Molecules and Cells

# Hypoxia Mediates Runt-Related Transcription Factor 2 Expression via Induction of Vascular Endothelial Growth Factor in Periodontal Ligament Stem Cells

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**Periodontitis is characterized by the loss of periodontal tissues, especially alveolar bone. Common therapies cannot satisfactorily recover lost alveolar bone. Periodontal ligament stem cells (PDLSCs) possess the capacity of self-renewal and multilineage differentiation and are likely to recover lost alveolar bone. In addition, periodontitis is accompanied by hypoxia, and hypoxia-inducible factor-1α (HIF-1α) is a master transcription factor in the response to hypoxia. Thus, we aimed to ascertain how hypoxia affects runt-related transcription factor 2 (RUNX2), a key osteogenic marker, in the osteogenesis of PDLSCs. In this study, we found that hypoxia enhanced the protein expression of HIF-1α, vascular endothelial growth factor (VEGF), and RUNX2 *ex vivo* and *in situ*. VEGF is a target gene of HIF-1α, and the increased expression of VEGF and RUNX2 proteins was enhanced by cobalt chloride ($CoCl_2$, 100 μmol/L), an agonist of HIF-1α, and suppressed by 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1, 10 μmol/L), an antagonist of HIF-1α. In addition, VEGF could regulate the expression of RUNX2, as RUNX2 expression was enhanced by human VEGF ($hVEGF_{165}$) and suppressed by VEGF siRNA. In addition, knocking down VEGF could decrease the expression of osteogenesis-related genes, i.e., RUNX2, alkaline phosphatase (ALP), and type I collagen (COL1), and hypoxia could enhance the expression of ALP, COL1, and osteocalcin (OCN) in the early stage of osteogenesis of PDLSCs. Taken together, our results showed that hypoxia could mediate the expression of RUNX2 in PDLSCs via HIF-1α-induced VEGF and play a positive role in the early stage of osteogenesis of PDLSCs.**

**Keywords:** hypoxia, hypoxia-inducible factor-1α, mesenchymal stromal cells, runt-related transcription factor 2, vascular endothelial growth factor

## INTRODUCTION

Periodontitis is a prevalent chronic inflammatory disease that destroys the supporting periodontal tissues, including periodontal ligament (PDL) and alveolar bone (Kassebaum et al., 2014). Data from the National Health and Nutrition Examination Survey (NHANES) showed that the incidence rate of peri-

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odontitis was 46% in American adults ≥ 30 years (Eke et al., 2015). Common therapies such as scaling and root planning (SRP) could remove pathogenic agents (Smiley et al., 2015), and photodynamic therapy has been applied to improve the efficacy of SRP (Meimandi et al., 2017); however, the lost alveolar bone could not be satisfactorily rehabilitated. It has been reported that the PDL contains stem cell-like cells capable of differentiating into cementum-like and PDL-like structures *in vivo*, and these cells have been termed periodontal ligament stem cells (PDLSCs) (Seo et al., 2004). Whether or not it is possible for PDLSCs to rehabilitate the lost alveolar bone needs further study.

In addition, periodontitis is accompanied by hypoxia. A distinctly reduced oxygen saturation (from 77% to 71%) in periodontitis was reported by Gölz et al. (2015). Hypoxia modulates oxygen homeostasis in mammals via hypoxia-inducible factor-1 (HIF-1) (Prabhakar and Semenza, 2015), which is a master transcription factor in the response to reduced oxygen (Loenarz et al., 2011). HIF-1 is composed of two subunits: HIF-1β is constitutively expressed, and HIF-1α functions as the critical regulator for oxygen homeostasis (Wang and Semenza, 1995). In periodontitis, the expression of HIF-1α was detected by immunohistochemistry to be significantly enhanced in the periodontium (Vasconcelos et al., 2016), indicating the relevance between HIF-1α and periodontitis. However, the role of HIF-1α in periodontitis is complicated and still not entirely clear. It has been reported that hypoxic treatment for a short time could increase the expression of osteogenic markers, e.g., runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), and osteopontin (OPN), enhancing the osteogenic differentiation of PDLSCs (Zhang et al., 2014). Furthermore, a deferoxamine-mimicked hypoxia could enhance the osteogenic differentiation of periodontal ligament cells (PDLCs) (Mu et al., 2017), indicating a positive role of hypoxia in the rehabilitation of lost alveolar bone by PDLSCs. However, the mechanisms involved are not clear.

RUNX2 is a transcription factor that is crucial for osteoblast differentiation (Xu et al., 2015). RUNX2 initiates osteoblast differentiation by inducing osteogenesis-related genes, including OCN, OPN, type I collagen (COL1), and bone sialoprotein (BSP) (Goldring et al., 2006). Deletion of RUNX2 in genetically engineered mice severely suppressed osteoblast differentiation and delayed chondrocyte maturation (Lee et al., 2012). Thus, the role of RUNX2 in bone formation by PDLSCs merits close attention. In addition, of the HIF-1α-targeted genes, vascular endothelial growth factor (VEGF) is essential for angiogenesis and osteogenesis, wherein it directs oxygen and minerals for mineralization to sites of bone formation (Hu and Olsen, 2016; Wu et al., 2013a). Knockdown of VEGF in mice impaired angiogenesis and caused delayed bone formation (Hu and Olsen, 2016), indicating the important role of VEGF in osteogenesis. Overall, whether hypoxia, VEGF, and RUNX2 play a role in the osteoblast differentiation of PDLSCs requires further study.

In addition, the relationships among HIF-1α, VEGF, and RUNX2 are rather complicated. It has been reported that exposure to hypoxia suppressed the mRNA expression of RUNX2 in osteoblasts (Ontiveros et al., 2004), while hypoxia enhanced the mRNA expression of RUNX2 in human mesenchymal stromal cells (Wagegg et al., 2012). RUNX2 can affect the expression of HIF-1α during bone formation (Kwon et al., 2011; Lee et al., 2012). In addition, the interaction between VEGF and RUNX2 is complicated. RUNX2 is an essential component for regulating VEGF production (Kwon et al., 2011; Lee et al., 2012), while VEGF has also been reported to increase the expression of RUNX2 in MDPC-23 odontoblast-like cells (Rahman et al., 2014). In this study, we hypothesized that the osteoblast differentiation of PDLSCs may be affected by hypoxia via the effects of HIF-1α-induced VEGF on RUNX2.

