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microRNA-132 inhibits osteogenic differentiation of periodontal ligament stem cells via GDF5 and the NF-κB signaling pathway

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ABSTRACT

Background: Periodontal ligament stem cells (PDLSCs) could differentiate into osteoblasts and have a great prospect in treating bone diseases. microRNAs (miRs) and nuclear factor kappa-B (NF-κB) signaling pathway have proved pivotal in regulating osteogenic differentiation. This study intended to discuss the mechanism of miR-132 and NF-κB in PDLSC osteogenesis.

Methods: PDLSCs were firstly cultured, induced, and identified by detecting the surface markers and observing cell morphology. Levels of osteogenic markers alkaline phosphatase (ALP), bone morphogenetic proteins 2 (BMP2), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN), along with miR-132 expression were measured. The osteoblast activity and mineral deposition were detected by ALP and alizarin red S (ARS) stainings. The targeting relationship between miR-132 and growth differentiation factor 5 (GDF5) was verified. The gain-and loss-of-function was performed to discuss roles of miR-132 and GDF5 in osteogenic differentiation of PDLSCs. Besides, levels of NF-κB signaling pathway-related proteins were measured.

Results: In osteogenic differentiation of PDLSCs, levels of ALP, BMP2, Runx2 and OCN were upregulated while miR-132 was downregulated. Overexpressing miR-132 reduced levels of osteogenic markers, osteoblast activity, ALP and ARS intensity and the activation of NF-κB axis. GDF5 is a target of miR-132 and GDF5 overexpression reversed the inhibitory effects of overexpressed miR-132 on PDLSC osteogenesis.

Conclusion: Together, miR-132 could inhibit PDLSC osteogenesis via targeting GDF5 and activating NF-κB axis. These data provide useful information for PDLSC application in periodontal therapy.

1. Introduction

Periodontal diseases are the main chronic infectious diseases of the oral cavity, resulting in periodontal supporting tissue destruction and ultimately tooth loss [19]. The ultimate goal of periodontal therapy is to bring about periodontal tissue regeneration [15]. Periodontal ligament, kind of hypervascularized connective tissue, where osteoblasts are located and proliferate and migrate when tissues damaged, helps to maintain the stability of periodontal tissues by releasing stem cells or progenitor cells [12]. Periodontal ligament stem cells (PDLSCs), which are located around periodontal blood vessels and originated from periodontal ligament tissues, have the features of mesenchymal stem cells as indicated by their capacity for multilineage differentiation and periodontal tissue regeneration [33]. PDLSCs can reconstruct periodontal ligament tissues that have been damaged by periodontal diseases and can regulate T-cell immunity, which is important in osteogenic differentiation [20]. Zajdel A et al. have revealed osteogenesis induced by mesenchymal stem cells may be an important treatment for bone injury [32]. Thus, comprehending the molecular mechanisms of PDLSC osteogenesis could help the development of regenerative therapies for periodontal and bone diseases.

MicroRNAs (miRs) are implicated in regulation of gene expression during stem cell differentiation [11], and their function in mesenchymal stem cell osteogenesis has been verified [7]. As a member of miRs, miR-132 is extensively studied in neuronal differentiation, axon growth, neural migration, and plasticity in the nervous system and its dysregulation results in neural development and degenerative diseases [23]. A recent research has reported that miR-132 is downregulated during osteogenic differentiation of umbilical cord mesenchymal stem cells [30]. In this study, it found miR-132 could target growth

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differentiation factor 5 (GDF5) and inhibit its expression. GDF5, a member of the transforming growth factor beta (TGF-β) superfamily [22], is a major regulator of osteogenesis and bone repair, which induces bone formation by improving chondrocyte and osteocyte differentiation and provoking angiogenesis [5]. Additionally, GDF5 deficiency dramatically blocks PDLSC osteogenic differentiation [17]. Interestingly, it has previously justified that TGF-β could inhibit nuclear factor kappa-B (NF-κB) activity in normal cells, but activate its activity in cancerous cells [25]. Surprisingly, receptor activator of NF-κB ligand is abundantly expressed by activated T lymphocyte and B lymphocyte in periodontal disease and induces osteoclast bone resorption [18]. Besides, osteogenic differentiation of PDLSCs is inhibited by inflammation, which is regulated by NF-κB [4]. These findings offer a fundamental insight for the mechanism of periodontal disease and support the hypothesis that osteogenic differentiation of PDLSCs can be regulated via the co-work of miR-132, GDF5 and NF-κB.

2. Materials and methods

2.1. Ethics statement

This study was approved and supervised by the ethics committee of the Beijing Stomotological Hospital Affiliated to Capital Medical University. All the subjects signed the informed consent.

2.2. Cell culture and mineralization induction

The healthy and intact premolars were extracted immediately from volunteers aged 18 ~ 24 years who took physical examination in the Beijing Stomotological Hospital Affiliated to Capital Medical University, and the periodontal ligament tissues were isolated under aseptic conditions. The tissues were detached for 45 min by adding 4 mg/L type I collagenase (500 μL) and suspended in α-modified Eagle's medium (α-MEM) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). The tissues were then seeded into 35 mm petri dishes, and incubated at 37℃ with 5% CO_2. When cell confluence reached 80%, the monoclones were selected by limited dilution method, and then expanded for subsequent experiments. The morphological changes and growth of cells were observed and photographed using an inverted phase contrast microscopy (Olympus, Tokyo, Japan).

