CircRNA FAT1 Regulates Osteoblastic Differentiation of Periodontal Ligament Stem Cells via miR-4781-3p/SMAD5 Pathway

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The ability of human periodontal ligament stem cells (PDLSCs) to differentiate into osteoblasts is significant in periodontal regeneration tissue engineering. In this study, we explored the role and mechanism of circRNA FAT1 (circFAT1) in the osteogenic differentiation of human PDLSCs. The proliferation capacity of PDLSCs was evaluated by EdU and CCK-8 assay. The abilities of circFAT1 and miR-4781-3p in regulating PDLSC differentiation were analyzed by western blot, reverse transcription-polymerase chain reaction (RT-PCR), alkaline phosphatase (ALP), and Alizarin red staining (ARS). A nucleocytoplasmic separation experiment was utilized for circFAT1 localization. A dual-luciferase reporter assay confirmed the binding relationship between miR-4781-3p and circFAT1. It was showed that circFAT1 does not affect the proliferation of PDLSCs. The osteogenic differentiation of PDLSCs was benefited from circFAT1, which serves as a miRNA sponge for miR-4781-3p targeting SMAD5. Both knockdown of circFAT1 and overexpression of miR-4781-3p suppressed the osteogenic differentiation of PDLSCs. Thus, circFAT1 might be considered as a potential target of PDLSCs mediated periodontal bone regeneration.

1. Introduction

Periodontitis is a chronic oral infectious disease characterized by the disruption of periodontal supporting tissues integrity, including the destruction of alveolar bone, periodontal ligament (PDL), and cementum [1]. As one of the most common infection-driven diseases, Periodontitis can affect 90% of the global population [2]. Periodontitis can lead to loss of periodontal attachment and, if left untreated, can eventually lead to early tooth loss [3]. At present, the biggest challenge in the treatment of periodontitis is periodontal regeneration. Human periodontal ligament stem cells (PDLSCs) are mesenchymal stem cells (MSCs) derived from tooth tissues with high osteogenesis potential. Therefore, studying the molecular mechanism of osteogenic differentiation of PDLSCs is the keystone of the clinical application of tooth regeneration and osteogenic tissue engineering.

Noncoding RNA (ncRNA) is a kind of RNA transcribed from the genome, which can function at the RNA level rather than traditionally encode protein [4]. ncRNA can be classified into three types: (1) microRNA (miRNAs), siRNAs, and new noncoding small RNAs (piRNAs) with a length less than 50 NT; (2) the length ranges from 50 to 500 NT, including ribosomal RNA (rRNA) and transfer RNA (tRNA); and (3) longer than 500 NT, including long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs) [4, 5]. miRNA is an endogenous noncoding small single-stranded RNA with a length of about 22 nucleotides [6]. It is worth noting that microRNAs participate in bone metabolism by acting on target genes related to osteogenic differentiation.
Many miRNAs have been proved to participate in the osteogenic differentiation of PDLS Cs [7–9].

The TGF-β signaling pathway is an essential pathway for the osteogenic differentiation process. It relies on multiple SMAD proteins, such as receptor-regulated SMAD (R-SMAD), common SMAD (co-SMAD), and inhibitory SMAD (I-SMAD) [10]. SMAD family member 5 (SMAD5) is a R-SMAD protein. As a transcription factor, it takes part in the osteogenic differentiation of BMSCs [11]. When osteogenic signals are transmitted to the cytoplasm of BMSCs, phosphorylated-SMAD5 is directed to the nucleus and then regulates the expression of osteogenesis-related target genes [10]. The nuclear translocation of SMAD5 is key to osteogenesis signal transduction. LncTUG1 may inhibit the osteogenic differentiation of BMSCs by targeting SMAD5 [12]. miR-21, miR-17-5p, and miR-106b-5p inhibit bone cells (BMSCs) by targeting SMAD5 [13]. miR-24-3p targets SMAD (I-SMAD) [10]. SMAD family member 5 (SMAD5) is a R-SMAD protein. As a transcription factor, it takes part in the osteogenic differentiation of BMSCs [11]. When osteogenic signals are transmitted to the cytoplasm of BMSCs, phosphorylated-SMAD5 is directed to the nucleus and then regulates the expression of osteogenesis-related target genes [10]. The nuclear translocation of SMAD5 is key to osteogenesis signal transduction. LncTUG1 may inhibit the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) by targeting SMAD5 [12]. miR-24-3p targets SMAD5 to promote the osteogenic potential of PDLS Cs [13]. miR-21, miR-17-5p, and miR-106b-5p inhibit bone formation by targeting SMAD5 [14, 15]. In another study, miR-222-3p depressed osteogenic differentiation of BMSCs, regulating the expression of SMAD5-RUNX2 signal axis [11].