## MATERIALS AND METHODS

### Isolation and characterization of PDLSCs

Healthy premolars were collected from 16 patients (aged 12-18 years old) undergoing orthodontic treatment in Daping Hospital after approval by the Medical Ethics Committee of the Army Medical University (2018-14) and with the informed written consent of both the patients and their parents. The PDL attached to the middle third of the root was dissociated with 3 mg/ml type I collagenase and 4 mg/ml dispase (Sigma-Aldrich, USA) for 1 h at 37°C and then transferred to a 6-well plate containing α-minimum essential medium (α-MEM) (HyClone, USA) with 10% foetal bovine serum (Excell, China), 100 U/ml penicillin (Solarbio, China), and 100 mg/ml streptomycin (Solarbio). Cover slides were applied carefully. The plate was then incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. Culture medium was refreshed every 2 days. Putative stem cells were purified by a single-cell colony formation assay and stained with crystal violet (Beyotime, China). Only cells at passages 2 to 4 were used.

The expression of mesenchymal stem cell (MSC)-associated surface markers was analysed by flow cytometry. Cells in the third passage ($1.0 \times 10^6$ cells) were incubated with specific antibodies for CD34, CD45, CD44, CD29, and isotype control for 1 h at room temperature. All antibodies were purchased from BD Biosciences (USA). The percentage of CD44-, CD29-, CD34-, and CD45-positive cells was calculated using a FACS Calibur, and the results were analysed using CellQuest Pro software (all from BD Biosciences).

Next, the differentiation ability of the putative stem cells was assessed. PDLSCs (passage 3) were plated in 6-well plates at a density of $1 \times 10^5$ cells/well and cultured in basal medium until subconfluent. The medium was then changed to osteogenic medium, i.e., basal medium supplemented with 50 μg/ml L-ascorbic-2-phosphate, 0.1 μmol/L dexamethasone, and 10 mmol/L β-glycerophosphate, or adipogenic medium, i.e., basal medium supplemented with 200 μmol/L indomethacin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 10 μmol/L insulin, and 1 μmol/L dexamethasone (all from Sigma-Aldrich). Media were refreshed every 3 days. After 3 weeks, cells were stained with 2% Alizarin Red S (pH 4.2) or 0.3% Oil Red O (Sigma-Aldrich).

### Hypoxia

Hypoxia was induced by transferring the cells to a tri-gas in-

cubator with 3% $O_2$. Cobalt chloride ($CoCl_2$, 100 μmol/L) was used to induce HIF-1α and mimic hypoxic conditions. 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1, 10 μmol/L) was used to antagonise the effects of HIF-1α.

### Cytotoxicity analysis

The viability of PDLSCs treated with $CoCl_2$ or YC-1 was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were plated in 96-well plates at $2\times10^3$ cells/well and then incubated with $CoCl_2$ (50, 100, 200, or 400 μmol/L) for 72 h under normoxia or with YC-1 (10, 20, 30, or 40 μmol/L) for 24 h under hypoxia. MTT (Solarbio) at 5 mg/ml was added to each well. After incubation for 4 h, media were replaced with dimethyl sulfoxide (DMSO), and the optical density (OD) values were detected with a micro-plate reader (BioTek, USA).

### Osteogenesis assay

PDLSCs were cultured in osteogenic medium under normoxia or hypoxia. At the indicated time points (0, 3, and 9 days), total RNA was extracted to measure the expression of osteogenesis-related genes. The osteogenic medium is formulated as listed above.

### Western blot

Total protein was extracted using ice-cold lysis buffer composed of 10% RIPA (Solarbio) and proteinase inhibitor cocktail (Roche, USA). Proteins were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Roche) and blocked with 3% bovine serum albumin (BSA; Solarbio) for 1 h at room temperature. Primary antibody incubation was performed with the following antibodies: anti-HIF-1α (1:1,000), anti-VEGF (1:1,000), anti-RUNX2 (1:1,000), and anti-β-actin (1:1,000) (all from Abcam, USA) at 4°C overnight. Secondary antibody incubation was performed with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5,000) (Beyotime) at room temperature for 1 h. Finally, immunoblotting was visualized using an enhanced chemiluminescence detection kit (Millipore, USA).

### Quantitative polymerase chain reaction

Total RNA was extracted from PDLSCs using TRIzol reagent (Invitrogen, USA). Reverse transcription was performed with 1,000 ng of total RNA and SuperScript II Reverse Transcriptase (Roche) according to the manufacturers' instructions. Quantitative polymerase chain reaction (qPCR) was performed with SYBR Ex Taq (Roche) according to the manufacturers' instructions. Sequences of the primers are listed in Table 1.

### Immunofluorescence

Cells ($5 \times 10^4$ cells/well) were seeded into a 24-well plate and cultured under normoxic or hypoxic conditions for 24 h. After 4% paraformaldehyde (PFA) (Sigma-Aldrich) fixation for 30 min, cells were incubated with blocking buffer (Dako, Denmark) at 37°C for 1 h, followed by incubation with anti-HIF-1α antibody (1:300), anti-VEGF antibody (1:300), or anti-RUNX2 antibody (1:300) (all from Abcam) at 4°C overnight. After washing with phosphate-buffered saline (PBS), cells were incubated with goat anti-rabbit or mouse IgG antibody conjugated with Alexa 488 or Alexa 588 (1:500) (Invitrogen) for 1 h at room temperature. Cells were then stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope (Olympus, Japan). For tissue immunofluorescence, rat periodontal tissues of the right maxillary first molar were excised, frozen, sectioned, and antigen retrieved, which was followed by the steps listed above.

