

# Simvastatin Promotes Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells by Regulating the BMP-2/Smads Signaling Pathway

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## ABSTRACT

**Objectives:** This study aimed to investigate whether simvastatin could promote the osteogenic differentiation of Bone Marrow Mesenchymal Stem Cells (BMSCs) by modulating the BMP-2/Smads signaling pathway and to elucidate its underlying mechanisms. **Materials and Methods:** Rat BMSCs were cultured *in vitro* and divided into five groups. The control group received no simvastatin intervention, while the other four groups were treated with different concentrations of simvastatin (0.00005 µg/mL, 0.0005 µg/mL, 0.005 µg/mL, and 0.05 µg/mL). Alkaline Phosphatase (ALP) staining and alizarin red staining were used to observe the morphological changes in each group of BMSCs. Western blotting was employed to detect the expression levels of BMP-2/Smads signaling pathway-related proteins BMP2, Smad2, and Smad3 in each group of BMSCs. **Results:** Compared to the control group, the expression of ALP and the formation of calcified nodules in BMSCs treated with simvastatin were significantly increased, indicating that simvastatin can promote the osteogenesis and differentiation of BMSCs. Moreover, as the concentration of simvastatin increased in the four experimental groups, the concentration of ALP and the number and size of calcified nodules significantly increased, suggesting that higher concentrations of simvastatin are more conducive to the osteogenesis and differentiation of BMSCs. Additionally, the Western blot results showed that the expression of BMP2, Smad2, and Smad3 in BMSCs of the experimental groups was significantly higher than that in the control group, and the expression level of these proteins increased with the concentration of simvastatin. This suggests that simvastatin may promote the osteogenesis and differentiation of BMSCs by regulating the BMP-2/Smads signaling pathway. **Conclusion:** This study suggests that simvastatin can promote the differentiation of rat BMSCs into osteoblast-like cells, and its mechanism of action may be related to the upregulation of the expression levels of related protein factors in the BMP-2/Smads signaling pathway.

**Keywords:** Simvastatin, Bone Marrow Mesenchymal Stem Cells (BMSCs), Bone Morphogenetic Protein-2 (BMP-2), Smads.

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## INTRODUCTION

Chronic Apical Periodontitis (CAP) is a prevalent oral disease characterized primarily by the formation of inflammatory granulation tissue and the destruction of alveolar bone.<sup>1</sup> Clinically, extensive bone resorption associated with chronic apical periodontitis is common.<sup>2</sup> In addition to routine root canal treatment, such conditions often require apical surgery.<sup>2,3</sup> Due to the large area of bone resorption, the natural healing process is slow, posing challenges for clinical repair work. The medical

community is continuously exploring methods to treat bone resorption caused by chronic apical periodontitis and seeking new strategies to promote bone healing.<sup>4,5</sup>

Mesenchymal Stem Cells (MSCs), particularly those derived from Bone Marrow (BMSCs), are widely used as seed cells in bone tissue engineering and are currently a focus of research.<sup>6</sup> BMSCs have characteristics such as ease of procurement, separation and culture, abundant sources, good stability, continuous proliferation, and the ability to differentiate under specific conditions.<sup>7,8</sup> Current methods to induce the differentiation of BMSCs into bone include drug induction, physical induction, co-culture with bone cells, induction with cytokines and growth factors, and transgenic induction, among which drug induction is a commonly used method.<sup>9,10</sup>

Statins, widely used for their clinical effect of lowering cholesterol, have been found in recent research to induce the



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differentiation of BMSCs into osteoblasts, thereby promoting bone formation.<sup>11</sup> Specifically, statins can increase the expression of Bone Morphogenetic Protein-2 (BMP-2) in BMSCs, upregulate the level of osteocalcin, and promote the differentiation of undifferentiated MSCs into osteoblasts, thus stimulating the process of bone formation, which is dose-dependent.<sup>12-14</sup> The BMP-2/Smads signaling pathway is one of the most important pathways regulating osteogenic differentiation.<sup>15</sup> However, the mechanism by which statins regulate the BMP-2/Smads signaling pathway is not yet clear.