CircRNAs are characterized by a special structure of continuous covalent closed loop, which has higher conservation and stability [16, 17]. CircRNAs have been applied in clinical treatment or disease diagnosis as biomarkers or targets [18]. Evidence has been demonstrated that circRNAs played a key role in developing numerous diseases by regulating key steps such as gene transcription, translation, and splicing [19, 20]. Studies on the accumulation of circRNAs revealed their important role in bone metabolism-related diseases [21]. Recently, circRNAs are discovered to be involved in maintaining the pluripotency of human embryonic stem cells (ESCs) [22], self-renewal ability of intestinal stem cells [23], the differentiation potential of osteoblasts and osteoclasts [24, 25], and even the rat liver regeneration [26]. So far, circRNAs have been well known as miRNA sponges. Thus, circRNAs work as posttranscriptional regulators, sponging with miRNAs and producing important biological effects [27]. Accumulating evidence shows that circRNAs play a nonnegligible role in many diseases, including periodontitis. Acting as a miR-7 sponge to upregulate Kruppel-like factor 4 (KLF4) expression, circCDR1 promoted PDLS Cs stemness [28]. circCDK8 inhibited the osteogenic differentiation of PDLS Cs by triggering autophagy activation in a hypoxic microenvironment [29].

CircRNA FAT1 (circFAT1), as a relatively new circRNA, has been reported to increase cell stemness of cancer cells through upregulation of miR-21 [30]. In breast cancer, circFAT1 may regulate miR-525-5p/SKAI resistance through Notch and Wnt pathways, providing a potential target for breast cancer treatment [31]. circFAT1 has not been reported in any published literature about its effect on the osteogenic differentiation of PDLS Cs. In our previous study, it was found that the expression of circFAT1 decreased in the hypoxic microenvironment, while the osteogenic differentiation ability of PDLS Cs decreased. Thus, it can be inferred that circFAT1 may be positively correlated with the osteogenic differentiation of PDLS Cs. miR-4781-3p was shown to be enriched in circFAT1 and miR-4781-3p, which may be involved in regulating the osteogenic differentiation of PDLS Cs. Combined with bioinformatics predictions, this study investigates the mechanism by which circFAT1 may act as a sponge of miR-4781-3p to regulate SMAD5 and then affect the osteogenic differentiation of PDLS Cs. It is expected to provide a new therapeutic target for exploring the periodontal regeneration mediated by PDLS Cs.

2. Materials and Methods

2.1. Animals. Male SD rats (5 weeks old) were purchased from the experimental animal center of Nanjing Medical University and raised at the SPF level. All animal experiments were conducted according to the regulations of the ethics committee of Nanjing Medical University (IACUC-20100501). SD rats were anesthetized by intraperitoneal injection (1% sodium barbital) to establish a skull defect model. Make a 10 mm incision in the rat head and perform a trephine osteotomy on the cranial platform. Make two 5 mm diameter holes symmetrically on both sides of the midline of the skull. Implant the cell mass and the corresponding control group into the pores. Then, suture the wound. At week 8, skull tissue was collected for further analysis.

2.2. Microcomputed Tomography (micro-CT), Hematoxylin-Eosin (H&E), and Masson Staining. The rats were sacrificed eight weeks after surgery, and the cranial tissue was collected. The tissues were fixed with 4% paraformaldehyde (PFA) for one week and 75% ethanol for micro-CT evaluation. 3D images of the mineralized tissues were reconstructed using Sky scan software. The bone volume/tissue volume (BV/TV) of each sample was collected for analysis. After micro-CT evaluation, the tissues were dehydrated in 14% EDTA solution for eight weeks, dehydrated by an automatic dehydrator, and embedded in paraffin. Paraffin sections were cut into tissue sections 5 mm thick for H&E and Masson staining.

