Article

Discovery Medicine 2025; 37(194): 486–495
https://doi.org/10.24976/Discov.Med.202537194.40

Notoginsenoside R1 Promotes Osteogenic Differentiation of Dental Pulp Stem Cells via MAPK Pathway

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Published: 20 March 2025

Background: Notoginsenoside R1 (NGR1) is a bioactive compound of *Panax notoginseng* (Burk.) F.H. Chen (PNS), which possesses desirable properties in bone fracture healing and osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs). Whether NGR1 can promote osteogenic differentiation of human dental pulp stem cells (DPSCs) is still unknown. This study aimed to assess the biocompatibility of NGR1 and its impact on DPSCs.

Methods: DPSCs were obtained from human wisdom teeth. Flow cytometry and multilineage differentiation were applied to determine stem cell properties. Then, the cells were treated with NGR1 for 1, 2 and 3 days, and its efficacy was detected by means of a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Alizarin red staining (ARS), alkaline phosphatase (ALP) activity, quantitative calcium node analysis, western blot and reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) were executed to detect osteogenic differentiation-related proteins and genes. Western blot was also performed to assess the activation levels of the p38 mitogen-activated protein kinase (p-38 MAPK), c-Jun N-terminal kinase mitogen-activated protein kinase (JNK MAPK), and extracellular signal-regulated protein kinase mitogen-activated protein kinase (ERK MAPK) pathways in DPSCs following treatment with NGR1.

Results: DPSCs were positive for CD105 and CD166, while negative for CD34 and CD45. NGR1 at concentrations of 10 and 100 µg/mL did not exhibit cytotoxicity ($p > 0.05$), the group of cells receiving 200 µg/mL and 500 µg/mL NGR1 exhibited proliferation inhibition on the second day as well as on the third day ($p < 0.05$). Compared to the control group (no treatment), the cells treated with 100 µg/mL NGR1 exhibited significantly higher ALP expression and calcium deposition. The 100 µg/mL NGR1 group also showed higher expression of Osterix (OSX), Runt-related transcription factor 2 (RUNX2), Collagen Type I (COL-1), and Osteocalcin (OCN) at both protein and gene levels. Western blot analysis revealed that NGR1 activated the MAPK pathway by upregulating p38 and ERK, but not JNK, in DPSCs. When the p38 and ERK signaling pathways were inhibited by SB203580 and U0126, the gene expression levels of *OSX*, *RUNX2*, *COL-1*, and *OCN* were significantly decreased ($p < 0.05$), but such alterations were not observed with the inhibition of the JNK pathway.

Conclusion: At the concentration of 100 µg/mL, NGR1 enhances DPSC osteogenic differentiation by regulating the MAPK pathways.

Keywords: dental pulp stem cells; notoginsenoside R1; osteogenesis; mineralization

Introduction

Human dental pulp stem cells (DPSCs) are derived from enzymatically decomposed adult dental pulp [1]. These cells are well-proliferative, self-renewing, and capable of multilineage differentiation [2]. Notably, DPSCs proliferate more rapidly than bone marrow-derived mesenchymal stem cells (BMSCs) and can differentiate into odontoblasts, osteoblasts, adipocytes, and neuroblasts under laboratory conditions [3,4]. The mineralized nodules formed by DPSCs exhibit structural similarities to dentin but differ from bone structure [5]. These characteristics make DPSCs a promising cell source for both fundamental research and clinical applications.

Panax notoginseng (Burk.) F.H. Chen (PNS) is a commonly used herb in several Eastern Asian countries. In China, it has been employed to promote the healing of bone fractures for thousands of years [6]. The root of this herb is the widely used part of PNS, and notoginsenoside R1 (NGR1) is its primary active ingredient [7]. PNS has been shown to enhance alkaline phosphatase (ALP) activity, promote osteoblast proliferation, and inhibit osteoclast activity *in vitro* [8]. Additionally, it has been found to improve blood circulation and reduce blood clotting [9]. Recent research has suggested that PNS is a potential therapeutic agent for treating conditions like osteoporosis and osteonecrosis [10]. However, no previous studies have investigated the effect of NGR1 on the odontogenic and osteogenic capacity of DPSCs.

Prior research has revealed that the DPSCs possess osteogenic and odontogenic differentiation capacity, which is enabled by the activation of the mitogen-activated protein kinase (MAPK) pathway [11]. The MAPK cascade comprises three main pathways: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK [12,13]. Each of these pathways consists of a linear, three-tiered series of kinases, including MAP kinase kinase kinase/MAPKKK (MAPK3 or MKK), MAP kinase kinase/MAPKK (MAPK2 or MEK), and MAPK [14]. However, it remains unclear whether NGR1 stimulates these MAPK pathways during DPSC differentiation.

This study aims to investigate the effect of NGR1 on the osteogenic differentiation of DPSCs and its modulation of MAPK signaling pathways.