In this study, we used different concentrations of simvastatin for *in vitro* intervention on rat BMSCs. By comparing the morphological changes of BMSCs under different concentrations of simvastatin and the expression levels of BMP-2/Smads signaling pathway-related proteins BMP2, Smad2, and Smad3, we aim to explore whether statins can promote the osteogenic differentiation of BMSCs via the regulation of the BMP-2/Smads signaling pathway and the possible mechanisms involved.

## MATERIALS AND METHODS

### Resuscitation and culture of BMSCs

Rat BMSCs (Cyagen, Guangzhou, China) were cultured *in vitro* using high-glucose DMEM culture medium (Solarbio, Beijing, China) with 10% fetal bovine serum (Oricell, Guangzhou, China) and 1% penicillin-streptomycin in an incubator at 37°C with 5% CO<sub>2</sub>. When the cells reached the exponential growth phase, the culture medium was replaced with osteogenic induction medium to induce osteogenesis.

### Grouping

The cytotoxicity of simvastatin to BMSCs was first assessed using the CCK-8 assay (YEASEN) to ensure that the drug did not affect cell proliferation and to establish the safe concentration range for simvastatin. The groups were divided into one control group and four experimental groups. Each group performs five repetitions. The control group received no simvastatin treatment, while the four experimental groups were treated with different concentrations of simvastatin (0.00005 µg/mL, 0.0005 µg/mL, 0.005 µg/mL, and 0.05 µg/mL). All groups were simultaneously supplemented with osteogenic induction medium (complete medium, 10 mmol/L β-glycerophosphate, 50 µg/mL vitamin C, and 108 mol/L dexamethasone).

### Alkaline Phosphatase (ALP) staining of BMSCs

Cells in good growth condition were seeded into 6-well plates (with sterile 24 mm × 24 mm cover slips placed at the bottom of each well). After adding different treatment factors according to the experimental group requirements mentioned above, the cells were cultured on the cover slips for 7 days. The cover slips were then rinsed several times with PBS according to the instructions. The staining results were observed under an inverted microscope:

the cell nuclei appeared blue, and those positive for ALP contained red-brown or red granules in the cytoplasm, with some showing coffee-colored granules. The absorbance at 570 nm was measured using a UV spectrophotometer, with three replicate samples per group.

### Alizarin Red staining of BMSCs

Alizarin Red staining was used to visualize the formation of calcified nodules. Cells in good growth condition were seeded into 6-well plates (with sterile 24 mm × 24 mm cover slips placed at the bottom of each well). After adding different treatment factors according to the experimental group requirements mentioned above, the cells were cultured on the cover slips for 21 days. The staining kit was used to color the calcified nodules according to the instructions.

### Western blot test of BMP2, Smad2, and Smad3 proteins

Cells in good growth condition were seeded into 6-well plates. Once the cells had adhered to the plate, they were treated with different concentrations of simvastatin (0.00005, 0.0005, 0.005, 0.05 µg/mL) for 24 hr. After the treatment, total cellular protein was extracted and the expression of BMP2, Smad2, and Smad3 proteins was assessed using Western blotting test.

### Statistical analysis

All experiments were repeated three times, and the data are expressed as the Mean ± Standard deviation (x ± s). Statistic Package for Social Science (SPSS) 21.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. The intergroup comparison was conducted using the Student's *t*-test. A *p*-value < 0.05 was considered to indicate statistical significance.

## RESULTS

### CCK.8 assay confirms safe concentrations of simvastatin for BMSCs

The CCK.8 assay was utilized to analyze the impact of simvastatin on the viability of rat BMSCs within a concentration range of 0.00005 to 5 µg/mL (Figure 1). The cell viability was assessed on days 3, 5, and 7 following simvastatin intervention. Concentrations were considered safe if the cell viability exceeded 80%. The results indicated that simvastatin at concentrations of 0.00005, 0.0005, 0.005, and 0.05 µg/mL is considered to be safe for BMSCs, with all repetitions in each group showing cell viability exceeding 80%. Moreover, there was no significant difference in cell viability and cell count between 0.00005 group and the control group on days 3, 5, and 7 (*p* > 0.05), and the simvastatin at concentrations of 0.0005 and 0.005 groups also showed no significant difference in cell viability and cell count compared to the control group on day 3 (*p* > 0.05). However, in the groups of simvastatin at concentrations of 0.5 µg/mL and 5 µg/mL, the

number of BMSCs was significantly lower than that of the control group, with an average cell viability below 80%. Therefore, the concentration range of 0.00005 to 0.05  $\mu\text{g}/\text{mL}$  is considered to be a safe concentration for simvastatin on BMSCs and is applied to subsequent studies.