PDLSCs of the 3 passage were inoculated into 6-well culture plates at 2×10^5 cells/well. After induction in mineralization inducing solution (10% FBS, 10 nmol/L dexamethasone, 50 mg/L vitamin C and 10 mmol/L β-glycerophosphate) for two weeks, the expression of osteogenesis-related genes and miR-132 was measured.

2.3. Flow cytometry

Cells in each group were detached using trypsin and washed with phosphate buffer saline (PBS), then incubated at 4 ℃ for 30 min in the dark. Fluorescein isothiocyanate (FITC) was bound to antibodies STRO-1, CD14, CD34, CD45, CD105, human leukocyte antigen D-Related (HLA-DR), CD19, CD90 and CD146 (eBiolegend, San Diego, CA, USA). Then the cells were cleaned and suspended by a flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, Fullerton, CA, USA) for further identification. Homotypically matched normal immunoglobulin G (IgG) antibody was used as a control.

2.4. Alkaline phosphatase (ALP) staining and activity detection

ALP staining was performed using a nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining kit (CoWin Biotech, Beijing, China). After osteogenic induction for 7 days, cells were fixed in 4% paraformaldehyde for 10 min, and then ALP staining was performed following the manufacturer's instructions.

A commercialized ALP activity colorimetric assay kit (BioVision, Milpitas, CA, USA) was used to analyze ALP activity [17]. The cultured cells were washed with cold PBS, then lysed with 1% Triton X-100 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and scraped into distilled water. The absorbance was detected at 405 nm. Total protein concentration was measured by the bicinchoninic acid method using the Pierce protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). ALP activity was calculated from the absorbance levels relative to protein concentration.

2.5. Alizarin red S (ARS) staining and quantitative analysis

ARS staining was applied to determine ARS intensity and quantitate the mineralized nodule formation. After osteogenic incubation for 14 days, cells were fixed in 95% ethanol for 30 min at room temperature. Subsequently, cells were washed with distilled water and then stained with 0.1% ARS (pH = 4.2; Sigma-Aldrich) for 20 min [3]. To quantitatively evaluate the mineralized nodules, the staining solution was dissolved in 1 mL 10% cetylpyridinium chloride (Sigma-Aldrich) for 1 h and the absorbance at 570 nm was detected by spectrophotometric methods.

2.6. Cell transfection and grouping

PDLSCs of the 3 passage in logarithmic growth phase were detached and inoculated into 6-well plates. After that, cells were allocated into blank group, miR-132 mimic negative control (NC), miR-132 mimic, miR-132 inhibitor NC, miR-132 inhibitor and miR-132 + GDF5 groups. All the plasmids were purchased from RiboBio (Guangzhou, China). PDLSCs were transfected as per the instructions of Lipofectamine™ 2000 (Invitrogen Inc., Carlsbad, CA, USA).

2.7. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from lysed cells, and then reversely transcribed into cDNA using RT primers in the miR kit. After osteogenesis induction, mRNA expression of miR-132, and osteogenic genes ALP, bone morphogenetic protein-2 (BMP2), osteocalcin (OCN) and runt related transcription factor-2 (Runx2) was detected by Prime Script™ RT-PCR Kit with U6 as an internal reference for miR-132 and β-actin for other genes. The primer sequences are displayed in Table 1.

2.8. Western blot analysis

After osteogenic induction for 7 days, cells were collected and lysed

Table 1
Primer sequences for RT-qPCR.

Primer	Sequence
miR-132	F: GCGCGCGTAACAGTCTACAGC R: GTCGTATCCAGTGCAGGGTCC
U6	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTCAT
ALP	F: GGACCATTCCCACGTCTTCAC R: CCTTGTAGCCAGGCCCATTG
BMP2	F: GAGGATTAGCAG GTCTTTG R: GGAGTTCAGGTGGTCAGC
OCN	F: CCCAGGCGCTACCTGTATCAA R: GGTCAGCCAACTCGTCACAGTC
Runx2	F: CCCGTGGCCTTCAAGGT R: CGTTACCCGCCATGACAG TA
β-actin	F: GTCATTCCAAATATGAGAGATGCGT R: GCTATCACCTCCCCTGTGTG

Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; miR-132, microRNA-132; ALP, alkaline phosphatase; BMP2, bone morphogenetic protein-2; OCN, osteocalcin; Runx2, runt related transcription factor-2; F, forward; R, reverse.

Table 2
Antibodies used for western blot analysis.

Antibody	Item No.	Ratio
ALP	ab83259	1 μg/ml
BMP2	ab14933	1/500
Runx2	ab76956	1/500
OCN	ab76956	1/500
GDF5	ab38546	1/500
P-P65	ab86299	0.5 μg/ml
p65	ab16502	1/2000
P-IκBα	ab63510	1/500
IκBα	ab7547	1/500
β-actin	ab8227	1/1000

Note: GDF5, growth differentiation factor 5; ALP, alkaline phosphatase; BMP2, bone morphogenetic proteins 2; Runx2, runt-related transcription factor 2; OCN, osteocalcin. All antibodies were from Abcam Inc (Cambridge, MA, USA).

using radio-immunoprecipitation assay lysis buffer. Cells were then oscillated three times by ultrasound, each for 5 ~ 10 s, and centrifuged at 1: 200 r/min for 15 min. The supernatant was absorbed and the protein concentration was measured. The proteins were added with loading buffer and boiled, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation. The membranes were transferred with semi-dry method and then sealed with 10% dried skimmed milk for 2 h. Afterwards, the membranes were incubated with primary antibodies at 4℃ overnight. After washing in tris-buffered saline tween (TBST), the membranes were supplemented with secondary antibodies at room temperature for 2 h. Afterwards, the membranes were washed with TBST, and proteins were visualized and scanned for quantitative analysis with β-actin as an internal reference. The antibodies are exhibited in Table 2.

