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. 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. In 2004, Seo 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). 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. PDLSCs can differentiate into osteoblasts, adipocytes, and chondroblasts under special culture conditions. 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 (lncRNAs) or the functioning of multiple lncRNAs 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 expression. In addition, the IncRNA ZFAS1 was found to suppress osteogenic differentiation and encourage adipogenic differentiation. Wang et al. 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 lncRNA 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
Materials and Methods

Isolation and primary culture of PDLSCs

The ethics committee of Renmin Hospital of Wuhan University approved the isolation and culture of human PDLSCs (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 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 type I 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 × 10^6/ml of PDLSCs were seeded into 100-mm dishes (37°C, 5% CO₂) and the α-MEM medium was changed once in 3–5 days. Then, the suspension was digested at a 1:2 ratio until the cells reached 80% confluence.

Osteogenic induction

PDLSCs cultured to the third passage were prepared as single-cell suspensions and seeded into 12-well plates at a density of 8 × 10^4 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. All cells were incubated at 37°C under 5% CO₂ and then collected for subsequent experiments.

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

PDLSCs were fixed with 4% paraformaldehyde after repeated rinses with PBS for 30 min. Subsequently, PDLSCs 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 PDLSCs and three normal cultured PDLSCs. The differences between the osteogenic and normal cultured PDLSCs 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 znf710-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-ZNF70-AS1/sh2-ZNF70-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% confluency, 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% CO₂ 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

| sh1-ZNF70-AS1 (5’-3’) | GTCCTCAGACCTTGGTACAGA |
| sh2-ZNF70-AS1 (5’-3’) | GTCCCTCAACGCGATGTACAACC |
teocalcin (1:1,000, Abcam, ab93876), anti-Collal (1:1,000, Abcam, ab58776), 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 $2^{-\Delta\DeltaCT}$ method.

**Cell Counting Kit-8 (CCK-8) assay**

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

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Table 2. Sequences of PCR primers used in this study

| Primer | Forward (5'-3') | Reverse (5'-3') |
|--------|----------------|----------------|
| ZNF701-AS1 | AGGTCCAAAAGCTGAACATGG | CACAAGTCCAGTCTTGGAA |
| miR-146a-5p | TGGTGCTGAGATC | GAGCAGCAACAAAGTTCTGA |
| miR-146b-5p | GCCCTGAGGACTGCTTTG | GGGGCTGCTGAAACCC |
| BMP6 | TGGTGTCGCTGGGTGAGAACT | GAATTCA |
| U6 | GCCCTGAGGACTGCTTTG | GGGGCTGCTGAAACCC |
| GAPDH | TTGTCAAGGATGGAACGA | CAGCCAGTTGGGTGACAG |

![Image](image_url)
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. Subsequently, 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 incubated with 1× Apollo staining solution and DAPI in the dark for 20 min. Then, the fluorescent staining of cells was observed under a fluorescence 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 labeled as pmirGLO-ZNF710-AS1/WT, pmirGLO-ZNF710-AS1/MIT, pmirGLO-BMP6/WT, and pmirGLO-BMP6/MIT, respectively. PDLSCs were co-transfected with pmirGLO-ZNF710-AS1/WT, pmirGLO-ZNF710-AS1/MIT or pmirGLO-BMP6/WT, pmirGLO-BMP6/MIT, and miR-146a-5p/miR-146b-5p mimics, and miR-146a-5p/miR-146b-5p NC. Luciferase activity was assessed using the dual luciferase reporter assay system (Promega Corp., WI, USA) kit following the manufacturer’s instructions.

**Statistical analysis**

SPSS 22.0 (IBM Corp., NY, USA) and GraphPad Prism 7.0 (GraphPad Software Inc., CA, USA) were used for data analysis and mapping. Student’s t-test was performed to compare two sample means, and analysis 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. 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 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 PDLSCs 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. Here, dataset GSE159507 downloaded from the GEO repository revealed significantly differentially expressed lncRNAs in three PDLCs undergoing osteogenic differentiation compared with three normal cultured PDLCs. 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 PDLCs 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 PDLCs and found that it was lower in old PDLCs than in young PDLCs (Fig. 2D, P < 0.05). These results suggest that upregulation of ZNF710-AS1 could induce PDLC osteogenic differentiation.