### ALP staining tests revealed increased ALP expression in BMSCs with increasing simvastatin concentrations

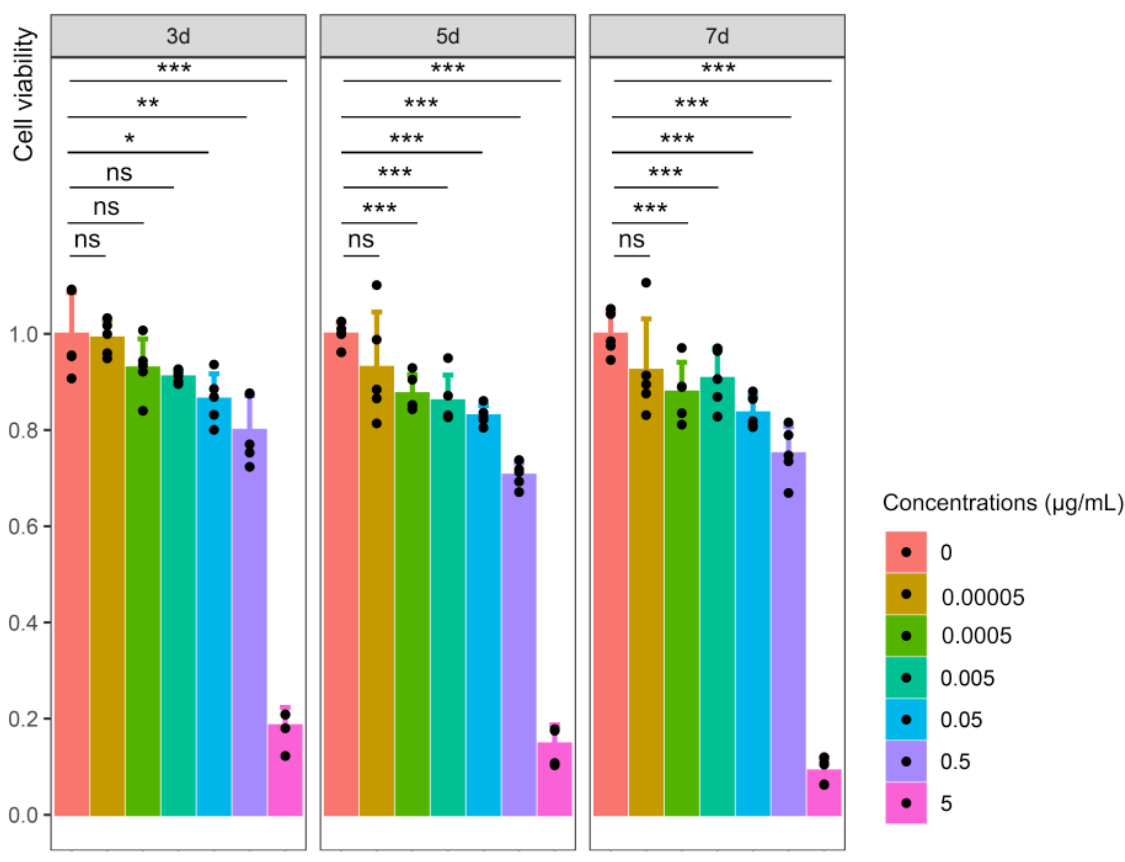
After a 7-day osteogenic induction culture, positive particles were observed in both the control and experimental groups following ALP staining (Figure 2a). The average absorbance values for the control group and the 0.00005, 0.0005, 0.005, and 0.05  $\mu\text{g}/\text{mL}$  simvastatin groups were 0.37, 0.72, 0.76, 0.86, and 1.28, respectively. The absorbance values of the experimental groups were significantly higher than the control group ( $p < 0.001$ ), indicating a marked increase in ALP content in the experimental groups. Moreover, an increasing trend in absorbance values among the experimental groups suggested that the ALP expression level of BMSCs also increased with rising simvastatin concentrations (Figure 2a and b).

