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Molecules and Cells

Hypoxia Inducible Factor-1α Directly Induces the Expression of Receptor Activator of Nuclear Factor-κB Ligand in Periodontal Ligament Fibroblasts

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During orthodontic tooth movement, local hypoxia and enhanced osteoclastogenesis are observed in the compression side of periodontal tissues. The receptor activator of nuclear factor-κB ligand (RANKL) is an osteoblast/stromal cell-derived factor that is essential for osteoclastogenesis. In this study, we examined the effect of hypoxia on RANKL expression in human periodontal ligament fibroblasts (PDLFs) to investigate the relationship between local hypoxia and enhanced osteoclastogenesis in the compression side of periodontal tissues. Hypoxia significantly enhanced the levels of RANKL mRNA and protein as well as hypoxia inducible factor-1α (HIF-1α) protein in PDLFs. Constitutively active HIF-1α alone significantly increased the levels of RANKL expression in PDLFs under normoxic conditions, whereas dominant negative HIF-1α blocked hypoxia-induced RANKL expression. To investigate further whether HIF-1α directly regulates RANKL transcription, a luciferase reporter assay was performed using the reporter vector containing the RANKL promoter sequence. Exposure to hypoxia or overexpression of constitutively active HIF-1α significantly increased RANKL promoter activity, whereas dominant negative HIF-1α blocked hypoxia-induced RANKL promoter activity. Furthermore, mutations of putative HIF-1α binding elements in RANKL promoter prevented hypoxia-induced RANKL promoter activity. The results of chromatin immunoprecipitation showed that hypoxia or constitutively active HIF-1α increased the DNA binding of HIF-1α to RANKL promoter. These results suggest that HIF-1α mediates hypoxia-induced up-regulation of RANKL expression and that in compression side periodontal ligament, hypoxia enhances osteoclastogenesis, at least in part, via an increased RANKL expression in PDLFs.

INTRODUCTION

The periodontal ligament (PDL) is a highly collagenous structure that serves a supportive function by attaching the tooth to the surrounding alveolar bone and a shock-absorbing function by providing resistance to mechanical force (Shuttleworth and Smalley, 1983). Mechanical force-induced orthodontic tooth movement is characterized by remodeling changes in the PDL and alveolar bone. Orthodontic tooth movement exerts different effects on two opposing sides, the compression and tension sides. Bone resorption is dominant on the compression side, whereas bone formation is dominant on the tension side (Cattaneo et al., 2005). Compressive force reduces blood flow in the PDL and alveolar bone, which leads to local hypoxia (Kitase et al., 2009; Kubota, 1989). Orthodontic tooth movement causes compression side PDLs to release various inflammatory mediators such as interleukin-1 (IL-1), tumor necrosis factor-α, IL-6 and prostaglandin E_2 (Kanzaki et al., 2002). These molecules can cause various cellular responses in and around moving teeth, providing a favorable microenvironment for bone resorption and tooth movement. Although these inflammatory mediators are known to stimulate osteoclastogenesis, the underlying mechanisms regulating osteoclastogenesis in compression side PDL tissues have not been fully elucidated. Receptor activator of nuclear factor-κB ligand (RANKL) is an essential factor for osteoclastogenesis that is produced by osteoblasts and bone marrow stromal cells (Wada et al., 2006). RANKL induces osteoclast differentiation from hematopoietic precursors and stimulates the bone-resorbing activity of osteoclasts (Suda et al., 1999; Udagawa et al., 1999). It has been reported that continuous compression up-regulates RANKL expression in rat PDL *in vivo* (Ogasawara et al., 2004). Compressive force significantly increases the expression of RANKL but decreases osteoprotegerin, a decoy receptor for RANKL, in human PDL fibroblasts (PDLFs) cultured *in vitro* in a time- and magnitude-dependent manner (Nishijima et al., 2006). It has also been demonstrated that intermittent force up-regulates

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RANKL expression via IL-1 in human PDLFs (Nakao et al., 2007). The results from these studies suggest that enhanced osteoclastogenesis in compression side PDLs may be partly caused by compressive force-induced RANKL expression in PDLFs. However, the regulatory role of local hypoxia *per se* in RANKL expression in compression side PDLs remains unclear.

