LIN28A alleviates inflammation, oxidative stress, osteogenic differentiation and mineralization in lipopolysaccharide (LPS)-treated human periodontal ligament stem cells

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Abstract. Periodontitis is a complex dental condition that has a number of different etiologies. Lin-28 homolog A (LIN28A) has been previously reported to regulate inflammation, where its expression levels have been indicated to be lower in periodontal tissues following periodontitis. However, there is a lack of evidence to indicate the precise role of LIN28A in periodontitis. In the present study, LIN28A and Runt-related transcription factor 2 (RUNX2) expression were measured in human periodontal biopsy tissues using reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR and western blot analyses were also used to measure LIN28A and RUNX2 expression in human periodontal ligament stem cells (hPDLSCs) following lipopolysaccharide (LPS) induction. Following construction of the LIN28A overexpression plasmid, the expression of LIN28A, RUNX2, osteopontin, osterix and osteocalcin were detected using RT-qPCR and western blotting. Additionally, RT-qPCR was used for the detection of proinflammatory biomarkers (IL-8, IL-1β and IL-6) and alkaline phosphatase (ALP) expression. Protein expression of intranuclear and cytoplasmic NF-κB p65 and NF-κB p65 phosphorylation were assessed using western blot analysis. The expression of antioxidant factors including SOD and GSH were determined using corresponding commercial assay kits. ALP and the mineralization capacity of hPDLSCs were detected by ALP activity assay and Alizarin red staining. The expression of LIN28A was found to be decreased in periodontal biopsy tissues from periodontitis patients compared with normal tissues and LPS-induced hPDLSCs compared with untreated hPDLSCs, which was positively correlated with RUNX2 expression. LIN28A overexpression was revealed to attenuate inflammatory damage and oxidative stress whilst improving ALP active damage, restoring RUNX2 expression and osteoblastic mineralization in LPS-induced hPDLSCs. In conclusion, the present study suggests that LIN28A serves a key role as a mediator of osteoblast differentiation and mineralization. In addition, LIN28A was able to alleviate inflammatory injury and oxidative stress in LPS-induced hPDLSCs.

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Introduction

Periodontitis is a chronic inflammatory disease and is the sixth most common human disease, with a prevalence of 45-50% worldwide (1). Although this disease can manifest either during childhood or adolescence, it most commonly occurs early adulthood, with a small number of cases also occurring in later life (2). Periodontitis occurs due to the accumulation of microbial plaque, such as that caused by *Porphyromonas gingivalis* and *Tannerella forsythia*, which have cell walls containing lipopolysaccharide (LPS), a major cause of the disease (3). The main features of periodontitis include clinical attachment loss, which is defined by the loss of the connective tissues and bone that support teeth, loss of alveolar bone (which can be assessed using radiology), periodontal pockets and bleeding gums (4). There is evidence to suggest an independent association between severe periodontitis and a number of different non-communicable diseases, including diabetes, atherosclerotic cardiovascular disease, chronic obstructive pulmonary disease and chronic kidney disease (1). The current treatment methods of periodontitis including scaling, surgery, and systemic antibiotics have made great progress, but the treatment effect remains unsatisfactory (2). If left untreated or addressed adequately, periodontitis can lead to the loss of the supporting tissues and teeth whilst also adversely obstruct chewing function, severely impairing the quality of life and significantly increasing the cost of dental treatment (5,6). Therefore, an effective treatment for periodontitis is in urgent demand.

Lin-28 homolog A (LIN28A) is an RNA-binding protein that has been found to regulate multiple aspects of cellular

activity, including stem cell self-renewal, metabolism and cell proliferation (7). Additionally, LIN28A serves an important role in the maintenance of pluripotency in embryonic stem cells (8). LIN28A has been indicated to promote the expression of stem cell markers CD133, CD44, Oct4 and Nanog homeobox in ovarian cancer (9). In addition, LIN28A has been reported to enhance the therapeutic possibilities of cultured neural stem cells in a rat model of Parkinson's disease (10). Overexpression of LIN28A can also potentiate osteogenic differentiation and its expression is downregulated during human embryonic stem cell differentiation (11). LIN28A mainly acts by inhibiting the maturation of lethal (Let)-7 microRNA (miRNA) precursors (12). Let-7a and Let-7b expression has been revealed to be regulated by LPS (13). LPS, a major component of the Gram-negative cell wall, induces inflammation and oxidative stress to inhibit osteogenic differentiation and stimulate mineralization (14-18). Additionally, a number of studies have demonstrated that the expression of LIN28A is decreased in periodontal tissues affected by periodontitis, whereby following the inhibition of LIN28A expression, the osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs) was also suppressed (3,19). However, the precise mechanistic role of LIN28A in periodontitis and osteoblast physiology remains poorly understood.

Therefore, the present study aimed to determine the expression profile of LIN28A and its potential effects on the inflammatory damage, oxidative stress, osteoblast differentiation and mineralization in LPS-induced hPDLSCs.

Materials and methods

Tissue sample collection and isolation of hPDLSCs. All the experimental protocols required for the present study were approved by the Ethics Committee of Meizhou People's Hospital, Meizhou Academy of Medical Sciences (Meizhou, China). In total, eight patients who were diagnosed with periodontitis (between the ages of 24 and 30, with a mean of 27.1±2.9 years; four male and four female) and eight healthy individuals (between the ages of 12 and 16, with a mean of 14.1±1.9 years; four male and four female) who wanted to receive orthodontics were selected for the present study and enrolled at the Meizhou People's Hospital between January 2016 and January 2018. All the patients had no history of chemotherapy and did not have other cancers, infectious diseases, or autoimmune diseases. All participants and their guardians signed written informed consent to take part in the study. The periodontal ligament tissues were extracted from the aforementioned patients and stored at -80˚C until further processing.

