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The function of miR-218 and miR-618 in postmenopausal osteoporosis

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Abstract. – OBJECTIVE: Postmenopausal osteoporosis (POMP) is a serious disorder with significant physical, psychosocial, and financial consequences, which greatly reduce the postmenopausal women's life quality. The related issues of postmenopausal osteoporosis are increasingly concerned by society. Past researches have shown that miRNAs play an important role in the occurrence and development of postmenopausal osteoporosis. However, the role of miR-218 and miR-618 in the osteoporosis regulation is still unclear.

MATERIALS AND METHODS: First of all, we investigated the alteration of miR-218 and miR-618 during osteoclastogenesis of RAW264.7 cells. Next, we transfected RAW264.7 cells with miR-218 or miR-618 mimics and inhibitors to explore the influences of miR-218 and miR-618 on osteoclast differentiation. Then, we conducted bioinformatics analysis and luciferase reporter assay to identify and test the target gene of miR-218 and miR-618.

RESULTS: MiR-218 and miR-618 were down-regulated when RAW264.7 cells differentiated into osteoclasts. In addition, overexpression of miR-218 or miR-618 attenuated RAW264.7 cells differentiated into osteoclasts *in vitro*, whereas inhibition of miR-218 or miR-618 promoted this progress. This was demonstrated by increased expression of osteoclast-specific genes and TRAP staining. TLR-4 was confirmed to be the direct target of miR-218 and miR-618 by bioinformatics and luciferase reporter assay.

CONCLUSIONS: These results suggested that miR-218 and miR-618 play an important role in osteoclastogenesis via TLR-4/MyD88/NF-κB signaling pathway. Thus, targeting miR-218 and miR-618 promise a therapeutic potential in the treatment of osteoporosis.

Key Words:
Postmenopausal osteoporosis, miR-218, miR-618, TLR-4, Nf-kB, Osteoclast differentiation.

Introduction

Postmenopausal osteoporosis is a systemic metabolic bone disease characterized by decreased bone mass, impaired bone microstructure and increased skeletal fragility, resulting in an increased incidence of fractures. The main cause of postmenopausal osteoporosis is the decreased estrogen level, leading to the impaired balance between bone resorption and bone formation broken[1-3]. MicroRNAs (miRNAs) are a class of endogenous, small RNAs with a length of about 19-25 nucleotides, which have variety of important regulatory functions in cells. MiRNAs regulate the expression of target genes by participating in post-transcriptional regulation, playing an important regulatory role in tumor development, biological development, organ formation, viral defense, epigenetic regulation and metabolism. This complex regulatory network can regulate the expression of multiple genes through a miRNA, and can also regulate the expression of a gene through several combinations of miRNAs[4-6].

MicroRNAs have been reported to be a crucial component in the development of osteoporosis and participated in osteoclast formation, differentiation, apoptosis, and resorption[7-11]. For example, miR-124 regulated osteoclast differentiation by regulating NFATc1 expression[12]; miR-34a blocks osteoporosis by inhibiting Tgif2[13] while miR-29 controls the osteoclast differentiation[14]. Emerging evidence indicates several miRNAs might be involved in osteoclasts differentiation. Here, we reported that miR-218 and miR-618 were down expressed in postmenopausal osteoporosis and intrinsically regulated osteoclastogenesis.

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Materials and Methods

Osteoclast Differentiation of RAW264.7 Cells

The RAW264.7 cell line was obtained from FuHeng Cell Center (Shanghai, China) for osteoclastogenesis. RAW264.7 cells were induced into osteoclasts at different times (0, 1, 3, 5 days) by stimulating with RANKL (receptor activator of nuclear factor Kappa-B ligand) (50 ng/mL, R&D Systems Inc., Minneapolis, MN, USA). The cells were cultured under standard cell culture conditions (5% CO_2 and 95% humidity). Osteoclasts induced in the culture were proved by tartrate-resistant acid phosphatase (TRAP) staining and osteoclast differentiation marker genes, including TRAF6 and NFATc1 mRNA detection. TRAP+ multinuclear osteoclasts (>3 nuclei) were counted by light microscopy.