Hypoxia activates hypoxia inducible factor 1 (HIF-1), a heterodimeric transcription factor comprised of α (inducible) and β (ubiquitous) subunits (Loboda et al., 2010). Under hypoxic conditions, HIF-1α is stabilized and translocated to the nucleus. In the nucleus, HIF-1α binds to its dimerization partner, HIF-1β, and stimulates the expression of its target genes, such as vascular endothelial growth factor (VEGF). Hypoxia stimulates bone resorption by promoting both the osteoclastic differentiation of hematopoietic precursor cells and the bone-resorbing activity of osteoclasts (Arnett et al., 2003; Knowles and Athanasou, 2009). In addition to direct regulation of osteoclast precursor cells, hypoxia enhances osteoclast differentiation indirectly by increasing the secretion of VEGF and insulin-like growth factor 2 from osteoblasts and non-osteoclastic bone marrow cells, respectively (Fukuoka et al., 2005; Knowles and Athanasou, 2008). However, the regulatory role of hypoxia in RANKL expression remains unknown.

In the present study, we investigated whether hypoxia regulates RANKL expression in human PDLFs. Here we show that hypoxia induces RANKL expression in human PDLFs and that HIF-1α mediates hypoxia-induced RANKL expression by directly binding the RANKL promoter.

MATERIALS AND METHODS

Materials

Alpha-modified Eagle's medium (α-MEM), fetal bovine serum (FBS) and other cultural reagents were obtained from Hyclone (USA). A GasPak™ EZ CO_2 Pouch System was purchased from BD (USA). The easy-BLUE™ and *i-Star*Taq™ reagents and WEST-ZOL (plus) were purchased from iNtRON Biotechnology (Korea). The AccuPower RT-PreMix was from Bioneer (Korea), and the SYBR *Premix Ex Taq™* was from TaKaRa (Japan). The PCR primers were synthesized by CosmoGenetech (Korea). Anti-HIF-1α antibody was purchased from NOVUS (USA), anti-RANKL antibody was from R&D Systems (USA), anti-actin antibody was from Santa Cruz Biotechnology (USA) and HRP-conjugated secondary antibodies were from Thermo Fisher Scientific (USA). LipofectAMINE 2000 reagent was purchased from Invitrogen (USA). The human RANKL ELISA kit pink-ONE was obtained from Koma Biotech (Korea). The Dual-Glo™ luciferase assay system was ordered from Promega (USA).

Cell culture and hypoxic treatment

Human PDLFs (ScienCell™ Research Laboratories, USA) were maintained in α-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. To induce hypoxia, the PDLFs were transferred to a GasPak pouch, where the total oxygen concentration was reduced to less than 1% (Steinbach et al., 2004), and incubated for the periods indicated. To induce HIF-1α, the PDLFs were incubated for 24 h in the presence of desferoxamine (DFO, 200 μM), a high affinity iron chelator (Hamrick et al., 2005). PDLFs were used between passages four to six for the experiments.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Semi-quantitative RT-PCR or quantitative real-time PCR was performed to evaluate mRNA expression. Total RNA was isolated using easy-BLUE™ RNA Extraction Reagents. Complementary DNA was synthesized from 1 μg of total RNA using the AccuPower RT-PreMix and was subsequently used for PCR amplification. Semi-quantitative RT-PCR was performed using *i-Star*Taq™ in the range of linear amplification. The PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The primer sequences used for the RT-PCR were as follows: RANKL-forward (f) 5′-AGAGCG CAGATGGATCCTAA-3′, RANKL-reverse (r) 5′-TTCCTTTTGC ACAGCTCCTT-3′; and GAPDH-f 5′-TGAAGGTCGGAGTCAA CGGATTTGGT-3′, GAPDH-r 5′-CATGTGGGCCATGAGGTCC ACCAC-3′. Real-time PCR was performed using the SYBR *Premix Ex Taq™* and an AB 7500 Fast Real-Time system (Applied Biosystems, USA). Each sample was analyzed in triplicate, and target genes were normalized to GAPDH. Fold differences were then calculated for each treatment group using normalized C_T values for the control. The primer sequences used for the real-time PCR were as follows: RANKL-f 5′-AGAGCGCA GATGGATCCTAA-3′, RANKL-r 5′-TTCCTTTTGCACAGCTCC TT-3′; VEGF-f 5′-GCTGTCTTGGG TGCATTGGA-3′, VEGF-r 5′-ATGATTCTGCCCTCCTCCTTC T-3′; and GAPDH-f 5′-CCATCTTCCAGGAGCGAGATC-3′, GAPDH-r 5′-GCCTTCT CCATGGTGGTGAA-3′.

