Original

IncRNA ZNF710-AS1 Acts as a ceRNA for miR-146a-5p and miR-146b-5p to Accelerate Osteogenic Differentiation of PDLSCs by Upregulating the BMP6/Smad1/5/9 Pathway

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Abstract: Multiple experimental pieces of evidence have confirmed that fully understanding the regulatory mechanisms of osteogenic differentiation of periodontal ligament stem cells (PDLSCs) can better promote and improve the ability of periodontal tissues to regenerate and alleviate periodontal diseases. This study aimed to reveal whether the long noncoding RNA (IncRNA) ZNF710-AS1 plays a role in the osteogenic differentiation of PDLSCs and its molecular mechanisms. Microarray datasets GSE159507 and GSE159508 were retrieved from the Gene Expression Omnibus database and differentially expressed genes were identified using R language (limma package). The results revealed that the expression of ZNF710-AS1 and bone morphogenetic protein 6 (BMP6) was upregulated whereas that of miR-146a-5p/miR-146b-5p was downregulated during the osteogenic differentiation of PDLSCs. PDLSCs were successfully isolated and cultured in vitro. Osteogenic and adipogenic differentiation abilities were evaluated by performing alizarin red staining and oil red O staining, respectively. Overexpression of ZNF710-AS1 significantly increased the osteogenic differentiation ability of PDLSCs by upregulating the expression of BMP6 and phosphorylation-Smad family member 1/5/9 (p-Smad1/5/9) and competitively sponging miR-146a-5p/miR-146b-5p and acting as a competing endogenous RNA (ceRNA). This study demonstrated that ZNF710-AS1 promotes the osteogenic differentiation of PDLSCs by upregulating BMP6/Smad1/5/9 expression and acting as a ceRNA for miR-146a-5p and miR-146b-5p.

Key words: Cell differentiation, ceRNA, IncRNA, Osteogenic differentiation, PDLSCs

Introduction

Periodontal disease is one of the most common inflammatory diseases in humans caused by the chronic infection of periodontal supporting tissues by bacteria. This disease is the most important cause of tooth loss in adults\textsuperscript{1}. Normally, the periodontium has a good regenerative capacity. The alveolar bone and periodontal membrane are involved in dynamic remodeling and maintain the integrity of the periodontium\textsuperscript{2}. In 2004, Sco et al., using the single-cell cloning technique, successfully isolated and identified a novel adult stem cell from the periodontal ligament, namely, periodontal ligament stem cells (PDLSCs)\textsuperscript{3}. PDLSCs have high clonogenic capacity. These cells can undergo osteogenic and adipogenic differentiation after appropriate induction in vitro, which are considered to play essential roles in periodontal remodeling, regeneration, and repair\textsuperscript{4-6}. PDLSCs can differentiate into osteoblasts, adipocytes, and chondroblasts under special culture conditions\textsuperscript{7-9}. Therefore, several studies have been conducted on the osteogenesis and tissue regeneration of PDLSCs to deeply understand the biological processes underlying the osteogenic differentiation of PDLSCs. These studies have revealed multiple regulatory mediators and behavioral patterns during this differentiation process, which may provide a substantial theoretical basis for better regeneration of periodontal tissues and other tissue structures.

Many investigators have found that the abnormal expression of long noncoding RNAs (IncRNAs) or the functioning of multiple IncRNAs simultaneously is closely related to the biological behavior and activities of human stem cells. The IncRNA MCM3AP-AS1 was markedly upregulated during the induction of osteogenic differentiation of PDLSCs, and its level was positively correlated with ALP and Runx2\textsuperscript{10}. In addition, the IncRNA ZFAS1 was found to suppress osteogenic differentiation and encourage adipogenic differentiation\textsuperscript{11}. Wang et al.\textsuperscript{12} demonstrated that interference of the IncRNA THAP9-AS1 could suppress the osteogenic differentiation of PDLSCs through the miR-652-3p/VEGFA axis. In this study, ZNF710-AS1 level was found to be increased during the osteogenic differentiation of PDLSCs based on Gene Expression Omnibus (GEO) data analysis (GSE159507 dataset). ZNF710-AS1, which is approximately 7.6 kb in length, is located on human chromosome 15q26.1 and is widely distributed in various tissues of the human body. However, there is no current research on ZNF710-AS1.

