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Periostin inhibits hypoxia-induced apoptosis in human periodontal ligament cells via TGF-β signaling

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ABSTRACT

Periostin (POSTN) is an extracellular matrix protein expressed predominantly in periodontal ligament (PDL) cells. The aim of this study was to investigate the effects of POSTN on human PDL cell apoptosis under hypoxic conditions. The percentage of apoptotic PDL cells under hypoxia was increased significantly when the endogenous *POSTN* gene was silenced using siRNA, but decreased when cells were treated with recombinant human POSTN (rhPOSTN), or when mouse *Postn* was overexpressed *in vitro*. Silencing *POSTN* during hypoxia decreased the expression of *HIF prolyl-hydroxylase 2* (*PHD2*), but increased HIF-1α protein level. Conversely, treating hypoxic cells with rhPOSTN or overexpressing *Postn* increased *PHD2* expression but decreased HIF-1α levels. The addition of rhPOSTN in the absence of a TGF-β receptor inhibitor (SB525334) significantly decreased hypoxia-induced apoptosis, while the effects of rhPOSTN were abolished when cells were co-treated with SB525334. Consistent with this, the phosphorylation of SMAD2 was increased in hypoxic PDL cells by the knockdown of *POSTN*, but decreased by treatment with rhPOSTN. Under normoxia, the *PHD2* expression, HIF-1α level, and apoptosis were unaffected by *POSTN* siRNA, rhPOSTN, or *Postn* overexpression. These findings suggest that, under hypoxic conditions, POSTN regulates *PHD2* expression and HIF-1α levels by modulating TGF-β1 signaling, leading to decreased apoptosis.

1. Introduction

Hypoxia-inducible factor-1α (HIF-1α) is an important transcription factor that responds to changes in oxygen (O_2) concentrations in the cellular environment [1], and which regulates a wide variety of physiological processes, including cellular metabolism, proliferation, autophagy, and apoptosis [2,3]. The regulation of HIF-1α levels under normoxic and hypoxic conditions has been extensively studied. In normoxia, HIF-1α is hydroxylated at conserved proline residues by HIF prolyl-hydroxylases (PHDs), leading to its recognition and ubiquitination by the von Hippel-Lindau tumor suppressor protein (VHL)-E3 ubiquitin ligase, labeling it for rapid proteasomal degradation [4]. Under hypoxic conditions, PHD activity is inhibited since this enzyme utilizes O_2 as a co-substrate [5]. HIF-1α is then no longer degraded, and stabilized HIF-1α induces the expression of multiple genes, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), glyceraldehyde phosphate dehydrogenase (GAPDH), and Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3), by allowing it to bind to hypoxia-responsive elements (HREs) in proximal promoter region [2].

During orthodontic treatment, the periodontal ligament (PDL), which is a fibrous connective tissue with vascular and neural components that surrounds the tooth root in the alveolar socket, undergoes mechanical stress from orthodontic appliances. Compression of the PDL alters blood vessel morphology and the vascular response in the alveolar socket [6,7]. A reduction in blood volume is also observed under pressure [8], which can eventually induce local hypoxia and apoptosis in PDL cells [9].

Periostin (POSTN) is a disulfide-linked 90-kDa secreted protein whose expression pattern is restricted in specific tissues such as the PDL [10], periosteum [10], cardiac valves [11], and several types of cancer [12,13]. POSTN is not only essential for the integrity and function of the PDL during occlusal loading in mice [14], it also promotes cellular tolerance against stress and inhibits cell death [15]. Although mechanical loading can increase the expression of POSTN in rat PDL [16], little is known about the effects of POSTN in PDL cells under hypoxic conditions.

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The aim of this study was to elucidate the effects of POSTN on human PDL cell apoptosis under hypoxic conditions, and to determine the biological mechanisms that regulate the susceptibility of human PDL cells to hypoxia-induced apoptosis.

2. Materials and methods

2.1. Cell culture and hypoxic treatments

Human PDL (hPDL) fibroblasts (Clonetics™ CC-7049 HPdLF; Lonza, Walkersville, MD) were maintained at subconfluency in α-MEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Life Technologies Corp., Carlsbad, CA), and 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) at 37 °C in a humidified 5% CO_2/95% air atmosphere. The hPDL cells were plated onto 6-well cell culture dishes at 5×10^5 cells/well for mRNA and protein experiments, or into Lab-Tek chamber slides (Thermo Fisher Scientific) at 5×10^3 cells/cm^2 for the assessment of apoptosis. Hypoxia was induced by transferring cells to an airtight container containing AnaeroPack for Cells (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan), a disposable O_2-absorbing and CO_2-generating agent, to reduce the O_2 concentration to ~1%.

