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Simvastatin promotes osteogenic differentiation of mesenchymal stem cells in rat model of osteoporosis through BMP-2/Smads signaling pathway

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Abstract. – OBJECTIVE: By establishing osteoporosis (OP) model in rats, the specific regulatory effect of simvastatin on promoting the differentiation of mesenchymal stem cells (MSCs) into osteoblasts through the bone morphogenetic protein 2 (BMP-2)/Smads signaling pathway was investigated.

MATERIALS AND METHODS: A total of 45 Sprague-Dawley rats were selected to establish the OP model by performing ovariectomy. The rats were divided into OP model group (OP group, n=15), 10-7 mmol/L simvastatin treatment group (SIM group, n=15), and normal control group (Control group, n=15). After the experimental period, the enzyme-linked immunosorbent assay (ELISA) was applied to observe the serum levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was adopted to detect the contents of the differentiation-associated genes [runt-related transcription factor 2 (RUNX2) and Osterix (Osx)]. Later, the bone marrow MSCs (BMSCs) were selected and divided into Control group, 10-7 mol/L simvastatin group (SIM group), and osteoinduction medium group (OM group). Cell morphology in each group was observed. The Cell Counting Kit-8 (CCK-8) was performed to determine the proliferation activity of BMSCs. ELISA was performed to measure the level of alkaline phosphatase (ALP). RT-PCR was conducted to examine the levels of key differentiation-associated gene RUNX2 and those in BMP-2/Smads pathway. Moreover, the Western blotting was adopted to analyze the expressions of RUNX2 and genes in BMP-2/Smads pathway.

RESULTS: The serum levels of TNF-α, IL-6, and IL-1 in OP group were remarkably higher than those in the Control group, and their levels in the SIM group were close to those in the Control group. The elevated messenger ribonucleic acid (mRNA) levels of the key differentiation-associated factors RUNX2, osteoprotegerin (OPG), osteopontin (OPN), and Osx were observed in the SIM group. In vitro cell culture revealed that the cells were in a favorable growth status in the SIM group and OM group, mostly manifesting in fusiform or spindle shape, and proliferated rapidly. In addition, the ALP level notably increased in the two groups compared with that in the Control group ($p<0.05$). Both SIM group and OM group had evidently higher mRNA expression levels of RUNX2, OPG, OPN, and Osx than those in the Control group ($p<0.05$), consistent with the expression trends of the genes in BMP-2/Smads pathway. The Western blotting indicated that the expression levels of RUNX2 and genes in BMP-2/Smads pathway in the SIM group were significantly higher than those in the Control group.

CONCLUSIONS: Simvastatin can promote the differentiation of MSCs into osteoblasts in the OP rat model through the BMP-2/Smads signaling pathway.

Key Words:

Simvastatin, BMP-2/Smads signaling pathway, Rats, Osteoporosis, Mesenchymal stem cells, Osteogenic differentiation.

Introduction

Osteoporosis (OP) is a common debilitating skeletal disease characterized by low bone mineral density and impaired bone structure, especially among elderly people. It has become a major public health problem threatening the health of elderly around the world[1]. Aging-induced osteopenia in women results from declined ovari-

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an function[2,3]. The extracellular matrix proteins, such as osteocalcin (OCN), produced by osteoblasts can be moderately controlled by the body to maintain stable secretion. Hence, they are the basis to maintain bone homeostasis[4]. The cytokines released the following OP damage and initiated osteogenic differentiation, which helps to recover the fracture-induced trauma and support benign homeostasis. This complex and dynamic process involves multiple genes, regulatory factors, cell components, and cytokines. Triggering the generation of osteoclasts can accelerate cartilage reabsorption and promote the formation of synostosis at the same time, while repressing the differentiation of osteoblasts or osteoclasts inhibits the healing of OP[5,6]. Once the good balance between bone formation of osteoblasts and bone resorption of osteoclasts is destroyed, it will lead to osteopenia and structural changes in bone tissues, thus leading to OP[7]. Therefore, it is essential to deeply understand the molecular regulatory networks of osteoblast proliferation and differentiation, so as to develop effective treatments for OP and other bone diseases. The development of drugs targeting these genes or proteins will provide new ideas for therapeutic and healing measures.