Plasmid constructs and site-directed mutagenesis

The constitutively active and dominant negative HIF-1α expression plasmids were generous gifts from Prof. J.-W. Park at Seoul National University (Chun et al., 2002). The reporter construct containing the mouse RANKL promoter (RANKL-luc) was prepared as follows: the DNA sequence spanning -2174 to +1 bp was amplified by PCR using the following primers: f 5′-CGAGCTCAGAATGAGGTGGTGGTCTTGCAGAC-3′ and r 5′-CCAAGCTTGGCGCGGCGCCCGGAGTTCG-3′. The amplicons were ligated into the *Sac*I and *Hin*dIII sites of pGL3-Basic to generate RANKL-luc. To produce the function-defective reporter construct (RANKL-MT-luc), which contains mutations in the putative HIF-1α binding site, a site-directed mutagenic PCR was performed at -971 to -968 and -965 to -962 bp (CGTG->AAAA) (Amarilio et al., 2007). The PCR primers used for the site-directed mutagenesis were as follows: f 5′-TGTGCGCG CGCGAAAATGAAAACTTGCGCACATGCCGGAGGA-3′ and r 5′-TCCTCCGGCATGTGCGCAAGTTTTCATTTTCGCGCGCG CACA-3′. PCR products were then used to replace the wild-type counterpart of the reporter vector.

Western blot analysis

For RANKL detection, PDLFs were scraped into lysis buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM leupeptin and 1 μM pepstatin) and sonicated briefly. Protein concentrations were determined using a modified Bradford method. The proteins were separated using a 10% SDS-PAGE gel and then electro-transferred onto a PVDF membrane. For HIF-1α detection, confluent cells were lysed in 2× Laemmli sample buffer, and the proteins were separated using a 6% SDS-PAGE gel. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with the relevant primary antibody, followed by incubation with the corresponding HRP-conjugated secondary antibody. Immune complexes were visualized using WEST-ZOL (plus) reagent and luminescence was detected with a LAS1000 (Fuji PhotoFilm; Japan).

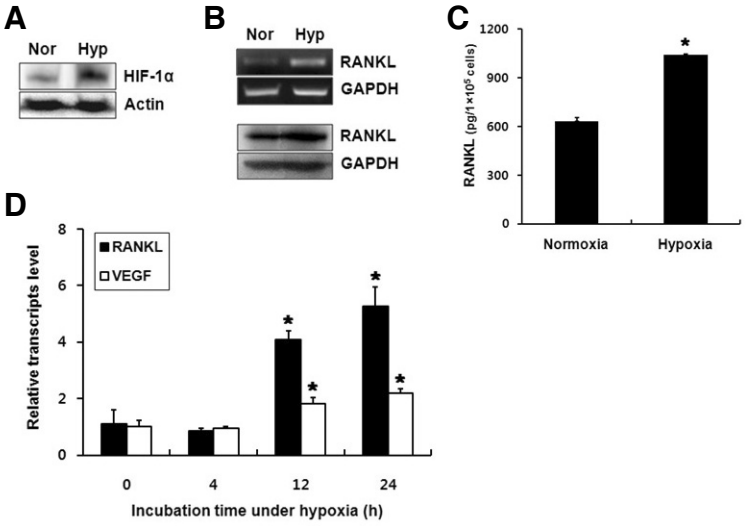

Fig. 1. Hypoxia enhances the levels of RANKL mRNA and protein in human PDLFs. (A-C) PDLFs were incubated in the GasPak pouches for 24 h to induce hypoxia. The Western blot results showed that hypoxia increased the levels of HIF-1α (A) and RANKL (B, lower panel) protein. Semi-quantitative RT-PCR demonstrated that the level of RANKL mRNA was also up-regulated by hypoxia (B, upper panel). (C) The ELISA results showed that hypoxia enhanced RANKL protein levels. The data represent the mean ± S.D. of six independent experiments. *$p < 0.05$, compared to normoxia. (D) The real-time PCR results showed that hypoxia increased the levels of RANKL and VEGF mRNA in a time-dependent manner. The data represent the mean ± S.D. of three independent experiments. *$p < 0.05$, compared to 0 h. Nor, normoxia; Hyp, hypoxia.

