Inhibiting PHD2 in bone marrow mesenchymal stem cells in an inflammatory microenvironment facilitates periodontal repair in SD rats

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Abstract

Background: Both seed cells and the oxidative stress-induced inflammatory microenvironment are crucial in periodontal regeneration. The inhibition of prolyl hydroxylase domain-containing protein 2 (PHD2), which activates the HIF pathway, can regulate the inflammatory response and improve wound healing during tissue repair. The present study focused on the ability of bone marrow mesenchymal stem cells (BMMSCs) with PHD2 gene silencing to modulate periodontal regeneration of the inflammatory microenvironment through the PHD2/HIF-1α signalling pathway.

Methods: In this study, PHD2 expression in BMMSCs was downregulated under normoxic conditions via lentiviral vector-mediated RNA interference to promote HIF-1α accumulation. We evaluated the osteogenic differentiation of BMMSCs with PHD2 gene silencing in an inflammatory microenvironment *in vitro*. Then, the PHD2 gene-modified BMMSCs were transplanted into rats with experimental periodontitis to observe their effects on periodontal repair.

Results: PHD2 gene silencing resulted in stable expression of HIF-1α and further upregulated the expression of osteogenic genes in BMMSCs. More importantly, the PHD2-silenced group exhibited a significantly enhanced osteoinductive effect and increased VEGF levels in an inflammatory environment, which were weakened by blocking the VEGF receptor. In addition, PHD2-modified BMMSC transplantation elevated osteogenic parameters and the expression of both HIF-1α and VEGF in periodontal tissue.

Conclusion: PHD2 gene silencing is a feasible approach to combat inflammatory bone loss by rescuing the dysfunction of seed cells and has a promising therapeutic effect on periodontal regeneration.

1 Introduction

Periodontitis is an inflammatory and destructive disease in periodontal tissue caused by plaque biofilms[1]. Patients with severe periodontitis experience loss of periodontal tissue, often accompanied by tooth loosening, displacement, and even tooth loss[2]. Strategies based on mesenchymal stem cells (MSCs) to achieve periodontal regeneration have attracted widespread attention[3, 4]. Accumulating evidence indicates that bone marrow mesenchymal stem cells (BMMSCs) have major advantages, especially immunomodulatory properties, in promoting new periodontal tissue[5–7]. However, the interaction between MSCs and their niche microenvironment, including inflammatory and low oxygen microenvironments, is crucial to self-renewal and protection[8–10].

As αβ-heterodimeric transcription factors, hypoxia inducible factors (HIFs) are closely correlated with angiogenesis, glucose metabolism, the cell cycle and apoptosis. Hypoxia-inducible factor-1 (HIF-1) has been widely studied as it was the first identified HIF isoform and enhances self-renewal, proliferation and post-homing differentiation of stem cells[11, 12]. The stability of HIF-1 and the changes in gene expression induced by hypoxia have a profound impact on the microenvironment of inflammatory tissues and disease outcomes[13, 14]. Cramer et al. found that aggregation, mobility, invasiveness, and bacterial inactivation of myeloid cells were decreased in mice in which HIF-1α was knocked out[15]. It was reported that a pharmacological agent (AKB-4924) could enhance skin innate defences against bacterial infection via HIF-1 stabilization[16]. Moreover, HIF-1 is the main regulator of hypoxic signalling, which regulates many downstream angiogenic growth factors, such as vascular endothelial growth factor (VEGF), erythropoietin (EPO) and basic fibroblast growth factor (bFGF), further promoting angiogenesis and osteogenesis[17, 18].

Prolyl hydroxylases (PHDs) induce proteasomal degradation of HIFs, and PHD2 is the main enzyme that downregulates HIF-1α expression in normoxia[11]. Recently, PHD inhibitors have shown promise in cell-based therapy

and bone tissue engineering[12, 19]. Mesoporous bioactive glass scaffolds with ionic cobalt, a kind of hypoxia-mimicking agent, could cause deactivation of HIF-specific prolyl hydroxylase and subsequently maintain HIF stabilization in a normoxic environment, indicating promising osteogenic properties[20]. Rios et al. found that transplantation of MSCs with siRNA against PHD2 into bone defects yielded good bone regeneration[21]. Earlier studies by our team found that PHD2 gene interference in stem cells induced resistance to oxidative stress and enhanced periodontal tissue repair, suggesting potential applications of the PHD2/HIF-1 signalling pathway in periodontal regeneration[22, 23]. Nonetheless, the regulation of the periodontal inflammatory microenvironment and the subsequent effects on periodontal tissue regeneration of PHD2-silenced BMMSCs in periodontitis treatment are unclear. Thus, we hypothesized that the PHD2/HIF-1 signalling pathway might also modulate the osteogenic process of PHD2-silenced BMMSCs in an inflammatory microenvironment.

In this study, PHD2 gene lentivirus RNA interference vectors were successfully constructed. We used *Porphyromonas gingivalis* lipopolysaccharide (*Pg*-LPS) to stimulate the inflammatory microenvironment *in vitro*[24, 25] and investigated whether the osteogenic differentiation of PHD2 gene-modified BMMSCs was improved in an inflammatory environment. Finally, the potential effect of rat BMMSCs with PHD2 gene modification on periodontal regeneration was evaluated in ligature-induced experimental periodontitis models.

2 Materials And Methods

Cell culture

Primary BMMSCs of SD rats were isolated and purified by AllCells (Alameda, CA, USA), and their phenotype was identified. The cells were recovered and cultured in low glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (HyClone, USA) at 37 °C in 5% CO_2. The cells were used between passages 2 and 4.

Lentiviral vector infection of BMMSCs

The short hairpin RNA (shRNA) interference sequence was designed for rat PHD2 according to our previous report[23]. Construction and sequencing of plasmids, packaging and purification of lentiviral vectors were performed by a commercial source (GenePharma, Ltd., Shanghai, China). All lentiviral vectors carried no other exogenous gene except for the green florescent protein (GFP) label. Interference sequences of shRNA are shown in Table 1.

Table 1

shRNA Interference sequences

shRNA		5'-3'
sh-PHD2	sense	GATCCGTGACTCTTCCAAGGACATCCTTCAAGAGAGGATGTCCTTGGAAGAGTCACTTTTTTG
	antisense	AATTCAAAAAAGTGACTCTTCCAAGGACATCCTCTCTTGAAGGATGTCCTTGGAAGAGTCACG

Third-generation BMMSCs were seeded in 6-well plates at a density of 1×10^5 cells/well and cultured at 37 °C and 5% in an incubator. The cells were divided into 3 groups: the sh-PHD2 group (lentiviral RNA interference vector), the NC group (negative control of the lentiviral vector) and the CON group (no lentiviral vector). After 24 h of incubation, the lentivirus was added to plates according to the number of cells per well and a multiplicity of infection (MOI) of 100,

150, 200 and 300. Supernatant cell fluid containing the virus was discarded 24 h after infection, and fresh medium was added. Then, the cells were cultured for 48 h. The infection and status of BMMSCs were observed by routine optical microscopy and inverted fluorescence microscopy. PHD2 gene silencing of BMMSCs was assayed for HIF-1α and PHD2 expression by western blots.