2.9. Dual luciferase reporter gene assay

The 3'-untranslated region (3'UTR) of GDF5 sequence containing the binding sites of miR-132 was synthesized. GDF5 3'UTR wild type (WT) plasmids and GDF5 3'UTR mutant type (MUT) plasmids were constructed, and then transfected into HEK293 T cells (American Type Culture Collection, Manassas, VA, USA) with mimic NC and miR-132 plasmids respectively. After transfection for 48 h, cells were collected and lysed, and luciferase activity was determined with luciferase assay kit (BioVision, San Francisco, CA, USA) and Glomax20/20 luminometer (Promega, Madison, Wisconsin, USA).

2.10. Statistical analysis

SPSS 19.0 (IBM Corp., Armonk, NY, USA) was employed for data analysis. Kolmogorov-Smirnov test checked whether the data were normally distributed. The results were exhibited in mean ± standard deviation. The *t* test was applied for comparisons between two groups, while one-way analysis of variance (ANOVA) or two-way ANOVA for multi-groups. Sidak's multiple comparisons test or Tukey' multiple comparisons test was used for post-test *p* was obtained by two-tailed test and $p < 0.05$ meant statistical differences.

3. Results

3.1. Downregulation of miR-132 is found during PDLSC osteogenesis

To begin with, PDLSCs were cultured for mineralization induction. After that, cell morphology was observed, and levels of PDLSC surface markers and miR-132 were detected for PDLSC identification. Under the microscope, it was observed that the primary cells gradually assembled to form fibrous meshes on the 4th to 5th day of culture, and were spindle-shaped and closely adjacent. When culturing to the 3rd passage, cells grew rapidly and evenly, with relatively simple morphology in long spindle shape. After induction, PDLSCs were confirmed competent in differentiating into osteoblasts and adipocytes, evidenced by the formation of mineralized nodules and lipid droplets (Fig. 1A ~ C). The cell surface markers STRO-1, CD73, CD90, CD105 and CD146 were confirmed positive and CD14, CD19, CD34, CD45 and HLA-DR were confirmed negative (Fig. 1D). The mRNA expression of osteogenic markers ALP, BMP2, Runx2 and OCN in PDLSCs cultured in osteogenic medium increased significantly with time ($p < 0.05$, Fig. 1E), indicating that PDLSCs were successfully induced into osteoblasts. Overexpression of miR-132 has been reported to inhibit osteogenic differentiation and ALP activity [8]. Therefore, we speculate miR-132 may have the possibility to affect PDLSC osteogenesis. In order to justify our speculation, we measured miR-132 expression in PDLSCs during osteogenesis. It turned out that miR-132 was noticeably downregulated during PDLSC osteogenesis ($p < 0.05$). Besides, miR-132 expression was notably increased when transfection with miR-132 mimic, but decreased when transfection with miR-132 inhibitor ($p < 0.05$, Fig. 1E), indicating the successful transfection.

3.2. Overexpression of miR-132 inhibits PDLSC osteogenesis

Given the downregulation of miR-132 during PDLSC osteogenesis, PDLSCs were transfected with miR-132 mimic or miR-132 inhibitor to further elucidate its roles in PDLSC osteogenesis. When transfecting with miR-132 mimic, PDLSCs exhibited notably reduced intensity and activity of ALP and ARS ($p < 0.05$, Fig. 2A, B), and mRNA and protein levels of ALP, BMP2, Runx2 and OCN in PDLSCs decreased substantially (all $p < 0.05$, Fig. 2C–D), which were opposite to the conditions when PDLSCs were transfected with miR-132 inhibitor. To sum up, overexpression of miR-132 could inhibit osteogenic differentiation of PDLSCs.

3.3. miR-132 targets GDF5

To deeply investigate the molecular mechanism of miR-132 in PDLSC osteogenesis, the potential target of miR-132 was predicted. According to a recent literature, upregulation of GDF5 may promote PDLSC osteogenic differentiation [17]. The biology website available at http://www.microrna.org/ predicted there was a binding site between miR-132 and GDF5 3'UTR (Fig. 3A). The luciferase activity of GDF5-WT in the miR-132 group was markedly decreased by the dual luciferase reporter gene assay (Fig. 3B). In addition, compared with the mimic NC group, GDF5 levels were obviously decreased in PDLSCs transfected with miR-132 mimic ($p < 0.05$) (Fig. 3C, D). These results suggested that miR-132 could target GDF5 and inhibit its expression.

3.4. GDF5 blocks the inhibitory effect of overexpressed miR-132 on PDLSCs osteogenesis

Considering the targeting relationship between miR-132 and GDF5, now the focus of our study shifted to investigating the mechanism of GDF5 in PDLSC osteogenesis. Following the treatment of overexpression of GDF5 and miR-132, PDLSCs exhibited enhanced intensity and activity of ALP and ARS, and increased mRNA and protein levels of ALP, BMP2, Runx2 and OCN (all $p < 0.05$, Fig. 4A ~ D). Briefly, we concluded that GDF5 could block the inhibitory effects of overexpressed miR-132 on osteogenic differentiation of PDLSCs.

