Original Paper

miRNA-125b Regulates Osteogenic Differentiation of Periodontal Ligament Cells Through NKIRAS2/NF- κ B Pathway

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Key Words

Mirna-125b • Osteogenic differentiation • NKIRAS2 • Periodontal ligament cells

Abstract

Background/Aims: Osteogenesis of periodontal ligament cells (PDLCS) is essential for alveolar bone repair. Varieties of factors have been found involved in the regulation of PDLCS osteoblast differentiation. Aim of this study was to identify microRNA as a regulator of the osteogenic differentiation of PDLCS. **Methods:** The CD markers were analyzed by flow cytometry analysis. Osteoblast differentiation of PDLCS was induced by treatment with dexamethasone, β -glycerol phosphate and α -ascorbic acid. The expression of osteoblastic phenotype was evaluated after the induction by simultaneous monitoring of alkaline phosphatase activity, the expression of genes involved in osteoblastic differentiation by RT-qPCR and Western Blot, and mineralization at the same time. MicroRNA and NKIRAS2 expression was determined by RT-qPCR. Luciferase reporter assays were performed to test whether miR-125b is capable of interacting with the 3'UTR sequence of NKIRAS2. The possible signaling pathway was determined by Western Blot. **Results:** In this study, we found that the expression of miR-125b was down regulated during the process of osteoblast differentiation of PDLCS. When the expression of miR-125b was up regulated, the osteogenic differentiation of PDLCS was inhibited. During this process, the over-expressed miR-125b led to the activation of NF- κ B. NF- κ B inhibitor interacting RAS-like 2 (NKIRAS2) is one of target gene of miR-125b, and it is a regulator of NF- κ B signaling that plays various roles in osteoblastic differentiation. We demonstrate that miR-125b is involved in osteogenic differentiation of PDLCS. **Conclusion:** Our data support the hypothesis that miR-125b attenuates PDLCS osteoblastic differentiation by targeting NKIRAS2 and enhancing NF- κ B signaling.

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Introduction

Periodontal ligament (PDL), the most important tissue influencing the orthodontic tooth movement, is composed of heterogeneous multipotent cell populations, which has the ability to undergo osteoblast differentiation [1]. Osteoblast differentiation of periodontal ligament cells (PDLs) results in periodontal tissue remodeling and regenerating therefore it plays a vital role in maintaining homeostasis of periodontium. Varieties of factors have been found involved in the regulation of PDLs osteoblast differentiation, however, the molecular mechanisms and signaling pathways are still unclear. A better understanding of the molecular mechanisms that govern osteogenesis might provide us with new perspectives on treatment of orthodontic teeth movement and periodontitis [2].

MicroRNAs consist of a group of endogenous small, non-coding RNAs of approximately 22 nucleotides that exert post-transcriptional effects on gene expression [3]. They are thought to take part in several biological processes [4]. Some microRNA can inhibit osteoblast differentiation (e.g. miRNA-214 and miRNA-138) by targeting *Osx* [5] and *Runx2* [6]; while the others (e.g. miRNA-378; miRNA-17; miRNA-21; miR-101; miRNA-146a; miR-132) can promote osteoblast differentiation via several pathways, by targeting BMP2 [7], *Smurf1* [8], *PLAP-1* [9], NF- κ B signaling [10] or mTOR signaling pathway [11]. MiR-125b, one of the early discovered miRNAs, has been shown to inhibit osteoblast differentiation in the mouse MSC line ST2 [12], and it is a key regulatory factor of osteoblastic differentiation by directly targeting *Cbfb* [13] and indirectly acting on *Runx2* at an early stage MSCs osteoblastic differentiation. Moreover, it has been proved that miR-125b is significantly down regulated during osteogenic differentiation of PDLs [14]. However, the role of miR-125b in the osteogenic differentiation of PDLs and the molecular targets of miR-125b are still not identified.

As a key transcription factor, nuclear factor kappa B (NF- κ B) comprises two subunits, commonly p50 (NF κ B1)/p65 (RelA), which is held in the cytoplasm by the inhibitor of NF- κ B (I κ B) in its inactive state [15]. It has been found involved in bone development and bone remodeling [16]. Suppressing activation of the NF- κ B signal transduction pathway can potentially augment osteoblast differentiation [17, 18]. NF- κ B inhibitor interacting RAS-like 2 (NKIRAS2) can interfere with proteosomal degradation of I κ B [19, 20] and disturb NF- κ B activity.