2.2. POSTN silencing, mouse Postn overexpression, and recombinant human POSTN peptide treatment

For *POSTN* silencing, 10 nM POSTN siRNA (5′-CCAUGGGAACCA-GAUUGCAACAAAU-3′ and 5′-AUUUGUUGCAAUCUGGUUCCC-AUGG-3′) (Stealth RNAi; Life Technologies Corp.) and a negative control were transfected to the cells using Lipofectamine RNAiMax transfection reagent (Life Technologies Corp.) following the manufacturer's instructions. To overexpress POSTN, cells were transfected with mouse POSTN (pCMV-SPORT6-Postn; MGC: 25368, IMAGE: 445722) or mock plasmid vector containing the CMV promoter, using X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Penzberg, Germany). Quantitative PCR (qPCR) was used to monitor the overexpression or knockdown of *POSTN* 24 h after transfection, as described below. Cells in the hypoxia group were then transferred to the hypoxic chamber. Where appropriate, recombinant human POSTN peptide (rhPOSTN) (OSF2) (RD1720451000; BioVendor R&D, Brno, Czech Republic) was added to cells at a final concentration of 100 ng/ml, and those in the hypoxia group were immediately transferred to the hypoxic chamber.

2.3. Latent and active TGF-β1 treatment with or without rhPOSTN during hypoxia

The growth medium was changed to serum-free medium 24 h before treatment with latent or active TGF-β1 (R&D Systems, Minneapolis, MN) or a TGF-β receptor type I blocker (SB525334; Wako Pure Chemical Industries, Ltd., Osaka, Japan). To assess the effects of POSTN on the activation of TGF-β1 in hypoxic cells, the medium was supplemented with 5 ng/ml latent or active TGF-β1, concomitant with the addition of 100 ng/ml rhPOSTN. The role of TGF-β1 in hypoxia-induced apoptosis was further investigated by treating hPDL cells with active TGF-β1 in the presence or absence of 100 ng/ml rhPOSTN, and/or 0.5 μM SB525334.

2.4. TUNEL staining

Apoptosis was assessed in hPDL cells 48 h after the induction of hypoxia using an *in situ* Cell Death Detection Kit (Roche Applied Science), except for the cells shown in Fig. 1, which were assessed at 48 and 72 h. Four randomly selected visual fields in each experimental group were photographed, and the apoptotic cell population (percentage of TUNEL-positive cells) from four independent experiments was calculated.

2.5. Western blotting

Whole-cell lysates were prepared 24 h after the induction of hypoxia using RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (cOmplete Mini and PhosSTOP; Roche Applied Science). Cell lysates (15 μg) were separated on 4–12% Bis–Tris gels using MOPS-SDS running buffer (NuPage; Life Technologies Corp.). The separated proteins were transferred to nitrocellulose membranes and blocked for 3 h in phosphate-buffered saline with 0.1% Tween-20 containing 5% skimmed milk at room temperature. The blocked membranes were incubated overnight at 4 °C with rabbit anti-POSTN (ab14041; Abcam, Cambridge, UK), rabbit anti-HIF-1α (NB100-497; Novus Biologicals, Littleton, CO), mouse anti-α-tubulin (T9026; Sigma–Aldrich, St. Louis, MO), rabbit anti-SMAD2/3 (SC-8832; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-phospho-SMAD2 (SAB4300251; Sigma–Aldrich) in blocking solution. After incubation with goat anti-rabbit or goat anti-mouse IgG-HRP (SC-2004 or SC-2005; Santa Cruz Biotechnology) secondary antibodies for 1 h, proteins were visualized and recorded using an LAS-3000 Image Reader (Fujifilm, Tokyo, Japan).

2.6. qPCR

Total RNA extracted 24 h after the induction of hypoxia was used for cDNA synthesis (RNeasy mini and Quantitect RT kits; Qiagen Inc., Valencia, CA). The expression of *BNIP3*, *FIH-1*, *PHD1–3*, *TGF-β1*, *human POSTN*, and *mouse Postn* in hPDL cells was assessed by qPCR using Quantifast SYBR Green (Qiagen Inc.) and an ABI7500 system (Life Technologies Corp.), and quantified relative to the housekeeping gene *60S ribosomal protein L27* (*RPL27*). All primer sequences were obtained from previous publications or PrimerBank (http://pga.mgh.harvard.edu/primerbank/), as shown in Supplementary Table 1.

2.7. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Student's *t*-test was used to compare the expression levels of *BNIP3* (Fig. 1D), *FIH-1*, and *PHD1–3* (Fig. 2C). For the other quantitative experiments, a two-way analysis of variance was performed followed by Bonferroni's multiple comparison as a post hoc test to identify statistically significant differences between groups. A p-value <0.05 was considered to be statistically significant.

