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Hypoxia-regulated human periodontal ligament cells via Wnt/β-catenin signaling pathway

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Abstract

Background: The aim of this study is to investigate the effects of hypoxia on the proliferation, morphology, migration ability, hypoxia inducible factor (HIF) 1 (HIF-1) expression, and the relationship with Wnt/β-catenin signaling of human periodontal ligament cells (hPDLCs) in vitro.

Methods: hPDLCs (4th passage) cultured by the tissue culture method were randomly assigned to slight (5% O_2), severe hypoxia (1% O_2) groups, and the control (21% O_2) group, respectively. From 1st to 7th day, the optical density values were detected, and the growth curve was described. Wound healing assay was done to observe the migration ability of hPDLCs under various O_2 conditions. Then reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was used to detect the expression of cementum-related genes and Wnt signaling pathway-related genes. Further, RT-qPCR, Western blot, and immunofluorescence staining method were constructed to show HIF expressions under different O_2 concentrations in hPDLCs.

Results: The growth rate of hPDLCs decreased with the reduction of O_2 content by degree, and the morphology of hPDLCs changed in different O_2 contents. Besides, hPDLCs migrate faster in 21% and 5% O_2 than in 1% O_2, and both the expressions of cementum-related genes and Wnt signaling pathway-related genes were raised under hypoxic conditions. In addition, with the reduction of O_2 concentration, the messenger RNA and protein level expression of HIF were all increased, and HIF was gradually transported from cytoplasm into the nucleus and in 1% O_2 concentration, it was mainly expressed in the nucleus.

Conclusion: This finding demonstrated that hypoxia was capable of suppressing the proliferation and migration ability, changing the morphology of hPDLCs, and stabilizing HIF-1α against degradation and promoting its translocation to the nucleus. Meanwhile, hypoxia may regulate hPDLCs proliferation and cementogenic differentiation via Wnt/β-catenin signaling pathway, which may potentially provide a novel insight into the etiology and treatment of periodontal diseases.

Abbreviations: ALP = alkaline phosphatase, AXIN2 = axis inhibition protein 2, DMEM = Dulbecco Modified Eagle's Medium, GAPDH = glyceraldehyde-3-phosphate-dehydrogenase, HIF = hypoxia inducible factor, hPDLC = human periodontal ligament cell, OD = optical density, PDL = periodontal ligament, PDLC = PDL cell, RT-qPCR = reverse transcription quantitative real-time polymerase chain reaction.

Keywords: HIF, human periodontal ligament cells, hypoxia, Wnt/β-catenin

1. Introduction

Oxygen is necessary to body's growth and development, and it is critical to functional metabolism.[1] Hypoxia happens when the blood flow is interrupted or reduced in tissues, disrupting oxygen homeostasis.[2] As part of the periodontium, the periodontal ligament (PDL) connects the tooth and the surrounding tissues, acting as a buffer between alveolar bone and tooth. PDL fibroblasts, also called PDL cells (PDLCs), dominated in the periodontium, play an important role in extracellular matrix synthesis, principal fibers and dental cement production, cell conglutination, transportation, and mineralization.[3–5] In normal periodontium, the oxygen content ranges from 2.9% to 5.7%,[6] but many causes can lead to local hypoxia microenvironment in periodontal tissues, like severe periodontitis,[7] smoking,[8] and orthodontic treatment.[9] So far specific mechanisms are still unknown about the behaviors of PDLCs under hypoxic conditions in vitro.

The Wnt signaling pathway is a highly complex, evolutionarily conserved, encompassing 19 Wnt ligands, and more than 15 receptors.[10] Generally, the Wnt pathway includes canonical Wnt/β-catenin signaling and noncanonical Wnt/Ca^{2+} signaling and has been revealed to play critical roles in cell proliferation and differentiation during tooth development.[10] The relationship between hypoxia and Wnt signaling has been paid more and more attention, including PDLCs. However, the results are

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contradictory: For instance, Meldrum et al[11] found that followed the activation of the canonical Wnt signaling, cementogenic markers increased significantly in primary PDLCs cultured in osteogenic induction medium. But Braunschweig et al[12] demonstrated that the canonical Wnt signaling pathway inhibited cementoblast differentiation by using an immortalized murine cementoblast cell line (OCCM-30; University of Washington, Wahington). Therefore, whether the effects of hypoxia on proliferation and cementogenic differentiation of PDLCs are modulated by Wnt/β-catenin signaling remain to be elucidated.

Therefore, the aim of this study was to investigate the effects of hypoxia on proliferation and cementogenic differentiation of human PDL cells (hPDLCs) in vitro and elucidate the role of Wnt/β-catenin signaling underlying this process, which is important in improving our understanding of the etiology and treatment of periodontal diseases.

2. Materials and methods

2.1. Culture and identification of hPDLCs

hPDLCs were isolated and cultured according to previous published protocols.[13] Teeth were obtained from premolar teeth extracted from healthy patients (mean 13 years old) for orthodontic treatment. Informed consent was given to all the patients involved, and the research protocol had been approved by the Human Ethics Committees of Chongqing Medical University and Tongji University. The patients were asked to gargle with chlorhexidine before the extraction of the tooth. Then, the tooth was disinfected with 1% iodine and 72% alcohol. Following extraction, the tooth was repeatedly washed with sterile phosphate buffer saline (PBS) and placed into Dulbecco Modified Eagle's Medium culture solution (DMEM; Gibco-Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (FBS). The tooth was subsequently sent to the super clean bench. The tooth was placed in a sterile culture dish, and a small quantity of DMEM containing antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL amphotericin B; North China Pharmaceutical, Shanghai, China) was added in order to keep the root face moist. Then PDL tissues were separated from the middle 3rd of the root surface using a scalpel and were cultured in a T25 flask in DMEM (Gibco-Invitrogen) supplemented with 10% v/v FBS (Thermo Scientific, Massachusetts), 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco-Invitrogen, Carlsbad, CA) at 37° C in a humidified CO_2 incubator. The medium was changed after 5 days and the outgrown cells growing around the PDL tissues were passaged at approximately 80% confluence and expanded through 2 passages to obtain a sufficient number of cells for the in vitro assays. Cells at passages 4th to 7th were used for the study.

