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KLF2 Promotes Osteogenic Differentiation of Human Periodontal Ligament Stem Cells by Regulating Nrf2 Expression

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ABSTRACT

Objective: Periodontal ligament stem cells (PDLSCs) are emerging as a promising source for periodontal regeneration and to manage periodontitis. This study aims to investigate the roles of Krüppel-like factor 2 (KLF2) and nuclear factor erythroid 2-related factor (Nrf2) in mediating osteogenic differentiation of human PDLSCs (hPDLSCs) in the context of lipopolysaccharide (LPS) stimulation.

Methods: The osteogenic differentiation potential of hPDLSCs isolated from human premolar root samples were examined by alkaline phosphatase (ALP) staining and ALP activity assay, Alizarin red S staining and quantitative analysis of mineralised matrix. Intracellular reactive oxygen species (ROS) production and glutathione (GSH) concentration were assessed to reflect oxidative stress.

Results: KLF2 overexpression influenced Nrf2-regulated transcription, leading to significant increases in GSH concentration, ALP activity, mineralised matrix formation, and RUNX2 expression in LPS-stimulated hPDLSCs, as well as significant reductions in ROS production and cell apoptosis. The subsequent Nrf2 knockdown impaired the protective effect of KLF2 on hPDLSCs against LPS stimulation.

Conclusion: The findings of the study demonstrate KLF2 overexpression has the ability to promote the osteogenic differentiation of hPDLSCs by promoting Nrf2 activation, suggesting that KLF2 mediating Nrf2 could be a promising target to facilitate the efficacy of PDLSC-based bone regeneration in periodontitis.

Introduction

Periodontitis is a chronic inflammation condition that contributes to progressive degradation and loss of tooth-supporting structures, particularly the periodontal ligament (PDL), until tooth loss occurs.[1] The ultimate goal of periodontal regenerative therapy is to achieve functional regeneration of periodontally diseased tissues, and stem cell-based tissue engineering has emerged as the key component of periodontal tissue engineering technology.[2] Periodontal ligament stem cells (PDLSCs) exhibit strong self-renewal abilities and multidirectional differentiation potential, are considered as the most ideal cell source among dental stem cells for tissue engineering to repair the degeneration of periodontal ligament.[3] The osteogenic differentiation potential of PDLSCs is of great significance for successful periodontal regeneration, creating a critical need to maintain or enhance this differentiation of PDLSCs by gene-addition/editing therapy or combing with the usage of biomaterials.[4,5] For example, injectable and self-setting calcium phosphate cement scaffold delivering human PDLSCs (hPDLSCs) and metformin demonstrated excellent efficacy for dental and craniofacial applications.[6] Cell sheet engineering based on recombinant human BMP-2 pre-treated hPDLSCs could effectively promote the regeneration of fibrous tissues and mineralised tissue in periodontal complexes.[7]

Krüppel-like factor 2 (KLF2) is a transcription factor belonging to the KLF family that are involved in regulating proliferation, cell adhesion, differentiation, and migration processes of

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various cell types.[8-10] KLF2 has been extensively studied as an important indicator of the stemness of human mesenchymal stem cells (hMSCs) and KLF2+ stemness-maintained hMSCs show more efficiently osteogenic differentiation potential.[11] Additionally, KLF2 overexpression could promote osteogenic differentiation of dental pulp-derived stem cells by inducing mitophagy and autophagy.[12,13] Epigenetic silencing of KLF2 was previously reported to impair the osteogenic differentiation of PDLSCs, and improved osteogenic differentiation was found in PDLSCs after KLF2 overexpression.[14] KLF2 primes the activation of nuclear factor erythroid 2-related factor (Nrf2), an antioxidant transcription factor, in a variety of cells, such as endothelial cells[15] and vascular smooth muscle cells.[16] The activation of Nrf2-mediated antioxidant pathway has been implicated in osteogenic differentiation of PDLSCs, which were previously proved in several studies.[17-19] However, limited evidence uncovers the key role of KLF2 in hPDLSCs in response to inflammatory stimuli and the underlying mechanism. Accordingly, we proposed an interesting hypothesis that hPDLSCs benefit with improved osteogenic differentiation from KLF2 gene modification and the Nrf2-mediated antioxidant pathway may be involved in the effects of KLF2. To prove this hypothesis, we mimicked an inflammatory condition by lipopolysaccharide (LPS) stimulation to investigate the effects of KLF2 mediating Nrf2 on proliferation, apoptosis, and osteogenic differentiation of hPDLSCs.

Materials and methods

Isolation of hPDLSCs

Four premolars were obtained from 4 systemically healthy patients (2 males and 2 females, with age ranging from 12 to 17 years) who received orthodontic treatment at our hospital on the premise of obtaining informed consent from the legal guardians. The premolar extraction was approved by the Ethics Committee of our hospital. The extracted premolars were raised repeatedly with precooled phosphate buffered saline (PBS, Corning Inc.) containing 100 U/mL penicillin and 100 μg/mL streptomycin (Beyotime). The periodontal ligament tissues were scraped off from the middle root of the teeth by using a sterile scalpel and placed into the centrifuge tube in the super-clean worktable. Following treatment with PBS containing 3 mg/mL collagenase I (Sigma Aldrich) for 40 minutes and shaking at a 10-minute interval, the tube was added with α-MEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) to terminate collagenase I treatment. After centrifugation at 700 $\times$ *g* for 10 minutes for removal of the supernatant, the tube was added with α-MEM containing 20% FBS. The mixture in the tube was inoculated into a culture flask which was placed in the incubator (37°C, 5% CO_2, saturated humidity), with the medium replaced every 3 days. Once reaching approximately 90% confluent, the cells were subcultured for 3 generations.