The teeth were extracted for orthodontic reasons from eight healthy individuals before being washed using PBS supplemented with 10% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, periodontal ligament tissues were scraped from the middle third of the tooth root and sectioned, because the periodontal ligament tissues in middle third were the thinnest, making it easier to digest to obtain cell suspensions and the rest, including coronal and apical portion of the periodontal ligament fibers, were discarded. These slices were then digested using collagenase/trypsin (3 mg/ml; MilliporeSigma) for 1 h at 37˚C. hPDLSCs were isolated from third-passage periodontal ligament cells using a cluster of differentiation (CD)146 microbead kit (Miltenyi Biotec GmbH) according to the manufacturer's instructions. The hPDLSCs were cultured in α-modified Eagle's medium (α-MEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Beyotime Institute of Biotechnology) and 100 mg/ml streptomycin and 100 U/ml penicillin in an incubator at 37˚C and 5% CO_2. Morphological changes of hPDLSCs were observed under a Labomed Inc. LB-601 metallurgical microscope (cat. no. 50-193-8116; Thermo Fisher Scientific, Inc.) at days 7, 10 and 14 during the cell culture. The hPDLSCs from passages 3-5 were used for the subsequent experiments.

Cell transfection. The full length of the LIN28A sequence was cloned into the pcDNA3.1 vector by Shanghai GenePharma Co., Ltd. to construct LIN28A overexpression (Ov-LIN28A) and negative control (Ov-NC) plasmids and cells were transferred into six-well plates. After 24 h, the LIN28A overexpression and negative control plasmids at a concentration of 20 μM were stably transfected into the hPDLSCs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were incubated with 5% CO_2 at 37˚C for 8 h and were used in subsequent experiments 48 h post-transfection.

Cell treatment. The hPDLSCs were treated with different concentrations (0.1, 1 and 10 μg/ml) of LPS (cat. no. ST1470; Beyotime Institute of Biotechnology) at 37˚C for 3 days. In another experimental protocol, hPDLSCs were treated with LPS at 10 μg/ml at 37˚C for 3 days 48 h post-transfection. Each experiment was repeated three times.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from periodontal ligament tissues and hPDLSCs using TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The extracted RNA was reverse transcribed into complementary DNA using a PrimeScript RT Reagent kit (Takara Bio, Inc.) following the manufacturer's protocol as follows: 25˚C for 5 min, 42˚C for 60 min and 70˚C for 5 min. mRNA expression quantification was performed via qPCR using One step SYBR Green RT-qPCR with MMLV and hot-start Taq DNA Polymerase (cat. no. QR0100; MilliporeSigma). The thermocycling conditions were: 95˚C for 5 min, followed by 40 cycles at 95˚C for 10 sec, 60˚C for 30 sec and 72˚C for 30 sec. All mRNA expression was normalized to that of GAPDH. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Cq}$ method (20). The experiments performed were performed in triplicate and repeated twice. The following primers were used for qPCR: LIN28A forward, 5'-TGCGGGCATCTGTAAGTGG-3' and reverse, 5'-GGAACCCTTCCATGTGCAG-3'; runt-related transcription factor 2 (RUNX2) forward, 5'-CCTGAACTCTGCACC AAGTC-3' and reverse, 5'-GAGGTGGCAGTGTCATCA TC-3'; IL-8 forward, 5'-ATGACTTCCAAGCTGGCCGTG GCT-3' and reverse, 5'-TCTCAGCCCTCTTCAAAAACT TCTC-3'; IL-1β forward, 5'-GCGGCCAGGATATAACTG ACTTC-3' and reverse, 5'-TCCACATTCAGCACAGGACTC TC-3'; IL-6 forward, 5'-ATGAACTCCTTCTCCACAAGC

GC-3' and reverse, 5'-GAAGAGCCCTCAGGCTGGACT-3'; alkaline phosphatase (ALP) forward, 5'-ACTGGTACTCAG ACAACGAGAT-3' and reverse, 5'-ACGTCAATGTCCCTG ATGTTATG-3'; osteopontin (OPN) forward, 5'-GAAGTT TCGCAGACCTGACAT-3' and reverse, 5'-GTATGCACC ATTCAACTCCTCG-3'; osterix (OSX) forward, 5'-GGC GTCCTCCCTGCTTGA-3' and reverse, 5'-TGCTTTGCC CAGAGTTGTTG-3'; osteocalcin (OCN) forward, 5'-CCC AGGCGCTACCTGTATCAA-3' and reverse, 5'-GGTCAG CCAACTCGTCACAGTC-3' and β-actin forward, 5'-GAC CTCTATGCCAACACAGT-3' and reverse, 5'-AGTACT TGCGCTCAGGAGGA-3'.