MiRNA Mimic/inhibitor Transfection

Both miR-218 and miR-618 mimic, inhibitor, and negative control (NC) with fluorescent tags, were synthesized by Guangzhou RiboBio (Guangzhou, China). MiR-218, miR-618 mimic, inhibitor and NC were transfected into the RAW264.7 cells separately in two different 6 well plates (20×10^4 cells/pore) by Lipo-3000 (Life Technologies Co., Gaithersburg, MD, USA). The transfection efficiency was observed under fluorescence microscope after being incubated for 36 h. Next, the cells were cultured in fresh medium incubating with RANKL for 3 days.

MiRNA extraction

The total RNA was extracted from the collected cells by TRIzol (Ambion by Life Technologies, Gaithersburg, MD, USA). In short, the cells were gathered in an Eppendorf (EP) tube, lysed with 1 mL/pore TRIzol and then mixed with chloroform. Cells were centrifuged (12,000 rpm) at 4°C for 15 min, the supernatant liquid was separated into a new EP tube and mixed with cold isopropanol. The mixture was centrifuged (12,000 rpm) at 4°C for 10 min; after that, the upper water of precipitated phase was discarded and the RNA precipitation was obtained. The RNA precipitation was washed in 75% ethanol and centrifuged (at 12,000 rpm) at 4°C for 15 min. After taking out the upper aqueous phase, the precipitation of RNA was dried at room temperature naturally; each tube added 20-50 uL diethyl pyrocarbonate (DEPC) water to dissolve the RNA precipitation, and the total RNA was received for RT-qPCR analysis.

RT-qPCR

Reverse transcription was conducted by a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). For miRNAs, Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) specific primers for miR-218, miR-618 and the internal reference U6 were obtained from Sangon Biotech, (Shanghai, China). These following primers were used for RT-qPCR detection: U6 (forward: 5′-AGAGAAGATTAGCATGGCCCCTG-3′, reverse: 5′-ATCCAGTGCAGGGTCCGAGG-3′); miR-218 (forward: 5′-TTGCGGATGGTTCCGTCA AGCA-3′, reverse: 5′-ATCCAGTGCAGGGTCCGAGG-3′); miR-618 (forward: 5′-CGGCGGAAACTCTACTTGTCCTT-3′, reverse: 5′-ATCCAGTGCAGGGTCCGAGG-3′); NFATc1 (forward: 5′-GACCCGGAGTTCGACTTCG-3′, reverse: 5′-TGACACTAGGGGACACATAACTG-3′); TRAF6 (forward: 5′-AAAGCGAGAGATTCTTTCCCTG-3′, reverse: 5′-ACTGGGGACAATTCACTAGAGC-3′); GAPDH (forward: 5′-TGGCCTTCCGTGTTCCTAC-3′, reverse 5′-GAGTTGCTGTTGAAGTCGCA-3′). Amplification and detection were conducted by the SYBR Premix Ex Taq™ II kit (TaKaRa, Dalian, China) and the Applied Biosystems StepOnePlus™ Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA), respectively. U6 and GAPDH are selected for using as internal reference.

Target Gene Prediction

We predicted the potential targets of miR-218 and miR-618 by using miRanda, TargetScan, and PicTar. We found that TLR4, with a miR-218 and miR-618 binding site in the 3′ UTR among the candidate target genes, were closely correlated with the osteoclast-signaling pathway.

Luciferase Reporter Assay

The TLR4 3′-UTR luciferase reporter vector was purchased from Genechem (Shanghai, China). Site directed mutagenesis was performed using the Stratagene QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). To test suppression by miR-218 or miR-618, miR-218 or miR-618 of interest was co-transfected into HEK293T cells with the indicated wild type or mutated 3′-UTR luciferase reporter and with Renilla luciferase as a transfection efficiency control. The luciferase activity was measured using Dual-Glo® Luciferase assay kit (Promega, Madison, WI, USA).