Bone morphogenetic proteins (BMPs) are the most efficacious growth factors that promote osteogenesis[8]. BMP-2 has been applied in the clinical treatment of spinal injury, bone transplantation, OP and fracture, which stimulates bone regeneration in patients[9]. An experimental study[10] on human bodies has revealed that induction of recombinant human BMP-2 (1.5 mg/mL) enhances the bone repair capacity in patients with open tibial fracture. BMP-2 plays a unique and vital role in bone formation after birth because it mainly activates some important osteogenic transcription factors such as OCN and osteoprotegerin (OPG) by activating Smads and related downstream kinases[11,12]. However, the expression level of BMP-2 significantly declines in the bone tissues and osteoblasts of OP patients[13,14], suggesting that the up-regulation of BMP-2 in the bone tissues of OP patients has certain therapeutic effects. Bone marrow mesenchymal stem cells (BMSCs) are common progenitor cells of osteocytes and adipocytes. The human MSCs (hMSCs) derived from the bone marrow are capable of differentiating into many lineage cells, including osteoblasts. The differentiation of hMSCs into osteoblast lineage depends on the local availability of the growth factors and hormones that can activate specific transcription regulators[15]. There has been conclusive evidence indicating that the impaired differentiation of BMSCs can result in an imbalance between osteogenesis and adipogenesis. In the case of OP, adipogenesis in the bone marrow is very active, and hMSCs capable of differentiating into osteoblasts simultaneously decreased, leading to attenuated bone formation. The recovery of the osteogenic differentiation of BMSCs is an effective method for the treatment of OP[16]. Related pathways to BMP-2 can induce bone regeneration by advancing the osteogenic differentiation of BMSCs[17]. However, no favorable progress has been achieved in the extensive clinical application of the BMP/Smad signaling pathway. Moreover, the molecular mechanism of BMP/Smad in regulating endogenous MSCs still remains unclear, thus hindering the advancement of the OP treatment.

Simvastatin possesses hypolipidemic effects, and is utilized for the treatment of cardiovascular diseases. In recent years, the pharmaceutical field has been committed to research and develop new effects of simvastatin, which may be conducive to OP treatment[18-20]. Therefore, this research proposed that simvastatin may have impacts on OP through the BMP/Smad signaling pathway. The classical OP rat model was established, and the content of the inflammatory factors was determined *via* biochemical index detection and enzyme-linked immunosorbent assay (ELISA). Moreover, *in vitro* cell culture and functional assays were adopted to clarify the changes in osteogenic differentiation-related genes and those in the BMP/Smad pathway. This research provides experimental bases for developing new drugs and treatment methods of OP.

Materials and Methods

Animal Modeling and Grouping

A total of 45 rats were selected to establish the OP model by performing ovariectomy after adaptive feeding. They were divided into OP model group (OP group, n=15), 10^{-7} mmol/L simvastatin treatment group (SIM group, n=15), and normal control group (Control group, n=15). After the experimental period, the blood was collected by removing the eyeballs of the rats, followed by centrifugation to collect the serum. The serum samples were preserved at -80°C to detect the biochemical indexes. Subsequently, the pentobar-

bital sodium was injected for anesthesia, and an appropriate amount of rat bone tissues were taken and stored at -80°C to determine the expression levels of the genes. This study was approved by the Animal Ethics Committee of Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine Animal Center.

Cell Culture and Grouping

MSCs purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) cell bank were quickly taken out from a liquid nitrogen tank and rapidly thawed in sterile water at 60°C prepared in advance. Later, the cells were centrifuged, and the supernatant was discarded. The cells were resuspended in the medium after repeated centrifugation. Next, the cells were seeded into a 6-well plate at a calculated density and cultured in a constant-temperature incubator, and the medium was changed every other day. Then, the second-generation MSCs in a good growth status was divided into Control group (with normal medium change), simvastatin (SIM) group (with 10^{-7} mmol/L simvastatin in the medium), and osteoinduction medium (OM) group (with different concentrations of OM).