Enzyme-linked immunosorbent assay (ELISA)
After hypoxic treatment for 24 h, PDLFs were lysed in phosphate-buffered saline containing protease inhibitors and sonicated briefly. The concentration of soluble RANKL in whole cell lysates was determined using a human RANKL ELISA kit according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay
The ChIP assay was performed as previously described (Cho et al., 2009). Briefly, human PDLFs were incubated for 24 h under hypoxic conditions or under transient transfection with constitutively active HIF-1α. Cells were cross-linked with 1% formaldehyde, lysed and sonicated to obtain DNA fragments of 200-800 bp. After preclearing with blocked protein G agarose, immunoprecipitation was carried out with anti-HIF-1α antibody (10 μg) or an equivalent concentration of mouse IgG as a negative control. DNA was eluted from the immune complexes, and cross-links were reversed. DNA was then purified and subjected to PCR amplification of human RANKL promoter (-926 to -817 bp) using the following primers: f 5′-GCAATGAAACTAA CATTTA-3′ and r 5′-GGGTAAAGGGGCGTGTAGCC-3′.

Luciferase reporter assay
Cells were seeded in a 96-well plate at a density of 5×10^3 cells/well. After overnight culture, the cells were transiently transfected with plasmids using the LipofectAMINE™ reagent. In each transfection, 0.2 μg expression vectors (pcDNA, constitutively active or dominant negative HIF-1α), 0.2 μg reporters (pGL3, RANKL-luc or RANKL-MT-luc) and *Renilla* luciferase plasmid were used as indicated. After 24 h incubation under hypoxic or normoxic conditions, the cells were harvested and the luciferase activity was measured using the Dual-Glo luciferase assay kit. Relative luciferase activity was calculated after normalizing the transfection efficiency to *Renilla* luciferase activity.

Statistical analysis
The data from the real-time PCR, ELISA and reporter assays were expressed as the mean ± S.D. The statistical significance was analyzed by Student's *t*-test. A *p* value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Hypoxia increases the levels of RANKL mRNA and protein in PDLFs
PDLFs in the compression side are exposed to hypoxia and compressive force during orthodontic tooth movement. Because the regulatory effect of hypoxia on RANKL expression in PDLFs is unclear, we first examined whether hypoxia regulates the expression of RANKL. To induce hypoxia, PDLFs were incubated in the GasPak pouch system for 24 h. To confirm that PDLFs were exposed to hypoxia, we assessed HIF-1α protein levels by Western blot analysis. As expected, stabilization of HIF-1α was observed in hypoxia-induced PDLFs (Fig. 1A), suggesting that incubation for 24 h in the GasPak pouch was sufficient to induce hypoxic responses in the PDLFs. We then evaluated the effect of hypoxia on RANKL expression in the same culture conditions. The results of semi-quantitative RT-PCR and Western blot analysis showed that hypoxia enhanced RANKL expression (Fig. 1B). To quantitatively analyze RANKL protein levels, we also performed an ELISA using whole cell lysates. Similar to the western blot results, higher amounts of RANKL were detected in PDLFs exposed to hypoxic conditions compared to the RANKL levels in PDLFs exposed to normoxic conditions (Fig. 1C). When we observed RANKL mRNA expression after incubation for shorter periods, the hypoxia-induced increment was also detectable at 12 h (Fig. 1D). These data indicate that hypoxia rapidly enhances RANKL expression in PDLFs.

