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Original Article

MiR-218 promotes osteogenic differentiation of periodontal ligament stem cell through activation of Wnt signaling by targeting SFRP2

Fucai Sun[1], Yike Ma[1], Zhibin Cai[2], Zhaoan Yang[1]

[1]Department of Stomatology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China; [2]Department of General Stomatology, The Affiliated Stomatology Hospital of Wenzhou Medical University, Wenzhou 32500, Zhejiang, China

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Abstract: Increasing evidence supports that microRNAs (miRNAs) play an important role in the control of osteoblastic differentiation such as human adipose-derived stem cells and embryonic stem cells. However, the role of miRNAs in periodontal ligament stem cell (PDLSCs) differentiation remains poorly understood. Here, we present evidence that miR-218 acts as a positive regulator of PDLSCs osteogenesis. Real-time PCR shows that miR-218 was increased during PDLSCs differentiation. Moreover, ectopic expression of miR-218 promoted PDLSCs cells differentiation, whereas inhibition of miR-218 would suppress cell differentiation. Furthermore, we verified that miR-218 directly targeted SFRP2, which is a Wnt signaling pathway antagonist. Western blot analysis showed that the expression level of miR-218 was negatively correlated with that of SFRP2. Taken together, miR-218 is an important mediator of osteoblast differentiation, thus offering a new target for the development of preventive or therapeutic agents against osteogenic disorder.

Keywords: MicroRNA-218, periodontal ligament stem cells, osteogenic differentiation, Wnt, SFRP2

Introduction

Previous studies have characterized progenitor cells residing in periodontal ligament (PDL) tissues, known as periodontal ligament stem cells (PDLSCs), which exhibit self-renewal capacity and expressing cell surface markers similar to bone marrow MSCs [1-3]. Further, PDLSCs possess unique properties compared with other MSC-like populations, which can differentiate into many kinds of cells derived from other systems and form osteogenic tissue; thus, these cells play important roles in tooth regeneration [4]. Subsequently, the regenerative potential of PDLSCs has been shown in preclinical large-animal studies and human clinical pilot trials for periodontal regeneration [5-10]. However, how to regulate and control their potency of osteogenic differentiation remains to be an unsolved problem.

During the osteoblast differentiation, numerous regulatory pathways play important roles in regulating osteoblast replication and cellular differentiation [11, 12]. For instance, the Wnt and BMP pathways have prominent and synergistic roles in osteoblast phenotype commitment [13]. In particular, Wnt signaling is very important for differentiation of human mesenchymal stem cells into osteoblasts [14]. Wnt signaling promotes mesenchymal stem cells proliferation during early differentiation [15, 16]. Canonical Wnt signaling then drives the differentiation of osteochondral progenitors toward the osteoblastic lineage [17]. In addition, Wnt signaling inhibits osteoblast and osteocyte apoptosis [18].

MicroRNAs, which function at the post-transcriptional level, are a group of small and non-coding RNAs that are 18-25 nucleotides in length and generally repress target gene expression by degrading mRNA or preventing translation [19]. Increasing evidence supports that miRNAs play indispensable roles in many biological processes, including proliferation,

Table 1. Primers used for qPCR

Gene name	Real time qPCR primers (5'-3')
Runx2	Forward: GTC TCA CTG CCT CTC ACT TG
	Reverse: CAC ACA TCT CCT CCC TTC TG
ALP	Forward: CGG ACA TCA TGA GGG TAA GG
	Reverse: GAG ACA TTT TCC CGT TCA CC
OCN	Forward: ACA GAC AAG TCC CAC ACA GCA GC
	Reverse: TGA AGG CTT TGT CAG ACT CAG GGC
BSP	Forward: GCCAGAGGAGCAATCACCAA
	Reverse: CAGGCTGGAGGTTCACTGGT
SFRP2	Forward: CGT GGG CTC TTC CTC TTC G
	Reverse: ATG TTC TGG TAC TCG ATG CCG
miR-218	TTG TGC T TG ATC TAA CCA TGT
U6	Forward: CGC TTC GGC AGC ACA TAT AC
	Reverse: AAA ATA TGG AAC GCT TCA

differentiation, apoptosis and cancer [20]. And, kinds of miRNAs have been confirmed to participate in human MSC differentiation [16, 21]. Zhang WB et al. presented evidence that miR-218 acts as a positive regulator of hASCs osteogenesis [22]. However, the specific roles ofmiR-218 in the differentiation of PDLSCs are largely unknown and its function remains to be characterized [23].