For osteogenic differentiation, the growth medium was replaced with osteogenic differentiation medium (α-MEM with 10% FBS, 0.1 μM dexamethasone, 50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphate). On Day 4 and Day 7 of osteogenic induction, the mRNA expression of osteogenesis-related parameters, including Runt-related transcription factor 2 (Runx 2), alkaline phosphatase (ALP), osteocalcin (OCN) and collagen type I (COL-1), was assayed by quantitative real-time polymerase chain reaction (q-PCR).

RNA preparation and q-PCR

The BMMSCs were treated as previously described. After PHD2 gene silencing, the cells treated with *Pg*-LPS (1 μg/mL) [24] were cultured in osteogenic culture medium. Osteogenic culture medium with *Pg*-LPS was replaced every 3 days. On Day 4 of osteogenic induction, the mRNA levels of Runx-2, COL-I, HIF-1α and VEGF were assayed by q-PCR.

Total RNA was extracted by TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, USA), and cDNA was prepared by the PrimeScript RT Reagent kit (TaKaRa Bio, Otsu, Japan). Amplification and detection of cDNA were performed using a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, USA) with primers (GenScript, China) and Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The primers used in the experiments are shown in Table 2. The relative gene expression level was normalized to that of the internal control (GAPDH) based on the $2^{-\Delta\Delta Ct}$ method.

Table 2

Primer sequences

Primer name	Forward primer sequence(5′-3′)	Reverse primer sequence(5′-3′)
GAPDH	TGAAGGGTGGAGCCAAAAG	AGTCTTCTGGGTGGCAGTGAT
HIF-1α	AAGCCCAGAGTCACTGGGACT	GTACTCACTGGGACTGTTAGGCTC
Runx-2	CAGACACAATCCTCCCCACC	GCCAGAGGCAGAAGTCAGAG
ALP	GGAGATGGATGAGGCCATCG	CGTCCACCACCTTGTAACCA
OCN	TGACCCATCTCAGAAGCAGA	ATGGCTTTCATTGGAGTTGC
COL-I	TCTGACTGGAAGAGCGGAGAG	GAGTGGGGAACACACAGGTCT
VEGF	GGCTCTGAAACCATGAACTTTCT	GCAATAGCTGCGCTGGTAGAC

Enzyme-linked immunosorbent assay (ELISA)

BMMSCs from different groups were cultured with *Pg*-LPS in osteogenic culture media as previously described. The supernatant of cells was collected on Day 4 and centrifuged at 3000 rpm/min for 10 min to remove dead cells and debris. A Quantikine ELISA kit was used to detect the concentration of VEGF (Neobioscience, China).

Western blot

The cell culture protocol was the same as that in 2.3. Total protein was extracted for western blotting on Day 7 of osteogenic induction. Protein expression levels in different groups were measured with PHD2 (Cell Signaling Technology, USA), HIF-1α (Abcam, USA), ALP (Santa Cruz, USA), Runx 2 (Abcam, USA) and COL-I (Proteintech, USA) primary antibodies, and GAPDH (Bioworld Technology, USA) expression served as an internal control. Membranes were exposed to an ECL reagent (Vazyme Biotech, China), and antibody binding was visualized using a Tanon 5200 Luminescent Imaging Workstation (Tanon, China).

ALP staining and alizarin red S (ARS) staining

BMMSCs were cultured and treated as described in previous steps at a density of 5×10^4 cells/well in 12-well plates. The cells were washed with PBS and then fixed with 4% paraformaldehyde for 30 min. ALP staining was performed on Day 7 of osteogenic differentiation. Then, the plates were stained with the BCIP/NBT alkaline phosphatase staining kit (Beyotime Institute of Biotechnology, China). Mineral deposition was performed on Day 14 of osteogenic differentiation. The cells were examined by alizarin red S (Sigma-Aldrich, USA) according to the manufacturer's instructions. The unbound dyes were washed with distilled water. All plates were examined using an inverted optical microscope (Olympus IMT-2, Tokyo, Japan), and digital images were saved.

VEGFR inhibitor treatment

After PHD2 gene silencing, the sh-PHD2+LPS group was treated with 1 μM of the VEGFR inhibitor tivozanib (Selleck, US) for 30 min; samples without inhibitor treatment served as controls. The inhibitor treatment continued in subsequent osteogenic induction experiments. Western blots and mineral deposition were measured to assess osteogenic differentiation.

Ligation-induced experimental periodontitis model

Five-week-old female Sprague–Dawley (SD) rats (weighing approximately 200 g) were maintained under specific pathogen-free conditions. All experimental procedures described in this study were approved by the Animal Ethics Committee of Nanjing University (IACUC-2003053). SD rats were randomly divided into six study groups (n = 5/group): 1) the CON group, without ligature or injection; 2) the Lig group, with ligature alone; 3) the NaCl +Lig group, with ligature and 100 μL of 0.9% NaCl injection; 4) the MSC + Lig group, with ligature and 100 μL of transplanted BMMSCs; 5) the NC + Lig group, with ligature and 100 μL of BMMSCs infected with negative control of lentiviral vector; 6) the sh-PHD2 + Lig group, with ligature and 100 μL of BMMSCs infected with lentiviral RNA interference vector. Briefly, 4-0 silk ligatures were ligated firmly and subgingivally around the left maxillary second molars of SD rats. After 2 weeks, experimental periodontitis was determined by clinical examination and HE staining. Then, the BMMSCs with different treatments were dissociated in 0.9% NaCl (1×10^6 cells/mL), and the cell suspensions were injected into the mesial, middle and distal sites of the palatal gingival tissues around the ligatured molar with a 100 μL microsyringe (Hamilton, Switzerland). After 24 h, we collected the relevant gingival tissues and processed them into frozen slices to confirm sh-PHD2 BMMSC (with green fluorescent protein) transplantation. Stem cell treatment was performed every two days. After 2 weeks of cell transplantation, all rats were sacrificed, and the left mandibles were collected for further experimental analyses.

Microcomputed tomography scanning (micro-CT)

After being placed in 4% paraformaldehyde fixative solution for 48 h, the maxillary bones of the SD rats were scanned with a micro-CT machine (Bruker, Karlsruhe, Germany). The scanning parameters were based on an acquisition protocol (70 kV, 353 μA and 18 μm voxel size). The data were reconstructed and imported into CTVox and CTAn software to obtain 3D model reconstruction and osteogenic parameters.

Histological examination

After fixation with 4% paraformaldehyde, all specimens were placed in 10% EDTA decalcifying solution (EDTA, Servicebio, China) for 2 months at room temperature. Histological sections (5 μm) were cut buccolingually for HE (Servicebio, China), Masson trichrome (Servicebio, China) and immunohistochemistry staining (Servicebio, China). Sections were scanned with Pannoramic MIDI (3DHistech, Ltd., Budapest, Hungary) and browsed with CaseViewer software (3DHistech, Ltd., Budapest, Hungary). For HIF-1α and VEGF staining, the positive area of each section was identified and quantified with ImageJ software.

Statistical analysis

All experimental data are presented as the mean ± standard deviation (SD). The differences were evaluated by unpaired t tests or one-way ANOVAs as appropriate. A two-tailed $p<0.05$ was considered statistically significant. The statistical graphs were produced with GraphPad Prism 8 (GraphPad Software, Inc., USA).