Western blot analysis. The protein samples were extracted from periodontal ligament tissues using RIPA buffer (Beyotime Institute of Biotechnology) containing 40 mM NaF, 20 mM Tris, 2.5 mM EDTA, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM $Na_4P_2O_7$ and 1 mM phenylmethylsulfonyl fluoride on ice and centrifuged at 14,000 x g for 30 min at 4˚C. Protein concentration was measured using a BCA Protein Assay kit. Equal amounts of protein (40 μg per lane) were loaded into each well of an 8% SDS-PAGE gel and separated using electrophoresis. The Separated proteins were then transferred onto PVDF membranes (MilliporeSigma). After blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with primary antibodies LIN28A (cat. no. ab279647; dilution, 1:1,000; Abcam), RUNX2 (cat. no. ab236639; dilution, 1:1,000; Abcam), phosphorylated (p-)-NF-κB p65 (cat. no. ab76302; dilution, 1:1,000; Abcam), NF-κB p65 (cat. no. ab32536; dilution, 1:1,000; Abcam), Lamin B1 (cat. no. ab16048; dilution, 1:1,000; Abcam), OPN (cat. no. ab214050; dilution, 1:1,000; Abcam), OSX (cat. no. ab209484; dilution, 1:1,000; Abcam), OCN (cat. no. ab133612; dilution, 1:1,000; Abcam) and GAPDH (cat. no. ab9485; dilution, 1:2,500; Abcam) overnight at 4˚C. These membranes were then washed with PBS three times and probed with the HRP-conjugated goat anti-rabbit IgG antibody (cat. no. ab6721; dilution, 1:2,000; Abcam) for 1 h at room temperature. The protein bands were visualized using enhanced chemiluminescent (ECL) substrate (cat. no. WBKLS0050; MilliporeSigma) and imaged using a ChemiDoc MP imager (Bio-Rad Laboratories, Inc.). Protein expression was quantified using the Image J 1.51 software (National Institutes of Health).

Measurement of reactive oxygen species (ROS), superoxide dismutase (SOD) and glutathione (GSH). The levels of ROS, SOD and GSH were measured to assess the extent of oxidative stress in hPDLSCs. A 2',7'-Dichlorofluorescin diacetate (DCFH-DA) measurement kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.) was used to detect ROS generation. Briefly, hPDLSCs cells at $1x10^6$ cells per well were washed twice with pre-warmed serum-free α-MEM and then stained with 5 μM DCFH-DA in serum-free medium at 37˚C for 20 min in the dark according to the manufacturer's protocol. SOD activity was evaluated using a SOD assay kit-WST (cat. no. S311-10; Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. GSH activity was assessed using a GSH assay kit (cat. no. KA3779; Abnova) following the manufacturer's protocol.

Induction of osteogenic differentiation in hPDLSCs. hPDLSCs were maintained at 37˚C in six-well plates loaded with osteogenic differentiation medium (Cyagen Biosciences, Inc.) containing 50 μg/ml ascorbic acid, 1 μM dexamethasone, 3 mM β-glycerophosphate from Guangzhou Saiguo Biotech Co., Ltd. and 5% FBS when they reached 80% confluence. The medium was refreshed every 3 days. For the control group, standard culture medium was loaded into the plates. The differentiation of hPDLSCs was observed through subsequent ALP activity assay and Alizarin red staining after culturing the cells for 21 days.

Alkaline phosphatase (ALP) activity assay. ALP activity was evaluated using an ALP assay kit (cat. no. P0321S; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. Cells were washed with PBS and homogenized using 50 μl ALP assay buffer on ice for 5 min and centrifuged (2,000 x g) at room temperature for another 5 min following 14 days of osteoblastic induction. Subsequently, 30 μl cell lysate, 50 μl assay buffer and 50 μl p-nitrophenyl phosphate were added into a 96-well plate. After incubation for 1 h at 25˚C, 20 μl stop solution was added into each well. Finally, the absorbance was measured at 450 nm using a microplate reader (cat. no. 11-120-533; Thermo Fisher Scientific, Inc.).

Alizarin red staining. The mineralization capacity of osteoblasts was assessed using Alizarin red staining. Cells were seeded into 24-well plates at a density of $1x10^5$ cells. Cells were grown in the osteogenic differentiation medium (Cyagen Biosciences, Inc.) for 21 days at 37˚C. Subsequently, cells were fixed with 4% cold methanol for 15 min at room temperature and stained with Alizarin red (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at 37˚C. Subsequently, the mineralization capacity of hPDLSCs was imaged using an inverted light microscope (magnification, x200) in five fields.

Statistical analysis. All experimental data are presented as the mean ± SD and all experiments were repeated at least three times. Statistical analysis was performed using the SPSS 13.0 Statistics Software (SPSS, Inc.). An unpaired Student's t-test was used to analyze results between two groups and one-way analysis of variance followed by a Tukey's post hoc test was applied to analyze results among ≥ three groups. Pearson's correlation analysis was utilized to confirm the correlation between LIN28A and RUNX2. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LIN28A expression is reduced in periodontal biopsy tissues and is positively associated with RUNX2 expression. To determine the expression levels of LIN28A and RUNX2, samples were divided into two groups of control and pathological tissues. The measurements were subsequently performed using RT-qPCR. Fig. 1A indicates a significant decrease in LIN28A expression in the periodontitis group compared with that in the control group. Similarly, a significant decrease in RUNX2 expression in the periodontitis group was found compared with that in the control group (Fig. 1B). In addition, the expression of LIN28A and RUNX2 was demonstrated to be positively associated in the periodontal biopsy tissues (Fig. 1C).

