

Original Research Article

MiR-452 negatively regulates osteoblast differentiation in periodontal ligament stem cells by targeting the polycomb-group protein, BMI1

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Sent for review: 19 November 2020

Revised accepted: 26 March 2021

Abstract

Purpose: To determine whether miR-452 regulates osteoblast differentiation (OD) in human periodontal ligament stem cells (hPDLSCs) by targeting polycomb-group protein BMI1.

Methods: hPDLSCs were stimulated to differentiate upon treatment with mineralization liquid. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting were used to measure mRNA and protein expressions, respectively. Alkaline phosphatase (ALP) activity and Alizarin red staining were used to determine the osteogenic differentiation (OD) of hPDLSCs. The bioinformatics software, TargetsCan, was used to predict the potential target of miR-452, while luciferase assay, qRT-PCR, and western blot were employed to verify the target gene of miR-452, BMI1.

Results: MiR-452 was downregulated during the OD of hPDLSCs, but miR-452 overexpression inhibited the OD of hPDLSCs. BMI1 was identified as a direct target gene of miR-452 during the OD of hPDLSCs, while miR-452 overexpression correlated inversely with BMI1 expression during OD of hPDLSCs.

Conclusion: Overexpression of miR-452 suppresses the OD of hPDLSCs by targeting BMI1. This study may provide potential diagnostic and therapeutic basis for OD in hPDLSCs.

Keywords: MiR-452, Osteogenic differentiation, Periodontal ligament stem cells, BMI1

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INTRODUCTION

Periodontal tissue damage causes oral organ defects and has adverse effects on health for many people [1]. Human periodontal ligament stem cells (hPDLSCs) from periodontal ligament tissue can form new periodontal supporting tissues and exert effects on the functional regeneration and biological repair of periodontal tissue [2]. MiRNAs are classified of short

noncoding RNAs (18–25 nucleotides), which function as gene suppressors to downregulate the stability or translational efficiency of target genes by the 3'-untranslated region (3'-UTR), and can regulate cell proliferation, migration, and differentiation [3,4].

Numerous studies have revealed the key roles of miRNAs in osteogenic differentiation (OD) of stem cells. For instance, in bone marrow

mesenchymal stem cells (BMSCs), miR-204 promotes adipogenic differentiation by regulating expression of Runx2 and thereby inhibiting OD [5], whereas miR-26a promotes OD of adipose-derived mesenchymal stem cells (ADMSCs) [6]. This study showed that miR-452 expression was downregulated during OD in PDLSCs, indicating that miR-452 could play an important role in the progression of OD.

B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) is a key component of the polycomb repressive complex 1. It has been shown that knockdown of BMI1 could reduce alveolar bone mass in mice [7]. However, whether the binding interaction between BMI1 and miR-452 could play a role in the pathological process of OD remains unknown. Therefore, this experimental study aimed to determine the role of miR-452 in the progression of OD in PDLSCs, and explore the underlying mechanism of action.

EXPERIMENTAL

Reagents

Radio-immunoprecipitation assay (RIPA) buffer, Revert Aid RT Reverse Transcription kit, antibodies against Runx2, osterix, OCN, BMI1, and β -actin, Lipofectamine 2000, the Alkaline phosphatase (ALP) activity kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell culture materials were from Gibco Life Technologies (Grand Island, NY, USA). The primers, BMI1-3'UTR wild type (BMI1 3'UTR-WT) and BMI1-3'UTR mutant (BMI1 3'UTR-MUT) were purchased from Sangon Biotech Company (Shanghai, China). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The mRNA expression was quantified using the SYBR-Green RT-PCR kit (Takara Bio, Inc., Otsu, Japan). Locked nucleic acid (LNA)-miR-452 and LNA-miR-NC were from IBS Solutions (Shanghai, China). The miR-452 mimic, miR-452 inhibitor, negative controls (NC) of miR-452 mimic and inhibitor, and the short hairpin (shRNA) sequences targeting the BMI1 gene were synthesized by Shanghai Gene Pharma Corporation (Shanghai, China). Alizarin red and cell lysate buffer were purchased from Sigma-Aldrich (St Louis, MO, USA). The dual luciferase reporter assay system was purchased from Promega (Madison, WI, USA). ECL detection reagents were from Amersham (Tokyo, Japan).

