Comparison of HIF1A-AS1 and HIF1A-AS2 in regulating HIF-1α and the osteogenic differentiation of PDLCs under hypoxia

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Abstract. Hypoxia-inducible factor-1α (HIF-1α) is essential for regulating the osteogenic differentiation of periodontal ligament cells (PDLCs). The regulatory mechanism of HIF-1α transcription is still not clear. Recently, two long non-coding RNAs, HIF1A antisense RNA 1 (HIF1A-AS1) and HIF1A antisense RNA 2 (HIF1A-AS2), were found to regulate HIF-1α mRNA, but the regulatory mechanisms among HIF-1α, HIF1A-AS1 and HIF1A-AS2 have not been well studied. We hypothesized that HIF1A-AS1 and HIF1A-AS2 play important roles in the osteogenic differentiation of PDLCs by regulating HIF-1α. In the present study, we showed that expression levels of HIF1A-AS1, HIF1A-AS2, HIF-1α and osteogenic biomarkers were time-dependent under hypoxia. Even though both HIF1A-AS1 and HIF1A-AS2 were complementary to HIF-1α mRNA, only HIF1A-AS2 showed an inhibitory effect on HIF-1α in PDLCs. Moreover, HIF-1α had positive regulatory effects on HIF1A-AS1 and HIF1A-AS2. HIF-1α promoted the osteogenic differentiation of PDLCs, and HIF1A-AS2 had a negative effect on the osteogenic differentiation of PDLCs. Altogether, the present study revealed the complex relationships among HIF1A-AS1, HIF1A-AS2 and HIF-1α, as well as their roles in regulating the osteogenic differentiation of PDLCs. These findings provide a theoretical basis for promoting periodontal tissue regeneration and repair during orthodontic tooth movement.

Introduction

Periodontitis and malocclusion along with caries are significant public oral diseases with extremely high incidence

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rates. Epidemiologic studies show that more than 50% of adults suffer from periodontitis and severe periodontitis is estimated to occur in approximately 5-20% of adults worldwide (1-3). The prevalence of malocclusion among children is also more than 50%. These two oral diseases are major public health issues that require attention. Periodontal ligament (PDL) is a complex tissue with abundant blood vessels and cells, including periodontal ligament cells (PDLCs), periodontal ligament stem cells, fibroblasts, osteoblasts and osteoclasts, which play a vital role in periodontal tissue regeneration (4). Periodontitis and orthodontic tooth movement can cause hypoxia around the PDL, which triggers a series of molecular responses in PDLCs for hypoxic adaptation (4). The most sensitive and important molecule is functional hypoxia-inducible factor-1 (HIF-1), composed of HIF-α (HIF-1α, HIF-2α and HIF-3α) and HIF-β subunits. HIF-1α is the dominant functional subunit that is unstable and tends to be degraded under normoxia, but it can be highly increased in short-term hypoxia (5,6). After translocating into the nucleus and dimerizing with HIF-β, HIF-1α binds to hypoxia response elements (HREs) of >100 genes and regulates their production, such as vascular endothelial growth factor (VEGF), transforming growth factor-β, erythropoietin (EPO) and microRNAs (7,8). Meanwhile, HIF-1α has been confirmed to regulate osteogenic differentiation, yet there is still controversy over whether HIF-1α promotes or inhibits osteogenic differentiation (9-12).

Since HIF-1α was discovered, its regulatory mechanism has aroused much interest. It is known that HIF-1α protein can be rapidly degraded under normoxia by the Von Hippel-Lindau protein-mediated ubiquitin-proteasome pathway. The degradation of HIF-1α protein is regulated by many factors, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (13-16). However, the regulatory mechanism of HIF-1α mRNA has not been well studied. Thrash-Bingham and Tartof (17) firstly named an antisense non-coding RNA as aHIF that originated from the 3' region of the *hif-1α* gene and could bind to the HIF-1α mRNA 3' untranslated region (UTR), and aHIF was also named as HIF1A antisense RNA 2 (HIF1A-AS2) or 3'αHIF-1α. Then in 2010, Baranello *et al* (18) identified another antisense non-coding RNA that originated from the 5' region of the *hif-1α* gene, named HIF1A-AS1 or 5'aHIF-1α. Both HIF1A-AS1 and HIF1A-AS2 are longer than 200 nt and belong to the family

of long non-coding RNAs (lncRNAs). Yet, they are different in structure and loci. HIF1A-AS1 has both 5'cap and poly (A+) tail, while HIF1A-AS2 has neither of them. HIF1A-AS1 accumulates at the nuclear membrane, while HIF1A-AS2 locates only in the nucleus (19).

HIF1A-AS2 can downregulate HIF-1α mRNA so that during long-term hypoxia HIF-1α protein is suppressed (20). Moreover, the putative HIF-1α protein binding sites-HREs are found in the HIF1A-AS2 promoter region by analyzing its RNA sequence, which indicates that HIF-1α might also regulate HIF1A-AS2, but this is not yet confirmed (21). Overall, HIF1A-AS2 and HIF-1α have a complicated regulatory mechanism. For the first time, we demonstrated that HIF1A-AS1 and HIF1A-AS2 exist in PDLCs. We then explored the differences between HIF1A-AS1 and HIF1A-AS2 in regulating HIF-1α and the osteogenic differentiation of PDLCs under hypoxia. Runt-related transcription factor 2 (Runx2) is a key factor initiating and regulating the early osteogenesis and late mineralization of bone (22). Alkaline phosphatase (ALP) activity can also describe the early cell differentiation of osteoblastic cells (23). Therefore, Runx2 and ALP activity were selected as the osteogenic biomarkers in the present study. Given that the osteogenic differentiation mechanism in PDLCs is essential to periodontal tissue regeneration, the present research can provide a theoretical basis for promoting periodontal tissue remodeling, regeneration and repair during orthodontic tooth movement and periodontitis.

