SPANDIDOS PUBLICATIONS

EXPERIMENTAL AND THERAPEUTIC MEDICINE 29: 33, 2025

Effect of overexpression of *GRN* on the proliferation and osteogenic capacity of human periodontal cells

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Received July 2, 2024; Accepted October 16, 2024

DOI: 10.3892/etm.2024.12783

Abstract. A human periodontal ligament cell line stably overexpressing the granulin precursor gene (*GRN*) was established through lentiviral mediation to explore the effects of *GRN* on the proliferation and osteogenic capacity of human periodontal ligament cells (hPDLCs). In the present study, a homologous recombinant lentiviral plasmid, pLV-GRN, was constructed and transfected into the hPDLCs. The expression levels of *GRN* and progranulin were assessed using reverse transcription-quantitative (RT-q)PCR and western blotting and the effect of *GRN* on the proliferative capacity was determined using the MTT assay. The osteogenic capacity of human periodontal cells overexpressing *GRN* was evaluated. The results showed that successful construction and transfection of the homologous recombinant lentiviral plasmid pLV-GRN led to the development of a stable periodontal cell line overexpressing *GRN*. The MTT assay results revealed an enhanced proliferative capacity in the pLV-GRN group compared with that in the hPDLCs and pLV-puro groups (P<0.05). Alizarin red staining and alkaline phosphatase (ALP) activity assays indicated a significantly increased osteogenic capacity in the pLV-GRN group compared with the hPDLCs and pLV-puro groups (P<0.01). RT-qPCR demonstrated strong expression of the osteogenic genes ALP, runt-related transcription factor 2 (Runx2) and osteopontin (OPN) in the periodontal cells of the pLV-GRN group (P<0.05), whereas western blotting results corroborated the high expression of the osteogenic genes Runx-2 and OPN in the periodontal cells of the pLV-GRN group (P<0.05). In summary, the overexpression of *GRN* significantly enhanced the proliferation and osteogenic capacity of hPDLCs. These findings provide an experimental foundation for periodontal tissue regeneration.

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Key words: periodontal ligament cells, granulin precursor, lentivirus, cell proliferation, osteogenic differentiation

Introduction

Periodontitis is a chronic and progressive inflammatory disease that affects the supporting tissues of the periodontium and primarily results from the invasion of specific microorganisms. This microbial intrusion into the periodontal tissues ultimately leads to tooth loosening and loss (1,2). Periodontitis is a risk factor for various systemic diseases, including diabetes mellitus and coronary heart disease (3), which has a prevalence of 45-50% overall, with the severe form affecting 11.2% of the general population (4). Consequently, there has been a growing focus on the prevention and treatment of periodontitis.

The advent of tissue engineering techniques has opened new avenues for addressing periodontitis (5,6). Tissue engineering involves three crucial elements: Seed cells, growth factors and scaffolding materials, with seed cells as the core components (7). In 2004, Seo *et al* (8) first isolated multipotent stem cells from the periodontal ligament, confirming the multipotential differentiation, robust self-renewal and self-repair capacity of human periodontal ligament cells (hPDLCs). These cells can differentiate into various cell types under specific conditions, thereby facilitating true periodontal tissue regeneration. Additionally, hPDLCs exhibit low immunogenicity, post-implantation stability, adaptability to the implant environment and minimal harm to the body (9), making them the most promising stem cell populations for regenerative periodontal therapy. Currently, hPDLCs are the most widely used seed cells for periodontal tissue engineering. However, the inflammatory microenvironment caused by cytokines, such as TNF-α and IL-1, limits the proliferation and osteogenic capacity of hPDLCs (10). Therefore, targeted antagonism of TNF-α or its receptors is crucial for reducing inflammatory factors and enhancing the proliferation and osteogenesis of hPDLCs.

Progranulin (PGRN), also known as pro-epithelin, 88 kDa glycoprotein (GP88) or PC cell-derived growth factor and granulin-epithelin precursor (11), is a growth factor encoded by the *granulin precursor* (*GRN*) gene with anti-inflammatory and osteogenic effects. It plays pivotal roles in various physiological and pathological processes such as early embryonic development, inflammation (12,13), wound healing (14), tumorigenesis and neurological disorders. In inflammatory response, PGRN inhibits neutrophil activation and proteolytic enzyme secretion by antagonizing TNF-α. In the context of anti-inflammatory mechanisms, the macrophage-derived factor secretory leukocyte protease inhibitor interacts with the inner domain of PGRN, providing protection against cleaving enzymes, such as proteinase 3 and elastase (15-17). Furthermore, studies have revealed that recombinant PGRN proteins and TNF-α competitively bind to tumor necrosis factor receptor (TNFR), leading to the antagonism of TNF-α and inhibition of the inflammatory response (17,18).

PGRN can induce mesenchymal stem cells to differentiate into cartilage. As a major downstream molecule of bone morphogenetic protein 2 (BMP2) (19), PGRN activates ERK1/2 signaling and the transcription factor JunB, which play significant roles in cartilage formation. Additionally, PGRN knockout mice exhibit dwarfism and severe skeletal defects, emphasizing the essential role of PGRN in skeletal development (20-22).

Periodontal regeneration involves recruiting endogenous stem cells to the defect site and using bioactive factors with anti-inflammatory and tissue-repair properties to enhance stem cell proliferation and differentiation. In contrast to TNF-α inhibitors that directly stimulate osteogenic differentiation (23), PGRN acts by antagonizing TNF-α and serving as a downstream protein of BMP2, promoting the osteogenic differentiation of cells. However, the direct use of exogenous growth factors presents challenges, such as high cost, complex protein extraction, short *in vivo* half-life, susceptibility to degradation by proteases and limited functionality (24,25). This often necessitates the use of large quantities of recombinant proteins, leading to increased economic costs and potentially excessive dosages as a side effect.

To address these challenges, researchers have proposed gene therapy, which relies on effective gene transfection and expression methods, as an innovative approach for periodontal regeneration (26). Viral vectors, particularly lentiviral or adenoviral vectors, are highly efficient and safe delivery vehicles for transferring exogenous genes to target cells (27,28). By transfecting target cells with these vectors, specific genes can be overexpressed or knocked down, enabling cells to proliferate and undergo osteogenic differentiation at the genetic level. Consequently, the present study hypothesized that lentivirus-mediated *GRN* could promote the proliferation and osteogenesis of periodontal ligament cells and aimed to construct a human periodontal cell line stably overexpressing *GRN* using the lentiviral method. Subsequently, it focused on assessing the effect of *GRN* overexpression on the proliferation and osteogenic capacity of hPDLCs, offering a theoretical foundation for advancing the understanding of periodontal regeneration.