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

To further investigate whether ZNF710-AS1 plays a role in regulating PDLC proliferation, ZNF710-AS1 overexpression vector (ZNF710-AS1) and two interference vectors (sh1-ZNF710-AS1 and sh2-ZNF710-AS1) were stably transfected into PDLCs 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. 5E). The possible target genes obtained were intersected with 1222 mRNAs that were differentially up-regulated 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.
tained. 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). Next, we identified the expression of these target genes in the dataset and found that the expression of BMP6, COL1A1, COL6A3, TGFBR2, and SCARA3 were significantly upregulated in PDLSCs cultured in an osteogenic differentiation medium (Fig. 6).
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-

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**Figure 7.** miR-146a-5p and miR-146b-5p could promote BMP6 expression and activate the Smad1/5/9 pathway. A: RT–qPCR was used to examine the miR-146a-5p, miR-146b-5p, and BMP6 levels after overexpression of miR-146b-5p in PDLSCs. B: Western blotting was used to examine BMP6 and p-Smad1/5/9 protein levels after transfection of the miR-146a-5p mimic. C: RT–qPCR was applied to examine the miR-146a-5p, miR-146b-5p, and BMP6 levels after overexpression of miR-146b-5p in PDLSCs. D: Western blotting was used to examine BMP6 and p-Smad1/5/9 protein levels after transfection of the miR-146b-5p mimic. *P < 0.05, **P < 0.01. N = 3.
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
cells. 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. Zhang et al. 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. 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. 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, osterix, and osteocalcin), which are essential regulators of bone regeneration. 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. 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. In this study, we obtained a dataset (GSE159507) of differentially expressed lncRNAs 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. MiRNAs can specifically bind to target mRNA 3′-UTRs, thereby causing degradation of the target mRNA or inhibiting translation of the target mRNA. 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. 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. Besides, the BMP/Smad 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. Hakkil et al. used quantitative RT-PCR to evaluate the expression of IGF-1 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. 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.

