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Evaluation of Hypoxia on the Expression of miR-646/IGF-1 Signaling in Human Periodontal Ligament Cells (hPDLs)

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 **Jun Yang***
ABCDE 2 **Jing Zhou***
ABCDE 3 **BoMiao Cui**
ABCDEF 1 **TaiPing Yu**

1 Department of Stomatology, Leshan People Hospital, Leshan, Sichuan, P.R. China
2 Department of Psychosomatic Medicine, Leshan People Hospital, Leshan, Sichuan, P.R. China
3 Key Laboratory of Oral State of West China College, Sichuan University, Chengdu, Sichuan, P.R. China

* The first 2 authors are co-first authors and contributed equally to this work

Corresponding Author: TaiPing Yu, e-mail: taiping_2006@sina.com
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Background: This study aims to investigate the role of miR-646 in hypoxia conditions in human periodontal ligament cells (hPDLs), exploring the effect of hypoxia on hPDLs proliferation and apoptosis. In addition, this study aimed to explore the potential mechanism of miR-646/IGF-1 signaling in hPDLs in hypoxia conditions.

Material/Methods: hPDLs (fifth passage) cultured by the tissue culture method were randomly assigned to the severe hypoxia (1% O₂) group, the slight hypoxia (5% O₂) group or the control (21% O₂) group. Then reverse transcription quantitative real-time polymerase chain reaction and western blot analysis were used to detect the mRNA and protein expression of miR-646 and IGF-1. hPDLs infected with lentivirus (LV)-pre-miR-646 or LV-anti-miR-646, and negative controls were cultured. MTT assay, caspase-3 ELISA assay, and wound healing assay were performed to evaluate how miR-646 was influenced by hypoxia. In addition, the relationship between miR-646 and IGF-1 was explored.

Results: The expression of miR-646 was downregulated and IGF-1 was upregulated in hypoxia conditions. MiR-646 was able to suppress hPDLs proliferation and promote apoptosis in hypoxia conditions. The mRNA and protein expressions of IGF-1 were downregulated when miR-646 was overexpressed and upregulated when miR-646 was downregulated.

Conclusions: This finding identified a significant role of miR-646 in hPDLs in suppressing cell proliferation and promoting apoptosis by inversely regulating IGF-1 expression. Meanwhile, the regulation of hPDLs in hypoxia may be through the miR-646/IGF-1 signaling pathway, probably serving as a promising therapeutic target for periodontal diseases.

MeSH Keywords: Cell Hypoxia • Insulin-Like Growth Factor 1 • MicroRNAs

Abbreviations: **DAB** – 3, 3'-diaminobenzidine; **DMEM** – Dulbecco Modified Eagle's Medium; **FBS** – fetal bovine serum; **GAPDH** – glyceraldehyde-3-phosphate-dehydrogenase; **hPDL** – human periodontal ligament cell; **hPLF** – human periodontal ligament fibroblast; **IGF** – insulin-like growth factor; **IGF-1R** – IGF-1 receptor; **LV** – lentivirus; **miRNAs** – microRNAs; **MTT** – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **OD** – optical density; **PDL** – periodontal ligament; **RT-qPCR** – reverse transcription quantitative real-time polymerase chain reaction; **SDS-PAGE** – sodium dodecyl sulphate polyacrylamide gel electrophoresis

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Background

The periodontal ligament (PDL) is an important part of periodontium that not only attaches the tooth to the surrounding alveolar bone but also acts as a buffer by providing resistance to mechanical force [1,2]. Human periodontal ligament cells (hPDLs) are the most numerous cells in the periodontium and play important roles in principal fibers and dental cement production, cell conglutination, extracellular matrix synthesis, mineralization, and transportation [3–5]. Oxygen is very important to functional metabolism as well as body growth and development [6]. In the oral cavity, the local periodontium hypoxia microenvironment happens in many situations, such as smoking [7], orthodontic treatment [8], and several periodontitis conditions [9].

MiR-646 was first found to be expressed in human cerebral cortical white and grey matter. MiR-646 belongs to the miR-15/107 gene group and is mammalian specific, only appearing in humans and chimpanzees [10]. Accumulation evidence has shown that the miR-15/107 gene group members have functions in cell division, metabolic pathways, and angiogenesis. In addition, miR-103 and miR-107 can be included in the group of 7 miRNAs (miR-30a-5p, miR-30b,c,d, miR-103/107, miR-191, and miR-195) that are possibly involved in neuronal migration, through the regulation of BDNF expression in human cortex. MiR-107 dysfunction may lead to neurodegeneration, cardiovascular dysfunction, neoplasia, etc. [11]. However, the possible relationship between miR-646 and hypoxia in hPDLs has not yet been reported.

Insulin-like growth factors (IGFs) includes several family members such as IGF-1 and IGF-2 [12]. IGF-1 is involved in several kinds of cells and tissues. It plays important roles in cell proliferation, differentiation, survival, and cell cycle progression [13–16]. IGF-2 plays roles mainly during prenatal development [17]. Human periodontal ligament has been found to express the IGF-1 receptor, indicating that human periodontal ligament is able to stimulate IGF-1 [18]. Previous studies have reported that IGF-1 could enhance hPDLs survival by inducing antiapoptotic molecules and downregulating proapoptotic molecules [19]. However, the specific molecular mechanism between IGF-1 and hypoxia in hPDLs is still unknown.