3. Results

3.1. Hypoxia-induced apoptosis in hPDL cells, and increased HIF-1α protein and BNIP3 mRNA expression

We first confirmed that hypoxia induced apoptosis in hPDL cells. As expected, the percentage of TUNEL-positive cells in the hypoxic group increased significantly compared with the normoxic group after 48 and 72 h of culture, and increased in a time-dependent manner ($p < 0.05$; Fig. 1A and B). After 24 h of hypoxia, the level of HIF-1α increased in hypoxic cells compared with normoxic controls (Fig. 1C). Furthermore, transcription of the HIF-1α target *BNIP3* was significantly upregulated in hypoxic cells (Fig. 1D).

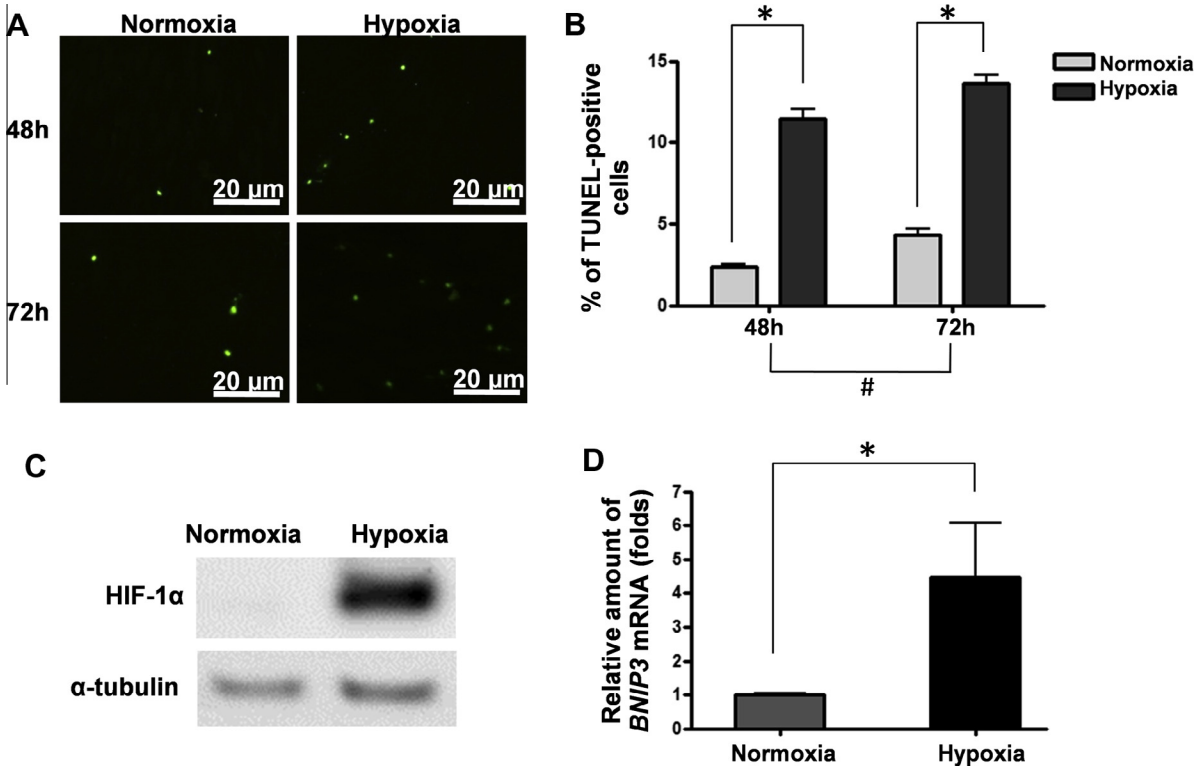

Fig. 1. Hypoxia induced hPDL apoptosis and increased HIF-1α and *BNIP3* expression. (A) TUNEL staining of hPDL cell cultures after 48 and 72 h of hypoxia. (B) Percentage of TUNEL-positive cells cultures after 48 and 72 h of hypoxia. Each column represents the mean ± SD ($n = 4$). $^{*}p < 0.05$ indicates a significant difference between groups. $^{\#}p < 0.05$ indicates a significant difference between times. (C) HIF-1α protein levels as assessed by Western blotting 24 h after the induction of hypoxia. (D) Relative *BNIP3* mRNA expression as assessed by qPCR in hPDL cells after 24 h of hypoxia ($n = 3$). $^{*}p < 0.05$.