Materials and Methods

Cell Culture

Six wisdom teeth were obtained from donors aged 18–25 years at the Affiliated Stomatological Hospital of Wenzhou Medical University. The human manipulation involved in this study has been approved by The Ethics Committee of school and Stomatology Wenzhou Medical University (the approval number: WYKQ2020022), and informed consent was obtained from all donors in accordance with the Declaration of Helsinki and its subsequent amendments. DPSCs were isolated from the healthy third molars, which were extracted due to impaction or orthodontic requirements. The extracted third molars were thoroughly disinfected. Under sterile conditions, dental pulp tissue was extracted and then trimmed into approximately 1 mm^3 tissue blocks and fully covered in type I collagenase solution (17018029, Gibco, Grand Island, NY, USA) for 60 minutes at 37 °C. The tissue was digested in a culture flask with α-minimum essential medium (α-MEM, 12571603, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, A5670701, Gibco, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin-streptomycin, 15140122, Gibco, Grand Island, NY, USA) at 37 °C in a 5% CO_2 humidified atmosphere. The medium was replaced every other day until DPSCs migrated out of the tissue block. Cells in passages 3 to 8, which had achieved 70% confluence, were used for experiments. Cellular mprphology can be seen in Fig. 1B, the cell shows fibrous. Cell surface markers CD34 and CD45 are negative, while CD105 and CD166 are positive. All cells used in this study were tested negative for mycoplasma.

Flow Cytometry

Cells from passage 3 were harvested, trypsinized with 0.25% trypsin, and resuspended in phosphate-buffered saline (PBS) to produce a cell suspension with a density of 1×10^6 cells/mL. After centrifuging for 5 minutes at 1000 rpm, 1 mL of cell suspension was discarded and 500 mL of PBS was added to each flow tube. Then, 500 µL of PBS was added to each tube. For each tube, CD34 (1:100; cat. no. 559882, Grand Island, NY, USA), CD45 (1:100; cat. no. 550989, Gibco, Grand Island, NY, USA), CD105 (1:100; cat. no. 566593, Gibco, Grand Island, NY, USA), and CD166 (1:100; 12-1668-42; Gibco, Grand Island, NY, USA) flow cytometry antibodies were added; for negative controls, a vehicle was added instead. The cell-antibody mixture was incubated in the dark for 30 minutes followed by two PBS washes. The mixture was resuspended in 200 mL of PBS, prior to analysis by flow cytometry (iQue® 3, Sartorius, Gottingen, Germany).

Multilineage Differentiation

Cells from passage 3 were harvested, trypsinized with 0.25% trypsin, and seeded in 6-well plates at a density of 12×10^4 cells per well (Corning, Corning, NY, USA). At 37 °C and in a 5% CO_2 atmosphere, the cells were cultured in α-MEM supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. Specific induction media made of α-MEM culture solution containing 10% FBS, 10 mmol/L β sodium glycerophosphate, 0.05 mmol/L vitamin C, and 100 mmol/L dexamethasone were used for multilineage differentiation for 2–3 weeks. Oil Red O and Alizarin Red stainings (Sigma-Aldrich, San Jose, CA, USA) were conducted to assess trilineage differentiation according to the manufacturer's instructions [3].

Cell Proliferation Assay

Cells from passage 3 were harvested, trypsinized with 0.25% trypsin, seeded in 96-well plates at a density of 5×10^3 cells per well. The cells were respectively incubated in 100 µL of medium containing different concentrations of NGR1 (0, 10, 100, 200, and 500 µg/mL NGR1 (80418-24-2, Sigma-Aldrich, St. Louis, MO, USA) for 24, 48, and 72 hours. A 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (M6494, Gibco, Grand Island, NY, USA) was used for cell proliferation assay. A microplate reader (AC3126, Biotek, Burlington, VT, USA) was used to measure the absorbance at 450 nm.

Alkaline Phosphatase and Alizarin Red S Staining

Cells from passage 3 were harvested, trypsinized with 0.25% trypsin, and seeded in 12-well plates at a density of 1×10^5 cells per well. After incubation for 24 hours, gradient concentrations of NGR1 were added on the 7th day for ALP staining and on the 21st day for Alizarin red S (ARS) staining. Cells were fixed with 4% paraformaldehyde, prior to staining by utilizing the BCIP/NBT staining kit (P0321S, Beyotime, Beijing, China) and 2% Alizarin Red (G1452, pH 4.2; Solarbio, Beijing, China). Stained cells were incubated in the dark for 30 minutes at room temperature and observed under an inverted fluorescence microscope. To quantify the calcium nodules and Ni-

Table 1. Primer sequences for RT-qPCR.

Gene	Reverse (5′–3′)	Forward (5′–3′)
RUNX-2	GGATCCTGACGAAGTGCCAT	CCTTTACTTACACCCCGCCA
COL-1	AAGACGAAGACATCCCACCAA	CAGATCACGTCATCGCACAAC
OCN	CCAGCCTCCAGCACTGTTTA	ATTGTGGCTCACCCTCCATC
OSX	TAGCATAGCCTGAGGTGGGT	TCTGCGGGACTCAACAACTC
GAPDH	GGCTGTTGTCATACTTCTCAATGG	GGAGCGAGATTCCCTCAAAAT

Abbreviations: *RUNX-2*, runt-related transscription factor 2; *COL-1*, collagen type 1; *OCN*, osteocalcin; *OSX*, osterix; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse-transcription-quantitative polymerase chain reaction.

trotetrazolium Blue chloride (NBT) formazan matrix, 10% cetylpyridinium chloride (CPC, IC5410, Solarbio, Beijing, China) was added for 20 minutes, and the extract was then subjected to absorbance reading in a microplate (AC3126, Biotek, Burlington, VT, USA) at 562 nm.

OCN Expression Assay

Cells from passage 3 were harvested, trypsinized with 0.25% trypsin, and seeded in 12-well plates at the density of 1×10^5 cells per well. After culturing for 24 hours, gradient concentrations of 100 µg/mL NGR1 were added to the culture. Supernatants were collected after 3 and 5 days of culture, and a human Enzyme-Linked Immunosorbent Assay (ELISA) kit (ab270202, Abcam, Shanghai, China) was employed to detect OCN concentrations according to the manufacturer's instructions.

