

Effect of BMP- 2, -4, and -7 on Proliferation and Osteogenic Differentiation in Cultured Human PDLSCs

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Research Article

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Abstract

Aim To investigate the effects of bone morphogenetic proteins (BMPs) 2, 4, and 7 on proliferation and osteogenic differentiation in human periodontal ligament stem cells (PDLSCs).

Methods PDLSCs were isolated by an immunomagnetic method. Expression of cell surface antigens CD146, CD44, and CD34, and pluripotency (osteogenic and adipogenic) were measured. Cultured PDLSCs were treated, in dose- and time-dependent experiments, with single BMPs, with 1:1 combinations, and with a mix of all three BMPs (1:3 each). For dose-dependent experiments, PDLSCs were incubated for 12 d with media containing BMPs at 0, 10, 25, 50, and 100 ng/ml. For time-dependent experiments, PDLSCs were treated with media containing 50 ng/ml BMPs for 0, 3, 6, 12, and 24 d. Cell growth and alkaline phosphatase activities were measured by MTT and an enzyme kit. Immunohistochemistry and western blotting were used to detect osteogenic differentiation-related proteins, i.e., osteocalcin, bone sialoprotein, collagen type I, and collagen type III.

Results PDLSCs displayed CD146 (93%) and CD44 (91.2%) positive expression; CD34 (1.8%) showed negative expression. All cells exhibited osteogenic and adipogenic potential. The proliferation and alkaline phosphatase activities of PDLSCs treated with the aforesaid single and combined BMPs increased in a dose- and time-dependent manner; proliferation and alkaline phosphatase activity were greater with the BMP combinations. Compared with the control group, the levels of osteogenic differentiation-related proteins increased markedly in PDLSCs treated with 50 ng/ml BMPs for 12 d, whereas no significant differences were observed between the different BMP treatments.

Conclusion BMP-2, -4, and -7, singly and in combination, promoted development and osteogenic differentiation of PDLSCs, and both cellular outcomes were more pronounced with BMP combinations.

Introduction

Periodontitis is a widespread chronic inflammatory disease characterized by irreversible disruption of the supporting periodontal tissues. If untreated, periodontitis may cause irreversible destruction of periodontal ligament, gingival tissue and supporting alveolar bone. Ultimately, tooth loss occurs (1), which may seriously affect an individual's physical and mental health. The goal of periodontitis treatment is complete reconstruction of disrupted periodontium to its original shape and function. Current conventional therapies, such as scaling and root planning (2), can prevent inflammatory progression and reconstruction of lost attachment to some extent; however, alveolar bone loss is not restored satisfactorily. In 2004, Seo et al. extracted human periodontal ligament stem cells (PDLSCs) that, for the first time, exhibited self-renewal and differentiation into various cell types (3). These cells have been considered a highly promising stem cell population for regeneration therapy in periodontium (4). However, the proliferation and osteogenic potential of PDLSCs were controlled by a variety of growth factors, extracellular matrix components, and transcription factors. Thus, there is still a challenge to simplify and improve induction of PDLSC growth and osteogenic differentiation for periodontal regeneration.

The bone morphogenetic proteins (BMPs), a 20-member family of secreted extracellular matrix-associated proteins, are widely expressed in PDLSCs while their physiological levels are insufficient for appropriate regeneration during periodontal disease (5). Among all the BMPs, BMP-2 is demonstrated to possess the greatest potential to promote bone formation (6, 7). However, exogenous BMP-2 protein is unstable and can lose bioactivity rapidly. In order to maintain the sufficient BMP-2, overdose BMP-2 was usually used in clinical practice, while a growing clinical complications have emerged, including postoperative inflammation, ectopic bone formation, and osteoclast-mediated bone resorption (8). Besides, another problem associated with the clinical application of BMP-2 is its high cost due to the need for high doses. BMP-4 has been shown to regulate bone and cartilage formation, morphogenesis, cell proliferation and apoptosis of a wide variety of tissue and cells(9). For clinical applications, recombinant human BMP-4 (rhBMP-4) is produced in Chinese hamster ovary (CHO) cells, while achieving a higher titer of functionally active rhBMP-4 in CHO cell cultures remains a challenge (10, 11). BMP-7 has been approved for clinical practice to promote bone formation in the United States, Europe, and Australia (12). However, the clinically effective doses of BMP-7 are extremely high, which leads to a heavy economic burden for patients and causes many potential side effects, such as overstimulation of osteoclastic differentiation and topical bone formation at unintended sites (13). Therefore, in the present study, we examined the effects of BMP-2, -4 and -7, singly and combined, on the proliferation and osteogenic differentiation of cultured PDLSCs to find a more effective method for periodontium regeneration.

Materials And Methods

PDLSCs isolation and culture

Human periodontal ligament (PDL) tissues were collected from the mid-root surface of third molars extracted for orthodontic reasons from six patients (3 male, 3 female, ages 18-25 years). The tissues were digested with collagenase Type I to generate single-cell suspensions. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin Solution at 37°C in a 5% CO₂ humidified atmosphere. The cells from one tooth were seeded onto a 60 mm plastic tissue culture dish. The medium was replaced every 2-3 days to enable further growth. The cells grown to 70% confluence were defined as passage zero (P₀). Later passages were named accordingly. P₃ PDLSCs were isolated by an immunomagnetic method using the marker stromatin-1 (STRO-1, R&D Systems, Minneapolis, MN, USA) (14). Briefly, 3×10⁷ cells/ml suspensions were incubated with mouse anti-STRO-1 antibody (1:200 dilution) at 4°C for 30 min. The cells were washed with PBS and incubated with rat anti-mouse IgM-conjugated magnetic beads (DynaL Biotech, Bromborough, Wirral, UK.) on a rotary mixer at 4°C for 1 h. The bead-bound cells were isolated by a magnet. After washing, the bead-bound cells were selected using a magnetic particle concentrator. Finally, STRO-1-positive cells were counted and harvested for further analysis.

