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Original Article

C4orf7 modulates osteogenesis and adipogenesis of human periodontal ligament cells

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Abstract: Periodontal ligament cells (PDLCs), which have potential for multilineage differentiation, are candidates for use in regeneration of periodontal tissue defects; however, our understanding of the mechanisms underlying the lineage commitment of PDLCs remains limited. C4orf7, which is specifically expressed in the periodontal ligament (PDL) tissue, may be crucial in deciding the fate of PDLCs and regulating the periodontal bone balance. In this study, we examined the expression of C4orf7 in PDL tissue, using immunohistochemical staining. We transfected PDLCs with lentiviral vectors expressing C4orf7 and examined the effect of C4orf7 on the balance of PDLC osteogenic and osteoclastogenic differentiation. Osteogenic induction resulted in the downregulation of mRNA and protein expression levels of the osteogenic/cementoblastic markers: *ALP*, *RUNX2*, *COL1*, *OPN*, *OPG, OSX*, *IBSP*, *CAP,* and *CEMP1.* Transfected cells also exhibited an increased *RANKL/OPG* ratio, which is an indicator of osteoclastogenic differentiation. *ALP* activity assays and Alizarin red staining confirmed the negative effect of C4orf7 on PDLC osteogenic differentiation. Finally, we investigated the effect of C4orf7 on the lineage commitment of PDLCs to adipocytes. We observed increased expression levels of *PPARγ2*, *GLUT4*, *ZFP423*, *FABP4*, and *LPL* mRNAs, as well as a gradual accumulation of lipid droplets in the C4orf7-overexpressing group compared with controls. In summary, our data confirm that C4orf7 has an important role in the regulation of periodontal bone remodeling through promotion of the adipogenic/osteoclastogenic, and inhibition of the osteogenic/cementoblastic, differentiation of PDLCs. Therefore, C4orf7 is a potential therapeutic target for the treatment of periodontal disease and other bone metabolic disorders.

Keywords: Periodontal ligament cells (PDLCs), C4orf7, osteogenesis, adipogenesis

Introduction

Periodontal bone defects, resulting from periodontitis, trauma, osteoporotic fractures, or tumor resection, represent a substantial challenge for dentists and dental researchers. Advances in tissue engineering and regenerative medicine have led to the development of mesenchymal stem cell-based therapy, a preferred option to address the challenges associated with the treatment of periodontal bone defects [1, 2].

The periodontal ligament (PDL), located in the alveolar socket, is a soft, vascularized, and cell-containing tissue that connects the cementum to the alveolar bone [3]. The PDL supports the teeth, buffers masticatory force, and contributes to tooth nutrition, homeostasis, and repair [4]. Mounting evidence suggests that PDL contains heterogeneous cell populations, which are not terminally differentiated and have the capacity to differentiate into different cell types, indicating the presence of stem/progenitor-like cells in the PDL [5, 6]. Under appropriate conditions, periodontal ligament cells (PDLCs) are capable of differentiating into different cell lineages, including osteoblasts, cementoblasts, chondrocytes, adipocytes, and neurons. Therefore, PDLCs are good candidates for application in regeneration of periodontal tissue.

Under physiological conditions, periodontal bone tissue is in a dynamic state of constant remodeling, which is a complex process involving close interactions between different progenitor cell lineages. Specific differentiation of PDLCs is essential for regenerating defective

periodontal bone tissue. Although adipocytes and osteoblasts share a common progenitor cell, adipogenesis and osteogenesis are mutually exclusive differentiation processes [7-9]. Despite the absence of adipocytes from human PDL, adipogenesis and osteogenesis are considered the two major pathways of mesenchymal stem cell differentiation of PDLCs [10, 11]. Elucidation of the mechanisms underlying the commitment of cells with multilineage potential to a specific differentiation pathway will facilitate improvements in the effective use of PDLCs for regeneration of periodontal bone tissue.

C4orf7, a small secreted peptide composed of 85 amino acids, was originally identified in tonsillar germinal center and follicular dendritic like cell lines [12]. It can exert an immunomodulatory function through its binding to the surface of B lymphoma cells [13, 14]. Recently, researchers unexpectedly discovered that C4orf7 was expressed in human PDL tissue, but not in enamel, dentin, dental pulp, alveolar bone, or cultured PDLCs [15]. Bioinformatics analyses demonstrated that C4orf7 has a structure similar to that of statherin, which is vital in the prevention of calcium salt deposition in saliva, and has binding affinity for hydroxyapatite. In light of these findings, investigators hypothesized that C4orf7 may prevent the accumulation of calcium salts, thus maintaining the unmineralized state of human PDL, facilitating its adsorption onto the surface of the cementum and alveolar bone [16]. Another study reported that overexpression of C4orf7 in human PDLCs can maintain their fibroblastic phenotype, while inhibiting osteogenic differentiation [17]. Although these studies have delineated the function of C4orf7 in osteogenesis, its role in determining the differentiation pathways of multipotent PDLCs is yet to be fully explored.

In the present study, we first confirmed the expression of C4orf7 in human PDL tissue. Next, we investigated the role of C4orf7 in the differentiation of human PDLCs *in vitro*. Our results demonstrate that C4orf7 strikingly enhances adipocyte, and attenuates osteoblast, differentiation. Therefore, C4orf7 may regulate the balance of osteogenic and adipogenic differentiation in human PDLCs.

Materials and methods

Primary culture of human periodontal ligament cells

Healthy third molars were collected from 18- to 25-year-old individuals who required tooth extraction. Informed consent was obtained from all study participants, and the study protocol was approved by the Ethics Committee, School & Hospital of Stomatology, Wuhan University. Extracted teeth were rinsed three times with phosphate-buffered saline (PBS) containing 10% penicillin and streptomycin. PDL tissue from the middle third of tooth roots was scraped and transferred to tubes containing collagenase and dispase (Roche, Basel, Switzerland). PDLCs were released by enzymatic digestion for 60 min. After centrifugation, single cell suspensions were prepared and cultured in α-modified essential medium (α-MEM, HyClone, South Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) and 1% antibiotics. Cultures were incubated at 37°C in a 5% CO_2 atmosphere, and passaged when cells were sub-confluent. PDLCs at passages 3-6 were used for subsequent experiments.

