# Metformin combats high glucose-induced damage to the osteogenic differentiation of human periodontal ligament stem cells via inhibition of MAPK pathway mediated through NPR3

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**Research Article**

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## Abstract

# Background

High glucose-induced damage to the osteogenic differentiation of human periodontal ligament stem cells (PDLSCs) has long been a challenge to periodontal regeneration for diabetic individuals. Metformin is an anti-hyperglycemic drug that exhibiting abundant biological activities associated with cell metabolism and downstream tissue regeneration. However, how metformin combats damage to PDLSC osteogenic potential under high glucose and the underlying mechanisms remain unknown.

# Methods

Osteogenic differentiation of PDLSCs was assessed by Alkaline phosphatase (ALP) staining, ALP activity, Alizarin red staining and quantitative assay, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. RNA-seq analysis was performed to screen target genes of metformin, and the effects of target genes were confirmed using lentivirus transfection. Western blot analysis was also used to detect the protein level of underlying signaling pathways.

# Results

Osteogenic differentiation of PDLSCs under high glucose was decreased and metformin addition enhanced the capacity of differentiation. Furthermore, natriuretic peptide receptor 3 (NPR3) was upregulated in PDLSCs under high glucose and downregulated after metformin addition. Additionally, we demonstrated that upregulation of NPR3 compromised the metformin-enhanced PDLSC osteogenic differentiation through activating the MAPK pathway, and that inhibition of p38 MAPK or Erk1/2 pathway enhanced the osteogenic differentiation of PDLSCs with NPR3 upregulation.

# Conclusions

The present study suggests that metformin may enhance the osteogenic differentiation of PDLSCs under high glucose via downregulation of NPR3 and inhibition of its downstream MAPK pathway. This is the first report identifying the involvement of NPR3-mediated MAPK pathway in the metformin-enhanced osteogenic differentiation, indicating that NPR3 antagonists, such as metformin, may be feasible therapeutics for periodontal tissue regeneration in diabetic individuals.

## Background

Periodontal disease constitutes a group of chronic inflammatory processes characterized by destruction of supporting structures of teeth [1]. This disease has been linked to more than 50 systemic diseases, mainly including cardiovascular disease and diabetes mellitus [2, 3]. In particular, periodontal disease has

been recognized as one of the most prevalent complications of diabetes mellitus [4]. Compared to individuals without diabetes, patients with diabetes are more susceptible to periodontal disease, and the damage caused by periodontal disease could possibly be exacerbated in these patients [5, 6]. In addition, evidence has suggested that the hyperglycemic environment in diabetic patients leads to delayed periodontal wound healing and impaired tissue regeneration [7–9]. Therefore, it is essential to enhance our knowledge about the impacts of diabetes on periodontal disease and indeed to identify new agents that can promote periodontal regeneration in diabetic individuals.

Mesenchymal stem cells (MSCs) are multipotent somatic stem cells that can be isolated from a variety of human tissues, including bone marrow, adipose tissue and periodontal ligament (PDL) [10]. These cells have become promising candidates for tissue engineering and regenerative medicine [11]. Periodontal ligament stem cells (PDLSCs) are important sources of MSCs that can be isolated from PDL tissues without significant safety or ethical concerns [12]. With their advantage of multilineage differentiation, PDLSCs are becoming attractive choices in regeneration of destroyed periodontal tissues [13]. However, high glucose-induced damage to PDLSCs presents a major challenge to their applications in diabetic individuals [14]. Accumulating evidence indicates that PDLSCs incubated in high glucose exhibit impaired stem cell performance, including reduced proliferation and multilineage differentiation potentials, which would compromise the therapeutic effect of these cells [15, 16]. Therefore, enhancing the stemness of PDLSCs under high glucose is important for periodontal tissue regeneration in diabetic individuals.

Metformin is a first-line drug extensively used for the treatment of diabetes [17]. In addition to its hypoglycemic effect, metformin also possesses anti-aging, anti- inflammatory, and immunoregulatory activities, indicating that this drug has great potential for the treatment of other disorders [18–20]. In recent years, the number of studies focusing on the role of metformin on the treatment of MSCs has increasing. This drug has been reported to improve cellular proliferation and multilineage differentiation potentials in *vitro* and to enhance the survival of stem cells in *vivo* [21–23]. In addition, several studies also demonstrate that metformin treatment rescued the reduced differentiation potential of rat adipose-derived stem cells under high glucose [24]. However, how metformin combats damage to PDLSC osteogenic potential under high glucose and the underlying mechanisms remain unknown.

In this study, we hypothesize that metformin may confer a protective effect on the osteogenic differentiation of PDLSCs under high glucose. To test our hypothesis, we first measured the osteogenic differentiation of PDLSCs under normal or high glucose. Next, metformin was added to the culture medium to investigate the biological function of metformin in PDLSC osteogenic differentiation under high glucose. Furthermore, we screened and identified the potential genes and signaling pathways involved in metformin-mediated PDLSC osteogenic differentiation under high glucose. Our findings provide new insight into the regulation of cellular osteogenic differentiation under high glucose, which is beneficial for optimizing current or developing new regenerative paradigms for diabetic individuals.