3.5. miR-132 inhibits PDLSC osteogenesis by targeting GDF5 and activating the NF-κB axis

As reported, NF-κB signaling pathway could inhibit odontoid process/osteogenic differentiation [29]. Therefore, we speculate that the NF-κB signaling pathway may also affect PDLSC osteogenesis. Western blot analysis revealed no notable differences in levels of IκBα and P65 (both $p > 0.05$), but levels of p-IκBα and p-P65 were increased

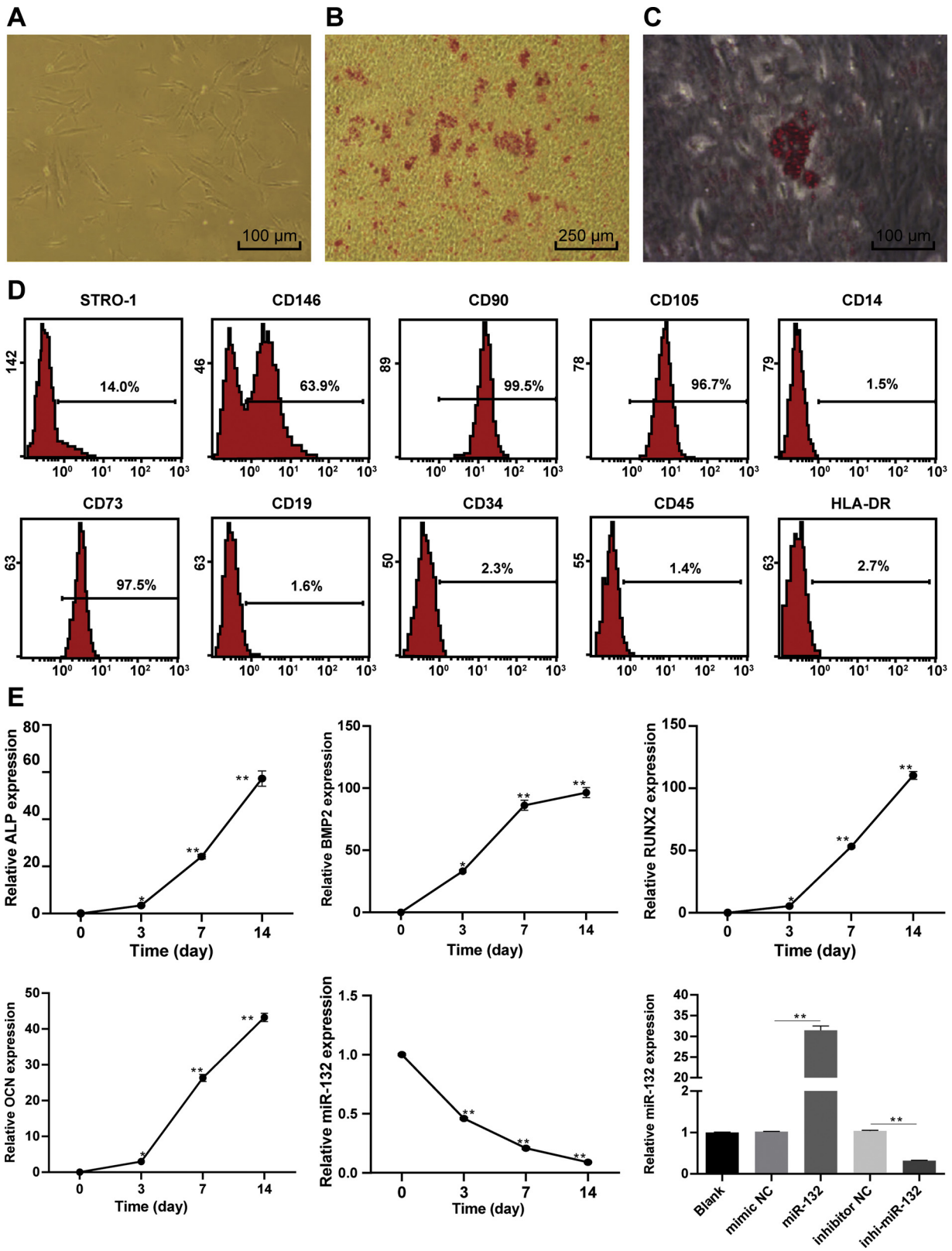

Fig. 1. miR-132 is downregulated during PDLSC osteogenesis. A. PDLSCs were spindle-shaped and closely adjacent under the invert microscope; B. Representative image of osteoblasts differentiated from PDLSCs; C. Representative image of adipocytes differentiated from PDLSCs; D. Levels of PDLSC surface markers detected by flow cytometry, among which STRO-1, CD73, CD90, CD105 and CD146 were confirmed positive and CD14, CD19, CD34, CD45 and HLA-DR were confirmed negative; E. Relative mRNA expression of ALP, BMP2, Runx2, OCN and miR-132 detected by RT-qPCR. ** $p < 0.01$, for pairwise comparison. Data in panel E were analyzed by one-way ANOVA, and Tukey's multiple comparisons test was used for post-test. All the experiments were repeated three times. miR-132, microRNA-132; PDLSCs, periodontal ligament stem cells; ALP, alkaline phosphatase; BMP2, bone morphogenetic proteins 2; Runx2, runt-related transcription factor 2; OCN, osteocalcin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ANOVA, analysis of variance.