Characterisation and multidifferentiation of hPDLSCs

The third generation of hPDLSCs were characterised by fluorescence-activated cell sorting (FACS) analysis using a CytoFLEX (Beckman Coulter) with fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies against CD73, CD90, and CD45 (eBioscience). For osteogenic potential detection of hPDLSCs, the third generation of hPDLSCs were treated with pancreatin to make single cell suspension. The suspension was inoculated into a 6-well plate (2×10^5/well) and cultured with osteogenic induction medium consisting of α-MEM with 10% FBS (Gibco), 50 μg/mL ascorbic acid (Solarbio), 10 mmol/l β-glycerophosphate sodium (Solarbio), and 100 nmol/L dexamethasone (Solarbio). The osteogenic induction medium was refreshed every 3 days. After 14 days, the cells were stained with 2% Alizarin red S (Sigma Aldrich). For adipogenic potential detection of hPDLSCs, the 6-well plate inoculated with 2×10^5 hPDLSCs each well was cultured with the adipogenic induction medium consisting of α-MEM medium containing 10% FBS (Gibco), 1 μM dexamethasone (Solarbio), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Aldrich), and 10 μg/mL insulin (Solarbio). The adipogenic induction medium was refreshed every 3 days. After 14 days, the cells were stained with oil red O (Sigma Aldrich).

Cell treatment

The third generation of hPDLSCs were subjected to different treatments including lipopolysaccharide (LPS) stimulation[20] (0.1, 1.0, and 2.0 μg/mL LPS, Sigma Aldrich) for 24 hours, transfection of oe-NC and si-NC followed by LPS (1.0 μg/mL) stimulation (oe-NC+si-NC+LPS group), transfection of oe-KLF2 and si-NC followed by LPS (1.0 μg/mL) stimulation (oe-KLF2+si-NC+LPS), transfection of oe-KLF2 and si-Nrf2 followed by LPS (1.0 μg/mL) stimulation (oe-KLF2+si-Nrf2+LPS). The hPDLSCs were transfected with the recombinant pcDNA3.1(-)/KLF2 cDNA vector (GenePharma, Shanghai, China) for 48 hours by using lipofectamine 3000 reagents (Invitrogen) according to the manufacturer's instructions, to achieve KLF2 overexpression, with vector-null (GenePharma) used as control. The hPDLSCs were transfected with siRNA against Nrf2 (si-Nrf2, GenePharma) for 48 hours by using lipofectamine 3000 reagents (Invitrogen) according to the manufacturer's instructions, to achieve Nrf2 knockdown, with corresponding nonspecific control (NC) siRNA (si-NC, GenePharma) used as control. After reaching 90% confluence, the cells were maintained in serum-free, antibiotic-free α-MEM for 24 hours and then added with the transfection mixture comprising 1 μg of DNA and 2 μL of Lipofectamine 3000 (Invitrogen) in 50 μL of Opti-MEM I (ThermoFisher). After 6 hours at 37°C in a CO_2 incubator, the medium of the cells was replaced with a complete medium, and the cells were incubated for another 48 hours. The hPDLSCs cultured with α-MEM medium containing 10% FBS only were set as a control group.

Alkaline phosphatase staining and ALP activity assay

The alkaline phosphatase (ALP) staining was performed by using a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime). Briefly, the hPDLSCs were seeded in 12-well plates (10^5 cells per well) and cultured in the osteogenic induction medium concurrent with different treatments. The osteogenic induction medium was refreshed every 3 days.

After 14-day osteogenic induction, the hPDLSCs were PBS-raised twice and paraformaldehyde-fixed, followed by addition of BCIP/NBT solution. After staining for 6 hours, the hPDLSCs stained by ALP were captured with the aid of an Olympus BX-53 microscope (Tokyo, Japan). To determine the ALP activity, protein extraction was performed in hPDLSCs undergoing 14-day osteogenic induction and analysed by using an Alkaline Phosphatase Assay Kit (Beyotime).

Alizarin red S staining and quantification

Following dual PBS washes and treatment of paraformaldehyde, the hPDLSCs undergoing 14-day osteogenic induction concurrent with different treatments were stained with 2% Alizarin red S (Sigma Aldrich). After staining for 6 hours, the mineralised nodules formed were captured with the aid of an Olympus BX-53 light microscope and then desorbed by addition of 10% cetylpyridinium chloride (Sigma–Aldrich) to quantify the staining (optical density at 570 nm).

Cell proliferation assays

The hPDLSCs undergoing different treatments were seeded in a 96-well plate (5000 cells for each well). Following an incubation at 3 time points (24, 48, and 72 hours), each well in the plate was added with 10 uL of CCK-8 reagent (Beyotime) and incubated for 4 hours. By using a microplate reader for absorbance measurement at 450 nm, cell proliferation rate was obtained.

Cell apoptosis assays

The Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) with the aid of the BD FACSCalibur flow cytometer (BD Biosciences) was utilised for cell apoptosis assay. The hPDLSCs undergoing 14-day osteogenic induction concurrent with different treatments were seeded in 12-well plates (10^5 cells per well) and resuspended in 300 mL of binding buffer followed by the addition of Annexin V-fluorescein isothiocyanate and propidium iodide, 5 μL for each stain.

Enzyme-linked immunosorbent assay

The contents of IL-1β (ab214025, Abcam), TNF-α (ab181421, Abcam), IL-6 (ab178013; Abcam) were analysed in cell culture supernatants of hPDLSCs after different treatments with the aid of enzyme-linked immunosorbent assay kits in keeping with the procedures recommended by the manufacturers.

Reactive oxygen species production measurement

The ROS levels were measured with the aid of the reactive oxygen species (ROS) assay kit (Beyotime). After incubation with the diluted fluoroprobe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 minutes at 37°C, the hPDLSCs undergoing 14-day osteogenic induction concurrent with different treatments were washed with serum-free culture medium and collected, with the fluorescence intensity read (excitation wavelength: 488 nm; emission wavelength: 535 nm) by using a fluorescent microplate reader (SpectraMaxM2, Molecular Devices Corp.).