Materials and methods

Bioinformatic analysis. UCSC Genome Bioinformatics (http://genome.ucsc.edu/) was used to locate the HIF-1α, HIF1A-AS1 and HIF1A-AS2 genes, as well as to obtain HIF-1α mRNA, HIF1A-AS1 RNA and HIF1A-AS2 RNA sequences. In addition, we used the basic local alignment search tool to obtain the complementary regions between HIF1A-AS1 and HIF-1α mRNA, as well as HIF1A-AS2 and HIF-1α mRNA. Furthermore, we examined the promoter regions of HIF1A-AS1 and HIF1A-AS2 to determine whether they have putative HRE sequences.

Sample collection and cell culture. Healthy premolars or third-molars were collected from patients (<25 years of age) at the Hospital of Stomatology, Sun Yat-sen University. Informed consent from each patient was obtained, and the present study was approved by the Ethics Committee of the Hospital of Stomatology, Sun Yat-sen University. PDL was separated from the middle-third of the root surface, washed with phosphate-buffered saline (PBS; Life Technologies, Grand Island, NY, USA), digested using type I collagenases (Life Technologies), and then cultured in primary culture medium containing 78% Modified Eagle's Medium α (MEMα), 20% fetal bovine serum (FBS) (both from Gibco BRL, Gaithersburg, MD, USA), and 2% antibiotics. Cells passaged to P_4 were used in the following experiments. For the hypoxia group, cells were incubated in a three-air hypoxia chamber (Galaxy 170R; Eppendorf Co., Ltd., Hamburg, Germany) with 2% O_2, 5% CO_2 and 93% N_2 at 37˚C, while the normoxia group was incubated in a chamber with 20% O_2,

Table I. Primer sequences used in this study.

Gene name	Primer sequence (forward and reverse)	Product size (bp)
HIF1A-AS1 (F)	5'-AGGCAGAGACGAGATGAACA-3'	100
HIF1A-AS1 (R)	5'-AGGCAGAGACGAGATGAACA-3'	
HIF1A-AS1 (RT)	5'-CTGGGTCTGGCCATTTCATT-3'	
HIF1A-AS2 (F)	5'-GACCTAAGGCTCTGGCACTT-3'	80
HIF1A-AS2 (R)	5'-CACTATGAATCCCTGCACCT 3'	
HIF1A-AS2 (RT)	5'-CACTATGAATCCCTGCACCT-3'	
U6 F	5'-CTCGCTTCGGCAGCACA-3'	94
U6 R	5'-AACGCTTCACGAATTTGCGT-3'	
U6 RT	5'-AACGCTTCACGAATTTGCGT-3'	
HIF-1α	5'-GTGGATTACCACAGCTGA-3'	115
	5'-GCTCAGTTAACTTGATCCA-3'	
Runx2	5'-TCTAAATCGCCAGGCTTCAT-3'	250
	5'-GAGGACCTACTCCCAAAGGA-3'	
β-actin	5'-TGGATCAGCAAGCAGGAGTA-3'	275
	5'-TCGGCCACATTGTGAACTTT-3'	

HIF1A-AS1, HIF1A antisense RNA 1; HIF1A-AS2, HIF1A antisense RNA 2; HIF-1α, hypoxia-inducible factor-1α; Runx2, runt-related transcription factor 2.

5% CO_2 and 75% N_2 at 37˚C (Shellab 2323-2; Shellab, Cornelius, OR, USA). Each group was divided into four subgroups (6, 12, 24 and 48 h).

Reverse transcription-quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Total RNA was extracted from PDLCs using TRIzol reagent (Life Technologies) at each time point. The quality and quantity of RNA were measured. Total RNA (1.0 μg) was reverse transcribed for cDNA synthesis (ImProm-II™ Reverse Transcription system; Promega Corp., Madison, WI, USA). Then cDNA was mixed with 2X SYBR-Green master mix (Life Technologies) and gene-specific primers in a final volume of 20 μl, and the real-time PCR was performed on the LightCycler® 480 platform (Roche, Basel, Switzerland) with the following reaction: 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 32 sec. The primers used in the present study are listed in Table I. U6 snRNA was used to normalize the expression level of HIF1A-AS1 and HIF1A-AS2, and β-actin was used to normalize the expression level of HIF-1α and Runx2.

Western blot analysis. PDLCs under 2% and 20% O_2 at each time point were lysed using lysis buffer (99% RIPA, 1% PMSF) and the protein concentration was determined by a bicinchoninic acid (BCA) protein assay kit (CWBio, Co., Ltd., Beijing, China). Equal amount of protein was separated by electrophoresis on duplicate 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, then transferred to polyvinylidene fluoride membranes and incubated with primary antibodies, mouse anti-human HIF-1α (1:1,000 dilution; cat. no. ab113642; Abcam, Cambridge, MA, USA) and

Table II. Sequences of the siRNAs used in this study.

Gene name/ siRNA	Sequences (5'→3')	Size (bp)
HIF1A-AS1		
siRNA1-sense	AUGGCCAGACCCAGAUGUUdTdT	21
siRNA1-antisense	AACAUCUGGGUCUGGCCAUdTdT	
siRNA2-sense	GCUCCAGAACGCAGAGGAAdTdT	21
siRNA2-antisense	UUCCUCUGCGUUCUGGAGCdTdT	
HIF1A-AS2		
siRNA1-sense	CUUAAAUUGUUGGUAAACAdTdT	21
siRNA1-antisense	UGUUUACCAACAAUUUAAGdTdT	
siRNA2-sense	GUAACAUUGUGACUAUAAUdTdT	21
siRNA2-antisense	AUUAUAGUCACAAUGUUACdTdT	
HIF-1α		
siRNA1-sense	CAAAGUUCACCUGAGCCUAdTdT	21
siRNA1-antisense	UAGGCUCAGGUGAACUUUGdTdT	
siRNA2-sense	GAUUAACUCAGUUUGAACUdTdT	21
siRNA2-antisense	AGUUCAAACUGAGUUAAUCdTdT	
NC		
siRNA-sense	UUCUCCGAACGUGUCACGUTT	21
siRNA-antisense	ACGUGACACGUUCGGAGAATT	

siRNA, small interfering RNA; HIF1A-AS1, HIF1A antisense RNA 1; HIF1A-AS2, HIF1A antisense RNA 2; HIF-1α, hypoxia-inducible factor-1α; NC, non-specific control.