Detection of Serum Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1) and IL-6 via ELISA

The serum stored at -80°C was thawed in gradient temperature, followed by centrifugation again and sub-packaging. The levels of TNF-α, IL-1, and IL-6 were detected according to the instructions and practical situations. Finally, the absorbance of each sample was determined using a microplate reader, and the standard curves were plotted.

Detection of Key Differentiation-Associated Genes and Pathway Molecules in Bone Tissues and Cells via Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

A proper amount of bone tissues frozen at -80°C were taken out carefully, weighed, added with liquid nitrogen, and ground in a mortar, followed by homogenization in low temperature at 2100 r/min for seconds. Then, the supernatant was discarded, the cells were washed, and the total ribonucleic acid (RNA) in the tissues and cells was extracted. Qualified RNAs were used for performing qRT-PCR. RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) *via* an Invitrogen reverse transcription kit. The primer sequences for the target genes and internal reference [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were designed as per those on GenBank. After that, an amplification system (20 μL) was prepared using cDNA (2 μL), qPCR Mix (10 μL), forward primer (1 μL), reverse primer (1 μL), and ddH_2O (6 μL), and PCR amplification was performed for 40 cycles. The specific primer sequences were shown in Table I. The relative expression levels of the related genes in each group of the bone tissues were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: RUNX2, F: 5'-CGAGGCAACCAAGCTCCTTACTC-3', R: 5'-GCCCTAGTTGTGACGACAGGA-3'; OPG, F: 5'-GCCTGTATACATCGGAGACTGC-3', R: 5'-ACCTTGCGTGGAGTGAGAGTCCG-3'; OPN, F: 5'-ACCGCAGCAAGGCATTTGCGACACA-3',

Table I. Primer sequences for target genes.

Target gene	Primer sequence (5'-3')
Runt-related transcription factor 2 (RUNX2)	GTCCAACCCGTAAGGT
	CGCTGCTGAGTCGATGCTAGCT
Osteopontin (OPN)	ATCGTAGCTAGCTAGTCGAGCA
	CCCCCTGTGCTAGCTAGCTAGC
Osteoprotegerin (OPG)	TGTGAAAGCAGTGTGCAACG
	CCAGGCAAGCTCTCCATCAA
Osterix (Osx)	GTGCTGATGTTAGCTAGCTAGCT
	AGCTAGTCGTAGCTAGCTGATCG
BMP-2	TTCCTGGTAACCGAATGCT
	GGGGCTTCATAACCTCATAA
Smad2	GCTTCTTGACGAGAGAGTCTACGG
	TACTAACACTGGTGGCAGCACTGG
GAPDH	GACATGCCGCCTGGAGAAAC
	AGCCCAGGATGCCCTTTAGT

R: 5'-CGGTGCGTACGCGAACAGTTC-3'; Osx: F: 5'-GAACTTCGGAGCACAATACAGAAGT-3', R: 5'-CGGCTTCAGCCTCTTGAGTAACAT-3'; GAPDH:F:5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Detection of Proliferation Activity via Cell Counting Kit-8 (CCK-8)

The cells in the logarithmic growth phase in each group were inoculated into a 96-well plate and cultured in the incubator with 5% CO_2 at 37°C for 0, 24, 48, 72, and 96 h. Then, the medium was discarded, and 100 μL of the color developer was added into each well. After incubation at 37°C for 2 h, the absorbance at 450 nm was measured by an ultraviolet spectrophotometer for depicting viability curves.

Detection of Alkaline Phosphatase (ALP) Level in the Supernatant via ELISA

The cells and supernatant in each group were collected using a cell scraper. The cells were lysed with strong RIPA lysis buffer, centrifuged, and separated to collect the supernatant. Next, the ELISA kit was applied to determine the levels of TNF-α, IL-6, and IL-1 in the cells in accordance with the practical situations and instructions. Finally, the absorbance in each group was detected using the microplate reader, and the raw data were recorded.

Western Blotting

The cells in suitable density were collected from the three groups to extract the proteins and measure the protein concentration. The total protein extracted from the cells was subjected to water bath and centrifugation. Then, the protein samples were electrophoresed on polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with the Tris-Buffered Saline and Tween 20 buffer solution (TBST). Then, the protein bands were scanned and quantified using an Odyssey membrane scanner, and the levels of the proteins to be detected were corrected *via* GAPDH. Ultimately, a grayscale scan was conducted.