PDLSCs identification

Surface marker evaluation: P₃ PDLSCs (n=6) were harvested and resuspended in culture medium at 1×10⁶ cells/ml. One ml of cell suspension was mixed with 10 µl of antibodies against CD146, CD44, or CD34 (1:100 dilution, R&D Systems, Minneapolis, MN, USA) and incubated at room temperature for 1 h. After washing with PBS, the cells were treated with FITC-conjugated goat anti-mouse IgG secondary antibody (1:100 dilution) for 30 min in the dark at room temperature. Finally, the cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Osteogenic differentiation: P₃ PDLSCs (n=6) at a density of 2×10⁴ cells/ml were seeded onto a 60 mm plastic tissue culture dish and incubated in DMEM containing 10% FBS, 10 mM β-glycerophosphate, 10 nM dexamethasone, 50 mg/l ascorbin-2-phosphate, and 200 mg/l CaCl₂. The medium was replaced every 7 days. On day 21, the cells were fixed with 4% paraformaldehyde and stained with alizarin red to assess mineral deposition.

Adipogenic differentiation: P₃ PDLSCs (n=6) at a density of 2×10⁴ cells/ml were seeded onto a 60 mm plastic tissue culture dish and incubated in DMEM containing 10% FBS, 0.5 mM 1-methyl-3-isobutylxanthine, 1 µM dexamethasone, 200 µM indomethacin, and 10 µg/ml insulin. The medium was replaced every 7 days. On day 21, the cells were fixed with 4% paraformaldehyde and subjected to oil red O staining to evaluate adipogenesis.

PDLSCs treatment

P₃ PDLSCs (n=6) were seeded onto a 96-well plate (5×10³ cells/ml) or 6-well plate (2×10⁴ cells/ml) and incubated. On day 2, they were divided into seven treatment groups: BMP-2, BMP-4, BMP-7, BMP-2+BMP-4, BMP-2+BMP-7, BMP-4+BMP-7, BMP-2+4+7 (R&D Systems, Minneapolis, MN, USA). The effect of BMP dose on the PDLSCs was determined by incubating in media containing 0, 10, 25, 50, and 100 ng/ml BMP for 12 d. For pairs of BMPs, the ratio was 50:50 and 33:33:33 for a mix of the three BMPs. For example, 25 ng/ml BMP-2 and 25 ng/ml BMP-4 was used as 50 ng/ml BMP-2+BMP-4, and 16.6 ng/ml BMP-2, 16.6 ng/ml BMP-4 and 16.6 ng/ml BMP-7 was used as 50 ng/ml BMP-2+4+7. For time-dependent experiments, PDLSCs were plated in medium containing 50 ng/ml BMPs for 0, 3, 6, 12, and 24 d.

MTT assay

P₃ PDLSCs (n=6) were incubated for indicated times in 96-well plates containing BMP concentrations as mentioned above. A 20 µl aliquot of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5mg/ml, Sigma-Aldrich, St. Louis, MO. USA) dye was added to individual wells followed by incubation for 4 h at 37°C. The supernatants were removed and 150 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO. USA) was added on a rotary mixer for 10 min. Cell proliferation was measured with a microplate reader at 570 nm. The data were recorded digitally.

Alkaline phosphatase (ALP) activity

The growth medium was removed and the PDLSCs (n=6) were washed with PBS, then incubated overnight at 4 °C in PBS containing 0.2% Triton X-100. The resulting lysates were incubated for 30 min at 37 °C with 100 µl ALP substrate solution, containing 2 mM MgCl₂ and 16 mM p-nitrophenol phosphate (Thermo Fisher Scientific, Waltham, MA, USA). The reaction was stopped with 50 µl 0.2 M NaOH, and the liberated p-nitrophenol was measured at 405 nm in a microplate reader. The change degree of absorbance was directly proportional to the ALP activity.

Immunohistochemistry

PDLSCs (n=6) were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.01% Triton X-100 for 15 min, and blocked with 1% bovine serum albumin for 30 min at room temperature. The cells were incubated with rabbit anti-osteocalcin (#73464), anti-collagen type I (#59772), anti-bone sialoprotein (#73630), and anti-collagen type III (#514601) antibodies (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight; incubation in normal rabbit serum served as negative controls. Subsequently, the cells were incubated with peroxidase-conjugated anti-rabbit secondary antibody (1:100 dilution) for 1 h, stained with 3-3-diaminobenzidine (DAB) substrate, and counterstained with hematoxylin. Finally, the cells were imaged with a Leica DM750 optical microscope, and photo micrographs were taken with a Leica DM2500 microscope.

Western blotting

Total protein (n=6) was collected with RIPA buffer, and the concentration was measured with a BCATM protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). Equal protein amounts (15 µg) were separated in 10-15% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Following blocking with 5% skim milk, the membranes were incubated overnight with anti-osteocalcin, anti-bone sialoprotein, anti-collagen type I, anti-collagen type III, and anti-β-actin antibodies (1:100 dilution) at 4°C. The membranes were then treated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (1:100 dilution) for 1 h at 37°C. The protein bands were developed with an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA).