2.2. Identification of hPDLCs using avidin–biotin complex immunohistochemistry

The hPDLCs (1×10^5/mL, 4th passage) were inoculated into a culture dish with a glass slide in order to create a cell climbing slide. Paraformaldehyde was used for fixation and exclusive serum (bovine serum albumin 1 g, phosphate-buffered saline 100 mL, sodium azide 0.08 g) for closure. The 1st, 2nd, and 3rd antibodies were added in sequence. 3,3′-Diaminobenzidine (DAB; Dako Denmark A/S, Denmark) was primarily used in the color reaction, which was terminated by PBS. Subsequently, hematoxylin was used for the counterstaining process, and neutral balsam was used in the sealing process. Images were captured under a microscope (Olympus, Tokyo, Japan). According to various hypoxic conditions, hPDLCs were assigned into 3 groups randomly as follows: slight hypoxia group, 5% O_2 content; severe hypoxia group, 1% O_2 content; and the control group, 21% O_2 content. hPDLCs in the 2 hypoxia groups were cultivated in a 3-gas (CO_2/O_2/N_2) incubator (NuAire Inc., Plymouth, MN), and the hPDLCs in the control group were cultivated in a common CO_2 incubator (NuAire, Inc.).

2.3. Proliferation of PDLCs on different O_2 conditions

hPDLCs were cultured in 96-well culture plates according to their corresponding circumstance. First, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, Solon, Ohio) solution (5 g/L) was added to each tested well from 1st to 7th day after cultivation. Subsequently, cultivation was continued for a further 4 hours at 37°C. The culture solution was discarded, and 20 μL dimethyl sulphoxide was added to each tested well. The optical density (OD) of each tested well was measured using an enzyme linked immunosorbent assay plate reader (Precision Microplate Reader, Molecular Devices, Bio-Rad Inc., Hercules, CA) at 490 nm following agitation for 10 minute.

2.4. Morphology of hPDLCs on different O_2 conditions

hPDLCs were cultured in a 96-well culture plate according to their corresponding circumstance, and their morphology were observed using an inverted microscope at 3rd and 7th day. At the same time, to assess the relative alkaline phosphatase (ALP) activity, the culture solution was discarded at 3rd and 7th day after cultivation, and the cells were washed with PBS. Triton X-100 (Sigma–Aldrich, St. Louis) was used to dissolve the cells. The lysates were maintained at 4°C overnight. The lysates from each well were moved to an Eppendorf tube (Eppendorf, Hamburg, Germany) for centrifugation at $997 \times g$ for 5 minute. Subsequently, 30 μL of supernatant fluid was extracted for the ALP activity detection using the ALP kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions in the. An ultraviolet spectrophotometer (Unico, Franksville, WI) was used to measure the OD at 520 nm.

2.5. Migration ability of the hPDLCs on different O_2 conditions

hPDLCs were plated on 24-well glass bottom plates under different O_2 contents. When confluent, the cells were mechanically scratched with a pipette tip (20 μL) similar to previous descriptions, yielding a linear cell-free area of approximately 300 to 400 μm. Then the scratch wounds were observed at 24, 48 hours, respectively.

2.6. Cementogenic-related gene expression of hPDLCs on different O_2 conditions

The effect of hypoxia on hPDLCs cementogenic differentiation was assessed by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) to measure the messenger RNA (mRNA) expression of mineralization-related gene (osteopontin [OPN], ALP) and cementum-related gene (cementum protein1 [CEMP1] and cementum attached protein [CAP]) of each group. The media was changed after 24 hours to osteogenic differentiation medium after 1×10^6 hPDLCs cultured in O_2 conditions. Cells were harvested on 3rd and 7th days, and then total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. From 1-mg total RNA, complementary DNA was synthesized using DyNAmoTM

Table 1

Nucleotide sequence of primers used in RT-qPCR.

Gene	Forward primer	Reverse primer
OPN	5′ TCACCTGTGCCATACCAGTTAA 3′	5′ TGAGATGGGTCAGGGTTTAGC 3′
ALP	5′ TCAGAAGCTAACACCAACG 3′	5′ TTGTACGTCTTGGAGAGGGC 3′
CAP	5′ CTGCGCGCTGCACATGG 3′	5′ GCGATGTCGTAGAAGGTGAGCC 3′
CEMP1	5′ GGGCACATCAAGCACTGACAG 3′	5′ CCCTTAGGAAGTGGCTGTCCAG 3′
β-CATENIN	5′GCTACTGTTGGATTGATTCGAAATC 3′	5′ CCCTGCTCACGCAAAGGT 3′
AXIN2	5′ CCCCAAAGCAGCGGTGC 3′	5′ GCGTGGACACCTGCCAG 3′
C-MYC	5′ CGCCAGCGGAAGGTATGAAG 3′	5′ CAACTTGTCCTTACACCTCTGAGTC3′
GAPDH	5′ TCAGCAATGCCTCCTGCAC 3′	5′ TCTGGGTGGCAGTGATGGC 3′

ALP = alkaline phosphatase, AXIN2 = axis inhibition protein 2, CAP = cementum attachment protein, CEMP1 = cementum protein 1, GAPDH = glyceraldehyde-3-phosphate-dehydrogenase, OPN = osteopontin.

cDNA Synthesis Kit (Finnzymes, Genesearch, Australia) following the manufacturer's instructions. RT-qPCR primers (Table 1) were designed on the basis of cDNA sequences from the NCBI Sequence database, and the primer specificity was confirmed by BLASTN searches. RT-qPCR was performed on an ABI Prism 7300 Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific) with SYBR Green detection reagent. Briefly, 2.5 mL of cDNA template, 2.5 mL of gene-specific reverse and forward primers (GeneWorks Pty Ltd, Australia), and 12.5 mL of 2 xSYBR Green qPCR Master Mix (Invitrogen) was mixed with 5-mL RNAse free water for a 25-mL final reaction volume in a 96-well PCR plate. The relative mRNA expressions of OPN, ALP, CEMP1, and CAP were assayed and normalized against the house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Each sample was performed in triplicate. The mean cycle threshold (C_t) value of each target gene was normalized against C_t value of GAPDH and the relative expression calculated using the following formula: $2^{-(\text{normalized average Cts})} \times 10^4$.