Based on the above research status, bioinformatics analysis was used to screen the significantly upregulated IncRNA ZNF710-AS1 in the osteogenic differentiation of PDLSCs. Subsequently, primary PDLSCs were successfully isolated and cultured, and cell models stably overexpressing or silencing ZNF710-AS1 were further constructed to investigate the effects of ZNF710-AS1 on the proliferation and osteogenic differentiation of PDLSCs at the gene and protein levels. Furthermore, bioinformatics methods and cell experiments were combined to predict...
and analyze whether ZNF710-AS1 could form a regulatory network with specific target genes during the osteogenic differentiation of PDLS cells as well as to reveal the molecular biological mechanism of ZNF710-AS1 that regulates the osteogenic differentiation of PDLS cells at the cellular and molecular levels.

Materials and Methods

Isolation and primary culture of PDLS cells

The ethics committee of Renmin Hospital of Wuhan University approved the isolation and culture of human PDLS (No. AF/ZN 2-14/5.0). Informed consent was obtained from the patients and their family members. Normal impacted third molars (n = 10) were collected from 10 individuals aged 16–35 years at the Renmin Hospital of Wuhan University. After tooth extraction, the teeth were immersed in α-MEM (Gibco Co., Ltd., NY, USA) culture solution. The isolated teeth were rinsed repeatedly with PBS (Gibco Co., Ltd.) containing dual antibodies. Subsequently, 1/3 of the periodontal membrane tissue in the tooth root was scraped using a sterile blade and transferred into a centrifuge tube. After centrifugation (1,000 rpm) for 1 min, a single-cell suspension was obtained by adding 3 g/l collagenase and 4 g/l dispase (Sigma Co., Ltd., MO, USA). This suspension was digested for 1 h at 37°C with shaking in a water bath. After pooling PDLSCs from different samples, 1 × 10^5 cells/ml of PDLSCs were seeded into 100-mm dishes (37°C, 5% CO_2). Glycophosphate, 10 mol/l dexamethasone, and 50 µg/ml vitamin C) for mineralization induction. The mineralization-inducing medium was replaced for mineralization induction. The mineralization-inducing medium was replaced with fresh medium. Cell morphological changes were observed under a microscope after PBS rinse.

Osteogenic induction

PDLS cells cultured to the third passage were prepared as single-cell suspensions and seeded into 12-well plates at a density of 8 × 10^3 cells/ml. Cells were cultured in a mineralization-inducing medium (containing 10% fetal bovine serum [FBS] at 10 mmol/l β-sodium glycerophosphate, 10 mol/l dexamethasone, and 50 µg/ml vitamin C) for mineralization induction. The mineralization-inducing medium was replaced with fresh medium. Cell morphological changes were observed under a microscope after every 3 days.

Flow cytometry analysis

Cell suspensions were collected and stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and rhodamine fluorescently labeled primary antibodies (CD45, CD34, CD90, CD105, CD146, and STRO) (BD Pharmingen Inc., CA, USA). Negative controls were mouse IgG fluorescently labeled with FITC, PE, or rhodamine. All antibodies were cleaned three times with PBS containing 2% FBS after treatment for 45 min in the dark at 4°C. Incubation continued for 45 min in the dark after the addition of secondary antibodies. Finally, cell surface stem cell marker molecules were detected using flow cytometry.

Alizarin red staining, oil red O staining, and alkaline phosphatase staining

PDLS cells were fixed with 4% paraformaldehyde after repeated rinses with PBS for 30 min. Subsequently, PDLS cells were subjected to alizarin red (Sigma Co., Ltd.) staining (ARS), 3% oil red O (Sigma Co., Ltd.) staining, or ALP (Sigma Co., Ltd.) staining for 30 min–1 h and photographed under an inverted phase contrast microscope after PBS rinse. Finally, a quantitative analysis was performed.

Bioinformatics analysis

Microarray data with the accession numbers GSE159507 and GSE159508 are available from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The datasets GSE159507 and GSE159508 were based on the GPL16956 and GPL29173 platforms, respectively. Both datasets included three induced osteogenic differentiation PDLS cells and three normal cultured PDLS cells. The differences between the osteogenic and normal cultured PDLS cells were analyzed using the limma package based on the R software (R Development Core Team 2011). This package uses the classical Bayesian t-test analysis method with the following filtering criteria: logarithmic (base 2) fold changes ≥ 0.5 in absolute value and adjusted P < 0.05. The volcano plot was drawn using the ggplot2 package in R to demonstrate differentially expressed genes (DEGs). Next, the heatmap was plotted against the significantly upregulated versus significantly downregulated DEGs using the pheatmap package in R. The samples were clustered on the upper part of the heatmap, while sites were clustered on the left side of the heatmap. Subsequently, we used the clusterProfiler package to perform a GO biological process (GO-BP) functional annotation analysis of the screened genes. Enrichment analysis of the transformed Entrez IDs was performed using the clusterProfiler package based on the R software with the following filtering criteria: false discovery rate (adjusted P) < 0.05. Bubble plots were drawn to visualize the enriched GO-BP results using the clusterProfiler package in R. PITA was used to predict the target genes of zn710-as1, miR-146a-5p, and miR-146b-5p as well as the corresponding binding sites.