3 Results

Lentiviral vector infection of BMMSCs and PHD2 gene silencing

BMMSCs were transfected with lentivirus to silence the PHD2 gene, which promoted the accumulation of HIF-1α under normoxia (Fig. 1A). After lentiviral infection for 72 h, no significant difference was observed in the third-generation BMMSCs with an MOI of 100, 150, and 200 by routine optical microscopy. The MOI 200 group had obviously higher expression of green florescence protein than the MOI 100, MOI 150 and MOI 300 groups (Fig. 1B). Hence, MOI 200 was chosen for the following experiments. Under normoxic conditions, western blot analysis showed that the expression of PHD2 in the sh-PHD2 group was lower than that in the other groups, while the expression of downstream HIF-1α protein was significantly increased (Fig. 1C). Therefore, the above results indicated that the constructed lentiviral RNA interference vector could successfully silence the PHD2 gene of BMMSCs and activate the downstream HIF-1α-related pathway.

After the lentiviral RNA interference vector was confirmed, we predicted that PHD2 gene silencing would promote osteogenic differentiation in BMMSCs. To verify this hypothesis, we infected cells with lentivirus for 72 h and then cultured them in osteogenic induction medium. Real-time quantitative fluorescence was performed to analyse osteoblast-related molecular expression in BMMSCs in different groups. On Days 4 and 7 of osteogenic induction, the mRNA expression levels of osteogenesis-related parameters, including Runx2, ALP, OCN and COL-I, in the sh-PHD2 groups were higher than those in the other groups ($P<0.05$) (Fig. 1D). The results indicated that PHD2 gene silencing could promote osteogenic differentiation of BMMSCs.

Effect of PHD2 silencing on osteogenesis and angiogenesis of BMMSCs under inflammatory conditions

In this experiment, all groups were assessed in the inflammatory microenvironment created with *Pg*-LPS. In an inflammatory environment, the mRNA levels of Runx 2, HIF-1α and VEGF were increased in the PHD2-silenced group compared with the other groups after 4 days of osteogenic differentiation ($P<0.05$) (Fig. 2A), while no significant difference was observed regarding the mRNA expression of COL-I among the different groups.

Under the same experimental conditions, the concentration of VEGF in the supernatant was assessed. The expression of VEGF in the sh-PHD2 +LPS group was significantly higher than that in the CON+LPS group and the NC+LPS group under *Pg*-LPS stimulation ($P<0.01$) (Fig. 2B).

Compared with those of the CON group, sh-PHD2 group and NC group, the total protein expression level, ALP staining and ARS staining were decreased in the CON+LPS group, sh-PHD2 +LPS group and NC+LPS group. However, these osteogenesis-related parameters in the sh-PHD2+LPS group were higher than those in the CON+LPS group and NC+LPS group under *Pg*-LPS stimulation (Fig. 2C-D).

The effect of VEGF on osteogenesis in an inflammatory environment

To verify the role of VEGF in the osteogenic differentiation of BMMSCs with PHD2 gene silencing in an inflammatory environment, we added the VEGFR inhibitor tivozanib during osteogenic induction. The protein levels of ALP, Runx 2 and COL-I in each group were detected on Day 7 during osteogenic induction, and the results showed that osteogenic proteins in the sh-PHD2 +LPS group were significantly increased without the addition of VEGFR inhibitors compared with those of the NC+LPS group but decreased after the addition of VEGFR inhibitors (Fig. 3A). Alizarin red staining also showed deeper staining, more mineralized nodules and a larger staining area in the sh-PHD2 +LPS group than in the NC+LPS group on Day 14 during osteogenic induction, but this effect disappeared with the addition of the VEGFR inhibitor (Fig. 3B).

Effects of the PHD2-silenced BMMSCs on bone repair in the SD rats with periodontitis

The rat periodontitis model was successfully established by silk thread ligation for 2 weeks. The BMMSCs with PHD2 gene silencing were implanted into the gingiva of the rats by local injection (Fig. 4A). Frozen sections of the injected gingival tissue were observed by confocal fluorescence after 24 h. The images of GFP carried by the PHD2-silenced BMMSCs indicated that the BMMSCs had been successfully transplanted into periodontal tissue (Fig. 4B). The reconstructed images showed apparent bone absorption and furcation involvement of the second molar in the Lig group compared with the CON group. The bone absorption in the sh-PHD2 + Lig group was significantly reduced compared with that in the Lig group and the NaCl +Lig group (Fig. 4C). The relative bone volume (BV/TV) in the sh-PHD2 +Lig group was lower than that in the CON group but significantly higher than that in the Lig group ($P<0.01$), and there was no significant difference between the other ligation groups and the Lig group. The bone mineral density (BMD) was significantly higher in the sh-PHD2 +Lig, MSC+Lig and NC+Lig groups than in the Lig group ($P<0.01$), and the increase in the sh-PHD2 +Lig group was more obvious (Fig. 4D). The experimental results indicated that the

transplantation of the PHD2 gene-silenced BMMSCs could reduce the absorption of alveolar bone under periodontal inflammation and potentially promote periodontal repair to some extent..

Haematoxylin and eosin (H&E) and Masson's trichrome staining (200 μm) results showed that the periodontal tissue destruction between the first and the second molars exhibited extensive damage, including the proliferation of gingival epithelial spikes, inflammatory cell infiltration, local degeneration and fracture of collagen fibres, and the significantly decreased height of alveolar bone, in the Lig group compared with the CON group. However, the periodontal tissue damage of the sh-PHD2+Lig group was significantly reduced, and this group exhibited a small amount of inflammatory cells infiltrating the gingival tissue and an increasingly thicker morphology of the alveolar bone in the ligation area, whereas bone defects were more apparent in the other periodontitis groups. Additionally, specimen sections with Masson's trichrome staining (100 μm) revealed neovascularization at the junction between epithelium and connective tissue in the sh-PHD2+Lig group, where blood vessels had smaller diameters and were restricted to the local region (Fig. 5A).

Furthermore, immunohistochemistry indicated that HIF-1α expression between the first and second molars in the Lig group was upregulated slightly compared with that in the CON group ($P< 0.05$), which may be caused by activation of the hypoxic pathway under local inflammatory stimulation after gingival ligation. Moreover, the expression of HIF-1α in periodontal tissues in the sh-PHD2 +Lig group was more obvious than that in the Lig group ($P< 0.001$), as well as the other ligation groups (Fig. 5B). These results suggest that local injection of the PHD2-silenced BMMSCs can promote the expression of HIF-1α in periodontal inflammatory tissues of rats. Further exploration found that downstream VEGF expression in local periodontal tissues changed after HIF-1 activation. The expression of VEGF between the first and second molars in the Lig group was higher than that in the CON group ($P< 0.01$), and the sh-PHD2 +Lig group expressed higher levels of VEGF than the ligation group ($P< 0.001$) (Fig. 5C).