3.2. Silencing of POSTN under hypoxic conditions decreased PHD2 expression and increased HIF-1α levels and apoptosis

To investigate the roles of POSTN in hypoxia-induced apoptosis, *POSTN* was silenced in hPDL cells. Endogenous *POSTN* mRNA levels were decreased more than 98% in cells treated with siRNA, as shown by qPCR (data not shown). The percentage of TUNEL-positive cells among the hypoxic cells was increased significantly compared with normoxic cells ($p < 0.05$; Fig. 2A). Under hypoxia, the number of TUNEL-positive hPDL cells treated with *POSTN* siRNA was increased significantly compared with the negative control ($p < 0.05$; Fig. 2A, Hypoxia). Conversely, treatment with *POSTN* siRNA did not affect the number of TUNEL-positive cells under normoxic conditions (Fig. 2A, Normoxia).

To assess the roles of POSTN in HIF-1α accumulation, HIF-1α protein levels in hypoxic and normoxic cells treated with *POSTN* siRNA were compared by Western blotting using α-tubulin as an internal control. The protein and mRNA expression of POSTN decreased in the negative control group under hypoxia compared with normoxia (Fig. 2B; mRNA data not shown). The level of HIF-1α under hypoxic conditions was increased in hPDL cells treated with *POSTN* siRNA compared with the negative control (Fig. 2B, Hypoxia). As expected, HIF-1α level was unaffected by *POSTN* siRNA under normoxia (Fig. 2B, Normoxia). Because HIF-1α is regulated by factor inhibiting HIF-1 (FIH-1) and PHDs, we assessed the mRNA levels of *FIH-1* and *PHD1–3* in hPDL cells under normoxic and hypoxic conditions. An analysis by qPCR revealed that only *PHD2* mRNA was increased significantly under hypoxic conditions ($p < 0.05$; Fig. 2C and D). To determine whether POSTN regulates HIF-1α, qPCR was carried out in hPDL cells treated with *POSTN* siRNA. Under hypoxic conditions, the mRNA expression of *PHD2* was decreased significantly in hPDL cells treated with *POSTN* siRNA compared with the negative control ($p < 0.05$; Fig. 2D). In contrast, treatment with *POSTN* siRNA did not affect the expression of *FIH1*, *PHD1*, or *PHD3* (data not shown).

3.3. Treatment of hypoxic cells with rhPOSTN or the overexpression of Postn increased PHD2 expression and decreased HIF-1α levels and apoptosis

To confirm the roles of POSTN in hypoxia-induced apoptosis, hPDL cells were treated with rhPOSTN or transfected with a mouse *Postn* expression vector. In both experiments, the percentage of TUNEL-positive cells was decreased significantly under hypoxic conditions compared with the control ($p < 0.05$; Fig. 3A and D). The presence of *Escherichia coli*-derived rhPOSTN was confirmed by a band at ~75 kDa by Western blotting (data not shown). This could be distinguished from endogenous or overexpressed POSTN, which was detected at 95–100 kDa in most experiments (Figs. 2B and 3B and E), presumably due to differences in post-translational modifications and/or splicing [17]. The expression of endogenous POSTN was not affected by the treatment of hPDL cells with rhPOSTN (Fig. 3B and by qPCR, data not shown). Under hypoxic conditions, the accumulation of HIF-1α was reduced in those groups either treated with rhPOSTN or transfected with *Postn* compared with the controls (Fig. 3B and E). Consistent with this, the mRNA expression of *PHD2* was increased significantly in the same treatment groups compared with the control ($p < 0.05$; Fig. 3C and F).

3.4. POSTN regulates the level of HIF-1α protein and gene expression of PHD2 by modulating TGF-β1 signaling under hypoxia

To understand the mechanisms underlying the regulation of *PHD2* expression and HIF-1α protein levels by POSTN, the phosphorylation of SMAD2, which is known to inhibit *PHD2* expression