2.3. Tissue Collection and Cell Culture. The premolars of healthy people extracted due to orthodontics were collected. Scrape the periodontal ligament tissue in the 1/3 area of the root, transfer it into a 10 cm sterile dish, and add α-minimum primary medium (α-MEM, Gibco, California, USA) to keep the tissue moist, sharply separate about 1mm^2 tissue blocks of periodontal ligament tissue, lay the separated tissue blocks in culture flask at a spacing of 1 mm, inverted the flask, and add α-MEM (including 100 ml/L fetal bovine serum, 100u/ml penicillin, and 100 μg/ml streptomycin). The culture flask was placed in an incubator at 37°C and 5% CO₂ for culture. Turn over the tissue after 4 hours. The solution was changed every three days. The cells grew up to 80% and were sub cultured when confluence. A monoclonal screening method was adopted to purify PDLS Cs. 3-5
generations of PDLSCs were cultivated for the follow-up experiments. The Ethics Committee of Nanjing Medical University School approved the relevant experiments (NJMU-2018202).

2.4. Adipogenic Differentiation. PDLSCs in the logarithmic growth stage were inoculated into the culture dish following the cell density of 2 × 10^4 cells/cm². The cells were cultured at 37°C, 5% CO₂ environment to the confluence of 90-100%, the supernatant was discarded, and the adipogenic induction differentiation medium induction solution (Cyagen, Guangzhou, China) was added. After three days, the culture medium was replaced with an adipogenic differentiation medium induction solution. After one day of culture, it was replaced with adipogenic differentiation medium maintenance solution for three days. The cells were induced for 14-21 days according to the above liquid exchange frequency; then, the medium was aspirated. After washing once with 1×PBS, PDLSCs were fixed with 4% PFA solution at room temperature for 30-60 minutes, and oil red O staining was performed.

2.5. Chondrogenic Differentiation. Transfer 3 × 10^5 PDLSCs to a 15 ml centrifuge tube and centrifuge at 250 g for 4 minutes. Discard the supernatant, add 0.5 ml of chondrogenic differentiation medium basal solution, resuspend the cells, and centrifuge at 150 g for 5 minutes. Carefully discard the supernatant, add 0.5 ml chondrogenic differentiation medium induction solution (Cyagen, Guangzhou, China), resuspend the cells, and centrifuge at 150 g for 5 minutes. Unscrew the 15 ml centrifuge tube cap slightly and place it at 37°C, 5% CO₂ incubator. After 24 hours, observe the deformation and accumulation of the cell pellets. The cell mass was transferred to a 24-well plate. Replace with induction solution every two days. After 21 days, the cartilage balls were fixed and sliced for Alcian blue staining.

2.6. Plasmid and siRNA Transfection. PDLSCs were inoculated in medium dishes and transfected when the fusion rate reached 50-60%. The transfected siRNA and plasmid were constructed by the company (RiboBio, Guangzhou, China). miR-4781-3p mimics (mimics, 50 nm), mimic negative control (NC, 50 nM), miR-4781-3p inhibitor (inhibitor, 100 nM), and inhibitor negative control (iNC, 100 nM) were mixed with Ribofect™ CP Kit (RiboBio, Guangzhou, China). Cells were stimulated for 24-72 hours. Similarly, PDLSCs transfected with circFAT1, SMAD5 siRNA, and negative controls (si-FAT1, si-SMAD5, NC, 100 nM) were performed similar operations. Luciferase reporter plasmids of circFAT1 and miR-4781-3p were constructed by predicting the binding sites. Lipofectamine 2000 was used as a transfection agent to transfect HEK-293 T cells.