Figure 1. Bioinformatic analysis of HIF-1α, HIF1A-AS1 and HIF1A-AS2 gene and RNA. (A) Relative gene positions of human HIF-1α, HIF1A-AS1 and HIF1A-AS2. (B) Complementary regions and sequences of HIF1A-AS1 with HIF-1α mRNA, as well as HIF1A-AS2 with HIF-1α mRNA. HIF-1α, hypoxia-inducible factor-1α; HIF1A-AS1, HIF1A antisense RNA 1; HIF1A-AS2, HIF1A antisense RNA 2.

rabbit anti-human Runx2 (1:1,000 dilution; cat. no. 12556; Cell Signaling Technology, Inc., Danvers, MA, USA), for 24 h at 4˚C. After washing, the membranes were incubated with the corresponding secondary antibodies (anti-mouse IgG, cat. no. M281; anti-rabbit IgG, cat. no. M283; both from Takara Bio, Inc., Otsu, Japan) for 1 h at 37˚C. Chemiluminescence was imaged and then the band intensity was quantified by ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA]. The relative protein levels were calculated as the ratio to the level of β-actin.

ALP activity. PDLCs were cultured in 6-well plates in 2 or 20% O_2. Cells were lysed by Triton X-100 (1% Triton X-100, 99% PBS). The protein concentration was determined using the BCA protein assay kit. The supernatant was then used for the ALP assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the absorbance was measured at a wavelength of 520 nm. The ALP activity was determined according to the manufacturer's instructions.

Transfection of small interfering RNAs (siRNAs). siRNAs targeting HIF1A-AS1, HIF1A-AS2, HIF-1α and non-specific control siRNAs (NC si) were designed by Sigma (St. Louis, MO, USA). NC si was used as a negative control. The sequences are listed in Table II. siRNA was dissolved to a concentration of 20 μM, and then HIF1A-AS1 siRNA, HIF1A-AS2 siRNA and HIF-1α siRNA (5 μl) were transfected using Lipofectamine™ RNAiMAX into PDLCs, respectively. In addition, HIF1A-AS1 siRNA (2.5 μl) and HIF1A-AS2 siRNA
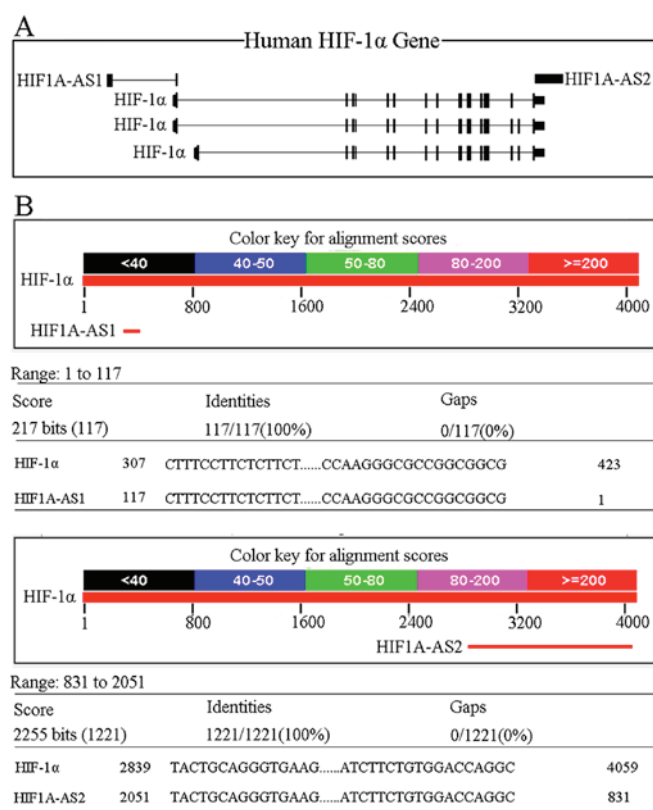
(2.5 μl) were transfected at the same time to knock down both HIF1A-AS1 and HIF1A-AS2. After transfection for 48 h, PDLCs were cultured under hypoxia for 12 h, and then total RNA and protein were collected for RT-qPCR, western blot analysis and ALP activity.

Statistical analysis. Statistical analysis was carried out using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). All of the results are expressed as the mean ± SEM. Data for the groups were compared by two-tailed Student's t-test and the significant level was set at P<0.05.

Results

Bioinformatic analysis of HIF-1α, HIF1A-AS1 and HIF1A-AS2. Gene locations for HIF1A-AS1 and HIF1A-AS2 in the human were from chr14:61681041-61695823 and chr14:61747039-61749089. HIF-1α had three transcripts, in which transcript 1 and 2 were from chr14:61695401-61748259 and transcript 3 was from chr14:61697622-61748259. The relative locations of HIF-1α, HIF1A-AS1 and HIF1A-AS2 are shown in Fig. 1A.