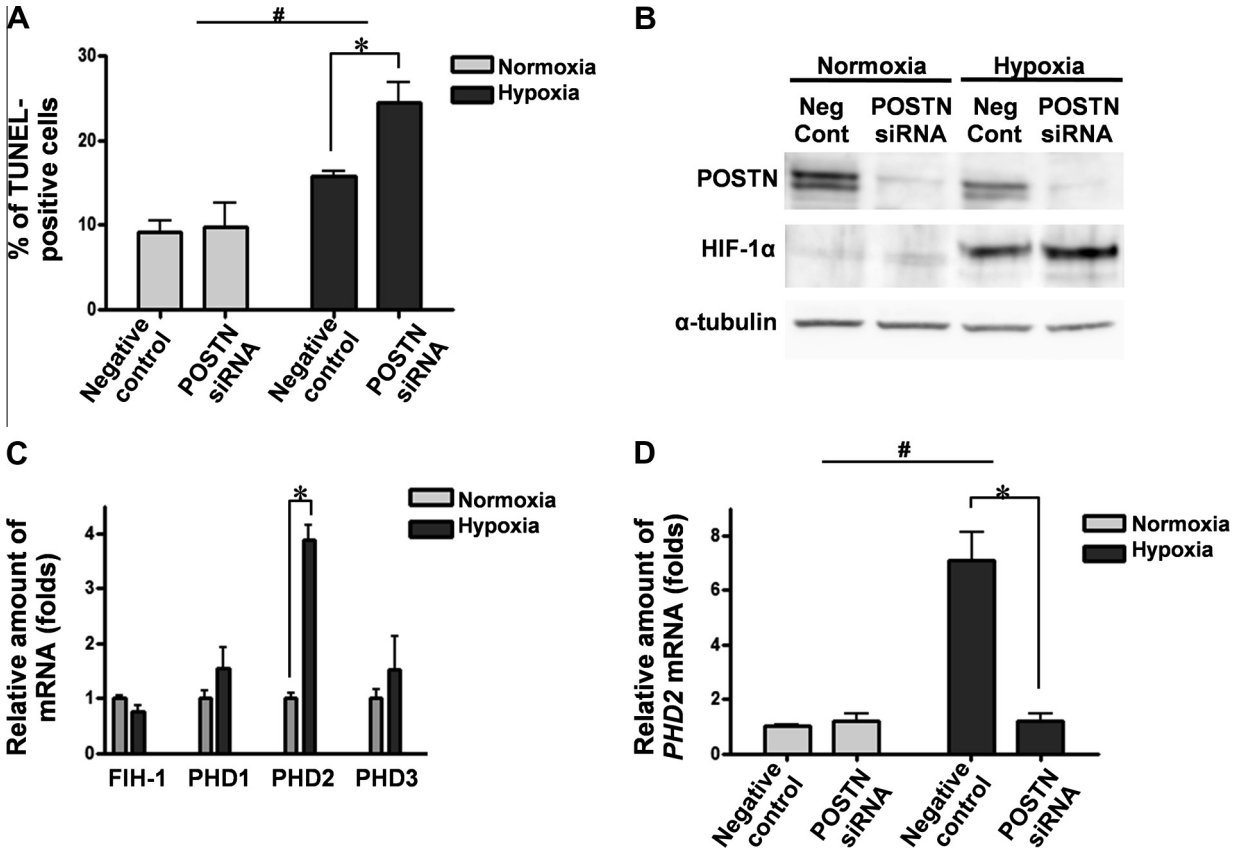

Fig. 2. *POSTN* silencing in hypoxic cells increased hPDL cell apoptosis and HIF-1α protein levels, but decreased *PHD2* transcription. (A) Percentage of TUNEL-positive hPDL cells with or without *POSTN* siRNA treatment after 48 h of hypoxia. Each column represents the mean ± SD ($n = 4$). (B) Western blotting for HIF-1α in hPDL cells treated with *POSTN* siRNA for 24 h after the induction of hypoxia. (C) Analysis of *FIH-1* and *PHD1–3* expression by qPCR after 24 h of hypoxia ($n = 3$) $^{*}p < 0.05$. (D) *PHD2* mRNA expression level as assessed by qPCR. $^{*}p < 0.05$ indicates a significant difference from the control. $^{\#}p < 0.05$ indicates a significant difference from the normoxic groups.

[18], was assessed. Under hypoxic conditions, the silencing of *POSTN* increased the phosphorylation of SMAD2 (pSMAD2; Fig. 4A). The role of TGF-β1 signaling in hypoxia-induced apoptosis and HIF-1α protein levels was therefore assessed in response to treatment with rhPOSTN. qPCR analysis revealed that the mRNA expression of *TGF-β1* was unaffected by rhPOSTN under either normoxic or hypoxic conditions after treatment for 24 and 48 h (data not shown). Next, TUNEL staining was performed in hPDL cells treated with rhPOSTN, active TGF-β1, and/or a TGF-β type I receptor blocker (SB525334; Fig. 4B). Treatment with SB525334 significantly decreased the number of TUNEL-positive cells during hypoxia compared with the SB525334 (−) groups ($^{\#}p < 0.05$; Fig. 4B). In contrast, active TGF-β1 significantly increased the percentage of TUNEL-positive cells compared with a TGF-β1 (−) control ($^{+}p < 0.05$; Fig. 4B) under hypoxic conditions. Importantly, treatment with rhPOSTN in the absence of SB525334 significantly decreased the number of apoptotic cells ($^{*}p < 0.05$; Fig. 4B), but these effects were abrogated by SB525334. These results strongly suggest that TGF-β1 signaling plays an important role in hypoxia-induced hPDL cell apoptosis, and is regulated by the POSTN.