Immunofluorescent staining

PDLCs at third passage were seeded on 15 × 15 mm coverslips placed in 12-well plates. After they reached confluence, cells were rinsed three times with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.1% Triton X-100 for 20 min. The cells were then blocked with bovine serum albumin (3% in PBS) for 1 h. Subsequently, the coverslips were incubated with primary antibody against vimentin (1:100), fibronectin (1:200), or pan cytokeratin (1:100) (all antibodies from Boster Biological Technology, Wuhan, China) at 4°C overnight. Negative controls were incubated with PBS instead of primary antibodies. After washing, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:200; Proteintech Group, Inc., Rosemont, USA) for 60 min at room temperature. Finally, coverslips were sealed with antifade fluorescence mounting medium containing DAPI and observed under a fluorescence microscope.

Table 1. Primer sequences for RT-qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ALP	CGAGATACAAGCACTCCCACTTC	CTGTTCAGCTCGTACTGCATGTC
COL1	CGATGGATTCCAGTTCGAG	TAGGTGATGTTCTGGGAGGC
OCN	GGTGCAGCCTTTGTGTCCAA	CCTGAAAGCCGATGTGGTCA
RUNX2	AACCCTTAATTTGCACTGGGTCA	CAAATTCCAGCAATGTTTGTGCTAC
CAP	CTGCGCGCTGCACATGG	GCGATGTCGTAGAAGGTGAGCC
IBSP	AAGGGCACCTCGAAGACAAC	CCCTCGTATTCAACGGTGGT
CEMP1	GGGCACATCAAGCACTGACAG	CCCTTAGGAAGTGGCTGTCCAG
FABP4	AAATGTGTGATGCTTTTGTAGGTACC	CCATGCCAGCCACTTTCC
GLUT4	CCGCTACCTCTACATCATCCAGA	CAGAAACATCGGCCCAGC
PPARγ2	CGTGGCCGCAGATTTGAA	CTTCCATTACGGAGAGATCCAC
ZFP423	GTCACCAGTGCCCAGGAAGAAGAC	AACATCTGGTTGCACAGTTTACACTCAT
LPL	TACACCAAACTGGTGGGACA	TGGATCGAGGCCAGTAATTC
OPG	AAGTGGACCACCCAGGAAAC	TGTGCCAGCTGTCTGTGTAG
RANKL	GCGTCGCCCTGTTCTTCTATT	GCAGTGAGTGCCATCTTCTGA
GAPDH	AACAGCGACACCCACTCCTC	CATACCAGGAAATGAGCTTGACAA

Overexpression of C4orf7 in PDLCs

The plasmid, C4orf7-CMV-MCS-EF1-copGFP, and vector, PCDH-CMV-MCS-EF1-copGFP, were purchased from Hanbio Biotechnology Co. Ltd., Shanghai, China, and both transfected into 293E cells along with PMD2G and PSPAX2 (Thermo, USA). Lentivirus particles in the culture supernatant were collected after 48-72 h. PDLCs seeded on 10-cm dishes were infected with the collected lentivirus particles for 6 h, using 5 μg/ml polybrene. The transfected cells were cultured in fresh α-MEM containing 10% FBS. After 48 h, transfected cells were observed and photographed under light and fluorescence microscopes. The expression efficiency of C4orf7 was determined by qPCR and western blotting. Transfected PDLCs were divided into C4orf7 overexpression and empty vector controls group for subsequent experiments.

Cell counting kit-8 (CCK8) assay

The effect of C4orf7 expression on human PDLCs was analyzed by CCK8 assay. Briefly, transfected cells were seeded in 96-well plates at a density of 1×10^4 cells/well. On days 1, 2, 3, and 4, cells were incubated for 3 h with 10% (v/v) CCK8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan), followed by measurement of absorbance at 450 nm.

Osteogenic differentiation

To explore the effect of C4orf7 overexpression on the osteogenic differentiation of PDLCs, we conducted osteogenic induction for different durations in mineralized medium containing 50 μg/ml ascorbic acid, 10 mmol/L β-glycerophosphate, and 10 nmol/L dexamethasone; medium was changed every 3 days.

Alkaline phosphatase (ALP) assay: *ALP* activity was assayed to determine the early osteogenic differentiation of PDLCs after C4orf7 transfection. PDLCs were cultured in 24-well plates at a density of 5×10^4 cells/well. After osteogenic induction for 4 days, cells were stained for ALP using a BCIP/NBT kit (Beyotime, China) following the manufacturer's instructions. ALP activity assays were performed on days 0, 4, and 7 of osteogenic induction. Briefly, cultures were washed three times with cold PBS and lysed with 0.1% Triton X-100 at 4°C overnight. Cell lysates were centrifuged at 14,000 g for 10 min. Total protein concentrations were determined using a BCA protein assay kit (Biosharp, Hefei, China) and ALP activity assayed using p-nitrophenyl phosphate as the substrate. Absorbance was measured at 405 nm and 562 nm, respectively. ALP activity in each group was calculated from the absorbance levels relative to the protein concentration.

Alizarin red staining: The formation of mineralized nodules is generally considered a late osteogenic marker. We performed Alizarin red staining after 18 days of osteogenic induction. PDLCs were seeded in six-well plates with mineralized medium. After rinsing with PBS, cells were fixed with 4% paraformaldehyde for 15 min, followed by staining with Alizarin red solution (pH 4.2) for 20 min at room temperature. Excess dye was washed away using distilled water. Images of stained mineral deposits were captured using an inverted microscope.