Statistical analysis

Data analysis was performed with SPSS 19.0 software and the data were expressed as the mean ± SEM. Differences between groups were analyzed by Student's *t* test, and one-way ANOVA with Tukey's test was applied for more than two groups. A value of *P* < 0.05 was considered statistically significant.

Results

Morphology, characterization, osteogenic, and adipogenic differentiation of PDLSCs

We cultured human PDL cell suspensions. On day 5, the cells attached to the flasks displayed typical fibroblastic morphology (Figure 1A). About 14 days was required to reach 70-80% confluence at P₀. We

used the marker STRO-1 and immunomagnetic beads to purified the PDLSCs at P₃ (Figure 1B). Subsequently, we used flow cytometry to characterize PDLSC surface markers. The cells expressed mesenchymal stem cell markers CD146 and CD44, with the positive rates were 93% and 91.2%, respectively. However, PDLSCs were negative for CD34 (1.8%) (Figure 1C, D and E). We used alizarin red and oil red O staining to assess osteogenic and adipogenic differentiation of PDLSCs. The PDLSCs had formed calcified deposits and developed into lipid-laden fat cells (Figure 1F and G).

Effect of BMPs on the proliferation of PDLSCs

To assess the effect of BMP-2, -4 and -7 on the proliferation of PDLSCs, we performed dose- and time-dependent experiments. With 0-100 ng/ml BMP-2, BMP-4, BMP-7, BMP-2+BMP-4, BMP-2+BMP-7, BMP-4+BMP-7, and BMP-2+4+7, proliferation of PDLSCs increased in a dose-dependent manner ($P<0.05$). Compared with BMP-4 and BMP-7 treatment, cells proliferated to a greater extent with the BMP combinations ($P<0.05$); only the BMP-2+4+7 treatment yielded greater proliferation compared with BMP-2 treatment ($P<0.05$) (Figure 2A). Compared with the control, proliferation of PDLSCs with a fixed amount of BMP (50 ng/ml) also increased in a time-dependent manner ($P<0.05$). Growth was greater with all the BMP combinations at 6, 12 and 24 d ($P<0.05$), and only the BMP-2+4+7 treatment resulted in markedly increased proliferation at 3 d ($P<0.05$) (Figure 2B).

Effect of BMPs on the ALP activity in PDLSCs

We performed concentration and time course experiments to assess the effect of BMP-2, -4 and -7 on ALP activity (ALP is an early marker of osteogenic differentiation). All BMP treatments produced dose-dependent increases in PDLSCs ALP ($P<0.05$). Compared with single BMP treatments, ALP activity was greater with the combined BMP treatments at 50 ng/ml and 100 ng/ml ($P<0.05$). At 10 ng/ml and 25 ng/ml, the ALP activity was significantly increased only with the BMP-2+4+7 treatment ($P<0.05$) (Figure 3A). ALP activity also increased in a time-dependent manner in PDLSCs treated with all BMPs ($P<0.05$). Compared with single BMP treatments, ALP activities for all BMP combination treatments were higher at 6, 12 and 24 d ($P<0.05$) whereas, at 3 d, only the BMP-2+4+7 group exhibited increased ALP activity ($P<0.05$) (Figure 3B).

Effect of BMPs on osteogenic differentiation-related proteins in PDLSCs

We used immunohistochemistry and western blotting to assess the effects of BMP-2, -4, -7 on PDLSCs osteogenic differentiation-related proteins. Compared with the controls, the levels of osteocalcin, bone sialoprotein, collagen type I, and collagen type III treated with 50 ng/ml BMPs for 12 d were all increased ($P<0.05$), and we did not observe any significant differences between the BMP treatment groups (Figure 4 and 5).

Discussion

Periodontitis may cause irreversible destruction of tooth attachment and the surrounding bone, eventually leading to tooth-loss. Complete and predictable reconstruction of periodontal tissue destroyed by periodontal diseases is still a major challenge. Ideal periodontal reconstruction demands consideration of many factors related to periodontal development, including the use of a stem cell population, signaling molecules, and scaffold materials in ordered, temporal, and spatial sequences (15). PDLSCs, which were implicated in the generation of alveolar bone, cementum and periodontal ligament, have shown self-renewal and the potential to differentiate into multiple cell types that could produce a periodontal ligament-like structure and cementum similar to natural periodontal complex. However, successful PDLSC proliferation, migration, and maturation depended on inclusion of growth factors and full contact with extracellular matrix to control gene expression. Conversely, healing may be compromised and occur by repair instead of regeneration (16, 17). In this study, we isolated and characterized PDLSCs from the periodontal ligament of human third molars. The cells were positive for mesenchymal stem cells markers CD146 and CD44, and displayed osteogenic and adipogenic differentiation potential. Therefore, we confirmed that the cells were PDLSCs, and they could be used for subsequent experiments.

One of the most promising approaches in periodontal regeneration has been the application of growth factors, especially BMPs. Most BMPs stimulate osteogenesis, with BMP-2, BMP-4 and BMP-7 being the most potent. Investigators have found that BMP-2, BMP-4 and BMP-7 separately may stimulate proliferation and osteoblast differentiation of PDLSCs (18-21). However, there were few studies that reported relationships between PDLSCs proliferation, osteogenic differentiation, and effects of BMP combinations. Thus, we first examined the effects of BMP-2, -4 and -7 on proliferation and ALP (an early marker of osteogenic differentiation) activity. Proliferation and ALP activity increased, in a dose- and time-dependent manner, for all single and combined BMP treatments. Notably, growth and ALP activity increased to greater extents with BMP combinations.