Several studies report that miR-125b plays a key role in inhibiting osteoblastic differentiation, but its role in regulating osteoblastic differentiation of PDLs still remains unclear. At same time, the function of NKIRAS2 in osteogenic process of PDLs is also unclear. And NKIRAS2 is one of the target gene of miR-125b [21] so whether osteogenic process of PDLs is regulated through miR-125b/ NKIRAS2 pathway? In this study, we used primary cultured PDLs as the host to explore the roles of miR-125b and the related mechanism.

Materials and Methods

Cell culture and Characterization of PDLs

The human permanent teeth were obtained from healthy individuals under 25 years of age for orthodontics reason at the School of Stomatology, China Medical University, under the approval of the Ethical Committee of School of Stomatology, China Medical University. After extraction, the teeth were immediately washed by phosphate-buffered saline (PBS; Sigma, USA) and Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) supplemented with antibiotics (300 U/ml penicillin and 300 mg/ml streptomycin; Sigma, USA). The periodontal ligament was separated from the middle third of the root surface and cultured by the combination of tissue explant and enzymatic digestion method. The PDL tissues were digested with 3ml 0.2% collagenase type I enzyme (Sigma, USA) at 37°C in 50 min and vortexed every 10 min into the smallest pieces. The cells containing mixture were supplemented with culture medium and centrifuged at 1,000 rpm. Precipitate was suspended with culture medium, then seeded onto a 25-cm² flask (Nunc, Denmark) and incubated in a humid environment. Cells growth and morphology were observed under an inverted microscope. PDLs were used at passages 3 for following study.

The characterization of PDLs was tested by flow cytometry (BD FACSVerser, USA). After the cells were

transferred, they were fixed for 15 min in 4% paraformaldehyde (Sigma, USA). The cells were incubated with 3% bovine serum albumin and then with primary antibodies (Abcam, UK) raised against CD45, CD90, CD146 for 1 h. Then we washed cells with buffer, and added the secondary antibody for 45 min. Finally, the cells were washed three times and analyzed with a flow cytometer. The identity of PDLCS was further confirmed based on their differentiation potential by Cell Counting Kit-8 (BestBio, China).

Osteoblast differentiation induction

Osteoblastic differentiation was initiated by seeding PDLCS at a density of 5,000 cells per cm² in the medium containing 10% FBS (Invitrogen, USA), 100nM dexamethasone (Baomanbio, China), 100 mM β-glycerol phosphate (Sigma, USA) and 50 μg/ml α-ascorbic acid 2-phosphate (Sanat Cruz, USA), 100 U/ml penicillin (Sigma, USA), and 100 mg/ml streptomycin (Sigma, USA) for 21d.

Alizarinred S staining and ALP staining

After 21 days of culture in the osteogenic medium, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 30 min at 4 °C. After washed by deionized water for three times, the fixed cells were stained with 2% Alizarin red S (Sanat Cruz, USA) and Alkaline phosphatase staining was monitored using ALP staining kit (Cosmo Bio, Japan) according to the manufacturer's instructions. Finally, the cells were washed and photographed.

ALP activity determination

After 21 days of culture in the osteogenic medium, the cells were washed three times with PBS and lysed by 0.1% TritonX-100. Lysates were clarified by centrifugation at 13,000×g, 4 °C for 20min and sonication, and the ALP substrate solution (Yeast, China) was added to each well (96-well plate) at 37 °C for 10-60 min. ALP activity was determined by absorbance measurement at 405 nm and normalized to total protein. ALP activity was normalized to total protein.

RT-qPCR Analysis

RT-qPCR was performed to measure the relative mRNA levels. Cultured cells were lysed by Trizol (Invitrogen, USA) for RNA isolation. Next, the RNA was reversed-transcribed into complementary DNA using the PrimeScript RT reagent kit (TaKaRa, JP). RT-qPCR amplification conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The relative amount of mRNA or microRNA was calculated by 2^{-ΔΔCt} method and normalized to the expression of U6 respectively. The primer pairs of the related genes are indicated in Table 1.