2.7. Wnt/β-catenin signaling related gene expression of hPDLCs on different O_2 conditions

To explore whether the effect of hypoxia on hPDLCs was modulated by Wnt/β-catenin signaling, its related genes axis inhibition protein 2 (AXIN2), β-catenin, c-Myc were analyzed by RT-qPCR as the same method in Section 2.6 and Western blot. Whole cell lysates harvested in 250-μL cell lysis buffer (2-mM Tris HCl, pH 7.5, 15-mM NaCl, 0.1-mM Na_2EDTA, 0.1-mM EGTA, 0.1% Triton, 0.25-mM sodium pyrophosphate, 0.1-mM β glycerophosphate, 0.1-mM Na3VO4, 0.1 μg/mL leupeptin). Protein lysates (15 μg per lane) were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Pall Life Sciences, Port Washington, NY). Membranes were blocked for 1 hour at room temperature in Odyssey blocking buffer (cat. no. 927 40000; LI COR, Inc., Lincoln, NE), then incubated overnight at 4 °C with primary antibodies against β-catenin (1:1000, rabbit antihuman/rat; cat. no. 9581; Cell Signalling Technology, Inc., Danvers, MA); hypoxia inducible factor (HIF) 1α (HIF-1α) (1:1000, mouse monoclonal antibody; cat. no. sc 13515; Santa Cruz Biotechnology, Inc.); c-Myc (1:1000), and α-Tubulin (1:2000, rabbit antihuman/rat; cat. no. ab15246; Abcam). The membranes were incubated with anti-mouse/rabbit fluorescently labeled secondary antibodies (P/N 925 32211 or P/N 925 68070; LI COR, Inc.) at 1:10,000 dilutions for 1 hour at room temperature. Protein bands were visualized using the Odyssey Infrared Imaging System (LI COR, Inc.). The relative intensity of protein bands compared with α-Tubulin was quantified using Image J software version 1.47 (National Institutes of Health, Bethesda, MD).

2.8. Statistical analysis

All the experiments were done in triplicate, and the results are given as the mean ± standard deviation of 3 independent experiments. Statistical comparisons were done using the single factor analysis of variance and a paired *t* test. All procedures were done using SPSS 19.0 (SPSS Inc., Chicago, IL) with $P < 0.05$ considered statistically significant.

3. Results

3.1. Cultivation and identification of hPDLCs

After being passaged, the hPDLCs were in fusiform shape and were arranged in a sarciniform or swirl pattern (Fig. 1A). Immunohistochemistry testing of the hPDLCs (4th passage) revealed that the cytoplasm was positive for vimentin with a yellow-brown color (Fig. 1B) while keratin was not found in the cytoplasm (Fig. 1C), indicating hPDLCs are mesenchymal cells derived from the embryonic mesoderm.

3.2. Proliferation of hPDLCs on different O_2 conditions

We detected the OD values under various hypoxic conditions from 1st to 7th day. The proliferation of hPDLCs increased in a time-dependent manner in each group, and the growth rate of hPDLCs decreased by degree with the reduction of O_2 content, but the hPDLCs growth curves were alike in 3 groups, which were similar to an 'S', and all these groups had the highest OD value at the 6th day, indicating hypoxia decreased the hPDLCs proliferation ability (Fig. 1D).

3.3. Morphology of hPDLCs on different O_2 conditions

The morphology of hPDLCs changed in different O_2 contents. Compared with normal hPDLCs, which had a high cell density, in the 1% O_2 concentration, the number of hPDLCs in 1 microscopy significantly reduced, and they were arranged much sparser. And cell bodies shrank, and processes were much shorter and slender. The morphology of hPDLCs in 5% O_2 conditions was between the control and 1% O_2 groups (Fig. 2A). In addition, the relative ALP activity of hPDLCs showed a significant increase with the reduction of O_2 concentration ($P < 0.05$) at 3rd and 7th day (Fig. 2B).