Materials and methods

Isolation, culture and identification of hPDLCs. The study protocol was approved by the Ethics Committee of Lanzhou University School of Stomatology (approval no. LZUKQ-2019-045). A 28-years-old volunteer with good oral health donated four intact caries-free premolars that were extracted due to orthodontic treatment and used for PDLCs isolation; written informed consent was obtained from the patient. Extracted teeth were placed in α-MEM (Gibco; Thermo Fisher Scientific, Inc.) containing 2% penicillin-streptomycin solution (PS; Gibco; Thermo Fisher Scientific, Inc.) and hPDLCs extraction was completed within 2 h. The teeth were rinsed from root to crown with PBS containing 10% double antibody and the periodontal membrane tissue from the middle 1/3 of the isolated root was scraped and collected in a tube, which was added to 1 ml of α-MEM and then centrifuged at 100 x g for 5 min at 4˚C. After discarding the supernatant, collagenase type I (Gibco; Thermo Fisher Scientific, Inc.) was added and the tissue was digested for 15 min in an incubator at 37˚C. Following centrifugation at 100 x g for 5 min at 4˚C, trypsin was aspirated and 5 ml of complete medium [1% PS; 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 89% α-MEM] was added. The cells were cultured for 3-5 days in a cell culture incubator at 37˚C and the cell culture flask was removed to observe cell growth under an optical electron microscope (magnification, x40). The medium was changed every 2 days after cell adhesion to the flask and these cells were recorded as primary cells (P_0) (19).

Finally, the third hPDLCs passage (P_3) exhibiting well-grown were seeded into six-well plates and cultured in an incubator for 1 day. After fixation with 4% paraformaldehyde for 10 min at 37˚C, 0.1% Triton X-100 (Millipore Sigma) was added and incubated for 20 min at room temperature. Cells were washed three times with PBS and each well was treated with 300 µl of 5% BSA blocking solution and allowed to stand for 1 h at room temperature. After aspirating the liquid, anti-vimentin (1:1,000 dilution; rabbit; cat. no. ab137321; Abcam) and anti-keratin (1:1,000 dilution; cat. no. ab8068 mouse; Abcam) antibodies were added and incubated overnight at 4˚C. Following antibody removal, the cells were rinsed three times with TBST (0.1% Tween-20) and the mouse anti-rabbit IgG (HRP) secondary antibody (1:5,000 dilution; cat. no. ab99697; Abcam) was added and incubated at room temperature for 1 h in the dark. Following TBST rinsing, cell nuclei were stained with DAPI at room temperature for 10 min. Subsequently, the DAPI solution was discarded, the cells were washed three times with PBS, the slides were mounted and the entire cell area was observed under an inverted fluorescence microscope (magnification, x20). Based on immunofluorescence staining, protein staining characteristics of hPDLCs were therefore observed in 15 random fields of view at x20 magnification.

Lentiviral construction of a stable periodontal cell line overexpressing pLV-GRN. Primers were designed according

Table I. Primer sequences.

Gene	Primer (5'-3')	Accession number
GRN forward	CCCTCGAGGCCACCATGTGGACCCTGGTGAGCTG	NM_002087.4
GRN reverse	GGTGCTAGCTTAAGCGTAGTCTGGGACGTCGTATGGGTACAGCAGCTGTC	
ALP forward	CCACGTCTTCACATTTGGTG	NM_000478.6
ALP reverse	AGACTGCGCCTGGTAGTTGT	
Runx2 forward	CACTATCCAGCCACCTTTAC	NM_001015051.4
Runx2 reverse	CACTCTGGCTTTGGGAAGAG	
OPN forward	TGAAACGAGTCAGCTGGATG	NM_000582.3
OPN reverse	TGAAATTCATGGCTGTGGAA	
β-actin forward	GAAACTACCTTCAACTCCATC	NM_001101.5
β-actin reverse	CTAGAAGCATTTGCGGTGGAC	

ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; OPN, osteopontin.

to the gene sequence of *GRN* in NCBI (NM_002087.4), as shown in Table I. The *Xho*1 restriction site and protective bases were added to the forward primer and the *Nhe*1 restriction site, HA tags and protective bases were added to the reverse primer. All components were added following the manufacturer's instructions for the PrimerSTAR Max kit (Invitrogen; Thermo Fisher Scientific, Inc.). Specific PCR amplification of the coding sequence of the *GRN* gene was conducted, followed by nucleic acid electrophoresis detection and the target bands were excised and recovered. The PCR products of *GRN* and pLV-puro plasmid (Addgene, Inc.) underwent double digestion with the restriction enzymes *Xho*1 (New England BioLabs, Inc.) and *Nhe*1 (New England BioLabs, Inc.) The gel mixture was incubated at 50-60˚C for 10 min, according to the instructions for the GeneJET gel extraction kit (Thermo Fisher Scientific, Inc.). The gel was recovered, ligated with T4 ligase (New England BioLabs, Inc.) and the ligated product was combined with DH5α (Invitrogen; Thermo Fisher Scientific, Inc.) receptor cells for transformation. The plasmid was extracted using the TIANprep Mini Plasmid Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The plasmid was identified by double digestion with *Xho*1 and *Nhe*1 and then sent to Tsingke Biotechnology Co., Ltd. for sequencing.

293T cells (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China) were cultured in T25 culture flasks. Upon reaching 80% confluence, cell transfection was performed using the jetPRIME Transfection Reagent Kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. A total of 300 μl of jetPRIME buffer was added to a sterile EP tube and combined with 6 μg of pLV-puro or pLV-GRN plasmid, 4 μg of psPAX2 plasmid (Addgene, Inc.) and 2 μg of pMD2.G plasmid (Addgene, Inc.). To this mixture, 12 μl of jetPRIME reagent was added, thoroughly mixed and left at room temperature for 15 min. The resulting solution was added dropwise to a 293T cell culture flask, followed by the addition of 5 ml of complete medium. The medium was changed after 12 and 36 h later, the supernatant was aspirated, centrifuged at 100 x g for 5 min at 4˚C, filtered through a 0.22 μm filter, collected into a centrifuge tube, aliquoted into 1 ml EP tubes and stored at -80˚C (29).