### *In situ* experiments

A hypoxic animal model was established on Sprague–Dawley (SD) rats from the Experimental Animal Center, Daping Hospital & Research Institute of Surgery, after the approval of the Medical Ethics Committee of the Army Medical University. The SD rats (n = 24, half female and half male) were 10-13 weeks old and 360-400 g at the start of the experiment. The experimental animals were randomly divided into normoxia

**Table 1**. Primers used for qPCR

| Primer | Sequence ID | Sequence (5′-3′) |
|---|---|---|
| HIF1A | NM_001530 4082 | F: CCGCTGGAGACACAATCATA<br>R: GGTGAGGGGAGCATTACATC |
| VEGF | NM_001171623 | F: GAGGAGGGVAGAATCATCAC<br>R: ATCAGGGGCACACAGGAT |
| RUNX2 | NM_001024630 | F: CCTCTGACTTCTGCCTCTGG<br>R: ATGAAATGCTTGGGAACTGC |
| GAPDH | NM_001289746 | F: CTCCTCCACCTTTGACGC<br>R: CCACCACCCTGTTGCTGT |
| ALP | XM_006710546.3 | F: TTGACCTCCTCGGAAGACACTC<br>R: CCATACAGGATGGCAGTGAAGG |
| OCN | NM_199173.5 | F: GGCGCTACCTGTATCAATGGC<br>R: TGCCTGGAGAGGAGCAGAACT |
| COL1 | NM_000088 | F: GAGGGCCAAGACGAAGACATC<br>R: CAGATCACGTCATCGCACAAC |

F, forward; R, reverse.

and hypoxia groups, with 12 rats in each group, and placed in normoxia or decompression chambers equal to 5,000 m above sea level, respectively, for 8 weeks (Oz and Puleo, 2011). The animals were then euthanized by intraperitoneal injection with 3% pentobarbital sodium. Then, 4% PFA was perfused through the left ventricle for 5 min (5 ml/min), and the right maxillary first molar segment was removed and fixed by 4% PFA.

## Immunohistochemistry

Rat periodontal tissues were fixed in 4% PFA for 48 h and decalcified with 12% ethylenediaminetetraacetic acid (EDTA, pH 5.6) at room temperature for 21 days. The sections were paraffin embedded and cut serially in the sagittal plane from the most lingual side. Semi-serial 5-μm sections of first molars were prepared and stained according to immunohistochemistry protocols. After antigen retrieval by heat-induced epitope retrieval, deparaffinized sections were immersed in 0.6% $H_2O_2$ for 20 min to quench endogenous peroxidase activity. Sections were then blocked in 5% BSA for 30 min and incubated with antibodies against HIF-1α (0.05 μg/ml), VEGF (0.05 μg/ml), or RUNX2 (0.05 μg/ml) (all from Abcam) overnight at 4°C. Sections were then incubated with goat anti-rabbit or mouse IgG antibodies for 1 h at room temperature and reacted with avidin-biotin-peroxidase complexes (Vector Laboratories, USA) in PBS for 30 min. After colour development with 0.05% 3,3′-diaminobenzidine, sections were counterstained with haematoxylin.

## Statistical analysis

Experiments were carried out in triplicate. Immunoblots were quantified using Quantity One 4.4.0 (Bio-Rad, USA). The immunoblot and mRNA quantifications are presented as the mean ± SD. Statistical differences were analysed by Student's *t*-test or one-way ANOVA in IBM SPSS Statistics (ver. 20.0; IBM, USA). A *P* value of less than 0.05 was considered statistically significant.

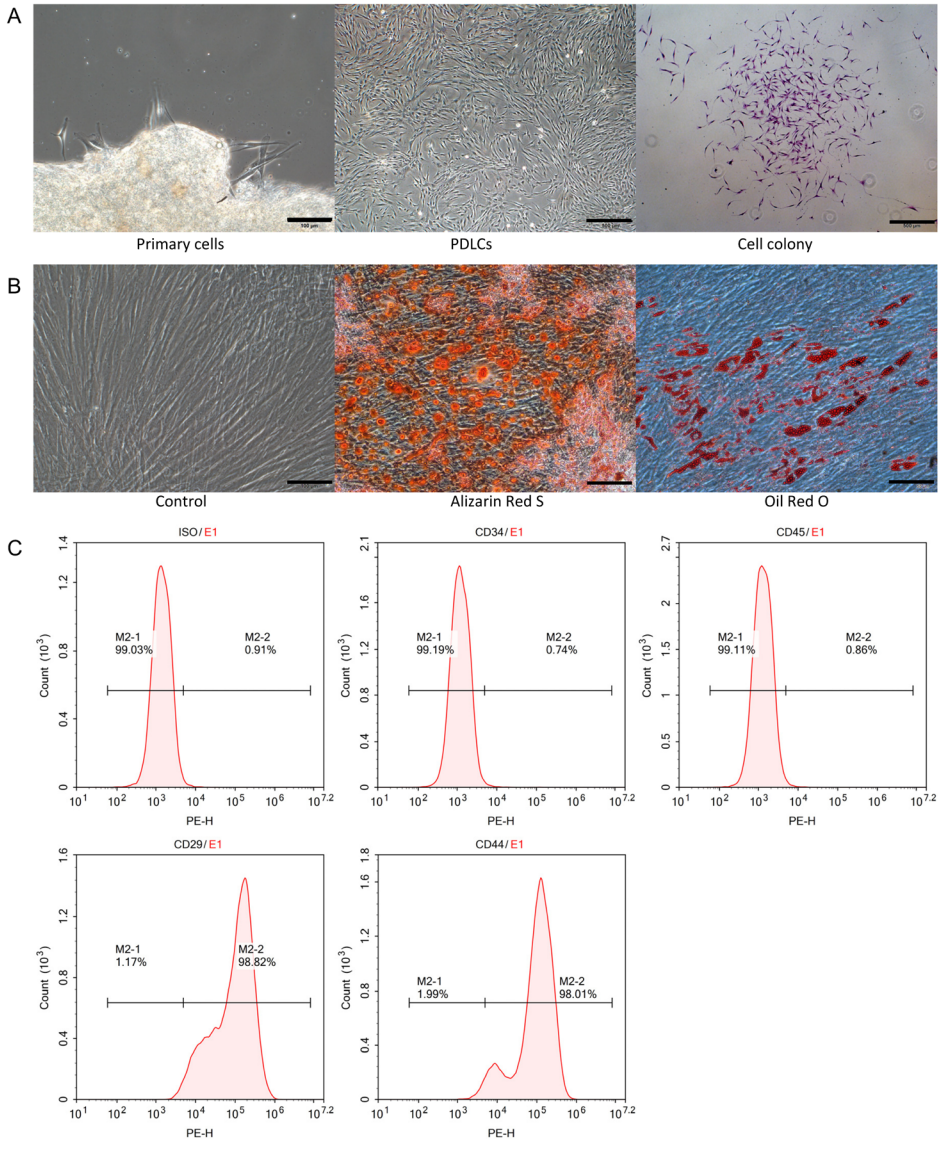


**Fig. 1. Isolation and characterization of human PDLSCs.** (A) Isolation and culture of primary periodontal ligament cells (PDLCs). Spindle-shaped adherent cells appeared from the primary explants. Scale bar = 100 μm. Confluence of 80% was reached before passage. Scale bar = 500 μm. Putative stem cells were identified by a single-cell colony formation assay and stained with crystal violet. Scale bar = 500 μm. (B) PDLCs showed multilineage differentiation potential. Compared to that of the control group, Alizarin Red S staining revealed several red calcified nodules, and Oil Red O staining showed several red fat droplets inside PDLCs, aside from the changed cell shapes. All scale bars = 100 μm. (C) Flow cytometry showed that the PDLCs expressed the MSC surface markers CD44 and CD29 and were negative for the haematopoietic cell surface markers CD34 and CD45.