Stem cell osteogenic differentiation and maturation of a mineralized bone matrix can be divided into early, middle, and late stages. Makers of the middle and late stages, such as collagen type I, collagen type III, osteocalcin, and bone sialoprotein, are often used to evaluate the effect of growth factors on osteogenic differentiation (22, 23). Thus, we further investigated the effect of BMPs on the levels of collagen type I, collagen type III, osteocalcin, and bone sialoprotein proteins in PDLSCs. Compared with the controls, all the maker proteins exhibited increased abundances in PDLSCs treated with 50 ng/ml BMPs for 12 d; further, we did not observe any significant differences between the BMP treatment groups. However, Qing et al. (24) reported that forced BMP-2 and BMP-7 activation with lentiviral vectors in rat adipose stem cells significantly increased expression of osteocalcin and osteopontin, and enhanced the rate of new bone formation. We speculate that the differences between our results and the findings of Qing et al. may have been due to the use of different types of stem cells. In addition, another interesting finding in our study was the difference in magnitude of response between early, middle, and late stage markers of osteogenic differentiation with combined BMP stimulation. This finding indicated that combined BMP stimulation of PDLSCs may be more important for inducing early markers compared with middle and late stage markers of osteoblastic differentiation. However, the mechanism of this effect requires further investigation.

Aoki et al. reported that combined BMP-4 and BMP-6 proteins had a synergistic effect on C2C12 myoblasts (25), which agrees with our results that BMP-2, BMP-4, and BMP-7 had a synergistic effect on PDLSCs proliferation and early osteoblastic differentiation, while Qing et al. showed in vitro that recombinant BMP-2 and BMP-7 enhanced osteoblastic differentiation under combined BMP gene transfer (24). The question of whether combined BMP gene transfer versus combined BMP protein have enhanced effects due to a similar mechanism should be a fruitful area of investigation that may yield important understanding of how BMP therapy may be improved.

In conclusion, we demonstrated that BMP-2, BMP-4, BMP-7, singly and in combination, promoted proliferation and osteogenic differentiation of PDLSCs, and the increases in both aspects of cellular behavior were greater with the various BMP combinations. Although the abundances of collagen type I, collagen type III, osteocalcin, and bone sialoprotein increased with all BMP treatments, there were no significant differences in protein expression between the treatment groups. However, all the experiments were conducted in vitro in the present study, and experiments in vivo are needed in the further study to confirm our results.

Declarations

Ethics approval and consent to participate The Ethics Committee of Xi'an Jiaotong University granted written approval to obtain human periodontal ligament samples, and all patients in this study provided written informed consent.

Consent for publication All Authors have agreed to publish the manuscript in *BMC Oral Health*.

Competing interests The Authors declare that no conflict of interest exists.

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Authors' Contributions This study is a product of the intellectual efforts of entire team; all members have contributed to various degrees. The contributions of each author are listed as follows: Juedan Li (study design, data acquisition and analysis, statistical analysis, manuscript draft); Min Wang (data acquisition and analysis, statistical analysis, manuscript revision for important intellectual content); Min Cui and Cheng Chen (data acquisition and analysis, statistical analysis); Zheng Cheng (study conception and design, final approval of the article, obtained funding, overall supervision). All authors read and approved the final manuscript.

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Availability of data and materials The datasets generated and/or analysed during the current study are not publicly available due to have no consent from all authors to share the data but are available from the corresponding author on reasonable request.