4 Discussion

BMMSCs are a potential resource for periodontal tissue regeneration due to their multidifferentiation capability, wide spectrum of immunoregulatory effects and ease of expansion[7]. Fair periodontal regeneration in a rat model of periodontitis was observed after local transplantation of allogeneic BMMSCs with tumour necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), and interleukin-1beta (IL-1β) expression in periodontal defects[27]. However, inflammatory cytokines and mediators released after infection or environmental stress may lead to a decrease in the osteogenic differentiation ability of stem cells[9]. *Pg*-LPS is an important pathogenic factor in the occurrence and development of periodontitis[25]. Based on the poor osteogenic differentiation and immunomodulatory properties of BMMSCs[24], 1.0 μg/mL *Pg*-LPS was chosen to create an inflammatory microenvironment in our study.

Hypoxia is a main component of the cell niche. As a master gene, HIF-1 regulates stem cell features such as multipotency and self-renewal[12]. Under normoxic oxygen levels, HIF-1α can be recognized by von Hippel-Lindau (VHL) E3 ubiquitin ligase and then degraded after hydroxylation on proline residues by PHDs. Under hypoxia, when PHD activity is suppressed, HIF-1α is stabilized and translocates to the nucleus, where it dimerizes with HIF-1β to promote the transcription of various hypoxia-inducible genes, including VEGF, EPO, and bFGF[11]. As the target gene of HIF-1, VEGF was found to be the most important growth factor for the regulation of vascular development and angiogenesis[17, 18]. The vascularization caused by VEGF can contribute to the formation of a neovascularization network and blood supply for periodontal tissue. VEGF not only promotes the secretion of cytokines such as bone morphogenetic protein (BMP) by endothelial cells but also regulates the differentiation of osteoblasts and osteoclasts and participates in osteogenesis[28]. Subsequent studies reported the effect of VEGF-VEGFR signalling pathways on the recovery from hypoxia-induced tissue damage[29], showing that HIF-1α has a possible effect on the NF-κB

pathway, thereby connecting natural immunity, inflammation, and ischaemia[30, 31]. Inhibition of PHD stabilizes and activates the hypoxia pathway by preventing proteasomal degradation of HIF-1. In proliferating chondrocytes, deletion of PHD2 elevated the expression of HIF signalling molecules and markers for chondrocyte hypertrophy and mineralization, resulting in an increased bone mass phenotype[32]. Therefore, PHDs are promising therapeutic targets. In our study, lentivirus vectors (MOI =200) silenced the PHD2 gene in BMMSCs, stabilized the expression of HIF-1α, and further upregulated the expression of osteogenic genes. However, osteogenic differentiation and mineralization of BMMSCs in the inflammatory microenvironment was significantly decreased which was improved after PHD2 gene silencing, and the difference was reduced when VEGFR inhibitors were used. These results indicate that lentiviral vector-mediated RNA interference can achieve persistent silencing of the PHD2 gene in BMMSCs and enhance the osteogenic differentiation of BMMSCs stimulated by *Pg*-LPS *in vitro*. In addition, VEGF plays a crucial role in promoting the osteogenic differentiation of BMMSCs in an inflammatory environment, but the further mechanism of VEGF on stem cells against inflammation remains to be further studied.

Based on previous *in vitro* results, we further explored the therapeutic effect of PHD2 gene-silenced BMMSCs on periodontal defects in rats. SD rats are commonly used as experimental animals in medical research due to their hardiness, rapid growth, and easy breeding. An experimental periodontitis model established by 2 weeks of silk ligation in rats could simulate realistic periodontal defects well due to local plaque accumulation[33]. Scaffold-free approaches, such as cell injection therapy, can eliminate the adverse effects of degradation scaffolds or complicated manipulation[34], which has been confirmed to effectively promote periodontal tissue regeneration in animal models[35, 36]. After sh-PHD2 BMMSCs containing GFP were locally injected into the gum of rats for approximately 24 h, green fluorescence in the local gingiva was observed to confirm the success of cell transplantation. With continuous transplantation of the cell suspension for 2 weeks, inflammatory periodontal tissue and bone resorption were significantly reduced, greater BMD and BV/TV values were observed, and higher levels of HIF-1α and VEGF in periodontal tissue were found in the sh-PHD2 + Lig group. On account of the study presented here, BMMSCs coupled with PHD2 gene silencing have the potential to generate a better outcome for periodontal repair.

To further develop the application of PHD2 gene silencing in BMMSCs, we note the limitations of our study. First, local injection of cell suspension could cause cell loss due to the fluidity of the suspension and requires repeated operation to obtain satisfactory results, which is time-consuming and laborious. Recent studies found that cell sheet tissue engineering can be used to harvest cells together with endogenous extracellular matrix (ECM) and intact cell–cell interactions, avoiding cell loss and retaining the microenvironment of the cells[37, 38]. Second, animals that are larger and more closely related to humans than SD rats should be used for more robust experiments. Finally, a variety of periodontal tissues, including the periodontal ligament and cementum, should be additionally explored.

5 Conclusion

In summary, our research demonstrated that lentiviral-mediated RNA interference had the potential to upregulate the expression of VEGF and promote the osteogenic differentiation of BMMSCs in an inflammatory environment. Additionally, PHD2-silenced BMMSCs may effectively inhibit periodontal inflammation and bone resorption. Further studies are needed to clarify the mechanism of PHD2-silenced BMMSCs on periodontal regeneration and immune regulation of the periodontal microenvironment.

Abbreviations

ANOVA: Analysis of variance; ALP: alkaline phosphatase; ARS: alizarin red S; BMMSCs: bone marrow mesenchymal stem cells; BV/TV: bone volume/ total volume; BMD: bone mineral density; bFGF: basic fibroblast growth factor; BMP:

morphogenetic protein; COL-I: collagen type I; DMEM: Dulbecco's modified Eagle' s medium; EPO: erythropoietin; ELISA: enzyme linked immunosorbent assay; ECM: endogenous extracellular matrix; FBS: fetal bovine serum; ; GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase; GFP: green fluorescent protein; HIFs: hypoxia inducible factors; HIF-1: Hypoxia-inducible factor-1; HE: hematoxylin-eosin; IL-10: interleukin-10; IFN-γ: interferon-gamma; IL-1β: interleukin-1beta; MOI: multiplicity of infection; Micro-CT: micro computed tomography; MSCs: mesenchymal stem cells; OCN: osteocalcin; PHDs: prolyl hydroxylases; PBS: phosphate buffer saline; *Pg*-LPS:porphyromonas gingivalis lipopolysaccharide; q-PCR: quantitative real-time polymerase chain reaction; Runx 2: runt-related transcription factor 2; SD rats: sprague-dawley rats; shRNA: short hairpin RNA; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor; VHL: von Hippel-Lindau; WB: western blot

Declarations

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Authors' contributions

BL was responsible for the design of the study, the acquisition and analysis of data, and the draft of the work; CC assisted in the design of the study and the revision of the manuscript; BT and DC contributed to the technique assistance and data analysis;MW and JQ assisted in the animal experiment; and FY made substantial contributions to the conception of the work and financed this research.All authors gave final approval of the manuscript and agreed to be accountable for all aspects of the work.

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Availability of data and materials

All data generated or analysed during this study are included in this article.

Ethics approval and consent to participate

All experimental protocols were approved by Medical School of Nanjing University

Consent for publication

Not applicable.

Competing interests

The authors declare no potential confict of interest.