Glutathione consumption measurement

Intracellular glutathione (GSH) levels were determined with a Glutathione Assay kit (Beyotime) by using the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) cycling method. The GSH level was spectrophotometrically detected at 412 nm with a microplate reader.

Quantitative real-time polymerase chain reaction

Extraction of total RNA from the hPDLSCs and the following reverse transcription into cDNA were completed by using Trizol reagent (Invitrogen) and PrimeScript RT reagent (Takara), respectively. The mRNA expression levels of KLF2 and Nrf2 were determined by using SYBR Premix Ex Taq (Takara) with the aid of a Bio-Rad CFX96 PCR system (Bio-Rad). The resulting mRNA expression level was normalised GAPDH mRNA and calculated by using the $2^{-\Delta\Delta Ct}$ method. Used primer sequences are listed in Table.

Western blotting

The hPDLSCs reacted in the RIPA lysis buffer to obtain total protein, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) separation, transfer onto polyvinylidene fluoride membrane, and blocking buffer treatment (5% skim milk in TBST). The membranes were incubated with anti-KLF2 (PA5-40591, Invitrogen), anti-Nrf2 (PA5-27882, Invitrogen), anti-HO-1 (#70081, Cell Signaling Technology), anti-NQO1 (MA1-16672, Invitrogen), anti-RUNX2 (#2556, Cell Signaling Technology), and anti-β-actin (#4967, Cell Signaling Technology), followed by rinse with TBST and incubation with secondary antibodies. The signal of immunoblots was visualised using an ECL chromogenic kit (Beyotime) and β-actin normalised the density of each immunoblot.

Statistical analysis

Results of mean with SD were yielded from 3 biological and technical replicates. One-way analysis of variance (ANOVA) plus Tukey's post-hoc test, and 2-way ANOVA plus

Table – The primer sequences used for the qRT-PCR analysis.

Target	Primer sequence
KLF2	Sense: 5'-CACCAAGAGTTCGCATCTGA-3'
	Antisense: 5'-CATGTGCCGTTTCATGTG-3'
Nrf2	Sense: 5'-AGTCCAGAAGCCAAACTATCT-3'
	Antisense: 5'-ACACTTCCAGGGGCACTATCT-3'
HO-1	Sense: 5'-TGCTAGCCTGGTGCAAGATA-3'
	Antisense: 5'-GCCAACAGGAAGCTGAGAGT-3'
NQO1	Sense: 5'-GTCCATTCCAGCTGACAACCA-3'
	Antisense: 5'-TTGCCCTGAGGCTCCTAATC-3'
RUNX2	Sense: 5'-AGGCAGTTCCCAAGCATTTCATCC-3'
	Antisense: 5'-TGGCAGGTAGGTGTGGTAGTGAG-3'
GAPDH	Sense: 5'-GGAGCGAGATCCCTCCAAAAT-3'
	Antisense: 5'-GGCTGTTGTCATACTTCTCATGG-3'

Bonferroni post-hoc test were performed with the aid of GraphPad Prism version 8.0 (GraphPad Software) for Windows. A value of $p < .05$ was regarded statistically significant.

Results

Identification of hPDLSCs

Following 5 to 7 days of cell culture, the primary cells merged from the edge of the periodontal tissue block, most of which presented a long spindle shape (Figure 1A). After subculture, the cells rapidly grew and arranged in bundles, with full cell body and clear nucleus (Figure 1B). The results of flow cytometry analysis indicated the isolated cells were positive for CD73 and CD90 as 2 known mesenchymal stem cell markers and negative for CD45 as a hematopoietic-specific cell marker (Figure 1C). The results of multidifferentiation assays, Alizarin red S staining and oil red O staining, indicated the osteogenic and adipogenic differentiation potential of isolated cells as the presence of Alizarin red-stained nodules (Figure 1D) and oil red O-stained lipid droplets (Figure 1E) observed. Therefore, the hPDLSCs were successfully obtained.

KLF2 influences Nrf2-regulated transcription in LPS-stimulated hPDLSCs

Results of qRT-PCR and Western blotting analysis showed that LPS administration dose at 1 and 2 μg/mL significantly reduced KLF2 expression in hPDLSCs (Figure 2A). No