### Alizarin red staining tests showed increased calcified nodule quantity and size in BMSCs with increasing simvastatin concentrations

Due to no significant difference in ALP concentration between the 0.00005 and 0.0005  $\mu\text{g}/\text{mL}$  simvastatin groups, the 0.00005  $\mu\text{g}/\text{mL}$  simvastatin group was not subjected to further experiments. After 21 days of culture, alizarin red staining was used to color calcified nodules in BMSCs for the control and three experimental groups (Figure 2c). The presence of calcified nodules in some cells indicated the differentiation of BMSCs into osteoblasts. The quantity and size of calcified nodules in the experimental groups significantly increased with the increase in drug concentration, demonstrating that simvastatin promoted the osteogenic differentiation of BMSCs.

### Quantitative detection of BMP2, Smad2, and Smad3 by western blotting

Western blotting results showed that the protein expression levels of BMP2, Smad2, and Smad3 in the four experimental groups were higher than those in the control group after a 7-day treatment with simvastatin, and these levels significantly increased with the drug concentration ( $p < 0.05$ ) (Figure 3a and b). The 0.05  $\mu\text{g}/\text{mL}$  simvastatin group exhibited the highest expression levels of these proteins, indicating that simvastatin promoted the osteogenic



**Figure 1:** The results of the CCK-8 assay for determining safe concentrations of simvastatin for BMSCs. The concentration range of 0.00005 to 0.05  $\mu\text{g}/\text{mL}$  is considered to be a safe concentration for simvastatin on BMSCs.

differentiation of BMSCs by upregulating the BMP2/Smads signaling pathway.