To date, few studies have investigated the osteogenic effect of miR-218 on PDLSCs. In this study, we aimed to analyze the bioactive effects of miR-218 on the osteogenic differentiation of PDLSCs and to determine their potential target genes that are related to osteogenic differentiation.

Materials and methods

Cell culture and isolation of PDLSCs isolation

Human premolars were obtained from five healthy patients for orthodontic reasons after obtaining the patients' approval and informed consent (donor age: 10-12 years). Tissue from the periodontal ligament was isolated as previously described [3]. Briefly, periodontal ligament tissues were gently scraped from the middle portion of the root surface, minced into 1 mm^3 cubes, and placed into 6-well culture dishes (Costar, Cambridge, MA). The explants were grown in a minimum essential medium (α-MEM; Gibco BRL, Rockville, MD) supplemented with 10% (v/v) fetal bovine serum (FBS) 0.292 mg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. $STRO\text{-}1^+$ stem cells were prepared using immunomagnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. After washing, bead-positive cells were segregated using a magnetic particle separator and subsequently seeded into 75-cm^2 culture flasks (Costar) at 37°C in 5% CO_2. The PDLSCs from this passage were used for further study.

Transfection

The PDLSCs were plated into six-well plates and grown to 30-50% confluence after 24 hours of incubation and were then transfected with miRNA mimics, miRNA inhibitor and negative control at a final multiplicity of infection of 10 using siLentFect™ Lipid reagent (Life Science Research). The cells were then diluted in DMEM/F12 without serum (GeneChem, Shanghai, China). After 4 h of incubation in a CO_2 incubator at 37°C, the medium was changed to 10% FBS containing DMEM. The efficiencies of miRNA mimics, miRNA inhibitor and negative control were tested by quantitative real time polymerase chain reaction (qRT-PCR).

MiRNA extraction and qPCR analysis

Total RNA and miRNA were purified from PDLSCs cell cultures treated with or without 50 μg/ml of ascorbic acid for d0, d2, d10 and d21 days using miRNeasy mini kit (Qiagen). Total RNA (0.5-1 μg) was reverse transcribed into cDNA using Oligo (dT) primers and was analyzed by real time qPCR. SYBR Green Master Mix (Applied Bio Systems Inc.) was used to detect the expression of gene markers. For miRNA detection, the isolation of small species-enriched RNA was performed as per the manufacturer's instructions (mirVana miRNA isolation kit, Ambion). MiRNA was reverse-transcribed with an Ncode miRNA first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer-specified guidelines. Real-time PCR was performed using a standard SYBR Green PCR kit (TAKARA, Osaka, Japan) protocol on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. U6 was used as a normalizing control.