Finally, P_3 hPDLCs were divided into hPDLCs, pLV-puro and pLV-GRN groups and inoculated into T25 culture flasks (n=3). The medium was changed when the cell confluence reached 70-80% and then 1 ml of the collected pLV-puro or pLV-GRN lentiviral plasmids was added dropwise to the corresponding flasks, mixed and cultured for 24 h at 37˚C. Based on the resistance screening concentration determined by the previous research, the final concentration of 5 μg/ml polybrene (Gibco; Thermo Fisher Scientific, Inc.) was added to the culture medium. After 3-4 days, when, upon microscopic investigation, most cells in the blank group appeared to have disintegrated and died, the polybrene concentration was halved and screening continued. After ~7 days, when all cells in the blank group had died, the cells that remained adherent to the wall in the PLV-puro and pLV-GRN groups were successfully infected and continued to be cultured and passaged. The experiment for constructing an hPDLCs line overexpressing *GRN* using lentivirus was repeated at least three times.

Reverse transcription-quantitative (RT-q) PCR of the mRNA expression level of GRN. After the confluence of cells reached 90%, total RNA was extracted from P_5 cells in the hPDLCs group, pLV-puro group and pLV-GRN group, followed by reverse transcription into cDNA using the PrimerScript RT Master Mix Reverse Transcription Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Gene primers were designed with β-actin serving as an internal reference (Table I). qPCR amplification was performed using 2X SYBR Green QPCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. PCR conditions were as follows: 95˚C for 5 min, followed by 30 cycles at 95˚C for 30 sec, 58˚C for 20 sec and 72˚C for 20 sec. Each experiment was repeated in three sets (n=3). Following the completion of the reaction, melting curves were analyzed and the data were processed based on $2^{-\Delta\Delta Cq}$ and the experiment was repeated three times (30,31).

Western blot analysis of GRN protein expression. Total protein from P_5 cells in the hPDLCs, pLV-puro and pLV-GRN groups was extracted (Laemmli buffer 2x; cat. no. S3401; MilliporeSigma) and protein quantification was performed using a BCA kit (n=3) following the manufacturer's instructions. The cells were evenly scraped with a cell scraper and the mixed solution was transferred to a 2 ml EP tube and ultrasonically lysed for 2 min and centrifuged at 16,000 x g for 10 min at 4˚C. The supernatant was added to a new 2 ml EP tube. Meanwhile, 5x loading Buffer (Thermo Fisher Scientific, Inc.) was added in a ratio of 1:4 and was denatured at 95˚C for 10 min, and finally the protein was stored at -20˚C. The pre-thawed protein was added to the electrophoresis tank (10% SDS-PAGE) at 12 µl per well and electrophoresed for 2.5 h (80 V 30 min, 120 V 120 min; n=3). Afterward, the membrane was transferred in the order of sponge-filter paper gel-PVDF membrane-filter paper-sponge, and the program was set to 2,000 mA for 120 min. Then, the PVDF membrane was placed in 15 ml of 5% skimmed milk powder and blocked on a shaker for 3 h. After blocking, the desired gene bands were cut into diluted anti-GRN (1:1,000 dilution; rabbit; cat. no. ab191211; Abcam) and β-actin (1:1,000 dilution; rabbit; cat. no. ab8227; Abcam) at 4˚C overnight. The next day, the strip was removed and washed three times with 15 ml TBST (0.1% Tween-20) for 10 min each time. After washing, the strip was placed in the goat anti-rabbit (IgG) secondary antibody HRP (1:5,000 dilution; cat. no. ab6721; Abcam) and incubated for 1 h at room temperature on a shaker. Then, the strip was washed three times with 15 ml TBST for 10 min each time. Finally, after adding the developer solution to the stripes, the image could be exposed and saved by the exposure instrument. The whole process of western blot experiment was replicated for three times. ImageJ 2.2.0-beta6 software (National Institutes of Health) was used to determine the protein grayscale values and the experiment was repeated three times (32).

MTT assay of cell proliferative capacity. P_2 hPDLCs were digested with 0.25% trypsin, counted and inoculated into 96-well plates at a density of $1x10^4$ cells/ml and 100 µl per well with three replicates per group (n=3). Culturing was conducted for 1-7 days at a constant temperature of 37˚C with 5% CO_2. After aspirating the medium, 10 µl of MTT labeling reagent (Abcam) was added to each well. Subsequently, 100 µl of lysis buffer was added to each well after continuous culture for 4 h and then mixed thoroughly on a shaker and the absorbance (OD) value at 450 nm was determined with a spectrophotometer. Each experiment was performed in triplicate (33).

Alizarin Red staining. P_6 cells from the hPDLCs, pLV-puro and pLV-GRN groups were inoculated into 35 mm^2 dishes, with three replicates per group (n=3). After the cells adhered to the surface, 2 ml of osteogenic induction solution was added and continuous culturing was performed for 21 days with the solution changed every 2 days. On the 7th, 14th and 21st day, the medium was aspirated and washed with PBS for three times and 300 µl of 4% paraformaldehyde was added to each group of cells for 30 min at room temperature. After aspirating the paraformaldehyde, the cells were washed three times with PBS and 1 ml of alizarin red solution (Procell Life Science & Technology Co., Ltd.) was added to each group of cells, allowed to stand for 20 min and observed the entire cell area under an optical electron microscope (magnification, x4) (19).

Determination of alkaline phosphatase (ALP) activity. Cells from the hPDLCs, pLV-puro and pLV-GRN groups were seeded into 96-well plates after 7, 14 and 21 days of induction and ALP activity was assessed using an ALP activity assay kit (Procell Life Science & Technology Co., Ltd.) according to the manufacturer's instructions. The absorbance (OD) of each well was measured at 405 nm using a spectrophotometer and a standard curve was constructed. The ALP activity of each cell group was calculated using an enzyme activity assay. Each experiment was performed in triplicate (34).

RT-qPCR determination of mRNA expression levels of osteogenesis-related genes. Total After the confluence of cells reached 90%, RNA was extracted from cells in the hPDLCs group, pLV-puro group and pLV-GRN group after induction for 7, 14 and 21 days, with three replicates per group (n=3) and reverse transcribed into cDNA using the PrimerScript RT Master Mix Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. PCR conditions were as follows: 95˚C for 5 min, followed by 30 cycles at 95˚C for 30 sec, 58˚C for 20 sec and 72˚C for 20 sec. Primers for osteogenesis-related genes, including ALP, runt-related transcription factor 2 (Runx2) and osteopontin (OPN), are listed in Table I. qPCR amplification was performed according to the instructions for 2X SYBR Green QPCR Master Mix. Each experiment for each gene was conducted in triplicate, with β-actin serving as an internal reference. The relative mRNA expression of each gene was calculated according to $2^{-\Delta\Delta Cq}$ (30,31).