References

1. Kinane DF, Stathopoulou PG, Papapanou PN. Periodontal diseases. Nat Rev Dis Primers. 2017;3:17038.
2. Tonetti MS, Jepsen S, Jin L, Otomo-Corgel J. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. J Clin Periodontol. 2017;44(5):456-62.
3. Ouchi T, Nakagawa T. Mesenchymal stem cell-based tissue regeneration therapies for periodontitis. Regen Ther. 2020;14:72-8.
4. Tassi SA, Sergio NZ, Misawa MYO, Villar CC. Efficacy of stem cells on periodontal regeneration: Systematic review of pre-clinical studies. J Periodontal Res. 2017;52(5):793-812.
5. Oryan A, Kamali A, Moshiri A, Baghaban Eslaminejad M. Role of Mesenchymal Stem Cells in Bone Regenerative Medicine: What Is the Evidence? Cells Tissues Organs. 2017;204(2):59-83.
6. Xu XY, Li X, Wang J, He XT, Sun HH, Chen FM. Concise Review: Periodontal Tissue Regeneration Using Stem Cells: Strategies and Translational Considerations. Stem Cells Transl Med. 2019;8(4):392-403.
7. Andrzejewska A, Lukomska B, Janowski M. Concise Review: Mesenchymal Stem Cells: From Roots to Boost. Stem Cells. 2019;37(7):855-64.
8. Zhou Y, Tsai TL, Li WJ. Strategies to retain properties of bone marrow-derived mesenchymal stem cells ex vivo. Ann N Y Acad Sci. 2017;1409(1):3-17.
9. Zhang J, Li ZG, Si YM, Chen B, Meng J. The difference on the osteogenic differentiation between periodontal ligament stem cells and bone marrow mesenchymal stem cells under inflammatory microenviroments. Differentiation. 2014;88(4-5):97-105.
10. Lee CC, Hirasawa N, Garcia KG, Ramanathan D, Kim KD. Stem and progenitor cell microenvironment for bone regeneration and repair. Regenerative medicine. 2019;14(7):693-702.
11. Strowitzki MJ, Cummins EP, Taylor CT. Protein Hydroxylation by Hypoxia-Inducible Factor (HIF) Hydroxylases: Unique or Ubiquitous? Cells. 2019;8(5).
12. Fan L, Li J, Yu Z, Dang X, Wang K. The hypoxia-inducible factor pathway, prolyl hydroxylase domain protein inhibitors, and their roles in bone repair and regeneration. Biomed Res Int. 2014;2014:239356.
13. Taylor CT, Colgan SP. Regulation of immunity and inflammation by hypoxia in immunological niches. Nat Rev Immunol. 2017;17(12):774-85.
14. Lin N, Simon MC. Hypoxia-inducible factors: key regulators of myeloid cells during inflammation. J Clin Invest. 2016;126(10):3661-71.
15. Cramer T, Yamanishi Y, Clausen BE, Förster I, Pawlinski R, Mackman N, et al. HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell. 2003;112(5):645-57.
16. Okumura CY, Hollands A, Tran DN, Olson J, Dahesh S, von Kockritz-Blickwede M, et al. A new pharmacological agent (AKB-4924) stabilizes hypoxia inducible factor-1 (HIF-1) and increases skin innate defenses against bacterial infection. J Mol Med (Berl). 2012;90(9):1079-89.
17. Yellowley CE, Genetos DC. Hypoxia Signaling in the Skeleton: Implications for Bone Health. Curr Osteoporos Rep. 2019;17(1):26-35.
18. Fu L, Zhang L, Zhang X, Chen L, Cai Q, Yang X. Roles of oxygen level and hypoxia-inducible factor signaling pathway in cartilage, bone and osteochondral tissue engineering. Biomed Mater. 2021;16(2):022006.

19. Esfahani M, Karimi F, Afshar S, Niknazar S, Sohrabi S, Najafi R. Prolyl hydroxylase inhibitors act as agents to enhance the efficiency of cell therapy. Expert Opin Biol Ther. 2015;15(12):1739-55.
20. Wu C, Zhou Y, Fan W, Han P, Chang J, Yuen J, et al. Hypoxia-mimicking mesoporous bioactive glass scaffolds with controllable cobalt ion release for bone tissue engineering. Biomaterials. 2012;33(7):2076-85.
21. Rios CN, Skoracki RJ, Mathur AB. GNAS1 and PHD2 short-interfering RNA support bone regeneration in vitro and in an in vivo sheep model. Clin Orthop Relat Res. 2012;470(9):2541-53.
22. Cui D, Chen C, Luo B, Yan F. Inhibiting PHD2 in human periodontal ligament cells via lentiviral vector-mediated RNA interference facilitates cell osteogenic differentiation and periodontal repair. Journal of Leukocyte Biology. 2021;110(3):449-59.
23. Chen C, Li H, Jiang J, Zhang Q, Yan F. Inhibiting PHD2 in bone marrow mesenchymal stem cells via lentiviral vector-mediated RNA interference facilitates the repair of periodontal tissue defects in SD rats. Oncotarget. 2017;8(42):72676-99.
24. Tang J, Wu T, Xiong J, Su Y, Zhang C, Wang S, et al. Porphyromonas gingivalis lipopolysaccharides regulate functions of bone marrow mesenchymal stem cells. Cell Prolif. 2015;48(2):239-48.
25. Bostanci N, Belibasakis GN. Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen. FEMS microbiology letters. 2012;333(1):1-9.
26. Kato H, Taguchi Y, Tominaga K, Umeda M, Tanaka A. Porphyromonas gingivalis LPS inhibits osteoblastic differentiation and promotes pro-inflammatory cytokine production in human periodontal ligament stem cells. Arch Oral Biol. 2014;59(2):167-75.
27. Du J, Shan Z, Ma P, Wang S, Fan Z. Allogeneic bone marrow mesenchymal stem cell transplantation for periodontal regeneration. J Dent Res. 2014;93(2):183-8.
28. Grosso A, Burger MG, Lunger A, Schaefer DJ, Banfi A, Di Maggio N. It Takes Two to Tango: Coupling of Angiogenesis and Osteogenesis for Bone Regeneration. Front Bioeng Biotechnol. 2017;5:68.
29. Ramakrishnan S, Anand V, Roy S. Vascular endothelial growth factor signaling in hypoxia and inflammation. J Neuroimmune Pharmacol. 2014;9(2):142-60.
30. Eltzschig HK, Bratton DL, Colgan SP. Targeting hypoxia signalling for the treatment of ischaemic and inflammatory diseases. Nat Rev Drug Discov. 2014;13(11):852-69.
31. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003;17(14):2115-7.
32. Cheng S, Xing W, Pourteymoor S, Schulte J, Mohan S. Conditional Deletion of Prolyl Hydroxylase Domain-Containing Protein 2 (Phd2) Gene Reveals Its Essential Role in Chondrocyte Function and Endochondral Bone Formation. Endocrinology. 2016;157(1):127-40.
33. de Molon RS, Park CH, Jin Q, Sugai J, Cirelli JA. Characterization of ligature-induced experimental periodontitis. Microsc Res Tech. 2018;81(12):1412-21.
34. Carmagnola D, Tarce M, Dellavia C, Rimondini L, Varoni EM. Engineered scaffolds and cell-based therapy for periodontal regeneration. J Appl Biomater Funct Mater. 2017;15(4):e303-e12.
35. Li G, Han N, Zhang X, Yang H, Cao Y, Wang S, et al. Local Injection of Allogeneic Stem Cells from Apical Papilla Enhanced Periodontal Tissue Regeneration in Minipig Model of Periodontitis. Biomed Res Int. 2018;2018:3960798.