Statistical Analysis

All the raw data obtained during the experiments were processed using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp.,

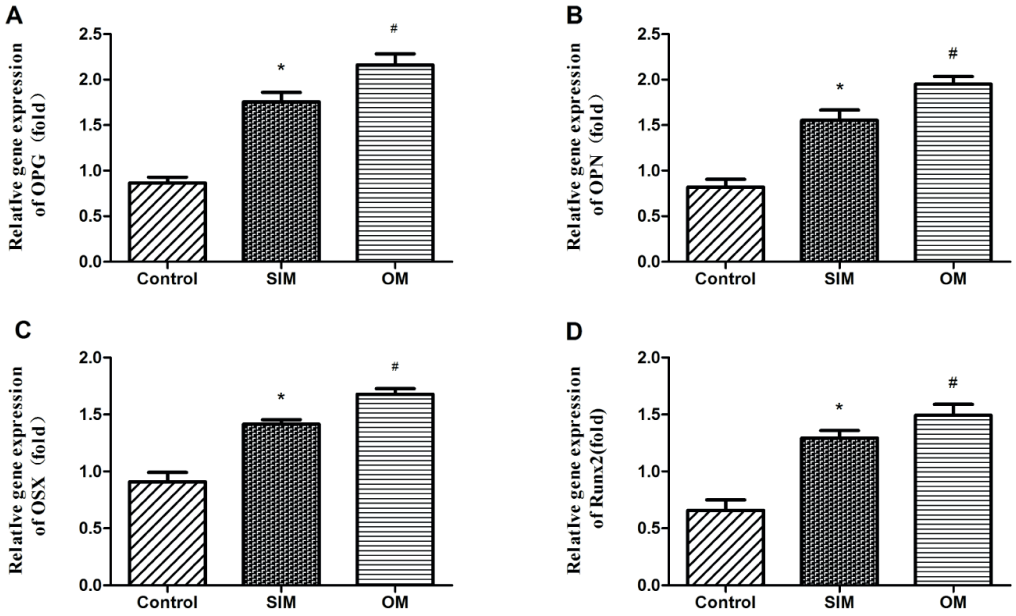

Figure 1. Key differentiation-associated genes in bone tissues detected *via* qRT-PCR. The mRNA levels of key differentiation-associated factors (RUNX2, OPG, OPN, and Osx) are markedly raised in the SIM group and notably reduced in the OP group ($p<0.05$). $^{*}p<0.05$ *vs.* Control group, $^{\#}p<0.05$ *vs.* OP group.

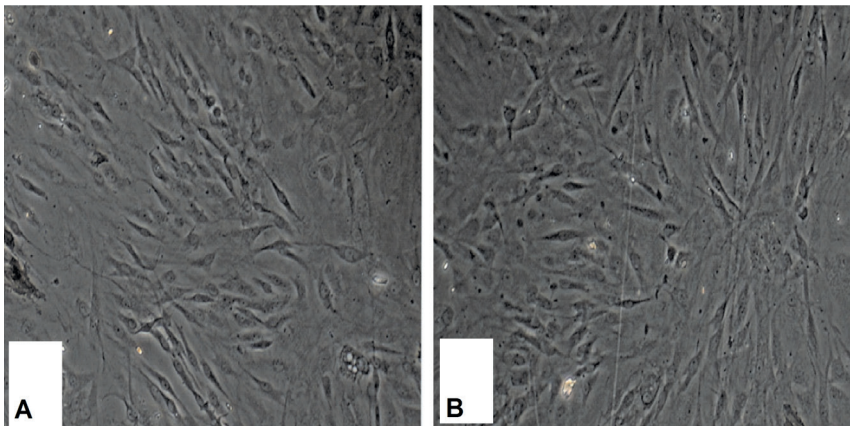

Figure 2. Observation of cell morphology. Most of cells in the SIM group and OM group are in fusiform or spindle shape, with apparent proliferation and colony growth (magnification × 40).

Armonk, NY, USA). The efficacy of the raw data was retained, and the data were subjected to multiple comparisons. The experimental results were presented as mean ± standard deviation ($\bar{\chi}$±SD), and $p<0.05$ suggested that the difference was statistically significant. The histograms were plotted by GraphPad Prism 7.0 (La Jolla, CA, USA).