2.7. Cell Proliferation Assay. Different stimuli were adopted to treat PDLSCs and then add 100 μl of cell suspension in a 96-well plate. Incubate the culture plate for 0, 1, 3, 5, and 7 days. Add 10 μl of CCK8 solution to each well and incubate the culture plate in the incubator for 1-4 hours. Measure the absorbance at 450 nm with a microplate reader. EdU was detected by Cell-Light™ EdU Apollo®567 In Vitro Imaging Kit (RiboBio, Guangzhou, China). The transfected PDLSCs were seeded in glass slides. After labeling with EdU, fixing the cells, staining with Apollo, and staining DNA with Hoechst 33342, the cells were observed with a fluorescence microscope. ImageJ software was used to calculate cell DNA replication efficiency.

2.8. Flow Cytometry. After the cells were treated differently, they were digested with trypsin without EDTA (Beyotime, Shanghai, China) and collected. The cells were resuspended in PBS and centrifuged twice, then resuspended in 100 μl of PBS, and added with CD34, CD45, CD29, CD90, and CD105 surface molecule antibodies. After incubating on ice for 30 minutes, the cells were centrifuged with PBS. Then, the supernatant was removed, and flow cytometry analysis (BD Biosciences, CA, USA) was performed.

2.9. Quantitative Real-time RT-PCR (RT-qPCR). TRizol (Invitrogen, CA, USA) method was used to extract RNA from cells. Then, the RNA was reverse transcribed to cDNA by a reverse transcription kit. Design the corresponding primers. Use the CHAMQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) reagent to set up the related program on the ABI QuantStudio 7 fluorescence quantitative PCR instrument (Applied Biological System) according to the instructions. The primer list is shown in Table 1.

2.10. Western Blot Analysis. The cell lysate was used to lyse the cells, the supernatant was taken after centrifugation, and the protein loading buffer was added; then, the protein sample was boiled. Perform electrophoresis experiments with 10% SDS-PAGE gel at 70 V constant pressure and transfer membrane at 300 mA constant current. After sealing with 5% milk for 2 hours, add primary antibody diluent (anti-COL1A (Proteintech, USA), anti-COL3A (Proteintech, USA), anti-RUNX2 (ABCAM, UK), anti-OSX (ABCAM, UK), anti-SMAD5 (Proteintech, USA), and anti-GAPDH (Cell signaling Technology, USA)). After 4°C overnight, protein bands were obtained by chemiluminescence gel imaging system. Then, the grey value analysis was performed.

2.11. Alkaline Phosphatase (ALP) Activity Assay and Alizarin Red Staining (ARS). After seven days of induction of PDLSCs by adding mineralization induction solution, the ALP activity was detected, and ALP staining was performed. The alkaline phosphatase detection kit (Jiancheng, Nanjing, China) and the alkaline phosphatase color reagent kit (Beyotime, Shanghai, China) were used for detection. After 14 days of induction, 4% PFA was added to fix PDLSCs and then wash the cells with PBS 3 times. The cells were observed under the microscope after being incubated with Alizarin Red dye solution (40 mM, pH = 4.2, Sigma-Aldrich) for at least 1 hour. Cetylpyridine chloride (CPC, 100 mM) was used to dissolve calcified nodules, and the relative calcium mass was calculated according to the absorbance at 562 nm.

2.12. Immunofluorescence Staining. The treated PDLSCs were digested and fixed with 4% PFA. Perforate the cells with Triton X-100 (Beyotime, Shanghai,
(1) Primary PDLSCs crawled out from around the tissue, mostly long spindle-shaped and growing densely (Figure 1(a)). After screening the cells cultured by the monoclonal method, multiple scattered cell colonies similar to clones could be seen (Figures 1(a)–1(c)). Flow cytometry results showed that CD29, CD73, CD90, and CD105 were positive in PDLSCs. In addition, both CD45 and CD34 were negative (Figure 1(b)).
Figure 1: Identification of PDLSCs and verification of PDLSC multidirectional differentiation ability. (a): (I) Primary cells migrated from PDL tissues on day 3, and 80% confluence was observed on day 12 (scale bar: 100 μm). (II, III) Cell colonies formed by PDLSCs observed in the dish and an amplified image of a representative colony captured by a microscope (scale bar: 100 μm). (b) Flow cytometry results showed that CD29, CD73, CD90, and CD105 were positive in PDLSCs. In addition, both CD45 and CD34 were negative. (c) Multiple differentiation potentials of PDLSCs (from left to right: osteogenesis, adipogenesis, chondrogenesis; scale bar: 100 μm). (d) Immunofluorescence assay revealed that cultured PDLSCs were positive for STRO-1 (scale bar: 100 μm).
3.2. circFAT1 Does Not Affect PDLSCs Proliferation