Adipogenic differentiation

For adipogenic differentiation, PDLCs were cultured in α-MEM in 6-well plates. Adipogenic differentiation medium was added into sub-confluent cell cultures, according to the manufac-

turer's instructions (Cyagen, Suzhou, China). Oil red O staining was used to detect lipid accumulation.

Quantitative reverse transcriptase-polymerase chain reaction (qPCR) analysis and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA preparation: Total RNA was extracted from PDLCs and PDL tissue samples using TRIzol reagent (Invitrogen, USA). The concentration of the extracted RNA was determined by optical density measurement using a NanoDrop2000 Spectrophotometer. One microgram of total RNA was reverse transcribed into cDNA, using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). The resulting cDNA was used as the template in subsequent quantitative and semi-quantitative PCR analyses.

qPCR analysis: qPCR procedures were performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Tokyo, Japan) on a BIO-RAD system, according to the manufacturer's instructions. Relative expression values were calculated by the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used are shown in **Table 1**. Experiments were repeated three times, and gene expression levels were normalized with respect to those of *GAPDH*.

RT-PCR: To compare the levels of expression of C4orf7 in human PDL tissue samples and cultured PDLCs at passages P0-P5, RT-PCR was carried out using an rTaq kit (Takara, Tokyo, Japan) following the manual. The primers for C4orf7 and *GAPDH* were the same as those used in qPCR. PCR products were separated on 2% agarose gels and photographed using a gel imaging system.

Western blots

Cell cultures were washed twice with cold PBS and lysed using RIPA buffer supplemented with PMSF (Roche, Germany) and phosphatase inhibitors (Roche, Germany). Lysates were centrifuged at 4°C, 12,000 g, for 10 min. Proteins in the supernatant were denatured by heating at 95°C for 10 min. Protein samples were separated by SDS-PAGE and electro-transferred onto 0.22-μm polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk for 1 h to eliminate non-specific binding. Subsequently, these membranes were incubated with primary antibodies against human C4orf7 (1:1000, Proteintech Group, Inc, Rosemont, USA); RUNX2 (1:500, ABclonal Biotechnology Co., Ltd, Wuhan, China); COL1 (1:1000, Abcam, Cambridge, UK); OPN (1:1000, ABclonal Biotechnology Co., Ltd, Wuhan, China); OSX (1:1000, Abcam, Cambridge, UK); and GAPDH (1:5000, Abcam, Cambridge, UK) at 4°C overnight. After washing three times with TBST, membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were developed using enhanced ECL reagent and captured on X-ray films. GAPDH was used as internal control for protein expression analysis.

Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining

Healthy donor teeth were collected clinically and fixed with 4% paraformaldehyde for 24 h at room temperature. Samples were decalcified using 10% EDTA for 8 months, dehydrated, and embedded in paraffin. All paraffin-embedded specimens were cut into 5-μm sections, dried at 65°C for 2 h, deparaffinized, and rehydrated, in that order. For histological and histomorphometric analysis, specimens were stained with hematoxylin and eosin. To detect the expression of C4orf7 in periodontal tissue, IHC was performed following the manufacturer's protocol (Zhong Shan Biotech, Beijing, China). Briefly, specimens were treated with gastric enzyme for antigen retrieval and incubated with 3% hydrogen peroxide to block endogenous peroxidases, followed by blocking of non-specific binding using 5% goat serum. Samples were then incubated overnight with primary rabbit polyclonal antibody against C4orf7 (1:1000, Sigma-Aldrich Corp. St. Louis, MO, USA) at 4°C, followed by treatment with secondary biotinylated goat anti-rabbit antibody for 30 min at 37°C. After washing three times with PBS, slides were stained using a DAB kit (Maixin Biotech. Co., Ltd, Fuzhou, China), and nuclei were counterstained with hematoxylin.

Statistical analysis

All assays were performed in triplicate and each experiment was repeated at least three times. Data were analyzed using SPSS version 16.0 or GraphPad Prism Version 5.0 and are presented as means ± standard deviation (SD).