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Figures

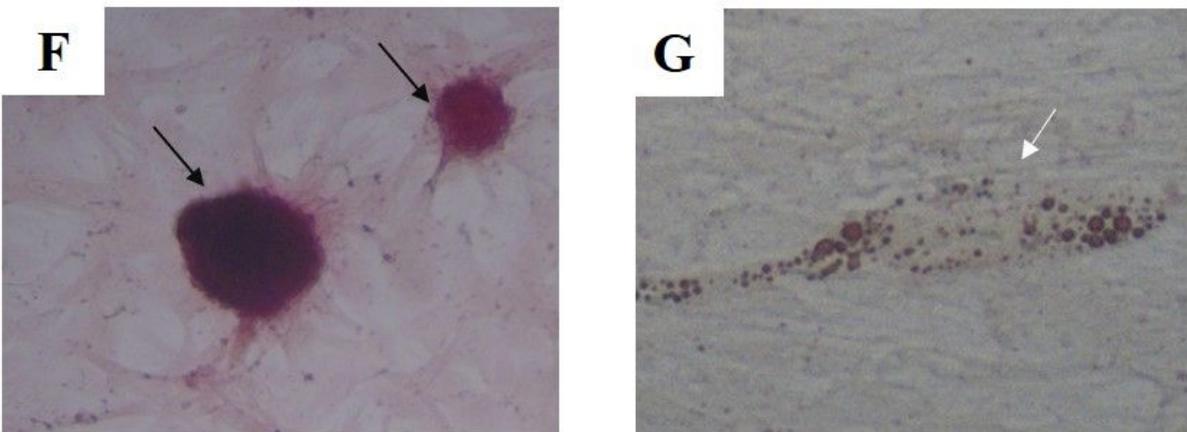
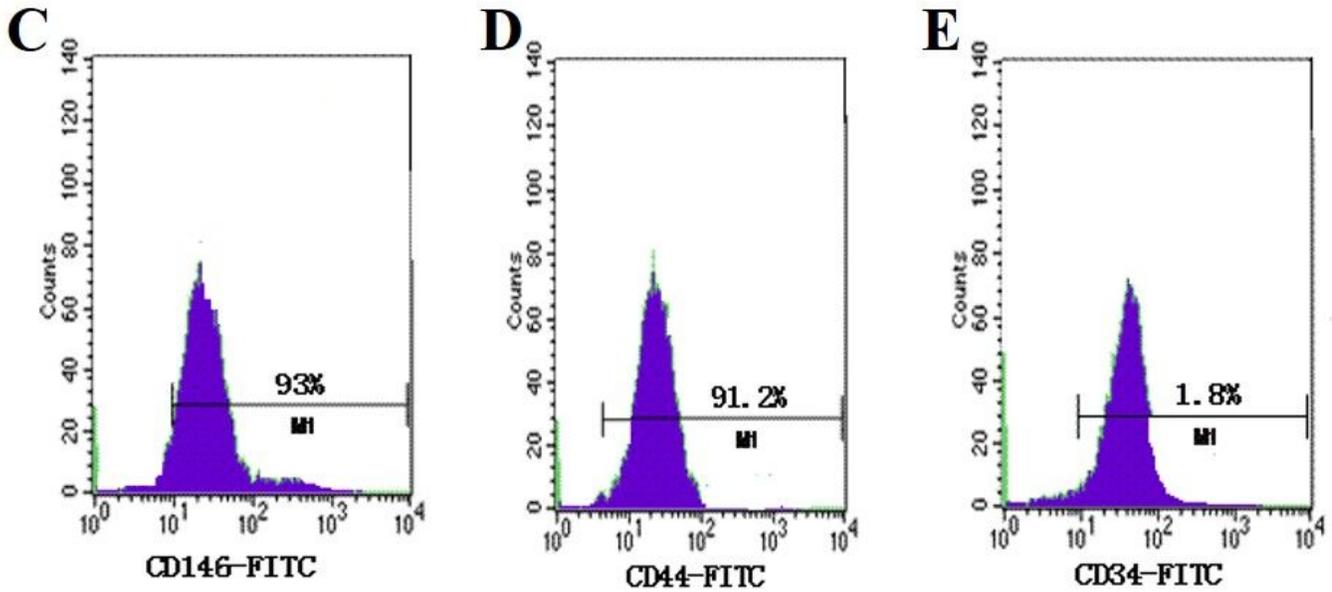
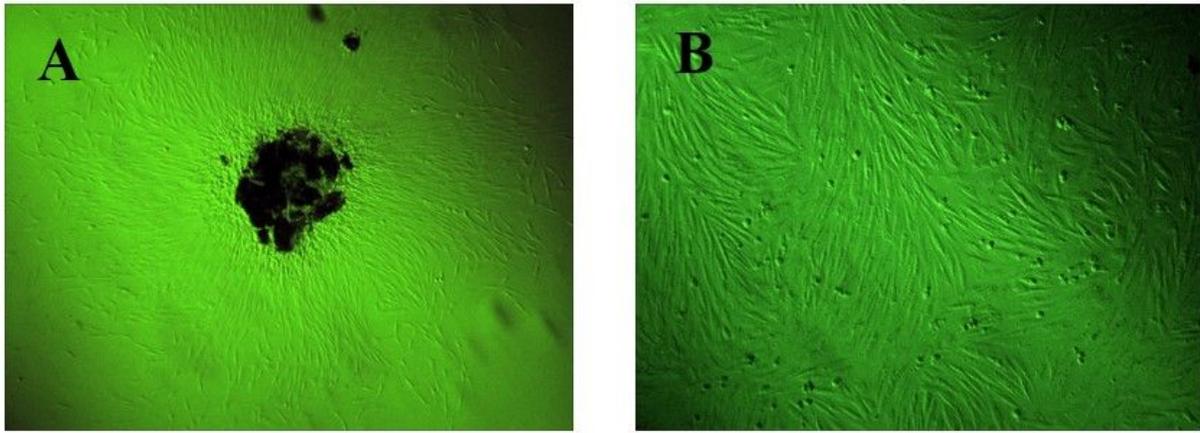


Figure 1

Morphology, characterization, and osteogenic and adipogenic differentiation of PDLSCs. PDL cell suspensions were cultured. On day 5, the cells attached to the flasks displayed typical fibroblastic morphology ($\times 100$) (A). PDLSCs were collected at P3 using the marker STRO-1 and immunomagnetic bead sorting ($\times 100$) (B). PDLSCs were positive for CD146 (C) and CD44 (D) at rates of 93% and 91.2%, respectively, but they were negative for CD34 (E). There were calcified deposits that stained with alizarin

red in the osteogenic differentiated PDLSCs 21 d after induction ($\times 200$) (F). PDLSCs developed into oil red O-positive, lipid-laden fat cells after 21 d of culture in an adipogenic-inductive medium ($\times 200$) (G). Black bars, calcified deposits that stained red; white bars, lipid-laden fat cells.