## RESULTS

### Isolation and characterization of PDLSCs

Adherent PDLCs grew from the edges of the explants in 3 to 5 days (Fig. 1A). These adherent cells resembled spindle-shaped fibroblast cells and reached 80% confluence in 10 to 12 days (Fig. 1A). Putative stem cells were screened out by single-cell colony formation assay and stained with crystal violet (Fig. 1A). Flow cytometry results showed that the isolated cells expressed cell surface markers of MSCs, i.e., CD44 and CD29, but not surface markers of haematopoietic cells, i.e., CD34 or CD45, confirming the origin of the isolated cells (Fig. 1C).

The multilineage differentiation potential of the PDLSCs was confirmed by induced differentiation. Alizarin Red S staining showed that there were considerably more irregular red calcified nodules scattered among cells treated with osteogenic medium compared with cells treated with basal medium (Fig. 1B), indicating the ability of PDLSCs to differentiate into osteoblasts. Differentiation of PDLSCs into adipocytes was confirmed by Oil Red O staining, which showed that there were many more red fat droplets in the cells cultured in adipogenic medium (Fig. 1B).

### Hypoxia increases the expression of HIF-1α, VEGF, and RUNX2

To assess the effects of hypoxia on the expression of HIF-1α, VEGF, and RUNX2, PDLSCs were cultured under hypoxia (3% $O_2$) for different time periods (0, 12, 24, 48, or 72 h). The 0 h group represented the normoxia control. Western blot results are shown in Figure 2A. HIF-1α was barely detected in the control group but was significantly induced by hypoxic stimulation ($P < 0.05$). VEGF and RUNX2 were present in the control group but were triggered to increase markedly by hypoxia ($P < 0.05$). In addition, qPCR results from total RNA extracted at different time periods (0, 6, 12, 24, 48, or 72 h) under hypoxia showed that VEGF mRNA was quickly induced by hypoxia ($P < 0.05$), while RUNX2 mRNA was slowly decreased by hypoxia ($P < 0.05$) (Fig. 2B).

To confirm the effects of hypoxia on HIF-1α, VEGF, and RUNX2, immunofluorescence was performed on PDLSCs under normoxic or hypoxic (3% $O_2$) conditions for 24 h. The results showed that HIF-1α was barely visible in the nuclei of untreated cells, but the green signal was intensified in the hypoxia group. VEGF and RUNX2 expression levels were increased by hypoxic stimulation (Fig. 2C). Thus, our results showed that hypoxia could mediate an increase in the expression of HIF-1α, VEGF, and RUNX2, which was consistent with previously published data (Steinbrech et al., 2000).

### HIF-1α increases the expression of VEGF and RUNX2

To study the putative effect of HIF-1α on inducing VEGF and RUNX2 expression, $CoCl_2$ or YC-1 (HIF-1α agonist and antagonist, respectively) were applied to PDLSCs under normoxic or hypoxic conditions.

