Article

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Progranulin Inactivates PI3K/AKT/mTOR Signaling Pathway to Promote Orthodontic Compression Force-induced Autophagy and Osteogenic Differentiation in Periodontal Ligament Stem Cells

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Background: Orthodontic tooth movement can be caused by the remodeling of periodontal tissues, which is initiated by mechanical forces. Understanding the response of periodontal ligament stem cells (PDLSCs) is crucial for enhancing orthodontic treatment methods. Autophagy serves as an intrinsic defense mechanism that protects cells from mechanical stress caused by environmental changes. This study aims to explore the autophagic mechanism of PDLSCs under orthodontic compression force.
Methods: In this study, static compression force was applied to stimulate PDLSCs. Cell viability and apoptosis were assessed through Cell Counting Kit-8 assay and flow cytometry. Alkaline phosphatase staining and Alizarin red staining assays were used to examine osteogenic differentiation. Autophagy and osteogenic differentiation-related proteins were measured by means of Western blotting.
Results: Progranulin (*PGRN*) was expressed at high levels in PDLSCs exposed to compression force ($p < 0.001$). Autophagy and osteogenic differentiation of PDLSCs could be induced by compression force ($p < 0.01$). Moreover, the autophagy and osteogenic differentiation of compression force-stimulated PDLSCs was further enhanced upon *PGRN* overexpression ($p < 0.01$), while silencing of *PGRN* contributed to diametrically opposite results ($p < 0.05$). Mechanistically, high levels of PGRN exerted an inhibitory impact on the expression of phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR) pathway-related proteins ($p < 0.01$). Furthermore, treatment with insulin-like growth factor-1 (IGF-1), a specific agonist, reversed the facilitating impacts of *PGRN* overexpression on autophagy and osteogenic differentiation ($p < 0.05$).
Conclusions: Our findings shed light on a novel autophagic mechanism of PDLSCs under orthodontic compression force and demonstrate that the inhibitory role of PGRN on the PI3K/AKT/mTOR pathway, thereby mediating the autophagy and osteogenic differentiation of compression force-stimulated PDLSCs. This may offer a new insight for periodontal remodeling during orthodontic treatment.

Keywords: orthodontic tooth movement; autophagy; osteogenic differentiation; periodontal ligament stem cells; compression force; progranulin

Introduction

Mechanical forces are responsible for initiating orthodontic tooth movement, which is facilitated by periodontal ligament (PDL) restructuring. Periodontal ligament stem cells (PDLSCs) are a type of mechanosensitive cell within PDL, and their balanced activation is essential for the effective transduction of orthodontic compression force [1]. The use of mild orthodontic force leads to the direct absorption of alveolar bone, whereas using excessive orthodontic force causes overwhelming pressure, leading to cell death and tissue ischemia in PDL [2,3]. Therefore, additional research is necessary to examine the impact of orthodontic compression force on PDLSCs in order to enhance orthodontic therapeutic strategies.

Autophagy is a highly conserved cellular process by which cells degrade and recycle their own unnecessary or dysfunctional components [4]. During the process, misfolded proteins and damaged organelles within cells are broken down, so as to ensure persistent survival [5]. The broken-down components are enclosed in autophagosomes, which then form autophagolysosomes upon fusion with lysosomes [6]. A recent study has suggested that autophagy may be responsive to mechanical stress and can be activated

in PDLSCs [7]. Mechanical stress applied to PDLSCs promotes their differentiation into osteoblasts in regions experiencing tension, ultimately leading to bone formation [3]. In fact, well-adjusted orthodontic forces have been shown to improve the proliferation potential and osteogenic differentiation of PDLSCs [8,9]. Progranulin (PGRN) is a type of glycoprotein consisting of 576 amino acids [10]. It is widely expressed in diverse tissues and cells, and exerts pivotal functions in numerous pathological and physiological processes such as tumorigenesis, inflammation, bone homeostasis, and wound repair [11–14]. The crucial role of PGRN in periodontal diseases has also been widely uncovered. For example, PGRN interacts with tumor necrosis factor receptor 2 (TNFR2) to mediate M2 macrophage polarization in periodontitis [15]. The osteogenic differentiation of PDLSCs during periodontitis progression can be promoted by *PGRN* overexpression [16]. More importantly, implication of PGRN for autophagy process has been reported in patients with metabolic syndrome or psoriasis [17,18]. However, whether PGRN is associated with the autophagy and osteogenic differentiation of PDLSCs induced by orthodontic compression force, and the detailed mechanism underlying their association, are still in the preliminary stage of exploration.

Therefore, the purpose of this study is to clarify the effect of PGRN on the autophagy and osteogenic differentiation of PDLSCs under orthodontic compression force and further elucidate the underlying mechanism. New findings would pave the way for exploring orthodontic therapeutic approaches.

Materials and Methods

Human PDLSCs Isolation and Culture

This study was approved by the Ethics Committee of Jinan Stomatological Hospital (approval ID: JNSKQYY20220216) and was conducted in accordance with the Declaration of Helsinki. From March 2022 to March 2023, a total of six healthy individuals were enrolled at the Jinan Stomatological Hospital for orthodontic therapy. The inclusion criteria are as follows: (i) patients without periodontal disease and bleeding or redness in the gums; (ii) patients without systemic diseases, such as diabetes, cardiovascular diseases, and autoimmune diseases; (iii) patients who had quit smoking for at least 3 months and did not engage in unhealthy habits such as excessive drinking. The healthy premolars were obtained upon informed consent. The brief procedure of isolating human PDLSCs from premolars is as follows: the periodontal connective tissues were scraped from the surface of the tooth root and cut into small pieces, followed by digestion in type I collagenase solution for half an hour. PDLSCs were cultured in alpha-modified Minimum Essential Medium (α-modified MEM) (cat.no. 12571063; Gibco, Rockville, MD, USA), followed by addition of 1% streptomycin/penicillin solution (cat.no. 15140122; Gibco) and 10% fetal bovine serum (cat.no. A5670701; Gibco). The cells were cultured in a culturing incubator set at 37 °C and supplied with 5% CO_2. The isolated PDLSCs (passage 3) were identified as previously reported [19]. The cells were tested negative for the presence of mycoplasma. For osteoblast differentiation induction, PDLSCs were incubated in complete medium containing 10 nM dexamethasone (cat.no. D7770; Solarbio, Beijing, China), 5 mM β-glycerophosphate (cat.no. G1485; Solarbio), and 25 µg/mL ascorbic acid (cat.no. A8100; Solarbio).