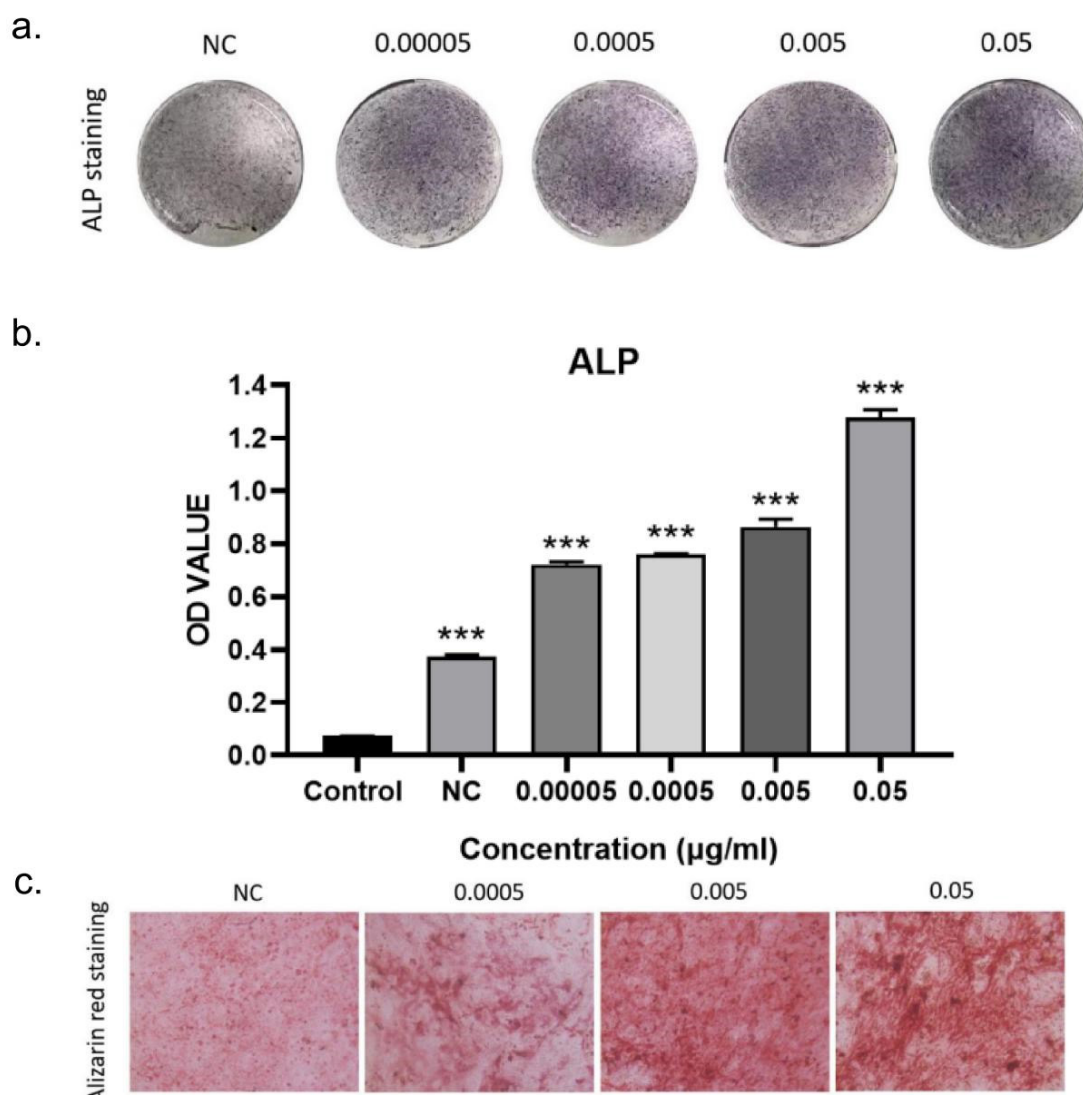
## DISCUSSION

This study employed *in vitro* experimental methods to investigate the effects of simvastatin on the osteogenic differentiation of BMSCs and its potential mechanisms of action. The results demonstrated that simvastatin within a certain concentration range could enhance the expression of ALP and the formation of calcified nodules in BMSCs, thereby promoting their osteogenic differentiation. This effect intensified with increasing drug concentrations, showing a clear dose-dependent relationship. The underlying mechanism is likely that simvastatin upregulates the expression of BMP2, Smad2, and Smad3, which are key components of the BMP2-Smads signaling pathway that plays a central role in osteoblast differentiation. Therefore, simvastatin