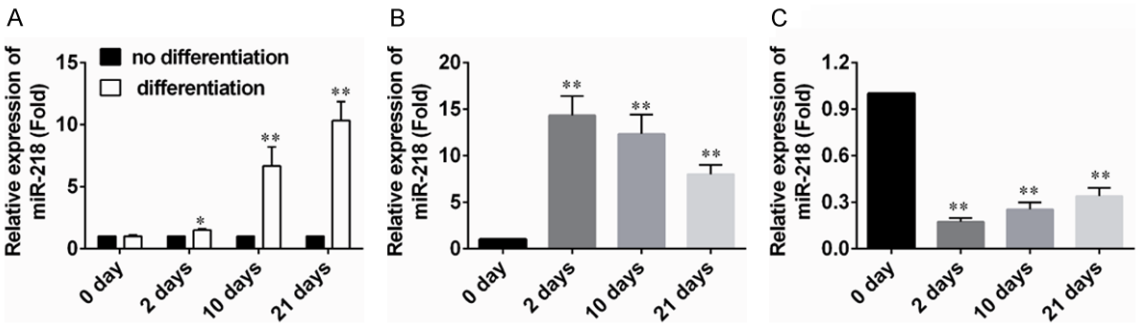

Figure 1. Expression level of miR218 during osteogenic differentiation of PDLSCs. A. Total RNA was analyzed for expression of precursor miR-218 by real time PCR at the indicated time points using PDLSCs. Absolute expression (y axis) was normalized to U6 small RNA. B. The expression of miR-218 in PDLSCs transfected with miR-218 mimic was assayed by real-time PCR and normalized to U6. C. The expression of miR-218 in PDLSCs transfected with miR-218 inhibitor was assayed by real-time and normalized to U6. The data are the mean ± SD of 3 independent experiments. **P < 0.01.

The mRNA expression of osteogenic differentiation marker alkaline phosphatase (ALP), osteocalcin (OCN), bone sialoprotein (BSP), the bone-specific transcription factor Runx2 and secreted frizzled-related protein 2 (SERP2) were analyzed. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta}$Ct value was used to determine the relative expression levels. The results were expressed as Log 10 ($2^{-\Delta\Delta}$Ct). Sequences of the primers are shown in **Table 1**.

Alizarin red staining and quantification

Cells were seeded into 24-well plates at a density of approximately 1×10^5 cells per well separately. After cells reached 80% confluence, the culturing medium was changed into standard osteogenic differentiation induction medium and then cultured for another 3 weeks. The induction medium was changed every 3 days. Finally cells were stained with Alizarin red (pH = 4.1) staining solution and were quantified according to the methods previously published.

DNA construction and luciferase assay

The 3'UTRs of the SFRP2 genes were amplified and cloned into the SacI/HindIII sites of the pMIR-Report luciferase vector. The seed region of the miR-218 target sites in the SFRP2 3'UTRs were mutated using the Quick change II site directed mutagenesis kit (Stratagene). The Luciferase assay was conducted by co-transfecting miR-218 miRNA, NS miR and miR-218 inhibitor with WT and mutant pMIR-REPORT-Luc DNA construct. The transfections were performed in duplicate and all experiments were repeated several times. Renilla Luciferase plasmid (Promega) was transfected to normalize the relative luciferase values. The transfected cells were incubated for 36 h to determine luciferase activity.

Western blots

Equal amounts of total protein were loaded into a 10% SDS-PAGE transferred onto a PVDF membrane probed with indicated primary antibody for SFRP2 (Abcam, ab86379) and β-actin (Santa Cruz). After incubation with secondary antibody, antibody-bound protein complex in the membrane was detected with chemiluminescence reagent.

Statistics

Data are expressed as mean ± SD. Student's t-test or one-way analysis of variance (ANOVA) was made for multiple comparisons. All experiments were repeated 3 times, and representative experiments are shown. *P*-values < 0.05 will be considered to be statistically significant.

Results

Expression level of miR-218 during osteogenic differentiation of PDLSCs

Previous studies have demonstrated that miR-218 positively regulated osteogenic differentiation of human adipose-derived stem cells (hASCs) [22]. Here, we utilized osteogenic differentiation medium to initiate the osteoblast