Lentivirus infection

Lentivirus vectors were designed and constructed by Genomeditech (Shanghai, China). These lentivirus vectors contain a target gene or empty lentiviral vectors. (1×10⁶/well) PDLCS were seeded into 24-well plates for 24 h and then treated with 5 μg/mL Polybrene (Sanat Cruz, USA) and lentivirus suspension, and cultured for 24h. Then the medium was replaced by fresh medium. The shRNA of NKIRAS2 were designed and synthesized by Genomeditech (Shanghai, China), and they were transfected into PDLCS according to the protocol.

Western blot

Cell fractionation was performed using the nuclear extract kit (Active Motif, USA). Cells lysates were extracted with lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.1%

Table 1. Sequences of primers for the RT-qPCR

Primer name	Sequence (5'-3')
β-actin	F : TGGCACCAGCACAATGAA
	R : CTAAGTCATAGTCCGCTAGAAGCA
Runx2	F : GCCACCACTCACTACCACACCTA
	R : TCCTGACGAAGTGCCATAGTAGAGATAT
Osterix	F : GCAAGAGGTTCACTCGTTCGGATG
	R : TTGGAGCAGAGCAGGCAGGT
OCN	F : CCCAGGCGCTACCTGTATCAA
	R : GGTCAGCCAACCTCGTCACAGTC
OPN	F : GCCGACCAAGGAAACTCACT
	R : GGCACAGGTGATGCCTAGGA
ALP	F : GGACCATCCCCACGTCTTCAC
	R : CCTTGATGCCAGGCCATTG
miRNA-125b	F : TCCCTGAGACCCTAATTGTGA
	R : Universal adaptor primer
NKIRAS2	F : GCTTCTCTTGCACTGATGGCTACG
	R : TGGTGACCTCCTTCTGTCTCTGG
18s rRNA	F : GCAGAATCCACGCCAGTACAAGAT
	R : TCTTCTCAGTCGCTCCAGGTCTT

sodium dodecyl sulfate, 2mM dithiothreitol (DTT), 0.5% NP-40, 1mM PMSF and protease inhibitor cocktail) on ice for 30 min. After centrifugation at 15,000g at 4 °C for 10 min, protein concentrations were collected and then subjected to SDS-PAGE gel and electro-blotted on to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% BSA (CST, USA) and incubated overnight with primary antibodies. Blots were then washed and incubated for 1 h with secondary antibody (goat anti-rabbit Alexa Fluor 680, Invitrogen, USA). Protein bands were visualized with chemiluminescent ECL reagent (Millipore, USA).

The primary antibodies used in this study were directed against NKIRAS2 (diluted 1: 500, Abcam, UK), IκB (diluted 1: 10000, Abcam, UK), phosphor-IκB (diluted 1: 2000, Cell Signaling, USA), p65 (diluted 1: 2000, Abcam, UK), Runx2 (1:500; Cell Signaling, USA), ALP (diluted 1: 5000, Abcam, UK), OPN (diluted 1: 1000, Abcam, UK), OCN (diluted 1: 500, Abcam, UK), LAMIN A and Osterix (diluted 1: 5000, Abcam, UK).

Luciferase assays

The luciferase assay is to determine whether miR-125b could directly regulate NKIRAS2. The binding sequences of NKIRAS2 mRNA 3'UTRs and miR-125b were amplified by RT-qPCR. Then the RT-qPCR product was inserted into the multiple cloning site of the pGL3 empty vector. Binding-region mutations were achieved from a Quik Change Site-Directed Mutagenesis Kit (Stratagene, USA). Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega, USA). Luciferase activity was normalized to β-galactosidase activity.

Statistical Analyses

Data are presented as the mean ± SD from at least three independent experiments. Statistical comparisons were made between two groups with the t-test by SPSS software. P values less than 0.05 were considered statistically significant.

Results

Characteristics and osteoblast differentiation of periodontal ligament cells

After 10 d, the cells began to appear spread the tissue and the cells reached confluence about 21d later. Cultured PDLs were spindle shaped, fibroblast-like appearance and they were grown to a near-confluent state from single cells (Fig. 1A). They are characterized by the absence of positive markers (CD146/CD90) and the expression of a specific pattern of surface antigens (CD45).