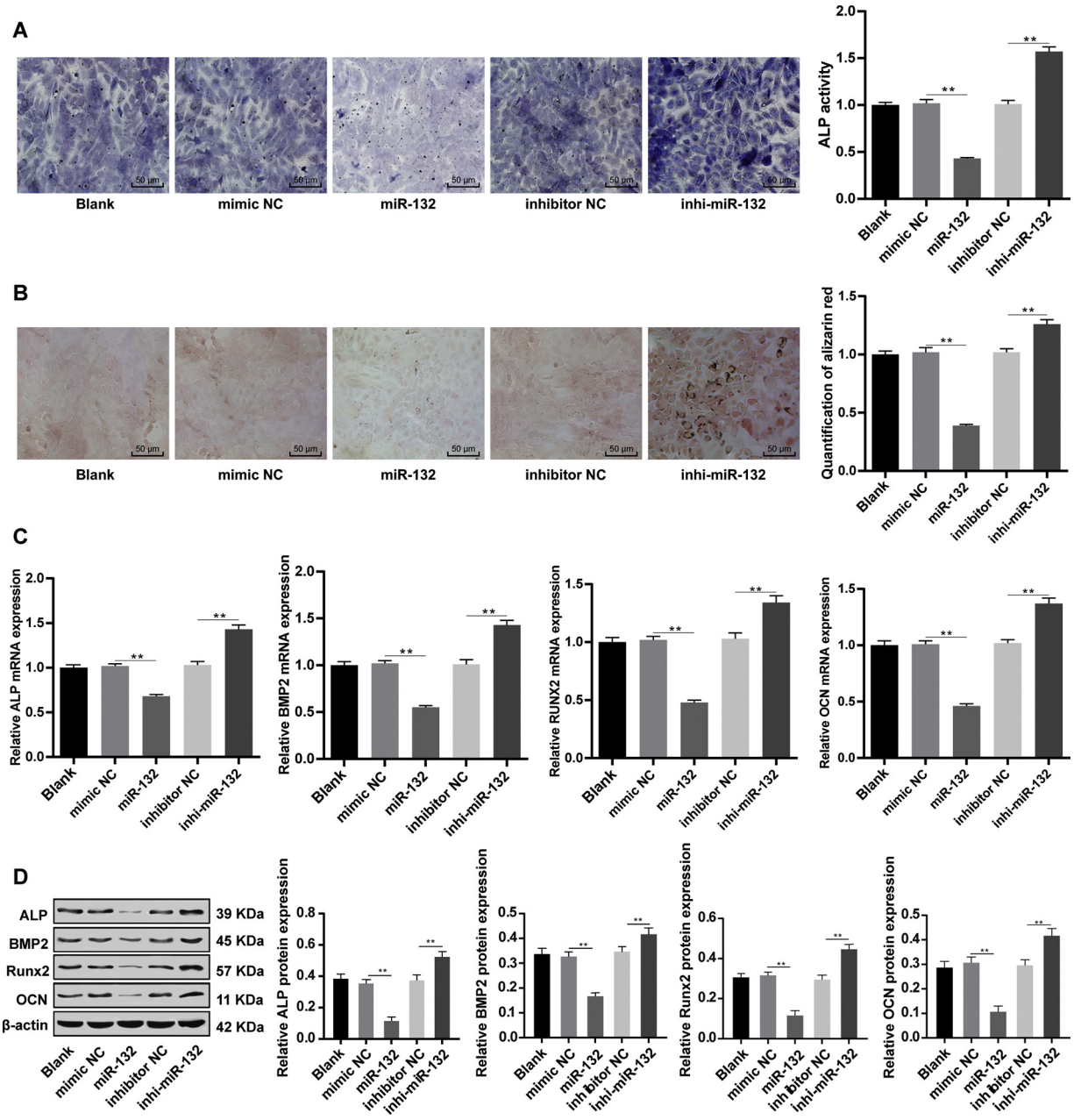

Fig. 2. Overexpression of miR-132 inhibits osteogenic differentiation of PDLSCs. A. Representative images and histogram of ALP activity measured by ALP staining; B. Representative images and histogram of ARS activity measured by ARS staining; C–D. Relative mRNA expression and protein levels of ALP, BMP2, Runx2 and OCN detected by RT-qPCR and western blot analysis. ** $p < 0.01$, for pairwise comparison. Data were analyzed by one-way ANOVA, and Tukey's multiple comparisons test was used for post-test. All the experiments were repeated three times. miR-132, microRNA-132; PDLSCs, periodontal ligament stem cells; ALP, alkaline phosphatase; ARS, alizarin red S; BMP2, bone morphogenetic proteins 2; Runx2, runt-related transcription factor 2; OCN, osteocalcin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ANOVA, analysis of variance.

significantly when miR-132 was overexpressed (both $p < 0.05$) (Fig. 5), which were contrary to the trends when miR-132 expression was inhibited. In summary, miR-132 could inhibit PDLSC osteogenesis by targeting GDF5 and activating the NF-κB signaling pathway.