Genetic overexpression and knockdown

The full sequence of ZNF710-AS1 was ligated into the pcDNA3.1 plasmid (GenePharma Co., Ltd., Shanghai, China). The shRNAs to knock down ZNF710-AS1 (sh1-ZNF710-AS1/sh2-ZNF710-AS1) were designed by Qiagen. shRNA sequences against specific targets are shown in Table 1. The miR-146a-5p mimic, miR-146b-5p mimic, and corresponding control NC mimic were obtained from Geneseed Biotech Co., Ltd. (Guangzhou, China). When cells reached 70% confluence, the cell monolayer was covered with serum-free DMEM medium. Plasmid transfection was performed using the TurboFect transfection reagent. All cells were incubated at 37°C under 5% CO_2 for a specified period and then collected for subsequent experiments.

Western blotting

Total cell protein was extracted with RIPA lysis buffer, and the protein concentration was determined using a BCA protein assay kit (Thermo Fisher Inc., MA, USA) in a microplate reader. After denaturation for 10 min with the addition of loading buffer, 50-µg protein samples were subjected to 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with blocking solution (5% nonfat dry milk) for 2 h, washed thrice with TBST, incubated with a specific primary antibody—horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (1:3,000, Abcam, ab6721)—at room temperature for 1 h, washed thrice with TBST again, and finally incubated with secondary antibodies on a shaker. After the membrane was cleaned and protein exposure was performed, ImageJ software (NIH Inc., MD, USA) was used to detect and analyze gray values of the protein bands on the membrane. The following primary antibodies were used: anti-GAPDH (1:2,500, Abcam, ab9485), anti-ALP (1:500, Abcam, ab229126), anti-Runx2 (1:1,000, Abcam, ab236639), anti-Osterix (1:1,000, Abcam, ab209484), anti-Os-

Table 1. Sequences of shRNA against specific targets

<table>
<thead>
<tr>
<th>sh1-ZNF710-AS1 (5'-3')</th>
<th>CTGCTTCAAGGCTTTGTACAGA</th>
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<tr>
<td>sh2-ZNF710-AS1 (5'-3')</td>
<td>GTCCCTCAACCGCATGTACAACC</td>
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Ying Liu et al.: ZNF710-AS1 Promotes Osteogenic Differentiation

teocalcin (1:1,000, Abcam, ab93876), anti-Collα1 (1:1,000, Abcam, ab58876), anti-BMP6 (1:1,000, Abcam, ab155963), and anti-Smad1/5/9 (1:500, Abcam, ab80255). GAPDH was used as an endogenous control.

RT–qPCR

PDLSCs from each group were collected. Total cellular RNA was extracted using TRIzol (Thermo Fisher Inc.) and reverse transcribed to cDNA using a reverse transcription kit. Using cDNA as a template, RT–qPCR was performed. The PCR products were detected using the steponeplus real-time PCR system (Thermo Fisher Inc.), with three replicate wells for each group and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 as the internal reference. The sequences of the PCR primers used in this study are listed in Table 2. The relative expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method.

Cell Counting Kit-8 (CCK-8) assay

After transfection, $1 \times 10^5$/ml PDLSCs from each group were resus-

Table 2. Sequences of PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<tr>
<td>ZNF710-AS1</td>
<td>AGGTCCAAACTCAGGCATCAG</td>
<td>CACAAGTTCAGGTCTCTAGCAAA</td>
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<tr>
<td>miR-146b-5p</td>
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<td>GCTGATGCTCCTGAAGACTTGA</td>
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<td>U6</td>
<td>GCCGCCGCTGGAACCTTCCTG</td>
<td>GTGCAGGGTGCCGAGG</td>
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<tr>
<td>GAPDH</td>
<td>TTGTCTAGGGAGTGAACGA</td>
<td>CAGGCAGTGGTGTACAGG</td>
</tr>
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</table>

Figure 1. Identification of PDLSCs. A: The shape of PDLSCs was observed under an inverted microscope (scale bar = 50 μm); B: The expression of CD45, CD34, CD90, CD105, CD146, and STRO was determined using flow cytometry; C: After incubating with osteogenic or adipogenic media for 14 days, the osteogenic and adipogenic abilities of PDLSCs were determined via alizarin red S (left) and oil red O staining (right), respectively (scale bar = 50 μm). N = 3.
pended and then press 100 μL of PDLSCs were seeded into each well of
96-well plates. After the completion of cell attachment, the plates were
removed from the oven. CCK-8 solution (10 μl/well) was added to each
well, following which the absorbance was measured at 450 nm. Subse-
dquently, the absorbance of cells in each group was measured after 24,
48, 72, and 96 h of culture using the same aforementioned method and
growth curves were plotted accordingly.