TRAP Staining

Osteoclasts were proved by tartrate resistant acid phosphatase (TRAP) staining, using a kit ac-

cording to the provider's instructions (Sigma, St. Louis, MO, USA). Briefly, cells were treated with the mixed solution containing 45 mL deionized water, 0.5 mL fast garnet GBC base solution, 0.5 mL sodium nitrite solution, 0.5 mL naphthalene AS-BI phosphate solution, 2 mL acetate solution and 1 mL tartrate solution and incubated for 1 h at 37°C. TRAP-positive cells containing at least 3 nuclei were observed by the microscope.

Statistical Analysis

All data were expressed as means ± SD. Comparison between groups was realized using One-way ANOVA test followed by Least Significant Difference (LSD). $p < 0.05$ was considered statistically significant.

Results

MiR-218 and miR-618 were Downregulated During Osteoclast Differentiation

To explore the changes of miR-218 and miR-618 during osteoclast differentiation of RAW264.7 cells, RAW264.7 cells were treated with RANKL (50 ng/ml) for a different time (0, 1, 3, 5 days) to induce osteoclast differentiation. Osteoclast formation was identified by increased expression of osteoclastogenesis-related genes (NFATC1 and TRAF6) (Figure 1A) and enhanced TRAP staining (Figure 1B). Further, qRT-PCR for miR-218 and miR-618 was conducted at days 0, 1, 3, and 5. As expected, results revealed a significant decrease of miR-218 and miR-618 in RAW264.7 cells after being differentiated into osteoclasts (Figure 1C).

MiR-218 and miR-618 Inhibited Osteoclast Differentiation

To investigate the role of miR-218 and miR-618 in osteoclast differentiation, RAW 264.7 cells were transfected with miR-218 or miR-618 mimics, inhibitors and empty vectors (negative control (NC) group). Then, they were induced into osteoclasts differentiation. Transfection efficiency was observed under fluorescence microscope and demonstrated by qRT-PCR of miR-218 and miR-618 (Figure 2A and 3A). At day 5 after osteoclastogenesis induction using RANKL, qRT-PCR was performed to explore expression profiles of NFATc1 and TRAF6 in different groups (miR-218 or miR-618 mimics, miR-218 or miR-618 inhibitors and empty) (Figure 2C and 3C). In addition, TRAP staining was also conducted at day 5 to detect osteoclast differentiation (Figure 2B and 3B). Results suggested that down-regulation of