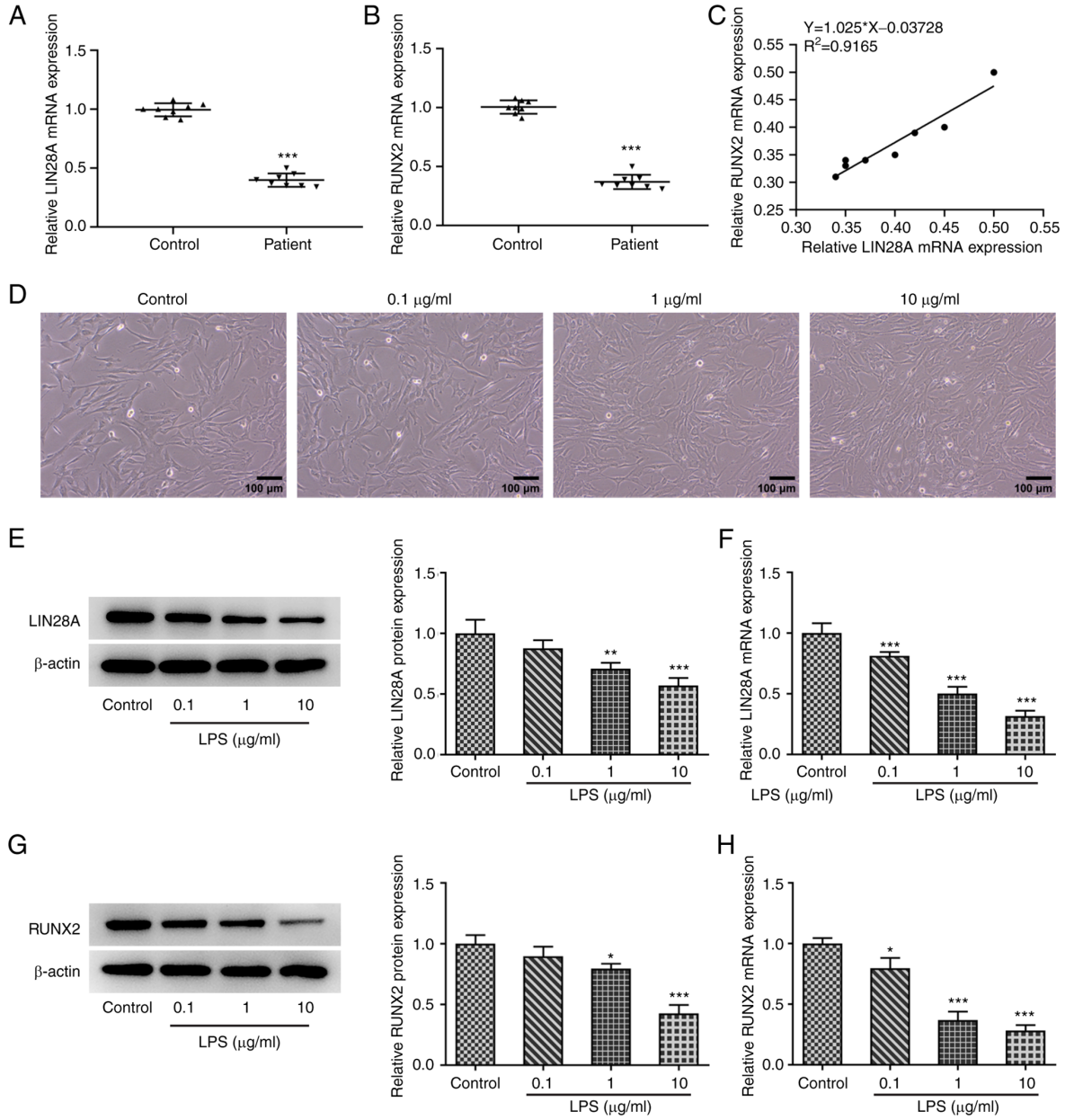

Figure 1. LIN28A expression is reduced in periodontal tissue biopsies and LPS-induced hPDLSCs. The expression of (A) LIN28A and (B) RUNX2 in periodontal biopsy tissues was examined by RT-qPCR. (C) The correlation between the mRNA expression level of LIN28A and RUNX2 in periodontal biopsy tissues was examined. (D) Cell morphology and number of hPDLSCs after treatment with different concentrations of LPS were observed by microscope. Scale bars, 100 μm. LIN28A expression in LPS-induced hPDLSCs was measured by (E) western blotting and (F) RT-qPCR. RUNX2 expression in LPS-induced hPDLSCs was determined by (G) western blotting and (H) RT-qPCR. *P<0.05, **P<0.01 and ***P<0.001 vs. Control. LIN28A, Lin-28 homeobox A; RUNX2, Runt-related transcription factor 2; RT-qPCR, reverse transcription-quantitative PCR; hPDSCs, human periodontal ligament stem cells; LPS, lipopolysaccharide.

LIN28A expression is reduced in LPS-induced hPDLSCs. The expression of LIN28A and RUNX2 were assessed in hPDLSCs using RT-qPCR and western blotting after 3 days of LPS treatment at concentrations of 0.1, 1 and 10 μg/ml. A marked increase in the number of hPDLSCs was observed following LPS induction (Fig. 1D). The expression of LIN28A was revealed to be significantly decreased in hPDLSCs with increasing concentration of LPS (Fig. 1E and F). The results also demonstrated progressively decreasing RUNX2 expression with increasing LPS concentrations (Fig. 1G and H). These results suggest that LIN28A expression was decreased in LPS-induced hPDLSCs.