5-Ethynyl-2′-deoxyuridine (EdU) staining assay
According to the manufacturer’s instructions, PDLSCs were incu-
bated with 1× Apollo staining solution and DAPI in the dark for 20 min.
Then, the fluorescent staining of cells was observed under a fluores-
cence microscope and analyzed quantitatively using the ImageJ software
(NIH Inc., MD, USA).

Luciferase reporter assay
Wild-type (WT) ZNF710-AS1 and BMP6 3′-UTR sequences were
synthesized using site-directed mutagenesis to generate WT and MT
pmirGLO dual luciferase reporter plasmids. These plasmids were labe-
led as pmiR-GLO-ZNF710-AS1/WT, pmiR-GLO-ZNF710-AS1/MUT,
pmiR-GLO-BMP6/WT, and pmiR-GLO-BMP6/MUT, respectively.
PDLSCs were co-transfected with pmiR-GLO-ZNF710-AS1/WT, pmiR-
GLO-ZNF710-AS1/MUT or pmiR-GLO-BMP6/WT, pmiR-GLO-BMP6/
MUT, miR-146a-5p/miR-146b-5p mimics, and miR-146a-5p/miR-146b-
5p NC. Luciferase activity was assessed using the dual luciferase report-
er assay system (Promega Corp., WI, USA) kit following the manufac-
turer’s instructions.

Statistical analysis
SPSS 22.0 (IBM Corp., NY, USA) and GraphPad Prism 7.0 (Graph-
Pad Software Inc., CA, USA) were used for data analysis and mapping.
Student’s t-test was performed to compare two sample means, and an-
alysis of variance was used to compare the means of multiple groups. A P
value of <0.05 was considered statistically significant.
Results

Identification of PDLSCs

In this experiment, we isolated primary PDLSCs from healthy human third molars. Microscopically, we observed that PDLSCs were spindle or spindle-shaped and had osteogenic and adipogenic differentiation properties (Fig. 1A). A previous study identified CD90 and CD105 as PDLSCs markers \(^1\). Subsequently, flow cytometry was performed to determine the percentage of cells positive for CD45, CD34, CD90, CD105, CD146, and STRO. The results showed that PDLSCs highly expressed CD90, CD105, CD146, and STRO but scarcely expressed CD45 and CD34 (Fig. 1B), suggesting that PDLSCs were successfully obtained. Further, the multidirectional differentiation ability of PDLSCs was determined using ARS and oil red O assays. The results showed that most cells formed mineralized calcium deposits after 3 weeks of culture in an osteogenic induction medium. Oil red O staining displayed the formation of lipid droplets in PDLSCs after adipogenic induction for 3 weeks (Fig. 1C). Taken together, the results suggest that the isolated and cultured PDLSCs exhibit osteogenic and adipogenic differentiation.

ZNF710-AS1 is highly expressed in PDLSCs undergoing osteogenic differentiation

Several studies have demonstrated that lncRNAs display molecular functions and have pathological significance in PDLSC osteogenic differentiation \(^1\). Here, dataset GSE159507 downloaded from the GEO repository revealed significantly differentially expressed lncRNAs in three PDLSCs undergoing osteogenic differentiation compared with three normal cultured PDLSCs (Fig. 2A). Subsequently, the top 50 significantly upregulated differentially expressed lncRNAs, including ZNF710-AS1, were selected for heatmap visualization (Fig. 2B). Further analysis revealed that ZNF710-AS1 expression was upregulated in a time-dependent manner in PDLSCs incubated in an osteogenic differentiation medium for 0–7 days, whereas it decreased at 14 days (Fig. 2C, \(P < 0.01\)). We then analyzed ZNF710-AS1 expression in young and old PDLSCs and found that it was lower in old PDLSCs than in young PDLSCs (Fig. 2D, \(P < 0.05\)). These results suggest that upregulation of ZNF710-AS1 could induce PDLSC osteogenic differentiation.