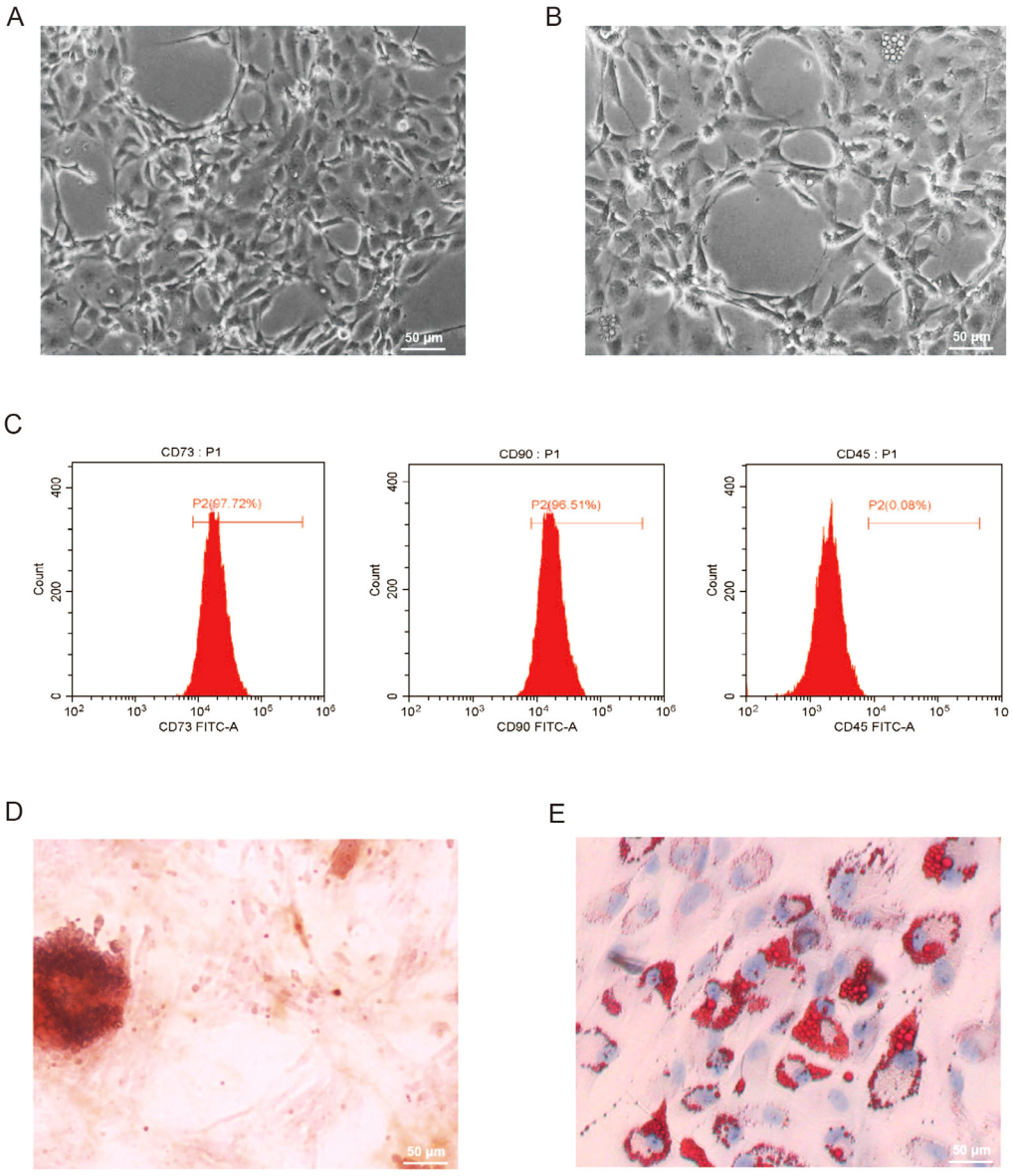

Fig. 1 – Identification of hPDLSCs. A, The primary culture of the periodontal tissue block for 5 to 7 days (× 100). B, The subculture of hPDLSCs (× 100). C, The flow cytometry detection of surface markers of hPDLSCs. D, A representative image (× 100) of osteogenic differentiation of hPDLSCs evaluated by Alizarin red S staining. E, A representative image (× 100) of adipogenic differentiation of hPDLSCs evaluated by oil red O staining.