4. Discussion

Inflammatory periodontal disease is very common and often overlooked by affected individuals [1]. What's worse, there is a link between periodontitis and systemic diseases such as diabetes and cardiovascular diseases, which seriously impact patients' quality of life [13]. Fortunately, researchers have confirmed PDLSCs with multi-lineage differentiation ability are effective in periodontitis treatment, and when combined with osteogenic differentiation-inducing proteins, the therapeutic potential increases [31]. Therefore, prior to the experiments, we made a hypothesis based on existing references that osteogenic differentiation of PDLSCs can be regulated via the co-work of miR-132, GDF5 and NF-κB. Collectively, we came to a conclusion that miR-132 could inhibit PDLSCs osteogenesis by targeting GDF5 and activating NF-κB axis.

The first major finding was that miR-132 was downregulated during PDLSC osteogenesis, indicating downregulated miR-132 was beneficial for PDLSC osteogenesis. Similarly, miR-132-3p expression was reduced in osteogenic differentiation of ligamentum flavum cells and played a negative regulatory role in osteogenesis [24]. To further investigate the molecular mechanism of miR-132 in osteogenesis of PDLSCs, we

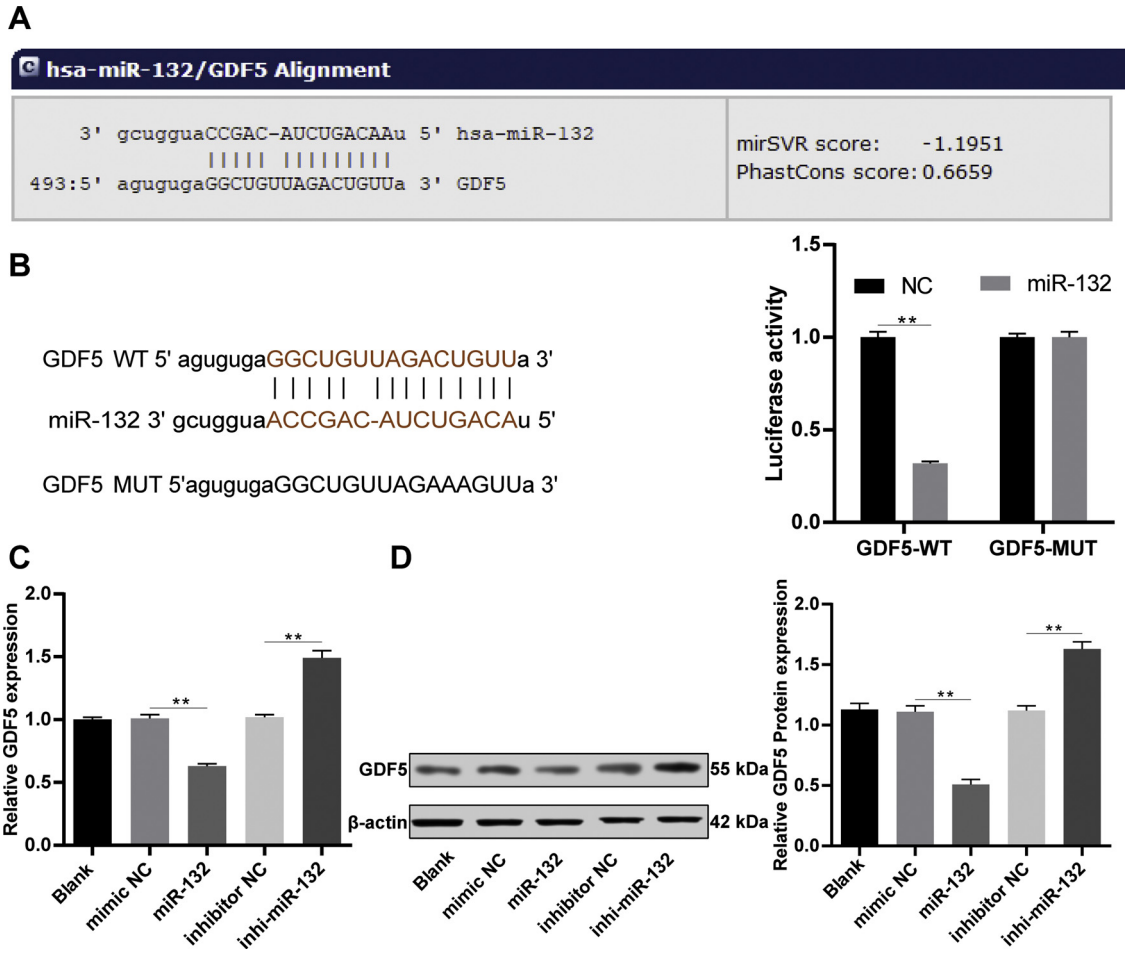

Fig. 3. miR-132 could target GDF5. A. The binding site between miR-132 and GDF5 3′UTR predicted by a biology website; B. The targeting relationship between miR-132 and GDF5 verified by dual luciferase reporter gene assay; C. Relative GDF5 mRNA expression measured by RT-qPCR; D. Relative GDF5 protein level measured by western blot analysis. ** $p < 0.01$, for pairwise comparison. Data in panel A were analyzed by two-way ANOVA, and data in panel C and D were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used for post-test. All the experiments were repeated three times. miR-132, microRNA-132; GDF5, growth differentiation factor 5; UTR, untranslated region; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ANOVA, analysis of variance.