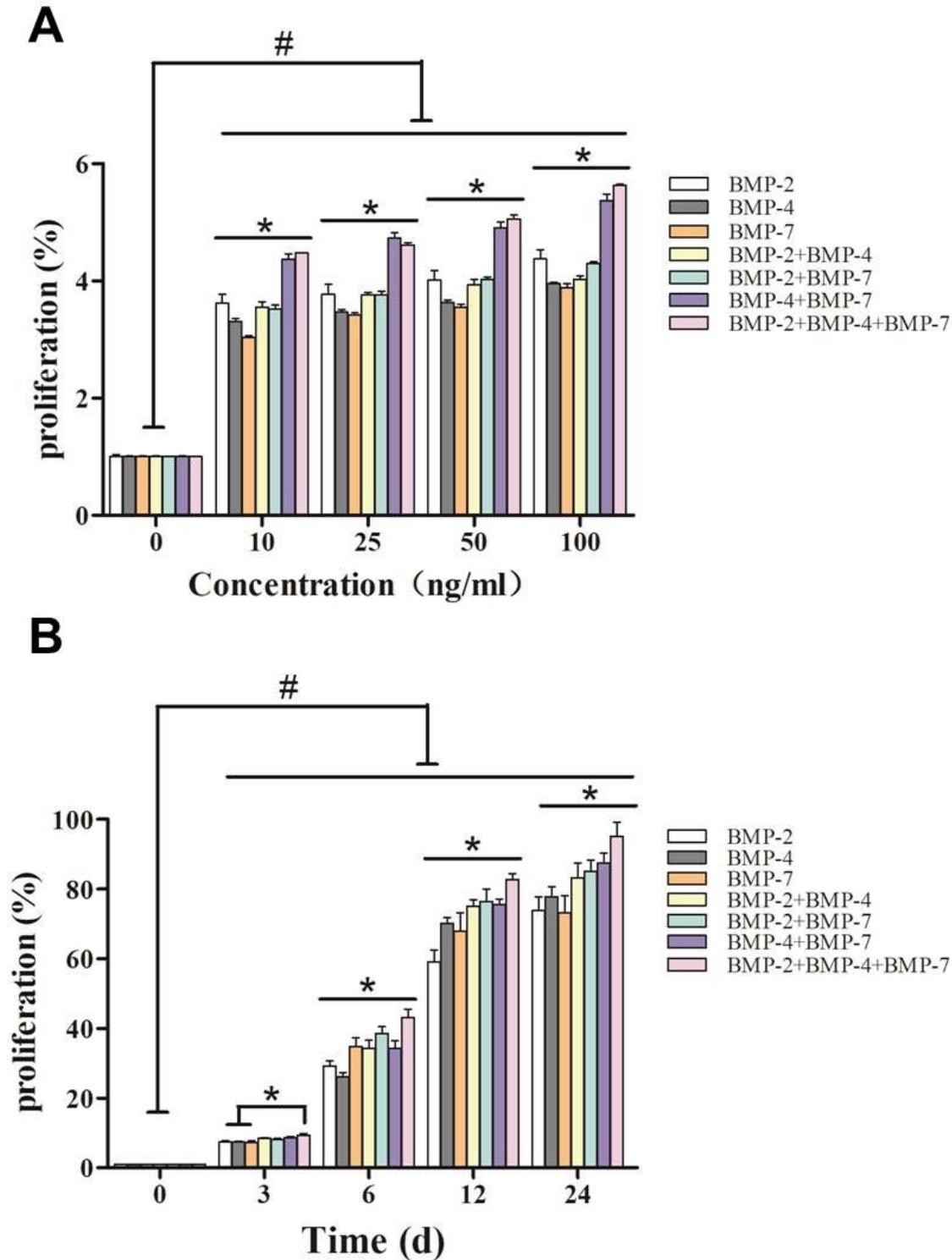


Figure 2

MTT results of the effect of BMP-2, -4, -7 on proliferation in PDLSCs. (A) PDLSCs were incubated with media containing increasing concentrations of BMPs (0, 10, 25, 50, and 100 ng/ml) for 12 d; (B) PDLSCs

were treated with media containing 50 ng/ml BMPs for 0, 3, 6, 12, and 24 d. Proliferation was measured by MTT assay. # $P < 0.05$, compared with the control groups (0 ng/ml and 0 d); * $P < 0.05$, compared with BMP treatment group; Data are presented as the mean \pm SEM.