# Methods

## Isolation and culture of PDLSCs

Human PDL tissue samples were obtained from third molars or teeth extracted for orthodontic reason. Written informed consent was provided by all donors (n = 5, aged 18 to 24 years), and the subsequent studies were approved by the Ethics Committee of the School of Stomatology, Fourth Military Medical University, Xi'an, Shannxi, China. In summary, freshly extracted teeth were washed using cold phosphate-buffered saline (PBS; Corning, New York, USA), and gingival tissue was excluded. PDL tissues were scraped from the middle third of the root surface and cut into small pieces (approximately 1 $mm^3$). Next, the periodontal samples were digested by type I collagenase (Sigma-Aldrich, St. Louis, USA) for 45 min in dark. After digestion, the PDL tissues were resuspended in complete Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Sijiqing, Hangzhou, China), 1% penicillin (Invitrogen, Carlsbad, CA, USA) and streptomycin (Invitrogen). The PDL tissues were finally transferred to a 6-well culture plate and cultured at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. The medium was refreshed every 2 days. Cells from passages 3 to 5 were used in the following experiments.

## Flow cytometry analysis

Flow cytometry analysis was conducted to identify the immunophenotypes of PDLSCs. PDLSCs were collected and then transferred into sterile Eppendorf tubes (Invitrogen) at $5\times10^5$ cells per tube. The cells were incubated with FITC-conjugated monoclonal antibodies against human CD31, CD35, CD45, CD90, CD105, and CD146 (all from eBioscience, San Diego, USA). A cell suspension without antibodies served as the blank control. After labeling for 1 h in dark, the PDLSCs were washed and resuspended with PBS. These procedures were performed on ice. The immunophenotypes of the cells were assessed with a Beckman Coulter Epics AL cytometer (Beckman Counter, Fullerton, USA).

## Colony forming unit (CFU) assay

To assess colony-forming efficiency of PDLSCs, cells in passage 4 were seeded in 100-mm-diameter culture dishes (Invitrogen) at $1\times10^3$ cells/dish. The medium was refreshed every 2 days. After 14 days of cultivation, the cells were fixed using 4% paraformaldehyde (Invitrogen) for 30 min and then stained with 0.1% toluidine blue (Sigma-Aldrich) for 20 min. Cell colonies were photographed with an inverted microscope (Olympus Optical, Tokyo, Japan). Cell aggregates containing more than 50 cells were recognized as colonies.

## Cell Counting Kit-8 (CCK-8) assay

Proliferation ability of PDLSCs was measured with Cell Counting Kit-8 assay. PDLSCs were seeded in 96-well culture plates at a density of $1\times10^3$. After 24 h of cell adhesion, 200 μL medium with 20 μL CCK-8 reagent (Invitrogen) was added to the test wells. The plate was then incubated at 37 °C for 2 h. After incubation, absorbance at 450 nm was detected with a microplate reader (TECAN, Männedorf,

Switzerland) to measure the proliferation capacity of PDLSCs. During the 8-day culture, all procedures mentioned were performed at proscribed time points every day.

### Osteogenic differentiation assay

For osteogenic induction, PDLSCs were seeded in 6-well plates at a density of $1 \times 10^5$ until 70~80% confluence. Cells were then cultured with osteo-inductive medium: complete DMEM or high glucose (25 mmol/L) DMEM supplemented with 10% (v/v) FBS, 1% penicillin and streptomycin, 50 μg/ml vitamin C, 10 nM dexamethasone and 10 mM β-glycerophosphate. The medium was refreshed every 2 days.

Alkaline phosphatase (ALP) staining was performed at 7 days after osteogenic induction. PDLSCs were stained using a BCIP/NBT ALP Color Development kit (Biotime, Shanghai, China) and then observed and photographed with an inverted microscope. ALP activity measurement was performed using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Alizarin red staining was conducted at 21 days after osteogenic induction. PDLSCs were fixed using 4% paraformaldehyde (Invitrogen) for 30 min. Calcium deposits were stained and then observed and photographed with an inverted microscope. The stained areas were then dissolved in 6% cetylpyridine (Sigma-Aldrich) for 15 min, and the absorbance at 560 nm was assessed using a microplate reader for quantitative assay.

### Adipogenic differentiation assay

For adipogenic induction, PDLSCs were seeded in 6-well plates at a density of $1 \times 10^5$ until 80~90% confluence. Cells were then cultured with adipo-inductive medium (Cyagen, Guangzhou, China). The medium was refreshed every 2 days.

Oil red O staining was performed at 21 days after adipogenic induction. PDLSCs were fixed using 4% paraformaldehyde (Invitrogen) for 30 min. Lipid droplets were stained and then observed and photographed with an inverted microscope. The stained areas were then dissolved in isopropanol (Sigma-Aldrich) for 15 min, and absorbance at 560 nm was measured using a microplate reader for quantitative assay.

### Chondrogenic differentiation assay

For chondrogenic induction, PDLSCs were collected in 15-mL centrifuge tubes (Invitrogen) at a density of $2 \times 10^5$ cells/tube. The cells were washed with PBS and then resuspended in chondro-inductive medium (Cyagen, Guangzhou, China). After 24 h, cell spheres were visible at the bottom of the tubes. The medium was refreshed every 2 days.

Alcian blue staining was performed at 21 days after chondrogenic induction to measure the chondrogenic differentiation ability of PDLSCs.

### Selection of the optimal concentration of metformin

To select the optimal concentration of metformin (Sigma-Aldrich), the toxic and protective effects of metformin were assessed by CCK-8 assay. For toxic effect, PDLSCs were seeded in 96-well culture plates at a density of $5\times10^3$ with different concentrations of metformin (0, 10, 100, 500 and 1000 μM). Cell viability was measured at 6, 12, 24, 48 and 72 h later. For protective effect, PDLSCs were seeded in 96-well culture plates at a density of $1\times10^3$ with the aforementioned concentrations of metformin. Cell viability was measured daily during the 6-day culture period. Accordingly, concentrations of metformin that induced cell toxicity were excluded. Ultimately, a concentration that conferred the maximal protective effect was selected.