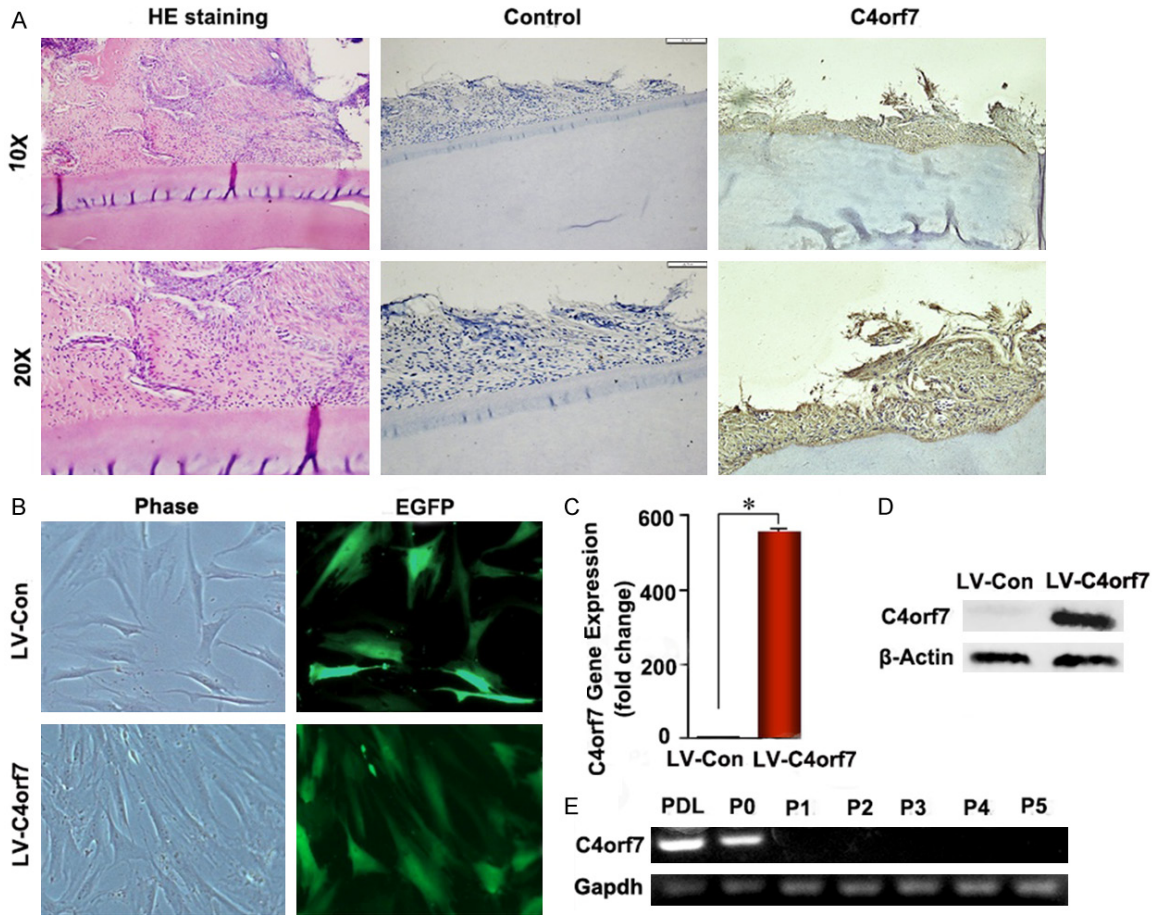

Figure 1. Expression of C4orf7 in human periodontal ligament (PDL) tissue and cultured periodontal ligament cells (PDLCs; P0-P5), and lentiviral-mediated overexpression of C4orf7 in human PDLCs. (A) PDL tissue sections stained with H&E (left), incubated with anti-C4orf7 antibody (right), or controls incubated with PBS instead of antibody (center). H&E-stained sections of PDL tissue show normal morphology. Immunohistochemical staining indicated widespread expression of C4orf7 in PDL tissue. Cell nuclei were counterstained with hematoxylin. (Previous row: original magnification × 100; next row: original magnification × 200). (B) PDLCs transfected with lentivirus for 48 h were photographed under light and fluorescence microscopes. (LV-C4orf7, cells transfected with lentiviral vector expressing C4orf7; LV-Con, control cells transfected with vector only). (C) The efficiency of C4orf7 overexpression from the lentiviral vector was confirmed by qPCR and (D) western blot analyses. (E) RT-PCR analysis of C4orf7 expression in PDL tissue and cultured PDLCs; images were taken using a gel imaging system.

Comparisons were performed using Student's t-tests for two groups and ANOVA for multiple groups. $P < 0.05$ was considered statistically significant.

Results

Expression of C4orf7 in human PDL tissue and cultured PDLCs

The expression of C4orf7 in human periodontal ligament was confirmed by histological assessment. H&E staining clearly showed that the morphology of PDL samples was normal, while IHC revealed extensive distribution of C4orf7 in PDL tissue (**Figure 1A**).

RT-PCR was used to detect differences in the expression of C4orf7 in PDL tissue samples and cultured PDLCs. As shown in **Figure 1E**, C4orf7 was expressed in PDL tissue, which was consistent with the results of IHC analysis. In contrast, C4orf7 expression was significantly lower in cultured primary PDLCs, with no expression detectable once cells were passaged (P1-P5).

C4orf7 overexpression in PDLCs

Human PDLCs infected with lentivirus were observed by light and inverted fluorescence microscopy. The ratio of GFP-labeled cells to total cells was almost 80% 48 h after lentiviral

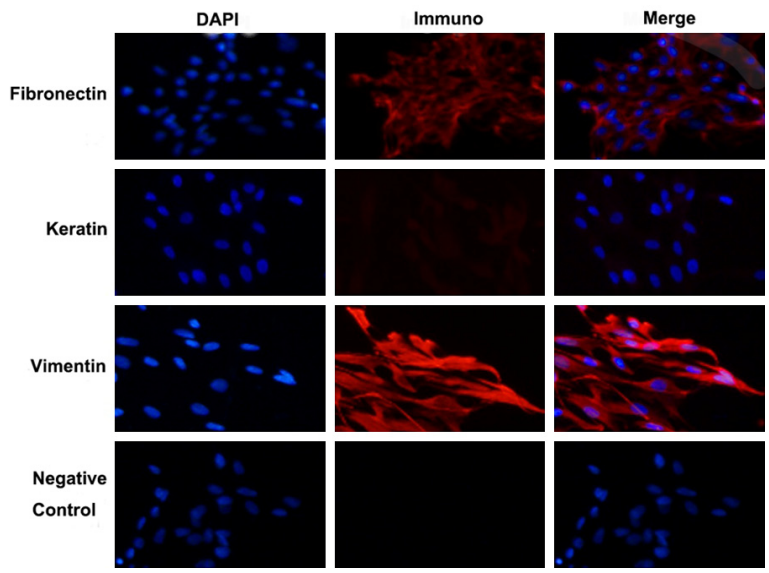

Figure 2. Identification of human periodontal ligament cells (PDLCs). Immunofluorescence staining showed that cultured PDLCs were positive for fibronectin and vimentin, and negative for pan cytokeratin, indicating their mesenchymal origin.

infection (**Figure 1B**). The efficiency of C4orf7 overexpression was confirmed by qPCR (**Figure 1C**) and western blotting (**Figure 1D**); See Supplementary Figure 1 for details. the expression of C4orf7 clearly increased at both mRNA and protein levels, with an increase at the mRNA level of approximately 600-fold. Cells transfected with empty vector were used as controls.