ZNF710-AS1 expression does not affect the proliferation ability of PDLSCs

To further investigate whether ZNF710-AS1 plays a role in regulating PDLSC proliferation, ZNF710-AS1 overexpression vector (ZNF710-AS1) and two interference vectors (sh1-ZNF710-AS1 and sh2-ZNF710-AS1) were stably transfected into PDLSCs to explore the potential biological function of ZNF710-AS1, and the transfection efficiency was validated using RT–qPCR (Fig. 3A, \(P < 0.05\)). CCK-8 and EdU staining assays revealed that there was no significant change in cell proliferation ability after ZNF710-AS1 overexpression or silencing (Fig. 3B, C, \(P > 0.05\)), suggesting that ZNF710-AS1 cannot regulate cell proliferation.
ZNF710-AS1 could encourage the osteogenic differentiation of PDLSCs

We further explored whether ZNF710-AS1 affects PDLSC osteogenic differentiation. The cells were treated as described above. PDLSCs were transfected with ZNF710-AS1 vector and ZNF710-AS1 overexpressing plasmid. ARS and ALP staining revealed that ZNF710-AS1 overexpression remarkably increased calcium deposition and alkaline phosphatase activity (Fig. 4A–C, \( P < 0.05 \)), whereas ZNF710-AS1 knockdown significantly inhibited these effects (Fig. 4A–C, \( P < 0.01 \)). In addition, ZNF710-AS1 overexpression significantly increased the protein levels of ALP, Runx2, Osterix, osteocalcin, and collagen I (colla1), whereas ZNF710-AS1 downregulation decreased the levels of these proteins. Thus, ZNF710-AS1 was able to accelerate the osteogenic differentiation of PDLSCs.

Prediction of ZNF710-AS1 target genes

Next, the underlying mechanism of ZNF710-AS1 in regulating the progression of PDLSC osteogenic differentiation was further investigated. We used RIP to determine whether ZNF710-AS1 and miRNAs were in the same RISC. We found higher ZNF710-AS1 levels in Ago2 immunoprecipitates compared with control IgG immunoprecipitates (Fig. 5A, \( P < 0.01 \)). Subsequently, we analyzed miRNA expression profiles during PDLSC osteogenic differentiation in the GSE159508 dataset. The differentially expressed up- and downregulated miRNAs after filtering are shown in Fig. 5B. Then, 111 significantly downregulated miRNAs were intersected with the 7 miRNAs predicted as potential targets by ZNF710-AS1, resulting in 2 common miRNAs, hsa-miR-146a-5p and hsa-miR-146b-5p (Fig. 5C). Similarly, the target genes of hsa-miR-146a-5p and hsa-miR-146b-5p were then predicted using the online bioinformatics tool PITA database, yielding 1958 hsa-miR-146a-5p target genes and 1957 hsa-miR-146b-5p target genes (Fig. 5D). The possible target genes were intersected with 1222 mRNAs that were differentially upregulated in the GSE159507 dataset, and 112 target genes were ob-

Figure 4. ZNF710-AS1 could cause the osteogenic differentiation of PDLSCs. A: ARS staining of osteogenic differentiation ability after overexpression or silencing ZNF710-AS1 of PDLSCs. B: ALP staining of osteogenic differentiation ability after overexpression or silencing ZNF710-AS1 of PDLSCs. C: Quantitative analysis of ALP activity. D: The protein expression of ALP, Runx2, Osterix, osteocalcin, and colla1 in PDLSCs after transfection with a ZNF710-AS1 overexpression vector and different doses of ZNF710-AS1 shRNA was employed by Western blotting. ARS: alizarin red S, ALP: alkaline phosphatase. N = 3.
Figure 5. Prediction of ZNF710-AS1 target genes. A: The relative enrichment level of znf710-as1 in PDLSCs was detected by RIP. **P < 0.01 versus Ago2. B: Volcano plot of differentially expressed miRNAs in the GSE159508 dataset, which included three induced osteogenic differentiation PDLSCs and three normal cultured PDLSCs. C: The Venn diagram exhibited the intersection of 111 significantly downregulated miRNAs with seven miRNAs adsorbed by ZNF710-AS1. D: Volcano plot of differentially expressed miRNAs in the GSE159507 dataset, which included three induced osteogenic differentiation PDLSCs and three normal cultured PDLSCs. E: The Venn diagram demonstrates the intersection of the predicted likely target genes obtained and the 1222 mRNAs differentially upregulated. F: GO entry enrichment bubble plot. The abscissa is the proportion of genes identified and the ordinate is the GO entry name. G: Visualization map of genes enriched in bone development and bone morphogenesis entries. H: The expression levels of BMP6, COL1A1, COL6A3, TGFBR2, and SCARA3 in osteodifferentiated cultured PDLSCs. I: Correlation analysis between ZNF710-AS1 and BMP6, COL1A1, COL6A3, TGFBR2, or SCARA3 expression. N = 3.
Subsequently, GO-BP functional enrichment analysis was performed. A bubble plot was drawn using the clusterProfiler R package for the 31 methylation-regulated genes, which were significantly enriched at bone development and bone morphogenesis entries (Fig. 5F). In addition, five target genes, including BMP6, COL1A1, COL6A3, TGFBR2, and SCARA3, were co-enriched in bone development and bone morphogenesis entries (Fig. 5G).