(1) CCK8 assay verified that PDLSCs transfection of circFAT1 siRNA had no significant effect on proliferation (Figure 2(a)). DNA replication ability of cells was detected by EdU assay, which further confirmed that the effect of circFAT1 on the proliferation of PDLSCs is not significant (Figures 2(a) and 2(b)).

(2) PCR gel electrophoresis verified that the primers were located at a single band of 449 kb. The cyclization site of circFAT1 was determined by plasmid vector construction (Figure 2(c)). The results of RNA nucleo-cyttoplasmic isolation proved that circFAT1 was mostly distributed in the cytoplasm (Figure 2(d)).

3.3. circFAT1 Silencing Inhibits Osteoblastic Differentiation Potential of PDLSCs. To detect whether circFAT1 affects the osteogenic differentiation potential of PDLSCs, cells were transfected with NC or circFAT1 siRNA (si-FAT1), respectively. Western blot and RT-qPCR showed that biomarkers of osteogenesis (RUNX2, OSX, COL1A, and COL3A) were downregulated, and RT-qPCR results showed that the circFAT1 expression was decreased (Figures 3(a)–3(c)).

The osteogenic induction medium was used for culturing two groups of cells, respectively. ARS confirmed that the mineralization nodules in the si-FAT1 group were decreased, and the calcium content calculated by the CPC assay was significantly decreased (P < 0.001) on 14 days. ALP activity was detected after seven days, which was used as an indicator for early observation of the osteogenic differentiation ability of PDLSCs. We found that the si-FAT1 group expresses less ALP, and meanwhile, the activity of ALP decreased significantly (P < 0.001) (Figures 3(d) and 3(e)). Immunofluorescence experiments confirmed the reduced expression of ALP in the si-FAT1 group (Figure 3(f)).

3.4. SMAD5 Regulates the Osteoblastic Differentiation Ability of PDLSCs. PDLSCs were transfected with NC or SMAD5 siRNA (si-SMAD5). Western blot and RT-PCR results showed decreased expression of SMAD5 and biomarkers of osteogenesis (RUNX2, OSX, COL1A, and COL3A) in the smad5 group. In addition, RT-qPCR results displayed less expression of circFAT1 and increased expression of miR-4781-3p (Figures 5(a)–5(c)). The mineralization induction medium was used for culturing two groups of PDLSCs, and ARS and CPC assay was performed at 14 days. It was found that the mineralization nodules and calcium content of the mimics group were significantly reduced (P < 0.001). After a week, the ALP activity was detected, less expression of ALP was found in the mimics group, and the ALP activity was downregulated significantly (P < 0.001) (Figures 5(d) and 5(e)). Immunofluorescence experiments confirmed that the expression of ALP in PDLSCs in the mimics group was significantly reduced (Figure 5(f)).

3.5. Overexpression of miR-4781-3p Decreases Osteoblastic Differentiation Tendency of PDLSCs. PDLSCs were transfected with NC for mimics or miR-4781-3p mimics (mimics), respectively. Western blot and RT-qPCR showed that SMAD5 and biomarkers of osteogenesis (RUNX2, OSX, COL1A, and COL3A) were reduced. In addition, RT-qPCR results displayed less expression of circFAT1 and increased expression of miR-4781-3p (Figures 5(a)–5(c)).

The mineralization induction medium was used for culturing two groups of PDLSCs, and ARS and CPC assay was performed at 14 days. It was found that the mineralization nodules and calcium content of the mimics group were significantly reduced (P < 0.001). After a week, the ALP activity was detected, less expression of ALP was found in the mimics group, and the ALP activity was downregulated significantly (P < 0.001) (Figures 5(d) and 5(e)). Immunofluorescence experiments confirmed that the expression of ALP in PDLSCs in the mimics group was significantly reduced (Figure 5(f)).