Fig. 1. Characteristics and osteoblastic differentiation of periodontal ligament cells. (A) Image of PDLs cultured for 10 days was shown here. (B) FACS was used to characterized the PDLs by cell-surface markers. (C) Growth curve of PDLs was plotted by CCK-8 method. (D) ALP and Alizarin Red S staining were performed at day 21 of osteoblastic differentiation. (E) Alkaline phosphatase activity was measured at day 21 of osteoblastic differentiation. (F) Osteoblastic differentiation was evaluated by determination of the osteoblast specific genes (ALP, RUNX2, OSX, OCN, and OPN) at different time points with RT-qPCR method. 2^{-ΔΔCt} method was used to process the data to calculate the relative gene expressions. 18S rRNA was used as an internal control and all experiments were done in triplicates. * means P<0.05 vs the control group/0 day.

FACS analysis demonstrates that PDLCS expressed positive markers CD146 (48.1%), CD90 (99.73%) and expressed negative makers CD45 (4.6%) (Fig.1B). The identity of PDLCS was further confirmed based on their differentiation potential by CCK-8 (Fig. 1C). After the cells being cultured in osteogenic differentiation media for 21 days, the osteogenic differentiation potential of PDLCS was evidenced by Alizarin red S staining and ALP staining (Fig. 1D), respectively. There was mineralized nodule formation inside the cells and the ALP activity increased significantly (Fig. 1E). Our results showed the expression of specific markers for the osteogenic differentiation were increased (Fig. 1F).

Expression of miR-125b and NKIRAS2 during osteoblastic differentiation

We performed RT-qPCR to determine the expression of miR-125b and NKIRAS2 during osteoblastic differentiation in PDLCS. After being cultured in osteogenic differentiation media for 1, 3, 7, 10, 14, 21days, the expression of miR-125b (Fig. 2A) was down regulated from day 1 and reached a bottom 14 days later. The gene NKIRAS2 was increased during osteoblast differentiation of PDLCS (Fig. 2B), similar to osteoblast marker genes (Fig. 1F) and coinciding with down-regulation of miR-125b (Fig. 2A).

The luciferase reporter assay showed that miR-125b targeted NKIRAS2

According to analysis, NKIRAS2 has a 7-nt seed match site for miR-125b within its 3'UTR, and this putative target site is highly conserved among the vertebrates (Fig. 3A). To test whether miR-125b is capable of interacting with the 3'UTR sequence of NKIRAS2, luciferase reporter assays were performed. The results demonstrate that co-transfection with pre-miR-125b resulted in concentration- dependent down-regulation of luciferase activity relative compared to that of co-transfection with pre-miR-125b negative control or pNKIRAS2-MUT, while co-transfection anti-miR-125b with NKIRAS2 3'UTR luciferase reporter resulted

Fig. 2. Changes of miR-125b and NKIRAS2 expression in PDLCS after osteoblastic differentiation. The cells were treated with the osteoblast-inducer reagents for different times. And the mRNA expressions of miR-125b (A) and NKIRAS2 (B) in the cells were quantified with SYBR Green-based RT-qPCR method at the different time points. $2^{-\Delta\Delta C_t}$ method was used to process these data to calculate relative gene expression for this experiment. 18S rRNA was used as an internal control and all experiments were done in triplicates. * means $P < 0.05$ vs the control group.

Fig. 3. NKIRAS2 is a potential target of miR-125b. (A) Computational analysis was performed for the complementarities of miR-125b seed sequence to the 3'UTR of NKIRAS2 and conservation of the putative binding site in vertebrates. (B) The wild-type NKIRAS2 3'UTR reporter and pNKIRAS2-MUT was co-transfected with pre-miR-125b negative control, pre-miR-125b, anti-miR-125b negative control, or anti-miR-125b into cells. The error bars represent the mean $\pm$ SD. All experiments were done in triplicates. * means $P < 0.05$ vs the mutant group.

in concentration-dependent up-regulation of luciferase activity compared to that of co-transfection with anti-miR-125b negative control or pNKIRAS2-MUT (Fig. 3B). These data indicate that NKIRAS2 is a direct target of miR-125b.