Western blot determination of osteogenesis-related protein expression levels. Total protein was extracted from cells in each group following osteogenic induction for 7, 14 and 21 d, with three replicates per group (n=3). Protein quantification was conducted using the BCA kit according to the manufacturer's instructions. Western blot analysis was performed using GADPH as an internal reference to assess the expression of osteogenesis-related proteins, including OPN (1:400 dilution; rabbit; cat. no. ab8448; Abcam), Runx-2 (1:200 dilution; rabbit; cat. no. ab114133; Abcam) and GADPH (1:1,000 dilution; rabbit; cat. no. ab263962; Abcam). The specific method was the same as that in Western blot analysis of GRN protein expression. ImageJ 2.2.0-beta6 software (National Institutes of Health) was used to determine the grayscale values of proteins (32).

Statistical analysis. SPSS Statistics for Windows (version 26.0; IBM Corp.) was used for the statistical analysis. One-way ANOVA and two-way ANOVA followed by Tukey's post hoc test were used to analyze the differences. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Culture and identification of primary hPDLCs. After 5 days of culture, black clusters of periodontal ligament tissue were observed under an inverted microscope. Cells could

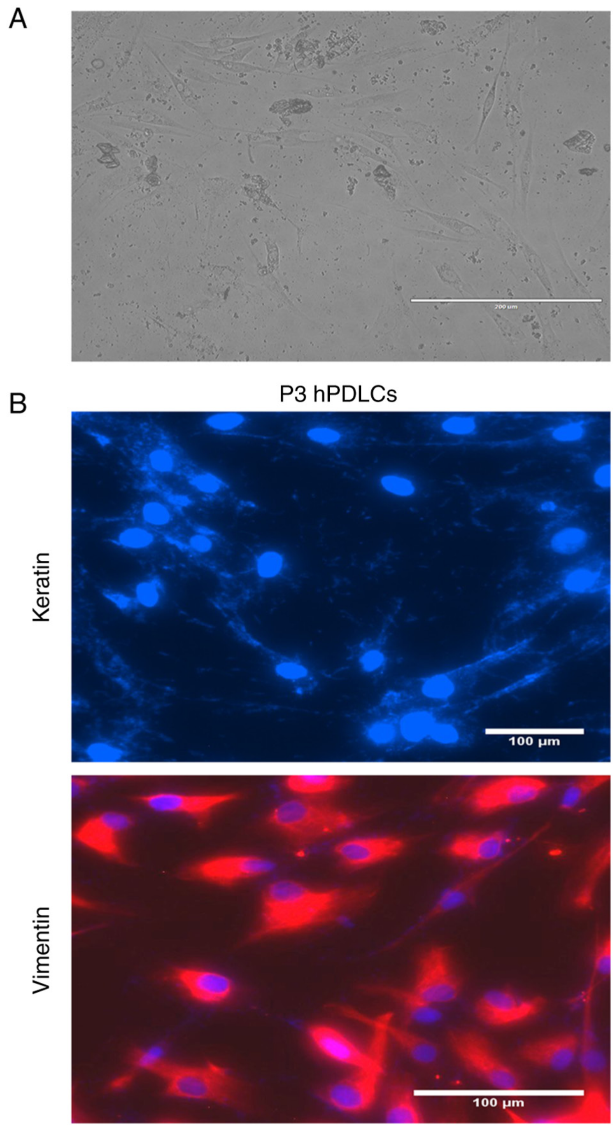

Figure 1. Culture and identification of hPDLCs. (A) Optical electron microscopy image of P_0 hPDLCs (magnification, x20). (B) Fluorescence microscopy images of vimentin and keratin, with vimentin being positive and keratin being negative (magnification, x20). hPDLCs, human periodontal ligament cells.

be observed expanding around the tissue mass, which were spindle or spindle-shaped, uniform in shape and full of cytoplasm (Fig. 1A). The immunofluorescence staining of P_3 hPDLCs was negative, indicating that the extracted cells were not contaminated with epithelial cells (Fig. 1B). Positive vimentin staining indicated that the cells were derived from the mesenchyme (Fig. 1B). The cells were characterized as hPDLCs based on immunofluorescence staining.

Identification of pLV-GRN recombinant plasmid by double digestion. The electrophoresis results showed a distinct band at ~1,866 bp (Fig. 2A). Following double digestion of the homologous recombinant plasmid pLV-GRN with *Xho*I and *Nhe*I, electrophoresis displayed clear bands at ~1,866 and 7,800 bp (Fig. 2B), which were consistent with the size of the 1,866 bp *GRN* gene along with its HA tag. The sequencing results were aligned with the cDNA sequence of the *GRN* gene (NM_002087.4) in the NCBI database (Fig. 2C). The sequence demonstrated 100% homology with the target sequence, confirming the successful construction of the recombinant plasmid.

Screening results of hPDLCs overexpressing GRN. After periodontal ligament cells were infected with the lentivirus for 24 h (Fig. 3A), the cell morphology in the hPDLCs, pLV-puro and pLV-GRN groups remained unchanged, maintaining a radial or spiral arrangement. After 7 days of puromycin resistance screening, microscopic examination revealed numerous cell fragments in the hPDLCs group, indicating the loss of intact cell morphology. Varying degrees of cell disintegration and death were observed in the pLV-puro and pLV-GRN groups. However, a small number of hPDLCs with surface adherence persisted, exhibiting an unaltered long spindle or fusiform shape. At this stage, cells with normal morphology, clear edges and continued adherence observed under the microscope were identified as hPDLCs that were successfully infected with the lentiviral vector.

RT-qPCR and western blot analysis of GRN expression. RT-qPCR results revealed robust expression of the *GRN* gene in the pLV-GRN group, which was 80-90 times higher than that in the hPDLCs and pLV-puro groups (P<0.01; Fig. 3B).

With β-actin as the internal reference, western blotting results demonstrated the expression of HA-tagged protein, indicating strong PGRN expression in periodontal cells transfected with *GRN*. Protein expression assays showed a significant enhancement in PGRN protein expression in the pLV-GRN group, which was ~1.8 times higher than that in the hPDLCs and pLV-puro groups (P<0.05; Fig. 3C).

Comparison of cell proliferation capacity. The MTT assay results indicated a similar increase in the cell proliferation rate for all groups cultured for 12-72 h. The hPDLCs group did not differ significantly from the pLV-puro group; however, the cell proliferation capacity of the pLV-GRN group was significantly higher (Fig. 4A; P<0.05).