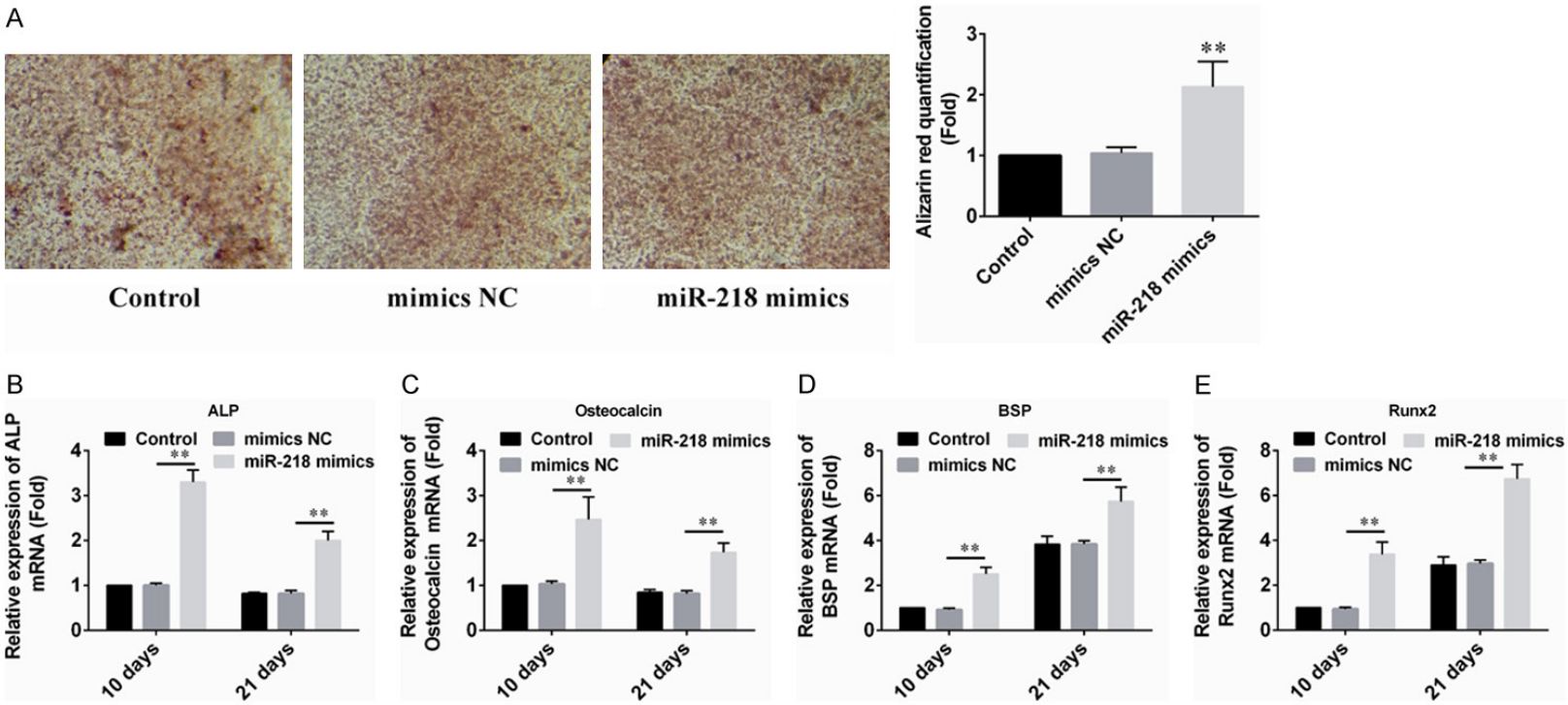

Figure 2. Up-regulation of miR-218 promoted PDLSC osteogenic differentiation. A. 10 days after induction, the cells were stained with alizarin red (pH = 4.1), and the results showed that PDLSCs transfected with miR-218 mimic formed more mineralized nodules than the control groups. B-E. PDLSCs were transduced with miR-218 mimic. Total RNA was analyzed by real time qRT-PCR for mRNA expression profile of bone marker genes (ALP, OCN, BSP and Runx2) at d10 and d21. The data are the mean ± SD of 3 independent experiments. **P < 0.01.

differentiation in PDLSCs. The RNA samples were collected and examined at indicated time points. According to the qPCR results, we found that miR-218 expression was gradually increased after the induction of osteogenic differentiation of PDLSCs (**Figure 1A**).