LIN28A attenuates LPS-induced inflammatory damage in hPDLSCs. To assess the potential effects of LIN28A on inflammatory damage in LPS-induced hPDLSCs, the expression of LIN28A, IL-8, IL-1β and IL-6, the protein levels of NF-κB p65 in the nucleus and cytoplasm and total NF-κB p65 phosphorylation in both the cytoplasm and nucleus were measured using RT-qPCR and western blotting. After the hPDLSCs were transfected with Ov-LIN28A, the expression of LIN28A was significantly increased compared with the Ov-NC group (Fig. 2A and B). LIN28A expression was discovered to be downregulated following LPS treatment and there was also a significant increase in the expression of LIN28A in LPS-induced hPDLSCs transfected with Ov-LIN28A compared

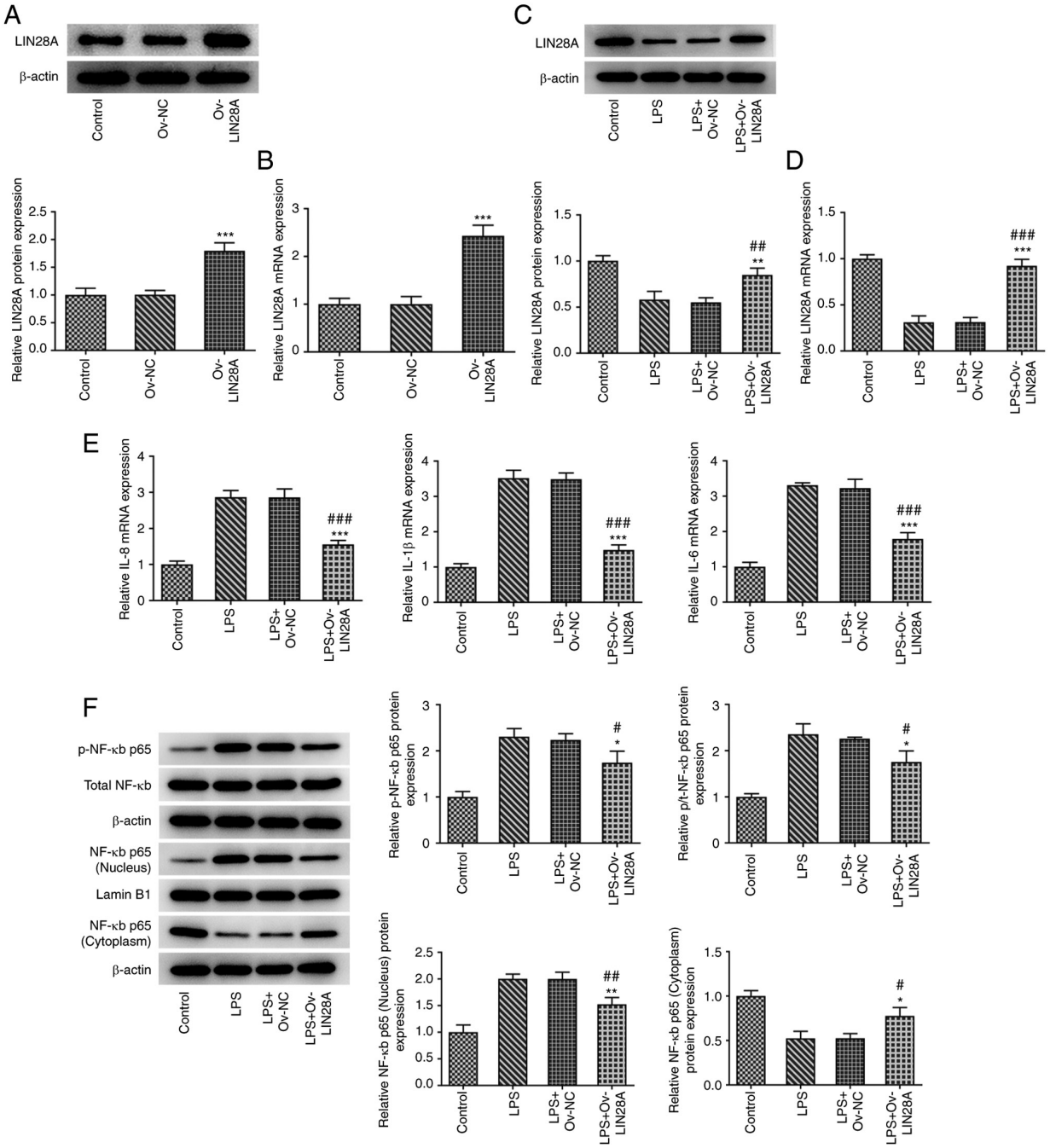

Figure 2. LIN28A overexpression attenuates LPS-induced inflammatory damage in hPDLSCs. (A) A plasmid of LIN28A was constructed for the overexpression of LIN28A and transfection efficiency in hPDLSCs was tested by (A) western blotting and (B) RT-qPCR. The expression of LIN28A in transfected hPDLSCs induced by LPS was measured by (C) western blotting and (D) RT-qPCR. (E) The expression of IL-8, IL-1β and IL-6 in transfected hPDLSCs induced by LPS were quantified by RT-qPCR. (F) The protein expression levels of NF-κB p65 in the nucleus and cytoplasm in addition to NF-κB p65 phosphorylation in transfected hPDLSCs induced by LPS were measured by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS + Ov-NC. LIN28A, Lin-28 homeobox A; LPS, lipopolysaccharide; hPDSCs, human periodontal ligament stem cells; RT-qPCR, reverse transcription-quantitative PCR; Ov, overexpression; NC, negative control.

with that in LPS-induced hPDLSCs transfected with Ov-NC (Fig. 2C and D). In addition, LPS treatment elevated the levels of IL-8, IL-1β and IL-6. The expression of IL-8, IL-1β and IL-6 exhibited a significant decrease following overexpression of LIN28A in comparison with the LPS+Ov-NC group (Fig. 2E). LPS treatment increased the protein levels of NF-κB p65 in the nucleus and p-NF-κB p65 and while decreased the protein level of NF-κB p65 in the cytoplasm. Furthermore, following the overexpression of LIN28A, there was a significant decrease in the protein levels of NF-κB p65 in the nucleus and p-NF-κB p65, in addition to a significant rise in the protein levels of NF-κB p65 in the cytoplasm compared with the LPS+Ov-NC group (Fig. 2F). Results from the present study revealed that the levels of inflammatory factors IL-8, IL-1β and IL-6, NF-κB p65 in the nucleus and p-NF-κB p65 were decreased following the overexpression of LIN28A. This suggests that LIN28A may serve a role in reducing inflammatory damage in LPS-induced hPDLSCs.