### RNA-seq analysis

RNA-seq analysis was performed to detect differentially expressed genes in PDLSCs cultured in different osteo-inductive mediums. In brief, total RNA was extracted using a TRIzol reagent kit (Invitrogen). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) and verified using RNase-free agarose gel electrophoresis. Then, eukaryotic mRNA was enriched with Oligo (dT) beads, and total RNA was fragmented into short fragments using fragmentation buffer and reversely transcribed into cDNA with random primers. Second-strand cDNA was synthesized by DNA polymerase I, RNase H, dNTP and buffer. The cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), and then, the ends were repaired, and poly(A) was added. The fragments were ligated to Illumina sequencing adapters. Desired genes were selected and compared to determine changes in the expression levels of relevant genes. After purification, cDNA fragments were sequenced using an Illumina Novasqr6000 by Gene Denovo Biotechnology CO (Guangzhou, China).

### Lentivirus transfection

Lentivirus transfection was performed to stably upregulate the expression of natriuretic peptide receptor 3 (NPR3) in PDLSCs. In summary, PDLSCs were cultured until 40~50% confluence and then transfected with lentivirus containing NPR3 sequence in the presence of polybrene (1 μL/mL). The medium was replaced 24 h after transfection. Cells transfected with lentivirus lacking NPR3 sequence were used as negative controls. Lentiviruses containing or lacking NPR3 sequence were designed and synthesized by Gene Pharma (Shanghai, China).

The efficiency of lentivirus-mediated upregulation of NPR3 expression was confirmed by qRT-PCR, Western blot analysis and Immunofluorescence staining.

### Enzyme-linked immunosorbent assay (ELISA)

After 7 days of osteogenic induction, the culture mediums were collected and centrifuged to remove the cells and debris. The concentrations of C-type natriuretic peptide (CNP) secreted into the cell culture supernates were detected with a ELISA kit (Rebiosci, Shanghai, China).

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymarese chain reaction (qRT-PCR) was conducted to measure mRNA expression levels following the manufacturer' s instructions. In brief, Total RNA was extracted from cells with TRIzol reagent (Invitrogen). Extracted total RNA was reverse transcribed to cDNA using Evo M-MLV RT Premix (Takala, Shiga, Japan). Quantitative real-time PCR was performed using the SYBR Green Premix Pro Taq HS qPCR kit (Tli RNaseH Plus; TaKaRa), and the results were analyzed with a CFX96 Real-time RT-PCR system (Bio-Rad, Hercules, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the expression levels. The expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences used for qRT-PCR are listed in Table 1.

### Western blot analysis

Western blot analysis was performed to measure protein expression levels in PDLSCs. Briefly, Prepared cells were first lysed in RIPA lysis buffer (Biotime) supplemented with protease and phosphatase inhibitors (Biotime). A bicinchoninic acid (BCA) assay kit was then used to measure protein concentrations. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Biotime) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk at room temperature for 2 h, the membranes were incubated with primary antibodies at 4 °C overnight. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000; goat anti-rabbit IgG, CST, #7074; goat anti-mouse IgG, CST, #7076) at room temperature for 2 h. Subsequently, the blots were visualized using enhanced chemiluminescence substrate (ECL kit, Millipore), and protein bands were analyzed with Image J software. GAPDH was used as the housekeeping gene for internal normalization. The following primary bodies were used for Western blotting: GAPDH (1:3000; Affinity, AF7021), ALP (1:10000; Abcam, ab108337), RUNX2 (1:1000; CST, #12556), BMP2 (1:1000; Abcam, ab214821), OCN (1:1000; Santa cruz; Sc-390877), NPR3 (1:1000; Abcam, ab177954), p38 MAPK (1:1000; CST, #9212), p-p38 MAPK (1:1000; CST, #4511), JNK (1:1000; CST, #9252), p-JNK (1:1000; CST, #4668), ERK1/2 (1:1000; CST, #9102) and p-ERK1/2 (1:1000; CST, #9101).

### Immunofluorescence staining

Immunofluorescence staining was performed to visualize NPR3 in PDLSCs. Briefly, cells were fixed with 4% paraformaldehyde for 30 min, followed by treatment with 0.5% Triton X-100 and 2% bovine serum albumin (BSA; Sigma-Aldrich). Then, the cells were incubated with primary antibodies against NPR3 (1:100; Abcam, ab97389) over night. The secondary antibodies for NPR3 were Alexa Fluor 488 AffiniPure donkey antirabbit IgG (1:100; Yeasen Biotech Company, Shanghai, China). Finally, the cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Abcam, ab104139), and the fluorescence images were acquired using a Beckman Coulter Epics XL cytometer (Beckman Coulter).

### Statistical analysis

All data are presented as the mean ± standard deviation of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 8 software. One-way analysis of variance

followed by Tukey's multiple comparisons tests, Sidak's multiple comparisons tests or Dunnett's multiple comparisons was used for comparing more than two groups, and unpaired two-tailed Student's t test was used for comparisons of two unpaired groups. Statistical significance was expressed as $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

# Results

# Characterization of human PDLSCs

The results of CFU assay confirmed that the PDLSCs we obtained possessed colony formation ability (Supplementary Fig. 1A). These cells continued to multiply, as suggested by the CCK-8 assay results (Supplementary Fig. 1B). The cells were also able to differentiate into multilineage cells when cultured in osteo-inductive, adipo-inductive and chondro-inductive medium, as evidenced by Alizarin red staining, Oil red O staining and Alcian blue staining, respectively (Supplementary Fig. 1C). In addition, flow cytometry analysis verified that these PDLSCs positively expressed MSC markers (CD90, CD105, and CD146) but not the markers of endothelial cells (CD31) or hematopoietic cells (CD34 and CD45) (Supplementary Fig. 1D).