Compressive Force Loading and Treatment

The identified PDLSCs were seeded in 6-well plates and subjected to compressive force for 12 h when 90% confluence was achieved. According to the guideline proposed in a previous report [20], the cell layer was squeezed by a cover glass with extra metal balls, and compressive force was adjusted to 2.0 g/cm^2. PDLSCs without compressive force application were the control group. Additionally, 5 mM of 3-methyladenine (3-MA; cat.no. SAE0107; Sigma–Aldrich, San Luis, MO, USA), an autophagy inhibitor to inhibit autophagy flux, was used to pretreat the compressive force-stimulated PDLSCs for 7 days [21], and how compressive force regulated cell autophagy was investigated. To ascertain the interactions between PGRN and phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR) pathway, PI3K/AKT/mTOR pathway-specific agonist insulin-like growth factor-1 (IGF-1) [22] (4 µM; cat.no. P5502; Beyotime, Shanghai, China) was added to compressive force-induced PDLSCs for 24 h.

Cell Transfection

Plasmids, namely the pcDNA3.1-NC (OE-NC), pcDNA3.1-PGRN (OE-PGRN) as well as si-NC, si-PGRN-1 or si-PGRN-2 were synthesized by Genechem (Shanghai, China). PDLSCs were transfected with these plasmids as required using Lipotransfectamine 3000 (Invitrogen, Karlsruhe, Germany). The sequence information of OE-/si-RNA is listed in **Supplementary Information 1**.

Cell Counting Kit-8 (CCK-8) Assay

Viability of PDLSCs under compressive force and control conditions was assessed by using CCK-8 assay. In brief, 5 $\times$ 10^3 cells/mL of PDLSCs were seeded in each well of a 96-well plate and subsequently cultured for 0, 24, 48, and 72 h. After that, CCK-8 solution (cat.no. C0037; Beyotime, Shanghai, China) was added and incubated for an additional 2 h, followed by measurement of cell viability using a microplate reader at the wavelength of 450 nm (Molecular Devices, San Jose, CA, USA).

Flow Cytometry Assay

A commercial apoptosis assay kit (cat.no. MA0220; Meilune, Suzhou, China) was used to measure the apoptosis rate of PDLSCs. PDLSCs were re-suspended in binding buffer, and then stained with annexin V-fluorescein isothiocyanate (V-FITC) and propidium iodide (PI) for 20 min in the dark. The apoptotic PDLSCs were measured using NovoCyte flow cytometer (Agilent Technologies, Santa Clara, CA, USA).

Flow cytometry was also used to analyze the cell-surface markers of PDLSCs. In brief, following reconstitution with fluorescence-activated cell sorting (FACS) buffer, the trypsinized PDLSCs (2.5×10^5 cells) were centrifuged ($300 \times g$ at 4 °C, 5 min). Primary antibodies fluorescein isothiocyanate (FITC)-labeled CD34 (cat.no. 48-0341-82), allophycocyanin (APC)-labeled CD45 (cat.no. 58-0459-42), FITC-labeled CD73 (cat.no. 11-0739-42), FITC-labeled CD90 (cat.no. 11-0909-42), and APC-labeled CD105 (cat.no. 17-1057-42), which were all acquired from eBiosciences (San Diego, CA, USA), were used to incubate with PDLSCs for 30 min. After washing twice with phosphate-buffered saline (PBS), the cells were re-suspended in 200 µL of PBS. NovoCyte flow cytometer was employed for the analysis of the proportions of specific stem cell markers.

Osteogenic Differentiation Assays

In accordance with the manufacturer's specifications, the osteogenic differentiation of PDLSCs was meticulously assessed using an alkaline phosphatase (ALP) assay kit (cat.no. P0321S; Beyotime, Shanghai, China) and an Alizarin red staining (ARS) kit (cat.no. C0148S; Cyagen Biosciences, Guangzhou, China). The ALP staining was performed 5 days after culture. Since extracellular matrix mineralization is the terminal step of osteoblast differentiation, ARS was performed after 14 days of culture. The rinsed PDLSCs were observed under a light microscope (Axio Scope A1; Carl Zeiss, Oberkochen, Germany). ALP viability and ARS viability were determined using a microplate reader (Molecular Devices, San Jose, CA, USA) at the wavelengths of 405 nm and 562 nm, respectively.

Total RNA Isolation and qRT-PCR

Total RNA from PDLSCs was obtained using a total RNA Extraction Kit (cat.no. LS1040; Promega, Madison, WI, USA). Afterwards, the RNA was reverse-transcribed to generate cDNA using the First Strand Kit (cat.no. 330401; Qiagen, Dusseldorf, Germany). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis was performed using the QuantiFast SYBR® Green PCR Kit (cat.no. 204756; Qiagen) in adherence to the manufacturer's instructions. The gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as internal reference for calculating expression of *PGRN*, *Beclin1*, and microtubule-associated protein 1 light chain 3 beta (*LC3II*). The sequences of primers used are shown in Table 1.

Table 1. Sequence of primers used in qRT-PCR.