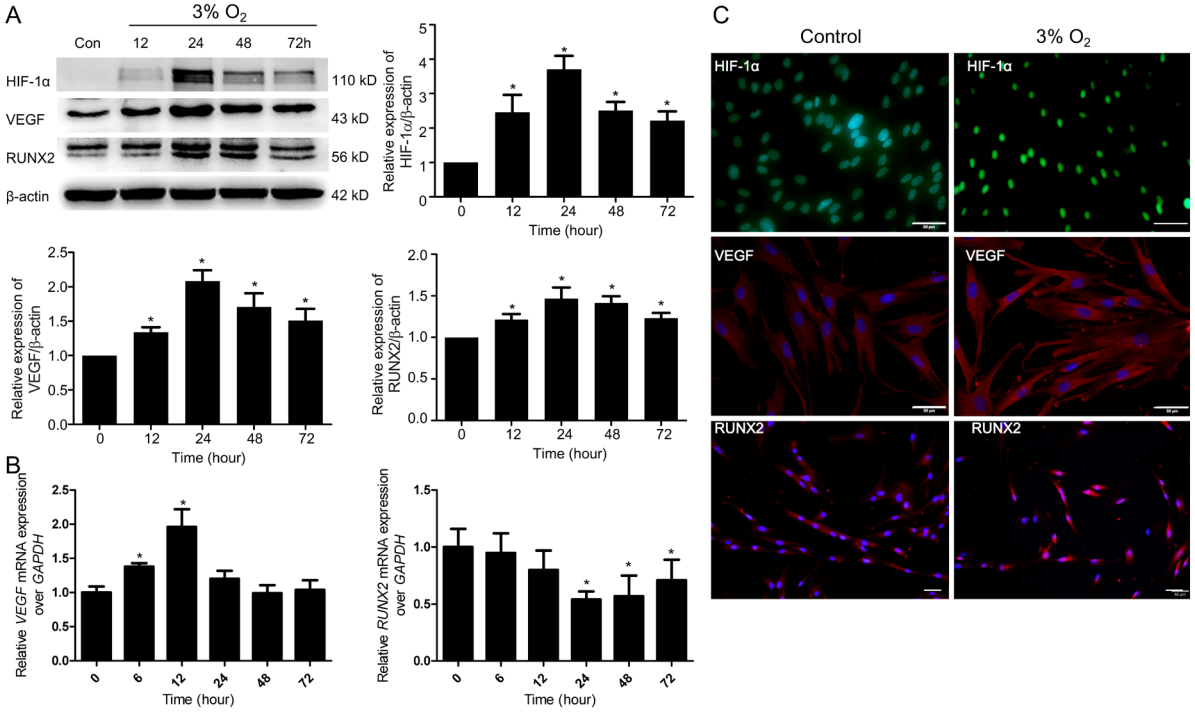


**Fig. 2. Hypoxia increased the expression of HIF-1α, VEGF, and RUNX2.** (A) Expression of HIF-1α, VEGF, and RUNX2 in PDLSCs at different time periods (0, 12, 24, 48, and 72 h) after hypoxic stimulation was analysed by Western blot. Visualization and quantification of Western blot results are shown; $*P < 0.05$ versus the control group. (B) The mRNA expression levels of VEGF and RUNX2 were measured by qPCR in PDLSCs cultured at 0, 6, 12, 24, 48, and 72 h under hypoxia; $*P < 0.05$ versus the control group. (C) Immunofluorescence confirmed the elevated expression of HIF-1α, VEGF, and RUNX2 in PDLSCs after 24 h of hypoxic stimulation. DAPI stain. Scale bars = 50 μm.

### *Cytotoxicity of $CoCl_2$ and YC-1 on PDLSCs*

MTT assay was used to analyse the proliferation of cells treated with $CoCl_2$ or YC-1. The concentration gradients were set as 0, 50, 100, 200, and 400 μmol/L for $CoCl_2$ and 0, 10, 20, 30, and 40 μmol/L for YC-1. $CoCl_2$-induced PDLSC death was statistically significant only at concentrations higher than 200 μmol/L (Fig. 3A; $P < 0.05$). For YC-1, the dose became toxic when changed from 10 to 20 μmol/L (Fig. 3F; $P < 0.05$). From our results, 100 μmol/L $CoCl_2$ and 10 μmol/L YC-1 were determined to be optimal doses for subsequent experiments.

### *Effects of the agonist and antagonist of HIF-1α on the expression of VEGF and RUNX2*

$CoCl_2$ (100 μmol/L) was applied to PDLSCs at different time periods (0, 12, 24, 48, and 72 h). Western blot analysis showed that HIF-1α expression was substantially increased after hypoxia stimulation ($P < 0.05$), VEGF expression was induced after 24 h and continued to increase ($P < 0.05$), and RUNX2 was steadily induced to a smaller extent ($P < 0.05$) and preceded VEGF expression (Figs. 3B-3E). Different mechanisms may be related to the differential hypoxia-induced expression of VEGF and RUNX2.

PDLSCs were cultured under hypoxia with or without 10 μmol/L YC-1. Western blot analysis showed that YC-1 inhibited the expression of HIF-1α under hypoxia. The expression of VEGF and RUNX2 was increased after stimulation by hypoxia and decreased after the addition of YC-1 ($P < 0.05$) (Figs. 3G and 3H).

In addition, PDLSCs were also cultured with $CoCl_2$ and with or without YC-1, and qPCR results indicated that, after the expression of HIF-1α was induced by $CoCl_2$, the mRNA level of VEGF was increased and that of RUNX2 was decreased, while the inhibition of HIF-1α did not increase the expression of VEGF mRNA but further reduced RUNX2 mRNA (Figs. 3I and 3J). Compared to hypoxia (3% $O_2$), $CoCl_2$ could strengthen the induction of VEGF mRNA and the suppression