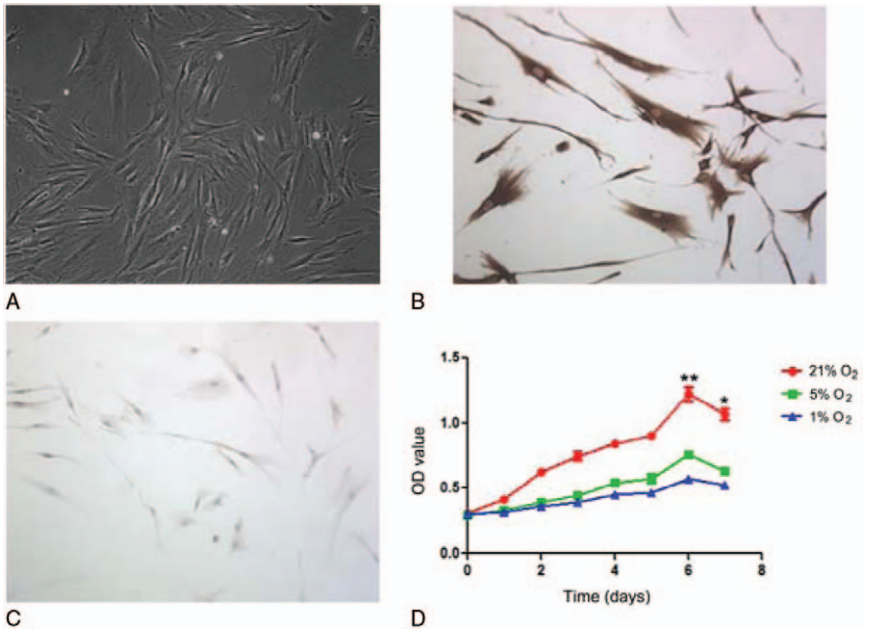

Figure 1. Cultivation and identification of human periodontal ligament cells (hPDLCs). (A) 4th passage of hPDLCs. Cells were fusiform in shape and were arranged in a sarciniform or swirl pattern. Original magnification, ×200. (B) Vimentin expression in hPDLCs. Vimentin was found in the cytoplasm with a yellow-brown color. Original magnification, ×200. (B) Keratin expression in hPDLCs. Keratin was not found in hPDLCs, indicating hPDLCs are mesenchymal cells derived from the embryonic mesoderm. Original magnification, ×200. (D) Growth curve of hPDLCs under various hypoxia conditions from 1st to 7th day. The growth rate of hPDLCs decreased by degree with the reduction of O_2 content, but the growth curves were alike in 3 groups, which were similar to an 'S', and all these groups had the highest optical density value at the 6th day. ($D6^{**}$, $P<.01$, $D7^{*}$, $P=.03$).

3.4. Migration ability of the hPDLCs on different O_2 conditions

Wound-healing assay demonstrated that after injuring the hPDLCs extended toward the cell-free scratch region and subsequently migrated and populated the scratch at a different speed under different O_2 conditions. In the first 24 hours, the scratched area of hPDLCs preconditioned in 1% O_2 was larger than the control group. After 48 hours, the difference was much more obvious, whereas the scratched area of hPDLCs preconditioned in 5% O_2 had no significant difference with the control group, suggesting that hPDLCs migrate faster in 21% and 5% than in 1% O_2 (Fig. 3).

3.5. The expression of cementum-related genes of hPDLCs on different O_2 conditions

RT-qPCR analysis showed that hypoxic conditions enhanced the cementogenic differentiation of hPDLCs. The promotion of cementogenic differentiation of hPDLCs is greater in the severe 1% O_2 concentration group than the slight 5% O_2 group. Mineralization-related gene expression of OPN and ALP for hPDLCs and the cementum-related CEMP1 and CAP genes were all significantly upregulated in 1% O_2 conditions compared to other 2 groups at both 3rd and 7th day (Fig. 4).

3.6. Wnt signaling pathway-related gene expression of hPDLCs on different O_2 conditions

RT-qPCR results showed that compared with the control group, the expression of Wnt-related genes AXIN2, β-catenin and c-Myc increased in hypoxic groups both at 3rd and 7th day (Fig. 5A–C). Similarly, the relative protein expression of Wnt-related genes also increased in 1% O_2 and 5% O_2 groups at both 3rd and 7th day (Fig. 5D).

3.7. HIF expression of hPDLCs on different O_2 conditions

Immunofluorescence staining results showed that under normal O_2 conditions, HIF-1α degraded rapidly and mainly expressed in cytoplasm. But with the concentration of O_2 reduced, HIF was gradually transported into the nucleus and mainly expressed in the nucleus, suggesting that hypoxia stabilizes HIF-1α against degradation and forms functional complexes in the nucleus (Fig. 6A). Western blot results also showed an increase of HIF-1α expression under hypoxic conditions (Fig. 6C).

4. Discussion

The PDL is a highly collagenous structure that not only plays a supportive role in attaching the tooth to the surrounding alveolar bone but also plays a shock-absorbing role in providing resistance to mechanical force.[14] hPDLCs are the most numerous cells in periodontium and function in periodontal tissue formation, regeneration, and maintaining integrity of the periodontal membrane. It's a kind of heterogeneous pluripotent cells with self-renewal ability, including mature cells that have different functions and undifferentiated mesenchymal cells that have differentiation potentials.[15–17] Hypoxia is a basic environment of life development, and it affects the survival of cells and the formation of blood vessels through a series of regulatory mechanism, which is critical to periodontal tissue reconstruction.[18] To better understand the mechanisms of hypoxia on hPDLCs and to provide novel therapies for periodontal diseases, there are increasingly researches to explore the effects of hypoxia on PDLCs. Zhou et al[19] have found that