Cell culture

hPDLSCs were isolated from 10 male patients (15–32 years old) who underwent molar

extraction. The isolated hPDLSCs were cultured in α -MEM supplemented with FBS (10 %), vitamin C sodium salt (50 μ g/mL), β -glycerophosphate disodium salt hydrate (10 mmol/L), dexamethasone (0.1 μ mol/L), and penicillin/streptomycin (100 U/mL) in an incubator at 37 °C under 5 % CO₂.

qRT-PCR assay

Total RNA was extracted from cultured hPDLSCs using the RNeasy Mini Kit, and converted to cDNA using the Revert Aid RT Reverse Transcription kit. The mRNA expression was quantified by qRT-PCR using the SYBR-Green RT-PCR kit and GAPDH was used as a control. The primers were listed.

Table 1: qRT-PCR primers

Gene name	Forward (5'-3')	Reverse (5'-3')
miR-452	GCGAACTGTTTG CAGAGG	CAGTGCCTGTTCG TGGAGT
BMI1	CAGTTAGGCAGT ATGTAGTTTTTC	GTTGTGGTGGAG TGTAAGAGTGT
GAPDH	CCTGGCACCCAG CACAAAT	GCCGATCCACAC G GAGTACT

Each qRT-PCR reaction was performed in triplicate using three RNA samples. The relative expression was determined using the $2^{-\Delta\Delta C_t}$ formula [8].

Cell transfection

The miR-452 mimic (miR-452), negative control (NC) of miR-452 mimic (miR-NC), miR-452 inhibitor (miR-452 inh), NC of inhibitor (miR-NC inh), and the short hairpin (shRNA) sequences targeting the BMI1 gene (5'-AAGGAGGAGGT GAATGATAAA-3'), LNA-miR-452 (50 nM) and LNA-miR-NC (50 nM) were transfected into hPDLSCs using Lipofectamine 2000.

Alizarin red staining

This assay was used to detect mineralization nodules in hPDLSCs. Briefly, hPDLSCs were washed (PBS), fixed with paraformaldehyde (4 % (w/v), 30 min, at room temperature (RT)), stained with Alizarin red (30 min, at RT), and washed (PBS) for three times. Staining was observed by microscopy.

Measurement of ALP activity

hPDLSCs were lysed with cell lysate buffer and then centrifuged (12,000 g, 10 min). ALP activity was measured using the ALP activity kit.

Luciferase reporter assay

The assay was used to evaluate the molecular interaction between miR-452 and BMI1 in hPDLSCs. hPDLSCs were seeded into 24-well plates (1.5×10^5 cells/well), and were co-transfected with 30 nM of miR-452 mimic or miR-NC and fluorescent plasmid (300 ng of BMI1 3'UTR-WT or BMI1 3'UTR-MUT) for 24 h using Lipofectamine 2000. The relative luciferase activity was determined.

Western blot

RIPA Buffer was used to lyse and extract total protein from hPDLSCs. Extracted protein was separated by SDS-PAGE, transferred to membranes, and blocked with milk (5 %). The membranes were incubated with anti-BMI1 (1:1000), anti- β -actin (1:2000), anti-Runx2 (1:2000), anti-osterix (1:1000), and anti-OCN (1:1000) antibodies (Thermo Fisher Scientific) at 4 °C overnight.

After washing with TBS-T solution 3–5 times, membranes were incubated with secondary antibody (1:5000 dilution) for 1 h at RT. ECL detection reagents were used to visualize the immune complexes. Image Pro software was used to calculate band intensities.

Statistical analysis

All experiments were repeated at least three times. The data are presented as mean \pm standard deviations (SD) and analyzed using SPSS 21.0 statistical software. ANOVA or *t* test was used to assess significance ($p < 0.05$). The Dunnett multiple comparison test was applied to identify differences between groups.

RESULTS

MiR-452 expression decreased in hPDLSCs during OD

Based on whether hPDLSCs were stimulated to differentiate upon treatment with mineralization liquid, hPDLSCs were divided into OD group and control group. The miR-452 expression in hPDLSCs was lower on days 7 and 14 as compared with that at day0 ($p < 0.05$; Figure 1). However, the miR-452 expression in Control group showed no significant difference at day0, days 7 and 14. These results indicated that the miR-452 expression was reduced in hPDLSCs during OD.