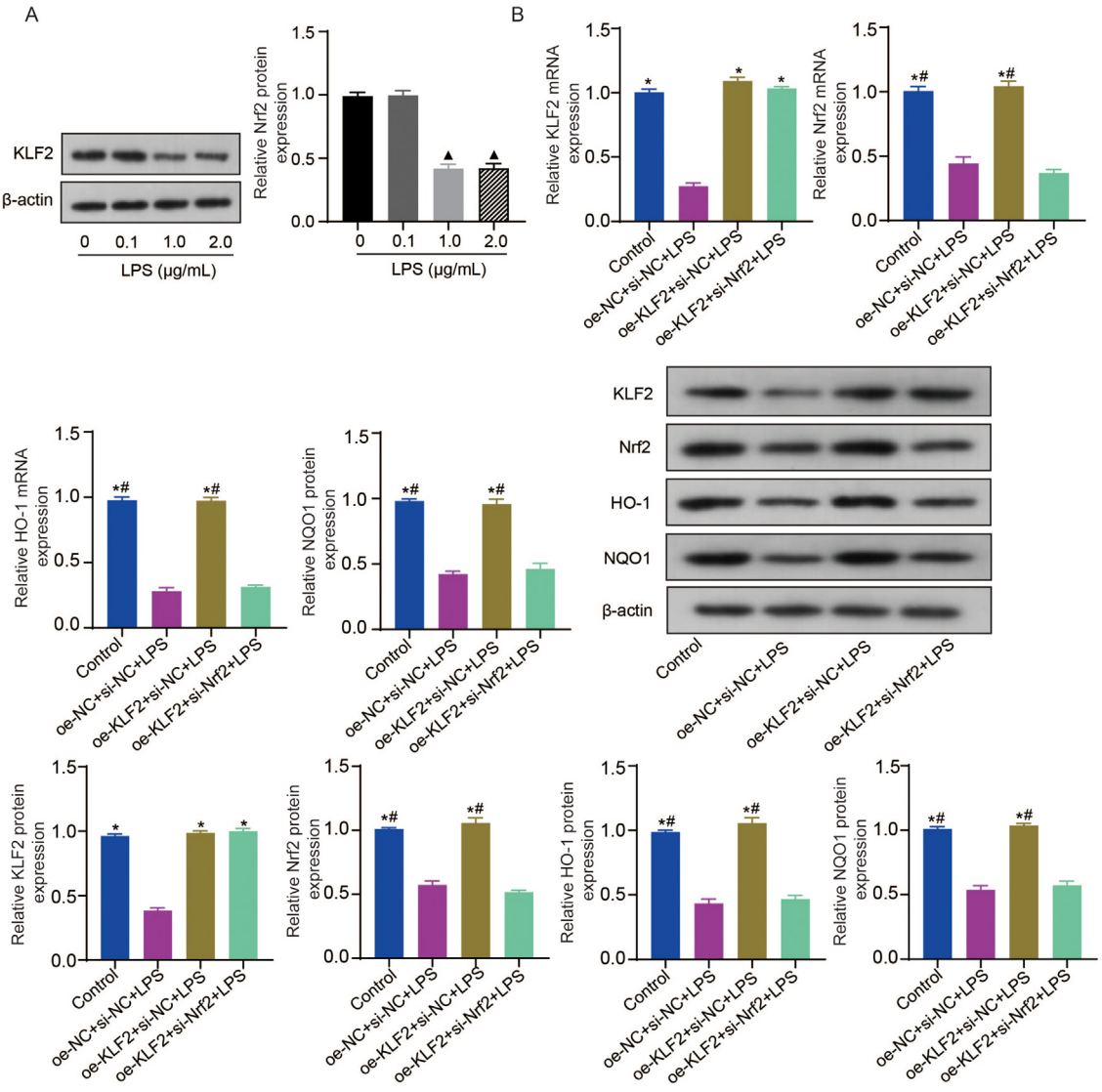

Fig. 2 – KLF2 influences Nrf2-regulated transcription in LPS-stimulated hPDLSCs. A, The Western blotting detection of KLF2 protein expression in hPDLSCs with or without LPS administration dose at 0.1, 1, and 2 μg/mL. B, The qRT-PCR and Western blotting detections of KLF2, Nrf2, HO-1 and NQO1 mRNA expressions in LPS-stimulated hPDLSCs after different treatments. Results of mean with SD were yielded from 3 biological and technical replicates and analysed by one-way ANOVA plus Tukey's post-hoc test for panel B. ▲ $P < .05$ compared to hPDLSCs without LPS administration, * $P < .05$ compared to the oe-NC+si-NC+LPS group, and # $P < .05$ compared to the oe-KLF2+si-Nrf2+LPS group.

significant difference was noted between 1 and 2 μg/mL. Thus, we selected LPS administration dose at 1 μg/mL for further experiments. To examine the effects of KLF2 on Nrf2-regulated transcription in hPDLSCs, we overexpressed KLF2 and knocked down Nrf2 in hPDLSCs by transfecting oe-KLF2 and si-Nrf2, with transfection efficiency > 90% as visualised by fluorescence microscopy for GFP (green fluorescent protein) expression (data not shown). As presented in Figure 2B, results of qRT-PCR and Western blotting analysis showed declined expressions of KLF2 and Nrf2 in hPDLSCs in LPS-stimulated inflammatory condition. KLF2 overexpression was found to effectively reduce expressions of Nrf2 in LPS-stimulated hPDLSCs. HO-1 and NQO1 as 2 known target genes of Nrf2 were also determined in LPS-stimulated hPDLSCs with different treatments. Reduced expressions of HO-1 and NQO1 were noted in LPS-stimulated hPDLSCs, which were prevented by KLF2 overexpression. The subsequent Nrf2 knockdown contributed to decreases in HO-1 and NQO1 in KLF2-overexpressed hPDLSCs under the LPS-stimulated inflammatory condition. These results reveal that KLF2 influences Nrf2-regulated transcription in LPS-stimulated hPDLSCs.

KLF2 overexpression prevent inflammation and oxidative stress in LPS-stimulated hPDLSCs by regulating Nrf2 expression

The results of enzyme-linked immunosorbent assay (Figure 3A) showed that KLF2 overexpression suppressed LPS