The sizes of HIF1A-AS1 mRNA and HIF1A-AS2 mRNA were 652 and 2,051 nt. As shown in Fig. 1B, HIF1A-AS1 was complementary upon 117 nt to the HIF-1α mRNA 5'UTR (HIF1A-AS1: 117 to 1, HIF-1α mRNA: 307 to 423), while HIF1A-AS2 was complementary upon 1,221 nt to
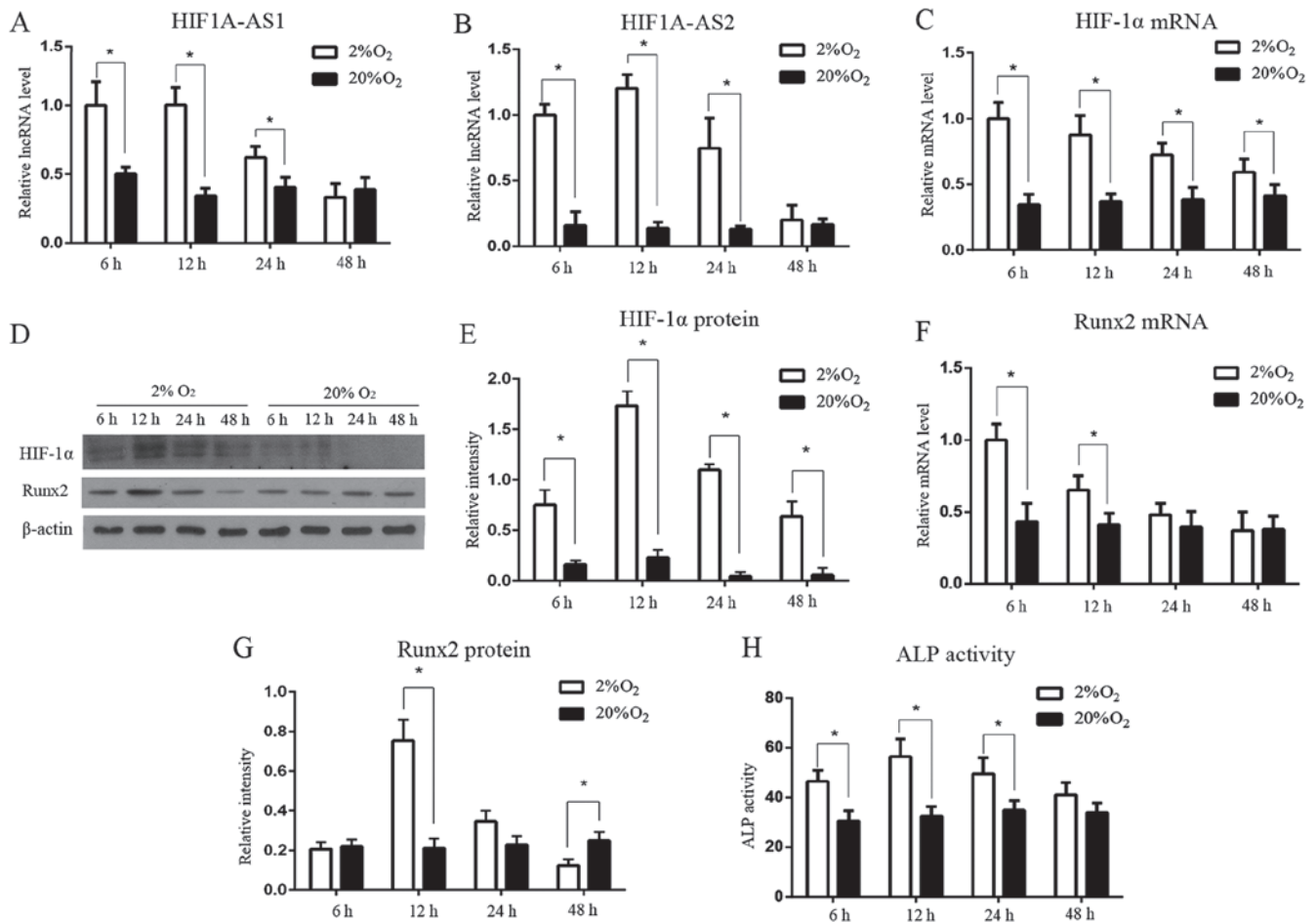
Figure 2. Expression patterns of HIF1A-AS1, HIF1A-AS2, HIF-1α, ALP and Runx2 in PDLCs under hypoxia and normoxia. (A and B) The lncRNA levels of HIF1A-AS1 and HIF1A-AS2 under hypoxia were significantly increased at 6, 12 and 24 h, and peaked at 12 h. (C) The mRNA level of HIF-1α was significantly higher under hypoxia than that under normoxia and gradually decreased from 6 to 48 h. (D and E) The protein expression of HIF-1α under hypoxia was notably higher than that under normoxia, and peaked at 12 h. (F) The mRNA level of Runx2 was induced by hypoxia before 12 h. (D and G) The protein expression of Runx2 under hypoxia was significantly induced at 12 h, and then was suppressed at 48 h. (H) The ALP activity under hypoxia was induced significantly at 6, 12 and 24 h, and peaked at 12 h. *P<0.05 vs. the normoxia group, n=3. HIF1A-AS1, HIF1A antisense RNA 1; HIF1A-AS2, HIF1A antisense RNA 2; HIF-1α, hypoxia-inducible factor-1α; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; PDLCs, periodontal ligament cells.

HIF-1α mRNA 3'UTR (HIF1A-AS2: 2,051 to 831, HIF-1α mRNA: 2,839 to 4,059). Consensus putative HRE sequence was 5'-(A/G)GTG-3', which has been identified in many hypoxia-inducible genes, such as VEGF and EPO (24,25). Commonly, the promoter region was from -2,000 to +200 bp from the RNA transcription initiation site. After searching for HIF1A-AS1 and HIF1A-AS2 promoter, we identified several putative HREs within the regions (HIF1A-AS1: -194, -492 and -661, HIF1A-AS2: -63 and -600), indicating that HIF-1α may also regulate the two long non-coding RNAs.

HIF1A-AS1, HIF1A-AS2, HIF-1α expression and the osteogenic differentiation of PDLCs under hypoxia at different times. Both HIF1A-AS1 and HIF1A-AS2 were expressed in the PDLCs. Hypoxia increased the level of HIF1A-AS1 to 2.00-fold, 2.26-fold and 1.53-fold (Fig. 2A) and HIF1A-AS2 to 6.33-fold, 8.77-fold and 5.79-fold (Fig. 2B) at 6, 12 and 24 h (P<0.05) in the PDLCs when compared with levels in the normoxia groups. Both HIF1A-AS1 and HIF1A-AS2 began to decrease under hypoxia after 12 h.

