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PHD2 shRNA-Modified Bone Marrow Mesenchymal Stem Cells Facilitate Periodontal Bone Repair in Response to Inflammatory Condition

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Objective: *To investigate whether bone marrow mesenchymal stem cells (BMMSCs) modulate periodontal bone repair through the hydroxylase domain-containing protein 2 (PHD2)/hypoxia-inducible factor-1 (HIF-1) signalling pathway in response to inflammatory conditions.*
Methods: *Osteogenic differentiation of PHD2 shRNA-modified BMMSCs and the possible mechanism were explored in an inflammatory microenvironment stimulated by porphyromonas gingivalis lipopolysaccharide (Pg-LPS) in vitro. The effect of PHD2 gene-modified BMMSCs on periodontal bone loss was evaluated with experimental periodontitis.*
Results: *Pg-LPS stimulation greatly impaired the osteogenic differentiation of BMMSCs, whereas the silence of PHD2 significantly enhanced the osteogenesis of BMMSCs. More importantly, increased level of vascular endothelial growth factor (VEGF) was detected under Pg-LPS stimulation, which was verified to be associated with the augmented osteogenesis. In experimental periodontitis, PHD2-modified BMMSCs transplantation elevated osteogenic parameters and the expression of VEGF in periodontal tissue.*
Conclusion: *This study highlighted that PHD2 gene silencing could be a feasible approach to combat inflammatory bone loss by rescuing the dysfunction of seed cells.*
Keywords: *BMMSCs, HIF-1, inflammation, PHD2, VEGF*
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Periodontitis is an inflammatory and destructive disease in periodontal tissue caused by microbial pathogens.[1,2] Most treatments for periodontitis have limited regeneration effectiveness due to the unpleasant inflammatory microenvironment.[3,4] Increasing evidence indicates that bone marrow mesenchymal stem cells (BMMSCs) have major advantages, especially immunomodulatory properties, in promoting new periodontal tissue.[5-7] However, patients with periodontitis suffer from an inflammatory environment that not only reduces the number of local mesenchymal stem cells (MSCs), but also impairs the function of autologous MSCs, hindering tissue regeneration.[8,9] Hence, it is imperative to improve the function and survival of MSCs in the periodontal microenvironment.[10]

The αβ-heterodimeric transcription factor, hypoxia-inducible factor-1 (HIF-1), has been investigated extensively due to its ability to enhance the self-renewal, proliferation and post-homing differentiation of stem cells.[11,12] The stability of HIF-1 and the alterations in gene expression caused by hypoxia have a significant impact on the microenvironment of inflammatory tissues and the outcomes of diseases.[13-16] Vascular endothelial growth factor (VEGF), which is regulated by HIF-1, is one of the earliest known angiogenic factors and functions as a pro-survival factor to safeguard cells damaged by inflammation.[17] The overexpression

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of VEGF in human MSCs led to an increase in the deposition of mineralised extracellular matrix and the promotion of tissue mineralisation through an autocrine pathway.[18]

In normoxia, prolyl hydroxylases (PHDs) induced proteasomal degradation of HIF-1, with PHD2 being the primary enzyme responsible for downregulating HIF-1α expression.[11] Recently, PHD inhibitors have shown promise in cell-based therapy and bone tissue engineering.[19-21] The use of lentivirus-based vectors has been found to provide stable expression of genes in transplanted cells. Earlier studies by the present authors' 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, the present authors hypothesised that the PHD2/HIF-1 signalling pathway might also modulate the osteogenic process of PHD2-silenced BMMSCs in an inflammatory microenvironment.

Porphyromonas gingivalis lipopolysaccharide (*Pg*-LPS) is known to play a significant role in the pathogenesis of periodontitis, exhibiting high toxicity and antigenicity towards periodontal tissue.[24-26] In the present study, *Pg*-LPS was utilised to induce an inflammatory microenvironment in vitro, with the aim of investigating whether the osteogenic differentiation of PHD2 gene-modified BMMSCs could be enhanced under such conditions. Furthermore, the potential impact of rat BMMSCs with PHD2 gene modification on periodontal regeneration was assessed in ligature-induced experimental periodontitis models. All experimental procedures described were approved by the Animal Ethics Committee of Nanjing University (IACUC-2003053).