performed a gain function of miR-132. When transfecting with miR-132 mimic, PDLSCs exhibited notably reduced intensity and activity of ALP and ARS, and substantially decreased expression of ALP, BMP2, Runx2 and OCN. Consistently, overexpressed miR-132 noticeably repressed levels of key markers of osteogenesis and ALP activity in high glucose-free fatty acids-treated MC3T3 cells [8]. Moreover, studies revealed miRs regulated osteogenesis via osteoblast-related genes, particularly Runx2 and BMP2, and via signaling pathways like TGF-β, BMP and Notch [10]. Serum ALP is mainly generated by osteoblasts [2]. Runx2 is a major regulator of osteoblast development by regulating key osteogenic genes, and Runx2 knockout in mice results in complete depletion of bone formation [14]. BMPs could exert potent function in the regulation of osteoblastic stem cells, among which recombinant human BMP2 is verified applicable in clinical adjuvant therapy for bone regeneration [31]. Runx2 and ALP are markers of early osteogenesis, OCN is a late period osteogenic marker, and calcium deposition is indicative of the final stage of osteogenesis [21,26]. Significantly, overexpression of miR-132-3p decreased ALP activity and levels of Runx2 and osterix, a key regulator of osteoblast mineralization, indicating inhibited osteoblast diff ;erentiation [9]. These results revealed that overexpression of miR-132 inhibited osteogenic differentiation of PDLSCs.

Furthermore, our in-depth investigation suggested the inhibitory effects of overexpressed miR-132 on PDLSC osteogenesis were achieved via GDF5 and the NF-κB signaling pathway. In addition to GDF5, the target genes of miR-132 included TIFB, VWA1, AIPLI, MSC, GPI, TLX3 and DUSP7 according to bioinformatics prediction. The reason why we chose GDF5 was that the up-regulation of GDF5 could promote the osteogenic differentiation of PDLSCS [17]. Consistent observation stated miR-132-3p could target GDF5 during the osteogenic differentiation of ligamentum flavum cells [24]. Belonging to the TGF-β family and subordinated group of BMPs, activating mutations in GDF5 led to increased function phenotype and chondrogenic activity [6]. Importantly, GDF5 upregulation was recently reported to reverse the inhibitory effects of miR-7 overexpression on PDLSC osteogenesis [17], which was highly in agreement with our results. GDF5 depletion blocked ligamentum flavum cell osteogenesis, presented with declined levels of Runx2, ALP, and OCN and decreased activity of ALP and ARS staining [24]. Nuclear factor p65 was expressed in gingival tissues of 75% of patients with chronic periodontitis [27]. A former research revealed a possibility that p65, mediated by NF-κB, was critical for osteogenesis but also protective of PDLSCs against tumor necrosis factor-alpha (TNF-α)-induced apoptosis [4]. NF-κB pathway-activated stem cells from apical papilla presented an enhanced migration capacity, ALP activity, mineralization capacity, and upregulated levels of osteogenic markers (ALP, Runx2, OCN) [16]. A similar research exhibited TNF-α directly stimulated miR-150-3p expression by activating NF-κB and inhibited mesenchymal stem cell osteogenesis [28].