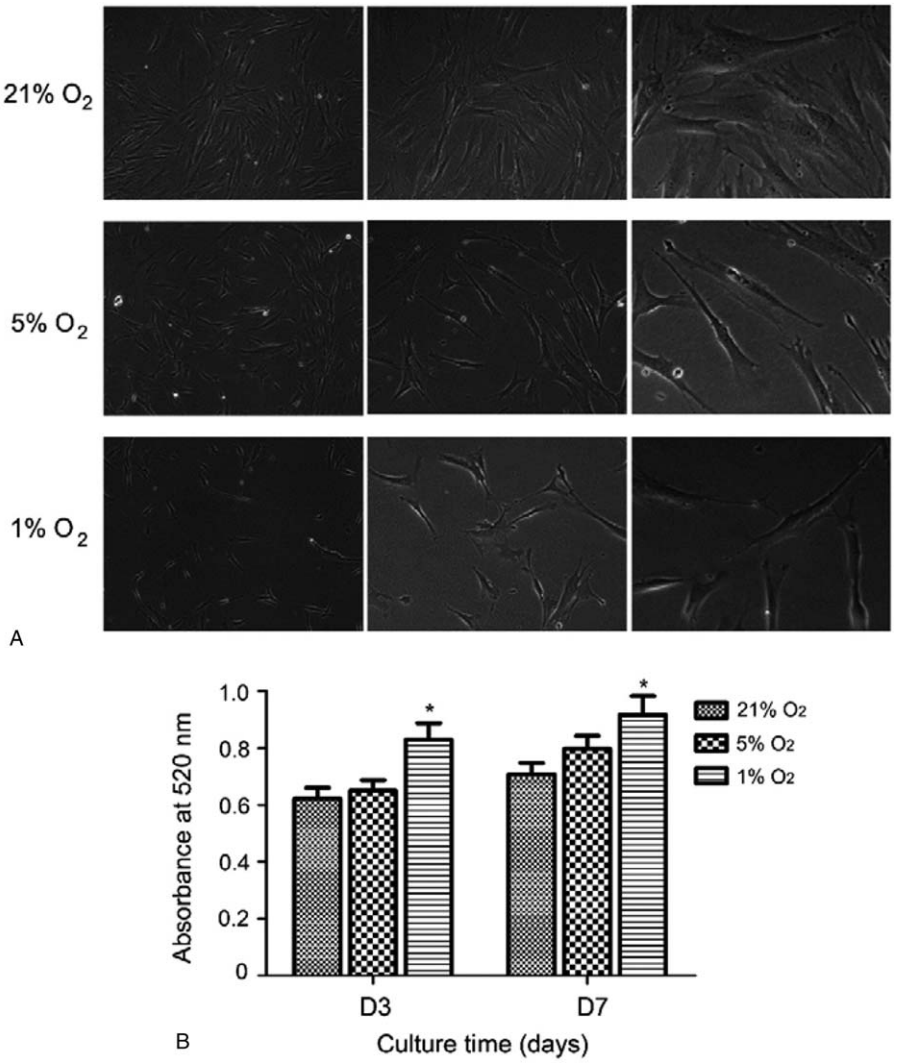

Figure 2. The morphology of human periodontal ligament cells (hPDLCs) changed in different O_2 contents. (A) In the 1% O_2 concentration, the number of hPDLCs in 1 microscopy significantly reduced, and hPDLCs were arranged sparser compared with normal conditions. Besides, cell bodies shrank, and processes were much shorter and slender. The morphology of hPDLCs in 5% O_2 conditions was between the control and 1% O_2 groups. The columns represented original magnification ×100, ×200, ×400, respectively. (B) The relative alkaline phosphatase activity. hPDLCs showed a significant increase with the reduction of O_2 concentration at 3rd and 7th day. (D3*, $P = .03$, D7*, $P = .03$).

hypoxia plays an important role in maintaining the stemness and differentiation capacity of PDLCs. Li et al[20] found that the differentiation of PDLCs into osteogenic and cementogenic lineages is partially regulated by the Wnt signaling pathway and that hypoxia is involved in this process. In present study, hPDLCs were isolated and cultured according to the tissue culture method and the cells in the 4th to 7th passages that had the best activity were selected. Cell proliferation is one of the most important factors in maintaining the balance of cell numbers and normal organism functions. We detected the OD values under various hypoxic conditions and found that the growth rate of hPDLC decreased with the reduction of O_2 content. Exposed to hypoxic conditions for various periods of time, migration ability was assessed, indicating that hPDLCs migrate remarkably faster in 21% and 5% than in 1% O_2.

To study cementogenic/osteogenic differentiation of hPDLCs, bone-related markers OPN, ALP and cementogenic-related markers CEMP1, CAP were detected. OPN is related to bone mineralization, while ALP is an early marker for osteoblastic differentiation.[20] CEMP1 and CAP expressed limitedly in mature cementum matrix of periodontium and cementoblasts.[21] In present study, cementogenic/osteogenic-related markers were all upregulated on hypoxic conditions, and the less the O_2 content was, the greater these markers were expressed. The results indicated that hypoxia play a critical role in enhancing cementogenic differentiation of PDLCs.

We further investigated the effect of hypoxia on canonical Wnt signaling pathways of hPDLCs to find possible underlying molecular mechanisms. Accumulating evidence has revealed that Wnt/β-catenin signaling pathway has a dual role in mineral tissue development and regeneration.[22,23] It could promote bone formation by enhancing both the proliferation and differentiation of bone marrow stromal cells, and it could also reduce bone formation during the terminal mineralization stages.[24] Now the Wnt signaling pathway has been investigated intensively in the mechanism study of hypoxia, including hPDLCs.[24] In present study, the expression of Wnt signaling related gene AXIN2, β-catenin, and c-Myc were all increased, suggesting that hypoxia