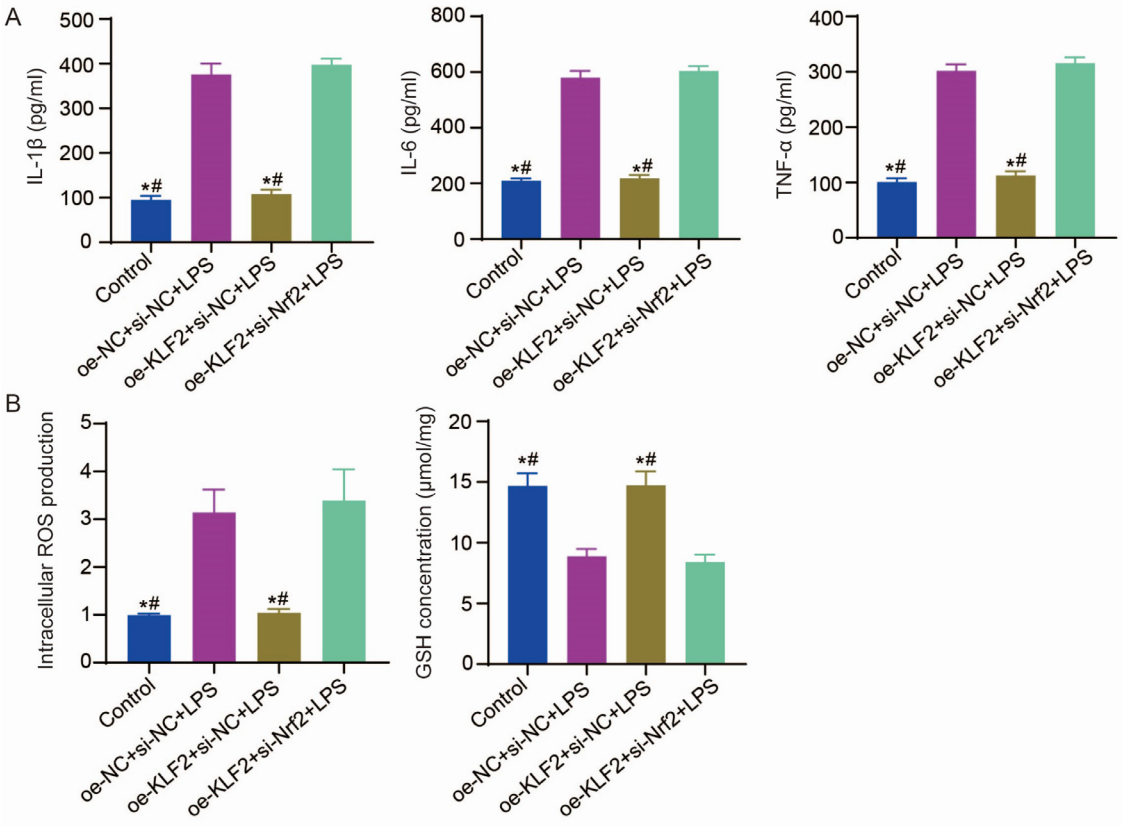

Fig. 3 – KLF2 overexpression prevent inflammation and oxidative stress in LPS-stimulated hPDLSCs by regulating Nrf2 expression. A, The enzyme-linked immunosorbent assay detection of IL-1β, IL-6, and TNF-α contents in the hPDLSCs. B, Measurements of intracellular ROS production in the hPDLSCs. C, Measurements of GSH concentration in the hPDLSCs. Results of mean with SD were yielded from 3 biological and technical replicates and analysed by one-way ANOVA plus Tukey's post-hoc test. * $P < .05$ compared to the oe-NC+si-NC+LPS group, and # $P < .05$ compared to the oe-KLF2+si-Nrf2+LPS group.

stimulation on the contents of IL-1β, IL-6, and TNF-α in the hPDLSCs, but the suppressive effects of KLF2 overexpression were quenched by the continuous Nrf2 knockdown. KLF2 overexpression resulted in a decline in intracellular ROS production (Figure 3B) with an elevation in the GSH concentration (Figure 3C) in LPS-stimulated hPDLSCs, whereas Nrf2 knockdown weakened the effects of KLF2 overexpression as the intracellular ROS level was increased and the GSH concentration was reduced. These results indicate that KLF2 overexpression could protect hPDLSCs against inflammation and oxidative stress following LPS stimulation by activating Nrf2 expression.

KLF2 overexpression restores osteogenic differentiation of hPDLSCs against LPS stimulation by regulating Nrf2 expression

As results of ALP staining (Figure 4A) and ALP activity test (Figure 4B) demonstrated, KLF2 overexpression improved the decrease of ALP activity caused by LPS stimulation in hPDLSCs. Nrf2 knockdown reduced the ALP activity, partially weakening the ALP activity improvement. Alizarin red S staining (Figure 4C) and quantitative results (Figure 4D) showed that LPS-stimulated hPDLSCs with KLF2 overexpression generated more mineralised matrix than hPDLSCs only with LPS stimulation, but the subsequent Nrf2 knockdown impaired the positive effects of KLF2 overexpression on the mineralised matrix formation. The results of qRT-PCR and Western blotting analysis (Figure 4E) proved elevations in the expression of RUNX2 mRNA and protein in LPS-stimulated hPDLSCs with KLF2 overexpression compared to those without KLF2 overexpression. However, Nrf2 knockdown reduced expression of RUNX2 mRNA and protein in LPS-stimulated hPDLSCs and counteracted the KLF2 overexpression. These results imply that KLF2 overexpression could enhance osteogenic differentiation of LPS-stimulated hPDLSCs by activating Nrf2 expression.

KLF2 overexpression promotes the proliferation while inhibiting the apoptosis of LPS-stimulated hPDLSCs by regulating Nrf2 expression

As shown in Figure 5A, LPS stimulation led to a significant reduction of proliferation of hPDLSCs and this reduction could be effectively improved after KLF2 overexpression. In LPS-stimulated hPDLSCs with KLF2 overexpression, Nrf2 knockdown inhibited the cell proliferation. As shown in Figure 5B, LPS stimulation significantly induced the apoptosis