Gene		Sequences (5′ – 3′)
PGRN	Forward	GAAGGCTCGATCCTGCGAGA
	Reverse	CTCAAGGCTGGGTCCCTCAA
GAPDH	Forward	CCACCCATGGCAAATTCCATGGCA
	Reverse	TCTAGACGGCAGGTCAGGTCCACC
Beclin1	Forward	AGGCGAAACCAGGAGAGAC
	Reverse	CCTCCCCGATCAGAGTGAA
LC3II	Forward	GACCGCTGTAAGGAGGTGC
	Reverse	CTTGACCAACTCGCTCATGTTA

qRT-PCR, quantitative reverse-transcription polymerase chain reaction; *PGRN*, Progranulin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *LC3II*, microtubule-associated protein 1 light chain 3 beta.

Western Blotting

Total proteins were extracted from the cells on the seventh day after inducing osteogenic differentiation in the PDLSCs to detect the expression of ALP and runt-related transcription factor 2 (RUNX2). The same protein extraction method was applied on the 14th day after inducing osteogenic differentiation to extract protein for detecting expression of osteopontin (OPN). Briefly, proteins were extracted from PDLSCs using radioimmunoprecipitation assay (RIPA) lysis buffer (cat.no. P0013B; Beyotime). The concentration of extracted proteins was determined using the bicinchoninic acid (BCA) Kit (cat.no. P0009; Beyotime). Then, the proteins were analyzed via the 10% polyacrylamide gel electrophoresis, and the separated protein bands were transferred to polyvinylidene difluoride (PVDF) membranes. Before incubation with the primary antibodies, the membrane was blocked using 5% nonfat milk. Primary antibodies include PGRN (1:2000; cat.no. AF2420; R&D, Minneapolis, MN, USA), Beclin1 (1:2000; cat.no. ab302669; Abcam, Cambridge, UK), LC3II (1:2000; cat.no. ab232940; Abcam), ALP (1:2000; cat.no. ab307726; Abcam), RUNX2 (1:2000; cat.no. ab192256; Abcam), OPN (1:2000; cat.no. ab214050; Abcam), PI3K (1:1000; cat.no. #4249; Cell Signaling Technology, Danvers, MA, USA), phosphorylated phosphoinositide 3-kinase (p-PI3K, 1:1000; cat.no. #17366; Cell Signaling Technology), AKT (1:1000; cat.no. #9272; Cell Signaling Technology), phosphorylated AKT (p-AKT, 1:1000; cat.no. #4060; Cell Signaling Technology), vinculin (1:1000; cat.no. #4650; Cell Signaling Technology), β-actin (1:1000; cat.no. #4967; Cell Signaling Technology) and GAPDH (1:1000; cat.no. #2118; Cell Signaling Technology). Subsequently, the membrane was incubated with the horseradish peroxidase (HRP)-linked secondary antibodies, namely goat anti-chicken (1:10,000; cat.no. ab6877; Abcam), rabbit anti-goat (1:10,000; cat.no.

ab97100; Abcam), and goat anti-rabbit (1:10,000; cat.no. ab6721; Abcam) for 1 h, and analyzed using enhanced chemiluminescence (ECL) kit (cat.no. P0018S; Beyotime). The protein bands were analyzed using ImageJ software (version 2.0; National Institutes of Health, Bethesda, MD, USA) installed on a BIO-RAD ChemiDoc imaging instrument (Bio-Rad Laboratories, Hercules, CA, USA). Vinculin, GAPDH and β-actin were utilized as the internal controls.

Statistical Analysis

Data analysis was conducted using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). The data are expressed as mean $\pm$ standard deviation (SD). Student's *t*-tests were used for comparison between two groups of normally distributed data, and one-way analysis of variance (ANOVA) coupled with Tukey's post-hoc test was used for comparison among multiple groups. A *p*-value of less than 0.05 was deemed to be statistically significant.

Results

Orthodontic Compression Force Induces Autophagy and Osteogenic Differentiation in PDLSCs

Flow cytometry was used to analyze the proportions of specific stem cell markers. The red and blue colors denote the control immunoglobulin G (IgG) staining of the same sample and the labeling of the corresponding antibody, respectively. The positive rates for CD34, CD45, CD73, CD90, and CD105 were 0.98, 0.32, 84.6, 91.5 and 98.8, respectively. As shown in Fig. 1A, detection of positive expression of markers (CD34, CD45, CD73, CD90, and CD105) that are related to the physical and behavioral traits specific to multipotent adult mesenchymal stem cells (MSCs) indicates the successful isolation of PDLSCs. To ascertain the impact of orthodontic compression force on PDLSC autophagy *in vitro*, 2.0 g/cm^2 of static compression force was imposed on PDLSCs for 12 h. As illustrated in Fig. 1B, there seemed to be no significant difference in cell viability between the control and compressive force groups. Flow cytometry analysis also showed that the percentages of apoptotic PDLSCs were similar between these two groups (Fig. 1C). The autophagy of PDLSCs was then determined by measuring the levels of autophagy markers. We observed that the mRNA expression of *Beclin1* and *LC3II* was at high levels in compressive force group by contrast to the control group (Fig. 1D, $p < 0.001$). 3-MA, an autophagy inhibitor, was added in PDLSCs to investigate how compressive force regulated cell autophagy. It was indicated that the protein levels of Beclin1 and LC3II were markedly decreased in PDLSCs when pre-treated with 3-MA (Fig. 1E, $p < 0.01$), while the application of compressive force significantly restored Beclin1 and LC3II protein levels in PDLSCs ($p < 0.05$). The influences of compressive force on osteogenic differentiation were simultaneously ascertained. As shown in Fig. 1F, by contrast to the control group, both ALP viability and ARS viability in the compressive force group were markedly increased ($p < 0.001$). Furthermore, compressive force also induced a notable increase in the levels of osteogenic protein (ALP, RUNX2 and OPN) (Fig. 1G, $p < 0.01$).

Pronounced Upregulation of PGRN is Observed in Compressive Force-induced PDLSCs

Following exposure to compressive force for 12 h, the mRNA expression of *PGRN* in PDLSCs was found to be overtly upregulated (Fig. 2A, $p < 0.001$). The subsequent Western blotting also showed a significant increase in PGRN protein level in the compressive force group as compared to the control group (Fig. 2B, $p < 0.001$).