Results

Results of TNF-α, IL-6, and IL-1

As shown in Table II, serum levels of IL-1, IL-6, and TNF-α were elevated in the OP group ($p<0.05$) and lowered in the SIM group ($p<0.05$).

Key Differentiation-Associated Genes in Bone Tissues Detected via qRT-PCR

According to the detection results of the crucial genes for osteogenic differentiation (Figure 1), the messenger RNA (mRNA) levels of the key differentiation-associated factors (RUNX2, OPG, OPN, and Osx) rose markedly in the SIM group and declined notably in the OP group ($p<0.05$), suggesting that simvastatin can promote the expressions of osteogenic differentiation-associated genes.

Observation of Cell Morphology

At about 14 d after osteogenic differentiation, the cells in the SIM group and OM group grew in relatively uniform morphology. Most of them were in fusiform or spindle shape, with apparent proliferation and colony growth. Cell body was enlarged with the prolongation of time (Figures 2A and 2B).

Results of Proliferation Activity Detected via CCK-8

Meanwhile, the CCK-8 proliferation assay was conducted to detect the cell proliferative capacity. It was indicated that the OM group and the SIM group had an evidently stronger proliferative capacity of osteoblasts at 48, 72, and 96 h than that in the Control group ($p<0.05$) (Figure 3).

ALP Level in the Supernatant

Since OP can be predicted by early biochemical indexes to prepare for the treatment and prognosis

Table II. Results of serum TNF-α, IL-1, and IL-6.

Group	TNF-α (fmol/mL)	IL-6 (mg/L)	IL-1 (mg/L)
Control	18.12±2.34	29.36±4.78	34.62±4.18
OP group	49.35±2.88[a]	88.51±6.25[a]	95.98±6.54[a]
SIM group	22.39±3.58[b]	33.25±5.27[b]	38.42±3.49[b]

Note: The levels of IL-1, IL-6, and TNF-α are elevated in OP group ($p<0.05$) and lowered in SIM group ($p<0.05$). [a]$p<0.05$ *vs.* Control group, [b]$p<0.05$ *vs.* OP group.

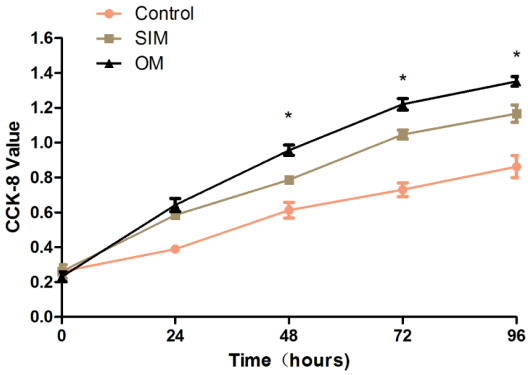

Figure 3. CCK-8 detection. Osteoblasts in the OM group and SIM group have evidently stronger proliferative capacity at 48, 72, and 96 h than those in the Control group ($p<0.05$). $^{*\#}p<0.05$ *vs.* Control group, with statistical differences.

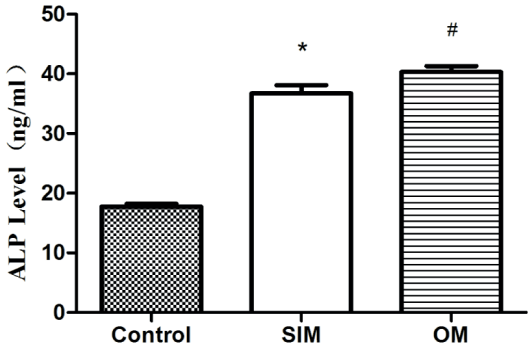

Figure 4. ALP content. The content of ALP is significantly raised in the SIM group and OM group ($p<0.05$). $^{*\#}p<0.05$ *vs.* Control group.

as early as possible, ALP content in the supernatant was determined, and the raw data recorded were analyzed. As shown in Figure 4, ALP content was raised significantly in the SIM group and OM group ($p<0.05$), illustrating that ALP gradually increased during induced osteogenic differentiation, further promoting osteogenic differentiation.