### Incubation of PDLSCs under high glucose impaired cellular osteogenic differentiation

The osteogenic differentiation of PDLSCs under normal or high glucose was measured respectively. We found that incubation of PDLSCs under high glucose led to fewer ALP staining-positive cells and lower cellular ALP activity (Fig. 1A). The results of Alizarin red staining further confirmed that less calcium deposits were formed by PDLSCs under high glucose compared to the control group (Fig. 1B). In addition, the results of qRT-PCR revealed that the osteoblast differentiation-related genes *ALP*, *RUNX2*, *BMP2* and *OCN* were downregulated in PDLSCs under high glucose (Fig. 1C). Similarly, the expression of osteoblast differentiation-related proteins ALP, BMP2 and OCN were also decreased in PDLSCs under high glucose, but no obvious change was observed in the protein level of RUNX2 (Fig. 1D).

### Metformin enhanced the osteogenic differentiation of PDLSCs under high glucose

To investigate the effect of metformin on the impaired PDLSC osteogenic differentiation under high glucose, metformin was added to the culture medium. We first selected the optimal concentration of metformin. During a 72-h incubation, cell viability was not significantly changed by different concentrations of metformin (0, 10, 100, 500 and 1000 μM) under high glucose (Supplementary Fig. 2A). In addition, PDLSCs treated with 100 μM metformin exhibited a higher proliferation rate than those in the other groups (Supplementary Fig. 2B). Hence, metformin at concentration of 100 μM was selected for further studies.

After metformin addition, the number of ALP staining-positive PDLSCs was increased, and ALP activity in cells was also enhanced (Fig. 2A). Consistently, the results of Alizarin red staining indicated that more calcium deposits were formed by PDLSCs with metformin addition than those without (Fig. 2B).

Furthermore, the results of qRT-PCR revealed that after metformin addition, the gene levels of *ALP*, *RUNX2*, *BMP2* and *OCN* were significantly increased in PDLSCs under high glucose (Fig. 2C). The protein levels of ALP, BMP2 and OCN in PDLSCs under high glucose were also elevated after metformin addition, but no obvious change was observed in the protein level of RNUX2 (Fig. 2D).

## Screening and identification of osteogenesis-related genes mediating the metformin-enhanced osteogenic differentiation under high glucose

To explore potential genes involved in the metformin-enhanced osteogenic differentiation under high glucose, RNA-seq analysis was performed to analyze differentially expressed osteogenesis-related genes in PDLSCs cultured in normal (CON group), high glucose (HG group) or high glucose with metformin addition (MET group). The threshold criteria for screening up- or downregulated genes was fold change >=2.0 and *p* value <=0.05. According to the results of RNA-seq analysis, 84 differentially expressed genes were screened out (Fig. 3A). Among these differentially expressed genes, 38 genes were highly expressed in HG group, and 46 genes were highly expressed in CON group and MET group (Fig. 3B). As we were specifically interested in osteogenic differentiation, these 84 genes were further examined to identify those coding osteogenesis-related proteins. Further analysis revealed that five of these 84 genes potentially contribute to cellular osteogenic differentiation. Among these five genes, *AREG*, *IGFBP5*, *GPR68* and *SFRP2* were upregulated in CON group and MET group but downregulated in HG group, while *NPR3* was upregulated in HG group but downregulated in the other two groups (Fig. 3C).

To further verify these results, we examined the expression of the five genes with qRT-PCR, and found that the expression patterns of *NPR3* and *SFRP2* were consistent with the results of RNA-seq analysis (Fig. 3D). We then performed Western blot analysis to validate the protein expression levels of NPR3 and SFRP2, and the results showed that the protein expression pattern of NPR3 was in line with the results of RNA-seq analysis and qRT-PCR (Fig. 3E).

## NPR3 played a role in the metformin-enhanced osteogenic differentiation of PDLSCs under high glucose

To further investigate the relationship between the metformin-enhanced osteogenic differentiation and the expression of NPR3, we constructed a lentivirus vector containing NPR3 gene to stably upregulate NPR3 expression in PDLSCs. The efficiency of lentivirus-mediated upregulation of NPR3 expression was confirmed by qRT-PCR, Western blot analysis and Immunofluorescence staining (Supplementary Fig. 3A-C). The expression of NPR3 in PDLSCs among the groups was visualized by Immunofluorescence staining (Fig. 4A). After NPR3 upregulation, fewer ALP-positive cells and lower cellular ALP activity were found in PDLSCs with metformin addition (Fig. 4B). The results of Alizarin red staining and quantitative analysis further revealed that less calcium deposits were formed by metformin-treated PDLSCs after NPR3 upregulation (Fig. 4C). In addition, we found that metformin-enhanced gene expression of *ALP*, *RUNX2*, *BMP2* and *OCN* were reduced upon upregulation in NPR3 expression (Fig. 5A-D). The results of Western blot analysis also confirmed that the protein levels of ALP, BMP2 and OCN in metformin-treated PDLSCs were decreased after NPR3 upregulation, and no significant difference was found in the protein level of RUNX2 among the groups (Fig. 5E-I).