Materials and methods

Cell culture

Primary BMMSCs of Sprague-Dawley (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 medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% foetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (HyClone, Logan, UT, USA) at 37°C in 5% CO_2. The cells were used between passages 2 and 4.

Lentiviral vector infection of BMMSCs

Construction and sequencing of plasmids, packaging and purification of lentiviral vectors were performed by a commercial source (GenePharma, Shanghai, China). The short hairpin RNA (shRNA) interference sequence and the method of lentiviral vector infection of BMMSCs are based on a previous report by the present authors.[23] 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. BMMSCs were divided into three 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). Then, the cells were cultured for 48 hours. 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 blot (WB).

Osteogenic differentiation

After PHD2 gene silencing, 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). The osteogenic culture medium was replaced every 3 days.

To stimulate the inflammatory microenvironment in vitro, BMMSCs were treated with *Pg*-LPS (1 μg/ml)[24] for 72 hours. The osteogenic culture medium with *Pg*-LPS was replaced every 3 days. On day 4 of osteogenic induction, the mRNA levels of VEGF were assayed by q-PCR. On day 7 of osteogenic induction, the total protein levels of osteogenesis-related parameters were measured by WB.

RNA preparation and q-PCR

Total RNA was extracted by TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA), and cDNA was prepared using 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) with primers (GenScript, Nanjing China) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The primers used in the experiments are shown in Table 1. The relative gene expression level was normalised to that of the internal control (GAPDH) based on the $2^{-\Delta\Delta Ct}$ method.

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Table 1 Primer sequences.

Primer name	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
GAPDH	TGAAGGGTGGAGCCAAAAG	AGTCTTCTGGGTGGCAGTGAT
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/minute for 10 minutes to remove dead cells and debris. A Quantikine ELISA kit was used to detect the concentration of VEGF (Neobioscience, Shenzhen, China).

WB analysis

Total protein was extracted by RIPA lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the concentration of protein was determined using a BCA protein Assay Kit (Beyotime Institute of Biotechnology). Proteins of the same quantity were separated by SDS-PAGE (Genscript) and transferred to a PVDF membrane, and the membranes were blocked with 5% bovine albumin (Sigma, Louis, MO, USA). Protein expression levels in different groups were measured with PHD2 (Cell Signaling Technology, Danvers, MA, USA), HIF-1α (Abcam, USA), ALP (Santa Cruz, CA, USA), Runx 2 (Abcam, USA) and COL-I (Proteintech, Rosemont, IL, USA) primary antibodies, and GAPDH (Bioworld Technology, St Louis Park, MN, USA) expression served as an internal control. Membranes were exposed to an ECL reagent (Vazyme Biotech, Nanjing, China), and antibody binding was visualised using a Tanon 5200 Luminescent Imaging Workstation (Tanon, Shanghai, China).

ALP 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 minutes. ALP staining was performed on day 7 of osteogenic differentiation, then the plates were stained with a BCIP/NBT alkaline phosphatase staining kit (Beyotime Institute of Biotechnology). Mineral deposition was performed on day 14 of osteogenic differentiation. The cells were examined by ARS staining (Sigma-Aldrich, St. Louis, MO, 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. The percentage of mineralized nodules and staining areas were calculated using Image J software (National Institutes of Health, Bethesda, MD, USA).

VEGFR inhibitor treatment

After PHD2 gene silencing, the sh-PHD2+LPS group was treated with 1 µM of the VEGFR inhibitor tivozanib (Selleck Chemicals, Houston, TX, USA) for 30 minutes[27]; samples without inhibitor treatment served as controls. The inhibitor treatment continued in subsequent osteogenic induction experiments. WB analysis and mineral deposition were measured to assess osteogenic differentiation.