ALP activity assay. The ALP activity assay results revealed that the ALP activity of the hPDLCs, pLV-puro and pLV-GRN groups peaked at 14 days. The ALP activity of the pLV-GRN group was significantly higher than that of the other two groups (P<0.01) and subsequently declined but remained higher than that of the hPDLCs and pLV-puro groups (P<0.05). No significant differences were observed between the hPDLCs and pLV-puro groups (Fig. 4B).

Alizarin red staining. At 7 days after osteogenic induction, no obvious orange-red mineralized nodules were observed in the hPDLCs (Fig. 4Ca), pLV-puro (Fig. 4Cb), or pLV-GRN (Fig. 4Cc) groups. After osteogenic induction for 14 days, orange-red mineralized nodules were observed in the hPDLCs (Fig. 4Cd), pLV-puro (Fig. 4Ce) and pLV-GRN (Fig. 4Cf) groups. The pLV-GRN group exhibited more mineralized nodules than the other two groups. After 21 days of osteogenic induction, the pLV-GRN group (Fig. 4Ci) produced more orange-red mineralized nodules than the hPDLCs (Fig. 4Cg) and pLV-puro groups (Fig. 4Ch) and there was no significant

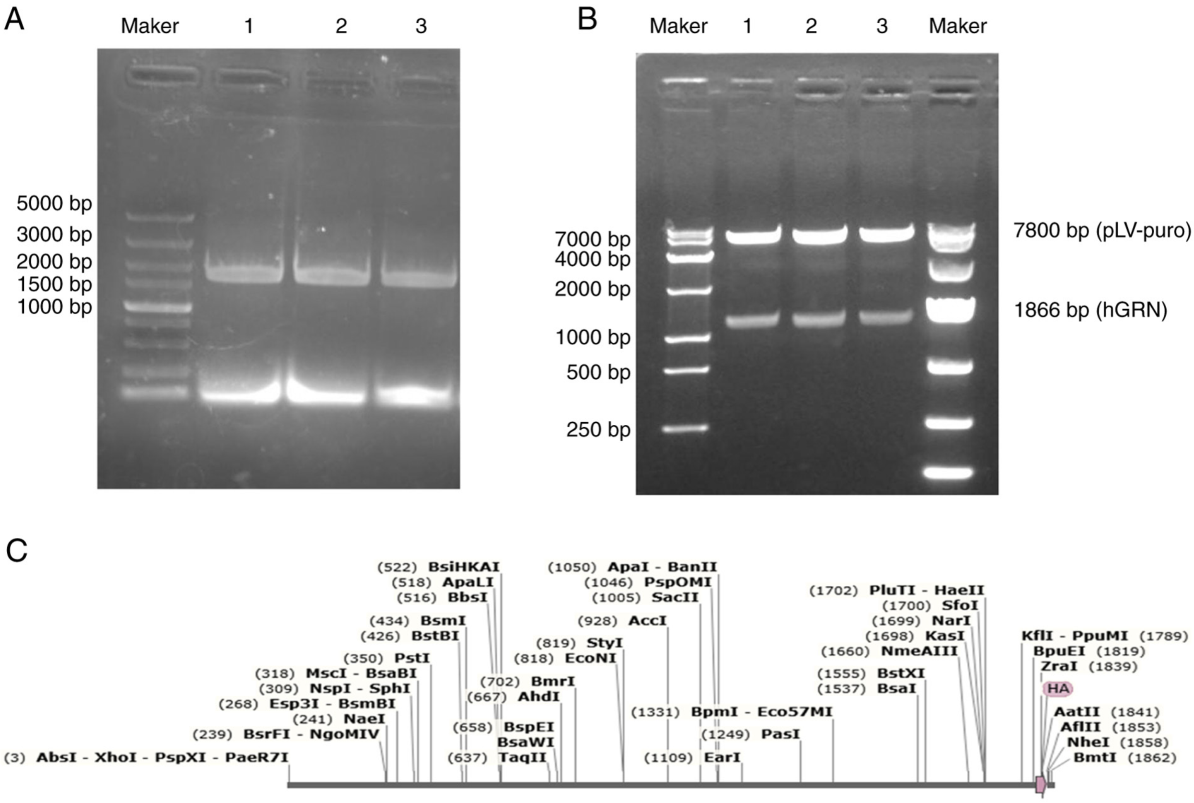

Figure 2. Identification of lentivirus recombinant plasmid pLV-GRN. (A) GRN gene amplification bands. (B) pLV-GRN double restriction enzyme electrophoresis map. (C) Sequencing results. pLV, lentivirus recombinant plasmid; GRN, granulin precursor gene.