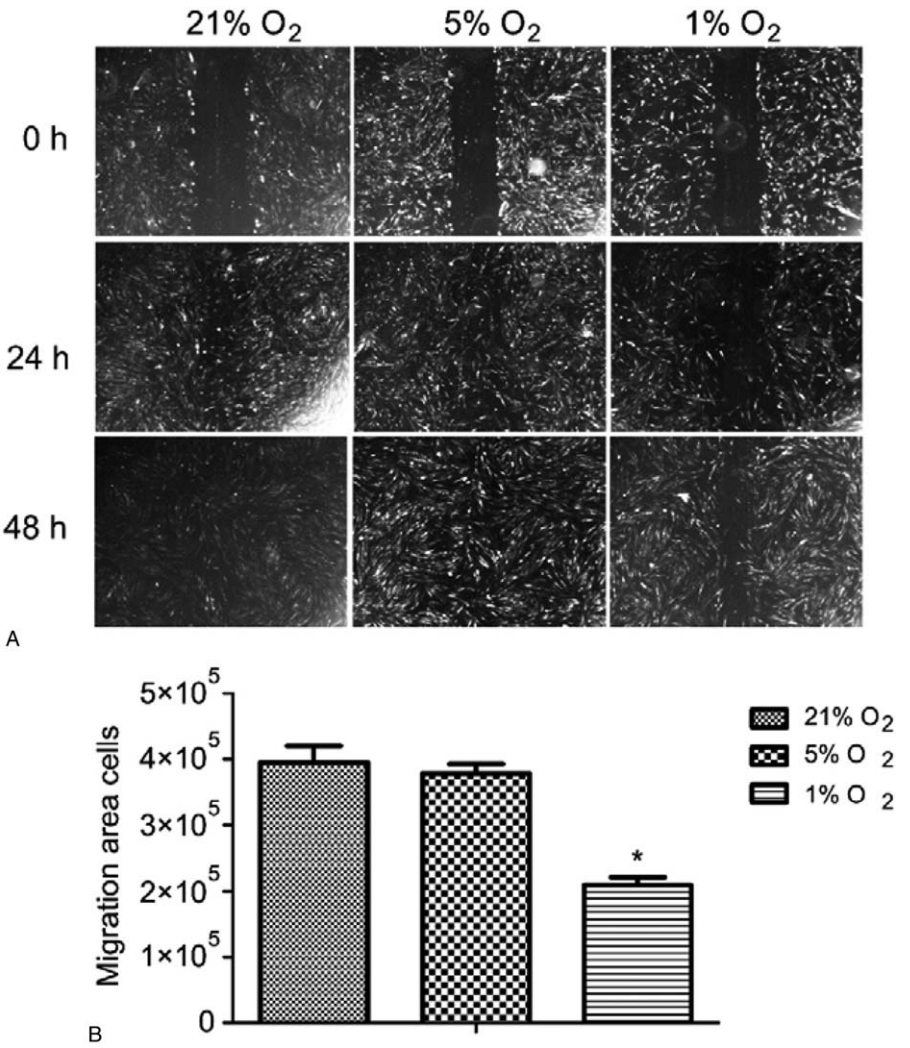

Figure 3. Migration ability of the human periodontal ligament cells (hPDLCs) on different O_2 conditions. (A) Wound-healing assay showed that in the first 24 hours, the scratched area of hPDLCs preconditioned in 1% O_2 was larger than the control group, but the scratched area of hPDLCs preconditioned in 5% O_2 had no significant difference with the control group. After 48 hours, the difference was much more obvious between the 1% O_2 group and the control group, whereas the scratched area of hPDLCs preconditioned in 5% O_2 still had no significant difference with the control group, suggesting that hPDLCs migrated faster in 21% and 5% than in 1% O_2. (B) Migration area cells on different O_2 conditions. The number of cells in the migration area was much less in 1% O_2 compared with the control group. ($^{*}P = .02$).

could activate Wnt signaling pathway. Then we hypothesized that the effect of hypoxia on proliferation and cementogenic differentiation of hPDLCs was mediated through Wnt signaling pathway. Li et al[20] reported that hypoxia could significantly suppress the proliferation of PDLCs in osteogenic medium either in Wnt stimulated or nonstimulated groups, which is consistent with our results. But further experiments to verify the necessity of Wnt signaling pathway on the effect of hypoxia on the proliferation of hPDLCs is needed. In our research, we found that hypoxia could promote hPDLCs cementogenic differentiation and induce activation of Wnt/β-catenin signaling pathway, consistent with previous studies that the Wnt signaling pathway promotes cementogenesis of hPDLCs cultured in osteogenic media. Though there were contradictions that Wnt signaling inhibits cementogenic differentiation of hPDLCs when cultured in nonosteogenic medium.[20]

Simultaneously, we explored the relationship between HIF expression and Wnt/β-catenin signaling pathway under hypoxic conditions. Usually, the effects of hypoxia on hPDLCs are determined by HIF-1.[25] HIF-1 is a heterodimeric transcription factor mostly composed of oxygen-sensitive (HIF-1α) and oxygen-insensitive subunits (HIF-1β).[25] The availability of HIF-1 is mainly determined by HIF-1α, which is regulated at the protein level in an oxygen-sensitive manner while HIF-1β is stably expressed.[26] When under hypoxic conditions, hydroxylation that degrades HIF-1α rapidly under normal O_2 conditions is halted, leading to HIF-1α stabilization and translocation to the nucleus, stimulating the expression of a broad range of genes in a hypoxic-dependent manner like glucose transporter-1, vascular endothelial growth factor, inducible nitric oxide synthase, and so on.[27,28] HIF-1α induced by hypoxia has a complex function on hPDLCs proliferation. Under short-term hypoxia, overexpression of HIF-1α could promote cell proliferation, but under a long time of hypoxia, it could oppositely restrain cell proliferation.[28] This study found that the proliferation of hPDLCs was decreased under hypoxic conditions and with the reduction of O_2