Ligation-induced experimental periodontitis model

Five-week-old female SD rats (weighing approximately 200 g) were maintained under specific pathogen-free conditions. They were randomly divided into four study groups (n = 5): 1) the Lig group, with ligature alone; 2) the MSC+Lig group, with ligature and 100 µL of transplanted BMMSCs; 3) the NC+Lig group, with ligature and 100 µL of BMMSCs infected with negative control of lentiviral vector; and 4) 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 rats' maxillary left second molars. The ligature process was conducted for up to 14 days in the periodontitis model while pathogenic bacteria of periodontitis were enriched in the ligature silk. 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, Bonaduz, Switzerland). Stem cell treatment was performed every 2 days. After 2 weeks of cell transplantation, all rats were sacrificed, and the left maxillary bones were collected for further experimental analysis.

Microcomputed tomography (microCT) scanning

After being placed in 4% paraformaldehyde fixative solution for 48 hours, the maxillary bones of the SD rats were scanned with a microCT machine (Bruker, Karlsruhe,

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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, Wuhan, China) for 2 months at room temperature. Histological sections (5 μm) were cut buccolingually for haematoxylin and eosin (HE), Masson trichrome and immunohistochemistry staining (all Servicebio). Sections were scanned with Panoramic MIDI (3DHistech, Budapest, Hungary) and examined with CaseViewer software (3DHistech). For VEGF staining, the positive area of each section was identified and quantified with Image J software.

Statistical analysis

All experimental data are presented as mean ± standard deviation (SD). After normalisation evaluation, the differences were evaluated by one-way ANOVA. A two-tailed $P < 0.05$ was considered statistically significant. The statistical graphs were produced with GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

Results

Effect of PHD2 silencing on osteogenesis of BMMSCs under inflammatory conditions

After lentiviral infection for 72 hours, the MOI 200 group had obviously higher expression of green florescence protein than the MOI 100, MOI 150 and MOI 300 groups, and cells grew well in radial shape (Fig 1a). Hence, MOI 200 was chosen for the following experiments. Under normoxic conditions, WB analysis showed that the constructed lentiviral RNA interference vector could successfully silence the PHD2 gene of BMMSCs and activate the downstream HIF-1α-related pathway (Fig 1b).

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, sh-PHD2+LPS and NC+LPS groups; however, these osteogenesis-related parameters in the sh-PHD2+LPS group were higher than those in the CON+LPS and NC+LPS groups under *Pg*-LPS stimulation (Fig 1c to e).

The angiogenic factor VEGF associated with the augmented osteogenesis in an inflammatory environment

In the inflammatory microenvironment, the mRNA levels of VEGF and the concentration of VEGF in the supernatant in the sh-PHD2+LPS group were significantly increased (Fig 2a). To explore the role of VEGF in sh-PHD2-promoted osteogenesis in BMMSCs in an inflammatory environment, the VEGFR inhibitor tivozanib was added during osteogenic induction. On day 7 during osteogenic induction, the osteogenesis-related protein expression in the sh-PHD2+LPS group was increased significantly compared with that of the NC+LPS group but decreased after the addition of VEGFR inhibitors (Fig 2b). ARS also showed deeper staining, more mineralised 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 2c).

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

The present authors applied a well-established model of chronic periodontitis in rats to examine the effect of PHD2 silencing on its onset, which showed extensive periodontal tissue destruction between the first and second molars, including the proliferation of gingival epithelial spikes and the significantly decreased height of alveolar bone (supplementary material, provided on request). Then, BMMSCs with PHD2 gene silencing were implanted into the gingiva of the experimental periodontitis rats by local injection (Fig 3a). The reconstructed images showed apparent bone resorption and furcation involvement of the second molar in the Lig group. The bone resorption in the sh-PHD2+Lig group exhibited a significant reduction in comparison to that of the Lig group and the remaining ligation groups (Fig 3b). The relative bone volume (BV/TV) in the sh-PHD2+Lig group was notably higher than that of the Lig group and the other ligation groups, with no variance observed between the remaining ligation groups and the Lig group. The results indicated that bone mineral density (BMD) was elevated in the sh-PHD2+Lig, MSC+Lig and NC+Lig groups compared with the Lig group. Specifically, the increase in BMD was most pronounced in the sh-PHD2+Lig group, followed by the MSC+Lig group and then the NC+Lig group (Fig 3c).