Identification and morphology of human PDLCs

Scattered single cells grew to confluence in 2 weeks with typical fibroblast-like morphology. Immunofluorescence staining of these cells was positive for vimentin and fibronectin and negative for pan cytokeratin, indicating that they were of mesenchymal origin, and the cells had fibroblast-like characteristics, demonstrating an absence of contamination with epithelial cells (**Figure 2**).

CCK8 assays

The effect of C4orf7 overexpression on PDLC proliferation and viability was analyzed by CCK8 assay. As illustrated in **Figure 3A**, the number of viable cells increased during the culture process. There was no significant difference in cell proliferation between the two groups at any of the time points studied.

Effect of C4orf7 overexpression on osteogenic/cementogenic/osteoclastogenic differentiation

To investigate the effect of C4orf7 overexpression on the osteogenic potential of PDLCs, cells were examined for different osteogenic markers at specific time points.

ALP, a key player in early osteogenic differentiation of PDLCs, was assayed at days 0, 4, and 7. The results indicated that *ALP* activity increased following the induction of mineralization, peaking on day 4 of induction. At day 4, alkaline phosphatase (*ALP*) staining was performed and showed that C4orf7 overexpression could reduce *ALP* expression levels in human PDLCs (**Figure 3B**). Also, as illustrated in **Figure 3C**, C4orf7 overexpression clearly inhibited *ALP* activity at days 0 and 4; however, on day 7, the effect was negligible ($P > 0.05$). The inhibitory effect of C4orf7 overexpression on that of *ALP* expression was verified by qPCR (**Figure 3G**).

All of the other osteogenic genes included in this study (*COL1*, *RUNX2*, and *OCN*) were downregulated in the C4orf7 overexpression group compared with controls (**Figure 3G**), and the protein expression levels of osteogenic markers, determined by western-blotting, showed a similar trend (**Figure 3E**). See the original western images in Supplementary Figure 2.

Calcium deposits and mineralized nodule formation were used as functional markers to determine the osteogenic capacity of cells *in vitro*. Microscopic images of C4orf7-expressing cells demonstrated a significant decrease in calcium nodules compared with controls, which was consistent with the results of quantitative analysis (**Figure 3D**).

The effect of C4orf7 overexpression on cementogenesis of PDLCs was examined by measuring the expression levels of the *IBSP*, *CAP*, and *CEMP1* genes by qPCR. Cementoblastic markers were also downregulated in response to C4orf7 overexpression, similar to markers of osteogenesis (**Figure 3F**).