PGRN Enhances Autophagy and Osteogenic Differentiation in Compressive Force-induced PDLSCs

In order to explore the role of PGRN in compressive force-induced PDLSCs, *PGRN* expression in PDLSCs was manipulated through either silencing or overexpression. As shown in Fig. 3A,B, *PGRN* expression levels were decreased remarkably when the cells were transfected with si-PGRN-1 or si-PGRN-2, and si-PGRN-1 was selected for subsequent functional experiments because of its relatively high silencing efficiency ($p < 0.001$). Meanwhile, *PGRN* expression was observed to be significantly enhanced in PDLSCs after OE-PGRN transfection (Fig. 3C,D, $p < 0.001$). The mRNA expression and protein levels of Beclin1 and LC3II, as depicted in Fig. 3E–H, were distinctly reduced when PGRN was silenced ($p < 0.01$), and were overtly elevated when PGRN was overexpressed ($p < 0.01$). Moreover, osteogenic differentiation assays indicated that in compressive force-induced PDLSCs, osteoblast differentiation was inhibited upon silencing of *PGRN* but was enhanced by *PGRN* overexpression (Fig. 3I,J, $p < 0.001$). Similar results were observed in the protein levels of osteogenic differentiation markers (Fig. 3K,L, $p < 0.05$).

PGRN Inhibits PI3K/AKT/mTOR Pathway in Compressive Force-induced PDLSCs

PI3K/AKT/mTOR pathway has been reported to be implicated in autophagic process of PDLSCs [23,24]. We therefore evaluated the impacts of PGRN expression on PI3K/AKT/mTOR pathway-related proteins. As indicated in Fig. 4A,B, compressive force showed a significant inhibitory effect on the protein levels of p-mTOR, p-PI3K and p-AKT. However, this inhibiting effect was dramatically restored through knockdown of *PGRN* ($p < 0.05$) but was further enhanced upon *PGRN* overexpression ($p < 0.01$).

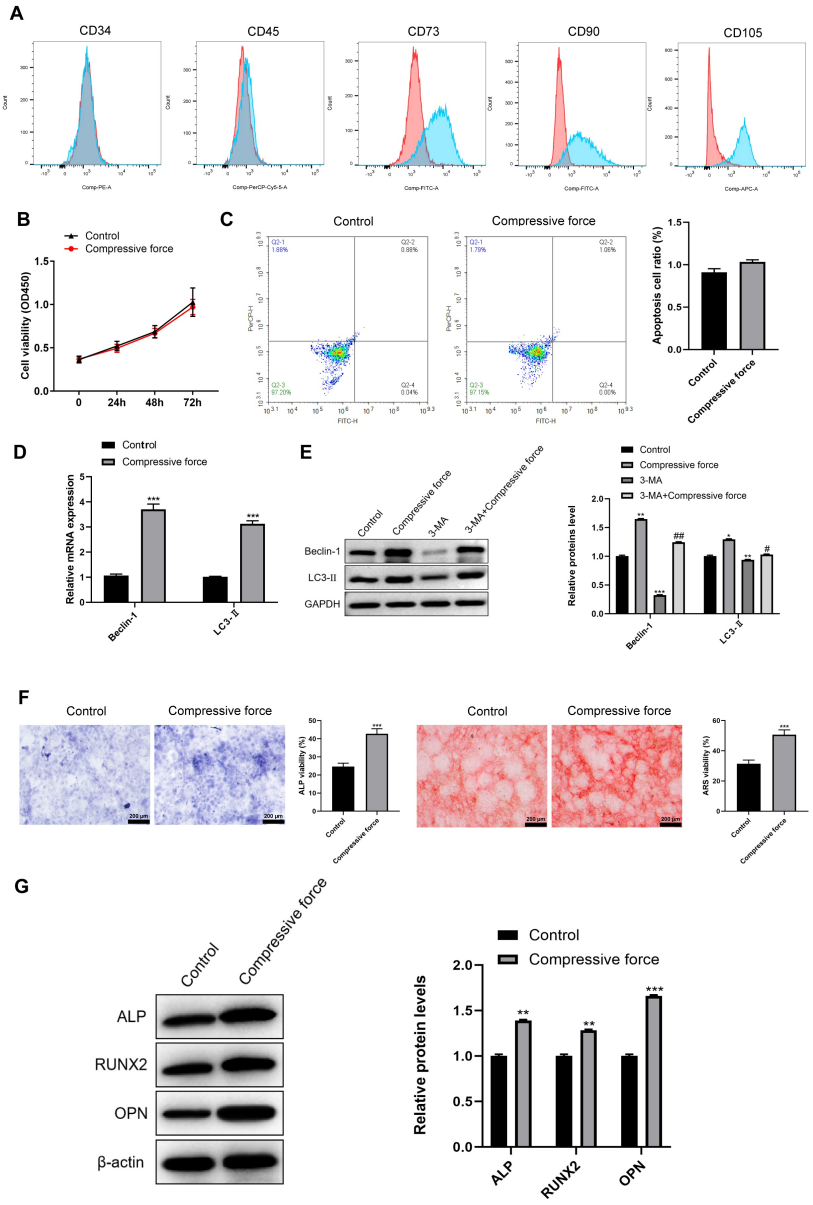

Fig. 1. Orthodontic compression force induces autophagy and osteogenic differentiation in PDLSCs. (A) PDLSCs were identified by means of flow cytometric analysis of cell-surface markers. (B) The relative cell viability of PDLSCs with or without compressive force. (C) The apoptosis of PDLSCs was analyzed by flow cytometry. (D) qRT-PCR analysis for the mRNA expression of Beclin-1 and LC3II. (E) The protein levels of Beclin-1 and LC3II determined by means of Western blotting, following the addition of 3-MA. (F) The ALP and ARS viability in PDLSCs with or without compressive force. scale bar = 200 μm. (G) The protein levels of osteogenic differentiation markers ALP, RUNX2 and OPN were determined by means of Western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. 3-MA. $n = 3$. Abbreviations: OPN, osteopontin; ALP, alkaline phosphatase; ARS, alizarin red staining; PDLSCs, periodontal ligament stem cells; 3-MA, 3-methyladenine; RUNX2, runt-related transcription factor 2.