HE and Masson trichrome staining showed that periodontal tissue damage was significantly reduced in the sh-PHD2+Lig group, which exhibited only a small amount of inflammatory cell infiltration and minor

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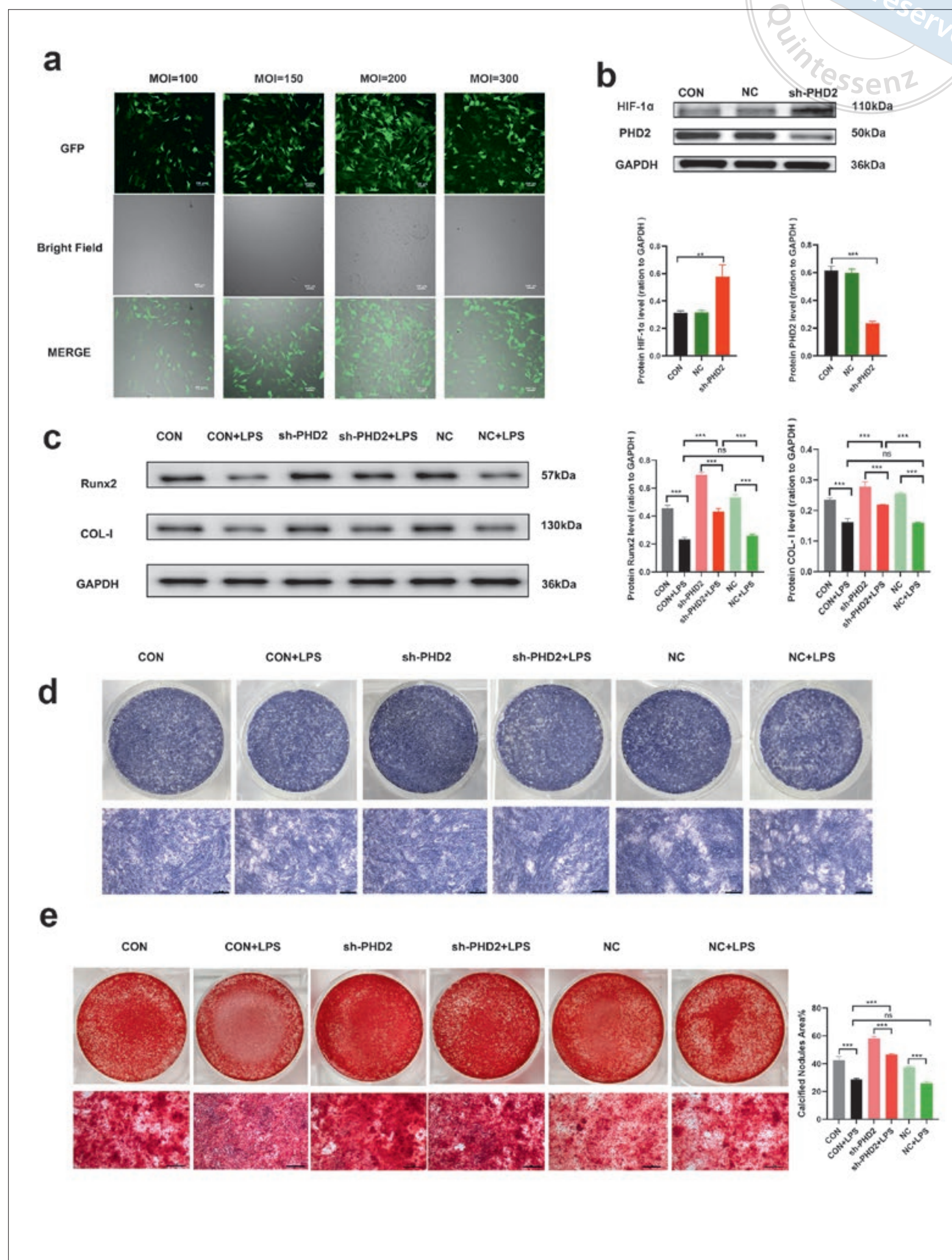