VEGF is the primary mediator of angiogenesis, and it serves various biological functions, such as promoting chemotaxis (Clauss et al., 1996) and stimulating osteoclastogenesis (Knowles and Athanasou, 2008). Because previous studies have shown that hypoxia is a strong inducer of VEGF expression in PDLFs (Motohira et al., 2007), we examined VEGF mRNA levels in hypoxic conditions. Hypoxia increased VEGF mRNA expression, confirming that the GasPak pouch system provided effective hypoxic conditions (Fig. 1D). Real-time PCR data showed that the fold change of hypoxia-induced RANKL expression was higher than that of hypoxia-induced VEGF expression, suggesting that RANKL is a sensitive target gene of hypoxia in PDLFs. Osteoblast-derived VEGF is known to act as a stimulator for the formation and activation of osteoclasts (Deckers et al., 2000). Given that both VEGF and RANKL were induced by

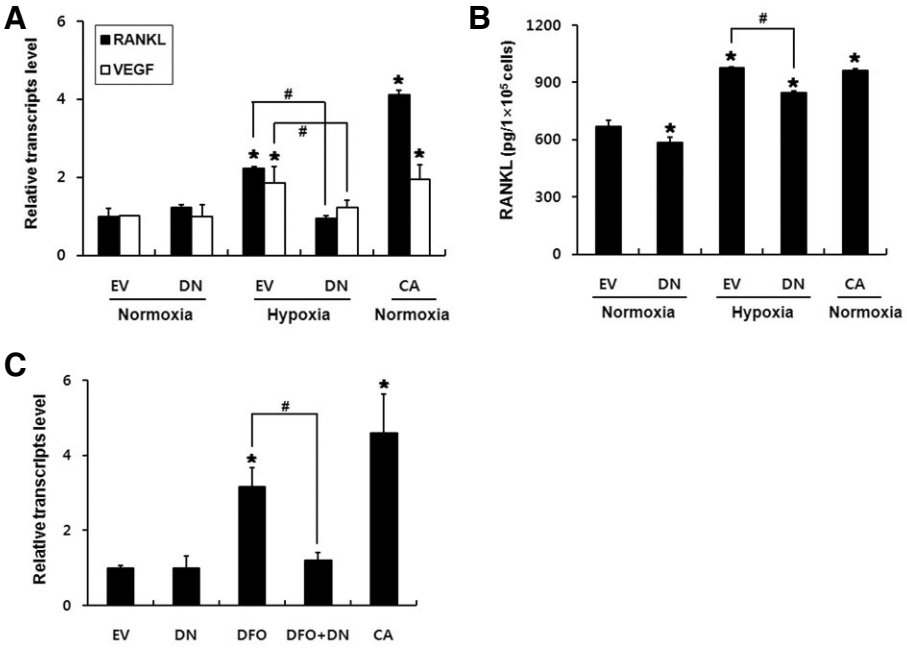

Fig. 2. Hypoxia-induced RANKL expression is HIF-1α-dependent. PDLFs were transiently transfected with pcDNA (EV), constitutively active HIF-1α (CA) or dominant negative HIF-1α (DN), and incubated for 24 h under the normoxic or hypoxic conditions or in the presence of desferoxamine (DFO, 200 μM). Then real-time PCR (A, C) or ELISA (B) was performed. Hypoxic treatment, DFO or CA overexpression increased the expression levels of RANKL and VEGF, whereas DN overexpression blocked hypoxia- or DFO-induced RANKL and VEGF expression. *$p < 0.05$, compared to EV-transfected cells under normoxia. #$p < 0.05$, compared to hypoxia alone (A, B) or DFO alone (C).

hypoxia in PDLFs, we infer that the additive action of RANKL and VEGF contributes to increased osteoclastogenesis under hypoxic conditions.

Hypoxia-induced RANKL expression is HIF-1α dependent

Given that HIF-1α is an important regulator of the cellular responses to hypoxia (Semenza, 2000; Wan et al., 2008), we next investigated whether HIF-1α mediates hypoxia-induced RANKL expression in PDLFs. PDLFs were transiently transfected with constitutively active HIF-1α, and the expression levels of RANKL mRNA and protein were examined. VEGF was also examined as a positive control. Constitutively active HIF-1α significantly increased the levels of RANKL and VEGF mRNA in PDLFs under normoxic conditions (Fig. 2A). Consistent with the real-time PCR results, constitutively active HIF-1α enhanced RANKL protein levels, suggesting that RANKL is the target gene of HIF-1α (Fig. 2B). To verify the role of HIF-1α in hypoxia-induced RANKL expression, PDLFs were transiently transfected with dominant negative HIF-1α and subjected to hypoxic conditions. Hypoxia increased mRNA levels of RANKL and VEGF in empty vector-transfected cells (Fig. 2A). When dominant negative HIF-1α was overexpressed, the hypoxia-induced increment of RANKL and VEGF mRNA was abolished (Fig. 2A). The hypoxia-induced increase in RANKL protein levels was also attenuated by dominant negative HIF-1α (Fig. 2B). To further confirm the role of HIF-1α, the PDLFs were exposed to DFO for 24 h and the level of RANKL expression was examined. Consistent with the results from hypoxia-induced cells, DFO increased RANKL expression whereas dominant negative HIF-1α blocked DFO-induced RANKL expression (Fig. 2C). These results indicate that stabilized HIF-1α enhances RANKL expression under hypoxic conditions.