### Metformin enhanced PDLSC osteogenic differentiation under high glucose via inhibition of the NPR3-mediated MAPK pathway

As indicated in previous studies, the Mitogen-activated protein kinase (MAPK) pathway is a downstream signaling pathway of NPR3 and can be activated by NPR3 in both CNP-dependent and CNP-independent way [25–27]. Moreover, MAPK pathway has been demonstrated to regulate the osteogenesis process and bone development [28, 29]. To determine whether NPR3-mediated MAPK pathway could alter the protective effect of metformin on cellular osteogenic differentiation under high glucose, we detected the protein expression levels of critical members of MAPK pathway. We first detected the expression of CNP. The results of qRT-PCR and ELISA showed that the expression of CNP was increased in PDLSCs treated with metformin compared to those not treated with metformin, but this increase was drastically reduced after upregulation of NPR3 (Fig. 6A, B). Western blot analysis showed that the expression of phosphorylated p38 MAPK and Erk1/2 was upregulated in PDLSCs under high glucose and downregulated after metformin addition. However, the metformin-mediated downregulation was further reversed by upregulation of NPR3. No significant difference was observed in phosphorylated JNK and total levels of p38 MAPK, Erk1/2 and JNK protein (Fig. 6C, D).

To further validate our findings, p38 MAPK and Erk1/2 was inhibited with specific inhibitor SB203580 (MedChemExpress, Monmouth Junction, NJ, USA) and U0126 (MedChemExpress) respectively. The NPR3-upregulated PDLSCs were treated with 20 μM inhibitors separately for 2 h before osteogenic-induction. Although upregulation of NPR3 decreased the mRNA expression levels of *ALP*, *RUNX2*, *BMP2* and *OCN* in PDLSCs with metformin addition, the treatment of SB203580 (Fig. 7A) or U0126 (Fig. 7B) significantly increased the expression of these genes. Similarly, the decreased protein expression levels of ALP, RUNX2 and OCN in NPR3-upregulated PDLSCs were also increased after inhibition of p38 MAPK (Fig. 7C) or Erk1/2 inhibitor (Fig. 7D). But no obvious change was found in the protein level of BMP2 after inhibition of p38 MAPK pathway (Fig. 7C).

## Discussion

The destruction of periodontal supporting tissues is initiated and aggravated during the pathogenesis of periodontal disease [1]. In the past decades, MSCs, particularly PDLSCs, have become promising candidates for repairing alveolar bone defect and mediating periodontal tissue regeneration [12, 13]. However, accumulating evidence suggests that long-term exposure to diabetic conditions might cause cell damage; and such damage has long been a challenge to tissue regeneration in individuals with diabetes [30, 31]. In addition, the development of a chronic hyperglycemic environment is accompanied by a range of diabetic complications and cell dysfunction [4]. Previous studies have revealed negative influences of high glucose on MSCs, including weakened mobilization and proliferation capacity, impaired differentiation potential and immunoregulatory capacity [14, 30, 32]. Thus, we first chose 25 mM glucose to mimic a high glucose condition according to previous studies [32–35]. Similarly, we found that incubation of PDLSCs under high glucose led to impaired osteogenic differentiation, evidenced by decrease in formation of calcium deposit and expression of osteoblast differentiation-related genes and

proteins. Therefore, our findings verify that long-term incubation in high glucose reduces the stem cell performance of PDLSCs, as was found in previous studies, and further indicate the need to protect cells from high glucose-induced damage.

Metformin is a potent insulin-sensitizing drug for the treatment of diabetes mellitus [36]. In addition to its anti-hyperglycemic effect, metformin has been found to exert protective effects on different cell types [37–39]. Moreover, metformin acts in a concentration-dependent manner; and the concentrations of metformin used for the treatment of MSCs typically range from 10 to 1000 μM [37, 40–44]. Therefore, we first selected a feasible concentration of metformin through CCK-8 assay. We found that metformin at concentrations ranging from 0 to 1000 μM induced no cytotoxicity and that metformin at the concentration of 100 μM conferred the maximal protective effect on cell viability under high glucose. Furthermore, we added 100 μM metformin into the culture medium. The results of qRT-PCR and Western blot analysis showed that the expression of osteoblast differentiation-related genes and proteins in PDLSCs under high glucose was increased after addition of metformin, suggesting that metformin can enhance the osteogenic differentiation ability of PDLSCs under high glucose. Similarly, previous studies have reported that 100 μM metformin can improve the differentiation potential of cells from aged rats [37]. In addition, metformin at concentrations ranging from 10 to 100 μM enhances the migration potential of PDLSCs; another study has also revealed that 1 mM metformin treatment protectes MSCs from apoptosis under inflammatory conditions [22, 42]. However, though previous studies have demonstrated that high glucose leads to decreased expression of RUNX2, we found in this study that the protein level of RUNX2 was not changed under high glucose or following metformin treatment [32, 45]. It has been reported that the expression of this transcription factor is increased at the early stage of osteogenic differentiation but is decreased at the late stage [46]. In our study, we detected the protein expression of RUNX2 at 14 days after osteogenic-induction, the late stage of osteogenic differentiation. These might explain why the protein level of RUNX2 was not obviously altered in the PDLSCs under high glucose or following metformin treatment. Taken together, the present and previous results indicate that metformin may exert a protective effect on the treatment of MSCs, including (but not limited to) PDLSCs. In addition, the effects of metformin are not only dependent on the drug concentration, but are also different in diverse cell lineages and under different incubation conditions. Moreover, metformin at the concentration of 100 μM may be feasible for treatment of cells under high glucose.