Fig 1a to e PHD2 gene silencing affects the expression of osteogenesis-related parameters in BMMSCs stimulated by *Pg*-LPS. Fluorescence images of BMMSCs transfected with the lentiviral vector at different MOI values ranging from 100 to 300 (scale bar 100 µm) **(a)**. The protein levels of PHD2 and HIF-1α after 72 hours of transfection under normoxic conditions **(b)**. 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 **(c)**. ALP staining was performed on day 7 (scale bar 200 µm) **(d)** and ARS staining was performed on day 14 of osteogenic induction (scale bar 50 µm) **(e)**. $^{**}P < 0.01$, $^{***}P < 0.001$.

changes in the gingival epithelium, periodontal ligament fibres and alveolar bone. In contrast, periodontal damage was more pronounced in the other ligation groups (Fig 4a).

Furthermore, immunohistochemistry indicated that HIF-1α and VEGF expression in local periodontal tissues changed. The sh-PHD2+Lig group expressed the highest levels of HIF-1α and VEGF between the first and second molars (Fig 4b and c).

Discussion

BMMSCs are a potential resource for periodontal tissue regeneration due to their multidifferentiation capability, wide spectrum of immunoregulatory effects and ease of expansion.[6,7] 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)

Fig 2a to c Effect of the angiogenic factor VEGF on the osteogenic differentiation potential of PHD2-silenced BMMSCs in an inflammatory environment. The mRNA expression levels and secretion of VEGF of different groups: after addition of the VEGF receptor inhibitor **(a)**, the expression of osteogenic proteins was performed on day 7 **(b)** and ARS staining was performed on day 14 of osteogenic induction (scale bar 50 µm) **(c)**. $^{**}P < 0.01$, $^{***}P < 0.001$.

and interleukin-1beta (IL-1β) expression in periodontal defects.[28] 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 µg/ml *Pg*-LPS was chosen to create an inflammatory microenvironment in the present 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] When PHD activity is suppressed, HIF-1α is stabilised and promotes the transcription of several hypoxia-inducible genes, including VEGF, erythropoietin (EPO) and basic fibroblast growth factor (bFGF).[11] As the target gene of HIF-1, VEGF was found to be the most important growth factor coupling angiogenesis and osteogenesis.[17,18] The vascularisation caused by VEGF can contribute