Therefore, the present study aimed to investigate the role of miR-646 in hypoxia conditions in hPDLs, exploring its impact on hPDLs proliferation and apoptosis. Furthermore, we explored the relationship between miR-646 and IGF-1 underlying this process, to provide a better understanding of the etiology of periodontal diseases and find potential treatment for it.

Material and Methods

Cell culture

All research protocol was approved by the College of Clinical Medicine of Henan University of Science and Technology. The hPDLs were isolated and cultured according to previous published protocols [20]. We obtained teeth from premolar teeth extracted from healthy patients (mean 14 years old) for orthodontic treatment. After extraction, the teeth were repeatedly washed with sterile PBS and placed into Dulbecco's Modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS; Thermo Scientific). Then the teeth were put on a super clean bench to obtain the periodontal ligament tissues from the middle third of the root surface. Subsequently, tissues were cultured in a T25 flask in DMEM (Gibco-Invitrogen, Carlsbad, CA, USA) containing 10% v/v FBS (Thermo Scientific), 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco-Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂ incubator. After the cells migrated from the tissue and became confluent, they were detached with 0.25% trypsin-EDTA and subcultured at a 1: 3 ratio. In each experiment, cells at the fifth to seventh passages were used.

Cell identification

The hPDLs were fixed with 4% paraformaldehyde. After first incubation with vimentin/keratin antibody (Cell Signaling Technology, Danvers, MA, USA, 1: 100), fluorescent secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added. Then the cells were washed and stained with propidium iodide (Santa Cruz Biotechnology). Fluorescence was analyzed with a Zeiss Axiophot Photomicroscope (Carl Zeiss, Oberkochen, Germany).

Hypoxic mimic condition

The hPDLs were randomly divided into 3 groups according to different hypoxia conditions: severe hypoxia group, 1% O₂ content; slight hypoxia group, 5% O₂ content; and the control group, 21% O₂ content. Hypoxic mimic condition was generated through a 3-gas (CO₂/O₂/N₂) incubator (NuAire Inc. Plymouth, MN, USA) while the normal O₂ condition was generated in a common CO₂ incubator (NuAire, Inc., Plymouth, MN, USA).

Total RNA extraction and quantitative real-time PCR

We investigated the mRNA expression of miR-646 and IGF-1 by quantitative real-time PCR (RT-qPCR). The hPDLs in various O₂ conditions were harvested on 7th day and then total RNA was isolated using TRIzol Reagent® (Molecular research Center, Cincinnati, Ohio, USA) according to the manufacturer's instructions. From 1 mg total RNA, complementary

DNA was synthesized using DyNAmo™ cDNA Synthesis Kit (Finnzymes, Genesearch, Australia) according to the manufacturer's instructions. RT-qPCR was performed on an ABI Prism 7300 Thermal Cycler (Applied Biosystems, Australia) with SYBR Green detection reagent. Each sample was constructed in triplicate. The mean cycle threshold (Ct) value of each target gene was normalized against Ct value of GAPDH (for mRNA) or U6 SnRNA (for miRNAs). The relative expression was calculated using the following formula: $2^{-(\text{normalized average Cts})} \times 10^4$. The results are shown as fold-change values relative to the control group. The oligonucleotide sequences were as follows: miR-646, forward 5'-GAAGCAGCTGCCTCTGAGC-3', reverse 5'-CAGAGCGCCAGCGAGGAGCC-3'; IGF-1, forward 5'-CATGCCTGCTCAGAAGGGTA-3', reverse 5'-GCCTCTGATCCTTGAGGTGA-3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3', reverse 5'-GAAGATGGTGATGGGATTTC-3'.

Protein extraction and western blot analysis

We extracted proteins from hPDLs in different O₂ conditions and investigated the protein expression by using western blot analysis. Cells were lysed in lysis buffer (2 mM Tris HCl, pH 7.5, 15 mM NaCl, 0.1 mM Na₂EDTA, 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) and resolved in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Then they were transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% nonfat milk in PBST for 1 hour at room temperature. The membrane was probed with primary antibody against IGF (1: 2000; Abcam, Cambridge, UK) or GAPDH (1: 5000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and was incubated overnight at 4°C. After extensive washing, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (1: 10 000; Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. Odyssey Infrared Imaging System (LI COR, Inc) was used to visualize protein bands. The relative intensity of protein bands compared with GAPDH was quantified using ImageJ software version 1.47 (National Institutes of Health, Bethesda, MD, USA).

Cell infection

Lentivirus (LV)-pre-miR-646, LV-anti-mR-646 and their negative controls were purchased from GenePharma (Shanghai, China). The cells were cultured in a normal medium for 24 hours and then cultured in a medium containing Polybrene (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for cell infection. Then hPDLs were infected with pre-miR-646, anti-miR-646, or their negative controls unit overnight. After that, the old medium was discarded and a new medium without polybrene was added and incubated overnight. Stable clones were selected with puromycin dihydrochloride (Santa Cruz Biotechnology, Inc.).

Plasmids constructs and dual-luciferase reporter assay

The cDNA fragment of 3'-UTR of IGF-1 containing the putative binding site of miR-646 was subcloned into a pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The cells which firmly expresses pre-miR-646 were transfected with 0.1 μg of pGL3-IGF-1-3'-UTR and incubated for 48 hours. The cells were collected and lysed. The Dual-Luciferase Reporter assay kit (Promega) was used to quantify the luciferase activities according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was detected by 3-(4,5)-dimethylthiazolyl(2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, USA) assay. The hPDLs infected with pre-miR-646, anti-miR-646, or their negative controls were seeded in 96-well culture plates according to their corresponding circumstance and 20 μL MTT solution (5 g/L) was added to each test well from 0 to 96 hours after cultivation. Subsequently, cultivation was continued for another 4 hours at 37°C. Then the culture solution was discarded, and 20 μL DMSO was added to each test well. The optical density (OD) of each tested well was measured with a microtiter plate reader (Thermo Electron Corporation, Vantaa, Finland).