Figure 6. ZNF710-AS1 upregulated BMP6 expression as a ceRNA of miR-146a-5p and miR-146b-5p. A: The binding sites of ZNF710-AS1 and miR-146a-5p were predicted with the PITA database. B: Relative luciferase activities of ZNF710-AS1 and miR-146a-5p were tested by luciferase reporter gene assay. **P < 0.01 versus NC mimic. C: The binding sites of ZNF710-AS1 and miR-146b-5p were predicted with the PITA database. D: The relative luciferase activities of ZNF710-AS1 and miR-146b-5p were checked by luciferase reporter gene assay. **P < 0.01 versus NC mimic. E: The binding sites of BMP6 and miR-146a-5p were predicted using the PITA database. F: Relative luciferase activities of BMP6 and miR-146a-5p were checked with a luciferase reporter gene assay. **P < 0.01 versus NC mimic. G: The binding sites of BMP6 and miR-146b-5p were predicted using the PITA database. H: Relative luciferase activities of BMP6 and miR-146b-5p were detected with a luciferase reporter gene assay, **P < 0.01 versus NC mimic. I: RT-qPCR was used to examine miR-146a-5p, miR-146b-5p, and BMP6 expression after overexpression or silencing of ZNF710-AS1 in PDLSCs. J: RT-qPCR was applied to examine BMP6 levels after transfection of ZNF710-AS1 or/and miR-146a-5p mimic or miR-146b-5p mimic. K: Western blotting was used to examine BMP6 and p-Smad1/5/9 protein levels after transfection of the ZNF710-AS1 or/and miR-146a-5p mimic or miR-146b-5p mimic. N = 3.
5H). We also found that ZNF710-AS1 and BMP6, COL1A1, COL6A3, TGFBR2, and SCARA3 gene expressions were significantly positively correlated with each other (Fig. 5I), thereby supporting our prediction.

**ZNF710-AS1 upregulated BMP6 expression as a ceRNA of miR-146a-5p and miR-146b-5p**

Using PITA, we discovered that miR-146a-5p and miR-146b-5p have binding sites with ZNF710-AS1 (Fig. 6A, C). Additionally, we constructed ZNF710-AS1 WT and ZNF710-AS1 MUT luciferase vectors based on the analysis results. The luciferase activity dramatically decreased in the ZNF710-AS1-WT group (Fig. 6B, D). Nevertheless, there was no significant change in the luciferase activity of the ZNF710-AS1-MUT group. Similarly, the binding of miR-146a-5p or miR-146b-5p and BMP6 was predicted and validated using online databases and dual luciferase assay (Fig. 6E–H). ZNF710-AS1 overexpression, sh1-ZNF710-AS1, and sh1-ZNF710-AS1 vectors were transfected into PDLSCs to further verify the regulatory relationship. The results showed that ZNF710-AS1 overexpression prominently suppressed miR-146a-5p and miR-146b-5p expression levels and upregulated BMP6 levels. In contrast, ZNF710-AS1 knockdown exhibited opposite results (Fig. 6I). Previous studies have shown that the Smad1/5/9 pathway plays a promoting role during osteogenic differentiation. Therefore, miR-146a-5p mimic or miR-146b-5p mimic were transfected alone or with ZNF710-AS1 into PDLSCs. The efficiency of the miR-146a-5p mimic or miR-146b-5p mimic on its level in PDLSCs was verified using RT–qPCR (Fig. 7A, C). The levels of BMP6 and Smad1/5/9 phosphorylation proteins decreased markedly after the overexpression of miR-146a-5p or miR-146b-5p (Fig. 7B, D). Moreover, upregulation of miR-146a-5p or miR-146b-5p remarkably reversed the promoting effect of ZNF710-AS1 overexpression on the protein levels of BMP6 and p-Smad1/5/9 (Fig. 6J, K). Interestingly, we excluded the effect of miR-146a-5p overexpression on miR-146b-5p levels and vice versa (Fig. 7A, C). The above findings revealed that ZNF710-AS1 could upregulate the expression of BMP6 as a ceRNA of miR-146a-5p and miR-146b-5p.