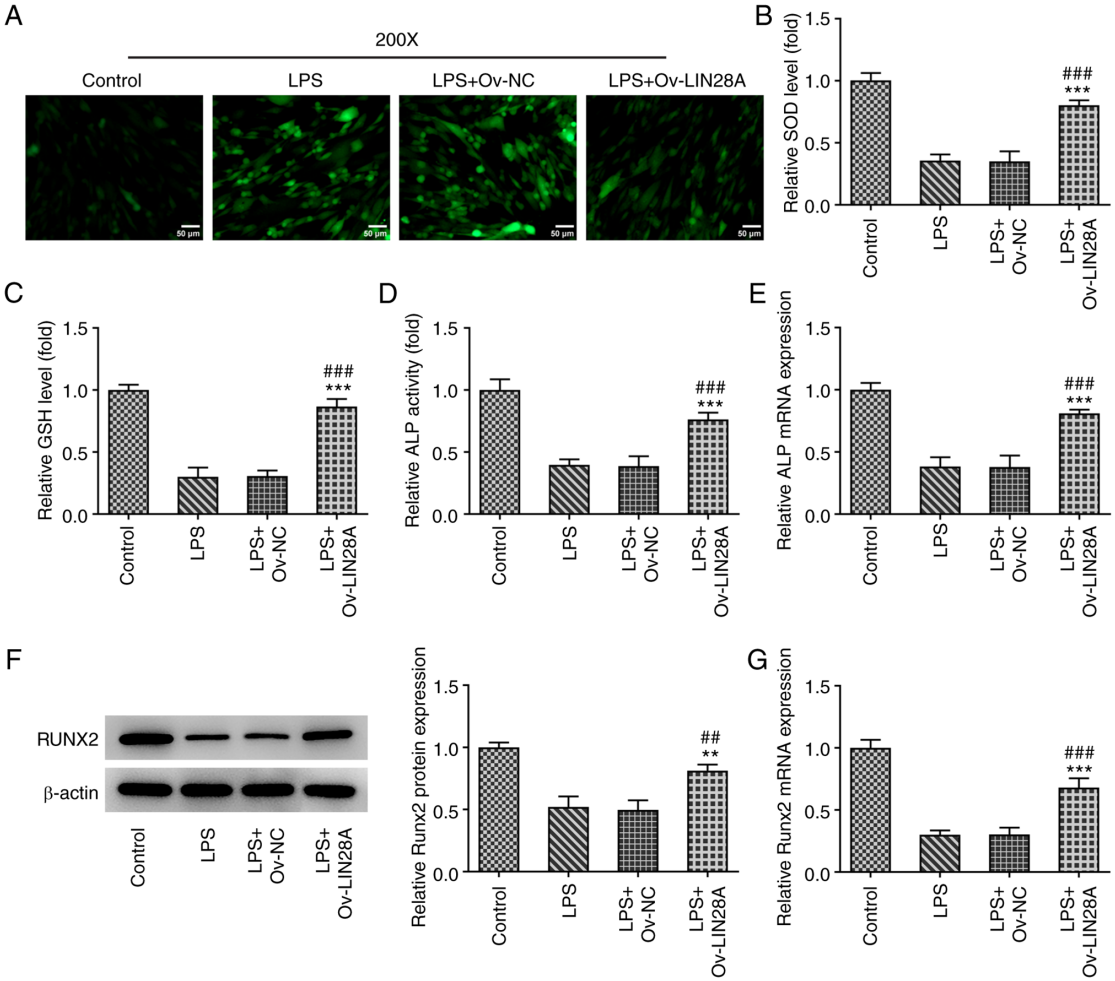

Figure 3. LIN28A overexpression alleviates oxidative stress damage and upregulates RUNX2 expression in LPS-induced hPDLSCs. (A) Reactive oxygen species levels in transfected hPDLSCs induced by LPS were measured using a 2',7'-dichlorofluorescin diacetate measurement kit. Scale bars, 50 μm. The levels of (B) SOD and (C) GSH in transfected hPDLSCs induced by LPS were determined using their corresponding kits. (D) An ALP kit was used for detecting the activity of ALP in transfected hPDLSCs induced by LPS. (E) RT-qPCR was used to measure ALP expression in transfected hPDLSCs induced by LPS. RUNX2 expression in transfected hPDLSCs induced by LPS was tested by (F) western blotting and (G) RT-qPCR. **P<0.01 and ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS + Ov-NC. LIN28A, Lin-28 homeobox A; RUNX2, Runt-related transcription factor 2; LPS, lipopolysaccharide; hPDSCs, human periodontal ligament stem cells; Ov, overexpression; NC, negative control; SOD, superoxide dismutase; GSH, glutathione; ALP, alkaline phosphatase.