Caspase-3 ELISA assay

Cell apoptosis was detected by caspase-3 ELISA assay. The hPDLs infected with pre-miR-646, anti-miR-646, or their negative controls were seeded in 96-well culture plates according to their corresponding circumstance. 48 hours later, by calculating the activity of caspase-3 using the kit (Aibosi, Shanghai, China) according to manufacturer's instructions, apoptosis of hPDLs was detected. OD values were measured using a microplate reader (Bio-Rad).

Cell apoptosis assay

The hPDLs infected with pre-miR-646, anti-miR-646, or their negative controls were seeded in 96-well culture plates according to their corresponding circumstance. The hPDLs were harvested 48 hours later and resuspended in fixation fluid. We added 2 mL propidium iodide and 5 mL Annexin V-FITC to 500 mL cell suspensions. Flow cytometry was used to determine cell apoptosis (EPICS, XL-4, Beckman, CA, USA).

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 (ANOVA) and a paired *t*-test. All procedures were done using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) with *P*<0.05 considered statistically significant.

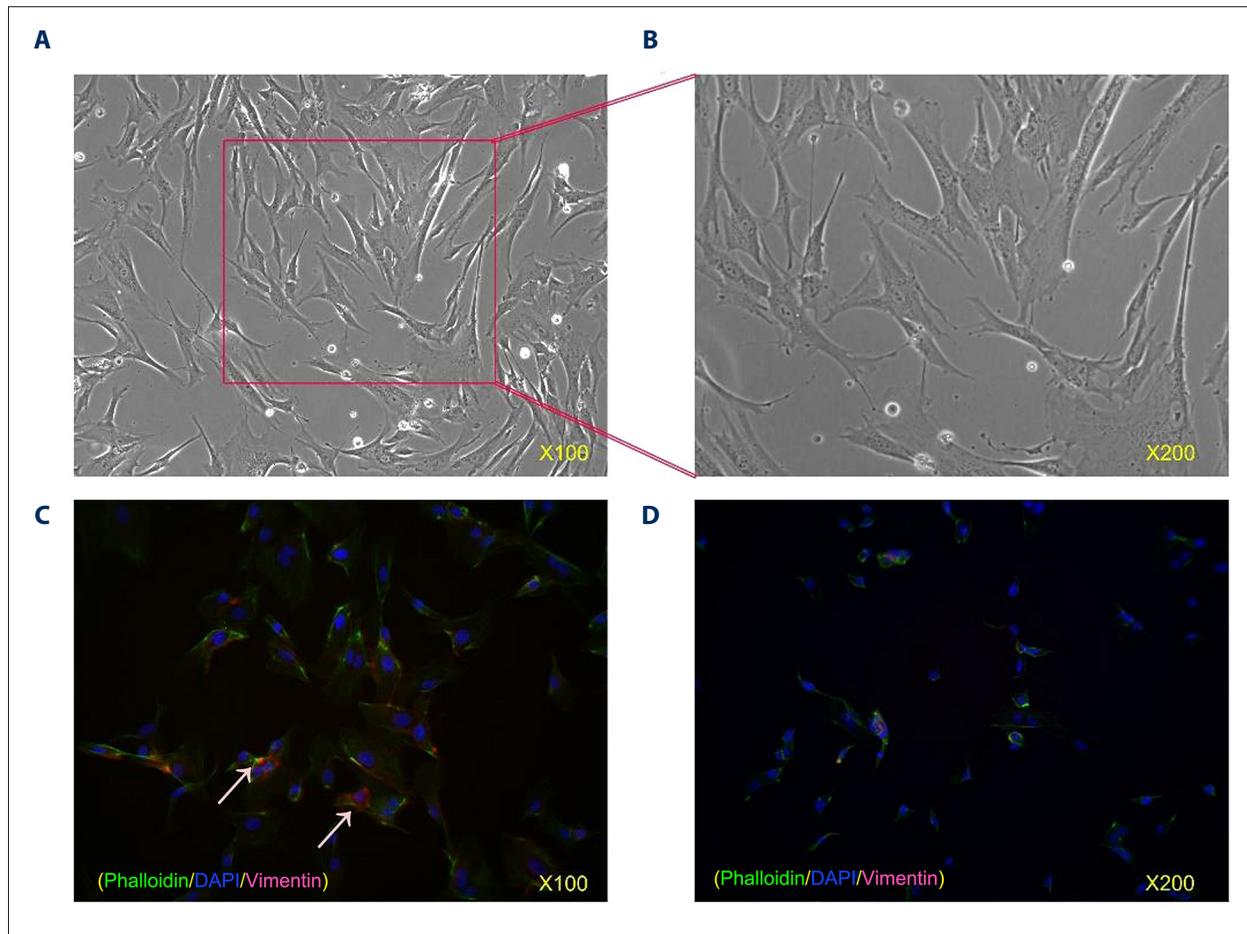


Figure 1. Cultivation and identification of hPDLs. **(A)** Fifth passage of hPDLs. Cells were arranged in a sarciniform or swirl pattern and were fusiform in shape. Original magnification, 100 \times . **(B)** Fifth passage of hPDLs. Original magnification, 200 \times . **(C)** Immunofluorescence strains of hPDLs. Vimentin was found in the cytoplasm with a red color whereas keratin was not found in hPDLs, indicating hPDLs are mesenchymal cells derived from the embryonic mesoderm. Original magnification, 100 \times . **(D)** Immunofluorescence strains of hPDLs. Original magnification, 200 \times .