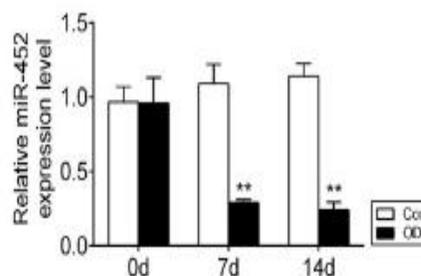


Figure 1. MiR-452 expression was decreased during OD of hPDLSCs; $n = 10$, ** $p < 0.01$ vs. day 0

MiR-452 overexpression inhibited OD of hPDLSCs

To investigate the effects of miR-452 on OD of hPDLSCs, hPDLSCs were stably transfected with LV-miR-452. qRT-PCR showed that hPDLSCs transfected with LV-miR-452 expressed higher levels of miR-452 than that transfected with LV-NC ($p < 0.01$; Figure 2 A), indicating the successful transfection. Compared with the control group, the ALP activity was increased in the OD group ($p < 0.01$). However, the induced ALP activity in OD group was inhibited by miR-452 overexpression ($p < 0.01$; Figure 2 B).

The results in Figure 2 C showed that treatment with mineralization liquid promoted matrix mineralization of hPDLSCs in OD group ($p < 0.01$). However, transfection with LV-miR-452 inhibited the progression of matrix mineralization of hPDLSCs in the OD group ($p < 0.01$). Consistently, the expression of OD markers, such as RUNX2, osterix, and OCN were upregulated in the OD group ($p < 0.01$), and transfection with LV-miR-452 reduced the expression of RUNX2, osterix, and OCN in the OD group ($p < 0.01$; Figure 2 D). These results showed that miR-452 inhibits OD of hPDLSCs.

BMI1 was the direct target of miR-452

The bioinformatics software Targetscan (http://www.targetscan.org/vert_72/) revealed that BMI1 was predicted as a potential target of miR-452 (Figure 3 A). The luciferase reporter assay was used to verify the binding affinity of miR-452 to the BMI1 3'UTR-WT in hPDLSCs. The luciferase activity in hPDLSCs that transfected with miR-452 mimic and BMI1 3'UTR-WT was decreased, but miR-452 mimics did not affect the luciferase activity in hPDLSCs that transfected with BMI1 3'UTR-MUT, indicating that BMI1-WT was a target of miR-452 (Figure 3 B).

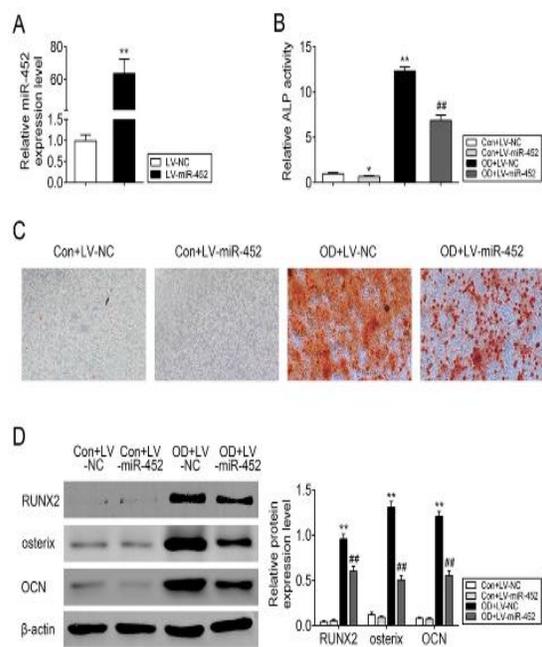


Figure 2. Overexpression of miR-452 inhibited OD of hPDLSCs. (A) qRT-PCR was used to measure miR-452 expression in hPDLSCs. (B) ALP activity was measured in hPDLSCs. (C) Alizarin red staining was used to evaluate OD of hPDLSCs. (D) Western blot was used to measure protein expression of RUNX2, osterix, and OCN in hPDLSCs. ** $p < 0.01$ vs. Con+LV-NC group and ## $p < 0.01$ vs. OD+LV-NC group

qRT-PCR showed that miR-452 expression was increased by miR-452 mimics and decreased by miR-452 inhibitor in hPDLSCs (Figure 3 C). To validate whether miR-452 can modulate BMI1 expression, qRT-PCR was used to measure BMI1 expression. The results showed that BMI1 expression was decreased by miR-452 mimics, and was increased by miR-452 inhibitor in hPDLSCs (Figure 3 D). In addition, the western blot assay showed that BMI1 protein expression was decreased in hPDLSCs transfected with miR-452 mimics, and was increased in hPDLSCs transfected with the miR-452 inhibitor (Figure 3 E). These data showed that miR-452 could negatively regulate BMI1 expression in hPDLSCs.