The functional activity of miR-218 was assessed in PDLSCs after forced expression of a miR-218 mimic and a miR-218 inhibitor. After transfection, miR-218 expression levels were examined through qPCR. qPCR analysis demonstrated that endogenous miR-218 level was increased more than 14-fold compared to the control cells after two day transfection, and remained 12-, 8-fold of the controls in 10 and 21 days after miR-218 mimic infected, respectively (**Figure 1B**). The transfected PDLSCs with miR-218 inhibitor had 5-, 4-, and 3-fold decrease of miR-218 expression at 2, 10 and 21 days respectively after transfection (**Figure 1C**). These results suggest that miR-218 may provoke osteoblast differentiation.

Over-expression of miR-218 promotes osteogenic differentiation of PDLSCs

To confirm the role of miR-218 in osteogenic differentiation of PDLSCs, we transfected miR-218 mimics into PDLSCs. Based on the results of Alizarin Red S staining assays, it was shown that over-expression of miR-218 significantly promoted PDLSCs osteogenesis when compared with control group (**Figure 2A**). We also illustrated that overexpression of miR-218 could activate the expression of several osteogenic marker genes such as ALP, the osteogenic transcription factor Runx2, and the mineralization marker (bone sialoprotein (BSP) and OCN), which are indicative of the osteoblastic differentiation status of PDLSCs (**Figure 2B-E**). The over-expression of miR-218 suggested potential roles for this miRNA in osteogenic differentiation of PDLSCs.

Knockdown of miR-218 inhibits osteogenic differentiation of PDLSCs

To further study the roles of miR-218 on the osteogenic differentiation of PDLSCs, we used the miR-218 inhibitor to transfect PDLSCs to retard the endogenous expression of miR-218. As shown in **Figure 3A**, Alizarin red staining and quantification results indicated that knockdown of miR-218 inhibited the formation of mineralized nodules after induction. The mRNA expression of the osteogenic differentiation markers ALP, OCN, BSP and Runx2 were all significantly decreased in PDLSCs transfected with miR-218 inhibitor (**Figure 3B-E**). Together, these experiments demonstrate the essential role of miR-218 for PDLSCs osteogenic differentiation.

MiR-218 is a positive regulator of Wnt signaling

To address the mechanism by which miR-218 regulates osteoblast differentiation, we examined predicted targets of miR-218 relevant to bone formation. More evidences have found that SFRP2 is a direct target of miR-218, which is potentially involved in differentiation through the Wnt signaling pathways [24]. To examine whether miR-218 directly targets SFRP2 in PDLSCs, we performed dual-luciferase reporter assays to confirm that miR-218 targets the SFRP2 3'UTR (**Figure 4A**). The luciferase activity was significantly inhibited when SFRP2-WT was co-transfected with miR-218 mimics compared with that after the mimic NC co-transfection, whereas the inhibitory effect was abolished when the SFRP2 3'UTR was mutated (**Figure 4B**).

To evaluate whether miR-218 regulated SFRP2 expression, we detected the protein expression level of SFRP2 in miR-218 mimic or miR-218 inhibitor infected cells. Western blot analysis showed that miR-218 overexpression markedly decreased the protein level of SFRP2 (**Figure 4C**), whereas miR-218 inhibition increased the protein expression of SFRP2 (**Figure 4D**). Together, these results indicated that SFRP2 is a direct target of miR-218 in PDLSCs, which suggested thatmiR-218 may regulate cell differentiation through activation of Wnt signaling by targeting SFRP2.

Discussion

In the present study, we observed that miR-218 was highly expressed during PDLSCs differentiation. Overexpression of miR-218 enhanced osteogenic differentiation, while inhibition of miR-218 has an opposite effect. More importantly, we found that SFRP2, the negative regulator of Wnt signaling, was a direct target of miR218. Taken together, our work reveals that miRNA-218 is among the regulators that drive PDLSCs differentiation by suppressing inhibitors of cell signaling pathways required for osteogenesis.