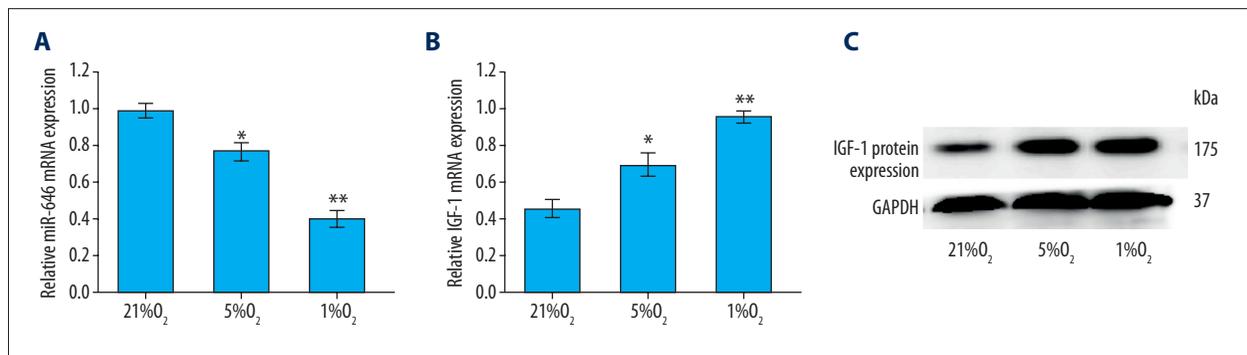


Figure 2. Downregulated expression of miR-646 and upregulated expression of IGF-1 in hypoxia conditions. **(A)** The downregulation of miR646 mRNA in hypoxia conditions was analyzed by RT-qPCR (** $P < 0.01$, * $P = 0.02$). **(B)** The upregulation of IGF-1 mRNA in hypoxia conditions was analyzed by RT-qPCR (** $P < 0.01$, * $P = 0.03$). **(C)** The results of western blot shown IGF-1 protein expression was significantly increased.

Results

hPDLCs identification

The cultivated hPDLCs were arranged in a sarciniform or swirl pattern and were fusiform in shape (Figure 1A). We first identified the immunofluorescence results of hPDLCs, finding that the cytoplasm of hPDLCs with a yellow-brown color was positive for vimentin (Figure 1B), while keratin was not found in the cytoplasm (Figure 1C). These results indicated that the hPDLCs were mesenchymal cells derived from the embryonic mesoderm.

Downregulated expression of miR-646 and upregulated expression of IGF-1 in hypoxia conditions

RT-qPCR results showed that compared with the control group, the mRNA expression of miR-646 in the hypoxia groups was markedly decreased and IGF-1 significantly increased (Figure 2A, 2B). Similarly, the relative protein expression of IGF-1 was upregulated compared with the control group with regards to hypoxia conditions (Figure 2C). These observations showed that in hypoxia conditions, the expression of miR-646 was downregulated and the expression of IGF-1 was upregulated.

miR-646 suppresses proliferation of hPDLCs in hypoxia conditions

Considering the downregulated expression of miR-646 in hPDLCs in hypoxia conditions, we investigated the function of miR-646 in hPDLCs by infecting with pre-miR-646, anti-miR-646, or their negative control units. The expression of miR-646 was significantly upregulated or downregulated in hPDLCs (Figure 3A, 3C, 3E). MTT assay results showed that hPDLCs with miR-646 that was upregulation significantly suppressed the proliferation in hypoxia conditions from the 0 to the 96 hours. On the contrary, hPDLCs with miR-646 that was downregulation remarkably promoted the proliferation in hypoxia conditions (Figure 3B, 3D, 3F). In each group, the proliferation of hPDLCs was increased in a time-dependent manner and the growth rate of hPDLCs was decreased by degree with the reduction of O₂ content. These data reveal that miR-646 suppressed hPDLCs proliferation in hypoxia conditions.

miR-646 promotes apoptosis of hPDLCs in hypoxia conditions

The relative caspase-3 activity was decreased when miR-646 was downregulated and increased when miR-646 was upregulated in hypoxia conditions by ELISA assay. In addition, cell apoptosis induction was observed in hPDLCs infected with pre-miR-646, anti-miR-646, or their negative controls by using flow cytometry analysis. The results were consistent with the

caspase-3 ELISA assay (Figure 4). These results indicated that miR-646 promotes hPDLCs apoptosis in hypoxia conditions.

IGF-1 was inversely regulated by miR-646

To investigate the potential interaction between miR-646 and IGF-1 in hPDLCs, we detected the mRNA and protein expressions of IGF-1 when miR-646 was overexpressed or downregulated in different O₂ conditions. We found that the mRNA expression of IGF-1 was upregulated when miR-646 was decreased whereas it was downregulated when miR-646 was increased (Figure 5A–5C). The results for protein expression of IGF-1 were similar to the results for mRNA expressions (Figure 5D–5F). These results indicated that IGF-1 was inversely regulated by miR-646.