Western Blot

Cells from passage 3 were harvested, trypsinized with 0.25% trypsin, and seeded in 6-well plates at a density of 1×10^5 cells per well. Once the cells reached approximately 70% confluence, an osteogenic induction medium containing 100 µg/mL NGR1 was added, and replaced every 2 to 3 days. After 1, 4, and 7 days, 100 µL of 1% Triton X-100 (HFH10, Sigma-Aldrich, St. Louis, MO, USA) was added to each well to induce cell lysis. After centrifugation at 12,000 rpm at 4 °C for 20 minutes, the supernatant of the lysates was collected to determine the total amount of protein using a BCA assay (P0010, Beyotime, Beijing, China). Separating and stacking gels were prepared, and equal amounts of protein were loaded into the wells in the gel. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the separated proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane. Blocking was subsequently performed using 50 g/L of nonfat dry milk. Afterward, the membrane was incubated with primary antibodies, including those targeting Osterix (OSX) (1:1000; PA5-115697, Invitrogen, Waltham, MA, USA), Runt-related transcription factor 2 (RUNX2) (1:1000; PA5-82787, Invitrogen, Carlsbad, CA, USA), collagen I (1:1000; 1048-MSM1-P0, Invitrogen, Carlsbad, CA, USA), β-actin (1:5000; MA5-11869, Invitrogen, Carlsbad, CA, USA), extracellular signal-regulated protein kinase (ERK) (1:1000; 13-6200, Invitrogen, Carlsbad, CA, USA), phosphor (p)-ERK (1:1000; MA5-15174, Invitrogen, Carlsbad, CA, USA), p38 (1:1000; MA5-15116, Invitrogen, Carlsbad, CA, USA), p-p38 (1:1000; MA5-15177, Invitrogen, Carlsbad, CA, USA), JNK (1:1000; MA5-32191, Invitrogen, Carlsbad, CA, USA), and p-JNK (1:1000; MA5-15228, Invitrogen, Carlsbad, CA, USA). Following an overnight incubation with primary antibodies at 4 °C, secondary antibodies (1:10,000) targeting OSX, RUNX2, β-actin, ERK1/2, p-ERK1/2, p38, p-p38, JNK, p-JNK (goat anti-mouse IgG H+L; 31430, Invitrogen, Carlsbad, CA, USA), collagen 1 (goat anti-rabbit IgG H+L; 31460, Invitrogen, Carlsbad, CA, USA) were added and incubated for 1 hour at room temperature in TBST buffer. The membranes were rinsed three times and developed using a chemiluminescence instrument (ELX800, Biotek, Burlington, VT, USA) following the instructions of the luminescence kit (ELX800, Biotek, Burlington, VT, USA). To investigate the role of the MAPK signaling pathway, the DPSCs were pre-treated with p38 MAPK inhibitor SB203580 (S1076, Selleck, Houston, TX, USA), JNK inhibitor SP600125(S1460, Selleck, Houston, TX, USA), and ERK inhibitor U0126 (S1102, Selleck, Houston, TX, USA) (20 µmol/L) for 1 hour, followed by treatment with NGR1 at target concentration. Each experiment was repeated three times. ImageJ software (v1.8.0.345, National Institutes of Health (NIH), Bethesda, MD, USA) was employed for analysis. The relative expression levels of target proteins were determined by determining the ratio of the gray value to the internal reference protein (β-actin).

Quantitative Reverse-Transcription Polymerase Chain Reaction

Cells from passage 3 were harvested, trypsinized with 0.25% trypsin, and seeded in 6-well plates at a density of 1×10^5 cells per well. The cells were treated with an osteogenic induction medium containing 100 µg/mL NGR1. After 1, 4, and 7 days of stimulation, total RNA was collected using Trizol, and reverse-transcribed to complementary DNA (cDNA). Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) was performed on the StepOne™ real-time PCR system (4376600, Thermo