To further explore the underlying mechanisms, we conducted RNA-seq analysis to investigate the potential genes mediating the effect of metformin on PDLSCs under high glucose. Five osteogenesis-related genes AREG, IGFBP5, GPR68, SFRP2 and NPR3 were detected. Among these genes, AREG, IGFBP5, GPR68 and SFRP2 were downregulated in PDLSCs under high glucose and upregulated after the addition of metformin, while NPR3 exhibited the opposite pattern. We further confirmed the mRNA and protein expression levels of these five genes and found that only the mRNA and protein expression of NPR3 was consistent with the RNA-seq results. Thus, NPR3 was considered a potential target of metformin in further experiments. NPR3 is a clearance receptor of natriuretic peptides and has been reported to be involved in the regulation of cell metabolism and the progression of metabolic diseases [47, 48]. Several studies suggest that NPR3 expression is upregulated in adipose tissues in diabetic individuals [49]. Moreover,

inactivating NPR3 can stimulate endochondral ossification and bone growth, indicating NPR3 might be involved in the process of osteogenic differentiation [25, 50]. To further investigate the role of NPR3 in metformin-enhanced osteogenic differentiation under high glucose, we used a lentivirus vector to stably upregulate NPR3 expression in PDLSCs. After upregulation of NPR3, the calcium deposit formation was decreased in PDLSCs treated with metformin, and osteoblast differentiation-related genes *ALP*, *RUNX2*, *BMP2* and *OCN* were downregulated. Similarly, after NPR3 upregulation, the protein levels of ALP, BMP2 and OCN were decreased in cells treated with metformin. These data suggest that upregulation of NPR3 can compromise the metformin-enhanced PDLSC osteogenic differentiation. Therefore, our results indicate that NPR3 might be a target of metformin and that metformin might enhance the osteogenic differentiation of PDLSCs under high glucose via downregulation of NPR3. In addition, regulation of NPR3 expression may be an effective strategy for maintaining the functionality of MSCs.

NPR3 possesses an intracellular Gi/o-binding domain, which enables NPR3 to increase the phosphorylation level of ERK1/2 and activate the MAPK pathway [27, 47]. In addition, NPR3 can increase the level of phosphorylated ERK1/2 through decreasing bioavailable CNP [26]. Thus, we further detected the changes in the three major subfamilies p38 MAPK, JNK and Erk1/2 of MAPK pathway during osteogenic differentiation of PDLSCs. After upregulation of NPR3, the metformin-mediated decrease in phosphorylation of p38 MAPK and Erk1/2 was reversed, but not JNK. These results indicate upregulation of NPR3 can activate the MAPK pathway in PDLSCs treated with metformin. We thus concluded that metformin enhances PDLSC osteogenic differentiation under high glucose may occur through inhibition of the NPR3-mediated MAPK pathway.

Several studies have confirmed that MAPK pathway plays a crucial role in cellular differentiation [51, 52]. Gold nanoparticles can enhance osteogenic differentiation of MSCs by the p38 MAPK pathway; and osteoblasts lacking p38 MAPK pathway-related proteins show decreased marker gene expression and defective mineralization [53, 54]. In addition, Erk1/2 pathway has also been suggested to be one of the key regulators in the osteogenic differentiation of PDLSCs [55, 56]. To further validate the role of NPR3-mediated MAPK pathway in the metformin-enhance PDLSC osteogenic differentiation, we blunted the p38 MAPK and Erk1/2 pathway with the corresponding inhibitors (the phosphorylation-specific inhibitor for p38 MAPK was SB203580 while that for Erk1/2 was U0126) and observed the corresponding effects on PDLSC osteogenic differentiation. To block the p38 MAPK and Erk1/2 pathway, the concentration (20 μM) and duration (2 hour) of SB203580 and U0126 on PDLSCs were selected according to previous similar investigations [56, 57]. From the results of qRT-PCR and Western blot analysis, we identified that the osteogenic differentiation of NPR3-upregulated PDLSCs was enhanced after the addition of p38 MAPK or Erk1/2 inhibitors, suggesting that the p38 MAPK and Erk1/2 pathways are closely related to the function of NPR3, which is consistent with the above speculation. However, although previous studies have also demonstrated that the JNK pathway is crucial for the osteogenic differentiation of MSCs, we found no obvious change in the JNK pathway after NPR3 upregulation, indicating that the JNK pathway might not be a target of NPR3 [58]. Taken together, our results suggest that metformin enhances PDLSC osteogenic differentiation under high glucose via downregulation of NPR3 and inhibition of the downstream MAPK pathway.

In summary, our study revealed for the first time that metformin can enhance the osteogenic differentiation of PDLSCs under high glucose and that metformin functions via downregulating NPR3 and inhibiting the downstream MAPK pathway (Fig. 8). These results suggest that metformin can be applied to as a regulator of the NPR3-mediated MAPK pathway to protect MSCs from cell damage, indicating the development of targeted strategies through regulation of NPR3 and its downstream MAPK pathway will pave the way for stem cell-based therapy. In addition, the limitations of our study should be acknowledged. We only used an in *vitro* model in this study to investigate the effect of metformin on PDLSCs under high glucose. Further in *vivo* studies are needed to fully elucidate the intracellular responses underlying the effect of metformin under diabetic conditions.

# Conclusions

In this study, we prove for the first time that the upregulation of NPR3 in PDLSCs was a significant mechanism of impaired osteogenic differentiation caused by high glucose. In addition, the function of metformin in combating high glucose-damaged osteogenic differentiation was related to its role as a regulator of NPR3. Further evidence suggests that MAPK pathway, particularly the p38 MAPK and Erk1/2 pathway, can be activated by NPR3, and the NPR3 -mediated MAPK pathway may participate in metformin-enhanced osteogenic differentiation under high glucose. Although the signals involved in metformin-enhanced osteogenic differentiation remain obscure, regulating NPR3 and its downstream MAPK pathway can be considered a key therapeutic target for effective periodontal regeneration in diabetic individuals.