IGF-1 was the direct target gene of miR-646

We further explored the potential mechanisms miR-646/IGF-1 signaling in hPDLCs in hypoxia conditions. We verified that IGF-1 was a likely target of miR-646 as it contained a putative miR-646 target site in the 3'-UTR by using TargetScan software (Lewis et al., 2005) (Figure 5H). Then to confirm whether IGF-1 was a direct target gene of miR-646, the wild-type and the mutants of 3'-UTR of IGF-1 in the predicted binding sequences were constructed into luciferase reporters. After that, these reporters were transfected into hPDLCs with miR-646 overexpression or negative controls by infection with and pre-miR-646 or pre-miR-Ctrl. The results showed that miR-646 overexpression significantly inhibited the luciferase activity in wild-type transfected cells. Additionally, in the mutated transfected cells, the altered expression of miR-646 had no obvious effect on the luciferase activity. These results indicate that IGF-1 was the direct target gene of miR-646. However, to determine whether miR-646 regulates hPDLCs proliferation and apoptosis through IGF-1, requires further experiments.

Discussion

Small non-coding RNA have drawn increasing attention in recent years because of their roles in gene transcription and post-transcription, especially in the study of cancer. It has been estimated that nearly 30% of gene expression in the human body is regulated by miRNAs [21]. PDL is an important part of periodontium, which connects the tooth and the surrounding tissues. hPDLs, also named as human periodontal ligament fibroblasts (hPLFs), is dominant in the periodontium. It is a type of heterogeneous pluripotent cell with self-renewal ability [22–24]. Hypoxia can seriously affect periodontal tissue reconstruction, leading to serious periodontal diseases, but the underlying mechanisms remain unknown. This study investigated the influence of hypoxia on miR-646 in hPDLs.