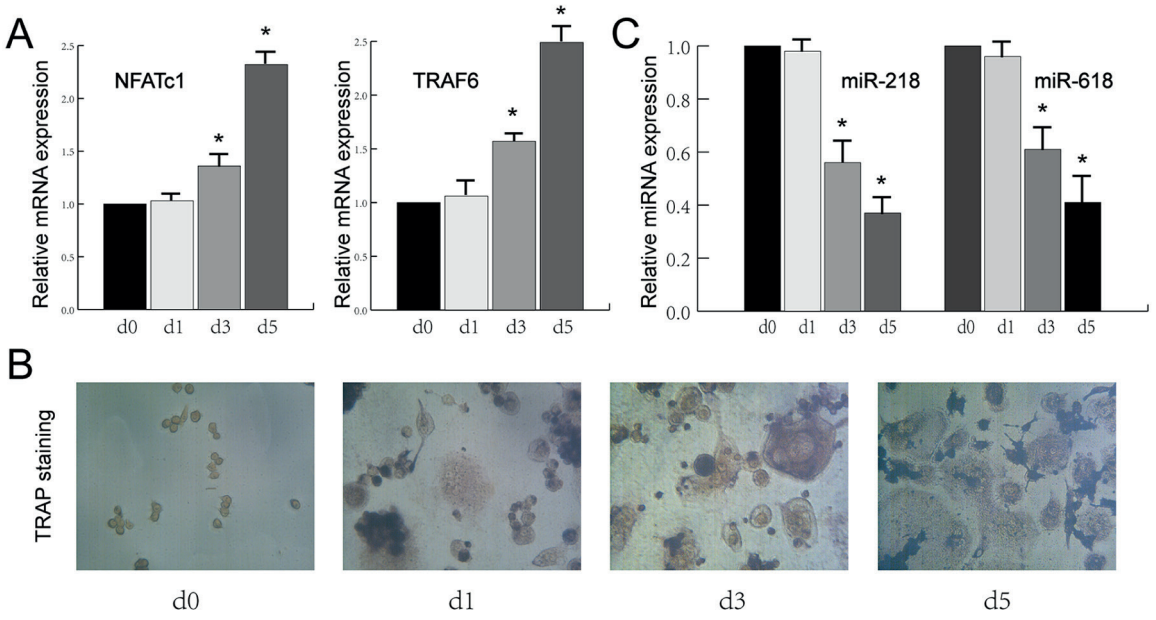

Figure 1. MiR-218 and miR-618 were downregulated in osteoclastogenesis. ***(A)*** mRNA expression of NFATC1 and TRAF6 at different time points (0, 1, 3 and 5 days). ***(B)*** Osteoclasts differentiation was confirmed by TRAP staining at different time points (0, 1, 3 and 5 days). ***(C)*** Expression of miR-218 and miR-618 were measured at different time points (0, 1, 3 and 5 days) during osteoclastogenesis. N=3 for the experiments (*p<0.05, compared with control group).