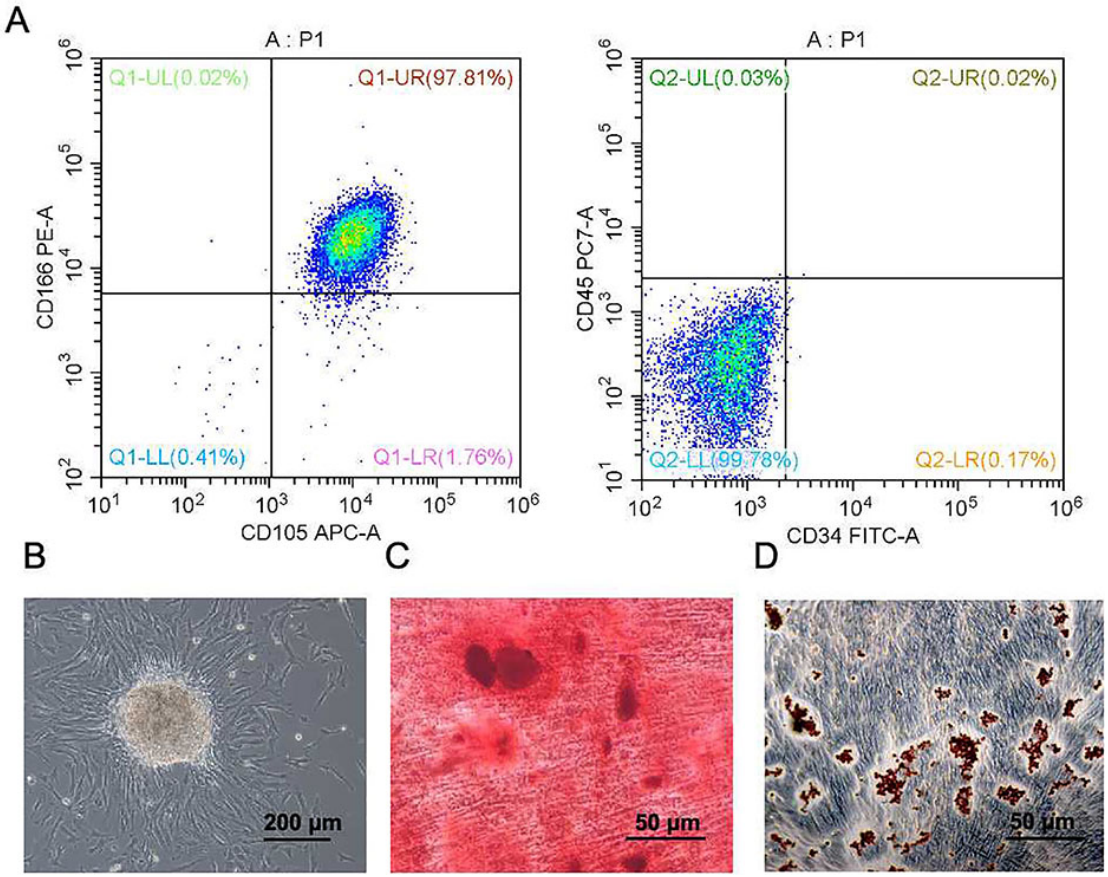

Fig. 1. Characterization of dental pulp stem cells (DPSCs). (A) Flow cytometric analysis of cultured DPSCs. (B) Morphology of DPSCs. (C) Alizarin red S staining after 21-day incubation and (D) Oil red O staining after 14-day incubation. N = 3.

Fisher Scientific, Shanghai, China) using SYBR Green Supermix (1708882, Bio-Rad, Hercules, CA, USA). The expression of related osteogenic genes, such as *OSX*, *RUNX-2*, *COL-1*, and Osteocalcin (*OCN*), in DPSCs was quantitated. A standard curve, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene, was generated using the $2^{-\Delta\Delta Ct}$ method [15]. Primer sequences are listed in Table 1.

Statistical Analysis

All data were analyzed by using GraphPad Prism5 software (GraphPad Software version 9.1, La Jolla, CA, USA). Quantitative data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with a completely random design was performed for comparing multiple groups, and two-way ANOVA coupled with Tukey's post-hoc test was employed for multiple comparisons. A *p*-value of less than 0.05 was considered statistically significant.

Results

DPSCs Shows Mesenchymal Stem Cell Properties

The DPSCs were successfully isolated and cultured using the tissue block enzyme digestion method. The cells exhibited a short spindle shape and possessed uniform cytoplasm and large nuclei (Fig. 1B). According to flow cytometry analysis, the expression levels of CD105 (99.57%) and CD166 (97.83%) for mesenchymal stem cells exceeded 90%, whereas the expression levels of CD34 (0.05%) and CD45 (0.19%) for hematopoietic stem cells were less than 1% (Fig. 1A). Alizarin red S staining revealed mineralized nodules after 21 days of osteogenic induction, and lipid droplets were observed following 14 days of adipogenic induction, confirming the multilineage differentiation potential of the cultured DPSCs (Fig. 1C,D).

NGR1 below 100 μg/mL is not Cytotoxic to DPSCs

NGR1 concentrations between 10 and 100 μg/mL showed no cytotoxic effect on the proliferation of DPSCs over time. The 500 μg/mL NGR1 inhibited cell proliferation on the first day ($p < 0.05$), while no statistically significant difference was found in the other groups ($p > 0.05$). The cell groups receiving 200 μg/mL and 500 μg/mL

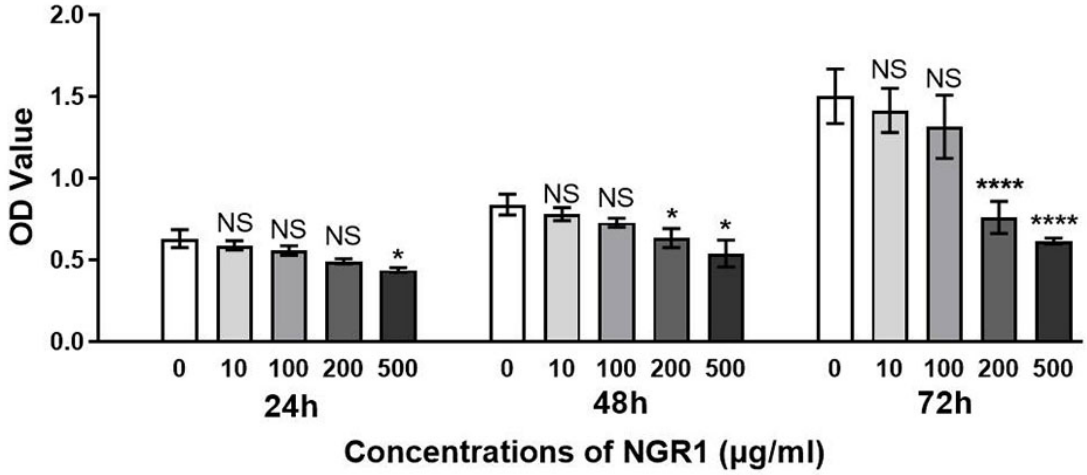

Fig. 2. NGR1 shows no toxicity in low concentrations. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was utilized for evaluating DPSCs viability at 24, 48 and 72 h. *$p < 0.05$, ****$p < 0.0001$ vs control group. NS, no statistically significant difference. OD, optical density. N = 3.