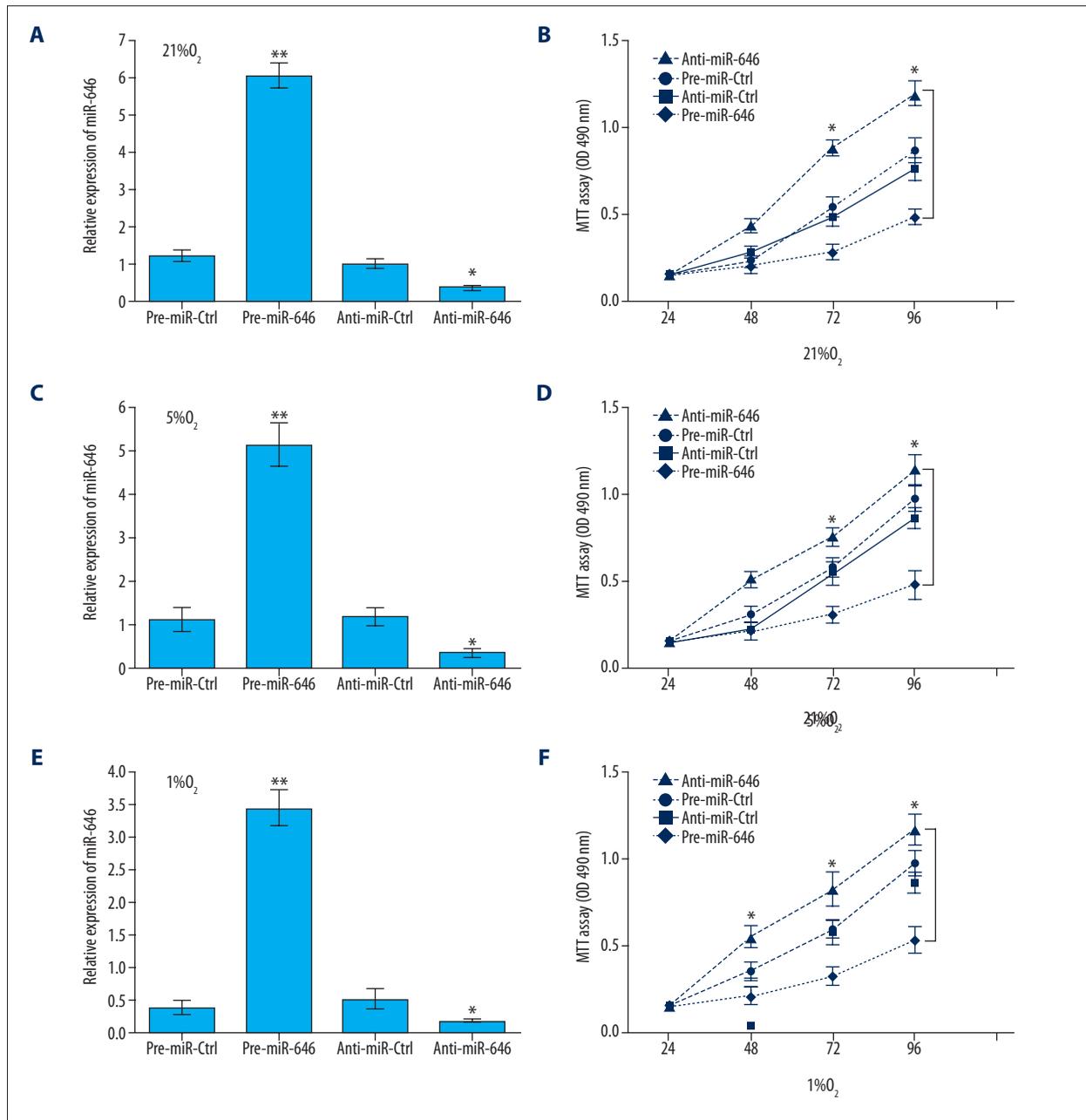


Figure 3. RT-qPCR analysis of miR-646 expression in (A) 21% O₂ condition (** $P < 0.01$ pre-miR-646 vs. pre-miR-Ctrl, * $P = 0.02$ anti-miR-646 vs. anti-miR-Ctrl); (C) 5% O₂ condition (** $P < 0.01$ pre-miR-646 vs. pre-miR-Ctrl, * $P = 0.02$ anti-miR-646 vs. anti-miR-Ctrl); and (E) 1% O₂ condition (** $P < 0.01$ pre-miR-646 vs. pre-miR-Ctrl, * $P = 0.02$ anti-miR-646 vs. anti-miR-Ctrl). The hPDLCs proliferation was significantly suppressed with miR-646 overexpression and remarkably promoted with miR-646 downregulation in (B) 21% O₂ condition (* $P = 0.03$ pre-miR-646 vs. pre-miR-Ctrl, * $P = 0.02$ anti-miR-646 vs. anti-miR-Ctrl); (D) 5% O₂ condition (* $P = 0.03$ pre-miR-646 vs. pre-miR-Ctrl, * $P = 0.02$ anti-miR-646 vs. anti-miR-Ctrl); and (F) 1% O₂ condition (* $P = 0.02$ pre-miR-646 vs. pre-miR-Ctrl, * $P = 0.02$ anti-miR-646 vs. anti-miR-Ctrl).

We reported that the expression of miR-646 was decreased in hypoxia conditions and miR-646 could suppress hPDLCs proliferation and promote apoptosis in hypoxia conditions. Furthermore, we found that IGF-1 was inversely regulated by miR-646. These findings suggest, for the first time, that the

interaction between hypoxia and miR-646 and the association between miR-646 and IGF-1 are mapped in hPDLCs, which may be involved in regulation of cellular functions and mechanisms of hPDLCs under oxygen conditions.