PGRN Promotes Compressive Force-induced PDLSC Autophagy and Osteogenic Differentiation by Regulating PI3K/AKT/mTOR Pathway

To further ascertain the interactions between PGRN and PI3K/AKT/mTOR pathway, 4 μM of PI3K/AKT/mTOR pathway-specific agonist insulin-like growth factor 1 (IGF-1) was added to compressive force-induced PDLSCs. The results showed that treatment with IGF-1 reversed the upregulation of mRNA expression and protein levels of Beclin1 and LC3II caused by *PGRN* overexpression (Fig. 5A,B, $p < 0.05$). Additionally, we further demonstrated that the osteogenic differentiation was partially counteracted following treatment with IGF-1, as evidenced by ALP staining, ARS and Western blot assays (Fig. 5C,D, $p < 0.05$).

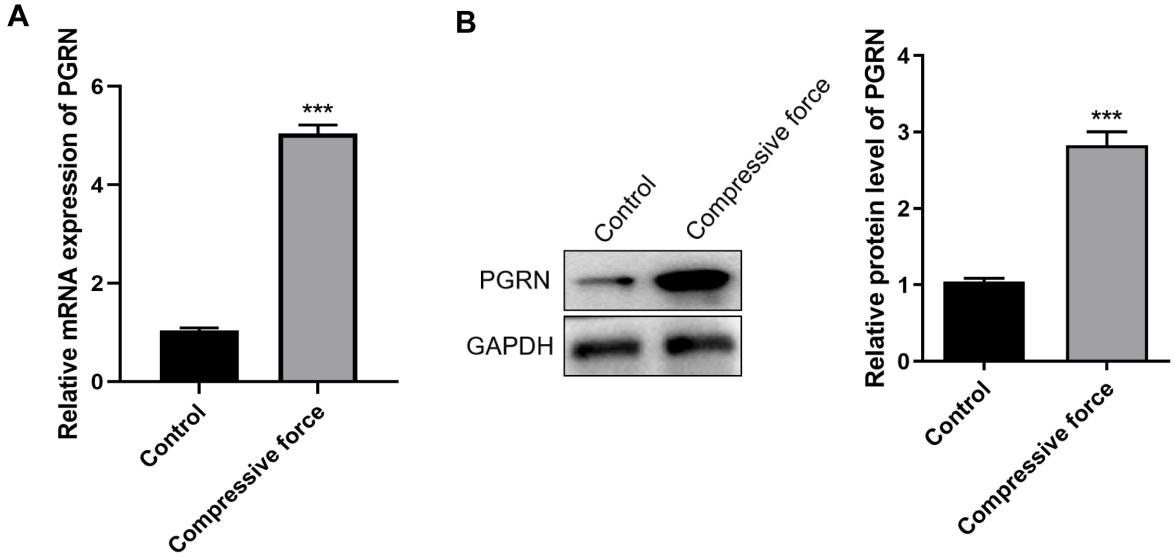

Fig. 2. Pronounced upregulation of PGRN is observed in compressive force-induced PDLSCs. The mRNA expression (A) and protein level (B) of PGRN in compressive force-induced PDLSCs were determined by means of qRT-PCR and Western blotting, respectively. $^{***}p < 0.001$ vs. control. $n = 3$.

Discussion

In 2004, researchers successfully isolated PDLSCs and found that they share similar characteristics with mesenchymal stem cells [25]. PDLSCs have the ability to modulate the immune response, as well as the potential to grow and form cementum/periodontal ligament-like complex [26], making them crucial for maintaining periodontal health. Furthermore, PDLSCs are likely influenced by mechanical forces and play a key role in reshaping both the periodontal ligament and bone during orthodontic tooth movement. Therefore, a deeper understanding of how PDLSCs respond to mechanical stress and the cellular signaling pathways involved could help address challenges in orthodontic treatment. In this study, we found that the overexpression of *PGRN* inhibited the activation of PI3K/AKT/mTOR signaling pathway, thereby facilitating the autophagy and osteogenic differentiation of PDLSCs induced by compressive force.

Static compression force is frequently employed in laboratory settings to simulate the pressure experienced on the compressed side during orthodontic tooth movement [27,28]. Studies have shown that this technique can lead to changes in the differentiation and morphology of PDLSCs [27,28]. For example, Zhang *et al.* [2] developed a hydraulic pressure-controlled device to apply 100 kPa static hydraulic pressure onto PDLSCs for 1 h, enhancing osteogenic differentiation potential of PDLSCs. Following application of static compression force at 1 g/cm^2 for 12 h, Feng *et al.* [29] observed repression of collagen expression and alteration to the cell morphology. Apart from the altered differentiation potential and cell morphology, autophagy has also been reported to be sensitive to compressive force [30]. Herein, an improved static compression force at 2 g/cm^2 was applied to PDLSCs, and its effect on the autophagy of PDLSCs was then explored. Our results support the previous research indicating that PDLSCs undergo autophagy in response to compressive force stimulation. Further analysis showed that autophagy flux was inhibited by 3-MA treatment, while application of compressive force augmented the protein levels of autophagy markers (Beclin1 and LC3II). Additionally, the experiments on ALP viability and ARS viability, as well as osteogenic differentiation markers, also revealed that compressive force can stimulate the osteogenic differentiation of PDLSCs. All these results confirmed the conclusion that compressive force can induce autophagy and osteogenic differentiation in PDLSCs.