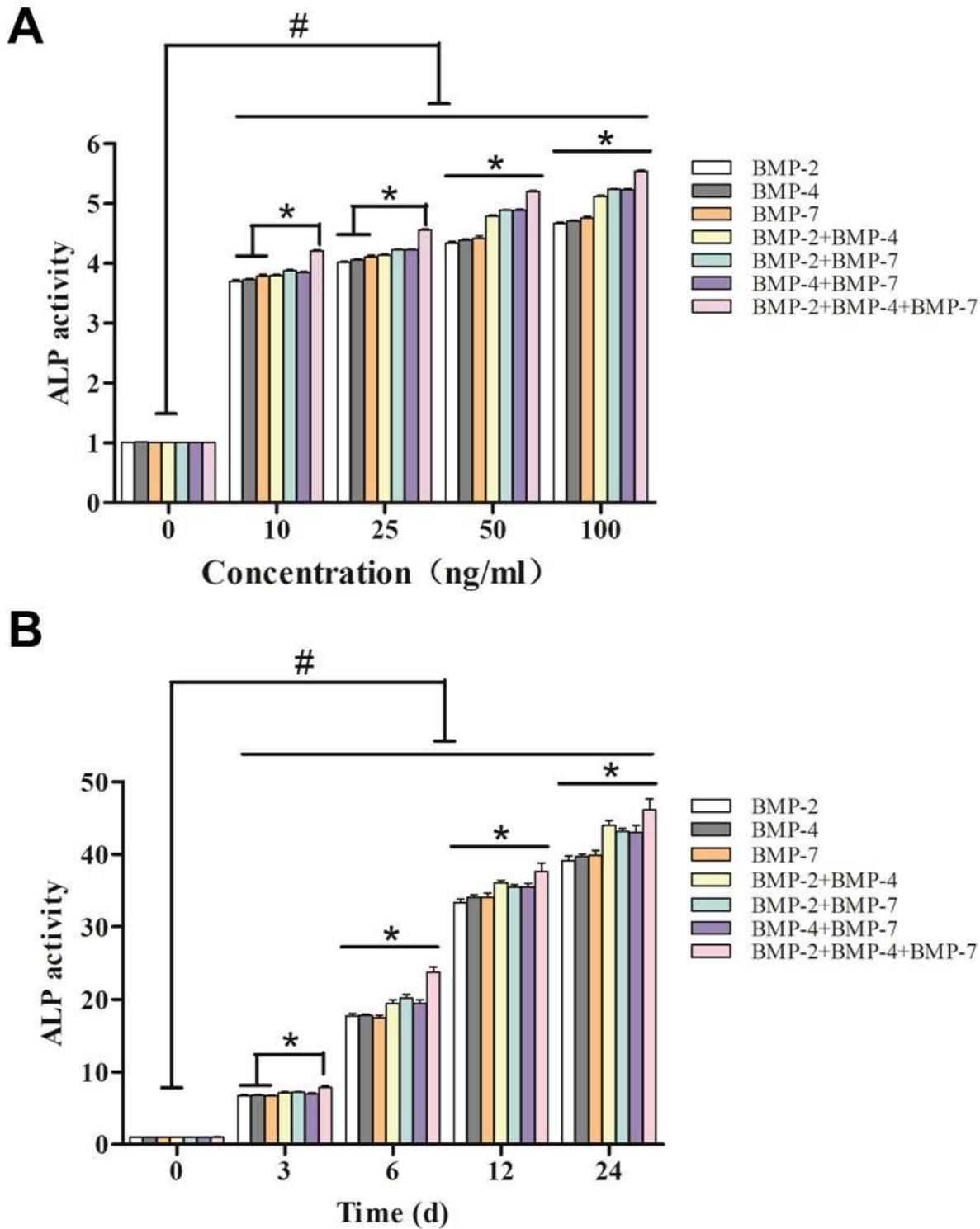


Figure 3

Statistical analysis of the effect of BMP-2, -4, -7 on ALP activity in PDLSCs. (A) PDLSCs were incubated with media containing 0, 10, 25, 50, and 100 ng/ml BMPs for 12 d; (B) PDLSCs were treated with 50

ng/ml BMPs for 0, 3, 6, 12, and 24 d. ALP activity was measured with ALP kits. # $P < 0.05$, compared with the control group (0 ng/ml and 0 d); * $P < 0.05$, compared with the BMP group; Data are presented as the mean \pm SEM.