Over-activity of miR-125b inhibits PDLcs osteoblastic differentiation

To determine the biological effect of miR-125b on osteogenic differentiation, miR-125b was overexpressed by transfecting lentivirus vectors. After transfected for 2 days, PDLcs were cultured in osteogenic differentiation media for additional 7 days, then ALP activity and the osteoblast marker gene expression were examined. Alizarin red S and ALP staining was examined after 21 days. RT-qPCR was used to confirm transfection efficiency (Fig. 4A). Alizarin red S (Fig. 4B), ALP staining (Fig. 4C) analysis indicated that overexpression of miR-125b attenuated the osteogenic differentiation of PDLcs. ALP activity (Fig. 4D) and the expression of osteoblast marker gene (Fig. 4E, F), such as ALP, OCN, and OPN were decreased in cells transfected with LV-miR-125b compared to LV-vector group. These results indicate that the miR-125b negatively regulates osteogenic differentiation in PDLcs.

Sh-NKIRAS2 inhibits PDLcs osteoblastic differentiation

To investigate the role of NKIRAS2 in the osteogenic differentiation of PDLcs, we use RNA interference technique for suppressing NKIRAS2 levels in PDLcs. PDLcs were transfected with sh-NKIRAS2 and control for 2 days then cultured in osteogenic differentiation media for additional 7 and 21 days. RT-qPCR was used to confirm transfection efficiency (Fig. 5A). Alizarin red S, ALP staining and ALP activity showed that suppression of NKIRAS2 decreased ALP activity and mineralized bone matrix formation of PDLcs (Fig. 5B, C). RT-qPCR analysis confirmed that osteoblast marker genes were decreased and western blot analysis showed that Runx2, Osterix, OCN, OPN, ALP protein levels were decreased (Fig. 5D, E). These results indicated that the suppression of NKIRAS2 negatively regulates osteogenic differentiation in PDLcs.

Fig. 4. Influence of transfection with miR-125b on osteoblastic differentiation. (A) The expression of miR-125b in PDLcs transfected with lentivirus vectors was determined by RT-qPCR. (B, C, D) ALP staining, Alizarin Red S staining and ALP activity showed the inhibited ALP activity and calcification of osteogenic differentiation after transfection with LV-miR-125b. (E) The expression of osteogenic specific genes RUNX2, ALP, OCN, Osterix and OPN after transfection with LV-miR-125b was analyzed by RT-qPCR. (F) Western blotting results showed the accordant protein expression with the mRNA expression. *means $P < 0.05$ compared with the LV-Vector group/NC group. $2^{-\Delta\Delta Ct}$ method was used to process these data to calculate relative gene expression for this experiment. 18S rRNA and β -actin were used as the internal control for gene and protein expressions, respectively. All experiments were done in triplicates.

Fig. 5. Suppression of NKIRAS2 negatively regulates osteogenic differentiation in PDLs. (A) The expression of NKIRAS2 in PDLs transfected with sh-NKIRAS2 was determined by RT-qPCR. * $P < 0.05$ compared with negative control (miR-NC)-transfected PDLs. (B, C) Alizarin Red S staining, ALP staining and ALP activity showed the inhibited calcification and ALP activity of osteogenic differentiation after transfection with sh-NKIRAS2. (D) The expression of osteogenic specific genes after transfection with sh-NKIRAS2 was analyzed by RT-qPCR. (E) the protein expression was determined with Western blot method and showed the similar tendency with that of the mRNA expression. β -actin protein was used as the inner control for Western Blot and 18S rRNA was used as an internal control for gene expression. All experiments were done in triplicates.

Fig. 6. NKIRAS2 is involved in the miR-125b regulated osteogenic differentiation in PDLs. (A) The relative expression of miR-125b was determined by RT-qPCR in PDLs transfected with anti-miR-125b. (B) anti-miR-125b was co-transfected with sh-NKIRAS2 into PDLs and the expressions of osteogenic-specific genes were determined and compared by RT-qPCR method. 18S rRNA was used as an internal control for gene expression. $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expressions for the experiments. (C) ALP staining and Alizarin Red S staining showed lower levels of mineralization in co-transfected group than that in anti-miR-125b transfection alone group. * means $P < 0.05$ compared with the control group. All experiments were done in triplicates.