PGRN is a crucial growth factor that is highly expressed in periodontitis tissues. It is involved in the osteogenic differentiation of PDLSCs and M2 macrophage polarization when periodontitis occurs [15,16]. In the current study, the pronounced upregulation of PGRN was observed in compressive force-stimulated PDLSCs, indicating its pivotal role during orthodontic tooth movement *in vitro*. Additionally, PGRN has been reported to be strongly associated with multiple human diseases such as metabolic syndrome and psoriasis [17,18]. We therefore speculated that the abnormal contents of PGRN may be implicated in the autophagy and osteogenic differentiation of PDLSCs. As expected, silencing of *PGRN* brought about an inhibitory impact on the autophagy marker expression, whereas PGRN overexpression contributed to diametrically opposite results. Similar patterns were observed in the results of osteogenic differentiation of PDLSCs. These findings validated our hypothesis that high levels of PGRN

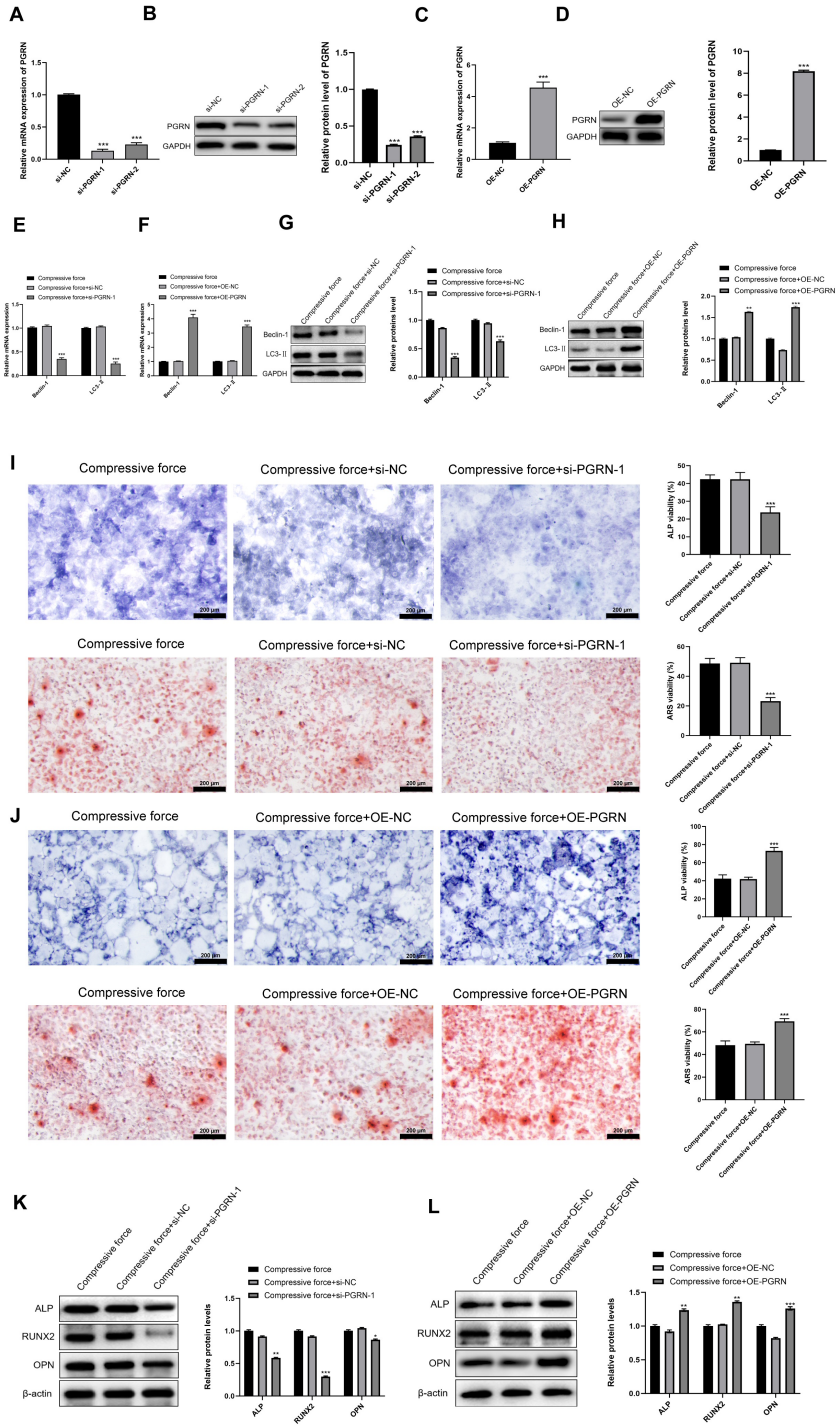

Fig. 3. PGRN enhances autophagy and osteogenic differentiation in compressive force-induced PDLSCs. (A) The mRNA expression and (B) protein level of PGRN in PDLSCs transfected with si-PGRN-1/-2 or si-NC were detected through qRT-PCR and Western blotting, respectively. $^{***}p < 0.001$ vs. si-NC. (C) The mRNA expression and (D) protein level of PGRN in PDLSCs transfected with OE-PGRN or OE-NC were detected through qRT-PCR and Western blotting, respectively. $^{***}p < 0.001$ vs. OE-NC. (E,F) The mRNA expression of Beclin-1 and LC3II in compressive force-induced PDLSCs was determined by means of qRT-PCR following transfection of si-PGRN-1 (E) or OE-PGRN (F). (G,H) The protein levels of Beclin-1 and LC3II, measured by Western blotting, in compressive force-induced PDLSCs transfected with si-PGRN-1 (G) or OE-PGRN (H). (I,J) The ALP and ARS viability following transfection with si-PGRN-1 (I) or OE-PGRN (J). scale bar = 200 µm. (K,L) The protein levels of ALP, RUNX2 and OPN, measured via Western blotting, in compressive force-induced PDLSCs following transfection with si-PGRN-1 (K) or OE-PGRN (L). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. compressive force + si-NC or OE-NC. $n = 3$.