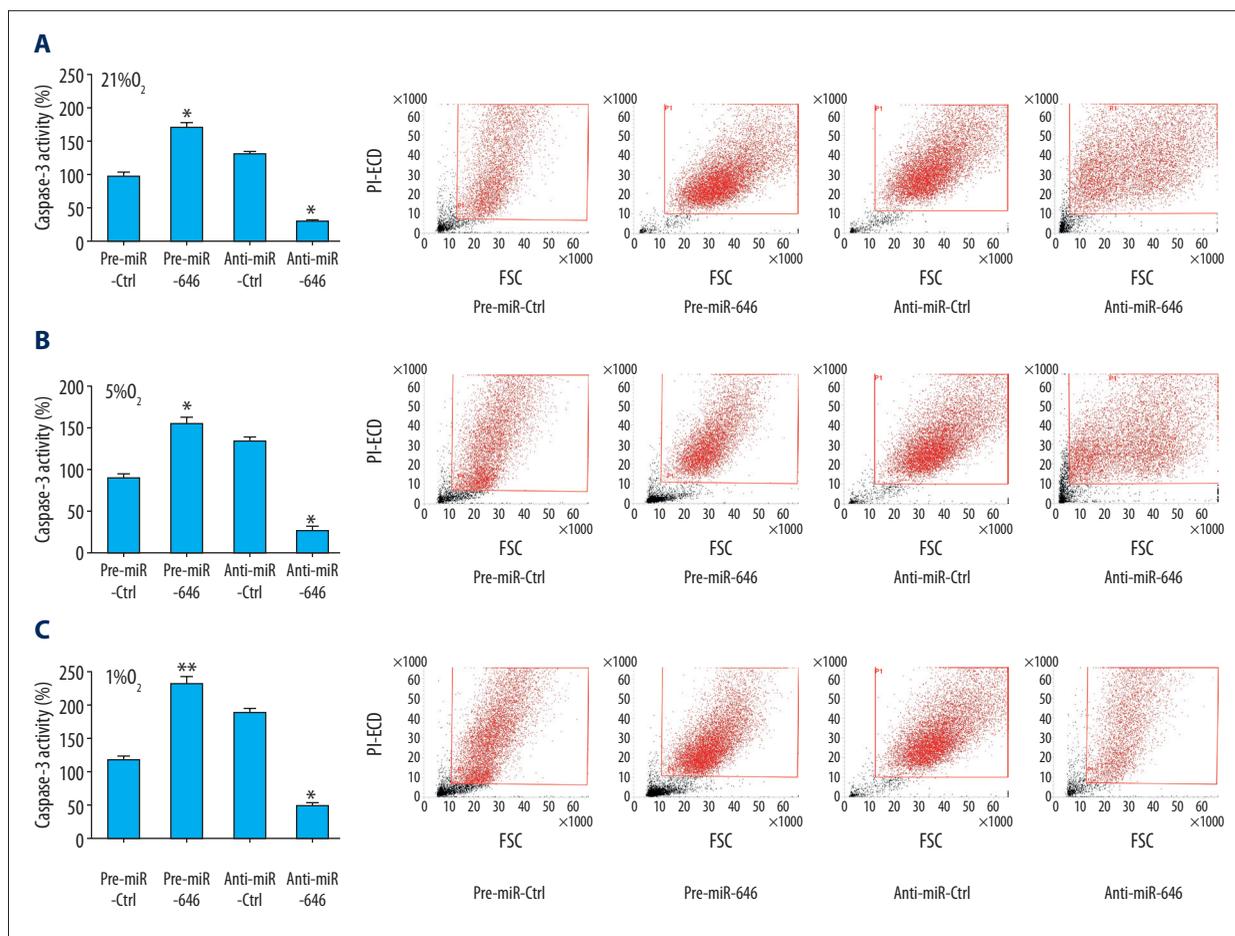


Figure 4. MiR-646 promote apoptosis of hPDLs in hypoxia conditions. The relative caspase-3 activity was decreased with miR-646 downregulation and increased with miR-646 overexpression in (A) 21% O₂ condition (* $P=0.03$ pre-miR-646 vs. pre-miR-Ctrl, * $P=0.02$ anti-miR-646 vs. anti-miR-Ctrl); (B) 5% O₂ condition (* $P=0.03$ pre-miR-646 vs. pre-miR-Ctrl, * $P=0.02$ anti-miR-646 vs. anti-miR-Ctrl); and (C) 1% O₂ condition (* $P<0.01$ pre-miR-646 vs. pre-miR-Ctrl, * $P=0.02$ anti-miR-646 vs. anti-miR-Ctrl). The results of flow cytometry analysis results were consistent with the caspase-3 ELISA assay.

MicroRNAs (miRNAs) which belong to small non-coding RNA, are capable of modulating the expression of many functional proteins. The expression level of miRNAs can be regulated by several microenvironments like hypoxia, oxidative stress, or nutrient deprivation. MiR-646 is one kind of miRNAs that has been reported to be aberrantly expressed in various human cancers like gastric cancer, lung cancer, renal cancer, hepatocellular carcinoma, and osteosarcoma [25–29]. However, the underlying molecular mechanisms of miR-646 and hypoxia in hPDLs have not yet been investigated. In the present study, we investigated whether hypoxia can have an impact on miRNA expression. We found that in severe, slight, and normal O₂ hypoxia conditions, the expression of miR-646 was decreased compared with a control group. In addition, the downregulation of miR-646 significantly suppressed proliferation and promoted apoptosis of hPDLs in hypoxia conditions, while on the contrary, overexpression of miRNA-646 markedly suppressed proliferation and promoted apoptosis of hPDLs in hypoxia conditions.

IGF-1 consists of 70 amino acid residues accompanied by 3 disulfide bridges [30]. Accumulating studies have illustrated that IGF-1 could bind with the IGF-1 receptor (IGF-1R) and then trigger multiple downstream signaling pathways. Among them, the MAPK/ERK and phosphatidylinositol-3-kinase PI3K/AKT pathways have been associated with cell proliferation and survival [31,32]. It has been proven that IGF-1 is one of the neurotrophins that modulate cell growth [33]. It has also been shown that IGF-1 can influence early embryonic development via increasing the number of cells [34]. Moreover, IGF-1 plays an important role in bone growth and development, promoting cell proliferation and osteogenic differentiation in human PDLs [13,35]. The expression of IGF-1 in hypoxia conditions has been studied recently. Zhao et al. illustrated that IGF-1 increased cell viability while decreasing apoptosis in hypoxic neural stem cells through the PI3K/AKT and the MAPK/ERK pathways [36]. Liu et al. reported that IGF-1R may increase cell viability under hypoxic conditions by promoting autophagy and scavenging