NGR1 demonstrated inhibited proliferation on the second day ($p < 0.05$). The inhibitory effect of 200 μg/mL and 500 μg/mL NGR1 on cell proliferation was significantly more pronounced on the third day ($p < 0.001$), whereas the 10 μg/mL and 100 μg/mL NGR1 did not elicit any significant effects ($p > 0.05$) (Fig. 2). Therefore, concentrations of NGR1 at or below 100 μg/mL were used in subsequent experiments.

NGR1 Significantly Increases Alkaline Phosphatase Activity and Mineralized Nodule Formation

The osteogenic differentiation of DPSCs was assessed by means of ALP and ARS staining. Results indicated that after 7 days of stimulation, both 10 μg/mL and 100 μg/mL NGR1 significantly increased intracellular ALP activity compared to the control ($p < 0.05$). After 21 days of stimulation, mineralized nodule formation in the 10 μg/mL and 100 μg/mL NGR1 groups was significantly enhanced ($p < 0.05$), with the 100 μg/mL group showing approximately a three-fold increase compared to the control ($p < 0.0001$) (Fig. 3A,B).

NGR1 Markedly Increases OCN Expression

The expression of OCN was measured after 4 and 7 days of treatment with NGR1. A dose-dependent increase in OCN expression was observed. On the fourth day, OCN expression was significantly increased only in the 100 μg/mL NGR1 group ($p < 0.05$), but no statistical difference between the 10 μg/mL group and the control group was detected ($p > 0.05$). However, on the seventh day of osteogenic induction, both the 10 μg/mL and 100 μg/mL NGR1 groups exhibited significantly increased OCN expression ($p < 0.05$). In the 100 μg/mL NGR1 group, OCN expression was much higher than that of the control group ($p < 0.01$) (Fig. 4D,H).

NGR1 Enhances Expression of Osteogenic Proteins and Genes

The impact of NGR1 on osteogenesis-associated markers, specifically OSX, COL-1, and RUNX2, was also investigated in this study (Fig. 4). The results revealed an upregulation of RUNX2 on both days 4 and 7 (Fig. 4A,E), while COL-1 (Fig. 4B,F) and OSX (Fig. 4C,G) were significantly upregulated at both the protein and mRNA levels on day 7 (Fig. 4) ($p < 0.05$).

These results confirmed that 100 μg/mL NGR1 significantly enhanced the osteogenic differentiation of DPSCs.

NGR1 Enhances Osteogenic Differentiation of DPSCs via MAPK Pathway

To further understand the molecular mechanism underlying the osteogenic differentiation of DPSCs, the study investigated the involvement of the MAPK pathway in osteogenic differentiation using Western blot. DPSCs treated with 100 μg/mL NGR1 showed increased phosphorylation levels of JNK MAPK (Fig. 5A), ERK MAPK (Fig. 5B) and p38 MAPK (Fig. 5C), whereas the total protein levels of p38 MAPK, JNK, and ERK did not experience significant changes. To validate these findings, specific inhibitors for the p38, JNK, and ERK pathways, namely SB203580, SP600125, and U0126, respectively, were used in subsequent experiments. Prior to NGR1 treatment, DPSCs were pretreated with 20 μmol/L of either inhibitor for 1 h. As a result, phosphorylation levels were downregulated in all groups (Fig. 5A–C). Both SB203580 and U0126 significantly inhibited the expression of *RUNX2*, *COL-1*, *OSX*, and *OCN* genes (Fig. 5D) in DPSCs. Further, ARS staining (Fig. 5E) revealed a significant decrease in calcium nodule formation, which was not observed in cells pretreated with the JNK-specific inhibitor SP600125.