Results of Key Differentiation-Associated Genes and BMP-2/Smads Pathway Molecules in Cells Detected via qRT-PCR

According to the detection results for crucial genes associated with osteogenic differentiation and pathway during *in vitro* cell culture (Figure 5), SIM group and OM group had remarkably elevated mRNA levels of key differentiation-associated factors (RUNX2, OPG, OPN, and Osx) ($p<0.05$) as well as mRNA levels of BMP-2 and Smad2 ($p<0.05$). It is suggested that simvastatin can enhance the expressions of the osteogenic differentiation-associated genes and BMP-2/Smads pathway-associated genes.

Results of Western Blotting Assay

The detection results for vital proteins associated with osteogenic differentiation and BMP-2/Smads pathway during *in vitro* cell culture man-

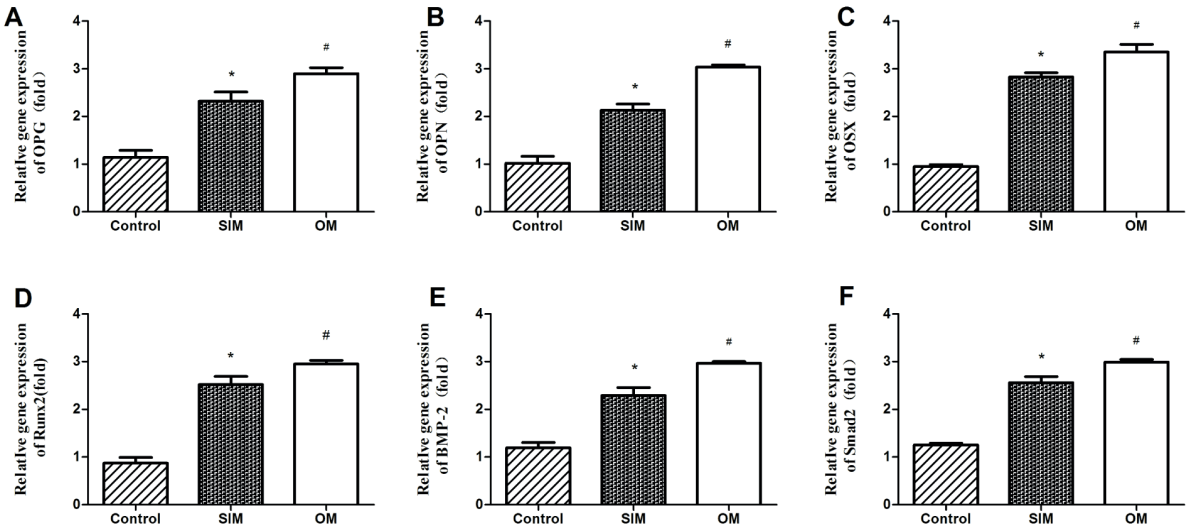

Figure 5. Key differentiation-associated and pathway-associated genes detected *via* qRT-PCR. The SIM group and OM group have remarkably elevated mRNA levels of key differentiation-associated factors RUNX2, OPG, OPN, and Osx ($p<0.05$), as well as mRNA levels of BMP-2 and Smad2 ($p<0.05$). $^{\#*}p<0.05$ *vs.* Control group.