**miR-146a-5p and miR-146b-5p overexpression counteracted the ZNF710-AS1-mediated promotion of osteogenesis**

To explore whether ZNF710-AS1 participated in PDLSC osteogenic differentiation through the miR-146a-5p/miR-146b-5p/BMP6 axis, we further assessed whether miR-146a-5p or miR-146b-5p and ZNF710-AS1 were functionally related. Therefore, the miR-146a-5p mimic, miR-146b-5p mimic, and ZNF710-AS1 vector were transfected alone or together into PDLSCs. Growth curves generated from the CCK-8 assay and EdU staining assay showed no significant changes in cell prolifera-
tion ability in each group (Fig. 8A, B, $P > 0.05$). Further, ARS and ALP staining revealed that miR-146a-5p and miR-146b-5p overexpression markedly restrained calcium deposition and alkaline phosphatase activity (Fig. 8C–E, $P < 0.05$) and the expression of osteogenic markers (ALP, Runx2, Osterix, osteocalcin, and Colla1) (Fig. 8F, $P < 0.05$). Furthermore, miR-146a-5p and miR-146b-5p upregulation also substantially reversed the promoting effects of ZNF710-AS1 upregulation on calcium deposition, alkaline phosphatase activity, and osteogenic marker levels (Fig. 8A–F, $P < 0.05$). These results suggest that miR-146a-5p and miR-146b-5p act downstream of ZNF710-AS1 and play a suppressive role during the osteogenic differentiation of PDLSCs.

**Discussion**

Cultured cells should be characterized for the corresponding biological characteristics and stem cell surface markers to ensure that the obtained cells have stable biological characteristics of mesenchymal stem...
Ying Liu et al.: ZNF710-AS1 Promotes Osteogenic Differentiation

It happens because of the large variety of cells in periodontal ligament tissues, of which only PDLSCs possess the potential to differentiate into other cells and the ability to achieve tissue regeneration. It has been demonstrated that MSCs derived from the human periodontal ligament, dental pulp, and bone marrow can express the surface antigen marker STRO-1, perivascular cell surface markers CD90, CD146, CD105, and CD44. Still, MSCs did not derive from leukocyte surface marker CD45 and vascular endothelial cell surface marker CD34.16 Zhang et al.7, suggest that PDLSCs were positive for CD90 and CD105 expressions, exhibiting more robust proliferative and osteogenic capacities. Therefore, antibodies against cell markers, such as CD45, CD34, CD90, CD105, CD146, and STRO-1, were selected to identify whether the cultured cells were PDLSCs. The cells cultured in vitro expressed CD90, CD105, CD146, and STRO-1 positively but lacked the expression of CD45 and CD34. It was confirmed that the experimentally cultured PDLSCs showed typical mesenchymal stem cell-like characteristics. Moreover, the appearance of mineralized nodules and lipid droplets after osteogenic and adipogenic induction of PDLSCs, indicating their multilineage differentiation capacity, further confirmed that our primary cultured cells were PDLSCs.

The stemness potential of pluripotent stem cells, as a critical factor in the regenerative field of tissue engineering, is influenced by certain specific IncRNAs.18,19 Hoxa-AS3 is the first known IncRNA to inhibit the differentiation of MSCs into osteoblasts. Its primary mechanism of action is that it can bind to EZH2 in the PRC2 protein complex to inhibit the transcription of Runx2, a critical osteogenic factor in MSCs. Finally, it affects the process of directed cell differentiation into bone cells.20 The IncRNA HOTAIRM1 has been reported to bind to the DNMT1 gene enhancer region in mesenchymal stromal cells and simultaneously promote the expression of key markers of osteogenesis (Runx2, osteric and osteocalcin), which are essential regulators of bone regeneration.21 The IncRNA ANRIL, as a ceRNA, can bind to miRNA-7-5p to target and regulate the gene expression of IGF-1R during the osteogenic differentiation of inflammatory PDLSCs to promote the functional recovery and regeneration of periodontal tissues in patients with periodontitis.22 Jia et al. studied the IncRNA expression profiles in PDLSCs and BMSCs of different tissue origins using IncRNA microarray and bioinformatics analyses. These authors found that many IncRNAs have noticeable expression differences in different MSCs, thus representing the biological activities of MSC diversity.23 In this study, we obtained a dataset (GSE159507) of differentially expressed IncRNAs during PDLSC osteogenic differentiation. Through screening, the expression of ZNF710-AS1 increased significantly. By performing CCK-8 and EdU staining assays, our results revealed that upregulation or knockdown of ZNF710-AS1 had no significant effect on cell proliferation. This suggests that ZNF710-AS1 is not involved in PDLSC proliferation regulation. ZNF710-AS1 overexpression increased calcium deposition, alkaline phosphatase activity, and osteogenic differentiation marker protein expression (ALP, Runx2, Osterix, osteocalcin, and Colla1). In contrast, ZNF710-AS1 knockdown had opposite effects. It has been reported that higher ALP activity during osteogenic differentiation indicates a higher degree of cell differentiation in the direction of osteogenesis and a greater ability to secrete mineralized matrix.24,25 Runx2, Osterix, and Colla1 are also essential transcription factors. Their high and low expression is a marker of high osteogenic differentiation capacity.26,27