This work puts forward a novel understanding of the interactions

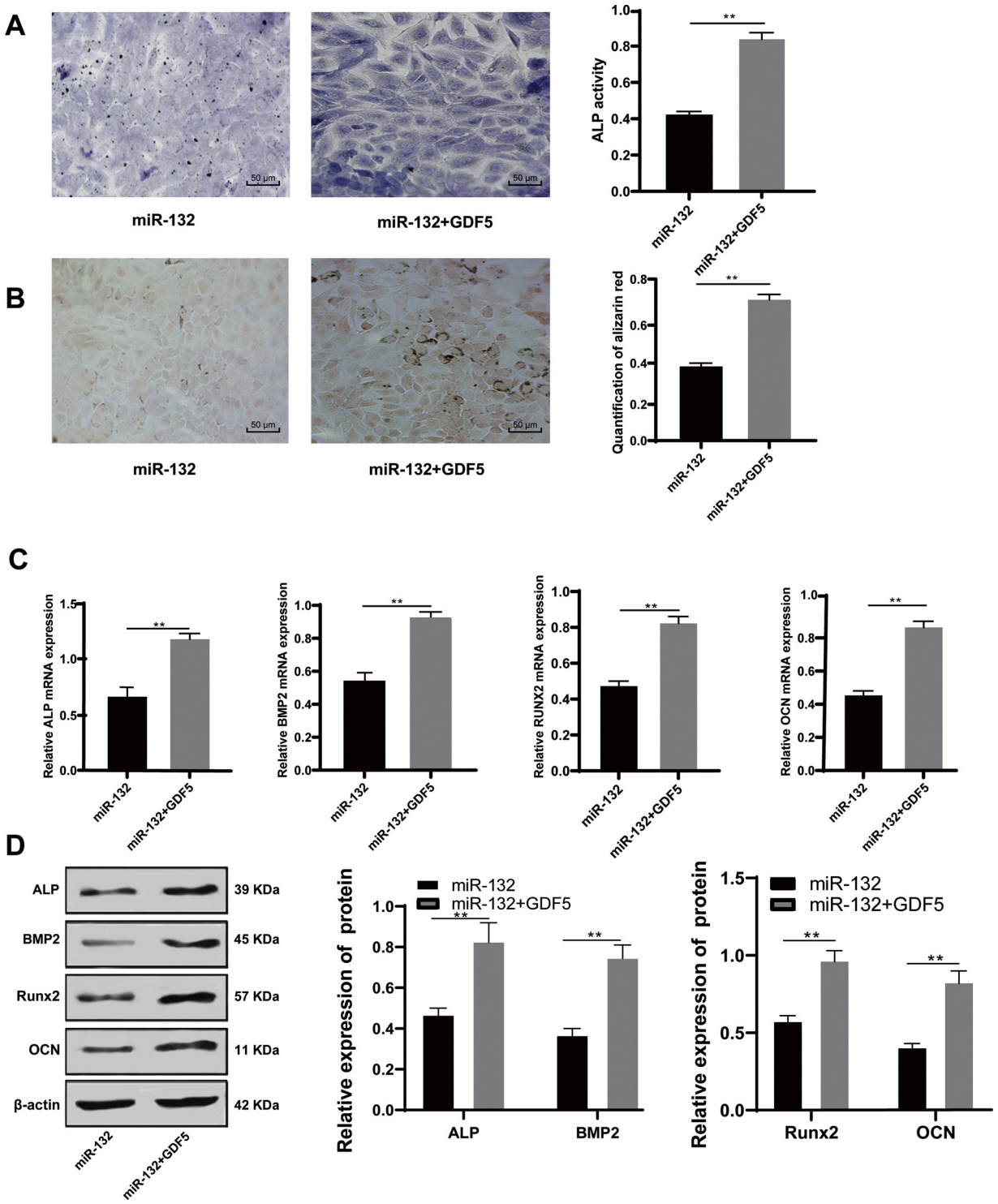

Fig. 4. GDF5 blocks the inhibitory effects of overexpressed miR-132 on osteogenic differentiation of PDLSCs. A. Representative images and histogram of ALP activity measured by ALP staining; B. Representative images and histogram of ARS activity measured by ARS staining; C–D. Relative mRNA expression and protein levels of ALP, BMP2, Runx2 and OCN detected by RT-qPCR and western blot analysis. ** $p < 0.01$, for pairwise comparison. Data in panel A, B and C were analyzed by the independent *t*-test, and data in panel D were analyzed by two-way ANOVA. Sidak's multiple comparisons test was used for post-test. All the experiments were repeated three times. GDF5, growth differentiation factor 5; miR-132, microRNA-132; PDLSCs, periodontal ligament stem cells; ALP, alkaline phosphatase; ARS, alizarin red S; BMP2, bone morphogenetic proteins 2; Runx2, runt-related transcription factor 2; OCN, osteocalcin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ANOVA, analysis of variance.

between PDLSCs, GDF5 and NF-κB mediated by miR-132, in which downregulated miR-132 accelerated PDLSC osteogenesis by targeting GDF5 and inhibiting the activation of NF-κB axis. Our results disclose the application of PDLSCs combined with GDF5, NF-κB and miR-132 is an useful approach to promoting periodontal tissue regeneration. However, this study is only on cell experiments. More extensive investigations should be performed to explore the potential applications of PDLSCs in clinical treatments for periodontal diseases and bone

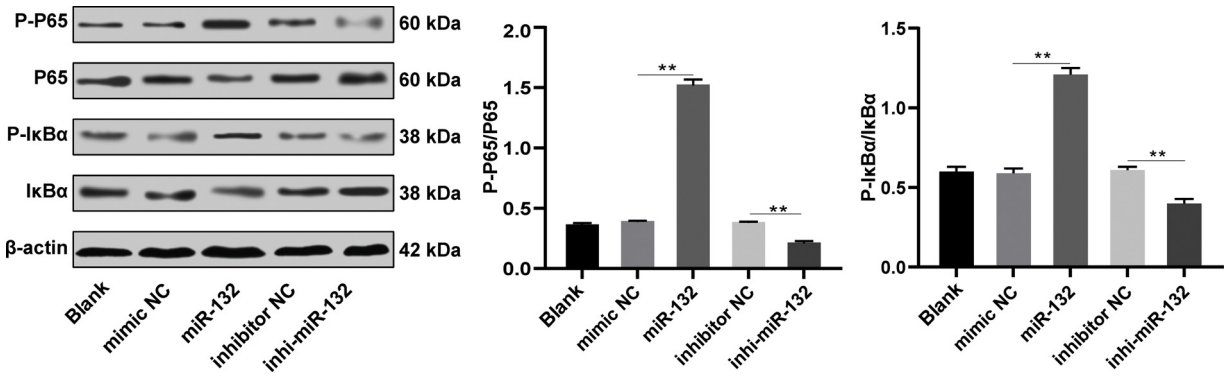

Fig. 5. miR-132 inhibits osteogenic differentiation of PDLSCs by targeting GDF5 and activating the NF-κB axis. Relative protein bands and levels of IκBα, P65, p-IκBα and p-P65 measured by western blot analysis. ** $p < 0.01$, for pairwise comparison. Data were analyzed by one-way ANOVA, and Tukey's multiple comparisons test was used for post-test. All the experiments were repeated three times. miR-132, microRNA-132; PDLSCs, periodontal ligament stem cells; GDF5, growth differentiation factor 5; NF-κB, nuclear factor κB; ANOVA, analysis of variance.

injury.

Declaration of Competing Interest

All authors declare that there is no conflict of interests in this study.

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