MiR-125b inhibits PDLs osteoblastic differentiation by target NKIRAS2

To demonstrate whether the increase of PDLs osteoblastic differentiation induced by the down-regulation of miR-125b expression was due to NKIRAS2, we co-transfected anti-miR-125b and sh-NKIRAS2 into PDLs cells. As shown in (Fig. 6A), the expression of miR-125b and NKIRAS2 was blocked after co-transfection. After 14d osteogenic differentiation, the extent of osteoblast marker genes increase was far less than that effected by anti-miR-125b transfection alone (Fig. 6B), and microscopic analysis of the Alizarin red S, ALP staining revealed lower levels of mineralization in NKIRAS2 knockdown PDLs than in control cells (Fig. 6C). All these data suggested that NKIRAS2 is essential for the role of miR-125b inhibitor to promote osteogenesis.

NKIRAS2 inhibits NF- κ B Signaling

PDLCs were transfected with sh-NKIRAS2 and cultured in osteogenic differentiation media for additional 7 days, the nuclear and cytoplasmic fractions of cells were analyzed by western blotting. The level of phosphorylated I κ B was higher in cells transfected with sh-NKIRAS2 than that were not. It suggests that NKIRAS2 can postpone phosphorylation process of I κ B and inhibit degradation of I κ B. The cells transfected with sh-NKIRAS2 were found to express higher level of nuclear p65 and lower level of cytoplasmic p65 relative to cells not transfected (Fig.7). The results suggest that NKIRAS2 may inhibit the translocation of p65 into the nucleus.

Fig. 7. NKIRAS2 inhibits NF- κ B activity. Expression of nuclear p65, cytoplasmic p65, I κ B and phospho I κ B in PDLCs transfected with sh-NKIRAS2 or pre-control were analyzed by Western blotting. Cells were treated with 10 ng/ml TNF α at 48h post transfection. Compared to the control, the cytoplasmic expression of I κ B and p65 in the sh-NKIRAS2 group were decreased but the pI κ B expression was increased (Fig. 6A); β -actin protein was used as the inner control of the cytoplasmic proteins; and the nucleic expression of p65 in the sh-NKIRAS2 group was increased (Fig. 6B); Lamin A protein was used as the inner control of nuclear proteins. * means P<0.05 vs the control group. All experiments were done in triplicates.

Fig. 8. MiRNA-125b affects the nuclear–cytoplasmic translocation of I κ B and p65 in PDLCs. (A) The protein expressions of I κ B and p65 in the cytoplasm and the nucleic of PDLCs transfected with LV-miR-125b were analyzed by Western Blot method. (B) The protein expressions of I κ B and p65 in the cytoplasm and the nucleic of PDLCs transfected with LV-anti-miR-125b were analyzed by Western Blot method. β -actin protein was used as the inner control of the cytoplasmic proteins; Lamin A protein was used as the inner control of nuclear proteins. *means P<0.05 compared to the control group. All experiments were done in triplicates.

miR-125b inhibits PDLCS osteoblastic differentiation by enhancing NF- κ B Signaling

The activation of the NF- κ B pathway is characterized by the stabilization of p50(NFKB1)/p65(RelA)(i), the translocation of p50/p65 into the nucleus (ii), the interaction of p50/p65 and its target genes(iii). It had been proved that NKIRAS2 can block the p50/p65 translocation into the nucleus and NKIRAS2 is the target gene of miR-125b. Therefore, we investigated the level of p65 in nucleus to demonstrate whether miR-125b inhibits PDLCS osteoblastic differentiation by enhancing NF- κ B signaling. Nuclear and cytoplasmic fractions of cells cultured in osteogenic differentiation media were analyzed by western blotting. When the miRNA-125b was up regulated, the level of I κ B was decreased and the level of nuclear p65 was increased (Fig. 8A). When the miRNA-125b was down regulated, the level of I κ B was increased and the level of nuclear p65 was decreased (Fig. 8B).

Discussion

In this study, we demonstrated that miR-125b and the related target NF- κ B pathway play an important role in the osteogenic differentiation of PDLCS. PDLCS have been regarded as an important candidate with capability of cyto-differentiation and mineralization to form mineralized tissue cells [22]. PDLCS consist of heterogeneous multipotent cell populations, which have the potential to undergo osteogenic differentiation. Osteoblast differentiation results in the expression of tissue-specific gene such as alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), periostin (PON), and others. Cell differentiation involves complex pathways regulated at both transcriptional and post-transcriptional levels. Recently, it has been reported that miRNAs have an influence on the cells osteoblast differentiation [23], through negative regulation of gene expression at the post-transcriptional level.