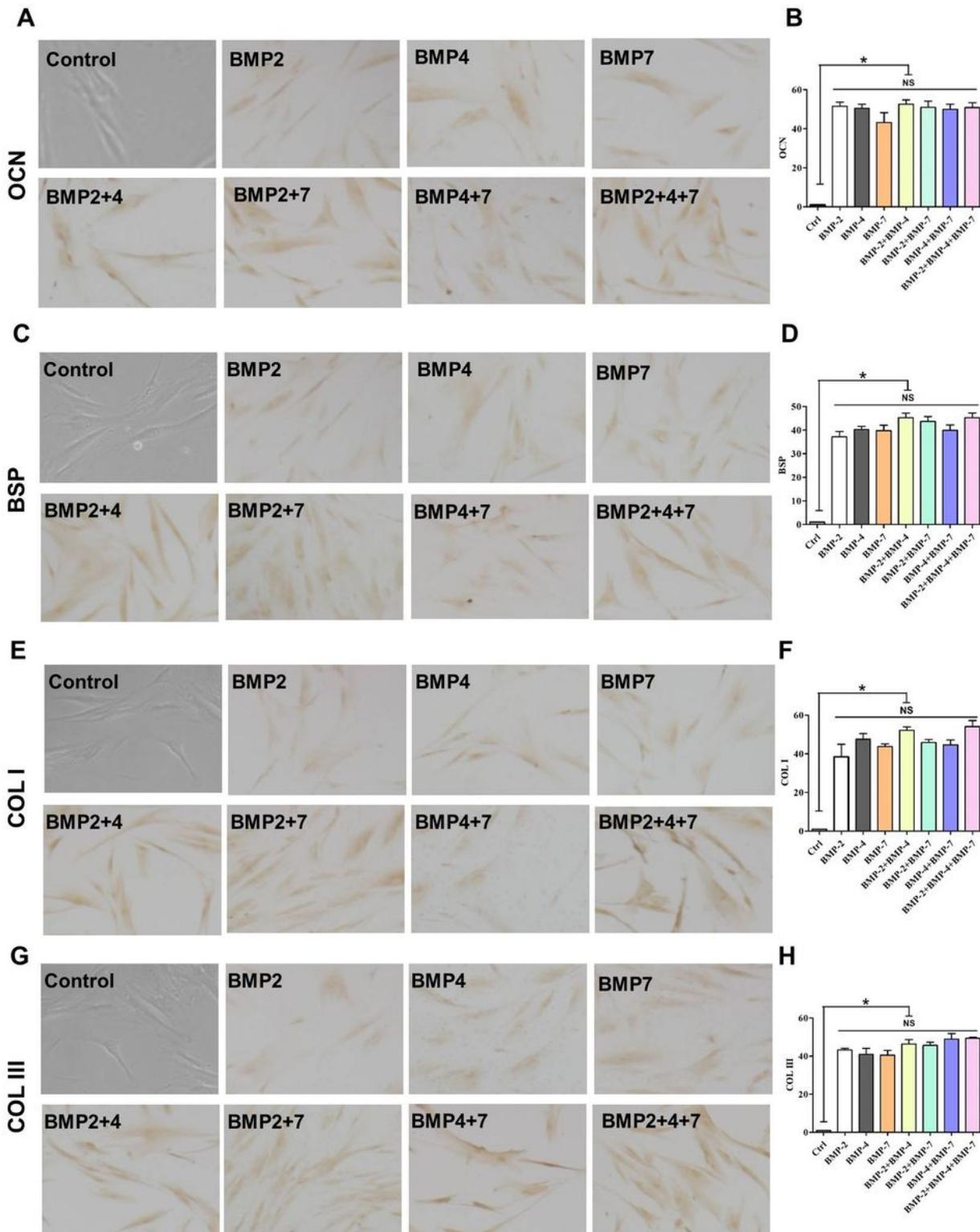


Figure 4

Immunohistochemistry of osteocalcin, bone sialoprotein, collagen type I and collagen type III proteins in PDLSCs treated with 50 ng/ml BMPs for 12 d ($\times 200$). Osteogenic differentiation-related proteins were

detected by immunohistochemistry. A: E: osteocalcin; B: F: bone sialoprotein; C: G: collagen type I; D: H: collagen type III. * $P < 0.05$, compared with the control group; NS: no significance; Data is presented as the mean \pm SEM.

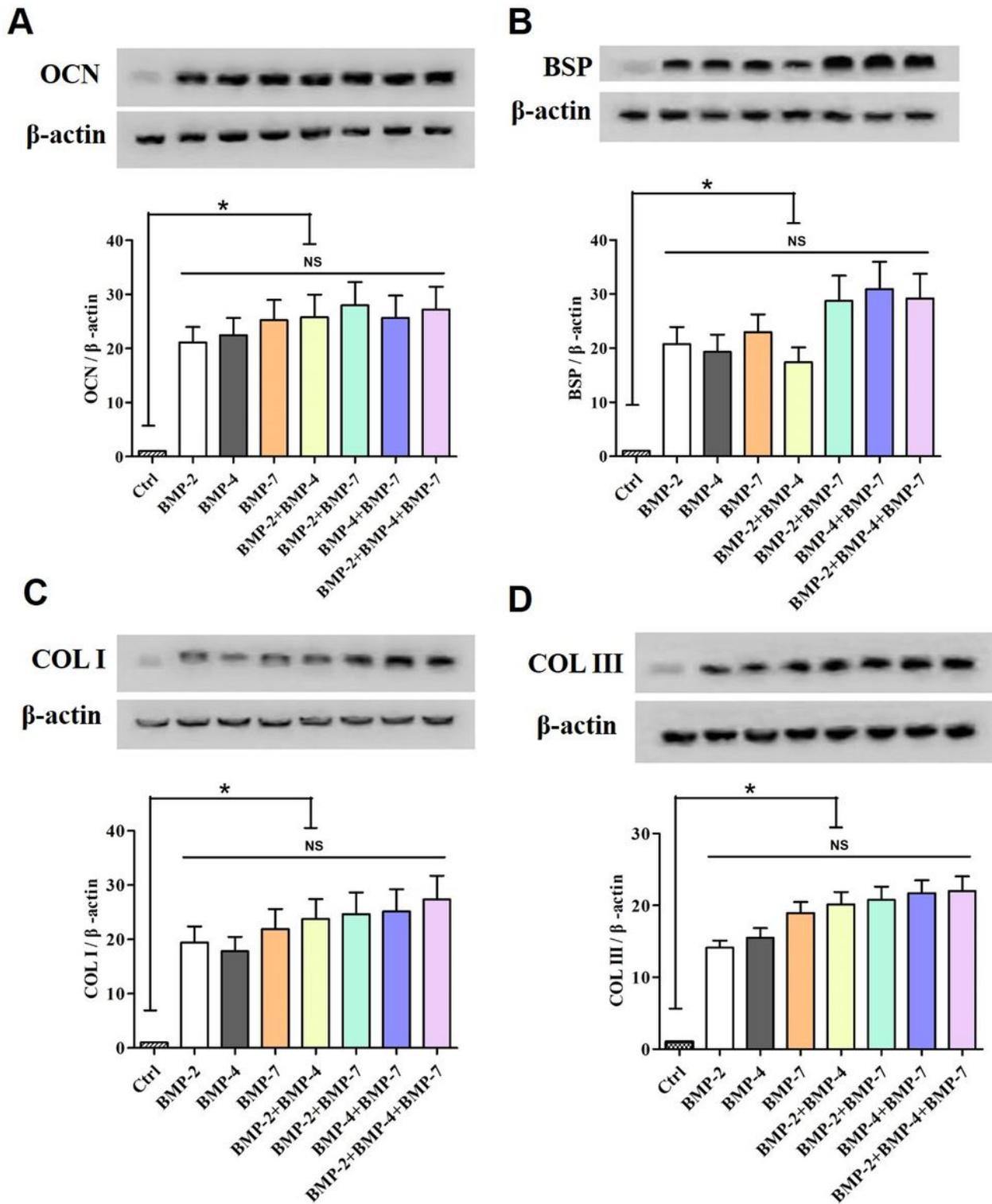


Figure 5

Western blotting of osteocalcin, bone sialoprotein, collagen type I and collagen type III proteins in PDLSCs treated with 50 ng/ml BMPs for 12 d. Osteogenic differentiation-related proteins were detected by

western blotting. β -actin was used as a loading control. A: osteocalcin B: bone sialoprotein C: collagen type I D: collagen type III. * $P < 0.05$, compared with the control group; NS: no significance; Data are presented as the mean \pm SEM.