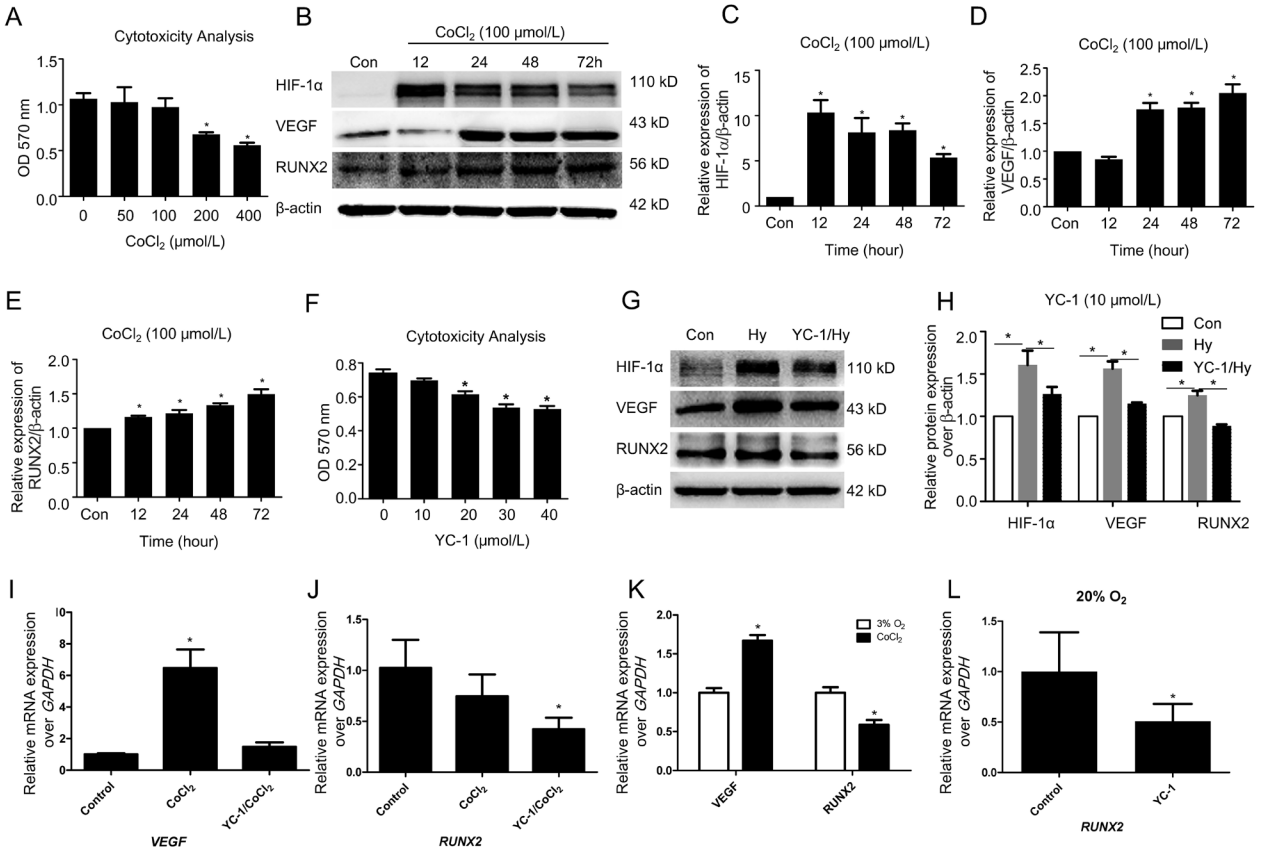


**Fig. 3. HIF-1α increased the expression of VEGF and RUNX2.** (A and F) Cytotoxicity of $CoCl_2$ and YC-1 in PDLSCs was detected using MTT assay, and the optimal concentrations of $CoCl_2$ and YC-1 were determined as 100 μmol/L and 10 μmol/L, respectively; $*P < 0.05$ versus the control group. (B-E) PDLSCs were cultured in normoxia and treated with 100 μmol/L $CoCl_2$ for different time periods (12, 24, 48, and 72 h), and the HIF-1α, VEGF, and RUNX2 protein expression levels were measured by Western blot. The results were quantified as shown; $*P < 0.05$ versus the control group. (G and H) PDLSCs were cultured in hypoxia with or without 10 μmol/L YC-1 (Hy or YC-1/Hy) for 24 h, and the expression of HIF-1α, VEGF, and RUNX2 proteins was measured by Western blot; $*P < 0.05$ among the groups. (I and J) $CoCl_2$ with or without YC-1 was applied to PDLSCs, and the expression of VEGF or RUNX2 was measured by qPCR; $*P < 0.05$ among the groups. (K) The effects of hypoxia or $CoCl_2$ on the mRNA expression of VEGF and RUNX2 were confirmed by qPCR in cells cultured for 6 h; $*P < 0.05$ versus the control group. (L) After inhibiting the stabilization of HIF-1α by YC-1, RUNX2 mRNA was measured by qPCR; $*P < 0.05$ versus the control group.

of RUNX2 mRNA (Fig. 3K). For RUNX2, basal mRNA expression was decreased by YC-1 in comparison with the normal condition (Fig. 3L), indicating that HIF-1α was involved in the basal mRNA expression of RUNX2.

## VEGF promotes RUNX2 expression

Exogenous recombinant human VEGF ($hVEGF_{165}$) (Peprotech, USA) at different concentrations (0, 1, 2.5, 5, and 10 nmol/L) was added to PDLSCs, and Western blotting showed a concentration-dependent increase in RUNX2 after adding $hVEGF_{165}$. Statistically significant results were observed when $hVEGF_{165} \geq 2.5$ ng/ml was added ($P < 0.05$) (Figs. 4A and 4B). Then, cells were transfected with VEGF siRNA (Santa Cruz Biotechnology, USA) to counteract the function of endogenous VEGF. qPCR results confirmed the concentration-dependent decrease in VEGF mRNA after VEGF siRNA transfection ($P < 0.05$), and RUNX2 mRNA exhibited a consistent decreasing trend ($P < 0.05$). Western blot analysis confirmed that VEGF protein expression was reduced after knockdown of the VEGF gene ($P < 0.05$), followed by a decrease in RUNX2 ($P < 0.05$; Figs. 4C-4E). Therefore, the results suggested that VEGF could mediate the expression of RUNX2 at the mRNA and protein levels.

## The expression of osteogenic genes under hypoxia

The mRNA expression of VEGF was successfully knocked down by transfection of PDLSCs with VEGF siRNA (50 nmol/L), and the expression of osteoblast markers such as RUNX2, COL1, and alkaline phosphatase (ALP) was obviously decreased ($P < 0.05$) (Fig. 5A); however, OCN mRNA expression did not show any obvious change. qPCR results on the effects of hypoxia on the osteogenic differentiation of PDLSCs confirmed that VEGF, RUNX2, ALP, COL1, and OCN were significantly increased at day 3 in comparison with those in the normoxic group ($P < 0.05$), and the expression of these genes was reversed and decreased at day 9, where there was still more COL1 mRNA expression in the hypoxic group than in the normoxic group (Figs. 5B-5F). These findings indicated that hypoxia could enhance the osteogenic differentiation of PDLSCs in the early stage.

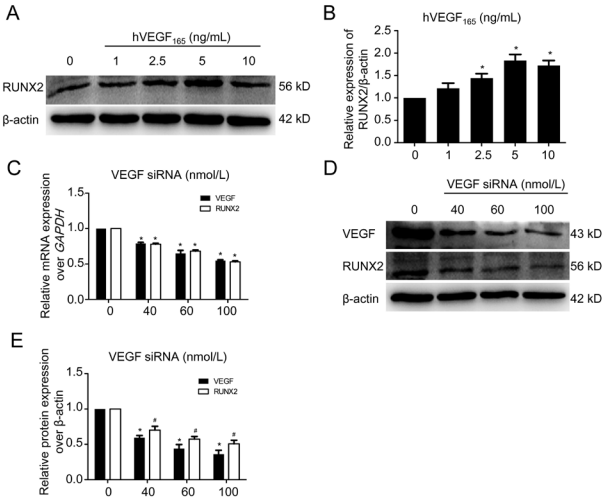


**Fig. 4. VEGF promoted the expression of RUNX2.** (A and B) PDLSCs were cultured under hypoxic conditions and treated with exogenous recombinant $hVEGF_{165}$ (0, 1, 2.5, 5, and 10 nmol/L). The expression of RUNX2 protein was measured by Western blot, and the results were quantified. (C-E) After transfection of cells with VEGF siRNA (0, 40, 60, and 100 nmol/L), the expression of VEGF and RUNX2 mRNA was detected by qPCR analysis, and the expression of VEGF and RUNX2 proteins was detected by Western blot. $*P < 0.05$ versus the control group for VEGF, and $^{\#}P < 0.05$ versus the control group for RUNX2.