In this study, we analyzed the expression of miR-125b in osteoblastic differentiation using PDLCS and found that miR-125b was decreased during the first 14 days and then elevated at the end of detection. The study also showed that the osteoblastic differentiation in PDLCS was inhibited by overactivity of miR-125b. They all demonstrated that miR-125b was a negative regulator in osteogenesis process. Furthermore, it has been reported NKIRAS2, which is a target gene of miR-125b, suppresses NF- κ B activation by inhibiting its transcriptional activation [24]. Since NF- κ B signaling is critical for PDLCS osteogenesis [9], NKIRAS2 caught our attention, and the osteogenic differentiation mechanism mediated by miR-125b was investigated. When we transfected the shRNA of NKIRAS2 into PDLCS, the osteoblastic differentiation had been inhibited. Cells transfected with sh-NKIRAS2 had decreased amounts of I κ B protein, but increased the phosphorylated form of I κ B protein. Furthermore, the decreased the amount of I κ B proteins in sh-NKIRAS2 transfected cells also have sequestered the increased p65 in the nucleus. These results suggest that NKIRAS2 associated with I κ B, slowing down the degradation of I κ B, and inhibited canonical NF- κ B signaling pathway.

The goal of this study was not only to identify miR-125b regulated the osteoblastic differentiation of PDLCS but also to predict whether miR-125b could regulate a specific signaling pathway. We silenced the NKIRAS2 and transfected the LV-anti-miR-125b to PDLCS simultaneously. The mRNA expression of Runx2, Osterix, OCN, OPN, and ALP increased and the protein expression level of NKIRAS2 reduced compared with the blank control group. However, the extent of osteoblast marker genes increase was far less than that effected by LV-anti-miR-125b transfection alone. All these data suggested that miR-125b inhibits PDLCS osteoblastic differentiation by target NKIRAS2.

Degradation of I κ B by a phosphorylation-dependent represents an important event for the unmasking of NF- κ B translocation sequences and the initiation of transcription by the nuclear factor [25]. To further investigate whether miR-125b inhibits PDLCS osteoblastic differentiation through enhancing NF- κ B signaling, we confirmed the effects of miR-125b induced I κ B degradation and nuclear translocation of p65. The results showed that miR-125b could inhibit PDLCS osteogenesis and the NF- κ B pathway was involved in this process. As

miR-125b expression was upregulated, the activity of NF- κ B pathway was increased. MiR-125b induces the degradation of I κ B and nuclear translocation of NF- κ B p65/p50 heterodimers.

The osteoblastic differentiation of PDLs can be influenced by many factors, such as inflammatory microenvironment. Liu [7] found that the multi-differentiation potential of mesenchymal stem cells (MSCs) isolated from periodontitis-affected periodontal ligament tissue was significantly lower than that of MSCs isolated from healthy human periodontal ligament tissue. Furthermore, inflammatory cytokines can regulate osteogenesis of PDLs through different signaling pathways, such as MAPKs and Smad signaling pathways [26]. The “One shot, multiple targets” signature of miRNA heralds that there are other signaling pathways regulating the osteogenesis of PDLs, for example, Smad4 is a target of miR-125b. In the future work, we will focus on the investigation of the roles of the other signaling pathways via which miR-125b regulates osteoblastic differentiation and whether miR-125b works in an inflammatory microenvironment.

When we analyzed the expression of miR-125b in osteoblastic differentiation, the result showed that the expression of miR-125b was decreased during early stage (≤ 14 days) while increased after 14 days of osteogenic differentiation of PDLs. It suggested the miRNA could modulate osteogenic function initiating differentiation rather than after the differentiation [27, 28].

Collectively, in the present study we have demonstrated a previously unknown role for miR-125b in inhibiting PDLs osteogenic differentiation. We have proved that this is likely to be due to the fact that NKIRAS2, a target of miR-125b, inhibits NF- κ B signaling and promotes osteogenic differentiation. Furthermore, we find that miR-125b activates the NF- κ B pathway by negatively regulating NKIRAS2 expression, inhibiting the osteogenic differentiation of periodontal ligament cells. Our results suggest that therapeutic inhibition of miR-125b in osteoblasts may promote bone formation and even reverse periodontitis by exerting an anabolic effect.

Disclosure Statement

The authors declare that they have no competing interests.

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