HIF-1α mRNA (Fig. 2C) was significantly upregulated at 6 h, under hypoxia (P<0.05), but it decreased gradually from

6 to 48 h. Meanwhile, HIF-1α protein (Fig. 2D and E) was barely detectable under normoxia, but it was sharply induced under hypoxia from 6 h, peaked at 12 h, then gradually diminished from 12 to 48 h, yet still higher than the normoxia groups (P<0.05).

The expression level of Runx2 mRNA (Fig. 2F) under hypoxia was also higher than the level in the normoxia groups at 6 and 12 h (P<0.05), then it gradually decreased to the baseline at 24 and 48 h (P>0.05). Runx2 protein (Fig. 2D and G) was notably induced at 12 h under hypoxia, peaked at 12 h, and was suppressed at 48 h when compared with the normoxia group (P<0.05). ALP activity (Fig. 2H) was facilitated significantly under hypoxia at 6, 12 and 24 h (P<0.05), reaching a peak at 12 h, but showing no significant difference at 48 h (P>0.05).

Silencing efficiency of HIF1A-AS1, HIF1A-AS2 and HIF-1α. To further investigate the regulatory mechanism among HIF1A-AS1, HIF1A-AS2 and HIF-1α, we silenced HIF1A-AS1, HIF1A-AS2 and HIF-1α. The siRNAs of HIF1A-AS1, HIF1A-AS2, HIF-1α and NC were successfully transfected into PDLCs. The transfection of NC si is
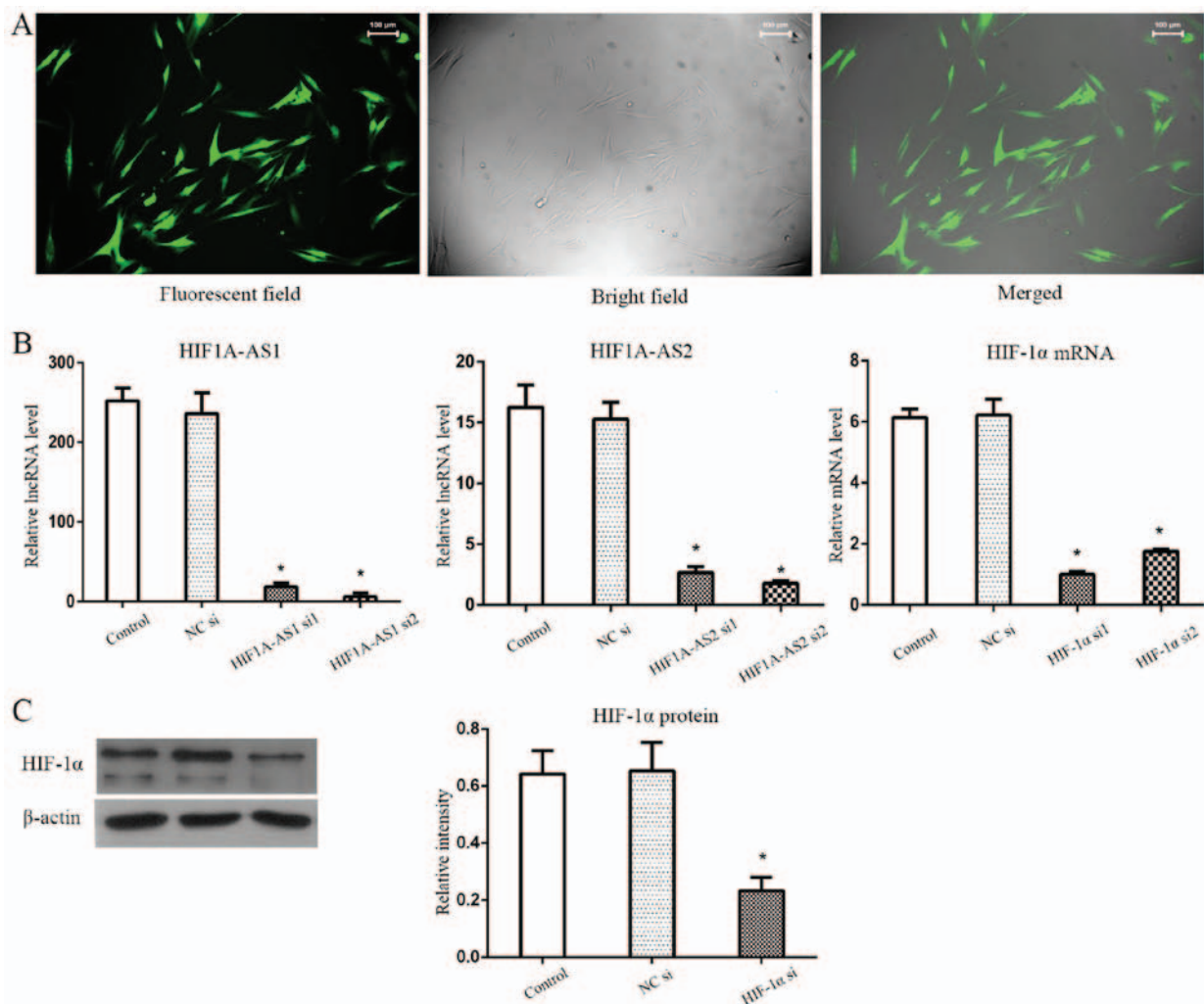
Figure 3. Silencing efficiencies of HIF1A-AS1, HIF1A-AS2 and HIF-1α siRNAs. (A) NC si shown as an example was successfully transfected into PDLCs. (B) HIF1A-AS1, HIF1A-AS2 and HIF-1α mRNA were effectively reduced by >90, 80 and 70% in PDLCs under hypoxia at 12 h, respectively. (C) HIF-1α protein was significantly inhibited by HIF-1α siRNA in hypoxia. *P<0.05 vs. the control group, n=3. HIF1A-AS1, HIF1A antisense RNA 1; HIF1A-AS2, HIF1A antisense RNA 2; HIF-1α, hypoxia-inducible factor-1α; NC si, non-specific control siRNA; PDLCs, periodontal ligament cells.

shown in Fig. 3A. Knockdown efficiencies were detected by RT-qPCR for HIF1A-AS1, HIF1A-AS2 and HIF-1α mRNA, and western blot analysis for HIF-1α protein. Results showed that the silencing efficiencies of HIF1A-AS1, HIF1A-AS2 and HIF-1α mRNA (Fig. 3B) were >90, 80 and 70% respectively. Furthermore, HIF-1α protein (Fig. 3C) was also successfully inhibited by HIF-1α siRNA under hypoxia.