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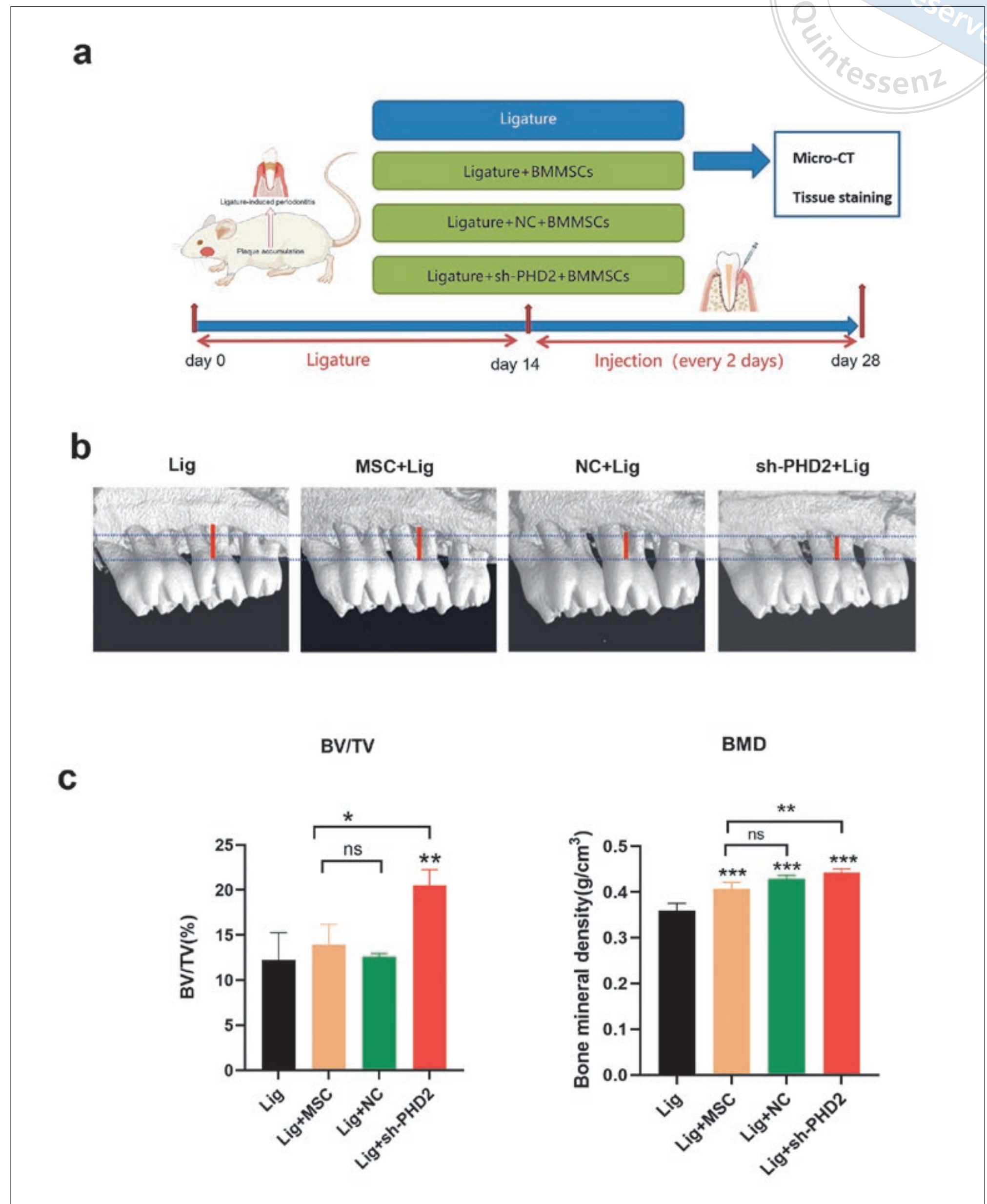

Fig 3a to c Ligature-induced experimental periodontitis and stem cell transplantation. Illustration of the animal experiments. After ligation of the maxillary left second molar in rats to establish the experimental periodontitis model, PHD2-silenced BMMSCs transplantation was performed locally **(a)**. MicroCT reconstruction images showed the alveolar bone loss of the maxillary second molar (red line: the cementoenamel junction to the alveolar bone crest distance) **(b)**. Parameters to evaluate new bone: BV/TV and BMD **(c)**. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