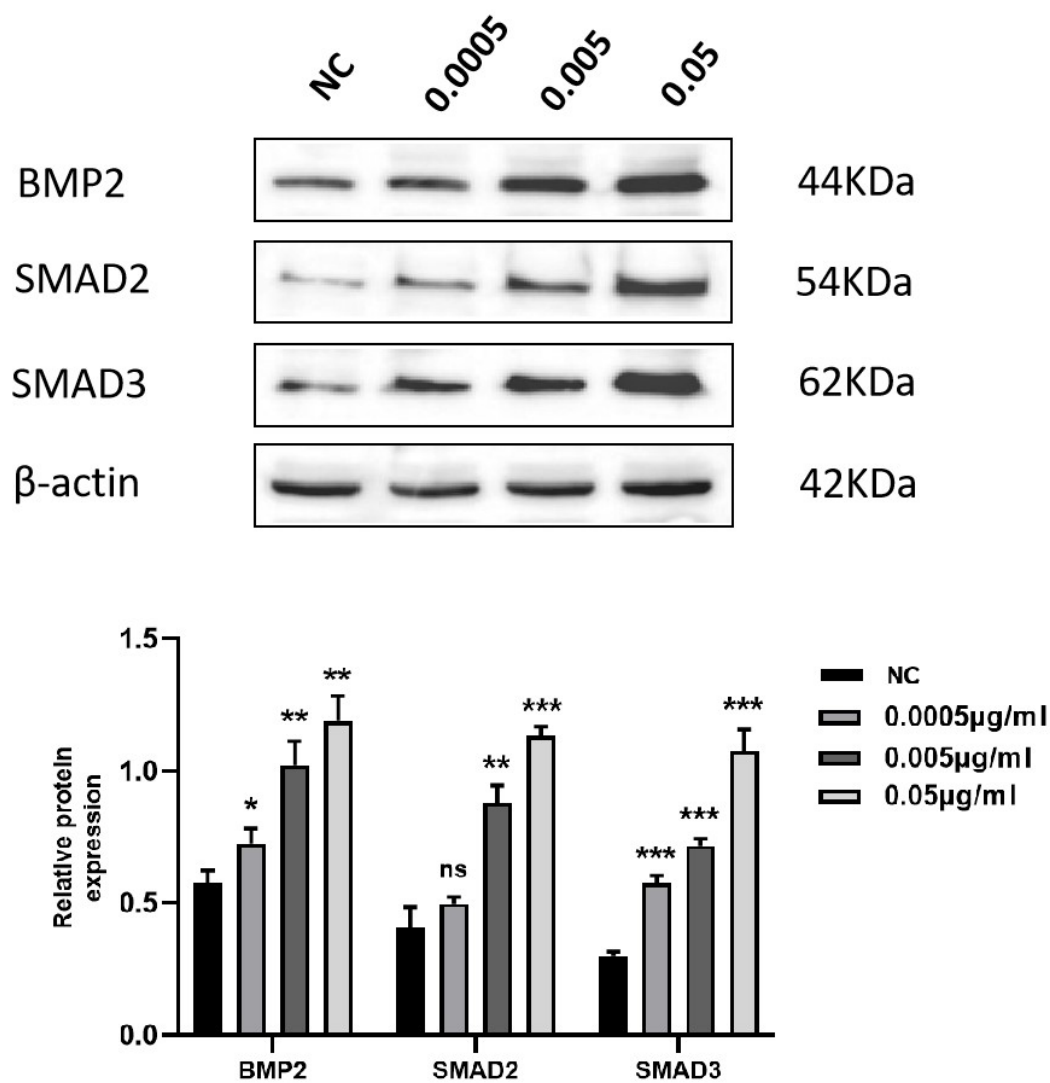
potentially upregulates the BMP2-Smads pathway to promote the osteogenic differentiation of BMSCs.

Osteoblasts release ALP, making it one of the early indicators of osteoblast differentiation.<sup>16</sup> The expression of ALP strengthens as osteoblasts develop, representing the status of bone formation. This study found that, compared to the control group, the 0.00005  $\mu\text{g}/\text{mL}$  simvastatin group exhibited diffuse brown-yellow granular positive expression in the cytoplasm. The 0.0005  $\mu\text{g}/\text{mL}$  simvastatin group showed more positive expression particles, and the 0.005  $\mu\text{g}/\text{mL}$  simvastatin and 0.05  $\mu\text{g}/\text{mL}$  groups also displayed more brown positive expression particles. The expression of ALP increased with the concentration of simvastatin, consistent with previous findings.<sup>12</sup>

Calcified nodules are an important sign of osteoblast maturation. This study used an alizarin red staining kit to stain calcified



**Figure 2:** ALP staining test and alizarin red staining test results on BMSCs after treatment with different concentrations of simvastatin. We found increased ALP expression and increased calcified nodule quantity and size in BMSCs with increasing simvastatin concentrations in this study.



**Figure 3:** Western blotting results of BMP2, Smad2, and Smad3 protein expression levels in BMSCs after treatment with different concentrations of simvastatin. (a). The protein expression levels of BMP2, Smad2, and Smad3 in the four experimental groups were higher than those in the control group after a 7-day treatment with simvastatin. (b). The 0.05 μg/mL simvastatin group exhibited the highest expression levels of these proteins.

nodules and found that osteogenesis became more apparent with increasing concentrations of simvastatin. The simvastatin-treated groups significantly enhanced the calcified nodule formation ability of BMSCs compared to the control group, and this ability increased with the concentration of simvastatin, making the osteogenic phenomenon more evident. This indicates that simvastatin has a promoting effect on the osteogenic differentiation of BMSCs, with increased concentration leading to more pronounced osteogenic phenomena.