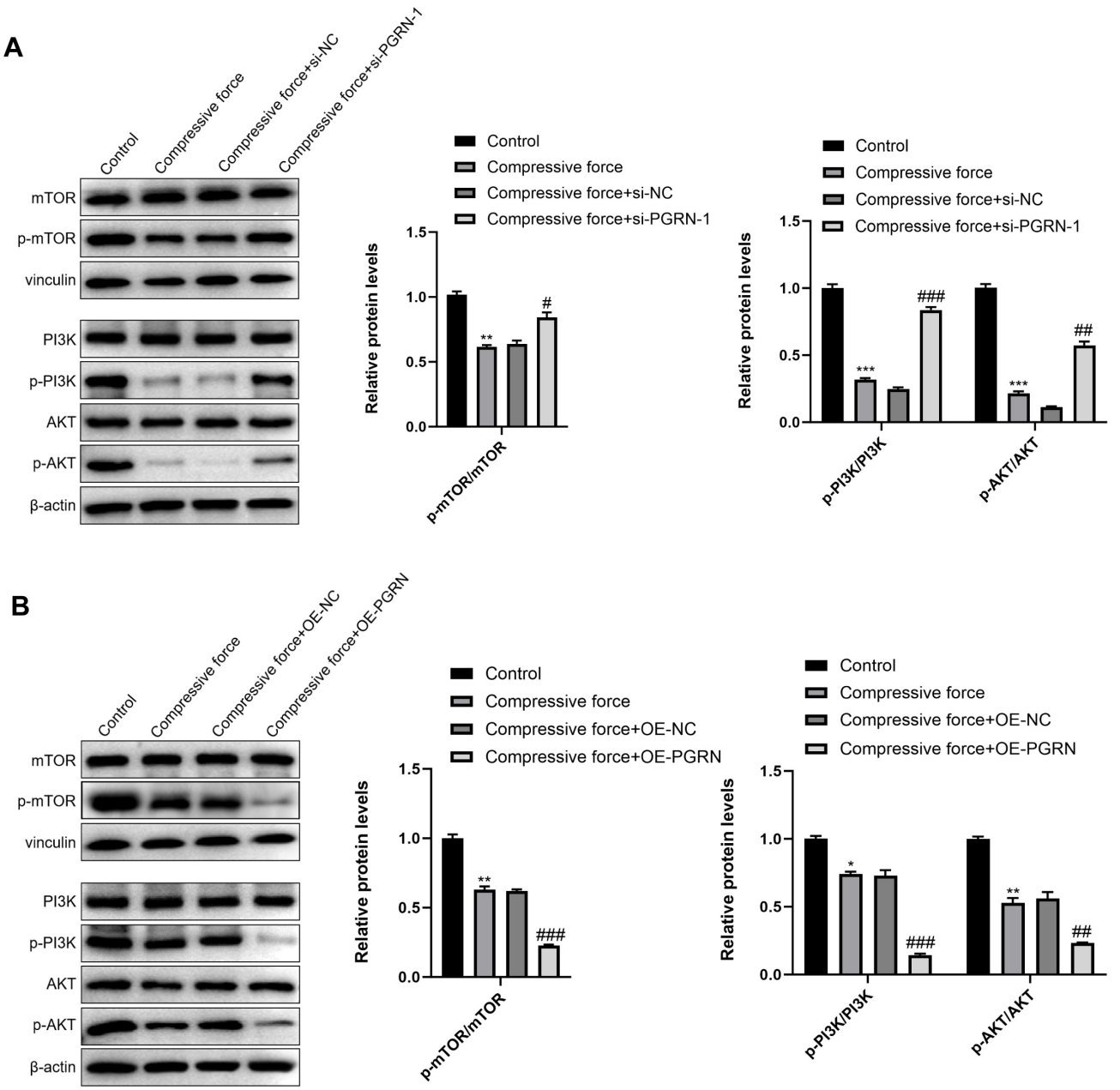

Fig. 4. PGRN inhibits PI3K/AKT/mTOR pathway in compressive force-induced PDLSCs. (A) The protein levels of mTOR, p-mTOR, PI3K, p-PI3K, AKT, and p-AKT, measured by means of Western blotting, in compressive force-induced PDLSCs transfected with si-PGRN-1 or si-NC. (B) The protein levels of mTOR, p-mTOR, PI3K, p-PI3K, AKT, and p-AKT, measured by means of Western blotting, in compressive force-induced PDLSCs transfected with OE-PGRN or OE-NC were. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. compression force + si-NC or OE-NC. $n = 3$. Abbreviations: PI3K/AKT/mTOR, phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin.

in compressive force-stimulated PDLSCs could trigger the progression of autophagy and osteogenic differentiation in PDLSCs.

The investigation into the mechanism of cell autophagy is increasingly recognizing the significance of the PI3K/AKT/mTOR signaling pathway. Autophagy is generally initiated by the Unc-51-like kinase (ULK) complex that is regulated by the PI3K/AKT/mTOR pathway [31]. The activated ULK complex is usually attached to the PI3K complex, while AKT phosphorylates autophagy protein Beclin1 at S295 site to inactivate Beclin1, preventing the occurrence of autophagy [32,33]. PI3K/AKT/mTOR pathway-mediated autophagy has been proposed to be of great importance for numerous human diseases including cancers [34,35], osteoarthritis [36], allergic asthma [37], and neurodegeneration [38]. A recent study con-