3.6. Knockdown of miR-4781-3p Increases Osteoblastic Differentiation Tendency of PDLSCs. PDLSCs were transfected with negative inhibitor control (iNC) or miR-4781-3p inhibitor (inhibitor), respectively. Western blot and RT-qPCR showed that the expression of SMAD5 and biomarkers of osteogenesis (RUNX2, OSX, COL1A, and COL3A) increased. In addition, RT-qPCR results showed that the circFAT1 expression was increased while the expression of miR-4781-3p was decreased (Figures 6(a)–6(c)).

The mineralization induction medium was used for culturing two groups of PDLSCs. ARS and CPC determination were performed on the 14th day. The results showed that mineralized nodules and calcium content increased significantly in the inhibitor group (P < 0.001). ALP activity was detected after a week, and the results showed that an increasing ALP expression in the inhibitor group and ALP activity was also upregulated (P < 0.001) (Figures 6(d) and 6(e)). Immunofluorescence analysis confirmed that the ALP expression was promoted in the inhibitor group (Figure 6(f)).

3.7. circFAT1 Acting as a miRNA Sponge for miR-4781-3p by Targeting SMAD5. To verify the sponge effect of circFAT1 as miR-4781-3p competitively combining SMAD5, circFAT1 wild-type (FAT1 WT) and mutant plasmid (FAT1 MT) were constructed, respectively. By cotransfection with miR-4781 negative control (NC) and miR-4781 mimics (mimics), dual-luciferase reporter assay showed that miR-4781 mimics significantly inhibited the luciferase activity of circFAT1 wild-type reporter gene and confirm the existence of binding sites (Figure 7(a)). SMAD5 wild-type (SMAD5 WT) and mutant plasmid (SMAD5 MT) were constructed, respectively. By cotransfection with miR-4781 negative control (NC) and miR-4781 mimics (mimics), dual-luciferase reporter assay showed that miR-4781 mimics significantly inhibited the luciferase activity of SMAD5 wild-type reporter.
gene and confirm the existence of binding sites between SMAD5 and miR-4781 (Figure 7(a)).

Meanwhile, a rescue experiment was carried out to further verify. The western blot results revealed that inhibiting miR-4781 could reverse the inhibitory effect of circFAT1 siRNA on the osteogenic ability of PDLSCs. And the miR-4781 inhibitor reversed the expression of SMAD5 (Figure 7(b)).
The osteogenic induction medium was used for culturing four groups of cells, respectively. CPC assay and ARS were performed at 14 days, and it was found that the mineralization nodules in the si-FAT1 group and si-FAT1 + miR-4781 iNC group decreased. Mineralized nodules were significantly increased in the si-FAT1 + inhibitor group, and calcium content was significantly increased ($P < 0.001$).

After seven days, less expression of ALP was found in the si-FAT1 group and si-FAT1 + miR-4781 iNC group, while the expression of ALP increased in the si-FAT1 + inhibitor group. ALP activity showed the same trend (Figures 7(c) and 7(d)). FISH experiments proved that circFAT1 was mostly located in the cytoplasm, and 18S and U6 were detected as the internal control (Figure 7(e)).
3.8. Silence of circFAT1 Inhibits the Osteogenic Differentiation Ability of PDLSCs In Vivo. To further understand the role of circFAT1, the skull defect model of SD rats was constructed (Figure 8(a)). PDLSC cell mass transfected with NC or circFAT1 siRNA (si-FAT1) was placed, respectively, and the tissue samples were taken eight weeks later. 3D reconstruction image of the rat skull showed that bone regeneration in the si-FAT1 group was inhibited (Figure 8(b)). H&E and Masson staining results indicated that the new bone mass in the si-FAT1 group was significantly reduced. The bone volume fraction was decreased in the si-FAT1 group by calculating the ratio of bone volume and tissue volume (BV/TV) ($P < 0.01$) (Figures 8(c) and 8(d)). Therefore, as shown in the pattern diagram, circFAT1 may regulate PDLSC osteoblastic regeneration by targeting SMAD5 through acting as a sponge for miR-4781-3p (Figure 8(e)).