36. Lu L, Liu Y, Zhang X, Lin J. The therapeutic role of bone marrow stem cell local injection in rat experimental periodontitis. J Oral Rehabil. 2020;47 Suppl 1:73-82.
37. Chen M, Xu Y, Zhang T, Ma Y, Liu J, Yuan B, et al. Mesenchymal stem cell sheets: a new cell-based strategy for bone repair and regeneration. Biotechnol Lett. 2019;41(3):305-18.
38. Kobayashi J, Kikuchi A, Aoyagi T, Okano T. Cell sheet tissue engineering: Cell sheet preparation, harvesting/manipulation, and transplantation. J Biomed Mater Res A. 2019;107(5):955-67.

Figures

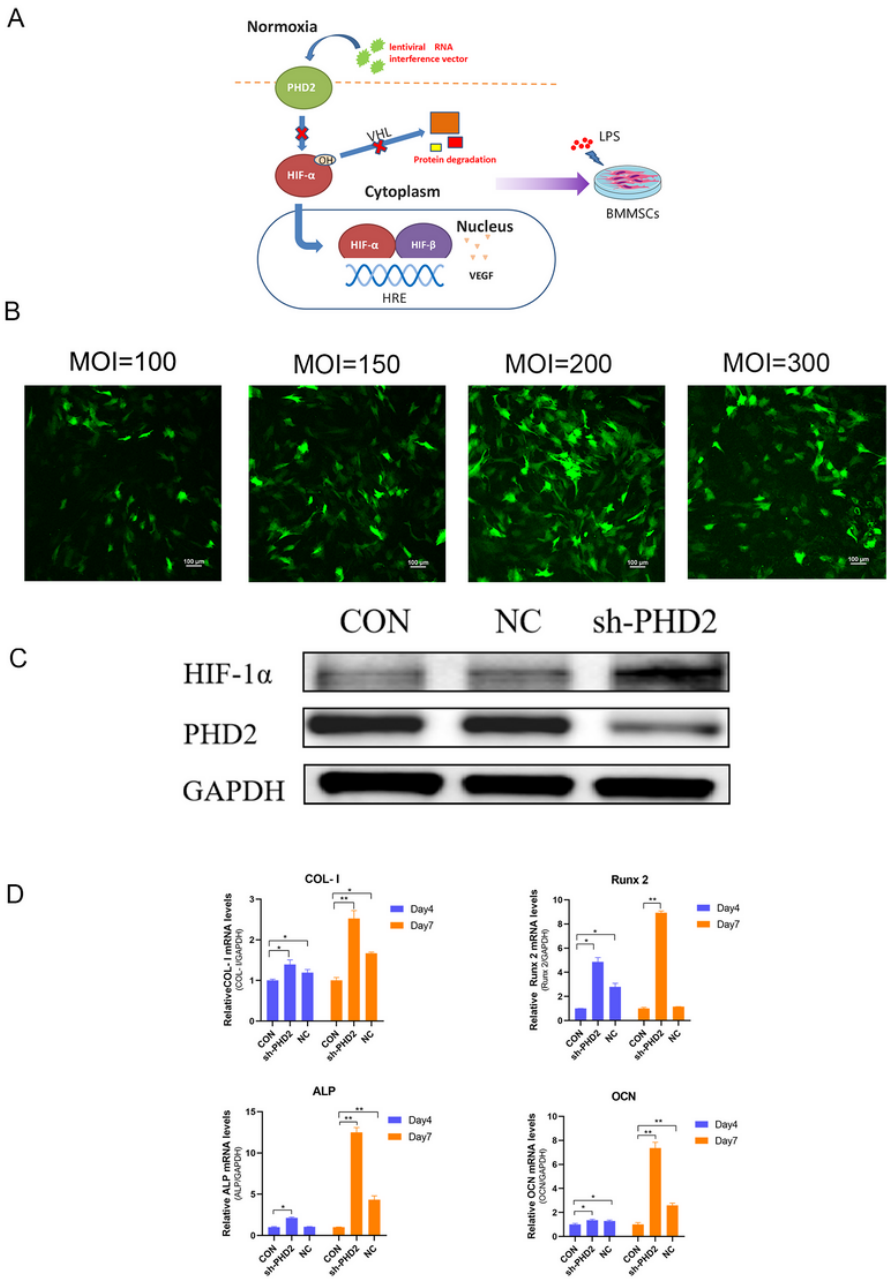

Figure 1

Prolyl hydroxylase domain-containing protein 2 (PHD2) gene silencing in bone marrow mesenchymal stem cells (BMMSCs). (A) Schematic diagram of the cell processing method. (B) Fluorescence images of BMMSCs transfected with the lentiviral vector at different MOI values ranging from 100 to 200 (×40). (C) The protein levels of PHD2 and HIF-1α after 72 h of transfection under normoxic conditions. (D) On Days 4 and 7 of osteogenic induction, mRNA expression of osteogenesis-related genes, including COL-I, Runx 2, ALP and OCN, was determined in the different groups. *, $P < 0.05$; **, $P < 0.01$.