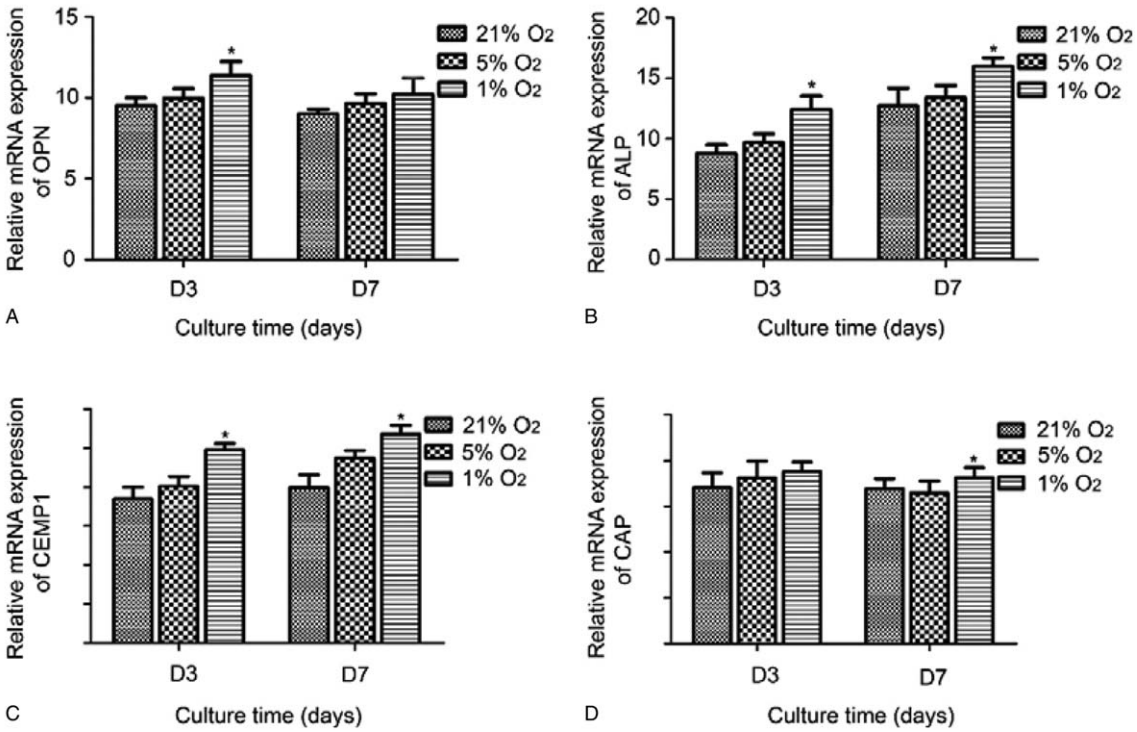

Figure 4. The relative gene expression of bone-related genes. Reverse transcription quantitative real-time polymerase chain reaction analysis showed that hypoxic conditions enhanced the cementogenic differentiation of human periodontal ligament cells (hPDLCs). The promotion of cementogenic differentiation of hPDLCs is greater in the severe 1% O_2 concentration group than the slight 5% O_2 group. Mineralization-related gene expression of osteopontin (OPN) and alkaline phosphatase for hPDLCs and the cementum-related cementum protein1 (CEMP1) and cementum attached protein (CAP) genes were all significantly upregulated in 1% O_2 conditions compared to other 2 groups at both 3rd and 7th day. (A) OPN. ($D3^*$, $P=.04$, $D7^*$, $P=.06$). (B) Alkaline phosphatase. ($D3^*$, $P=.02$, $D7^*$, $P=.03$). (C) Cementum-related genes of CEMP1 ($D3^*$, $P=.02$, $D7^*$, $P=.03$). (D) CAP. (D3, $P=.07$, $D7^*$, $P=.04$).

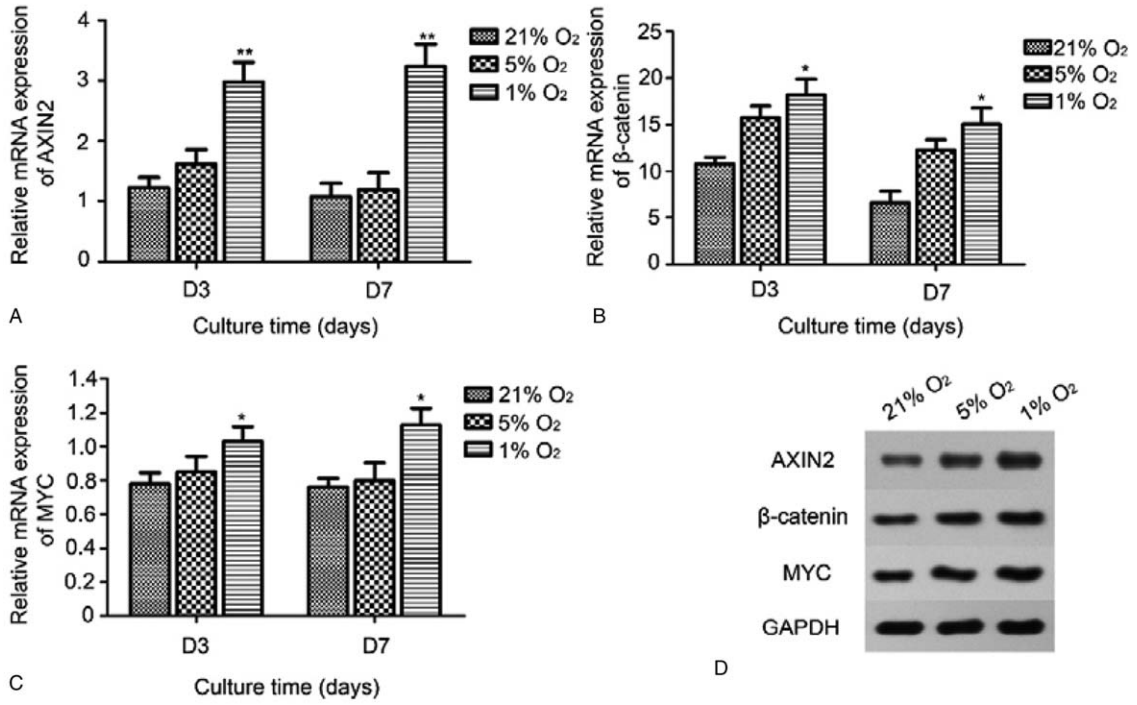

Figure 5. Wnt signaling pathway-related gene expression of human periodontal ligament cells on different O_2 conditions. (A–C) The expression of Wnt-related genes *AXIN2* ($^{**}P<0.01$), β-catenin ($D3^*$, $P=.03$, $D7^*$, $P=.03$), and MYC ($D3^*$, $P=.03$, $D7^*$, $P=.02$) increased slightly in hypoxic groups at both 3rd and 7th day. (D) The relative protein expression of Wnt-related genes increased in 1% O_2 and 5% O_2 concentration groups at both 3rd and 7th day.