Figure 4: SMAD5 regulates the osteoblastic differentiation ability of PDLSCs. (a)–(c) Western blot and RT-qPCR showed that osteogenic markers (RUNX2, OSX, COL1A, and COL3A) were downregulated in the SMAD5 siRNA-treated PDLSCs (si-SMAD5 group). (d, e) Silence of SMAD5 reduced the ALP activity of PDLSCs 7 days after osteogenic induction. ARS showed the mineralization of PDLSCs was decreased significantly in the si-SMAD5 group after osteogenic induction for 14 days. (f) Immunofluorescence experiments confirmed the decreased expression of ALP in the si-SMAD5 group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. 

staining results indicated that the new bone mass in the si-FAT1 group was significantly reduced. The bone volume fraction was decreased in the si-FAT1 group by calculating the ratio of bone volume and tissue volume (BV/TV) ($P < 0.01$) (Figures 8(c) and 8(d)). Therefore, as shown in the pattern diagram, circFAT1 may regulate PDLSC osteoblastic regeneration by targeting SMAD5 through acting as a sponge for miR-4781-3p (Figure 8(e)).
4. Discussion

As one of the most common oral inflammatory diseases, periodontitis is often related to human tooth loss. For periodontal tissue with complex structure, tissue regeneration and stable microenvironment are challenging for currently available treatments. In regenerative therapy, since MSCs can be obtained from various tissues, stem cell therapy has
attracted more and more attention. PDLSCs are considered the best cell source for periodontal tissue regeneration [33]. In our previous study, LncNEAT1 may target miR-214-5p/SMAD4 to regulate the cementogenic differentiation of PDLSCs (preprint) [34]. Seo et al. [35] found that PDLSCs can create a cementum/periodontal ligament-like structure.
**Figure 7:** circFAT1 acting as a miRNA sponge for miR-4781-3p by targeting SMAD5. (a) I: Dual-luciferase reporter gene assay was used to verify the binding sites between circFAT1 and miR-4781-3p. II: Dual-luciferase reporter gene assay was used to verify the binding sites between SMAD5 and miR-4781-3p. (b) Rescue experiment results showed that miR-4781 inhibitor could reverse the inhibition of circFAT1 siRNA on the osteogenic ability of PDLSCs. The expression level of SMAD5 was also reversed by miR-4781 inhibitor. (c) After seven days, less expression of ALP was found in the si-FAT1 group and si-FAT1 + miR-4781 iNC group, while the expression of ALP increased in the si-FAT1 + inhibitor group. The mineralization nodules in the si-FAT1 group and si-FAT1 + miR-4781 iNC group decreased. (d) I: ALP activity showed the same trend. II: Mineralized nodules were significantly increased in the si-FAT1 + inhibitor group, and calcium content was significantly increased ($P < 0.001$). (e) FISH experiments proved that circFAT1 were mostly located in the cytoplasm, 18S and U6 were the internal control. (Scale bar: 25 μm). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. 

**Table:**

| Compound          | ALP Activity | Calcium Content |
|-------------------|--------------|-----------------|
| si-NC             | 1.0          | 0.0             |
| si-FAT1           | 0.2          | 0.0             |
| si-FAT1 + miR-4781 iNC | 1.5        | 3.0             |
| si-FAT1 + miR-4781 inhibitor | 1.2       | 2.0             |
in vivo. Therefore, PDLSCs may be the key to periodontal tissue regeneration [35]. Although odontogenic stem cells can differentiate into various compartments, the number of cells that can perform the required functions is limited. Therefore, it is vital to induce PDLSCs to differentiate into osteoblasts and cementoblasts [36].