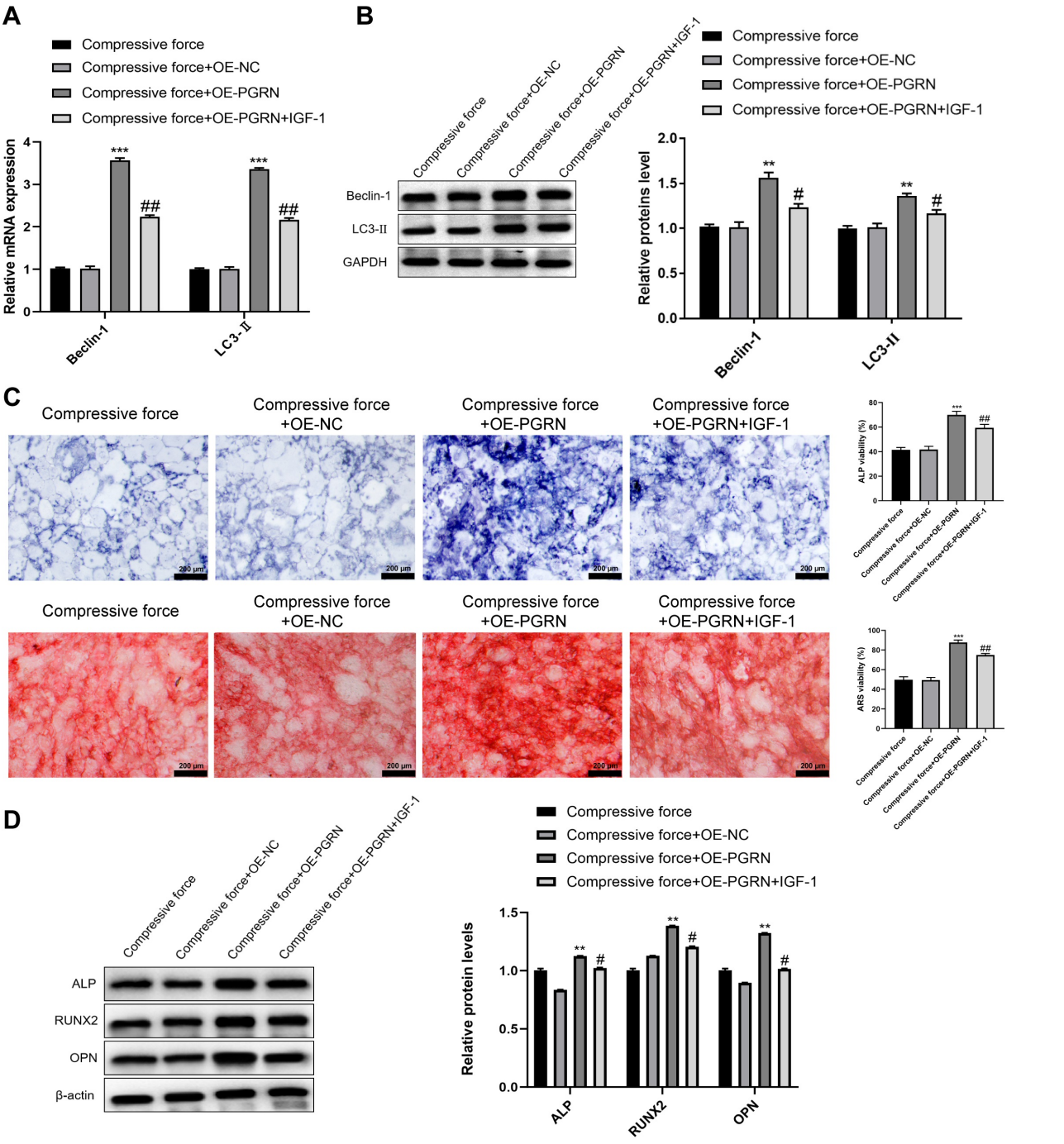

Fig. 5. PGRN promotes autophagy and osteogenic differentiation in compressive force-induced PDLSC by regulating PI3K/AKT/mTOR pathway. (A,B) The mRNA expression (A) and protein levels (B) of Beclin-1 and LC3II, determined by qRT-PCR and Western blotting, respectively, following transfection of the compressive force-induced PDLSCs with OE-PGRN or OE-NC and the IGF-1 addition. (C) The ALP and ARS viability was captured with a light microscope. scale bar = 200 µm. (D) The protein levels of ALP, RUNX2 and OPN, measured by means of Western blotting, following IGF-1 addition. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. compressive force + OE-NC. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. compressive force + OE-PGRN. $n = 3$. Abbreviations: IGF-1, insulin-like growth factor-1.

ducted by Yang *et al.* [23] reported new findings of PI3K/Akt/mTOR pathway-mediated autophagy during orthodontic tooth movement. Given the pronounced promoting impact of PGRN on autophagy and osteogenic differentiation in the compressive force-stimulated PDLSCs, we believe that interplays between PGRN and PI3K/AKT/mTOR pathway may exist. It was well demonstrated in our study that *PGRN* overexpression resulted in even a further drop in the levels of PI3K/AKT/mTOR pathway-related proteins due to the compressive force treatment, whereas op-

posite results were observed in the event of *PGRN* silencing. Moreover, we found that the high levels of Beclin1 and LC3II induced by *PGRN* overexpression could be suppressed by PI3K/AKT/mTOR pathway agonist IGF-1. Similarly, the enhanced ALP viability and ARS viability, as well as osteogenic differentiation marker, were also partially counteracted by the treatment with IGF-1. These findings further validated our assumption that PGRN can interact with PI3K/AKT/mTOR pathway.

Several limitations of this study should be acknowledged. First, animal models should be used to verify the experimental results *in vivo*. Second, how PGRN affects these cellular processes through the PI3K/AKT/mTOR pathway warrants further investigation to validate findings obtained in this study.

Conclusions

In conclusion, compressive force drives the autophagy and osteogenic differentiation processes in PDLSCs, with *PGRN* overexpression further stimulating these physiological processes by inhibiting the PI3K/AKT/mTOR signaling pathway. These findings provide a certain theoretical foundation for formulating potential molecular treatments targeting orthodontic tooth movement.

Availability of Data and Materials

The dataset that supports the results and findings of this research is available from the corresponding author on reasonable request.

Author Contributions

Substantial contributions to conception and design: YJ. Data acquisition, data analysis and interpretation: JY, XS, XL. Drafting the article or critically revising it for important intellectual content: All authors. Final approval of the version to be published: All authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Jinan Stomatological Hospital (approval ID: JNSKQYY20220216) and was conducted in accordance with the Declaration of Helsinki. All participants provided informed consent in the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537200.159.

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