# Abbreviations

MSCs
Mesenchymal stem cells
PDL
periodontal ligament
PDLSCs
Periodontal ligament stem cells
CFU
Colony forming unit
CCK-8
Cell Counting Kit-8
ALP
Alkaline phosphatase
NPR3
natriuretic peptide receptor 3
ELISA
Enzyme-linked immunosorbent assay

CNP
C-type natriuretic peptide
qRT-PCR
Quantitative real-time polymerase chain reaction
MAPK
Mitogen-activated protein kinase

# Declarations

### Additional material

Additional supporting information are available at the Additional material of this article.

### *Ethics approval and consent to participate*

The experimental protocol of this study was approved by the Ethics Committee of the Stomatological Hospital of FMMU (201203), and informed consent was signed by all the subjects who donated their extracted teeth for cell isolation.

### *Consent for publication*

Consent to participate/consent for publication is not applicable to this study.

### *Availability of data and materials*

All data generated or analyzed during this study are included in this published article.

### *Competing interests*

The authors declare that they have no competing interests.

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### *Author' contributions*

Y-LZ, FL and Z-BL contributed to the conception and design of the study. Y-LZ, XL, X-TH and R-XW did the job of acquisition, analysis, interpretation of data and manuscript writing. S-HG, H-HS, YA and F-MC contributed to the study conception and design, financial support and manuscript writing. All authors read and approved the final manuscript.

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# Tables

Table 1
Sequences of gene-specific primers used in the present study.

| Gene ID | Genes | Forward sequence | Reverse sequence |
|---|---|---|---|
| 632 | *OCN* | CCCAGGCGCTACCTGTATCAA | GGTCAGCCAACTCGTCACAGTC |
| 860 | *Runx2* | TGGTTACTGTCATGGCGGGTA | TCTCAGATCGTTGAACCTTGCTA |
| 249 | *ALP* | AACATCAGGGACATTGACGTG | GTATCTCGGTTTGAAGCTCTTCC |
| 2597 | *GAPDH* | GGAGTCCACTGGCGTCTTCA | GTCATGAGTCCTTCCACGATACC |
| 60 | *β-Actin* | CTGCTCATCCCACTAATGTC | CTTTATTAACTACCACCTGGTCCT |
| 650 | *BMP2* | ACCCGCTGTCTTCTAGCGT | TTTCAGGCCGAACATGCTGAG |
| 4880 | *CNP* | CTGACCGACCGACTCCAG | AAATCTCTCACCTCCGCCAG |
| 374 | *AREG* | GTGGTGCTGTCGCTCTTGATA | CCCCAGAAAATGGTTCACGCT |
| 3488 | *IGFBP5* | ACCTGAGATGAGACAGGAGTC | GTAGAATCCTTTGCGGTCACAA |
| 8111 | *GPR68* | TGTACCATCGACCATACCATCC | GGTAGCCGAAGTAGAGGGACA |
| 6423 | *SFRP2* | CTTAGAGGACAGCGGGGAAG | TCCAAGCATCTTGCCCTGAG |
| 4883 | *NPR3* | AGACTACGCCTTCTTCAACATTG | GCTTCAAAGTCGTGTTTGTCTCC |

# Figures

**Figure 1**

**Incubation of periodontal ligament stem cells (PDLSCs) under high glucose impaired cellular osteogenic differentiation.** (A) ALP staining and ALP activity assay of PDLSCs when they were cultured in normal (CON) or high glucose (HG) condition following a 7-day osteogenic induction. (B) Alizarin Red staining and quantitative analysis of the stained calcium deposits formed by PDLSCs when they were cultured in CON or HG condition following a 21-day osteogenic induction. (C) Expression of osteoblast differentiation-related genes (ALP, RUNX2, BMP2 and OCN) in PDLSCs when they were cultured in CON or HG condition following a 14-day osteogenic induction (mRNA expression levels detected by qRT-PCR). (D) Expression of osteoblast differentiation-related proteins (ALP, RUNX2, BMP2 and OCN) in PDLSCs when they were cultured in CON or HG condition following a 14-day osteogenic induction (protein expression levels detected by Western blot analysis). The displayed bands were cropped from the corresponding original blots. Experiments for each cell line were repeated independently for at least 3 times and data are

presented as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences between the indicated columns, while *NS* represents no significant difference.

## Figure 2

**Metformin enhanced the osteogenic differentiation of PDLSCs under high glucose.** (A) ALP staining and ALP activity assay of PDLSCs when they were cultured in normal (CON), high glucose (HG) or high glucose with metformin addition (MET) following a 7-day osteogenic induction. (B) Alizarin Red staining and quantitative analysis of the stained calcium deposits formed by PDLSCs when they were cultured in normal (CON), high glucose (HG) or high glucose with metformin addition (MET) following a 21-day osteogenic induction. (C) Expression of osteoblast differentiation-related genes (ALP, RUNX2, BMP2 and OCN) in PDLSCs when they were cultured in normal, high glucose or high glucose with metformin addition following a 14-day osteogenic induction (mRNA expression levels detected by qRT-PCR). (D) Expression of osteoblast differentiation-related proteins (ALP, RUNX2, BMP2 and OCN) in PDLSCs when they were cultured in normal, high glucose or high glucose with metformin addition following a 14-day osteogenic induction (protein expression levels detected by Western blot analysis). The displayed bands were cropped from the corresponding original blots. HG, high glucose. Met, metformin. Experiments for each cell line were repeated independently for at least 3 times and data are presented as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences between the indicated columns, while *NS* represents no significant difference.