SMAD5 is considered to be involved in regulating osteogenic differentiation [37]. After bone morphogenetic protein
2 (BMP-2) binds to the receptor in the bone morphogenetic protein (BMP) pathway, SMAD5 is activated by phosphorylation and binds to SMAD1 and SMAD8 to form polymers and then transferred to the nucleus and positively regulates the transcription of osteogenic genes [38]. This regulatory effect is mainly achieved by interacting with various transcription regulators, targeting several cis-acting promoter elements among osteogenic genes like ALP and osteocalcin (OCN). It is worth noting that the upregulation of RUNX2, as a SMAD5 transcription factor, largely determines this effect [39]. Many studies have confirmed that miRNAs targeting SMAD5 inhibit osteogenic differentiation. In this study, we demonstrated the direct regulatory effect of miR-4781-3p on SMAD5. After SMAD5 knockout, the osteogenic differentiation potential of PDLSCs was limited, and the overexpression of miR-4781-3p could reverse this effect.

Recent studies have shown that circRNAs participate in various biological processes such as miRNA sponges, protein binding regulation, and gene transcription and may regulate multiple diseases, including periodontitis. Both in vitro [29, 40, 41] and in vivo [42, 43] studies have confirmed that circRNAs are involved in regulating the osteogenic differentiation of PDLSCs. In the process of PDLSCs' osteoblastic induction and mechanical stimulation, specific circRNAs were detected upregulated or downregulated. circCDK8 was proved to impair the osteogenesis of PDLSCs under hypoxia [29]. It is found that circRNA function through the network relationship between circRNAs and miRNAs. CircRNA 3140 targets miR-21, and circRNA 436 may act as a sponge for miR-107 and miR-335 [41, 44]. CircMAP3K11 may promote the proliferation of PDLSCs and inhibit their apoptosis by acting as a sponge for miR-511-3p [42]. CircCDR1as acts as a sponge for miR-7 in PDLSC proliferation and differentiation, affecting ERK or MAPK signaling pathways [43]. In addition, circRNAs may also regulate bone formation by regulating extracellular matrix tissue and cell differentiation and affect the BMP signaling pathway, according to sequencing analysis of osteogenic-induced PDLSCs. Gu et al. [44] found that 766 circRNAs were upregulated, and 690 were downregulated in PDLSCs 7 days after osteogenic induction. It is reasonable to believe that the competing endogenous RNA (ceRNA) network seems to be the main mechanism for circRNAs to perform functions during the osteogenesis of PDLSCs [44]. However, it is still necessary to better understand all the potential mechanisms that may regulate circRNAs. Some studies have found circFAT1 can act as a sponge for miR-30a-5p in competitive combination with REEP3, thus influencing the progression of HCC [45], but there is no relevant research on the biological characteristics of circFAT1 and its role in oral diseases. It is worth noting that there seems to be no common circRNAs among different oral cell types. For instance, circ0081572 was found in gingival tissue (not cells) and PDLSCs [39]. These results suggest that circRNAs may be cell-specific in periodontal tissue. Because some circRNAs can change cell behaviors, it is promising to study it as a therapeutic target to regulate stem cell differentiation and optimize periodontal regeneration. Further research on circRNAs is needed to understand their role in periodontal diagnosis and regeneration.

5. Conclusions
In this study, SMAD5 was found positively regulated the osteogenic differentiation of PDLSCs. miR-4781-3p may participate in inhibiting the protein translation of SMAD5, blocking its expression, and then reducing osteogenic differentiation of PDLSCs. The binding sites between SMAD5 and miR-4781-3p and between circFAT1 and miR-4781-3p were confirmed by analysis of dual-luciferase reporter assay, respectively. The functional relationship between the three was verified by rescue experiments. In vivo, inhibiting the expression of circFAT1 reduced the bone regeneration of rat skull defect. Therefore, combined with in vivo and in vitro studies, we could conclude that circFAT1 may be involved in the regulation of PDLSCs' osteogenic differentiation through the ceRNA network of miR-4781-3p/SMAD5. Whether there are other regulatory mechanisms remains to be further explored. Our research may be helpful to explore the role of circFAT1 in periodontal regeneration and verify the mechanism of its possible regulation of SMAD5 through sponging with miR-4781-3p, aiming to investigate the potential target of PDLSCs mediated periodontal bone regeneration.

Data Availability
The raw data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest
The authors declare that they have no competing interests.

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