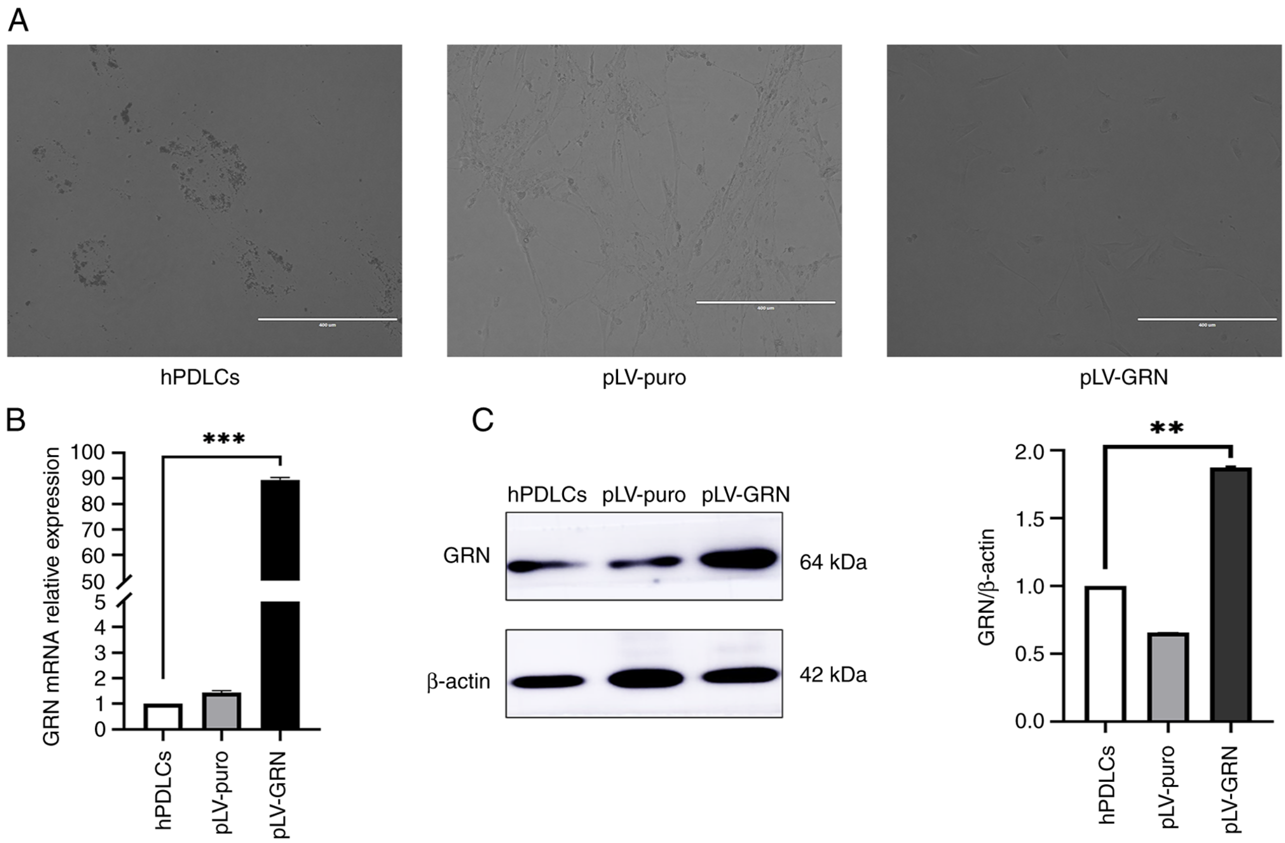

Figure 3. pLV-GRN transfection of hPDLCs. (A) Lentiviral infection screening results (magnification, x40). (B) RT-qPCR results of the relative expression of *GRN* in hPDLCs after transfection (***P<0.01). (C) Western blotting results of the relative expression of *GRN* in hPDLCs following transfection (**P<0.05). pLV, lentivirus recombinant plasmid; GRN, granulin precursor gene; hPDLCs, human periodontal ligament cells; RT-qPCR, reverse transcription-quantitative PCR.

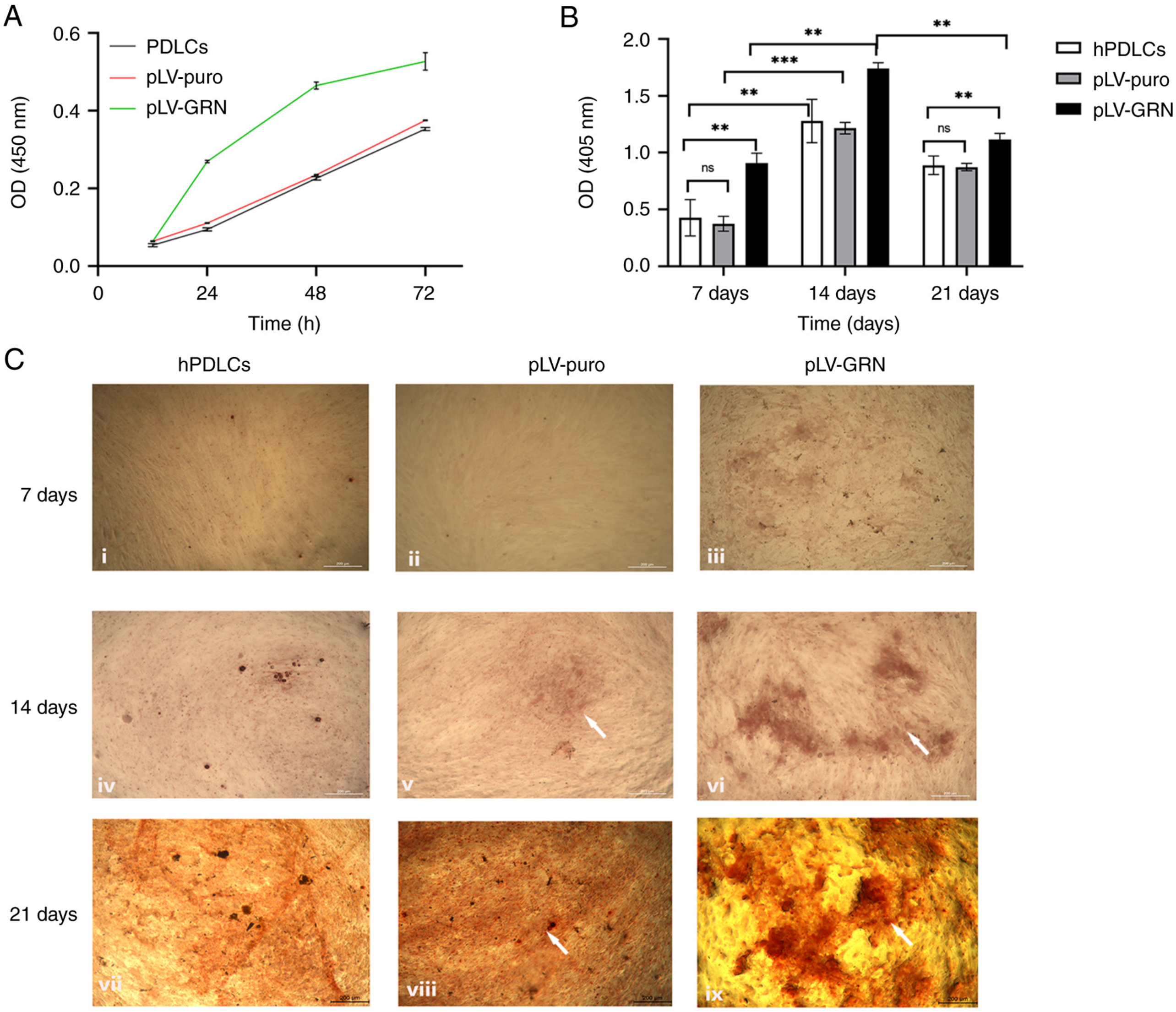

Figure 4. The effects of GRN on the proliferation and osteogenesis of hPDLCs. (A) MTT assay for determination of cell proliferation capacity. (B) ALP activity assay results (**P<0.05, ***P<0.01, ns, not significant). (C) Alizarin red staining results (magnification, x4; mineralized nodules are visible at the white arrows); (a, b and c) PDLCs group, pLV-puro group and pLV-GRN group after 7 days of osteogenic induction respectively; (d, e and f) PDLCs group, pLV-puro group and pLV-GRN group after 14 days of osteogenic induction respectively; (g, h and i) PDLCs group, pLV-puro group and pLV-GRN group after 21 days of osteogenic induction, respectively. GRN, granulin precursor gene; hPDLCs, human periodontal ligament cells; RT-qPCR, reverse transcription-quantitative PCR; ALP, alkaline phosphatase; pLV, lentivirus recombinant plasmid.

difference between the hPDLCs (Fig. 4Cg) and pLV-puro groups (Fig. 4Ch).