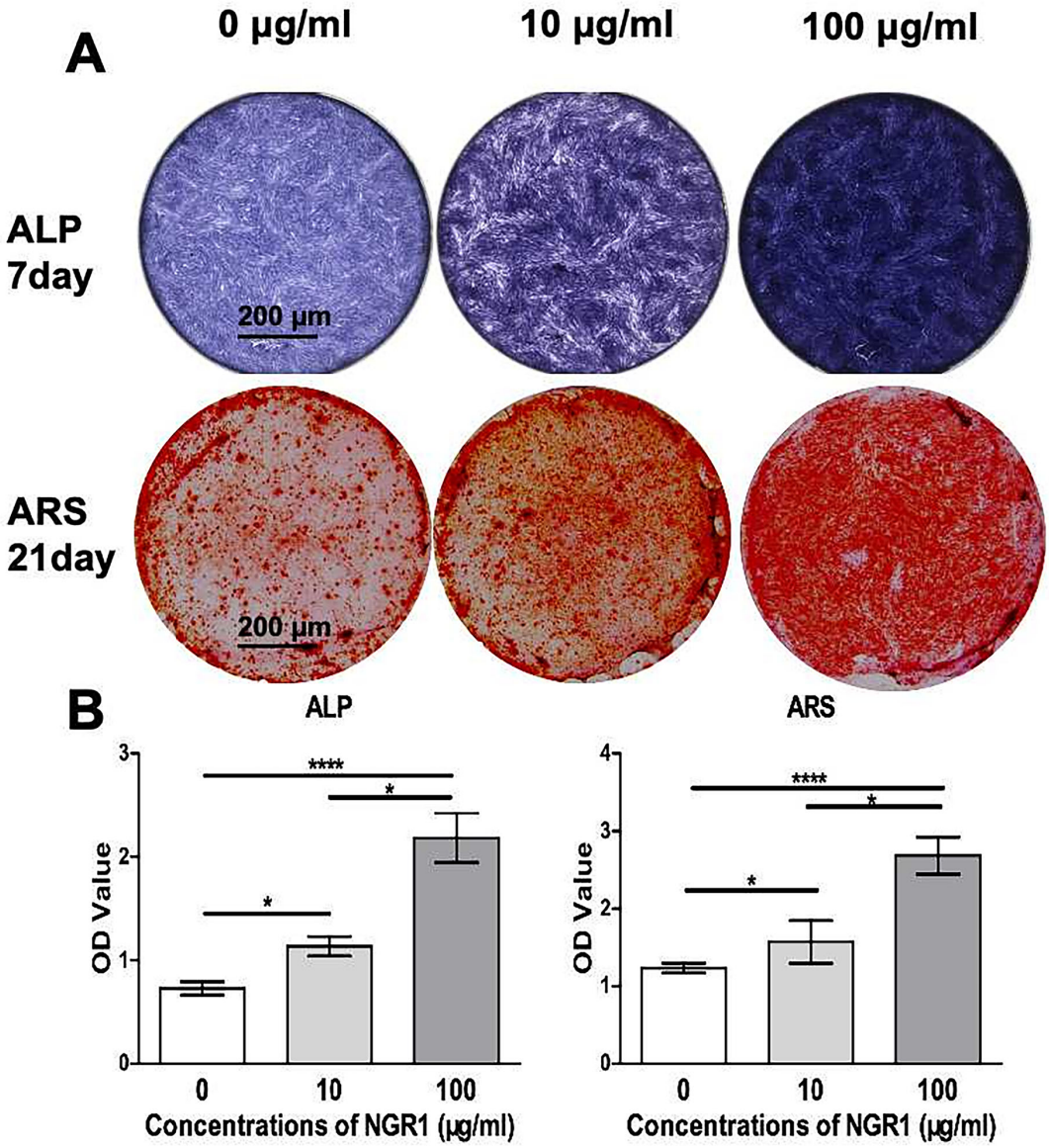

Fig. 3. NGR1 enhances osteogenic differentiation of DPSCs. (A) Alkaline phosphatase activity (ALP) staining for determining ALP activity after 7-day incubation and Alizarin red staining (ARS) staining for assessing mineralization after 21-day incubation in each group. (B) Quantification of Nitrotetrazolium Blue chloride (NBT) formazan matrix and mineralized nodules. *$p < 0.05$, and ****$p <$ 0.0001 vs control group. N = 3.

These findings suggest that the enhancement of osteogenic differentiation in DPSCs by NGR1 is hindered when the p38 MAPK or ERK pathways are inhibited (Fig. 5).

Discussion

Bone tissue engineering technology is a promising method for bone fracture healing and bone reconstruction, with its efficacy largely contingent on the osteoinductive capacity of materials. Numerous recent studies have focused on investigating small natural compounds by virtue of their ability to stimulate osteogenic differentiation, including dihydromyricetin [16], berberine [17], and catecholamine [18]. NGR1 is the main active compound of PNS, which has been applied to treat bone fractures for centuries, primarily by inhibiting pathological bone loss [19,20]. Previous studies have elucidated that NGR1 exerts a protective effect in many disorders, such as myocardial infarction and acute lung injury [21,22]. Importantly, several researchers have reported that NGR1 can directly enhance the osteogenic differentiation of MC3T3-E1 cells, human Periodontal ligament cells (hPDLCs) and bone marrow-derived mesenchymal stem cells (BMSCs) *in vitro*. But the optimal concentration of NGR1 for promoting osteogenic differentiation varies [23–25]. We speculated that differ-