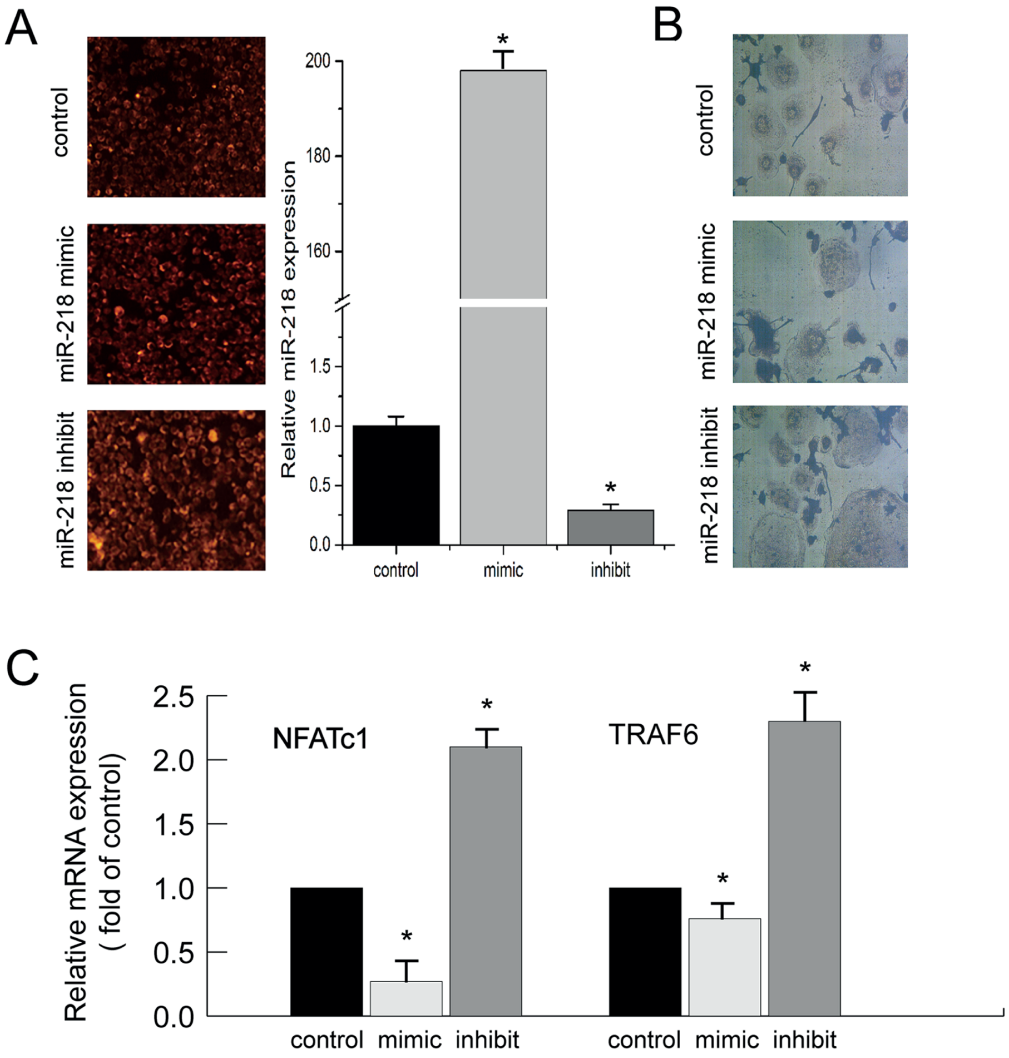

Figure 2. MiR-218 inhibited osteoclast differentiation. ***(A)*** Transfection efficiency was observed under the microscope and relative expression of miR-218 in RAW264.7 cells transfected with miR-218 mimic or inhibitor was detected by qRT-PCR. ***(B)*** TRAP staining in RAW264.7 cells from different groups (miR-218 mimic, inhibitor and control) was conducted after induction with RANKL for 3 days; ***(C)*** qRT-PCR was performed to explore expression profiles of NFATc1 and TRAF6 in 3 different groups (miR-218 mimic, inhibitor and control)). N=3 for the experiments (*p<0.05, compared with control group).

miR-218 or miR-618 remarkably stimulated osteoclast differentiation, demonstrated by increased expression of the osteoclast-specific genes NFATc1 and TRAF6 and increased TRAP staining. However, overexpression of miR-218 or miR-618 significantly inhibited osteoclastic differentiation of RAW 264.7 cells.

TLR4 was a Direct Target of miR-218 and miR-618

To investigate the further molecular mechanism of which miR-218 or miR-618 regulates osteoclast differentiation, we used databases of miRanda, TargetScan, and PicTar to look for the target genes with an established function of stimulating osteoclastogenesis. Among these candidate, osteoclast-related target genes, we discovered that the 3'UTR of TLR4 has miR-218 and miR-618 binding site (Figure 4A). To analyze whether miR-218 or miR-618 could inhibit TLR4, HEK293T cells were transfected with the TLR4 3′-UTR luciferase reporter vector. Then, we mutated the seed region in TLR4 for miR-218 or miR-618 binding and conducted the luciferase assays to evaluate whether the regulation was via targeting the predicted binding site in TLR4 identified by miR-218 or miR-618 rather than other non-specific behaviors (Figure 4A). Results showed that miR-218