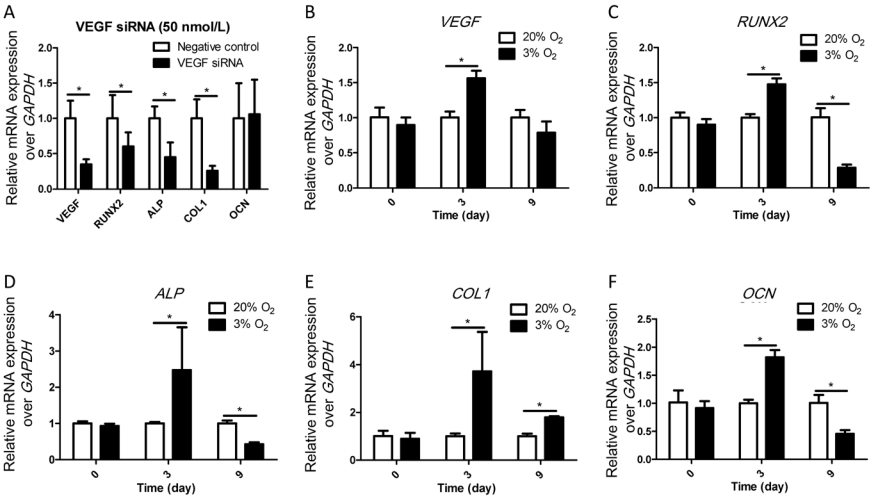


**Fig. 5. The expression of osteogenic genes under hypoxia.** (A) After transfecting VEGF siRNA (50 nmol/L) into PDLSCs, qPCR was performed to measure the mRNA expression of VEGF, RUNX2, ALP, COL1, and OCN. $*P < 0.05$ indicates statistically significant differences. (B-F) The mRNA expression of VEGF, RUNX2, COL1, and OCN was measured by qPCR at the indicated time points (0, 3, and 9 days) of osteogenic differentiation of PDLSCs under normoxia or hypoxia; $*P < 0.05$ indicates statistical significance versus the control group.

### The expression of HIF-1α, VEGF, and RUNX2 in rat periodontal tissues

Immunofluorescence of rat periodontal tissues showed that HIF-1α was slightly detected in the gingival epithelium, but the signal was stronger and more extensive in the gingival epithelium under hypoxia. VEGF was detectable in the epithelium and submucosa under normoxia, and hypoxia led to an increase in the VEGF signal, especially in the submucosa. RUNX2 was barely found in the epithelium in untreated rats; however, the RUNX2 signal was obviously increased in the epithelium and the submucosa (Fig. 6A). Immunohistochemistry of rat periodontal tissues further proved the increased expression of HIF-1α, VEGF, and RUNX2 in rat periodontal tissues, as indicated in Figure 6B.

## DISCUSSION

PDLSCs possess the capacity for self-renewal and differentiation (Abbayya et al., 2015; Feng et al., 2010). Studies have shown that the capabilities of PDLSCs remain unchanged after allogeneic or xenogeneic transplantation (Menicanin et al., 2014; Mrozik et al., 2013). Thus, it could be indicated that (i) the stemness of PDLSCs is likely quiescent in unfavourable conditions and can be triggered in conducive environments inside or outside the periodontium, and (ii) the acquisition of PDLSCs for treatment may not be a concern for dentists and patients.

Hypoxia is a characteristic of ischaemic cardiovascular diseases, bone or soft tissue injuries, and solid tumours (Manoochehri Khoshinani et al., 2016), and it has been reported that hypoxia is intimately correlated with periodontitis (Eltzschig and Carmeliet, 2011). Thus, the effects and mechanisms of hypoxia on the capabilities of PDLSCs to recover alveolar bone loss in periodontitis are worth studying. It has been proved that hypoxia promotes bone formation via the MEK/ERK and p38 MAPK pathways (Wu et al., 2013b). In this study, we found that hypoxia could increase the protein expression of the crucial osteogenic-related transcription factor RUNX2 *ex vivo* and *in situ*, indicating a positive role of hypoxia on the osteogenesis of PDLSCs. This mechanism needed the involvement of HIF-1α and HIF-1α–induced VEGF.

Our results were not consistent with some previously published works. It has been confirmed that RUNX2 interacts with HIF-1α and induces VEGF mRNA and protein expression, while HIF-1α mRNA is not influenced by RUNX2 (Kwon et al., 2011). Protein-protein interaction between HIF-1α and RUNX2 has been demonstrated through immunoprecipitation (IP), indicating a competitive binding of RUNX2 with the oxygen-dependent degradation domain (ODD) in HIF-1α, thus precluding ubiquitin-mediated proteolysis (Lee et al., 2012). Thus, RUNX2 was thought to affect VEGF through HIF-1α. However, a putative RUNX2 binding site in the murine *Vegfa* promoter was confirmed through chromatin immunoprecipitation (ChIP) in intact MC3T3-E1 cells (Kwon et al., 2011), suggesting a direct regulation of VEGF by RUNX2. In our study, RUNX2 mRNA and protein expression was inhib-