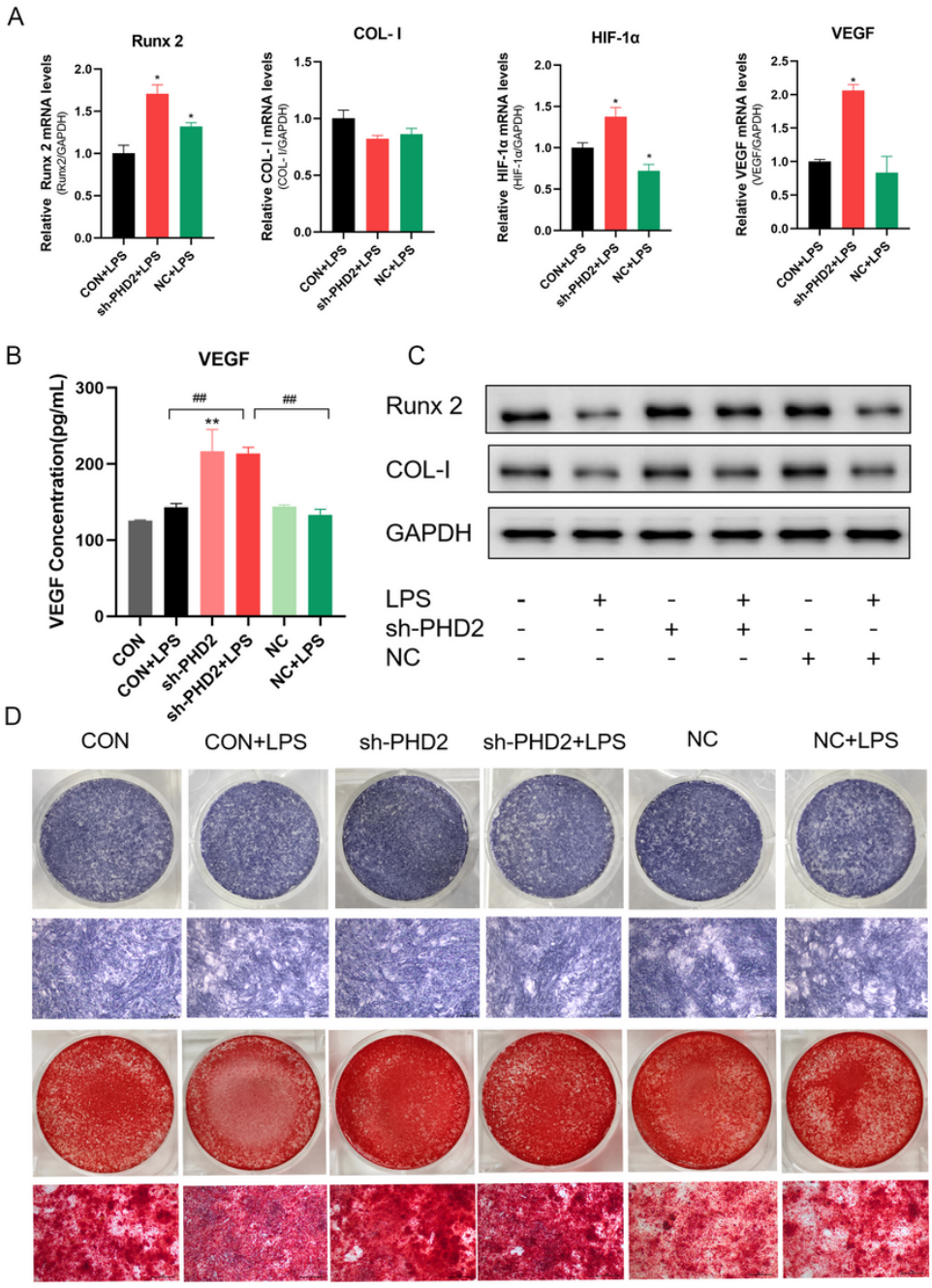

Figure 2

PHD2 gene silencing affects the expression levels of osteogenesis-related and angiogenesis-related parameters in BMMSCs stimulated by *Pg*-LPS. (A) On Day 4 of osteogenic induction, the mRNA expression levels of Runx 2, COL-I, HIF-1α and VEGF in the different groups were determined. (B) ELISAs showed VEGF secretion in the culture medium.

(C) On Day 7 of osteogenic induction, total protein levels of osteogenesis-related parameters, including COL-I and Runx 2, in the different groups were determined. (D) ALP staining on Day 7 and ARS staining on Day 14 of osteogenic induction was performed (×40). * $P < 0.05$, ** $P < 0.01$, compared with the control group; ## $P < 0.01$.

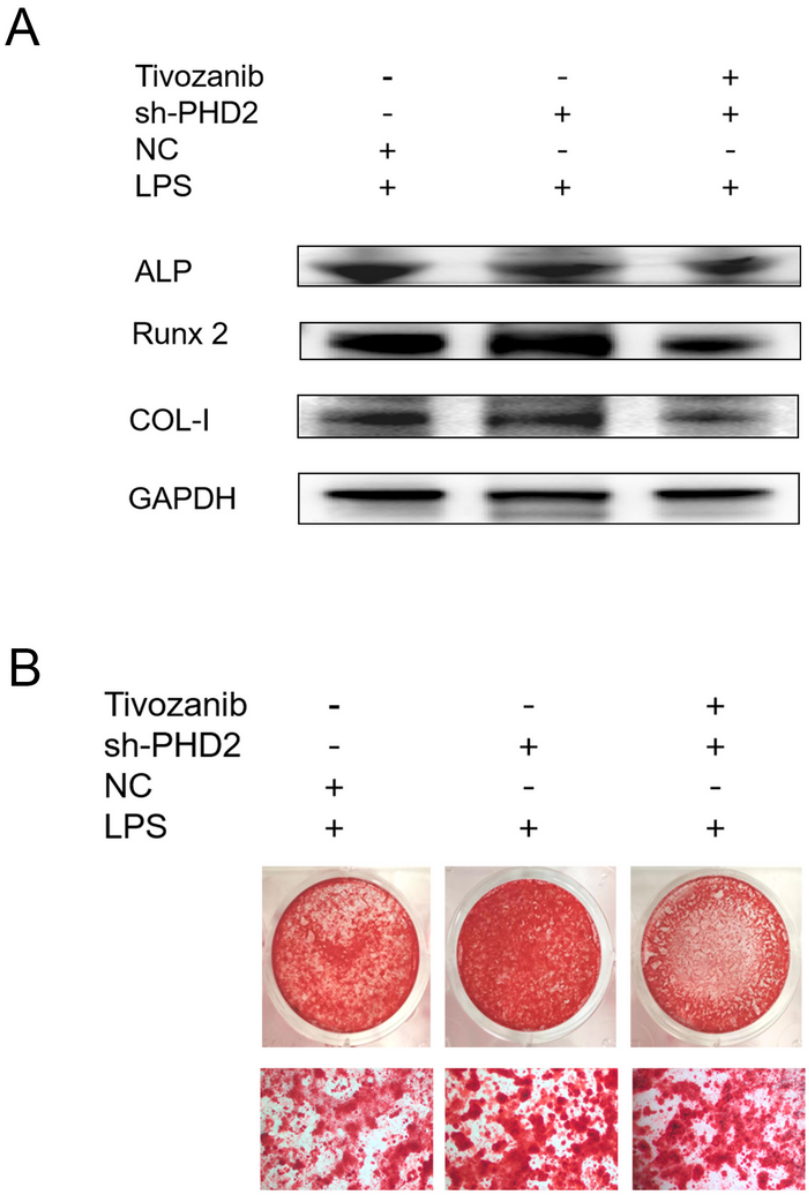

Figure 3

Effect of treatment with the VEGFR inhibitor tivozanib on the osteogenic differentiation of the PHD2-silenced BMMSCs in an inflammatory environment. After addition of the VEGF receptor inhibitor, (A) the expression of osteogenic proteins on Day 7 of osteogenic induction and (B) the formation of mineralized nodules on Day 14 of osteogenic induction in the different groups treated with *Pg*-LPS were determined.