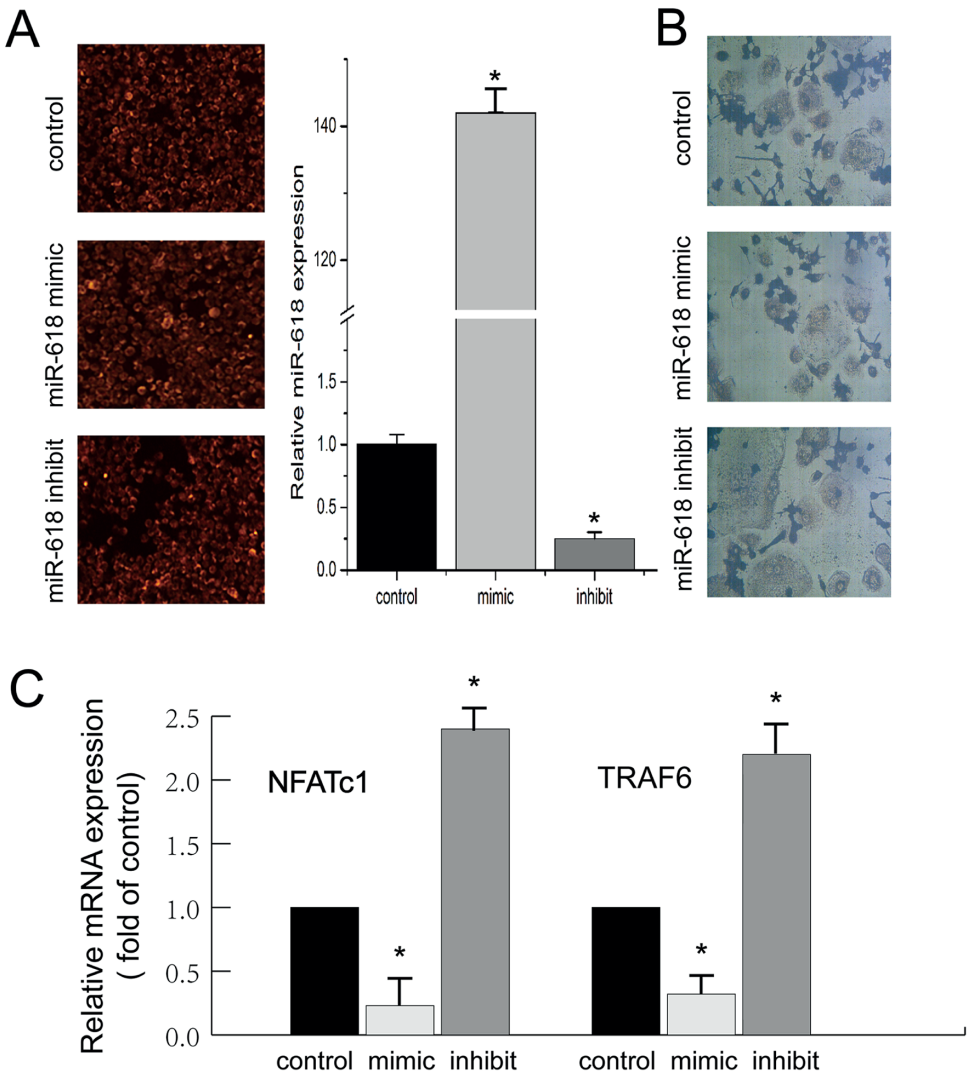

Figure 3. MiR-618 inhibited osteoclast differentiation. ***(A)*** Transfection efficiency was observed under the microscope and relative expression of miR-618 in RAW264.7 cells transfected with miR-618 mimic or inhibitor was detected by qRT-PCR. ***(B)*** TRAP staining in RAW264.7 cells from different groups (miR-618 mimic, inhibitor and control) was conducted after induction with RANKL for 3 days; ***(C)*** qRT-PCR was performed to explore expression profiles of NFATc1 and TRAF6 in 3 different groups (miR-618 mimic, inhibitor and control)). N=3 for the experiments (*p<0.05, compared with control group).

and miR-618 could significant inhibit the wild-type TLR4, but not the mutant TLR4 (Figure 4B).

Discussion

Postmenopausal osteoporosis is mainly caused by the decrease level of estrogen, which leads to the enhancement of bone immune function, resulting in imbalance of bone remodeling. The interaction between osteoblasts and osteoclasts is the key factor in bone remodeling[15-17].