Effects of silencing HIF1A-AS1 and HIF1A-AS2 on HIF-1α in PDLCs. Changes in HIF-1α mRNA (Fig. 4A) and HIF-1α protein (Fig. 4B and C) were consistent following the silencing of HIF1A-AS1 and/or HIF1A-AS2. Silencing of HIF1A-AS1 did not significantly alter the expression of HIF-1α protein and mRNA, whereas silencing of HIF1A-AS2 significantly induced the expression of HIF-1α mRNA and protein. In order to determine whether HIF1A-AS1 and HIF1A-AS2 had synergistic effects on HIF-1α expression and osteogenic differentiation, we knocked down HIF1A-AS1 and HIF1A-AS2 by co-transfecting PDLCs with HIF1A-AS1 siRNA and HIF1A-AS2 siRNA. Results showed that HIF-1α protein and mRNA were significantly elevated, similar to silencing HIF1A-AS2 alone.

Effects of silencing HIF-1α on HIF1A-AS1 and HIF1A-AS2 in PDLCs. Since putative HRE sequences were identified within the promoter regions of HIF1A-AS1 and HIF1A-AS2, we aimed to ascertain whether HIF-1α regulates HIF1A-AS1 and HIF1A-AS2 by silencing HIF-1α. Results demonstrated that both HIF1A-AS1 (Fig. 4D) and HIF1A-AS2 (Fig. 4E) were significantly inhibited by HIF-1α siRNA (P<0.05), indicating that HIF-1α acts as an upstream regulatory factor of HIF1A-AS1 and HIF1A-AS2.

Effects of the silencing of HIF-1α, HIF1A-AS1 and HIF1A-AS2 on the osteogenic biomarkers of PDLCs. To further investigate the effects of HIF-1α, HIF1A-AS1 and HIF1A-AS2 on the osteogenic differentiation of PDLCs, we investigated the changes in Runx2 mRNA, Runx2 protein and ALP activity following the silencing of HIF-1α, HIF1A-AS1 and HIF1A-AS2 under hypoxia. The levels of osteogenic biomarkers (Fig. 5), Runx2 protein, Runx2 mRNA and ALP activity, were significantly inhibited by the silencing of HIF-1α and induced by the silencing of HIF1A-AS2 and co-silencing of HIF1A-AS1 and HIF1A-AS2 (P<0.05), while there were no significant changes following the silencing of HIF1A-AS1 alone (P>0.05).
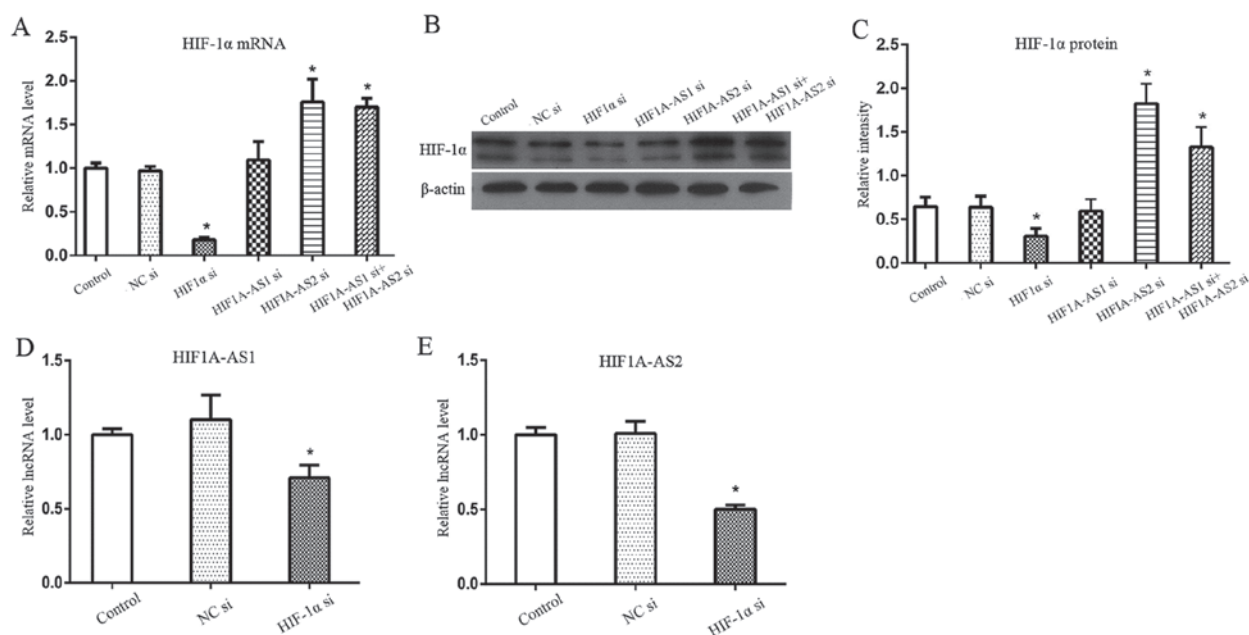
Figure 4. Effects of the silencing of HIF1A-AS1, HIF1A-AS2 and HIF-1α on the expression of each other in hypoxia. (A) HIF-1α mRNA showed no signifi-cant change following the silencing of HIF1A-AS1 in hypoxia, while it was significantly induced following the silencing of HIF1A-AS1 or co-silencing of HIF1A-AS1 and HIF1A-AS2. (B and C) Changes in HIF-1α protein were consistent with that of HIF-1α mRNA following the silencing of HIF1A-AS1 and HIF1A-AS2 in hypoxia. (D and E) HIF1A-AS1 and HIF1A-AS2 were significantly suppressed following the silencing of HIF-1α in hypoxia. *P<0.05 vs. the control group, n=3. HIF1A-AS1, HIF1A antisense RNA 1; HIF1A-AS2, HIF1A antisense RNA 2; HIF-1α, hypoxia-inducible factor-1α.
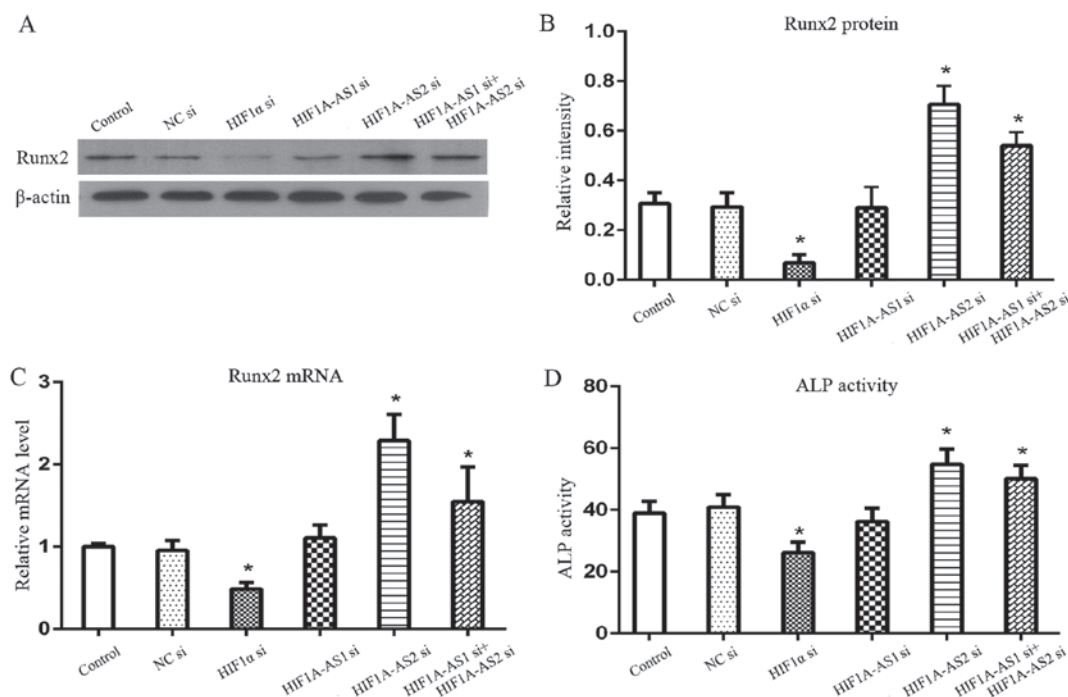