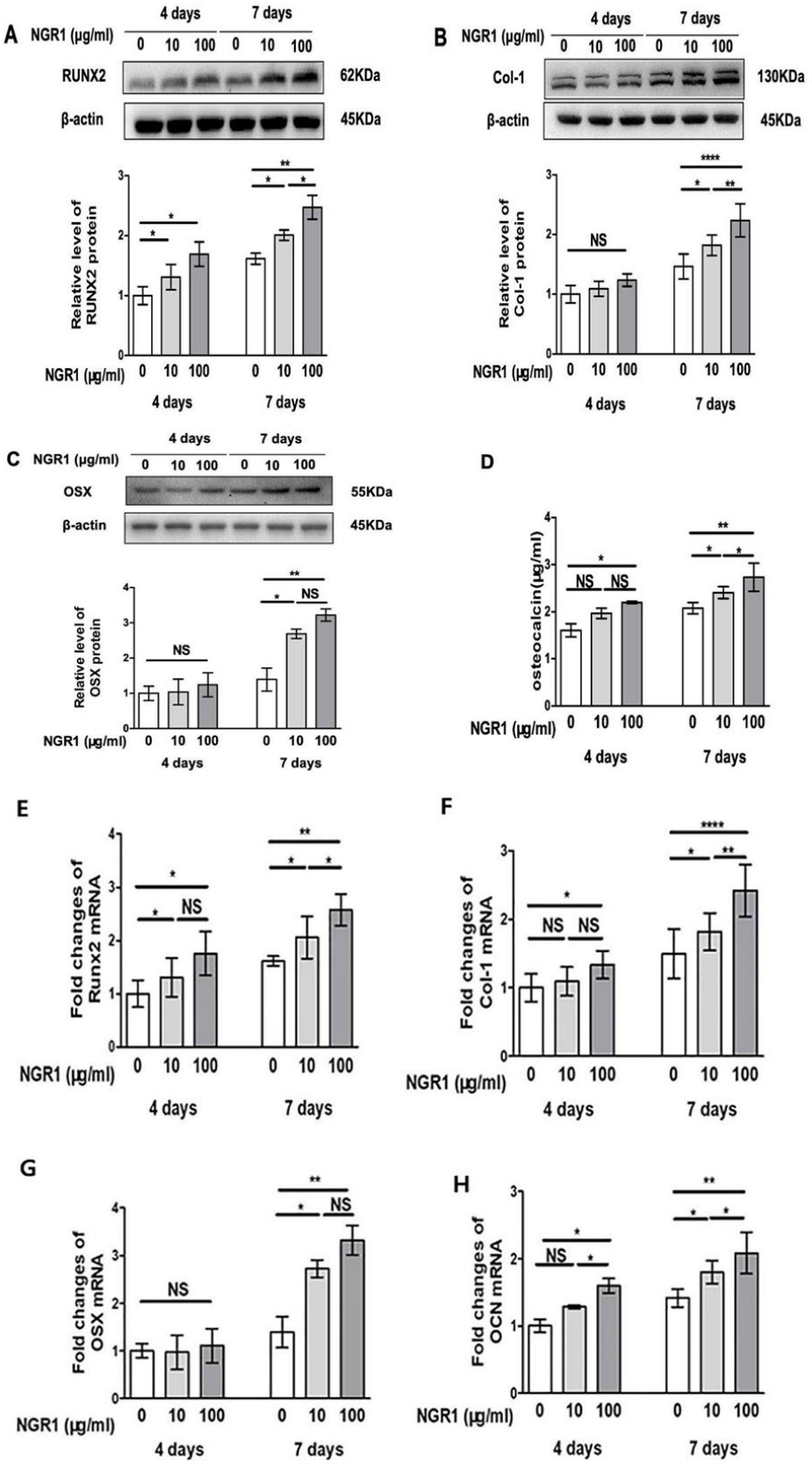

Fig. 4. NGR1 enhances osteogenic marker expression in DPSCs. Western blot for assessing protein expression levels of (A) RUNX2, (B) COL-1, and (C) OSX. (D) OCN protein expression level measured by a human Enzyme-Linked Immunosorbent Assay (ELISA) kit. (E–H) RT-qPCR analysis of *RUNX2*, *COL-1*, *OSX* and *OCN* mRNA levels. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.001$ vs the control group. NS, no statistically significant difference. N = 3.