Several researches[18-20] have reported the relationship between miRNA and osteoclasts. For example, a study demonstrated that p65 promoted osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway[18]. Another work found that MiR-142-3p was an inducer of cell death in osteoclasts[19]. Chen et al[20] discovered that reduction of miR-503 expression in $CD14^+$ PBMCs of postmenopausal women resulted in accelerated osteoclastogenesis and contributed to osteoporosis.

In the current study, we found that miR-218 and miR-618 expression in RAW264.7 cells were decreased after induction with RANKL. We used miR-218 or miR-618 mimics and inhibitors to upregulating or downregulating miR-218 or miR-

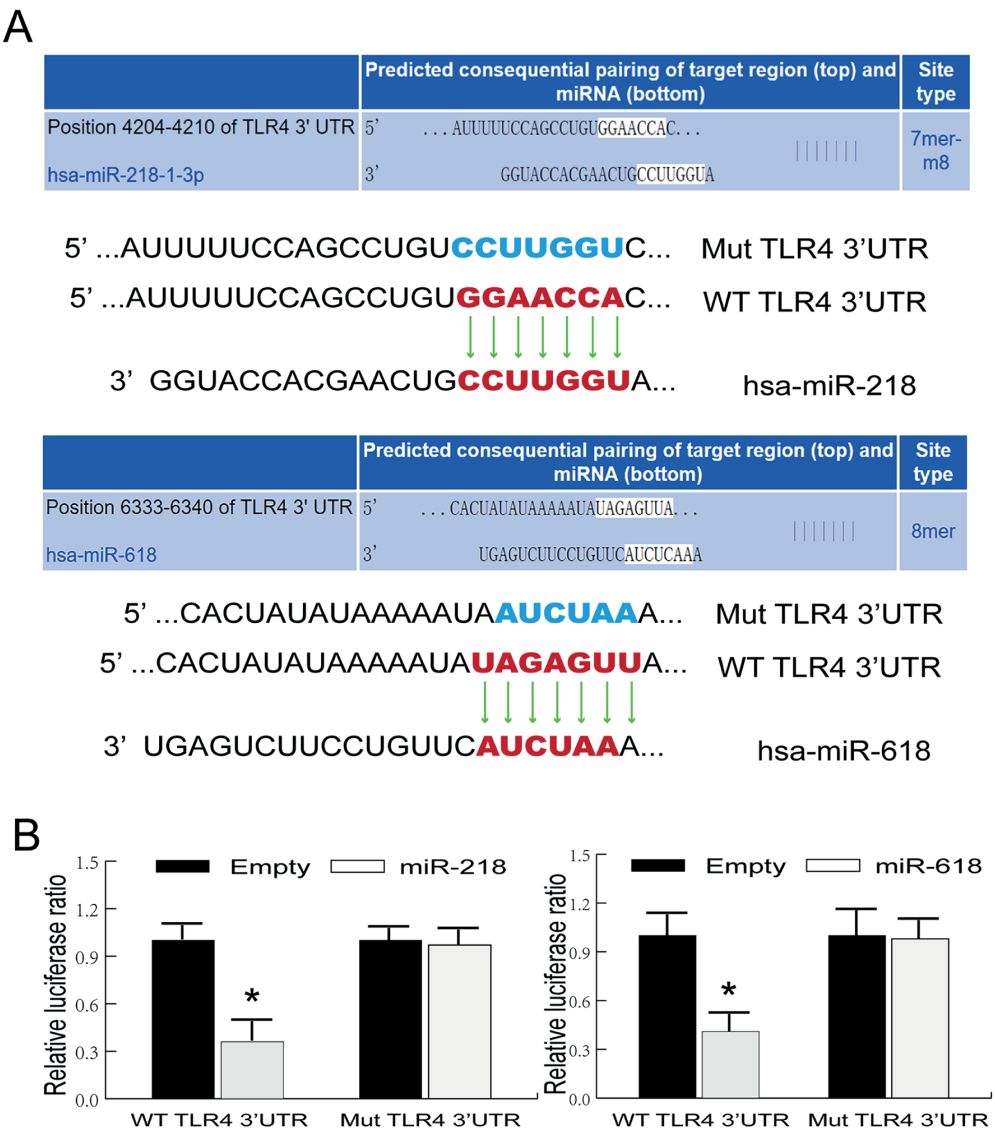

Figure 4. TLR4 was a direct target of miR-218 and miR-618. ***(A)*** The database was used for prediction of potential target genes that stimulate osteoclast differentiation and schematic representation showed the 3'UTR of TLR4 has miR-218 and miR-618 binding site. ***(B)*** HEK293T cells were co-transfected with the indicated TLR4 3′-UTR luciferase reporter (with either wild-type or mutant-type miR-218 or miR-618 binding sites), miR-218 or miR-618 significantly inhibited luciferase activity of wild type TLR4 3′-UTR. N=3 for the experiments (*p<0.05, compared with empty group).

618 expression. As we expected, overexpression of miR-218 or miR-618 both attenuated osteoclast differentiation, whereas inhibition of miR-218 or miR-618 had an opposite effect. These results indicated that miR-218 and miR-618 were negative regulators in RAW264.7 cells osteoclast differentiation. Next, we tried to look for the target genes of miR-218 or miR-618 in this process. Among the candidates, we found that TLR4 might be a potential target gene for miR-218 or miR-618. Previous investigations[21] have reported that TLR4 participated in the occurrence and development