HIF-1α directly binds to RANKL promoter and stimulates RANKL transcription

As described above, hypoxia rapidly induces RANKL expression in PDLFs. Therefore, we examined whether HIF-1α directly regulates RANKL transcription. *In silico* analysis of the human RANKL promoter using the Transcription Element Search System showed that a putative HIF-1α binding element (CGTG) resides at -891 to -888 bp within 2 kb of the RANKL promoter region (Fig. 3A) (Semenza et al., 1996). Mouse RANKL promoter also contains two putative HIF-1α binding elements (-971 to -968 and -965 to -962 bp, Fig. 3A), suggesting that the HIF-1α binding site is conserved in mammalian RANKL orthologs. Using the reporter constructs containing wild-type and mutant mouse RANKL promoter sequences (RANKL-luc, RANKL-MT-luc), we performed a luciferase reporter assay. Overexpression of constitutively active HIF-1α and hypoxia treatment significantly increased RANKL-luc reporter activity (Fig. 3B). Dominant negative HIF-1α overexpression decreased hypoxia-induced RANKL-luc reporter activity and basal RANKL-luc activity. Furthermore, mutations in the HIF-1α binding elements prevented hypoxia-induced increases in reporter activity. Consistent with the results from hypoxia-induced cells, DFO significantly increased RANKL-luc reporter activity, whereas overexpression of dominant negative HIF-1α or mutations in the HIF-1α binding elements blocked DFO-induced reporter activity (Fig. 3C). These results suggest that hypoxia-induced HIF-1α directly binds to and transactivates the RANKL promoter.

To confirm that HIF-1α binds to the human RANKL promoter, we performed a ChIP assay. Nuclear extracts from PDLFs with constitutively active HIF-1α overexpression or hypoxic treatment were used for immunoprecipitation with anti-HIF-1α antibody. PCR amplification of the putative HIF-1α binding element within the RANKL promoter region revealed that HIF-1α binds to the RANKL promoter and that constitutively active HIF-1α overexpression or hypoxia treatment increases HIF-1α binding to RANKL promoter (Fig. 4). Taken together, these results indicate that hypoxia increases RANKL transcription by stabilization and subsequent binding of HIF-1α to RANKL promoter in human PDLFs.

Although previous reports have shown that hypoxia induces osteoclastogenesis via direct action on osteoclast precursor cells or via indirect action on osteoblasts and bone marrow cells (Fukuoka et al., 2005; Knowles and Athanasou, 2008), there have been no reports on hypoxia-induced RANKL expression