References
1. Sedghi LM, Bacino M and Kapila YL. Periodontal disease: The good, the bad, and the unknown. Front Cell Infect Microbiol 11: 766944, 2021
2. Shaddox LM, Morford LA and Nibali L. Periodontal health and disease: The contribution of genetics. Periodontology 2000 85: 161-181, 2021
3. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Shaddox LM, Morford LA and Nibali L. Periodontal health and disease: The contribution of genetics. Periodontology 2000 85: 161-181, 2021
4. Lei M, Li K, Li B, Gao LN, Chen FM and Jin Y. Mesenchymal stem cell. J Cell Mol Med 25: 6217-6231, 2021
5. Zhai Q, Dong Z, Wang W, Li B and Jin Y. Dental stem cell and dental tissue regeneration. Front Med 13: 152-159, 2019
6. Yang C, Xu X, Lin P, Luo B, Luo S, Huang H, Zhu J, Huang M, Lei M, Li K, Li B, Gao LN, Chen FM and Jin Y. Dental stem cell characteristics of dental pulp and periodontal ligament stem cells after in vivo transplantation. Biomaterials 35: 6332-6343, 2014
7. Lei M, Li K, Li B, Gao LN, Chen FM and Jin Y. Mesenchymal stem cell characteristics of dental pulp and periodontal ligament stem cells after in vivo transplantation. Biomaterials 35: 6332-6343, 2014
8. Yang C, Xu X, Lin P, Luo B, Luo S, Huang H, Zhu J, Huang M, Peng S, Wu Q and Yin L. Overexpression of long noncoding RNA MCM3AP-AS1 promotes osteogenic differentiation of dental pulp stem cells via miR-143-3p/IGFBP5 axis. Human cell 35: 150-162, 2022
9. Wu J, Lin T, Gao Y, Li X, Yang C, Zhang K, Wang C and Zhou X. Long noncoding RNA ZFAS1 suppresses osteogenic differentiation of bone marrow-derived mesenchymal stem cells by upregulating miR-499-5p/VEGFA axis. Mol Cell Endocrinol 539: 111490, 2022
10. Wang J, Liu X, Wang Y, Xin B and Wang W. The role of long noncoding RNA THAP9-AS1 in the osteogenic differentiation of dental pulp stem cells via the miR-652-3p/VEGFA axis. Eur J Oral Sci 129: e12790, 2021
11. Foudah D, Monfrini M, Donzelli E, Niada S, Brini AT, Orciani M, Tredici G and Miloso M. Expression of neural markers by undifferentiated mesenchymal-like stem cells from different sources. J Immunol Res 2014: 987678, 2014
12. Jia L, Zhang Y, Li D, Zhang W, Zhang D and Xu X. Analyses of key mRNAs and IncRNAs for different osteo-differentiation potentials of periodontal ligament stem cell and gingival mesenchymal stem cell. J Cell Mol Med 25: 6217-6231, 2021
13. Lim YJ, Kim KM and Jang WG. Chrysophanol increases osteoblast differentiation via AMPK/Smad1/5/9 phosphorylation in vitro and in vivo. Clin Exp Pharmacol Physiol 48: 515-523, 2021
14. Lee E, Kim YS, Lee YM, Kim WK, Lee YK and Kim SH. Identification of stemness and differentially expressed genes in human cementum-derived cells. J Periodontal Implant Sci 51: 329-341, 2021
15. Aydin S and Şahin F. Stem cells derived from dental tissues. Adv Exp Med Biol 1144: 123-132, 2019
16. Kukreja BJ, Bhat KG, Kukreja P, Kumber VM, Balakrishnan R and Govila V. Isolation and immunohistochemical characterization of periodontal ligament stem cells: A preliminary study. J Indian Soc Periodontol 25: 295-299, 2021
17. Zhang Z, He Q, Yang S, Zhao X, Li X and Wei F. Mechanical force-sensitive IncRNA SNHG8 inhibits osteogenic differentiation by regulating EZH2 in hPDLSCs. Cell Signal 93: 110285, 2022
18. Guo CJ, Ma XK, Xing YH, Zheng CC, Xu YF, Shan L, Zhang J, Wang S, Wang Y, Carmichael GG, Yang L and Chen LL. Distinct processing of IncRNAs contributes to non-conserved functions in stem cells. Cell 181: 621-636.e622, 2020
19. Lu P, Li M, Zhang D and Jiang W. Lnc-ing pluripotency maintenance and early differentiation in human pluripotent stem cells. FASEB J 35: e21438, 2021
20. Zhu XX, Yan YW, Chen D, Ai CZ, Lu X, Xu SS, Jiang S, Zhong GS, Chen DB and Jiang YZ. Long non-coding RNA HoxA-AS3 interacts with EZH2 to regulate lineage commitment of mesenchymal stem cells. Oncotarget 7: 63561-63570, 2016
21. Chen Z, Zheng J, Hong H, Chen D, Deng L, Zhang X, Ling J and Wu L. IncRNA HOTAIR1 promotes osteogenesis of hDFSCs by epigenetically regulating HOXA2 via DNMT1 in vitro. J Cell Physiol 235: 8507-8519, 2020
22. Bian M, Yu Y, Li Y, Zhou Z, Wu X, Ye X and Yu J. Upregulating the expression of IncRNA ANRIL promotes osteogenesis via the miR-7-5p/IGF-1R axis in the inflamed periodontal ligament stem cells. Front Cell Dev Biol 9: 60400, 2021
23. Jia L, Zhang Y, Ji Y, Li X, Xing Y, Wen Y, Huang H and Xu X. Comparative analysis of IncRNA and mRNA expression profiles between periodontal ligament stem cells and gingival mesenchymal stem cells. Gene 699: 155-164, 2021