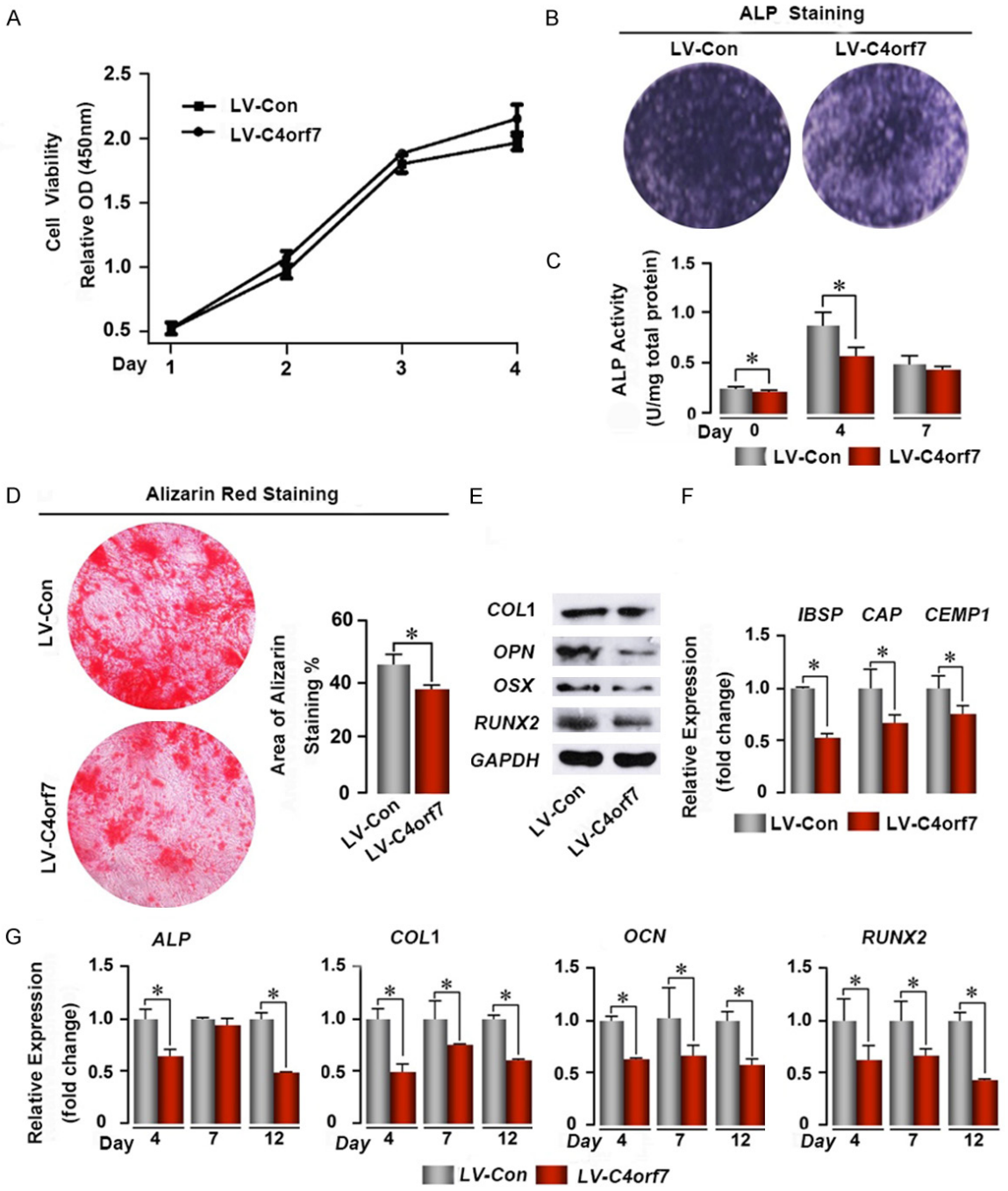

Figure 3. Effects of C4orf7 overexpression on viability, proliferation, and osteogenic/cementoblastic differentiation of cultured human periodontal ligament cells (PDLCs). A. Effect of C4orf7 transfection on cell proliferation. On day 1, we observed no significant difference among cells transfected with C4orf7 or empty vector ($P > 0.05$). The number of viable cells increased in both groups during culture, and there were no significant differences between the two groups on days 2, 3, and 4 ($P > 0.05$). B. Alkaline phosphatase (*ALP*) staining showed that C4orf7 overexpression could reduce *ALP* expression levels in human PDLCs. C. *ALP* activity assay in cells overexpressing C4orf7 and controls on days 0, 4, and 7. C4orf7 overexpression had a clear inhibitory effect on the *ALP* activity of human PDLCs on days 0 and 4 ($P < 0.05$); however, its effect was negligible on day 7 ($P > 0.05$). D. Matrix mineralization of PDLCs cultured for 18 days with osteogenic supplements was detected by Alizarin Red staining. Calcium deposition was quantified in both groups. The results demonstrated significantly fewer calcium nodules in PDLCs overexpressing C4orf7 compared with controls. E. Western blot analysis of the osteoblast markers, COL1, OPN, OSX, and RUNX2 in PDLCs; the group overexpressing C4orf7 showed a trend towards decreased expression levels compared with controls. F. The cementoblastic markers, *IBSP*, *CAP*, and *CEMP1*, were detected by qPCR analysis and exhibited

downregulation in response to C4orf7 overexpression. G. Relative mRNA expression levels of the osteogenic genes, *ALP*, *COL1*, *OCN*, and *RUNX2* in PDLCs overexpressing C4orf7 were lower than those in controls transfected with empty vector across the whole observation period ($P < 0.05$), indicating an inhibitory effect of C4orf7 on human PDLC osteogenesis. *, $P < 0.05$ vs. control.