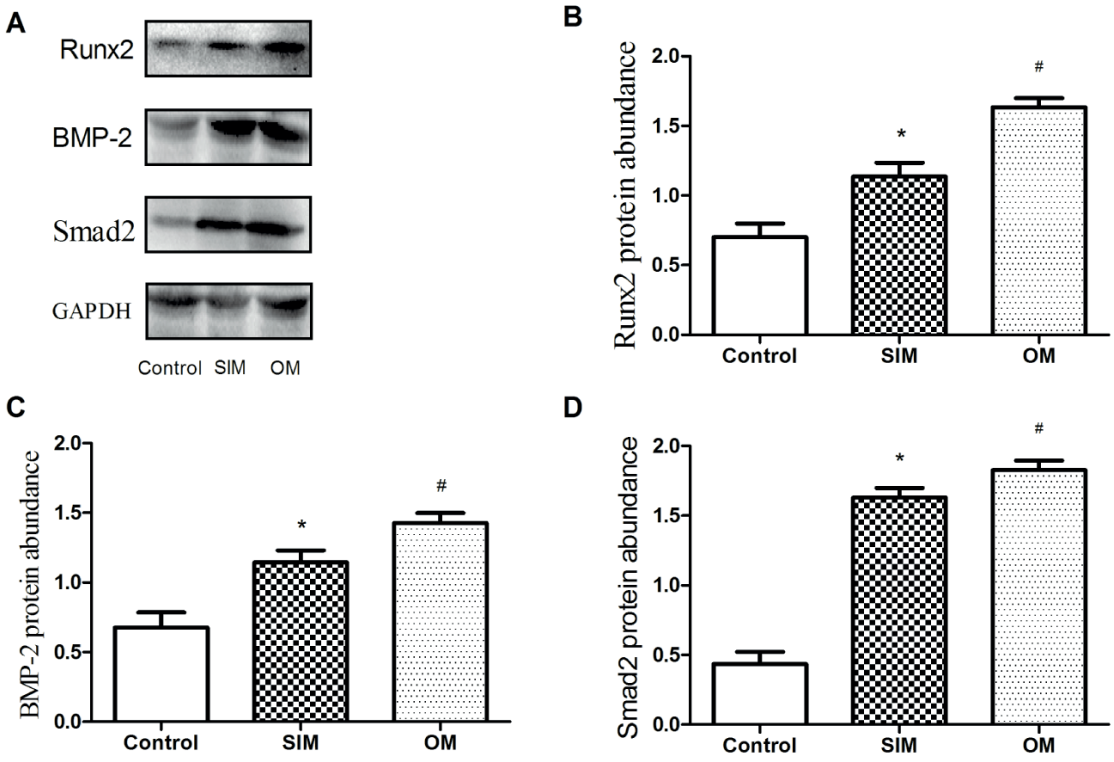

Figure 6. The results for key differentiation-associated and pathway-associated proteins. The level of the key differentiation-associated protein RUNX2 notably increases in the SIM group and OM group ($p<0.05$). The protein levels of BMP-2 and Smad2 also rise prominently in the SIM group and OM group ($p<0.05$). $^{\#*}p<0.05$ *vs.* Control group.

ifested that the level of the key differentiation-associated protein RUNX2 notably increased in the SIM group and OM group ($p<0.05$) (Figure 6). The protein levels of BMP-2 and Smad2 also prominently rose in SIM group and OM group ($p<0.05$), suggesting that simvastatin was capable of promoting the expressions of the osteogenic differentiation-associated proteins and BMP-2/Smads pathway-associated proteins.

Discussion

As a common disease among the elderly, OP seriously affects the life of the middle-aged and elderly people, with numerous causes and regulatory factors. It is a common complication of sarcopenia. As for women, the risk of menopause-induced OP increases markedly with aging. Currently, OP has become a major public health problem threatening the health of the elderly throughout the world[21]. Hence, effective treatment for bone diseases is urgent to be solved. This process involves the impacts of various genes and regulatory factors on the healing, in which some cytokines related to osteoblast proliferation and differentiation are activated[22]. OP is a complicated and dynamic process regulated by a variety of cell components and cytokines, during which the increased growth of osteoclasts can accelerate cartilage reabsorption and promote the formation of synostosis. The inhibition of the differentiation of osteoblasts or osteoclasts suppresses the improvement of OP[5]. Physical exercise is recommended as the most effective non-pharmacological prevention and treatment strategy probably because it can directly exert certain anabolic effects on human bone through a direct mechanical signal generated by muscle strength or indirect endocrine regulation[23]. Therefore, seeking the key genes and proteins leading to OP is of important significance for early prevention and precise treatment. In this research, a classical rat model of OP was established for *in vivo* studies. The levels of IL-1, IL-6, and TNF-α rose in the OP group ($p<0.05$) and declined in the SIM group ($p<0.05$). Moreover, mRNA levels of key differentiation-associated factors (RUNX2, OPG,