Figure 5. Effects of the silencing of HIF1A-AS1, HIF1A-AS2 and HIF-1α on the expression of osteogenic biomarkers in PDLCs in hypoxia. (A and B) Silencing of HIF-1α significantly inhibited Runx2 protein under hypoxia. Silencing of HIF1A-AS1 or co-silencing of HIF1A-AS1 and HIF1A-AS2 obviously promoted Runx2 protein in hypoxia, while silencing of HIF1A-AS2 alone did not significantly alter Runx2 protein. (C) Changes in the Runx2 mRNA level under different treatment conditions in hypoxia. (D) Changes in ALP activity under different treatment conditions in hypoxia. *P<0.05 vs. the control group, n=3. HIF1A-AS1, HIF1A antisense RNA 1; HIF1A-AS2, HIF1A antisense RNA 2; HIF-1α, hypoxia-inducible factor-1α; PDLCs, periodontal ligament cells; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase.

Discussion

The functions of HIF1A-AS1 and HIF1A-AS2 have not been studied in PDLCs. HIF1A-AS1 and HIF1A-AS2 play key roles

in cell proliferation and apoptosis, and could be used as predic-tive biomarkers for cancers (26-29). However, little is known concerning HIF1A-AS1 and HIF1A-AS2 in periodontal tissues under hypoxia. Thus, we explored the complex relationships

among HIF1A-AS1, HIF1A-AS2 and HIF-1α and investigated whether they regulate the osteogenic differentiation of PDLCs.

In the present study, we discovered that the two antisense lncRNAs, HIF1A-AS1 and HIF1A-AS2, were expressed in PDLCs. Our present findings showed that the expression levels of HIF1A-AS1, HIF1A-AS2, HIF-1α and osteogenic biomarkers were altered in a temporal manner under hypoxia. Both HIF1A-AS1 and HIF1A-AS2 were expressed at low levels under normoxia, but they were obviously induced in short-term hypoxia, reaching a peak at 12 h. Increased HIF1A-AS2 under hypoxia was found in the first few hours by researchers in various cancer cells (17,30). The expression patterns of HIF-1α protein and the osteogenic markers (Runx2 and ALP activity) were consistent with that of HIF1A-AS1 and HIF1A-AS2. HIF-1α mRNA was highly expressed under hypoxia and then declined gradually after 6 h. In addition, Huang *et al* (31) also observed that HIF-1α mRNA was elevated at 1 h and peaked at 6 h under hypoxia in human mesenchymal stem cells (hMSCs), and then the level was gradually declined from 6 to 24 h. Uchida *et al* (20) found that HIF-1α protein was highly induced during the first few hours in hypoxia, regulated by translation or post-translation pathways, and inhibited in prolonged hypoxia due to the decline in HIF-1α mRNA. With respect to the osteogenic differentiation ability under hypoxia, Wu *et al* (32) demonstrated that Runx2 mRNA and protein were immediately enhanced from 1 h and ALP activity was from 3 h in PDLSCs when exposed to hypoxia, and they still remained higher than the normoxia group at 24 h. Furthermore, Ding *et al* (33) found that continuous hypoxia after 3 days impaired the osteogenenic differentiation of hMSCs in hypoxia. Therefore, the above studies indicated that HIF1A-AS1, HIF1A-AS2, HIF-1α and the osteogenic biomarkers were altered but time-dependently under hypoxia. Our research revealed that HIF1A-AS1, HIF1A-AS2 and HIF-1α might have important roles in regulating the osteogenenic differentiation of PDLCs under hypoxia.