We further examined the effects of TGF-β1 signaling on hypoxic hPDL cells by Western blotting (Fig. 4C). Treatment with active TGF-β1 increased the levels of pSMAD2 in the absence of SB525334, whereas the presence of SB525334 abolished the effects of active TGF-β1 on SMAD2 phosphorylation. In addition, HIF-1α levels were increased slightly when TGF-β1 was added to hypoxic hPDL cells in the absence of SB525334. This suggests that TGF-β1-induced pSMAD2 is positively correlated with HIF-1α protein levels.

Previous studies demonstrated that inhibiting the maturation of TGF-β increases *PHD2* levels and decreases basal HIF-1α protein levels [18]. We therefore assessed the effects of rhPOSTN on TGF-β1 signaling by co-treatment with latent or active TGF-β1 and rhPOSTN. Treatment with either latent or active TGF-β1 increased pSMAD2 under normoxic and hypoxic conditions in the absence of rhPOSTN (Fig. 4D, rhPOSTN-). Interestingly, the addition of rhPOSTN inhibited not only TGF-β1-induced SMAD2 phosphorylation, but also HIF-1α levels only under hypoxic conditions (Fig. 4D, rhPOSTN+). However, no significant differences in SMAD2 phosphorylation were detected between groups treated with latent and active TGF-β1, either in the presence or absence of rhPOSTN. In addition, the HIF-1α protein level under hypoxic conditions was higher in cells treated with active TGF-β1 compared with those treated with latent TGF-β1.

4. Discussion

This study indicates that POSTN decreases hypoxia-induced apoptosis in human PDL cells *in vitro*. POSTN may regulate HIF-1α accumulation by blocking TGF-β type I receptor signaling and the subsequent phosphorylation of SMAD2, which leads to increased expression of *PHD2*. This represents a key mechanism for the inhibition of hypoxia-induced apoptosis in hPDL cells.

In human PDL cells, HIF-1α accumulation after $CoCl_2$ treatment not only upregulates the *BNIP3* expression, it also induces apoptosis and autophagic cell death [19]. Here, we induced severe hypoxia (~1% O_2) in hPDL cells, which increased the HIF-1α level and its