to the formation of a neovascularisation network and blood supply for periodontal tissue. More interestingly, osteoblast differentiation is directly affected by VEGF via autocrine and intracrine mechanisms. It was shown that VEGF stimulated osteoblast differentiation through an intracellular mechanism involving the transcription factors Runx 2 and PPARV2, but not through paracrine signalling.[29] Subsequent studies reported the effect of VEGF-VEGFR signalling pathways on the recovery from hypoxia-induced tissue damage,[30] showing that HIF-1α has a possible effect on the NF-κB pathway, thereby connecting natural immunity, inflammation and ischaemia.[31,32] In the present study, osteogenic differentiation of BMMSCs in the inflammatory microenvironment was significantly decreased, and was improved after PHD2 gene silencing. Meanwhile VEGF level was increased in BMMSCs after PHD2 silencing, which was confirmed to be associated with enhanced osteogenesis. These results indicate that persistent silencing of the PHD2 gene could 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 further mechanisms of VEGF on stem cells against inflammation remain to be studied.

Fig 4a to c Histological observation of the ligatured areas in SD rats. HE and Masson trichrome staining were performed 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; black arrow, alveolar ridge crest; scale bar 200 μm) **(a)**. Positive expression of HIF-1α in the different groups and their quantitative analysis (scale bar 200 and 50 μm) **(b)**. Positive expression of VEGF in the different groups and their quantitative analysis (scale bar 200 and 50 μm) **(c)**. $^{**}P < 0.01$, $^{***}P < 0.001$.

Based on previous in vitro results, the present authors further explored the therapeutic effect of PHD2 gene-silenced BMMSCs on periodontal defects in rats. The ligation-induced experimental periodontitis model is a traditional approach to induce periodontitis similar to that in humans caused by microbial plaque accumulation. An experimental periodontitis model established by 2 weeks of silk ligation in rats could simulate periodontal inflammation and bone loss due to microbiome shifts.[33] Cell injection therapy can eliminate the adverse effects of degradation scaffolds or complicated manipulation,[34] and has been confirmed to effectively promote periodontal tissue regeneration in animal models.[35,36] With continuous transplantation of the cell suspension for 2 weeks, inflammatory periodontal tissue and bone resorption were significantly reduced, greater BV/TV and BMD values were observed, and higher levels of HIF-1α and VEGF in periodontal tissue were found in the sh-PHD2+Lig group. For BMD, the difference between the sh-PHD2+Lig group and

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the NC+Lig group was not significant, probably due to the limited experimental time or the limited effect of PHD2 silencing on the mineralisation degree of the new alveolar bone during inflammation, which still needs further exploration. Moreover, the expression of HIF-1α in periodontal tissues in the sh-PHD2+Lig group was more obvious than that in the Lig group, as well as the other ligation groups. These results suggest that local injection of the PHD2-silenced BMMSCs can promote the expression of HIF-1α in periodontal inflammatory tissues in rats. Further exploration revealed that downstream VEGF expression in local periodontal tissues changed after HIF-1 activation. Based on the present study, 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, the present authors note the limitations of this study. 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 and intact cell–cell interactions, avoiding cell loss and retaining the microenvironment of the cells.[37,38] In addition, periodontal ligament and cementum regeneration, beyond the alveolar bone, are the two vital and challenging parts of periodontitis therapy that should be explored further.

Conclusion

In summary, the present study demonstrates 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. (This manuscript was preprinted on research square URL https://doi.org/10.21203/rs.3.rs-1291789/v1.)

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Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Bin Yan LUO and Shu Yu CHENG contributed to the conception, design, data acquisition, analysis and interpretation, drafting and revision of the manuscript; Drs Wen Zheng LIAO, Bao Chun TAN and Di CUI contributed to technical assistance and data analysis; Drs Min WANG and Jun QIAN contributed to the data acquisition and manuscript revision; and Drs Chang Xing CHEN and Fu Hua YAN contributed to the conception, design and manuscript revision. All the authors gave their final approval of the manuscript and agreed to be accountable for all aspects of the work.

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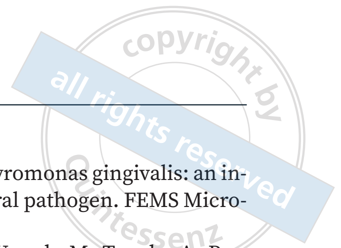

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