through a variety of pathways and interlocking issues. Latest reports[15] have shown that simvastatin has the effect of stimulating bone formation and improving bone mass. Maeda et al[16] pointed out that simvastatin could promote osteoblast differentiation as well as mineralization and enhance the alkaline phosphatase activity along with mineralization rate of osteoblast. In addition, there are investigations[17] revealing that simvastatin can promote the differentiation of MSCs cultured *in vitro* into osteoblast-like cells, but its specific mechanism is still unclear.

In this experiment, simvastatin was used as an inducer to investigate the role of Wnt/β-catenin signal pathway in the conduction of simvastatin biological signals. MSCs were isolated from rat

bone marrow specimens and their compositions were identified in this study. In the third generation of MSCs, the gradient concentration of simvastatin was used to induce the osteogenic differentiation of MSCs and the 0.3 nmol/L was chosen as the best treatment concentration. ALP, RUNX2, OCN, and OPN are important genes of osteogenic differentiation; we found that simvastatin could increase the expression level of ALP, RUNX2, OCN and OPN, promote the formation of calcium nodules and induce the differentiation of MSCS into osteoblast.

DKK1, which is produced by osteoblast and osteocyte, can block the binding of protein Wnt to its receptor and inhibit the conduction of Wnt/β-catenin signaling pathway[18]. In this work, DKK1 was added into MSCs before treatment of simvastatin, and then cell ALP activity and osteoblast-related protein levels were detected after osteogenic differentiation of MSCs for 7 days. The addition of DKK1 inhibited the osteogenic differentiation induced by simvastatin, indicating that simvastatin might affect the differentiation of MSCs into osteoblasts through Wnt/β-catenin signaling pathway. β-catenin is an important information molecule in Wnt signaling pathway, which can effectively promote the expression of target molecules at downstream of Wnt signaling pathway, thus plays an important role in the activation of Wnt/β-catenin signaling pathway. In further corroboration of the effect of simvastatin on Wnt/β-catenin signaling pathway, we used a β-catenin disturbing agent to study the effect of simvastatin on osteogenic differentiation of MSCs. The specific β-catenin shRNA could significantly reduce ALP activity and inhibit the expression of osteoblast-specific factors. These results further suggested that simvastatin could promote differentiation of MSCs into osteoblasts by activating Wnt/β-catenin signaling pathway.

In summary, we found that simvastatin can induce MSCs to differentiate into osteoblast by activating Wnt/β-catenin signaling pathway, which provides some theoretical basis and new research direction on simvastatin promoting fracture healing.

Conclusions

We showed that simvastatin can promote the differentiation of rat MSCs into osteoblast-like cells, and its mechanism may be related to the mediation of Wnt/β-catenin pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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