Expression of BMI1 during OD of hPDLSCs

The mRNA and protein levels of BMI1 in hPDLSCs was higher at day7 and day14 as compared with that at day0 ($p < 0.05$; Figure 4). These results indicated that the miR-452 expression was induced in hPDLSCs during OD, which may suggest a positive correlation between BMI1 expression and OD (Figure 4 A and 4 B).

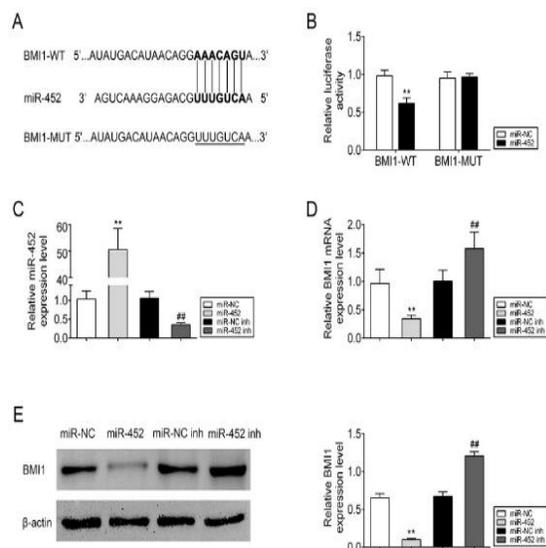


Figure 3: BMI1 was the direct target of miR-452. (A) This diagram showed binding sites between the miR-452 and BMI1-WT sequences. BMI1-WT represents the entire 3'UTR sequence (625-631) of wild-type BMI1. BMI1-MUT represents the mutated 3'UTR sequence and could not bind to miR-452. (B) The luciferase reporter assay was performed to evaluate binding affinity of miR-452 to BMI1-WT/BMI1-MUT. (C and D) qRT-PCR was used to measure miR-452 and BMI1 mRNA expression levels in hPDLSCs. (E) Western blot was used to measure BMI1 protein expression in hPDLSCs. ** $p < 0.01$ vs miR-NC, ## $p < 0.01$ miR-NC inh

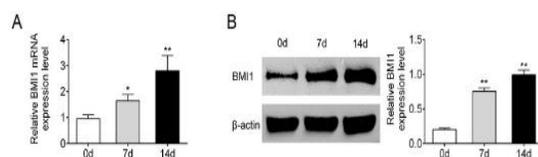


Figure 4. BMI1 expression in hPDLSCs during OD. (A) BMI1 mRNA expression was measured by qRT-PCR. (B) BMI1 protein expression was measured by western blot. * $P < 0.05$ and ** $p < 0.01$ vs. day 0, respectively

Knockdown of BMI1 inhibited OD of hPDLSCs

ShBMI1-transfected hPDLSCs were used to evaluate the functional effect of BMI1 during OD. The successful knockdown of BMI1 in hPDLSCs was confirmed (Figure 5 A). Compared with the control group (shNC-transfected hPDLSCs), the ALP activity was inhibited in shBMI1-transfected hPDLSCs ($p < 0.05$; Figure 5 B). In addition, Alizarin red staining showed that matrix mineralization was lower in shBMI1-transfected hPDLSCs when compared with shNC-transfected hPDLSCs ($p < 0.05$; Figure 5 C). Furthermore, the expression of OD markers, such as RUNX2, osterix, and OCN, were downregulated in shBMI1-transfected hPDLSCs

when compared with shNC-transfected hPDLSCs ($p < 0.05$; Figure 5 D). These data showed that BMI1 promoted OD of hPDLSCs.