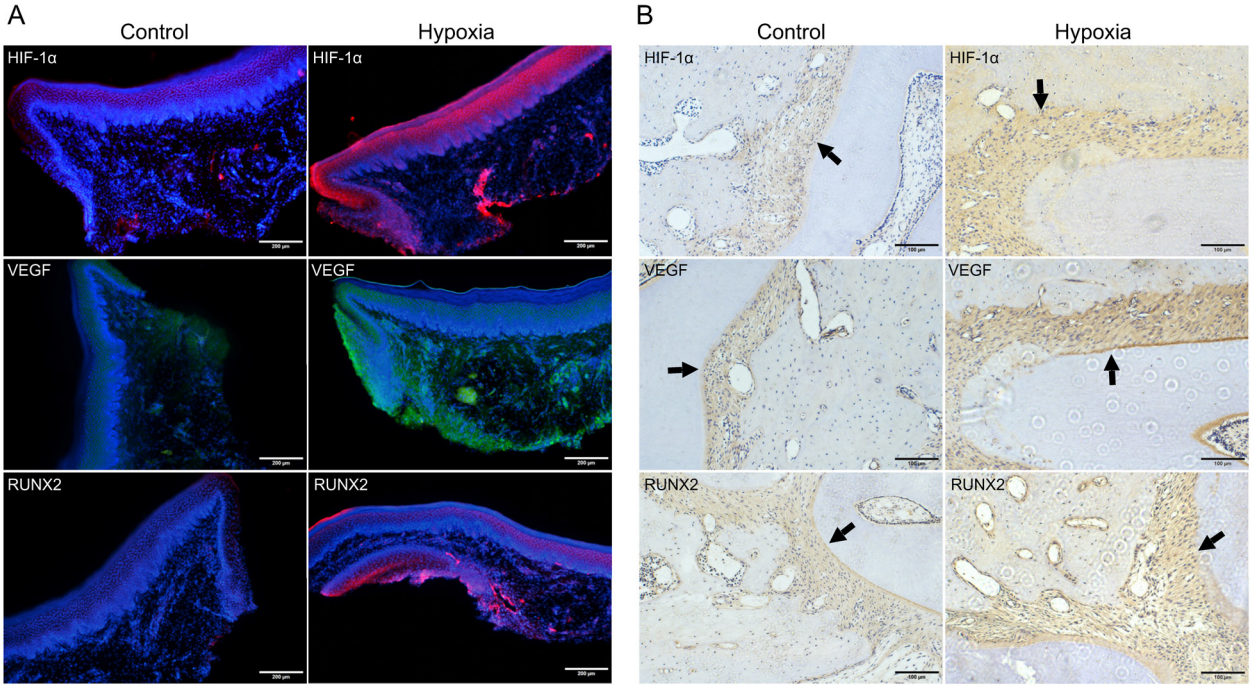


**Fig. 6. Expression of HIF-1α, VEGF, and RUNX2 in rat periodontal tissues.** After establishing the rat hypoxic model, SD rats were fed under normoxic conditions (Control) or in a decompression chamber equal to 5,000 m above sea level (Hypoxia) for 8 weeks. After the approval of the Medical Ethics Committee of the Army Medical University, SD rats were euthanized, and the periodontal tissues were removed. (A) HIF-1α, VEGF, and RUNX2 in periodontal tissues were visualized via immunofluorescence. Representative images of the control and hypoxic groups are shown. Scale bars = 200 μm. (B) Immunohistochemistry showed increased positive staining with HIF-1α, VEGF, and RUNX2 under hypoxia (arrows). DAB stain. Scale bars = 100 μm.

ited by transfection of VEGF siRNA into PDLSCs, and RUNX2 protein expression was induced by the addition of exogenous $hVEGF_{165}$, indicating a signal from VEGF to RUNX2. In addition, it has been confirmed that VEGF can activate the ERK/RUNX2 pathway and induce the osteogenic differentiation of human MSCs (Murakami et al., 2017). Hence, this reciprocal signal from VEGF to RUNX2 in PDLSCs under hypoxia implicates that: (i) angiogenesis and osteogenesis forms a reciprocal feedback cycle; (ii) in periodontitis, hypoxia influences RUNX2 directly or indirectly through VEGF, which may promote osteogenesis and partially counteract bone resorption; and (iii) this feedback cycle potentiates PDLSC-mediated alveolar bone recovery. Furthermore, our results demonstrated that knock down of VEGF could suppress the expression of osteogenic genes, e.g., RUNX2, ALP, and COL1, although the expression of OCN showed no obvious change, and hypoxia/HIF-1α could enhance the expression of ALP, COL1, and OCN in 3 days, suggesting a positive role of hypoxia/HIF-1α on PDLSCs in the early stage of osteogenesis. However, the different expression of OCN after treatment with VEGF siRNA or hypoxia may indicate that signals triggered by hypoxia are more complicated than signals triggered by VEGF alone, which warrants further investigation.

The effect of hypoxia on the mRNA expression of RUNX2 was not consistent with that on the RUNX2 protein. This inconsistency has been reported by some studies. It has been reported that short-term hypoxia could upregulate the mRNA and protein expression of RUNX2 in PDLSCs (Wu et al., 2013b), while hypoxia longer than 24 h downregulated the mRNA and protein expression of RUNX2 in human osteoblast-like cells (Park et al., 2002). In PDLCs, hypoxia induced the mRNA and protein expression of RUNX2 before 24 h and suppressed the protein expression of RUNX2 after 24 h (Chen et al., 2017). Thus, the regulation of the expression of RUNX2 by hypoxia is complicated in different cell types. In fact, the relative protein levels may be proportionate to the corresponding mRNA levels, and only approximately 40% of protein variations can be explained by mRNA abundances (Vogel and Marcotte, 2012); it has also been reported that the RUNX2 protein level is inconsistent with its mRNA expression, which may be associated with phosphorylation or interactions with cofactors (Franceschi et al., 2009). In addition, it seemed that HIF-1α plays a role in the stabilization of RUNX2 mRNA because the inhibition of HIF-1α by YC-1 also decreased RUNX2 mRNA, while hypoxia or $CoCl_2$ treatment led to reduced RUNX2 mRNA expression in PDLSCs. This inconsistency suggests that the regulation of RUNX2 by HIF-1α is not a solitary process and involves a complicated network.

Periodontitis is associated with the imbalance of pathogens and host immune responses (Campbell et al., 2016; Jia et al., 2017; Silva et al., 2015). It has been reported that hypoxia could promote the formation of blood vessels and bones (Guan et al., 2008; Kim et al., 2002). Here, we show that HIF-1α induced the expression of VEGF and RUNX2 in PDLSCs and periodontium and that HIF-1α-induced VEGF could regulate the expression of RUNX2 and enhance the osteogenesis of PDLSCs. However, it has been proved that VEGF alone cannot enhance the osteogenic activity in human bone marrow stromal cells (Sharma et al., 2018). Thus, the interactions among HIF-1α, VEGF, and RUNX2 could enhance the effects of VEGF on the osteogenesis of PDLSCs. It can be assumed that prolonged intermittent hypoxia may play a positive role in the recovery of alveolar bone by PDLSCs. However, the ligament where PDLSCs reside is tightly attached to the tooth root surface, and the ossification of the PDL and the fusion of tooth roots to alveolar bone would occur if PDLSCs were induced to differentiate into osteoblasts *in situ*. Further study is needed before applying PDLSCs to restore lost alveolar bone.

In conclusion, we found that hypoxia could regulate the expression of RUNX2 via HIF-1α-induced VEGF in PDLSCs and periodontium. The effects of HIF-1α-induced VEGF on RUNX2 could enhance the osteogenesis in hypoxic periodontium, suggesting a positive role for HIF-1α in the early stage of osteogenesis of PDLSCs. However, more studies are needed to investigate their interactions in periodontitis.

### Disclosure

The authors have no potential conflicts of interest to disclose.

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