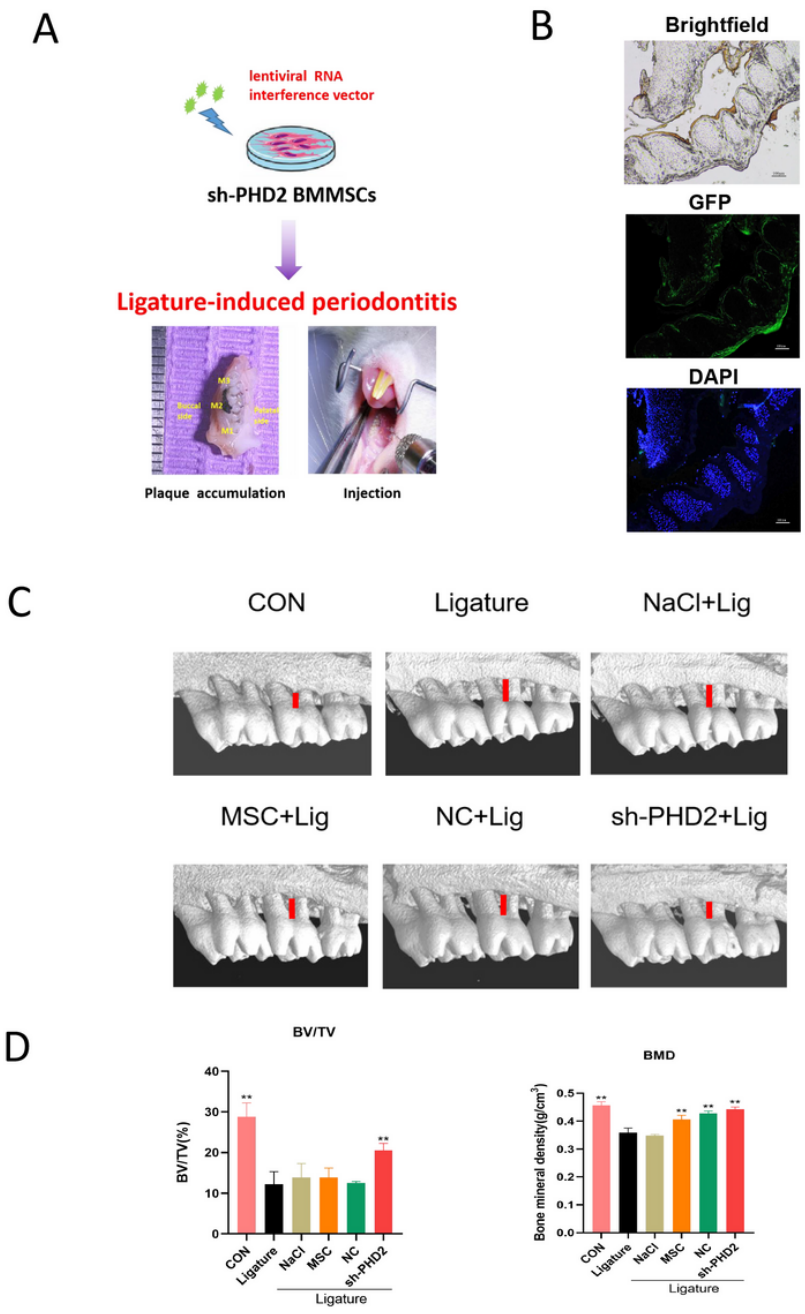

Figure 4

Ligature-induced experimental periodontitis and stem cell transplantation. (A) Illustration of the animal experiments. (B) Fluorescence images of gingiva after transfection. (C) Micro-CT reconstruction images. (D) The ratio of bone volume to tissue volume and bone mineral density. $^{**}P < 0.01$, compared with the ligature group.

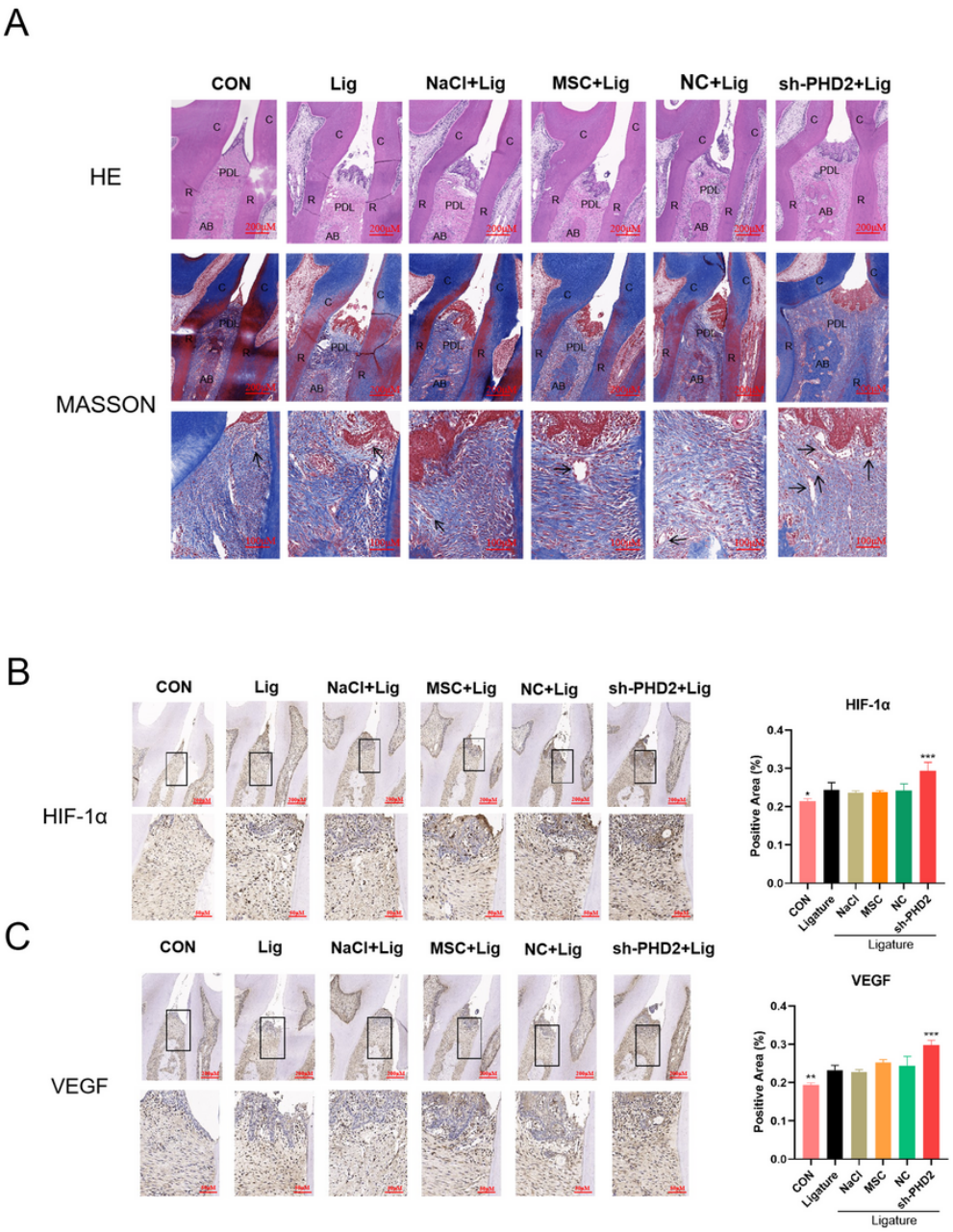

Figure 5

Histological observation of the ligatured areas in SD rats. (A) H&E and Masson's trichrome staining were conducted to observe the morphological changes of the periodontal tissues, including gingival tissues, alveolar bone, and the periodontal ligament (C: crown; R: root; AB: alveolar bone; PDL: periodontal ligament; scale bar 200 μm). And microscopic observation of neovascularization was futher observed. (black arrows: new blood vessels; scale bar 100 μm). Positive expression of HIF-1α (B) and VEGF (C) in the different groups and their semiquantitative analysis in the different groups (scale bar 100 μm and 50 μm).*P< 0.05, compared with the ligature group. **P< 0.01, compared with the ligature group. ***P< 0.001, compared with the ligature group.