The directed differentiation process of BMSCs is a series of orderly and precisely regulated programmed processes that require the regulation of various signal transduction pathways.<sup>17</sup> The BMP2/Smads pathway is one of the main signal transduction pathways involved in the induction of osteogenic cell differentiation.<sup>14,18</sup>

The Bone Morphogenetic Protein (BMP) family is a paracrine-autocrine factor for osteogenic cells, and BMP-2 is an important member of the BMPs family, which promotes the proliferation of osteogenic cells and their precursors and induces the differentiation of osteogenic cells.<sup>19</sup> Smads can be divided into three subfamilies: receptor-activated Smads (R-Smads), common-mediator Smads (Co-Smads), and inhibitory Smads (I-Smads). R-Smads include Smad 1, 2, 3, 5, and 8.<sup>20</sup> Among them, Smad2 and 3 can accelerate the osteogenesis of cartilage by promoting the proliferation of chondrocytes and increasing the secretion of extracellular matrix.<sup>21</sup> This study found that simvastatin could upregulate the expression of BMP2, Smad2, and Smad3 in the BMP-2/Smads signaling pathway, thereby promoting the osteogenic differentiation of BMSCs. The reason

why simvastatin can upregulate these related proteins is not yet clear. Some studies have shown that statins may inhibit the liver HMG-CoA reductase, reduce the production of the HMG-CoA reductase metabolite mevalonic acid, and thereby increase the expression of the BMP-2 gene. Yamashita *et al.*<sup>22</sup> reported that statins may counteract the- $\alpha$  (TNF- $\alpha$ )-Ras/Rho/MAPK pathway, amplifying the BMP-Smad signal. The specific mechanism requires further exploration.

This study has some limitations. Further *in vivo* experiments and clinical trials are needed to provide a theoretical basis for clinical application. On the other hand, if simvastatin is to be used *in vivo* or in clinical trials, the carrier of simvastatin needs further research.

## CONCLUSION

This study demonstrates that simvastatin promotes the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) in a dose-dependent manner. Treatment with simvastatin significantly enhanced alkaline phosphatase activity, calcified nodule formation, and the expression of BMP-2/Smads pathway proteins. These findings suggest that simvastatin exerts its osteogenic effects through upregulation of the BMP-2/Smads signaling pathway, thereby facilitating differentiation of BMSCs into osteoblast-like cells. The results provide new insights into the potential application of simvastatin in bone regeneration and the treatment of conditions such as chronic apical periodontitis. Further *in vivo* studies and clinical validation are needed to confirm its therapeutic value.

## CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial relations or financial relationships of interest that might be a constant of interest.

## ABBREVIATIONS

**BMSCs:** Bone Marrow Mesenchymal Stem Cells; **ALP:** Alkaline Phosphatase; **BMP-2:** Bone Morphogenetic Protein-2; **Smads:** Mothers Against Decapentaplegic Homologs (signal transduction proteins); **CAP:** Chronic Apical Periodontitis; **MSCs:** Mesenchymal Stem Cells; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **PBS:** Phosphate Buffered Saline; **CCK-8:** Cell Counting Kit-8; **SPSS:** Statistical Package for Social Science; **TNF- $\alpha$ :** Tumor Necrosis Factor-alpha; **MAPK:** Mitogen-Activated Protein Kinase; **R-Smads:** Receptor-regulated Smads; **Co-Smads:** Common-mediator Smads; **I-Smads:** Inhibitory Smads; **HMG-CoA:** 3-hydroxy-3-methylglutaryl coenzyme A.

## ETHICAL APPROVAL

The study protocol was approved by Changzhou Stomatological Hospital. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Written informed consent was obtained from the patients or their guardians.

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## AUTHOR CONTRIBUTIONS

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## SUMMARY

This study clarifies the mechanism by which simvastatin affects bone metabolism and confirms that an appropriate dose of simvastatin can promote the differentiation of BMSCs, providing a new strategy for the repair and regeneration of bone defects. This not only provides a theoretical basis for the study of the pathogenesis of chronic apical periodontitis and new treatment methods but also opens up new horizons for the clinical application of simvastatin.

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