of many diseases. For instance, TLR4-mediated inflammation promoted KSHV (Kaposi sarcoma herpesvirus)-induced cellular transformation. TLR4 promoted the generation of programmed cell death 1 ligand 2 $(PD\text{-}L2)^{+}$ dendritic cells in allergic asthma[22] and partly mediated AngII-induced cavernosal dysfunction[23]. A previous study[24] reported that the expression of TLR4 was increased in osteoclasts differentiation and TLR-4/MyD88/NF-κB signaling pathway was integral

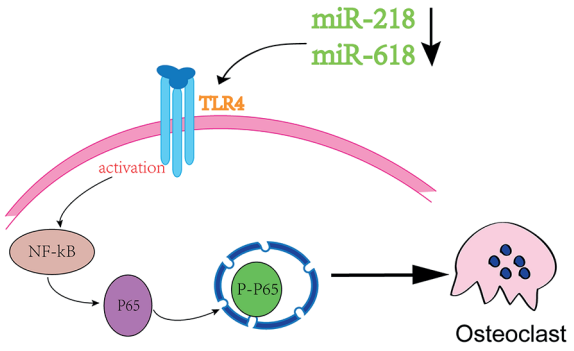

Figure 5. Possible mechanisms of miR-218 and miR-618 regulate the osteoclast differentiation. The decrease of miR-218 and miR-618 upregulated TLR4, thus activating TLR-4/NF-κB signaling pathway and consequently resulting in the osteoclast differentiation of RAW264.7 cell.

for osteoclast development. In the present investigation, we demonstrated that TLR4 was indeed a direct target of miR-218 and miR-618 by using luciferase report assay. Thus, we can make an explanation why overexpression of miR-218 or miR-618 attenuated osteoclastogenesis. We assumed that overexpression of miR-218 or miR-618 may result in a remarkably decrease of TLR4, thus inhibiting the TLR-4/MyD88/NF-κB signaling pathway and impairing the normal functions of osteoclasts. All the above results suggested that miR-218 or miR-618 inhibited osteoclastogenesis by repressing TLR-4/MyD88/NF-κB signaling pathway (Figure 5).

Conclusions

We proved that miR-218 and miR-618 inhibit osteoclastogenic differentiation by suppressing TLR-4/MyD88/NF-κB signaling pathway. What's more, the results revealed that functional overexpression of miR-218 or miR-618 can inhibit osteoclastogenesis, indicating that targeting miR-218 and miR-618 might be a potential therapy in the treatment of postmenopausal osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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