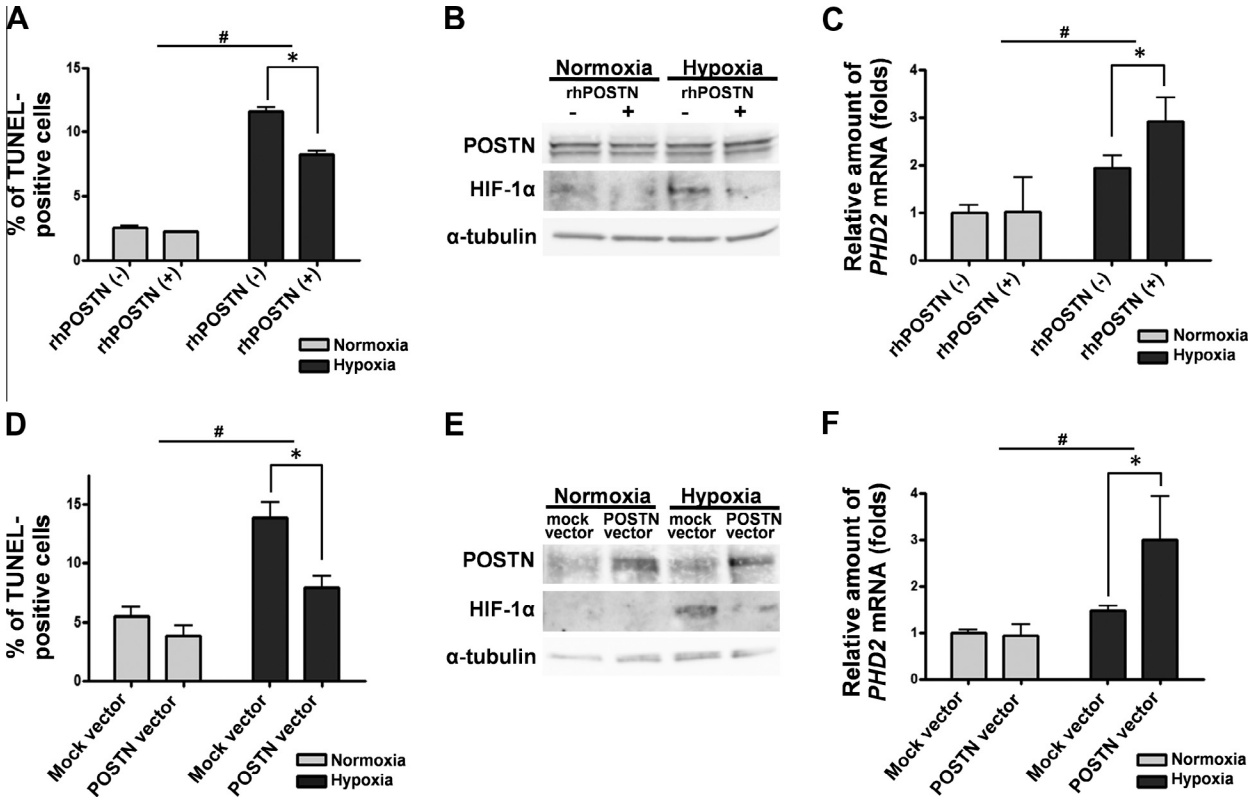

Fig. 3. Treatment with exogenous rhPOSTN or the overexpression of *Postn* increased *PHD2* expression and decreased HIF-1α levels and apoptosis under hypoxic conditions. (A and D) The percentage of TUNEL-positive hPDL cells after 48 h of hypoxia. (A) rhPOSTN treatment group; (D) *Postn* overexpression group. Each column represents the mean ± SD ($n = 4$). (B and E) Western blotting for HIF-1α in hPDL cells 24 h after the induction of hypoxia. (B) rhPOSTN treatment group; (E) *Postn* overexpressing group. (C and F) *PHD2* expression as assessed by qPCR after 24 h of hypoxia. (C) rhPOSTN treatment; (F) *Postn* overexpression. Each column represents the mean ± SD ($n = 3$). $^{*}p < 0.05$ indicates a significant difference from the control. $^{\#}p < 0.05$ indicates a significant difference from the normoxic groups.

target gene, *BNIP3*, concomitant with an increase in hPDL apoptosis (Fig. 1). The stabilization of HIF-1α during hypoxia induces *BNIP3* mRNA and protein levels due to an HRE in its proximal promoter [20]. Recent reports indicate that HIF-1α-induced BNIP3 promotes autophagy (mitophagy) for cell survival via the release of Beclin-1 from the BCl-2/BCL-xL complex under hypoxia (~3–0.1% O_2) [3,21]. However, HIF-1α also plays an important role in apoptotic cell death under hypoxic conditions [22,23]. Non-identical cellular responses to hypoxia may be due to the different types of cells and culture conditions such as pH, severity and duration of hypoxia, and nutrients. Although the functions of BNIP3 are not examined in our experiments, our data suggest that hypoxia induced apoptosis in hPDL cells possibly by increased HIF-1α protein.

FIH-1 interacts with HIF-1α and VHL to repress the transcriptional activity of HIF-1, and it modulates HIF-1α protein levels [24]. PHDs also promote the binding of VHL to HIF, and regulate the degradation of HIF-1α by the ubiquitin–proteasome pathway. The knockdown of *PHD2* (but not *PHD1* or *PHD3*) upregulated HIF-1α in many human cells under normoxic conditions [25]. In addition, PHD2 could regulate HIF-1α-induced gene transcription under hypoxic conditions [26]. We demonstrated that in hPDL cells, out of all of the factors that regulate HIF-1α levels, only *PHD2* transcription was induced by hypoxia (Fig. 2). PHD2 is therefore a key regulator of HIF-1α degradation in hPDL cells. In addition, PHD2 was regulated by the presence or absence of POSTN, modulating apoptosis (Figs. 2 and 3).

TGF-β1 significantly and specifically decreases both the mRNA and protein levels of PHD2 via SMAD signaling, resulting in the stabilization of HIF-1α [18]. In addition, the activation of TGF-β1 from the latent to the active form is important for regulating the expression of HIF-1α and PHD2 in hypoxic cells [18]. It could therefore be assumed that POSTN regulates the activation of TGF-β, and modulates the response of hPDL cells to hypoxia. However, we demonstrated that rhPOSTN suppressed the effects of both latent and active TGF-β on the phosphorylation of SMAD2 and HIF-1α accumulation (Fig. 4). Since latent and active TGF-β1 had similar effects on pSMAD2, it is unlikely that POSTN plays a role in the activation of TGF-β from the latent to the active form. In addition, our study indicates that the silencing of *POSTN* upregulated SMAD2 phosphorylation, resulting in decreased *PHD2* mRNA and increased HIF-1α protein levels. Conversely, overexpressing *Postn* or treatment with exogenous rhPOSTN inhibited hypoxia-induced apoptosis, possibly by decreasing pSMAD2 and increasing *PHD2* mRNA levels. Our data therefore strongly suggest that POSTN regulates TGF-β signaling by blocking TGF-β type I receptor signaling.

