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CircRNA_009934 induces osteoclast bone resorption via silencing miR-5107

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Abstract. – OBJECTIVE: We aimed to explore the expression of circRNA_009934 in osteoclast, as well as its potential roles in regulating osteoclastogenesis and bone resorption via regulating miR-5107.

MATERIALS AND METHODS: We performed qRT-PCR analysis to examine the expression of circRNA_009934 in osteoclast in distinctive stages. We used CCK-8 assay to detect the cell proliferation ability. Correlation analysis between the expression levels of circRNA_009934 and miR-5107 was performed using statistical analysis. Bioinformatics prediction was performed to predict the binding site of circRNA_009934 and miR-5107, subsequently followed by Luciferase assay for validation. The mice TRAF6 3′-UTR were cloned into the Luciferase reporter vector and miR-5107 binding mutants were constructed to validate the inhibited regulation of miR-5107 to the expression of TRAF6.

RESULTS: Our results showed that expression of circRNA_009934 was increased during osteoclast differentiation. CircRNA_009934 expression was closely correlated with osteoclastogenesis and bone resorption activity. Bioinformatics prediction and Luciferase assay demonstrated that circRNA_009934 served as a ceRNA of miR-5107 and regulated its downstream TRAF6 expression.

CONCLUSIONS: We first demonstrated that circRNA_009934 expression was increased in osteoclasts, which promoted osteoclastogenesis by serving as a ceRNA of miR-5107 and regulated the expression of TRAF6.

Key Words:
Osteoclasts, CircRNA_009934, MiR-5107, TRAF6.

Introduction

Bone is a dynamic organ continuously undergoing remodeling[1]. Bone remodeling is maintained by osteoblastic bone formation and osteoclastic bone resorption[2]. Osteoclasts, a type of bone specific multinucleated cells, play a vital role in bone remodeling process[3,4]. Osteoclasts are bone resorbing cells differentiated from monocyte/macrophage lineage hematopoietic stem cells[5]. Receptor activator of nuclear factor κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) are two important regulating factors in osteoclastogenesis[6,7]. Osteoclast differentiation and function dysregulation can cause various bone pathologies including osteoporosis, Paget's disease, and pycnodysostosis[8-10]. Therefore, finding the ways and mechanisms to regulate osteoclasts will help us to find potential targets for the treatment of bone diseases.

Noncoding RNAs play important roles in physiological or pathological processes. Circular RNA (circRNA) is a type of single-stranded RNA which is not the same as the well-known linear RNA, and forms a covalently closed continuous loop, i.e., in circular RNA the 3' and 5' ends normally present in an RNA molecule have been joined together[11-13]. CircRNAs were thought to be functionless due to their splicing errors. With the advent of next-generation sequencing, a growing number of circRNAs have been identified in distinctive cell lines and species[14-15]. CircRNA molecules are rich in microRNA (miRNA) binding sites, and act

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as miRNA sponges in cells[16-21], thereby lifting the inhibitory effect of miRNA on its target genes to increase its target genes expression level. This mechanism of action is called the competitive endogenous RNA (ceRNA) mechanism[22-25]. There is evidence[26,27] showing that circRNAs participate in various pathological processes by interacting with miRNAs associated with diseases, such as proliferation, invasion, and metastasis of various malignancies, including gastric cancer, breast cancer and pancreatic cancer.

In this study, we aimed to explore the functional roles of circRNA_009934 in osteoclastogenesis, as well as to disclose the molecular mechanisms. We detected the expression level of circRNA_009934 in osteoclasts at different stages during osteoclastogenesis. Besides, the osteoclast differentiation ability and bone resorption function were examined after transfection with lentiviral circRNA_009934. At last, we assessed the regulatory relationship between circRNA_009934 and miR-5107 and the miR-5107-mediated roles of circRNA_009934. Our study uncovered a critical function of circRNA_009934 in osteoclastogenesis and bone resorption function.

Materials and Methods

Cell Culture

Murine monocyte macrophage RAW264.7 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplied with 10% FBS (Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) and incubated at 37°C in an atmosphere of 5% CO_2.