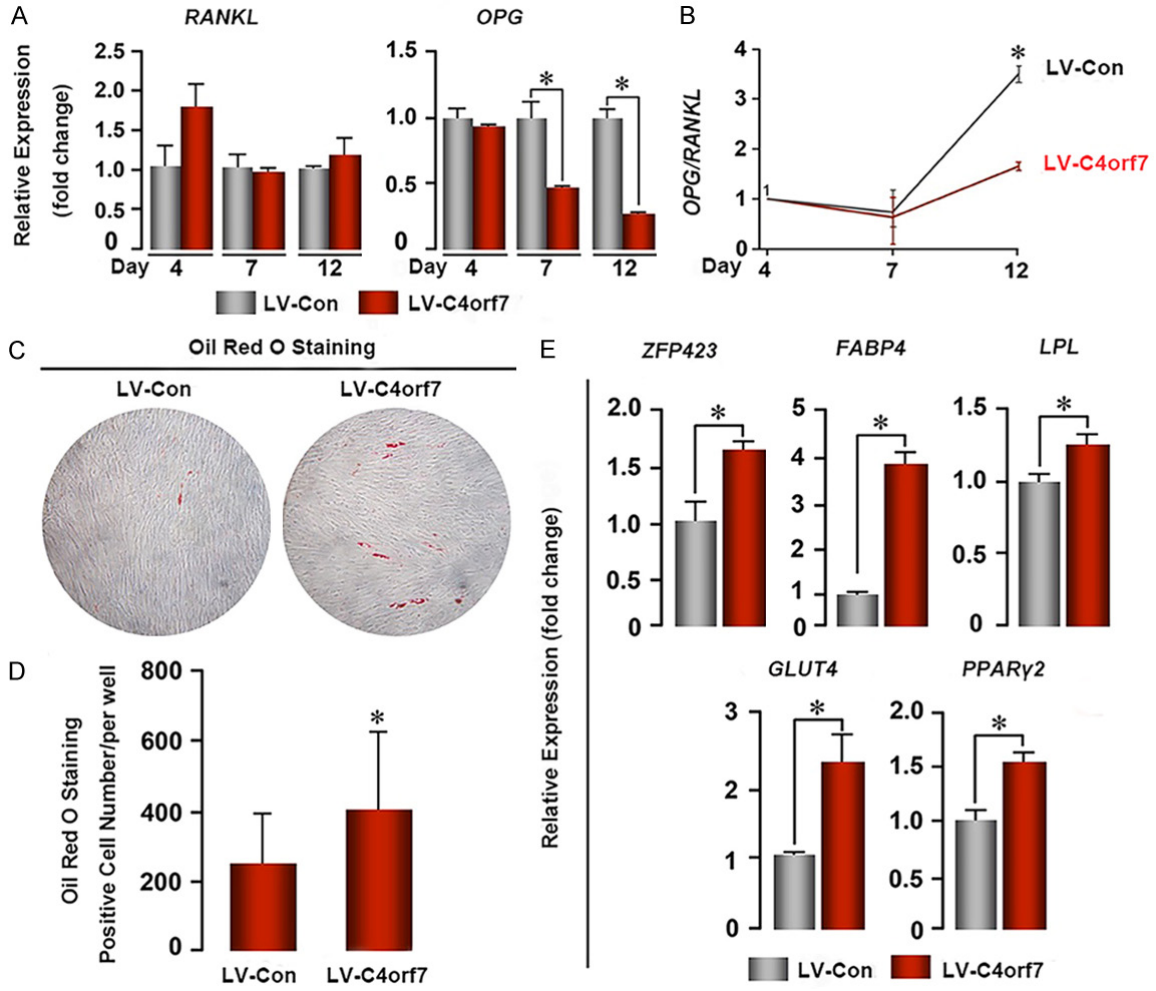

Figure 4. Effects of C4orf7 expression on the osteoclastogenic/adipogenic differentiation of PDLCs. A. The expression levels of the osteoclastogenesis-associated biomarkers, *RANKL* and *OPG*, were detected by qPCR at days 4, 7, and 12. There was no significant difference in the expression of *RANKL* after C4orf7 overexpression; however, the expression of *OPG* was downregulated in C4orf7 transfected cells on days 7 and 12. B. The higher *OPG*/*RANKL* ratio in the control group relative to that in the C4orf7 transfected group indicates that C4orf7 can promote osteoclastogenesis in human PDLCs. C. Lipid accumulation was detected by Oil Red O staining of PDLCs cultured for 21 days with adipogenic supplements. The number of lipid droplets significantly increased in C4orf7-transfected cells compared with controls. D. Quantitative analysis of lipid accumulation in the C4orf7 overexpression and empty vector control groups. E. The relative mRNA expression levels of the adipogenic markers, *ZFP423*, *FABP4*, *LPL*, *GLUT4*, and *PPARγ2,* were higher in PDLCs overexpressing C4orf7 than in controls transfected with empty vector ($P < 0.05$). *, $P < 0.05$ vs. control.

The ratio of *RANKL/OPG* is an indicator of osteoclastogenesis. We did not detect any difference in the expression levels of *RANKL* after C4orf7 overexpression (**Figure 4A**); however, the expression of *OPG* was downregulated, thereby increasing the ratio of *RANKL/OPG* in the C4orf7-overexpressing group compared with controls (**Figure 4A**, **4B**).

Effect of C4orf7 overexpression on adipogenic differentiation

qPCR analysis showed that the adipogenic markers, *PPARγ2*, *LPL*, *GLUT4*, *FABP4*, and *ZFP423* were significantly upregulated in the C4orf7 overexpression group relative to controls (**Figure 4E**). Moreover, staining with Oil red

O showed the formation of more lipid clusters in cells overexpressing C4orf7 compared with controls (**Figure 4C**, **4D**).

Discussion

PDL is an excellent source of cells with potential for self-renewal, high proliferation rates, and multilineage differentiation ability; therefore, this tissue is a candidate for use in tissue engineering and regenerative medicine applications [18, 19]. For PDLCs to be useful for regeneration of defective tissues, it is essential to understand the genes that exert a major influence on their multilineage differentiation potential. In the present study, we explored the effect of C4orf7 expression in human PDL tissue and cultured PDLCs. Based on our results, we conclude that C4orf7 is important in maintaining the equilibrium between adipogenic and osteogenic differentiation of PDLCs.

Our data confirm a previous report [15] that C4orf7 is expressed in PDL tissue, but not cultured PDLCs (P5/P7/P9). We also examined the expression of C4orf7 in cultured PDLCs at earlier passages (P0-P5), and unexpectedly found that it is expressed in primary PDLCs (P0). These results indicate that cultured PDLCs lose their PDL phenotype gradually during the culture process. In contrast to these observations, a recent study [20] showed that cultured PDLCs did express C4orf7 and the levels of expression increased after stimulation with lipopolysaccharide (LPS). Whether LPS stimulation can affect the expression profile of C4orf7 in cultured PDLCs warrants further investigation.

Periodontal bone remodeling involves a balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation [21, 22]. In physiological remodeling and healing processes, undifferentiated progenitor cells residing in the PDL are recruited to sites of periodontal injury and/or remodeling [23]. PDLCs are important in periodontal bone remodeling because of their multilineage differentiation potential. In the present study, we first explored the effect of C4orf7 overexpression on PDLC osteogenesis/cementogenesis. Consistent with the results of previous studies [24, 25], we found that the expression levels of the osteoblastic/cementoblastic phenotype markers, *ALP, RUNX2, OPN, COL1, CAP, CEMP1*, and *IBSP* were downregulated in PDLCs overexpressing C4orf7. Furthermore, the activity of alkaline phosphatase (*ALP*), which is involved in the mineralization of calcified tissue [26], also decreased on C4orf7 overexpression. Moreover, Alizarin red staining, an indicator of the accumulation of calcium and phosphate ions, confirmed the negative effect of C4orf7 on osteogenesis.

It is established that *OPG* is produced by osteoblasts and acts as a decay receptor that competes with *RANKL* for binding to RANK, and that this interaction can prevent bone resorption. The expression levels of *RANKL/RANK/OPG* can be used as an indicator to determine the degree of osteoclast-mediated bone resorption [27, 28]. In our study, the expression of *OPG* mRNA was downregulated, leading to an increase in the ratio of *RANKL/OPG* in PDLCs overexpressing C4orf7 over the duration of the experiment. Based on this result, we conclude that C4orf7 can promote PDLC osteoclastogenesis. However, in contrast to the results obtained by other researchers [20], the two experimental groups in our study did not differ significantly with respect to *RANKL* mRNA expression. This discrepancy may be due to the differences in the characteristics of human primary cells from different donors and/or differences in the durations of experiments. In light of these findings, we speculate that C4orf7 may skew the differentiation of PDLCs toward osteoclastogenesis, rather than osteogenesis, during periodontal bone remodeling.