LncRNAs have been found to have many functions, such as acting as miRNA sponges, RBP sponges, or mRNA regulators.28,29 In 2011, Salmena et al.30 proposed the hypothesis of competing endogenous RNAs (ceRNAs) that IncRNAs competitively bind miRNAs with coding RNAs and thereby play an interactive role. This mechanism is gradually becoming known as research progresses. There has been much evidence that IncRNAs can exert their functions by acting as ceRNAs in the osteogenic differentiation of PDLSCs.31,32 Inhibition of IncRNA KCNQ1OT1 repressed proliferation and PDLSC osteogenic differentiation by targeting upregulated miR-24-3p level. In this study, we obtained the possible target genes of ZNF710-AS1, hsa-miR-146a-5p, and hsa-miR-146b-5p, using bioinformatics prediction and analysis. A previous study performed differential expression analysis to identify dysregulated DEGs in the osteodifferentiation of DPSCs and BMSCs. Consequently, eight significantly downregulated DEGs were discovered, including miR-146a-5p and miR-146b-5p.33 MiRNAs can specifically bind to target mRNA 3′-UTRs, thereby causing degradation of the target mRNA or inhibiting translation of the target mRNA.34 Further, in the GSE159507 dataset, our screen obtained BMP6, a common target gene of miR-146a-5p and miR-146b-5p, and verified their binding by dual luciferin reporter assays.

Alveolar bone regeneration is a critical process in periodontal tissue regeneration. Therefore, growth factors that promote osteogenic differentiation are also integral. BMPs belong to one of the TGF-β members. More than 20 growth factors have been discovered, of which BMP2 and BMP7 are recognized for their significant osteogenic induction ability. For instance, such growth factors have already been applied in bone repair therapy in clinical settings.35,36 Previous studies have demonstrated that in the periodontal regeneration field, BMP2, BMP6, and BMP7 can detect different levels of osteogenic differentiation and mineralized matrix formation after acting on PDLSCs.37,38 Besides, the BMP/Smads pathway is one of the most classical signaling pathways. BMPs can activate the Smad 1/5/9 pathway by binding to transmembrane type I and II Ser/Thr receptors to form heterotrimers, subsequently translocating the signal into the nucleus upon Smad 4 action, causing the corresponding gene transcription.39,40 Hakki et al.41 used quantitative RT-PCR to evaluate the mRNA expression of type I collagen, bone sialoprotein, osteocalcin, osteopontin, and the osteoblast transcription factor Runx2 in PDLSCs treated with BMP2, BMP6, and BMP7. They revealed that the most apparent induction occurred in the BMP6 group. It has been confirmed in previous studies that BMP6 could increase the transcriptional level of the Smad1/5/9 pathway, promoting their translocation into the nucleus. This, in turn, causes osteogenic differentiation of MSCs.42 In the present study, BMP6 and p-Smad1/5/9 protein levels were up- or downregulated after overexpression of ZNF710-AS1 or miR-146a-5p/miR-146b-5p. Moreover, miR-146a-5p/miR-146b-5p overexpression reversed the promoting effect of ZNF710-AS1 upregulation on the osteogenic differentiation of PDLSCs. This situation was evidenced by decreased calcium deposition, alkaline phosphatase activity, and osteogenic protein expression. ZNF710-AS1 participates in PDLSC osteogenic differentiation by activating the BMP6/Smad1/5/9 pathway expression as a ceRNA of miR-146a-5p and miR-146b-5p.

The results of the present study demonstrate that ZNF710-AS1 can induce osteogenic differentiation of PDLSCs. The primary mechanism is that a ceRNA of miR-146a-5p and miR-146b-5p elevates the BMP6 level, thereby upregulating the Smad1/5/9 signaling pathway. These results contribute to a better understanding of the molecular biological mechanisms involved in PDLSC osteogenic differentiation. These results are expected to further clarify the mechanisms by which PDLSCs mediate and improve the biological remodeling of periodontal tissues. These results also provide a new molecular target and theoretical basis for regulating dental stem cells to promote oral tissue regeneration.
However, our present study only used PDLSCs isolated from normal impacted third molars of 10 individuals (primary cultures have individual differences), which lends some limitation to the translatability of the findings from our data alone. Besides, this research content is mainly based on in vitro cell experiments. Further validation of animal experiments, which will be the focus of our subsequent research, is still required.

Conflict of Interest
The authors have no conflicts of interest relevant to this article.

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