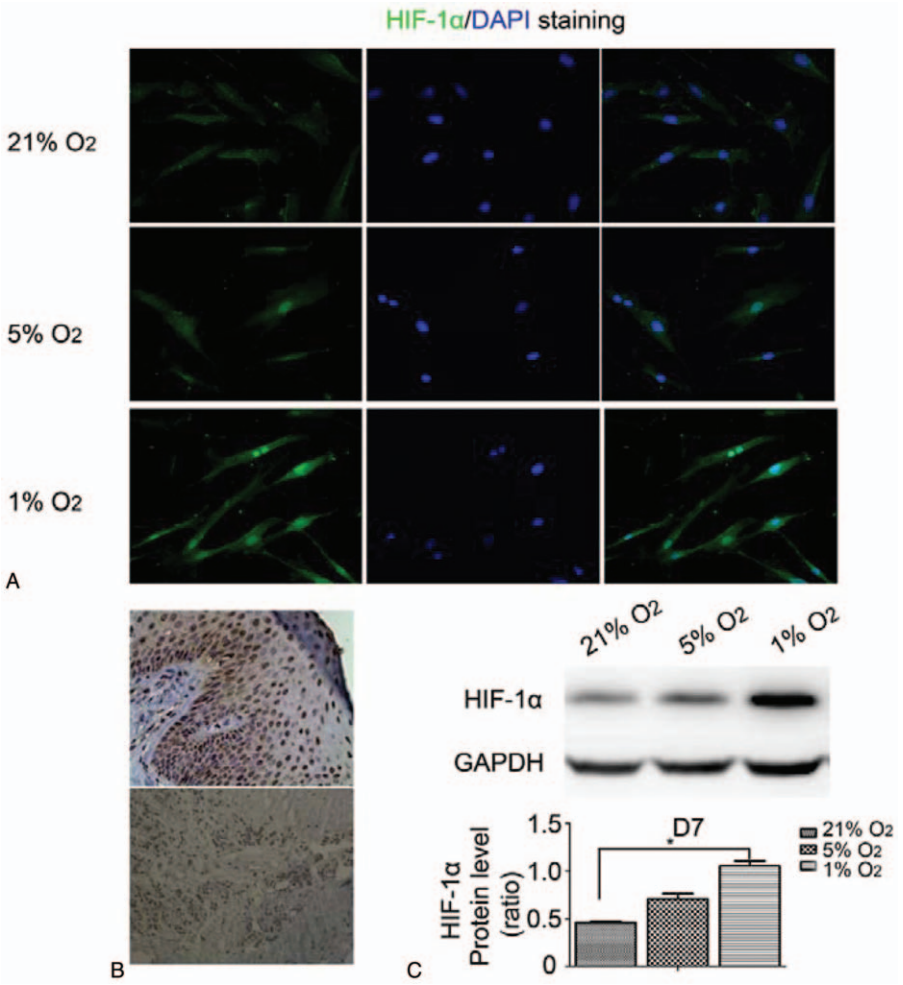

Figure 6. Hypoxia inducible factor (HIF) expression of human periodontal ligament cells on different O_2 conditions. (A) Immunofluorescence staining results showed that under normal O_2 conditions, HIF-1α degraded rapidly and mainly expressed in cytoplasm, but with the concentration of O_2 reduced, HIF was gradually transported into the nucleus and mainly expressed in the nucleus, suggesting that hypoxia stabilizes HIF-1α against degradation and forms functional complexes in the nucleus. (B) Immumohistochemical staining results of HIF in periodontitis tissues. (C) Western blot results showed an increase of HIF-1α expression in hypoxic conditions. ($^{*}P = .02$).

concentration, the expression of HIF increased and gradually transported from cytoplasm to nuclei. The HIF expression was obviously higher than the control group and mainly expressed in nuclei in the severe 1% O_2 condition. Both RT-qPCR and Western blot results showed an increase of HIF-1α expression in hypoxia. Thus, we predicate that HIF-1α could induce the activation of Wnt/β-catenin signaling. Previous studies have proved that HIF-1α could activate Wnt to increase the expression levels of c-Myc and other cytokines in osteoblasts.[29] However, the molecular mechanisms of HIF-1α and Wnt signaling pathway in hPDLCs under hypoxic conditions remain to be elucidated.

Generally, there are various causes leading to local hypoxia microenvironment in periodontal tissues. For example, periodontal diseases that are frequently observed in adult patients, with a prevalence of over 50% of patients older than 30 years can seriously damage the periodontal tissue blood circulation, leading to local hypoxic conditions. Then the severe hypoxia further aggravates the progression of periodontal diseases.[7,30] Smoking can badly affect the O_2 content of periodontal tissues, and it was reported that the O_2 content in periodontal tissues of smokers is only 2/3 of nonsmokers.[8] Besides, orthodontic tooth treatment can also lead to hypoxic conditions, and the orthodontic force could exert different effects on the compression and tension sides. Bone resorption is dominant on the compression side, whereas bone formation is dominant on the tension side.[9] Compressive force reduces blood flow in the PDL and alveolar bone, causing local hypoxia.[31,32] The periodontium is particularly sensitive to hypoxia, and a series of pathological and physiological reactions to hypoxia lead to the reduction of tissue defenses.[33] Furthermore, hypoxia results in the decrease of redox potential, which leads to the gathering of anaerobes.[34] The interaction of these factors accelerates the initiation and development of periodontal disease. Hence, the study of biological activities and specific mechanisms on the effect of hypoxia on hPDLCs improved our understanding of the etiology and treatment of periodontal diseases.

In conclusion, hypoxia was capable of suppressing the proliferation and migration ability, changing the morphology of hPDLCs, stabilizing HIF-1α against degradation and promoting its translocation to the nucleus, which may further initiate or aggravate periodontal diseases. Meanwhile, hypoxia may regulate hPDLCs proliferation and cementogenic differentiation via Wnt/β-catenin signaling pathway and may potentially provide a novel insight into the etiology and treatment of periodontal diseases.

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