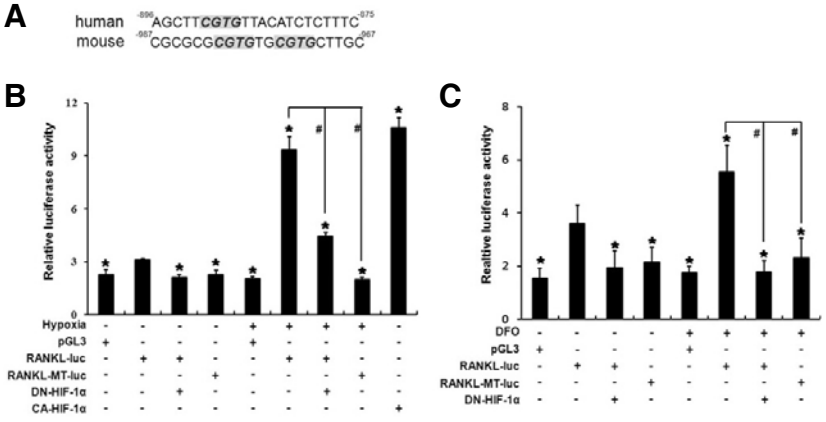

Fig. 3. Hypoxia enhances HIF-1α-dependent RANKL promoter activity. (A) DNA sequence alignments of human and mouse RANKL promoter region containing the putative HIF-1α binding site (CGTG). (B, C) Reporter plasmids containing the mouse RANKL promoter sequence (RANKL-luc) and mutated HIF-1α binding site (RANKL-MT-luc) were prepared. PDLFs were transiently transfected with reporter plasmids and expression vectors containing constitutively active (CA) or dominant negative (DN) HIF-1α and incubated for 24 h under the conditions of normoxia, hypoxia or DFO treatment. Hypoxia or CA-HIF-1α transfection significantly increased reporter activity, whereas DN-HIF-1α transfection or HIF-1α binding site mutation blocked hypoxia-induced reporter activity (B). DFO significantly increased reporter activity, whereas DN-HIF-1α transfection or HIF-1α binding site mutation blocked DFO-induced reporter activity (C). Data are shown as activity relative to *Renilla* luciferase activity and represent the mean ± S.D. of six independent experiments. *$p < 0.05$, compared to RANKL-luc alone under normoxic conditions. #$p < 0.05$, compared to RANKL-luc plus hypoxia (B) or RANKL-luc plus DFO (C).

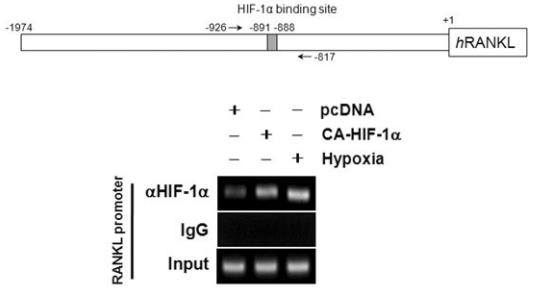

Fig. 4. Hypoxia increases DNA binding of HIF-1α to the RANKL promoter. (Upper panel) Schematic representation shows the human RANKL promoter region and putative HIF-1α binding site. Arrow indicates the primer binding sites for PCR amplification of the RANKL promoter region containing the HIF-1α binding element. (Lower panel) A chromatin immunoprecipitation assay was performed using cells under hypoxic conditions or those transfected with CA-HIF-1α. Cellular DNA fragments were immunoprecipitated with anti-HIF-1α antibody (αHIF-1α) or normal IgG, and DNA fragments spanning -926 to -817 bp were amplified by PCR. Hypoxia or transfection with CA- HIF-1α increased DNA binding of HIF-1α.

to our knowledge. In this study, we demonstrated for the first time that hypoxia induces RANKL expression in human PDLFs and that RANKL is a target gene of HIF-1α. A previous report has shown that no osteoclast precursor cells are found in PDLs before the application of orthodontic force but osteoclast precursor cells rapidly increase one day after the application of force (Xie et al., 2008). Given that VEGF is a well-known chemotactic factor for osteoclast precursor cells (Matsumoto et al., 2002), it is likely that hypoxia-induced VEGF secretion from compression side PDLFs may be involved in the recruitment of osteoclast precursor cells from the surrounding alveolar bone to the PDL tissue. Subsequently, osteoclast differentiation may be induced by the additive actions of RANKL and VEGF. In addition to hypoxia, compressive force also up-regulates RANKL expression in PDLFs (Kanzaki et al., 2002; Nakao et al., 2007), suggesting that in compression side PDLs, RANKL expression is strongly induced by both compressive force and local hypoxia. Given that hypoxia-induced HIF-1α down-regulates osteoprotegerin expression in chondrocytes (Shirakura et al., 2010), the ratio of RANKL to osteoprotegerin may be increased in compression side PDLs, creating favorable conditions for osteoclastogenesis.

In conclusion, our results suggest that RANKL is a direct target gene of HIF-1α and that hypoxia plays a role in enhanced osteoclastogenesis in compression side PDLs, at least in part, through the induction of RANKL expression in PDLFs.

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