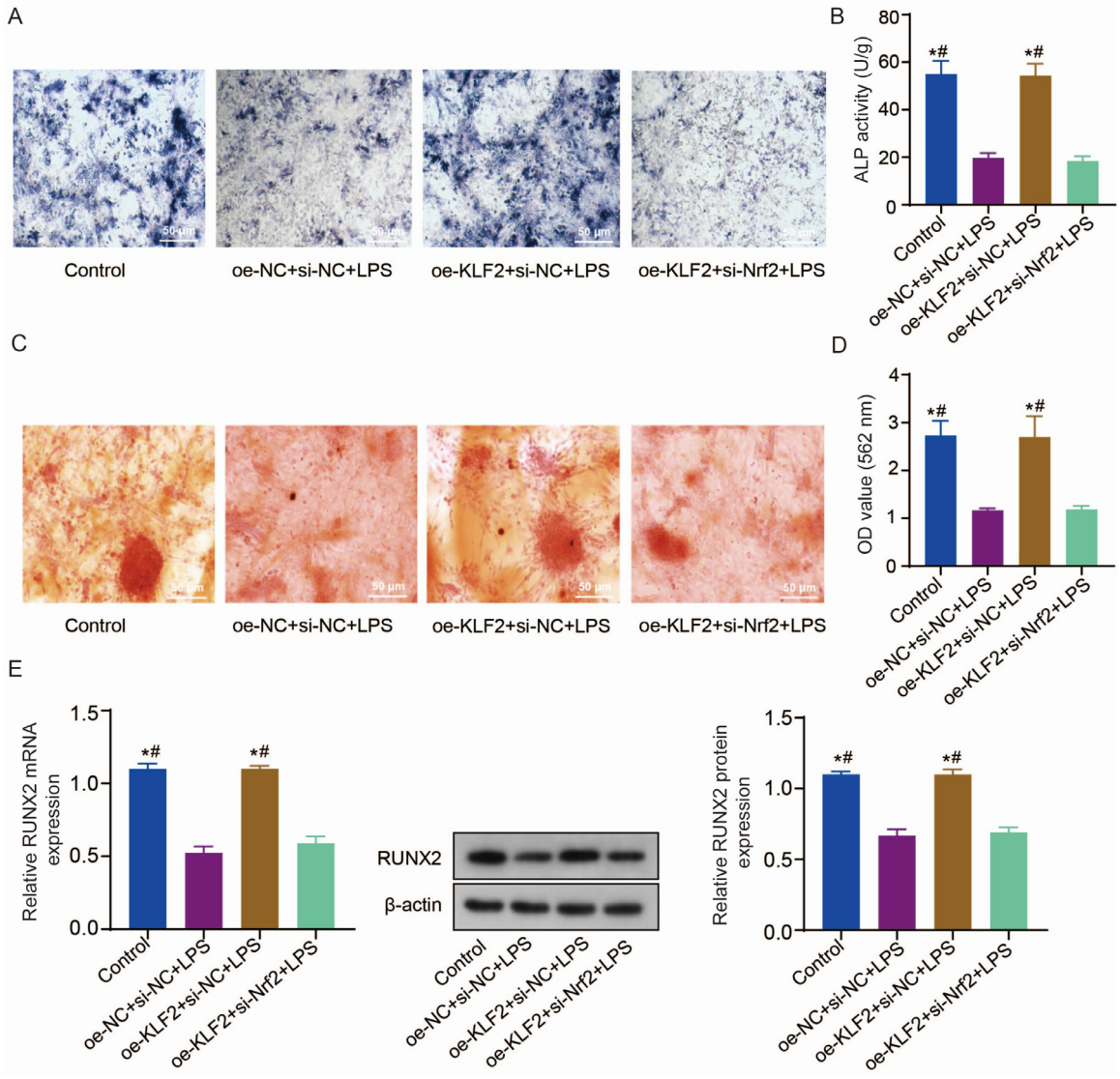

Fig. 4 – KLF2 overexpression restores osteogenic differentiation of hPDLSCs against LPS stimulation by regulating Nrf2 expression. A, Representative images (× 40) of ALP-positive cells in the hPDLSCs after 14-day osteogenic induction. B, The ALP activity in the hPDLSCs after 14-day osteogenic induction. C, Representative images (× 40) of Alizarin red-stained nodules in the hPDLSCs after 14-day osteogenic induction. D, The quantitative analysis (OD value at 562 nm) of mineralised matrix in the hPDLSCs after 14-day osteogenic induction. E, The qRT-PCR and Western blotting detections of RUNX2 mRNA and protein expressions in the hPDLSCs. Results of mean with SD were yielded from 3 biological and technical replicates and analysed by one-way ANOVA plus Tukey's post-hoc test. * $P < .05$ compared to the oe-NC+si-NC+LPS group, and # $P < .05$ compared to the oe-KLF2+si-Nrf2+LPS group.

of hPDLSCs, which was effectively inhibited by KLF2 overexpression. In LPS-stimulated hPDLSCs with KLF2 overexpression, Nrf2 knockdown induced the apoptosis of hPDLSCs. These results suggest that KLF2 overexpression could promote the proliferation while inhibiting the apoptosis of LPS-stimulated hPDLSCs by activating Nrf2 expression.

Discussion

The bacterial infections subsequently leading to host immune responses have contributed to a disordered immune microenvironment, which generally influences the osteogenic differentiation of resident PDLSCs and reduces their regenerative activity, thus disturbing periodontal homeostasis.[21] Exploring methods to enhance osteogenic differentiation of PDLSCs impaired by disordered immune microenvironment is critical for developing PDLSC-based tissue engineering in treating periodontitis. In this study, we overexpressed KLF2 in LPS-impaired hPDLSCs and found KLF2 overexpression facilitated the proliferation and osteogenic differentiation of hPDLSCs while preventing the apoptosis. We also found the activation of Nrf2 might participate in the positive and protective effect of KLF2 on hPDLSCs.