Recent reports suggested that inhibiting TGF-β1/SMAD3 signaling decreased the stability of HIF-1α protein by inducing PHD2 expression in human PDL cells under normoxic conditions [27]. However, the effects of POSTN on apoptosis were evident only under hypoxic conditions in our study. The mechanism by which POSTN is effective only under hypoxic conditions remains to be elucidated, and additional studies are needed to define the roles of POSTN and TGF-β in the regulation of SMAD phosphorylation during hypoxia.

In conclusion, our study reveals that POSTN decreases HIF-1α accumulation in hPDL under hypoxic conditions, possibly by inhibiting TGF-β/SMAD2 signaling. These results not only provide insight into the role of the extracellular matrix protein POSTN, but also provide novel evidence for the involvement of POSTN in TGF-β/SMAD signaling and the regulation of apoptosis.

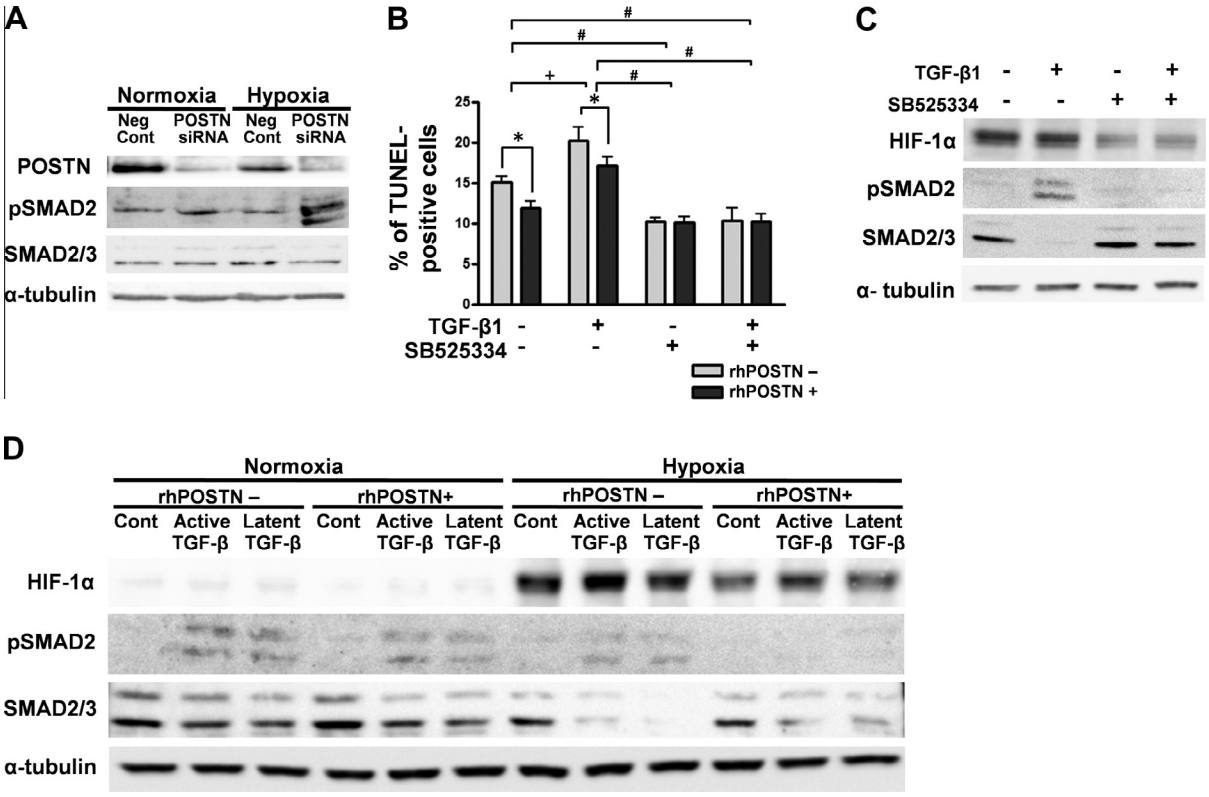

Fig. 4. Treatment with rhPOSTN inhibits TGF-β1/SMAD signaling by blocking the TGF-β1 receptor under hypoxic conditions. (A) Western blotting for pSMAD2 after treatment with *POSTN* or control siRNA after 24 h of hypoxia. (B) The percentage of TUNEL-positive hPDL cells after 48 h of hypoxia in the presence or absence of rhPOSTN, active TGF-β1, and a TGF-β receptor blocker (SB525334). The data are expressed as the mean ± SD ($n = 4$). (C) Western blotting for HIF-1α, pSMAD2, and SMAD2/3 in the presence or absence of active TGF-β1 and SB525334 after 24 h of hypoxia. (D) Western blotting for HIF-1α, pSMAD2, and SMAD2/3 in hPDL cells supplemented with or without rhPOSTN, and latent or active TGF-β1 after 24 h of hypoxia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.027.

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