LIN28A alleviates oxidative stress damage in LPS-induced hPDLSCs. To investigate further if LIN28A can reduce oxidative stress injury induced by LPS in hPDLSCs, the levels of ROS, SOD and GSH were examined using corresponding commercial kits. ROS level was noted to be enhanced following LPS treatment and a marked decrease in ROS levels was found following LIN28A overexpression compared with those in the LPS+Ov-NC group (Fig. 3A). However, the levels of anti-oxidants SOD and GSH were decreased following LPS induction but were significantly elevated after transfection with LIN28A compared with the LPS+Ov-NC group (Fig. 3B and C). These data suggest that LIN28A overexpression can mitigate oxidative stress injury in LPS-induced hPDLSCs.

LIN28A reverses ALP activity impairment and restores RUNX2 expression in LPS-induced hPDLSCs. To elucidate the effects of LIN28A on hPDLSCs, detection of ALP activity was performed using corresponding kits whereas the expression of ALP and RUNX2 was examined using RT-qPCR and western blotting. As a catalytic enzyme for osteoblast differentiation, the activity and expression of ALP were found to be markedly decreased following the treatment of LPS, but significantly increased following LIN28A overexpression relative to the LPS+Ov-NC group (Fig. 3D and E). Additionally, LPS treatment reduced the expression of the osteoblast transcription factor RUNX2 and there was a significant increase in the expression of RUNX2 after the hPDLSCs were transfected with the Ov-LIN28A plasmid (Fig. 3F and G). As a consequence, increased RUNX2 expression, osteoblast differentiation and the restoration of ALP activity all suggest an ameliorative effect of LIN28A overexpression on LPS-induced hPDLSCs.

LIN28A facilitates osteoblast mineralization in LPS-induced hPDLSCs. To assess the role of LIN28A on osteoblast mineralization, Alizarin red staining was used to evaluate the mineralization capacity of osteoblasts cultured for 21 days. RT-qPCR and western blotting were used for the measurement of the expression of osteogenic-specific