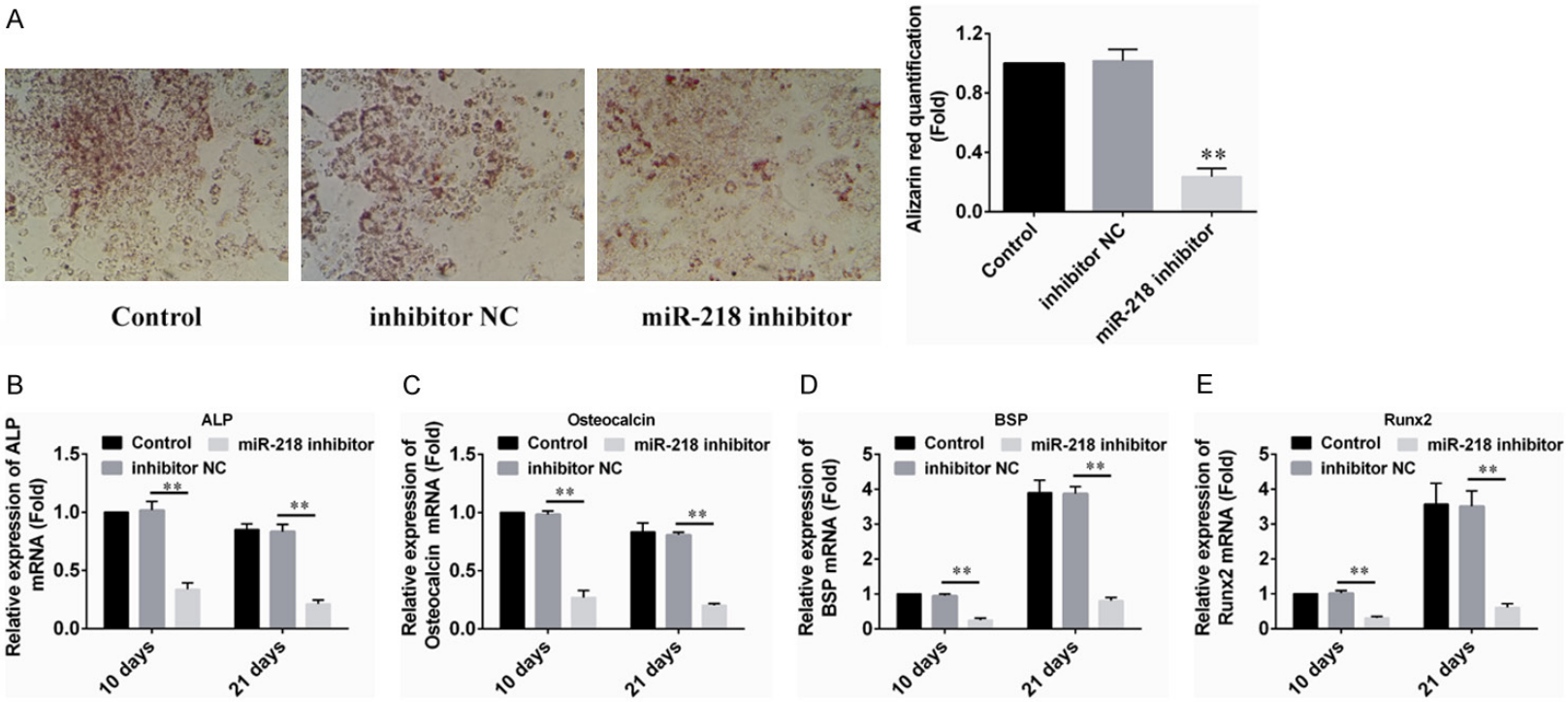

Figure 3. Down-regulation of miR-218 inhibited PDLSC osteogenic differentiation. A. 10 days after induction, the cells were stained with alizarin red (pH = 4.1), and the results showed that PDLSCs transfected with miR-218 inhibitor formed fewer mineralized nodules than the control groups. B-E. PDLSCs were transduced with miR-218 inhibitor. Total RNA was analyzed by real time qRT-PCR for mRNA expression profile of bone marker genes (ALP, OCN, BSP and Runx2) at d10 and d21. The data are the mean ± SD of 3 independent experiments. **P < 0.01.