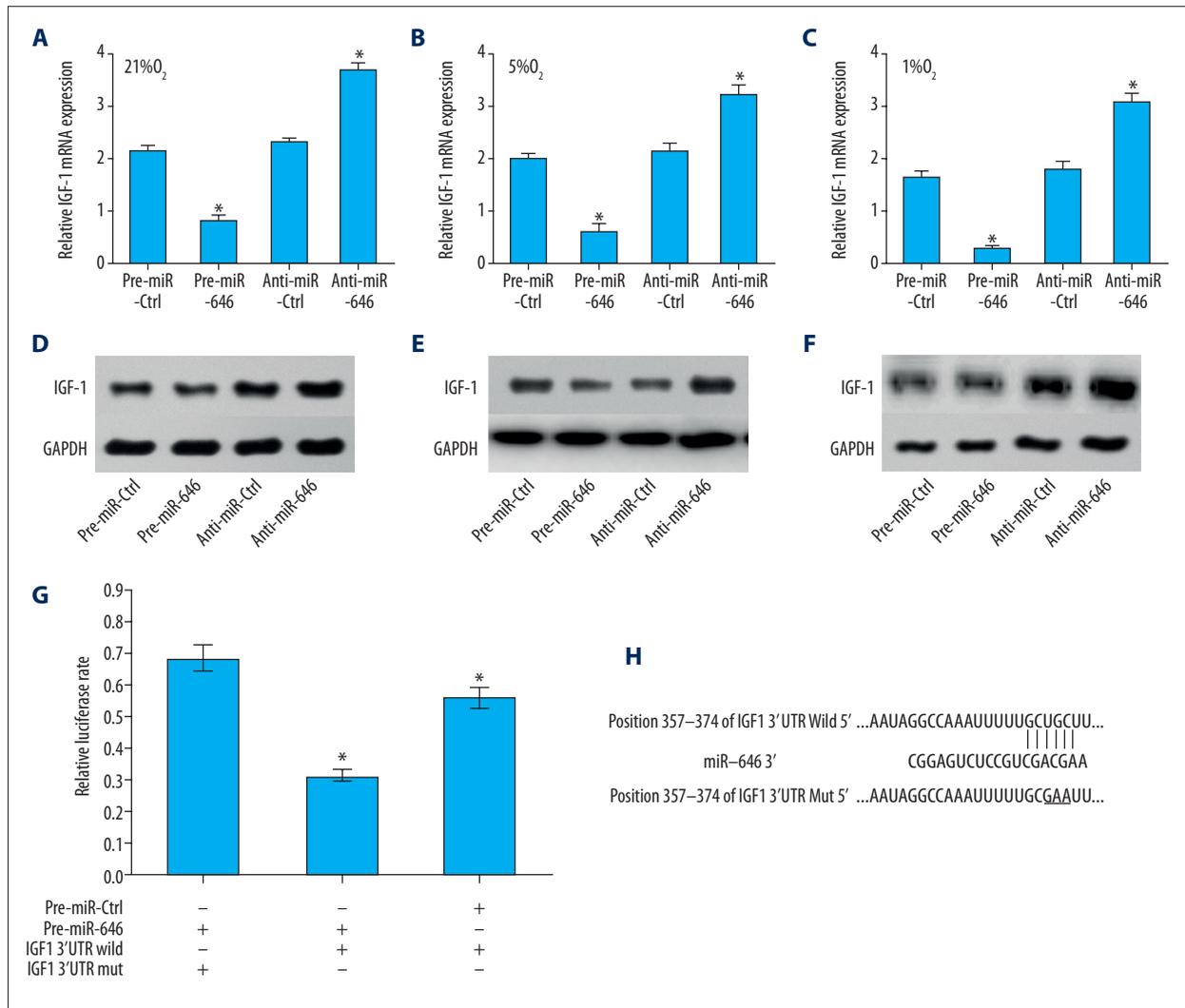


Figure 5. MiR-646 inversely regulated IGF-1 mRNA and protein expression. The mRNA expression of IGF-1 was upregulated with miR-646 downregulation and downregulated with miR-646 overexpression in (A) 21% O₂ condition (* $P=0.03$ pre-miR-646 vs. pre-miR-Ctrl, * $P=0.02$ anti-miR-646 vs. anti-miR-Ctrl); (B) 5% O₂ condition (* $P=0.03$ Pre-miR-646 vs. pre-miR-Ctrl, * $P=0.02$ anti-miR-646 vs. anti-miR-Ctrl); and (C) 1% O₂ condition (* $P=0.02$ pre-miR-646 vs. pre-miR-Ctrl, * $P=0.02$ anti-miR-646 vs. anti-miR-Ctrl). The results of western blot showed that miR-646 significantly repressed IGF-1 protein expression in (D) 21% O₂ condition; (E) 5% O₂ condition; and (F) 1% O₂ condition. (G) The direct binding between miR-646 and 3'-UTR of IGF-1 was detected by luciferase activity assay. The wild-type or mutated 3'-UTR of IGF-1 containing binding sites in luciferase reporters was transfected into hPDLs with miR-646 overexpression or negative control (* $P=0.02$ pre-miR-646 vs. pre-miR-Ctrl). (H) The predicted miR-646-binding site in the IGF-1 3'-UTR.

ROS production, which is closed with PI3K/Akt/mTOR signaling pathway [37]. In this study, we found the expression of IGF-1 was increased in hypoxia conditions, indicating that IGF-1 play a critical role in hypoxia conditions in hPDLs. Nevertheless, the underlying mechanisms of IGF-1 and hypoxia in hPDLs remain to be elucidated.

We further explored the interaction between miR-646 expression and IGF-1 expression in hypoxia conditions. Previous studies have reported on the association between microRNAs and IGF-1. For example, Ho et al. found that miR-181d exhibited

an inverse correlation with IGF-1 in gliomas [38]. Wang et al. reported that miR-422a was downregulated in glioma tissues and was negatively correlated with the expression levels of IGF1/IGF1R [39]. Li et al. demonstrated that miR-379 plays an important role in regulating vascular smooth muscle cells proliferation, invasion, and migration by targeting IGF-1 [40]. Budzinska et al. found that miR-96, miR-145, and expression were increased while IGF-1R was decreased in peripheral blood mononuclear cells of aging humans. The age-associated higher expression of miR-96 and miR-145 might contribute to the lower expression of IGF-1R [41]. In this study, we first

investigated the relationship between miR-646 and IGF-1, revealing that IGF-1 was the direct target of miR-646 and there was a converse relationship between them. However, further investigations are needed to explore whether miR-646 regulates proliferation and apoptosis of hPDLs in hypoxia conditions through directly targeting on IGF-1.

In addition, there are different new treatments for periodontitis. Recent Isola et al. study showed that diode laser therapy, and changes in microbial and inflammatory mediators are closely related to the efficacy of invasive periodontitis; also scaling and root planning (SRP) plus the desiccant resulted in a greater reduction in clinical, microbial, and inflammatory mediators compared to SRP alone [42,43]. It has been suggested that microRNAs (miRNAs) are involved in the immune regulation of periodontitis. Recent research shows miR-146a inhibits inflammatory cytokine production in B cells through directly targeting IRAK1, suggesting a regulatory role of miR-146a in B cell-mediated periodontal inflammation [44].

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Conclusions

We identified a significant role played by miR-646 in hPDLs in that it suppressed cell proliferation and promoted apoptosis by inversely regulating IGF-1 expression. Meanwhile, hypoxia may regulate hPDLs proliferation and apoptosis via the miR-646/IGF-1 signaling pathway, as such, interference with miR-646 may be a target for the treatment of periodontal disease.

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Conflicts of interest

None.

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