24. Nakamura T, Nakamura-Takahashi A, Kasahara M, Yamaguchi A and Azuma T. Tissue-nonspecific alkaline phosphatase promotes the osteogenic differentiation of osteoprogenitor cells. Biochem Biophys Res Commun 524: 702-709, 2020
25. Kanawa M, Igarashi A, Fujimoto K, Sasaki T, Nakashima A, Higashi Y, Kurihara H, Kato Y and Kawamoto T. The identification of marker genes for predicting the osteogenic differentiation potential of mesenchymal stromal cells. Curr Issues Mol Biol 13: 2157-2166, 2021
26. Meyer MB, Benkusky NA, Sen B, Rubin J and Pike JW. Epigenetic plasticity drives adipogenic and osteogenic differentiation of marrow-derived mesenchymal stem cells. J Biol Chem 291: 17829-17847, 2016
27. Liu Y, Sun X, Zhang W, Wang X, Zhang C and Zheng S. RUNX2 mutation impairs osteogenic differentiation of dental follicle cells. Arch Oral Biol 97: 156-164, 2019
28. Xu Y, Li D, Zhu Z, Li L, Jin Y, Ma C and Zhang W. miR-27a-3p negatively regulates osteogenic differentiation of MC3T3-E1 preosteoblasts by targeting osteix. Mol Med Rep 22: 1717-1726, 2020
29. Tang H, Yuan S, Chen T and Ji P. Development of an immune-related IncRNA-miRNA-mRNA network based on competing endogenous RNA in periodontitis. J Clin Periodontol 48: 1470-1479, 2021
30. Song J, Tian S, Yu L, Xing Y, Yang Q, Duan X and Dai Q. AC-Caps: Attention based capsule network for predicting RBP binding
sites of IncRNA. Interdiscip Sci 12: 414-423, 2020
31. Salmena L, Poliseno L, Tay Y, Kats L and Pandolfi PP. A ceRNA hypothesis: The rosetta stone of a hidden RNA language? Cell 146: 353-358, 2011
32. Pang M, Wei HX and Chen X. Long non-coding RNA potassium voltage-gated channel subfamily Q member 1 overlapping transcript 1 regulates the proliferation and osteogenic differentiation of human periodontal ligament stem cells by targeting miR-24-3p. Hua Xi Kou Qiang Yi Xue Za Zhi 39: 547-554, 2021 (in Chinese)
33. Gaus S, Li H, Li S, Wang Q, Kottek T, Hahnel S, Liu X, Deng Y, Ziebolz D, Haak R, Schmalz G, Liu L, Savkovic V and Lethaus B. Shared genetic and epigenetic mechanisms between the osteogenic differentiation of dental pulp stem cells and bone marrow stem cells. Biomed Res Int 2021: 6697810, 2021
34. Fabian MR, Sonenberg N and Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79: 351-379, 2010
35. Ghosh-Choudhury N, Harris MA, Feng JQ, Mundy GR and Harris SE. Expression of the BMP 2 gene during bone cell differentiation. Crit Rev Eukaryot Gene Expr 4: 345-355, 1994
36. Li J, Wu X, Shi Y and Zhao H. FGDS-AS1 facilitates the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells via targeting the miR-506-3p/BMP7 axis. J Orthop Surg Res 16: 665, 2021
37. Neumann K, Endres M, Ringe J, Flath B, Manz R, Häupl T, Sitterer M and Kaps C. BMP7 promotes adipogenic but not osteo-/chondrogenic differentiation of adult human bone marrow-derived stem cells in high-density micro-mass culture. J Cell Biochem 102: 626-637, 2007
38. Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY and He TC. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 85: 1544-1552, 2003
39. Xu WP, Shiba H, Mizuno N, Uchida Y, Mouri Y, Kawaguchi H and Kurihara H. Effect of bone morphogenetic proteins-4, -5 and -6 on DNA synthesis and expression of bone-related proteins in cultured human periodontal ligament cells. Cell Biol Int 28: 675-682, 2004
40. Kim KM, Jeon WJ, Kim EJ and Jang WG. CRTC2 suppresses BMP2-induced osteoblastic differentiation via Smurf1 expression in MC3T3-E1 cells. Life Sci 214: 70-76, 2018
41. Maung WM, Nakata H, Miura M, Miyasaka M, Kim YK, Kasugai S and Kuroda S. Low-intensity pulsed ultrasound stimulates osteogenic differentiation of periosteal cells in vitro. Tissue Eng Part A 27: 63-73, 2021
42. Bradford STJ, Ranghini EJ, Grimley E, Lee PH and Dressler GR. High-throughput screens for agonists of bone morphogenetic protein (BMP) signaling identify potent benzoxazole compounds. J Biol Chem 294: 3125-3136, 2019
43. Hakki SS, Bozkurt B, Hakki EE, Kayis SA, Turac G, Yilmaz I and Karaog E. Bone morphogenetic protein-2, -6, and -7 differently regulate osteogenic differentiation of human periodontal ligament stem cells. J Biomed Mater Res B Appl Biomater 102: 119-130, 2014
44. Cai C, Wang J, Huo N, Wen L, Xue P and Huang Y. Mx2 plays an important role in BMP6-induced osteogenic differentiation of two mesenchymal cell lines: C3H10T1/2 and C2C12. Regen Ther 14: 245-251, 2020