RT-qPCR analysis of osteogenesis-related genes. RT-qPCR results demonstrated that after 7, 14 and 21 days of osteogenic induction, the mRNA expression levels of osteogenesis-related genes, including ALP and Runx2, in the pLV-GRN group were 2-3 times higher than those in the hPDLCs and pLV-puro groups (P<0.05). The mRNA expression level of the osteogenesis-related gene OPN was ~1.5 times higher in the pLV-GRN group than in the hPDLCs and pLV-puro groups (P<0.05; Fig. 5A).

Western blot analysis of osteogenesis-related protein expression. Western blotting results indicated that the expression level of the osteogenic protein Runx-2 in the pLV-GRN group was ~1.5-fold higher than that in the hPDLCs and pLV-puro groups at 7, 14 and 21 days of osteogenic induction (P<0.05). The expression level of the osteogenic protein OPN in the pLV-GRN group did not differ significantly from that in the hPDLCs and pLV-puro groups on days 7 and 14 of osteogenic induction. However, on day 21 of osteogenic induction, the expression level of OPN in the pLV-GRN group was ~2-fold higher than that in the hPDLCs and pLV-puro groups (P<0.05; Fig. 5B).

Discussion

Periodontitis, characterized by chronic inflammation affecting the supporting periodontal tissues, poses a significant threat, leading to the progressive destruction of the alveolar bone and potential tooth loss in severe cases. Current clinical treatments primarily focus on addressing etiological factors and controlling inflammation through scaling and root planning.

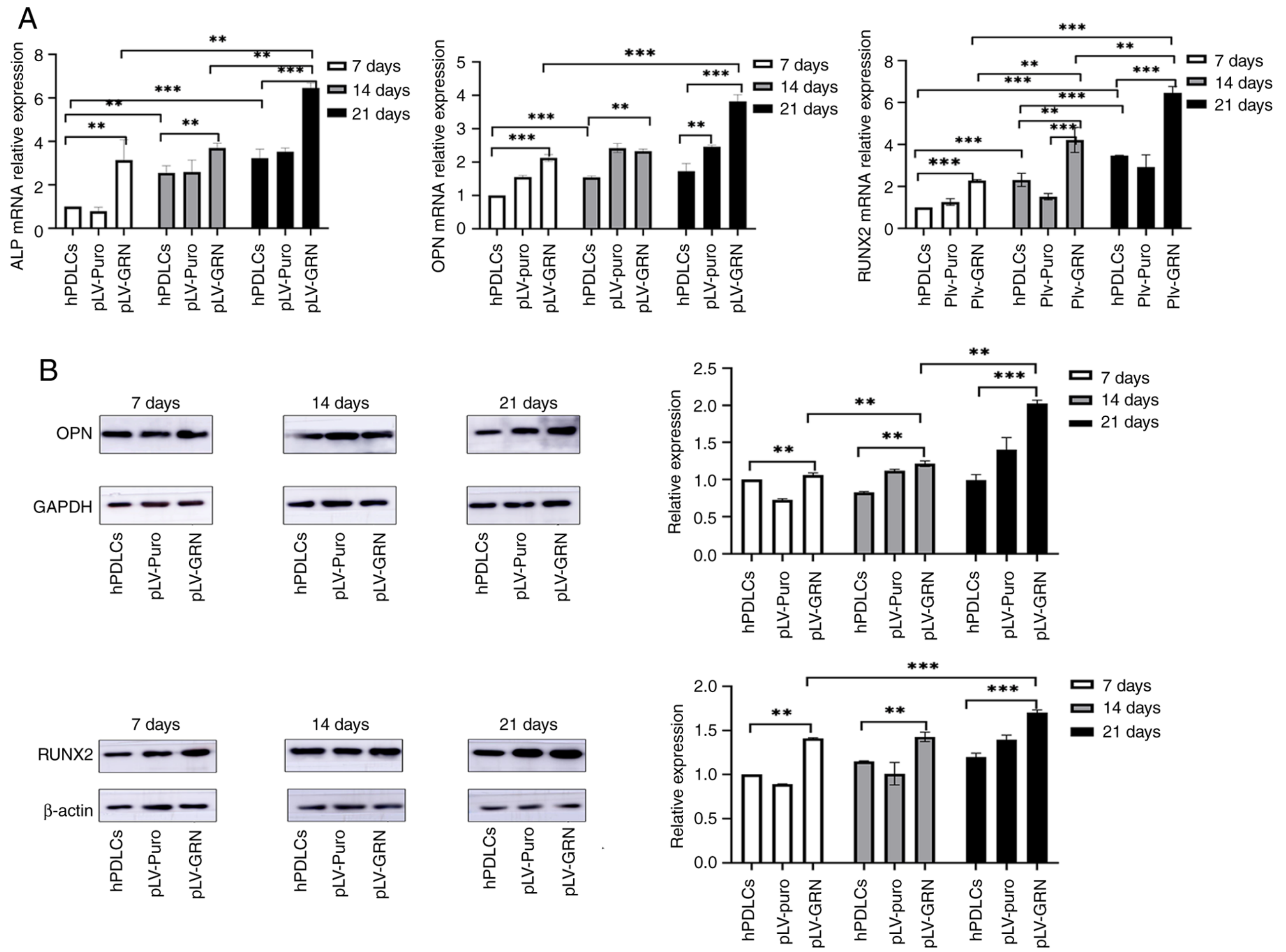

Figure 5. Histogram of the expression levels of osteogenic genes and proteins in hPDLCs after osteoinduction. (A) RT-qPCR determination of the expression of osteogenesis-related genes (***P<0.01, **P<0.05). (B) Expression levels of osteogenesis-related proteins detected by western blotting (**P<0.05). hPDLCs, human periodontal ligament cells; RT-qPCR, reverse transcription-quantitative PCR; GRN, granulin precursor gene; OPN, osteopontin; RUNX2; runt-related transcription factor 2; pLV, lentivirus recombinant plasmid.