Osteoblasts and adipocytes share a common precursor cell pool. Accumulating evidence indicates that there is an inverse relationship between osteogenesis and adipogenesis [29, 30]. Some researchers have demonstrated that a proportion of cells in PDL tissue have adipogenic potential. Therefore, we examined adipogenesis-associated markers, including *PPARγ2, LPL, ZFP423, FABP4*, and *GLUT4*, to investigate the role of C4orf7 in adipogenic differentiation of PDLCs. *PPARγ* is a master transcription factor and an early marker of adipogenic differentiation [31, 32]. It can regulate a wide range of genes expressed in developing and mature adipocytes, including *LPL*, *ZFP423*, *FABP4*, and *GLUT4*. We observed that upregulation of *PPARγ2* mRNA expression was accompanied by an increase in expression of the adipocyte markers, *GLUT4, ZFP423, FABP4*, and *LPL*, in the PDLCs overexpressing C4orf7. Oil

Red O staining, a marker of lipid maturation, demonstrated that the level of lipid accumulation in the cells overexpressing C4orf7 was higher than that in controls. Considering all these results together, we propose that C4orf7 may skew the osteogenic/adipogenic balance by favoring adipogenesis, while inhibiting osteogenesis.

Mounting evidence indicates that an imbalance between normal adipogenesis and osteogenesis of mesenchymal stem cells (MSCs) is associated with various human metabolic disorders, including obesity and osteoporosis [33, 34]. These metabolic disorders induce local and systemic low-grade inflammation, which is a risk factor for bone loss [35, 36]. More importantly, a recent study [37] reported that adipogenic differentiation of non-adipose-derived MSCs can result in the loss of their intrinsic immunoprotective capacity and alter the bioactivity of IL-6 or TGFβ, switching the function of these cytokines from an immunoprotective to a pro-inflammatory state. Such pro-inflammatory cytokines promote bone loss by activating osteoclastogenesis and decrease bone formation by inhibiting osteogenic differentiation. Adipocytes are not present in normal PDL tissue. Aberrant adipogenic differentiation of PDLCs may downregulate osteogenesis and directly reduces bone formation, and can also trigger inflammatory mediators, leading to further bone loss. Thus, C4orf7 may skew the periodontal remodeling balance via these direct and/or indirect pathways, and can, therefore, act as an indicator of periodontal bone loss; however, the precise mechanism by which C4orf7 regulates periodontal bone metabolism requires further exploration.

In conclusion, this is the first report on the novel role of C4orf7 as a key regulator of periodontal bone metabolism. C4orf7 represents a potential therapeutic target for the treatment of periodontal diseases and other bone metabolic disorders.

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Disclosure of conflict of interest

None.

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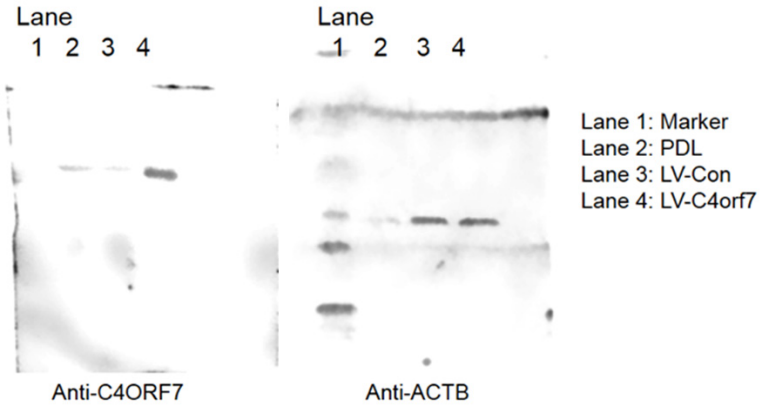
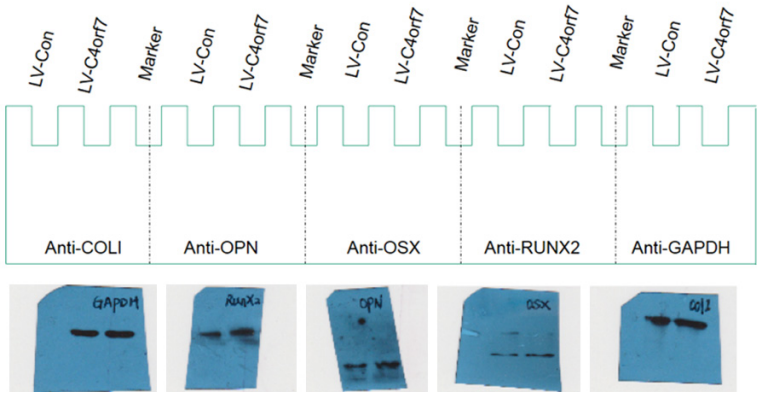


Supplementary Figure 1. Expression of C4orf7 in human periodontal ligament (PDL) tissue and cells transfected with lentiviral vector expressing C4orf7or empty vector only. Each protein sample was loaded on SDS-PAGE gel as above order. PDL tissue was used as positive control of C4orf7 expression, and ACTB was used as internal control. Protein bands were developed by using enhanced chemiluminescence (ECL) system (Biosharp, Hefei, China) and visualized by Odyssey® CLx imaging system (LI-COR Biotechnology, Lincoln, NE, United States).



Supplementary Figure 2. Western-blot analysis of relative osteogenic marker. Each protein sample was loaded on SDS-PAGE gel as above order and electro-transferred onto 0.22 μm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 1 h to eliminate non-specific binding. Subsequently, these membranes were cut off at the marker band and then incubated with primary antibodies against human COL1, OPN and OSX, RUNX2 respectively at 4°C overnight. After washing three times with TBST, the membranes were then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were developed by enhanced ECL reagent, impressed by X-ray films. The upper left corner of the film indicates the position of marker. GAPDH was used as internal control of protein expression analysis.