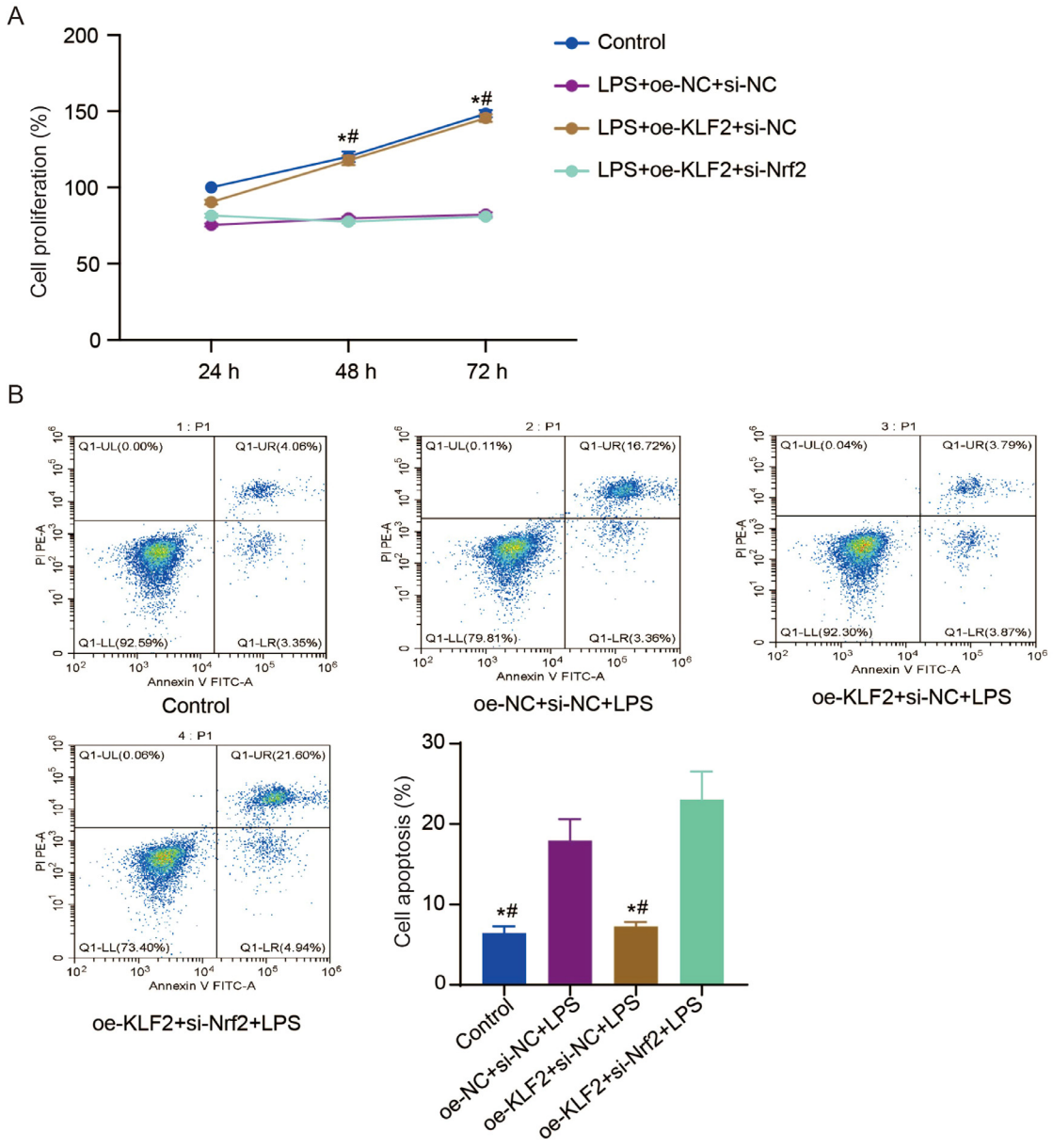

Fig. 5 – KLF2 overexpression promotes the proliferation while inhibiting the apoptosis of LPS-stimulated hPDLSCs by regulating Nrf2 expression. A, CCK-8 assays to detect the proliferation of hPDLSCs; results of mean with SD were yielded from 3 biological and technical replicates and analysed by two-way ANOVA plus Bonferroni post-hoc test. B, Flow cytometry analysis to evaluate the apoptosis of hPDLSCs; results of mean with SD were yielded from 3 biological and technical replicates and analysed by one-way ANOVA plus Tukey's post-hoc test. * $P < .05$ compared to the oe-NC+si-NC+LPS group, and # $P < 0.05$ compared to the oe-KLF2+si-Nrf2+LPS group.

The anti-inflammatory, antioxidant role of KLF2 have been reported in previous studies.[22,23] In line with these studies, our results showed KLF2-overxpressing hPDLSCs exhibited lower levels of inflammatory cytokines concomitant with reduced intracellular ROS production and GSH consumption than those control cells in the LPS condition. Although not in PDLSCs, Maity et al. proved the positive effects of KLF2 on osteoblast differentiation from dental pulp-derived stem cells.[13] The roles of KLF2 in maintaining or enhancing stemness and self-renewal of human bone marrow MSCs were demonstrated in Gong et al.'s study[24] and Wu et al.'s study.[25] KLF2 has been found to regulate runt-related transcription factor 2 (RUNX2) which is a predominant transcription factor involved in osteoblast differentiation and bone mineralisation.[26] Quite similarly, our results demonstrated an enhanced osteogenic differentiation of hPDLSCs in the LPS condition after overexpressing KLF2.

The underlying mechanism of KLF2's effect on osteogenic differentiation of hPDLSCs was also a focus of our study. In 2 recent reports,[15,16] KLF2 mediated activation of Nrf2 provides protection to endothelial cells or vascular smooth muscle cells. KLF2 could promote the nuclear import of Nrf2 and

prime the antioxidant activity.[27] Subsequently, the activity of the Nrf2 signaling pathway improved osteogenic differentiation and inhibited oxidative stress caused by H_2O_2 treatment in PDLSCs.[28] Like KLF2, Nrf2 was previously proved to modulate RUNX2 and thus enhanced osteogenic differentiation and mineralisation in human bone marrow-derived stromal cells.[29] Nrf2 activation induced by its activator had the ability to facilitate osteogenic differentiation of PDLSCs under cyclic mechanical stretch and improved the microstructure of alveolar bone.[30] An impaired osteogenic differentiation ability was observed in PDLSCs in response to Nrf2 knockdown or treatment with Nrf2 inhibitor ML385.[31,32]

To cautiously interpret the data in this study, several study limitations should be noted. First, a control group without LPS stimulation but with KLF2 overexpression and Nrf2 knockdown is missing, and this group will be performed in further studies to validate the specific effects of KLF2 overexpression on Nrf2 and osteogenesis independent of the inflammatory stimulus. Second, the lack of in vivo studies, such as using immunocompromised mice models, limits the clinical utility of KLF2 during PDLSC-based tissue regeneration, which should be centered in further studies.

In conclusion, the findings demonstrate a crucial role for KLF2-mediated activation of Nrf2 in providing considerable protection against inflammation, oxidative stress, and impaired osteogenic ability to hPDLSCs after exposure to LPS. The study suggests KLF2 may be a promising target to promote the therapeutic efficiency of hPDLSCs in cellular therapy for successful periodontal regeneration. A hPDLSC sheet engineering technology with pretreatment of recombinant human KLF2 can be grafted on the calcium phosphate cement scaffold to repair damaged periodontal complex.

Conflict of interest

None disclosed.

Author contributions

Jiujiu Chen: the conception and design of the study, acquisition of data, and drafting the article. *Lichao Deng:* analysis and interpretation of data and revising it critically for important intellectual content. All authors reviewed and approved the manuscript.

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