OPN, and Osx) were notably elevated in the SIM group and lowered significantly in the OP group ($p<0.05$), suggesting that simvastatin can promote the expressions of osteogenic differentiation-associated genes. Later, such an effect was further verified via *in vitro* cell culture. At 14 d after differentiation, the cells in the SIM group and OM group grew in relatively uniform morphology. Most of them were in fusiform or spindle shape, with apparent proliferation and colony growth, and the cell body was enlarged with the prolongation of culture time. ALP, a marker for osteogenesis of stem cells, can indicate the treatment effect of simvastatin on OP. Early-stage biochemical indexes could be fully utilized for early intervention of OP. Hence, the detection of ALP in the supernatant showed that the content of ALP rose remarkably in the SIM group and OM group ($p<0.05$), revealing that ALP gradually increased in the process of induced osteogenic differentiation, further advancing the process. In the meantime, the results of CCK-8 proliferation assay displayed that both OM group and SIM group had markedly enhanced the proliferative capacity of osteoblasts at 48, 72, and 96 h compared with that in the Control group ($p<0.05$), manifesting that simvastatin has preferable treatment effects on OP. The above-mentioned results indicated that simvastatin contributes to OP treatment by influencing numerous indexes such as serum ALP and inflammatory factors, which were consistent with the findings of Chen et al[24] and Misuzu et al[25].

The primary pathways promoting osteogenesis include the BMP-2/Smads signaling pathway, in which BMP-2 triggers the osteogenic signal by phosphorylating Smads, and then mediates the expressions of the genes associated with osteoblast differentiation[26]. The BMP-2/Smads signaling pathway can activate RUNX2, a basic gene of osteoblasts. Previous studies have demonstrated that RUNX2 is highly expressed in osteoblasts and osteosarcoma cells. In addition, the osteogenic differentiation of MSCs in OP is significantly impaired in the case of BMP-2-induced RUNX2 expression[27,28]. Furthermore, OPG plays a unique and critical role in bone formation after birth mainly by repressing the related kinases downstream RANKL, thereby activating several important osteogenic transcription factors such as RUNX2 and finally increasing the expressions of bone matrix proteins. In this signaling pathway, the downstream Smads and their transcription factors are crucial to osteogenic induction. BMPs can stimulate the phosphorylation and activation of Smad2/3 by combining with serine/threonine kinase receptors. BMPs can ultimately transcribe and activate proteins responsible for osteoblast proliferation and differentiation once they are activated[29,30]. The BMP-2/Smads signaling pathway can be affected by damaged actin microfilaments. Moreover, OCN1, a connexin that can be found in preosteoblasts, is capable of connecting Smad proteins to the actin microfilaments. The silence of OCN1 can prominently reduce the transcriptional activity of Smad proteins[31]. MSCs were cultured *in vitro* to detect the vital genes, proteins, and pathway molecules during osteogenic differentiation. It was displayed that the mRNA levels of the key differentiation-associated factors (RUNX2, OPG, OPN, and Osx) in the SIM group and OM group were markedly elevated ($p<0.05$), and the mRNA levels of BMP-2 and Smad2 were also raised in the two groups ($p<0.05$). Besides, the level of the key differentiation-associated protein RUNX2 notably increased in the SIM group and OM group ($p<0.05$). The protein levels of BMP-2 and Smad2 rose evidently in the SIM group and OM group ($p<0.05$), suggesting that simvastatin was able to promote the expressions of osteogenic differentiation-associated genes and BMP-2/Smads pathway-associated genes. Consistent with the aforementioned results, simvastatin can accelerate the differentiation of MSCs into osteoblasts in OP rats by activating the BMP-2/Smads signaling pathway. Although such an effect was testified *via* the results of this research, there were still some deficiencies. Firstly, only one type of classical BMP pathway was investigated. In subsequent studies, changes in the expressions of various factors during treatment can be dynamically observed, and more genes and proteins in the signaling pathways can be detected, so as to verify such effects.

Conclusions

Simvastatin treatment improves relevant inflammatory factors and serum biochemical indexes in OP rats. Moreover, simvastatin promotes the differentiation of MSCs into osteoblasts in OP rats by activating the BMP-2/Smads signaling pathway, providing experimental and theoretical bases for developing novel drugs and therapies for OP.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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