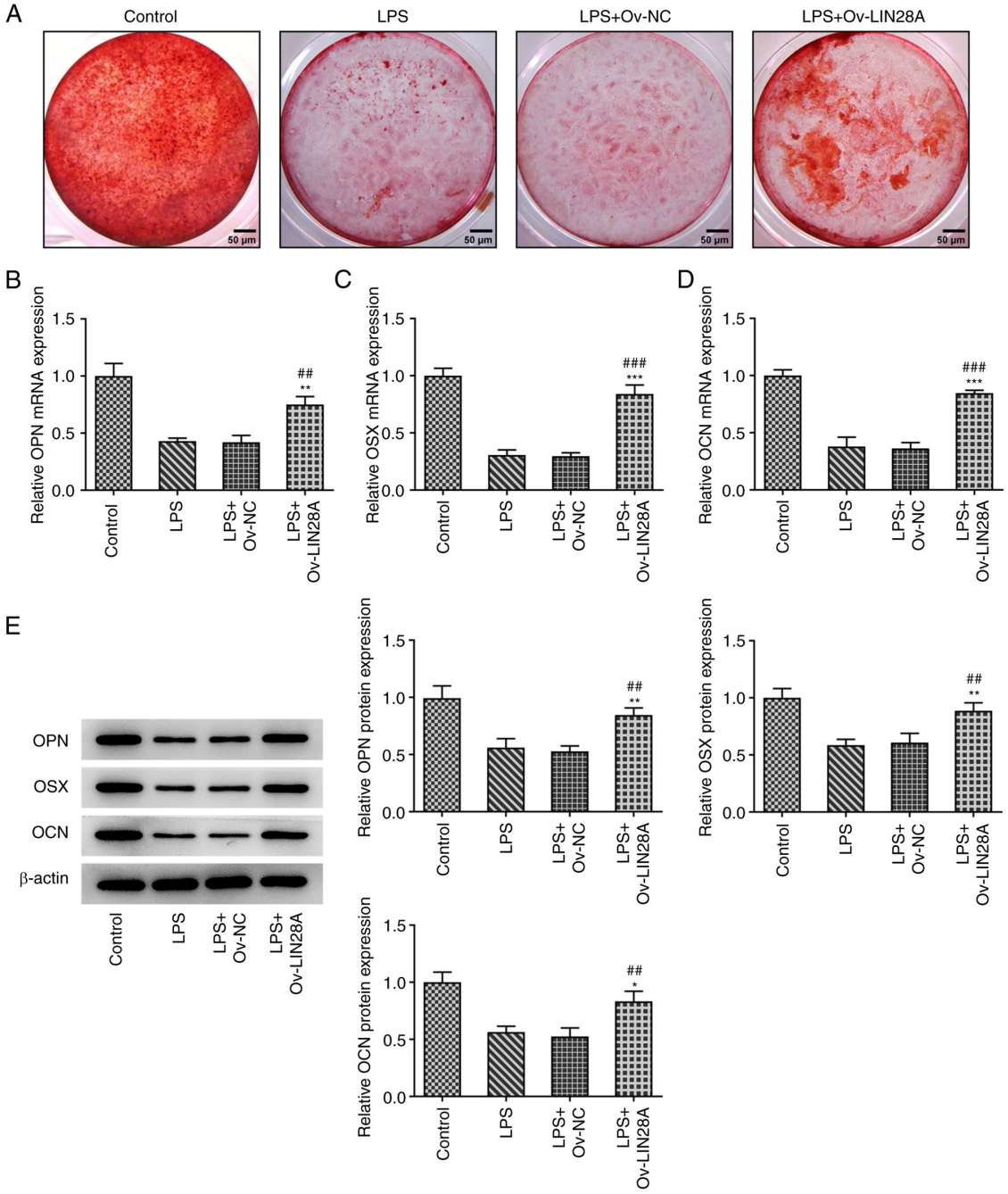

Figure 4. LIN28A facilitates osteoblast mineralization in LPS-induced hPDLSCs. (A) Alizarin red staining was used to evaluate the mineralization capacity of osteoblasts. Scale bars, 50 μm. The expression of (B) OPN, (C) OSX and (D) OCN in transfected hPDLSCs induced by LPS were assessed by reverse transcription-quantitative PCR. (E) The protein levels of OPN, OSX and OCN in transfected hPDLSCs induced by LPS were measured by western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. Control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS + Ov-NC. Lin-28 homeobox A; LPS, lipopolysaccharide; hPDSCs, human periodontal ligament stem cells; OPN, osteopontin; OSX, osterix; OCN, osteocalcin; Ov, overexpression; NC, negative control.

matrix proteins OPN and OCN, in addition to the osteoblast-specific transcription factor OSX. As shown in Fig. 4A, the degree of Alizarin red staining was lower following LPS induction compared with that in the control group, but was restored following LIN28A overexpression. In addition, LPS treatment reduced the expression of OPN, OSX and OCN while a significant elevation in OPN, OSX and OCN expression was observed compared with that in LPS+Ov-NC group (Fig. 4B-E). These results suggest that LIN28A facilitated osteoblast mineralization in hPDLSCs induced by LPS.

Discussion

A number of recent studies have demonstrated that LIN28A is a highly conserved RNA-binding protein that is closely associated with the differentiation of keratinocytes (21) and glial lineage cells (22). LIN28A has been previously found to regulate neuronal differentiation and regulate miR-9 expression (23). In addition, LIN28A expression was revealed to be downregulated during human and murine embryonic stem cell differentiation, where the balance between the expression of LIN28A and let-7 miRNAs was proposed to be important for

embryonic stem cell self-renewal and differentiation (24,25). However, the effects of LIN28A on osteogenic differentiation and mineralization in hPDLSCs remain poorly understood.

RUNX2 is a transcription factor that is essential for osteoblast differentiation (26), as it directs pluripotent mesenchymal cells towards an osteoblast lineage (27). ALP is a byproduct of osteoblast activity, where its elevation indicates the possible presence of active bone formation (28). The present study found that LIN28A expression was decreased in periodontal tissues effected by periodontitis, whereas a positive correlation was observed between RUNX2 and LIN28A expression. Consequently, these results suggest that LIN28A can improve osteoblast differentiation under LPS treatment. Downstream, RUNX2 can induce the expression of OPN, OCN and OSX (29), which have been revealed to regulate bone formation (30). The present study also demonstrated that LIN28A overexpression promoted the expression of RUNX2, ALP, OPN, OSX and OCN. In addition, Alizarin red staining also demonstrated that LIN28A enhanced the mineralization ability of osteoblasts. These results suggest that LIN28A may promote osteoblast differentiation and mineralization in LPS-induced hPDLSCs.

Previous studies have indicated that LIN28A is associated with the inflammatory response (31). LIN28 has been revealed to participate in the LPS-induced astrocyte inflammatory response through the NF-κB signaling pathway (32). Additionally, an inflammatory signal may initiate an epigenetic switch into the oncogenic gene expression program through a positive feedback loop involving NF-κB, LIN28, let-7 and IL-6 in cancer cells (33). It has been reported that NF-κB is a sequence-specific transcription factor that serves an important role in the inflammatory response (34,35). Activation of NF-κB leads to the production of proinflammatory cytokines and chemokines, such as IL-1, IL-6, IL-8 and TNF-α (3). In the present study, proinflammatory cytokines IL-8, IL-1β and IL-6, the levels of NF-κB p65 in the nucleus and p-NF-κB p65 in hPDLSCs induced by LPS were all significantly decreased following the overexpression of LIN28A. By contrast, the levels of NF-κB p65 was increased in the cytoplasm. Therefore, results from the present study suggest that LIN28A can attenuate inflammatory damage in LPS-induced hPDLSCs.

Oxidative stress refers to the imbalance between the oxidative and antioxidative systems within cells and tissues, resulting in the excessive accumulation of oxidative free radicals and associated with ROS (36). SOD and GSH are important antioxidant enzymes and antioxidants in the body that protect an organism from oxidative damage (37). A previous study has indicated that LIN28A overexpression can reduce ROS production in hypoxia/reoxygenation induced cardiomyocytes under high glucose/high fat conditions (38). In the present study, LIN28A overexpression reduced ROS levels whilst inducing an increase in SOD and GSH levels in hPDLSCs treated with LPS, suggesting that LIN28A can alleviate oxidative stress damage in LPS-induced hPDLSCs.

In conclusion, results of the present study revealed that LIN28A expression was decreased in periodontal biopsy tissues and LPS-induced hPDLSCs, which was also positively correlated with RUNX2 expression. Furthermore, LIN28A was found to attenuate inflammatory damage and oxidative stress, whilst improving osteoblast differentiation and mineralization in LPS-induced hPDLSCs. Therefore, LIN28A may serve as a potential therapeutic target in patients with periodontitis, which is potentially a novel mechanism underlying this disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LG and LL designed the study and analyzed the data. LL performed the experiments. LG and LL drafted the manuscript and interpreted the data. LG and LL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Meizhou People's Hospital, Meizhou Academy of Medical Sciences (Meizhou, China). All participants and their guardians signed written informed consent to take part in the study.

Patient consent for participation

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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