However, these methods failed to achieve comprehensive periodontal regeneration. Therefore, exploring effective strategies for complete periodontal regeneration is imperative for managing periodontitis. Tissue engineering techniques, which are gaining prominence in domestic and international research, offer a promising avenue for advancing periodontal regeneration (10).

hPDLCs, which are multipotent cells that can differentiate into fibroblasts, osteoblasts and cementoblasts, have emerged as the ideal seed cells for periodontal tissue engineering (10). In the present study, primary hPDLCs were successfully isolated and cultured from teeth extracted during orthodontic procedures and from third molars. Immunofluorescent staining for keratin and vimentin confirmed the mesenchymal origin of the cells, ensuring stable biological characteristics following passaging. Microscopic examination revealed a consistent fusiform or long-spindle morphology, indicating a robust growth status suitable for subsequent investigation (35).

The dual protective and regenerative attributes of PGRN make it a promising target for novel therapies to treat diseases associated with tissue defects (36). Periodontal regeneration promotes the proliferation and differentiation of stem cells by recruiting endogenous stem cells to the defect site and using bioactive factors with anti-inflammatory and tissue repair effects. Therefore, the effect of PGRN on hPDLCs is key to promoting periodontal tissue regeneration (37). While local application of exogenous PGRN has shown promise in promoting periodontal regeneration (37), direct administration of recombinant proteins presents challenges, such as frequent dosing and high quantities. To address these problems, a stable hPDLCs cell line overexpressing GRN was successfully constructed using a lentiviral vector. Western blotting results unequivocally indicated that the *GRN* gene was successfully constructed in the present study using a lentiviral vector and the protein expression of *GRN* protein in transfected cells of the pLV-GRN group was compared with that in the hPDLCs and pLV-puro groups. The RT-qPCR results demonstrated robust expression of the *GRN* gene in the pLV-GRN group, exhibiting a substantial difference from that in the hPDLCs and pLV-puro groups. Moreover, following passaging, the morphological consistency of hPDLCs transfected with the *GRN* gene, resembling primary cells with a fusiform or long spindle shape, confirmed the stable effect of *GRN* on hPDLCs. This stable research model provides a foundation for exploring the effects of *GRN* on the proliferation and osteogenic capacity of hPDLCs.

Exogenous PGRN has been shown to induce cell proliferation and increase ALP activity in hPDLCs (37,38). In the present study, the proliferative capacity of hPDLCs successfully transfected with pLV-GRN was assessed using MTT and ALP activity assays. The results revealed that the proliferative capacity of cells in the pLV-GRN group significantly surpassed that of cells in both the hPDLCs and pLV-puro groups, underscoring the role of *GRN* in promoting hPDLCs proliferation.

Studies have shown that the intricate process of osteogenic differentiation in hPDLCs involves the orchestration of various factors, including BMPs, Wnt family proteins and transcription factors (Runx-2, β-catenin), with BMP2 being a pivotal inducer in bone formation (5,6,10,39). PGRN, a downstream target of BMP2, plays a crucial role in inducing bone formation (19). In the present study, osteogenic induction spanning 21 days was conducted on cells from the hPDLCs, pLV-puro and pLV-GRN groups, followed by Alizarin Red staining on days 7, 14 and 21. Microscopic observations revealed a higher occurrence of orange-red mineralized nodules in the pLV-GRN group than in the other two groups. This observation supported the robust osteogenic-promoting capability of GRN in hPDLCs at the cellular level.

Runx-2 regulates osteoblast differentiation and maturation primarily through the Wnt and BMP signaling pathways (40,41). PGRN promotes the expression of Runx-2, exerting a pivotal role in the proliferation, maturation and differentiation of mesenchymal stem cells (19). Additionally, OPN plays a crucial role in bone metabolism, not only as a vital factor in the neuron-mediated and endocrine regulation of bone mass but also in various biological activities (42-44). ALP, recognized as the principal mineralizing enzyme in osteogenesis, metabolism and regeneration (45,46), serves as an early differentiation marker for osteoblasts and a characteristic indicator for evaluating differentiation toward osteogenic lineages. In the present study, RT-qPCR results revealed that the mRNA expression of osteogenesis-related genes, including OPN, Runx-2 and ALP, in the pLV-GRN group significantly surpassed that in both the hPDLCs and pLV-puro groups. No significant differences were observed between the hPDLCs and pLV-puro groups. Consistent with these findings, western blotting demonstrated higher protein expression levels of OPN and Runx-2 in the pLV-GRN group than in the hPDLCs and pLV-puro groups during osteogenic induction. Moreover, OPN expression in the pLV-GRN group was notably higher than that in the hPDLCs and pLV-puro group on the 21st day. These results further substantiated that *GRN* effectively enhanced the ability of hPDLCs to differentiate into an osteogenic lineage at both the molecular and protein levels.

The present study unequivocally demonstrated that *GRN* possessed a pronounced ability to enhance both the proliferation and osteogenic differentiation of hPDLCs. Importantly, it addressed the drawbacks associated with the frequent administration and high dosage of exogenous *GRN* observed in previous studies. Using lentiviral-mediated methods, hPDLCs cell lines that overexpressed *GRN* and exhibited stable and noteworthy osteogenic effects were successfully constructed. The present study provided a solid theoretical foundation for future investigations on periodontal regeneration.

To summarize, the present study successfully established a stable hPDLCs cell line overexpressing the *GRN* gene through the use of a lentiviral vector. The ability of *GRN* to promote proliferation and osteogenic differentiation of hPDLCs was confirmed. Further exploration of this signaling pathway is needed to comprehensively verify the role of *GRN* in promoting the proliferation and osteogenesis of hPDLCs. The present study not only served as a robust experimental basis for advancing the understanding of periodontal tissue regeneration but also charted a novel direction for preventing and treating periodontal disease.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Research Fund of Lanzhou University (grant no. 20JR10RA653-ZDKF20210103) and the Key Research Fund of Gansu Province (grant no. 21YF5GA100).

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

XY and RQ conducted the synthesis of recombinant plasmids and osteogenic differentiation experiments and were major contributors to writing the manuscript. ZC, DH and XS collected the clinical samples and experimental data. YS and XH designed the experiments and reviewed and edited the manuscript. XY and XH confirm the authenticity of all the raw data. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Human periodontal ligament cells were isolated and used in accordance with the ethical standards established in the Declaration of Helsinki. The present study was approved by the ethics committee of the School of Stomatology, Lanzhou University (approval no. LZUKQ-2019-045) and informed consent form was signed by the patient prior to participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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