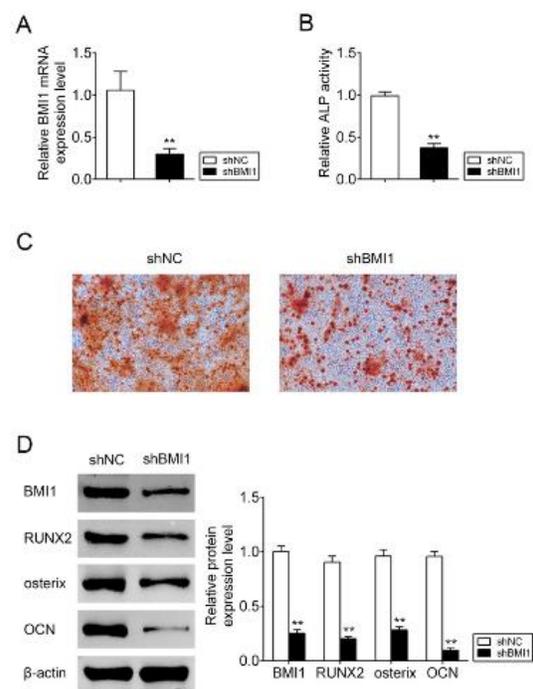


Figure 5. Knockdown of BMI1 inhibited OD of hPDLSCs. (A) BMI1 mRNA expression was measured in hPDLSCs by qRT-PCR. (B) ALP activity in hPDLSCs. (C) Alizarin red staining was used to evaluate OD of hPDLSCs. (D) Western blot was used to measure Runx2, osterix, and OCN protein expression in hPDLSCs; ** $p < 0.01$ vs. shNC group

DISCUSSION

Periodontitis is a serious chronic oral disease, of which the integrity of periodontal tissue is disrupted, and periodontitis significantly affects the health of the individual [9]. Periodontal ligament regulates the vascular balance, and nutrition and repairment of tooth tissue. hPDLSCs can differentiate into periodontal ligament, bone, and cementum [10]. Thus, understanding the molecular mechanism of OD of hPDLSCs is important for the practical application of osteogenic tissue engineering.

MiRNAs function in the progress of OD through multiple pathways. For example, miR-24-3p promotes OD by targeting Smad5, and miR-214 inhibits OD of hPDLSCs by binding to ATF4. MiR-452, a widely known miRNA, attracted our attention due to its regulatory role in cell proliferation and differentiation in many human cancers. However, little is known about the role of miR-452 in the progression of OD. In this study, qRT-PCR assay revealed that miR-452

expression was decreased during OD of hPDLSCs. Thus, miR-452 has potential as a biomarker for OD, and overexpression of miR-452 could inhibit OD. ALP activity and Alizarin red staining assays were used to evaluate progression of OD of hPDLSCs, and we found that miR-452 overexpression inhibited OD of hPDLSCs.

This study is the first evidence demonstrating that miR-452 regulates OD of hPDLSCs. In detail, the results showed that overexpression of miR-452 inhibits OD of hPDLSCs *in vitro*. Other studies have shown that miRNAs modulate OD via various molecular mechanisms. A previous study showed that miR-452-5p upregulated cell proliferation in colorectal cancer by interacting with CDKN1B [11], and miR-452 suppressed cell migration in breast cancer by targeting RAB11A [12]. Besides, in human cervical cancer, reduced miR-452 expression was related to vascular invasion, lymph node metastasis, poor tumor differentiation and short overall survival [13]. It has also been shown that miR-452 mediates tumor cell proliferation and invasion by targeting various genes via different signaling pathways [14]. However, the precise target of miR-452 in OD of hPDLSCs remains unclear.

To explore the molecular mechanism underlying the regulatory role of miR-452 in the progression of OD, the bioinformatics predicted miR-452 could bind directly to BMI1 3'UTR-WT, which was also verified by luciferase reporter assay. In addition, miR-452 overexpression inhibited BMI1 expression in hPDLSCs, thus, BMI1 is a downstream target of miR-452. Furthermore, qRT-PCR, western blot, ALP activity, and Alizarin red staining assays showed that knockdown of BMI1 inhibited OD of hPDLSCs. Taken together, our data demonstrated that miR-452 suppresses OD of hPDLSCs by targeting BMI1. BMI1 is a polycomb-group protein and evidence have suggested that it plays a role in various cancers, including bladder cancer [15] and lung cancer [16]. It has also been shown that BMI1 is regulated by numerous miRNAs that affect cell proliferation and differentiation. For example, overexpression of miR-27a and miR-155 inhibited cell proliferation and migration by directly targeting BMI1 [17]. Whether BMI1 is regulated by other miRNAs in the progression of OD needs further investigations.

CONCLUSION

MiR-452 suppresses OD of hPDLSCs *in vitro*, and also miR-452 suppresses OD of hPDLSCs by targeting BMI1. Thus, miR-452 is a potential biomarker for detecting molecular pathogenesis

of OD in hPDLSCs, and may ultimately lead to the development of novel therapies for periodontitis.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tiantian Mao and Jun Li designed the study and supervised the data collection. Ruobing Peng analyzed and interpreted the data. Linhua Liu and Youjian Peng prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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