Furthermore, we investigated whether both HIF1A-AS1 and HIF1A-AS2 participated in regulating HIF-1α mRNA by silencing HIF1A-AS1 and HIF1A-AS2. Silencing of HIF1A-AS2 prominently increased HIF-1α mRNA and protein, whereas the silencing of HIF1A-AS1 did not affect HIF-1α mRNA and HIF-1α protein, which indicated that only HIF1A-AS2 has a strong negative regulatory function on HIF-1α in PDLCs. The increase in HIF-1α was slightly lower following co-transfection of HIF1A-AS1 siRNA and HIF1A-AS2 siRNA than following transfection of HIF1A-AS2 siRNA alone, which might be that the amount of HIF1A-AS2 siRNA was half when co-transfecting HIF1A-AS1 siRNA (2.5 μl) and HIF1A-AS2 siRNA (2.5 μl). In previous studies, HIF1A-AS2 has been reported to be complementary on at least 1,027 nt of the HIF-1α mRNA 3'UTR which was rich in AU elements and HIF1A-AS2 could expose these AU rich elements to accelerate the degradation of HIF-1α mRNA (21). Kumar and Carmichael (34) speculated that HIF1A-AS2 suppressed HIF-1α mRNA translation by hybridizing to HIF-1α mRNA 3'UTR, therefore HIF-1α protein was suppressed. Our bioinformatic analysis revealed that both HIF1A-AS1 and HIF1A-AS2 could be strictly complementary to HIF-1α mRNA, in which HIF1A-AS1 was complementary upon 117 nt and HIF1A-AS2 was 1,221 nt. In addition, our experiment *in vitro* proved that only HIF1A-AS2 significantly downregu-

lated HIF-1α. By analyzing the regulatory mechanism of the HIF-system in human macrophages, elevated HIF-1α mRNA and protein were also observed when silencing HIF1A-AS2, which was in accordance with our results (35). Bertozzi *et al* (19) also reported that HIF1A-AS1 and HIF1A-AS2 had different responses to different stimulations. Since HIF1A-AS1 accumulates at the nuclear membrane, it might be associated with the export of mRNA from the nucleus into the cytoplasm. Therefore, we predicted that HIF1A-AS1 and HIF1A-AS2 may be involved in different types of regulatory mechanisms.

HIF-1 was speculated as the upstream factor of the antisense HIF-1α. Some evidence was provided by Uchida *et al* (20) to support this hypothesis. i) Cycloheximide, a protein synthesis inhibitor that restrains hypoxia-induced HIF-1α increase, also inhibited the augmentation of HIF1A-AS2 during long-term hypoxia; ii) HIF-1α was found to be bound to the putative HRE sequence, which could be displaced by the oligonucleotide sequence of HRE found in human HIF1A-AS2 gene promoter, not by a mismatch of putative HRE sequence. Moreover, in embryonic cells lacking the HIF-β subunit where HIF-1 could not be formed, hypoxia-induced HIF1A-AS2 was attenuated, indicating that HIF1A-AS2 was HIF-1 responsive (36,37). Consistently, the HIF1A-AS1 promoter also has putative HREs. To further investigate the effects of HIF-1α on HIF1A-AS1 and HIF1A-AS2, we silenced HIF-1α, and the results showed that HIF1A-AS1 and HIF1A-AS2 were downregulated, which confirmed that HIF-1α has a positive regulatory effect on both HIF1A-AS1 and HIF1A-AS2. This was also consistent with the observation that HIF1A-AS1 and HIF1A-AS2 were decreased after 12 h under hypoxia accompanied by the decline of HIF-1α protein.

HIF1A-AS1 and HIF1A-AS2 also had different roles in regulating the osteogenic differentiation of PDLCs under hypoxia, which might be related to HIF-1α. Inhibition of HIF-1α significantly suppressed osteogenic biomarkers, which demonstrated that HIF-1α positively regulated the osteogenic differentiation ability of PDLCs. Typically, HIF-1α-induced downstream proteins respond rapidly, usually occurring during the first several hours (17,38). Zhou *et al* (32,39) found that hypoxia-simulated osteogenesis was via ERK1/2 and p38-mediated HIF-1α pathway, in which the ERK1/2 pathway was the dominant one. ERK1/2 and p38, members of the MAPKs, were found to be involved not only in maintaining the stability and promoting the transactivation of HIF-1α, but also in regulating osteogenic differentiation. Furthermore, we found that silencing of HIF1A-AS2 significantly promoted the expression of osteogenic biomarkers, whereas silencing of HIF1A-AS1 had no significant effects on the expressions of osteogenic biomarkers. It should be noted that the changes in the osteogenic biomarkers were in accordance with the changes in HIF-1α following the silencing of HIF1A-AS2, since HIF-1α regulated the osteogenic differentiation of PDLCs. Therefore, we speculated that there may be an HIF1A-AS2/HIF-1α signaling pathway through which to regulate the osteogenic differentiation of PDLCs.

In conclusion, we found that both HIF1A-AS1 and HIF1A-AS2 were complementary to HIF-1α mRNA by bioinformatic analysis, but the *in vitro* study revealed that only HIF1A-AS2 had an inhibitory effect on HIF-1α in PDLCs. In addition, HIF-1α also induced HIF1A-AS1 and HIF1A-AS2, which have putative HREs in the promoters. Furthermore,

HIF-1α promoted the osteogenic differentiation of PDLCs and HIF1A-AS2 inhibited the osteogenic differentiation of PDLCs.

Acknowledgements

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