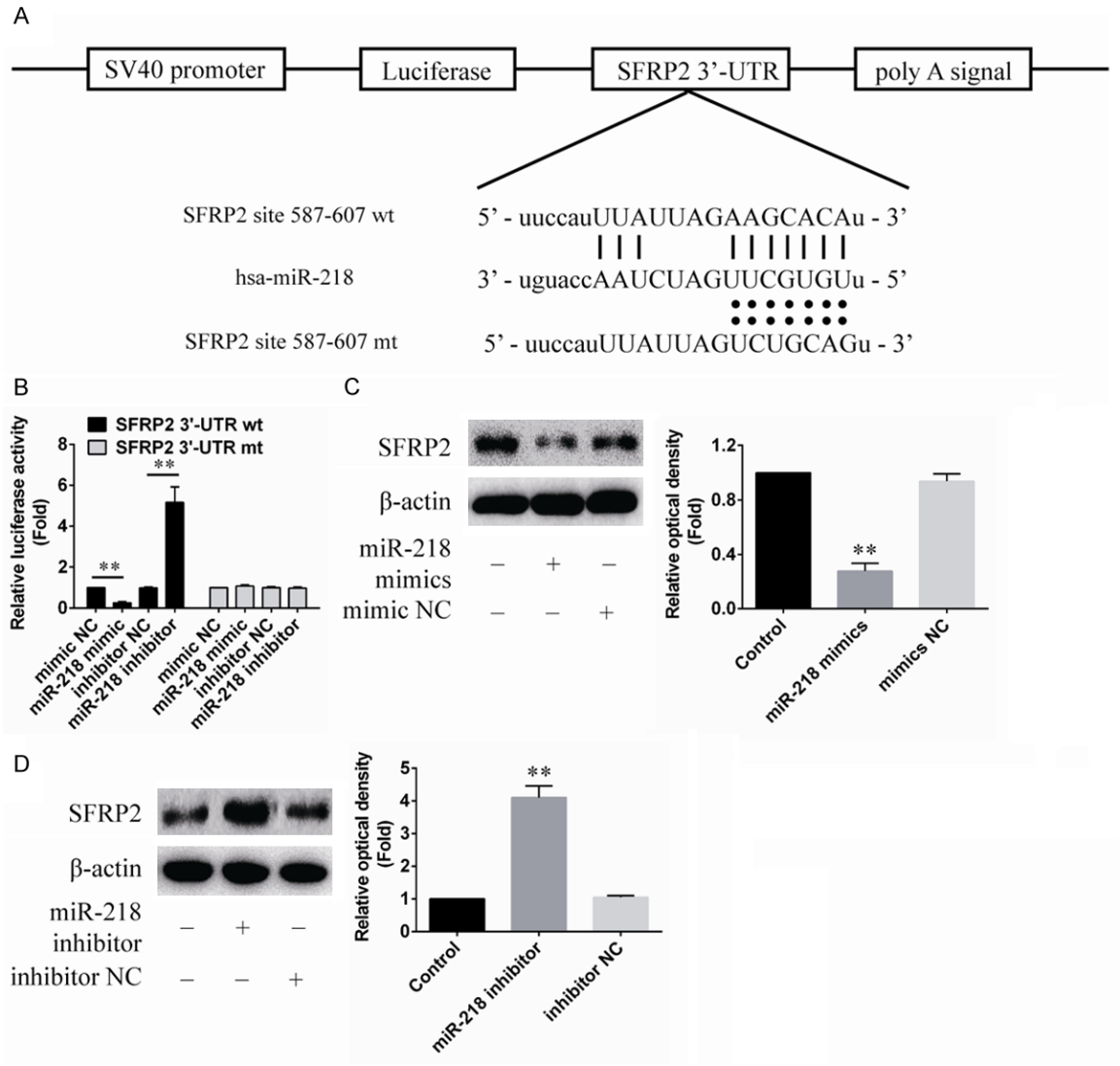

Figure 4. miR-218 directly binds and downregulates SFRP2. A. Schema of the firefly luciferase reporter constructs for SFRP2, indicating the interaction sites between miR-218 and the 3'-UTRs of the SFRP2. B. Luciferase activities. PDLSCs were co-transfected with firefly luciferase constructs containing the SFRP2 wild-type or mutated 3'-UTRs and miR-218 mimic, mimic NC, miR-218 inhibitor or inhibitor NC, as indicated (n = 6). C and D. Protein expression of SFRP2 after treatment with miR-218 mimic or miR-218 inhibitor (n = 6). All data represent the mean ± SD results of three independent experiments. **P < 0.01.