## Figure 3

**Screening and identification of osteogenesis-related genes mediating metformin-enhanced osteogenic differentiation under high glucose.** (A) Venn diagram showing the number of differentially expressed genes in PDLSCs when they were cultured in normal, high glucose or high glucose with metformin addition following a 14-day osteogenic induction. (B) Heatmap showing all the differentially expressed genes in PDLSCs when they were cultured in normal, high glucose or high glucose with metformin addition following a 14-day osteogenic induction. The color key (from purple to red) of Z-score value (-1.95-2.77) indicated low to high expression levels. (C) Heatmap showing the osteogenesis-related genes in PDLSCs when they were cultured in normal, high glucose or high glucose with metformin addition following a 14-day osteogenic induction. The color key (from purple to red) of Z-score value (-1.24-2.08) indicated low to high expression levels. (D) The statistical analysis of expressed levels of osteogenesis-related genes (AREG, IGFBP5, GPR68, SFRP2 and NPR3) in PDLSCs when they were cultured in normal, high glucose or high glucose with metformin addition following a 14-day osteogenic induction (mRNA expression levels detected by qRT-PCR). (E) Protein expression of SFRP2 and NPR3 in PDLSCs when they were cultured in normal, high glucose or high glucose with metformin addition following a 14-day osteogenic induction (protein expression levels detected by Western blot analysis). The displayed bands

were cropped from the corresponding original blots. HG, high glucose. Met, metformin. Experiments for each cell line were repeated independently for at least 3 times and data are presented as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences between the indicated columns, while *NS* represents no significant difference.

## Figure 4

**NPR3 played a role in the metformin-enhanced osteogenic differentiation of PDLSCs under high glucose.** (A) Representative confocal images of natriuretic peptide receptor 3 (NPR3) in PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition following a 14-day osteogenic induction (scale bar = 100 μm, ×20 magnification). (B) ALP staining and ALP activity assay of PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition following a 7-day osteogenic induction. (C) Alizarin Red staining and quantitative analysis of the stained calcium deposits formed by PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition following a 21-day osteogenic induction. HG, high glucose. Met, metformin. LV-NPR3, lentivirus containing NPR3 gene. Experiments for each cell line were repeated independently for at least 3 times and data are presented as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences between the indicated columns, while *NS* represents no significant difference.

## Figure 5

**NPR3 Upregulation decreased the expression of osteoblast differentiation-related genes and proteins that enhanced by metformin.** (A-D) Expression of osteoblast differentiation-related genes (ALP, RUNX2, BMP2 and OCN) in PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition following a 14-day osteogenic induction (mRNA expression levels detected by qRT-PCR). (E-I) Expression of osteoblast differentiation-related proteins (ALP, RUNX2, BMP2 and OCN) in PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition following a 14-day osteogenic induction (protein expression levels detected by Western blot analysis). The displayed bands were cropped from the corresponding original blots. HG, high glucose. Met, metformin. LV-NPR3, lentivirus containing NPR3 gene. Experiments for each cell line were repeated independently for at least 3 times and data are presented as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences between the indicated columns, while *NS* represents no significant difference.

## Figure 6

**Metformin enhanced PDLSC osteogenic differentiation under high glucose via inhibition of the NPR3-mediated MAPK pathway.** (A) Gene expression of C-type natriuretic peptide (CNP) in PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition (mRNA expression levels detected by qRT-PCR). (B) Total CNP content in PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition (total content in the cell culture supernates detected by ELISA). (C-D) Protein expression of MAPK pathway-related proteins (p38 MAPK, p-p38 MAPK, Erk1/2, p-Erk1/2, JNK, p-JNK) in PDLSCs transfected with or without LV-NPR3 when they were cultured in normal, high glucose, or high glucose with metformin addition (protein expression levels detected by Western blot assay). The displayed bands were cropped from the corresponding original blots. HG, high glucose. Met, metformin. LV-NPR3, lentivirus containing NPR3 gene. Experiments for each cell line were repeated independently for at least 3 times and data are presented as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences between the indicated columns, while *NS* represents no significant difference.

## Figure 7

**Effects of p38 MAPK and Erk1/2 pathway inhibitors on PDLSC osteogenic differentiation.** (A) Expression of osteoblast differentiation-related genes (ALP, RUNX2, BMP2 and OCN) in PDLSCs transfected with or without LV-NPR3 when they were cultured in high glucose with metformin addition or high glucose with metformin and SB203580 addition following a 14-day osteogenic induction (mRNA expression levels detected by qRT-PCR). (B) Expression of osteoblast differentiation-related genes (ALP, RUNX2, BMP2 and OCN) in PDLSCs transfected with or without LV-NPR3 when they were cultured in high glucose with metformin addition or high glucose with metformin and U0126 addition following a 14-day osteogenic induction (mRNA expression levels detected by qRT-PCR). (C) Expression of osteoblast differentiation-related proteins (ALP, RUNX2, BMP2 and OCN) in PDLSCs transfected with or without LV-NPR3 when they were cultured in high glucose with metformin addition or high glucose with metformin and SB203580 addition following a 14-day osteogenic induction (protein expression levels detected by Western blot assay). (D) Expression of osteoblast differentiation-related proteins (ALP, RUNX2, BMP2 and OCN) in PDLSCs transfected with or without LV-NPR3 when they were cultured in high glucose with metformin addition or high glucose with metformin and U0126 addition following a 14-day osteogenic induction (protein expression levels detected by Western blot assay). The displayed bands were cropped from the corresponding original blots. HG, high glucose. Met, metformin. LV-NPR3, lentivirus containing NPR3 gene. SB203580, p38 MAPK pathway inhibitor. U0126, Erk1/2 pathway inhibitor. Experiments for each cell line were repeated independently for at least 3 times and data are presented as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences between the indicated columns, while *NS* represents no significant difference.

**Figure 8**

**Schematic of the identified signaling molecules/pathways involved in the metformin-enhanced osteogenic differentiation of periodontal ligament stem cells under high glucose.**

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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