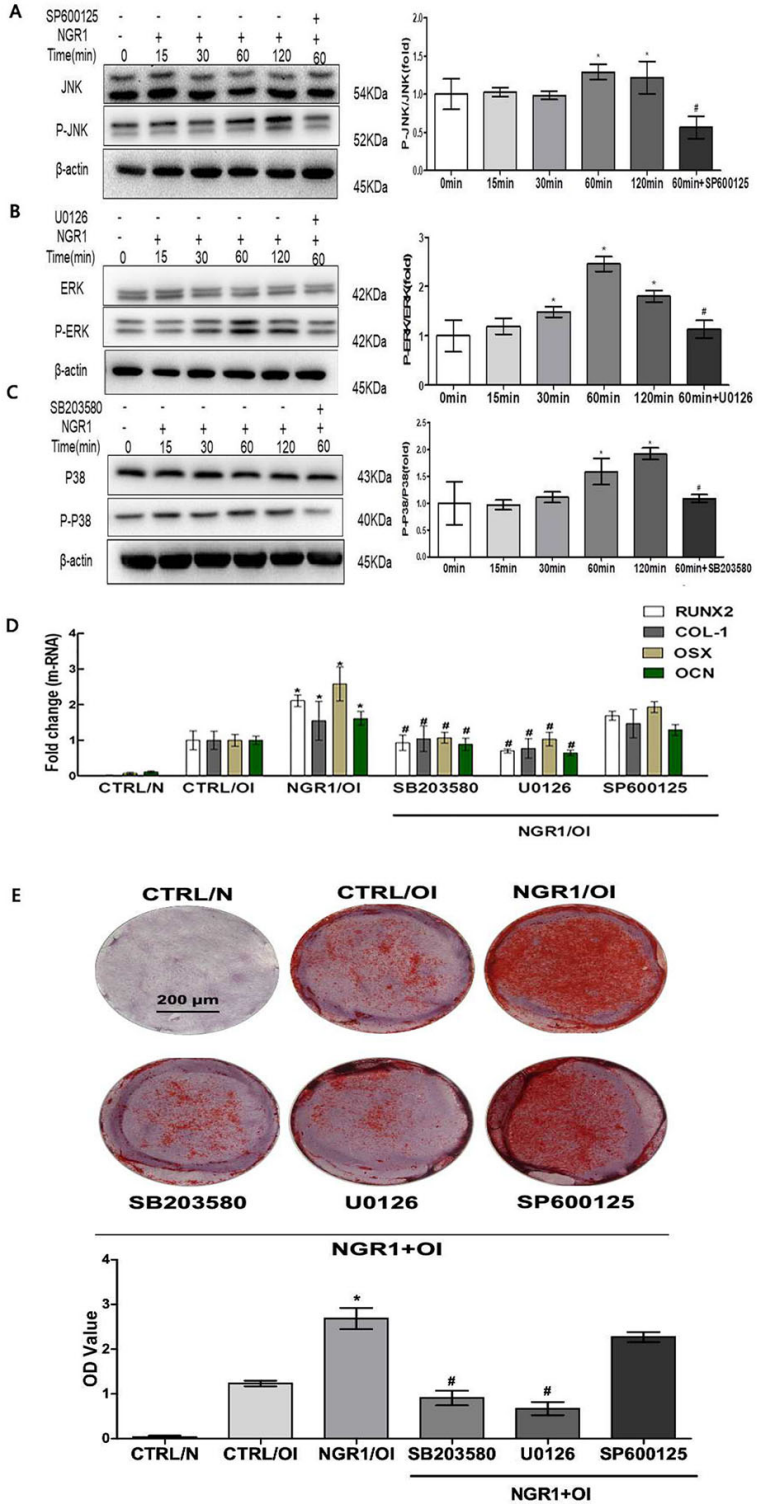

Fig. 5. Molecular phosphorylation analysis of NGR1 regulating osteogenic differentiation of DPSCs through mitogen-activated protein kinase (MAPK) pathway. Western blot analysis of p-JNK/total JNK (A), p-ERK/total ERK (B), and p-p38/total p38 (C). (D) mRNA level of *OCN*, *RUNX2*, *COL-1*, and *OSX* after treatment with p38 MAPK inhibitor SB203580, JNK inhibitor SP600125, and ERK inhibitor U0126 (20 μmol/L). (E) Alizarin red staining for detecting mineralized nodule formation and the corresponding quantification. *$p < 0.05$ vs the control group. $^{\#}p < 0.05$ vs the NGR1-treated 60 minutes group. Abbreviations: CTRL/N, control group without osteogenic induction; CTRL/OI, control group with osteogenic induction; p-JNK, phosphor-c-Jun N-terminal kinase; p-ERK, phosphor-extracellular signal-regulated protein kinase. N = 3.

ent cell types may respond differently to NGR1, and even a specific cell type may express varied effects upon treatment with different concentrations of NGR1. Thus, we hypothesize that the therapeutic effect of NGR1 is concentration-dependent and used different concentrations of NGR1 in our experiments. In this study, a low concentration of (100 μg/mL) NGR1 did not inhibit the proliferation of DPSCs and an expectedly high concentration of NGR1 (200 μg/mL) suppressed DPSCs proliferation.

Osteogenic differentiation comprises two stages: the initial stage involves matrix maturation and specific protein formation, while the second stage is characterized by the deposition of calcified nodules. ALP participates in the first stage, while calcium nodules are associated with the second stage [26]. Our study demonstrated that NGR1 can significantly promote osteogenic differentiation of DPSCs, which was evidenced by increased ALP activity, increased calcium nodule formation, and enhanced expression of proteins and genes related to osteogenic differentiation, such as RUNX-2, COL-1, OSX and OCN.

A burgeoning body of research provides an emerging picture of the strong connection between the MAPK pathway and osteogenic differentiation. MAPK, a cytoplasmic protein kinase, operates through cascading reactions mediated by p38 MAPK, ERK, and JNK pathways. These pathways regulate cell growth and respond to mitogenic substances, playing vital roles in cell proliferation and differentiation [27,28]. Chan *et al*. [29] demonstrated that activating the MAPK pathway and increasing p38 MAPK and ERK phosphorylation significantly enhanced dentin differentiation in DPSCs. Similarly, another study employing parathyroid hormone to induce DPSC differentiation found a marked increase in mineralized nodules and elevated amounts of protein bound to p-p38 MAPK and p-ERK, which was attenuated through the use of MAPK inhibitors [30]. In the current study, we observed a substantial upregulation in the phosphorylation of p38, ERK, and JNK signaling pathways. When specific inhibitors targeting the pathways of ERK MAPK and p38 were introduced, the expressions of *OSX*, *RUNX-2*, *COL-1*, and *OCN* genes were significantly downregulated. ARS staining further confirmed that DPSCs may be unable to differentiate into osteoblasts if the p38 and ERK MAPK pathways are inhibited. Notably, DPSCs were not differentiated into osteoblasts upon NGR1 treatment via the JNK pathway.

Our study revealed that 100 μg/mL NGR1 did not affect the proliferative capacity of human DPSCs. Instead, it promoted their osteogenic differentiation by activating the MAPK pathway, underscoring NGR1's pivotal role in this process. This study was limited by the lack of *in vivo* experiments to confirm the effect of NGR1 on DPSC osteogenic differentiation. Therefore, more robust evidence is required to validate our findings.

Conclusion

In this study, we demonstrated that 100 μg/mL NGR1 could enhance DPSC osteogenic differentiation through MAPK pathways. These findings offer new insights into the potential application of NGR1 in tooth and bone tissue engineering.

Availability of Data and Materials

All the data and materials supporting the findings of this study are available within the article. The raw data supporting the conclusion of this article will be made available by the corresponging author.

Author Contributions

JY and YP conceived the study. JY, XW and CC conducted the study and analyzed the data. YP supervised the study and compiled the data and figures. JY, XW, CC and YP wrote the manuscript. All the authors revised the manuscript critically for important intellectual content. The final manuscript was read by all authors and approved for publication. All the authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

The human manipulation involved in this study has been approved by The Ethics Committee of school and Stomatology Wenzhou Medical University (the approval number: WYKQ2020022), and informed consent was obtained from all donors in accordance with the Declaration of Helsinki and its subsequent amendments.

Acknowledgment

Not applicable.

Funding

This work received support from the National Natural Science Foundation of China (81870757) and the Wenzhou Technology Bureau Project (Y20210118).

Conflict of Interest

The authors declare no conflict of interest.

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