Several reports have demonstrated miRNAs were known to act as regulators in adipogenesis, myeloblasts differentiation and skeletal muscle development, and recently reported in regulating osteoblastogenesis. The function of miR-218 was recently reported. Zhang WB et al. found that miR-218 was up-regulated during osteogenic differentiation of Human adipose-derived stem cells (hASCs) and overexpression of miR-218 enhanced osteogenic differentiation in vitro by directly targeting SFRP2 and DKK2 [22]. Mohammad Q. Hassan et al. demonstrated that miR-218 stimulated the Wnt pathway by down-regulating three Wnt signaling inhibitors Sclerostin (SOST), Dickkopf2 (DKK2), and secreted frizzled-related protein2 (SFRP2) during the process of osteogenesis [25]. Therefore, we concluded that miR-218 might regulate osteoblastic differentiation of PDLSCs. The results shown that miR-218 levels were increased in differentiating PDLSCs, and miR-218 overexpression promoted PDLSCs differentiation. When miR-218 function was blocked, the formation of mineralized nodules was inhibited and the mRNA expression of the osteogenic differentiation markers ALP, OCN,

BSP and Runx2 were all significantly decreased. However, there has been no research on the regulatory functions of miR-218 in the field of osteogenic differentiation of PDLSCs.

In our study, we identified that miR-218 targeted the SFRP2 gene, which was a Wnt signaling pathway antagonist [26, 27]. The reporter assay showed that miR-218 was able to significantly repress luciferase contained SFRP2-3'-UTR expression. Western blot analysis also showed that miR-218 significantly inhibited the SFRP2 protein levels in differentiating PDLSCs cells. This result is in agreement with the report from Wei-Bing Zhang that SFRP2 is a direct target of endogenous miR-218 in hASCs [22]. sFRPs have been considered antagonists of canonical Wnt signaling by binding to Wnt proteins and preventing signal transduction based on their sequence homology with the Wnt-binding domain of the Fz receptors [16, 17]. Recent study from Jeffrey Schmeck peper et al. demonstrated that Sfrp2 treatment induced cardiac progenitor cells (CPCs) to exit the cell cycle and primed them for cardiac differentiation by inhibition of Wnt6 canonical signaling and activation of Wnt non-canonical pathways. In a pluripotent mouse embryonal carcinoma stem cell line, SFRP2 inhibits cardiomyogenic differentiation by regulating Wnt3a transcription [28]. Indeed, the expression of the negative regulators of Wnt signaling, sFRP2, is decreased in mature osteoblasts, providing a potential mechanism for increased Wnt signaling in more differentiated cells [29]. Therefore, it is possible that targeting of sFRP2 RNA by miR-218 is one mechanism contributing to PDLSCs differentiation. Because miR-218 is expressed in a wide array of tissues, it is likely that other miR-218 regulatory networks could exist [30, 31].

In conclusion, our data demonstrate that miR-218 regulates osteoblast differentiation by targeting SFRP2. Thus, miR-218 should be considered an important candidate as a molecular target of osteoblastic differentiation for the development of preventive or therapeutic agents against osteogenic disorders.

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Disclosure of conflict of interest

None.

Address correspondence to: Yike Ma, Department of Stomatology, The First Affiliated Hospital of Wenzhou Medical University, 2 Fuxue Lane, Ouhai District, Wenzhou, Zhejiang Province, China. Tel: +86 13968817548; E-mail: mayikemyk@yeah.net

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