Construction of Lentivirus and Cell Transfection

Lentiviral circRNA_009934 and circRNA_009934 shRNA were synthesized and constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For miR analysis, the miR-5107 mimic, miR-5107 inhibitor and the negative control were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). The knockdown of TRAF6, si-TRAF6 plasma and negative control plasma were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For transfection, 1×10^4 cells were seeded in 6-well plates and cultured with RANKL (100 ng/mL) and M-CSF (100 ng/mL). Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I reduced serum medium were used for transfection. For analysis of circRNA_009934, the cells were transfected with circRNA_009934 shRNA (referred as to sh) and negative control shRNA (referred as to nc), respectively. For analysis of miR-5107, the cells were transfected with miR-5107 inhibitor, and control cells were transfected with empty vector, respectively. The cells without transfection were used as the control (referred as to control). After incubation for 30 min, the cultures were replaced with DMEM containing 10% FBS. Then, at indicated time point after transfection, the cells were harvested for further study.

Osteoclast Differentiation

5×10^3 cells were seeded in 96-well plates uniformly. After one day, the medium was changed with DMEM containing M-CSF (100 ng/mL) and RANKL (100 ng/mL) according to the experiments. The culture medium was refreshed every day. After induction for 3 days, Tartrate-Resistant Acid Phosphatase (TRAP) stain was performed using TRAP stain kit. For Real Time-PCR, the cells were harvested.

RNA Extraction and qRT-PCR

After taking out the culture plates, the cells were washed with PBS. After treatment, total RNA of cells was extracted by using TRIzol reagent (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. The samples were stored at room temperature for 30 min. The reverse transcription of cDNA was performed with a PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. For qRT-PCR, PCR primers were synthesized by GenePharma (ShangHai Gene Pharma, Shang-Hai, China) and the sequences were listed in Table I. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression.

CCK-8 Assay

The CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) was used to measure the cells proliferation according to the manufacturers' instructions. In brief, 5×10^3 cells were seeded in 96-well plates uniformly. After treated with regulated medium, the medium was removed and

Table I. Primer sequences for qRT-PCR.

Genes	Forward	Reverse	Tm (°C)
NFATc1	5'-GACCCGGAGTTCGACTTCG-3'	5'-TGACACTAGGGGACACATAACTG-3'	61
CTSK	5'-GCTCCTCTTAGGGGCCACT-3'	5'-ATTGGGGACCCTTAGGCCAT-3'	62
MMP-9	5'-AAGTCTCAAGGTTATAGGGACGG-3'	5'-CCATGCTTGTCTGGGTATAGTGT-3'	62
TRAP	5'-CACTCCCACCCTGAGATTTGT-3'	5'-CATCGTCTGCACGGTTCTG-3'	60
C-fos	5'-TTGAGCGATCATCCCGGTC-3'	5'-GCGTGAGTCCATACTGGCAAG-3'	61
DC-STAMP	5'-CGGCGGCCAATCTAAGGTC-3'	5'-CCCACCATGCCCTTGAACA-3'	61
GAPDH	5'-TGGATTTGGACGCATTGGTC-3'	5'-TTTGCACTGGTACGTGTTGAT-3'	62
circRNA_009934	5'-AGCATGATCGAGCGTGCATTC-3'	5'-TAGCTCAGTCTGAGCTGACGT-3'	60
miR-5107	5'-GATCGATCGTCGTAGCTAGCT-3'	5'-ACGGTGGATCGAACGTTCGATC-3'	61

cells were washed with PBS solution for 3 times. Then, CCK-8 dilution was added to the 96-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 2 hours. After incubation, the plates were taken out, and cell proliferation was measured using multi-detection microplate reader. The absorbance (OD) value at 490 nm of each well was detected.

Luciferase Assay

After transfection for 48 h, the Luciferase activities were measured by using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Renilla Luciferase activities were normalized to the firefly Luciferase activities and the data were expressed as the fold change relative to the corresponding control groups which were defined as 1.0.

Statistical Analysis

Unless otherwise indicated, all data were processed by Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Each assay was applied at least three independent experiments or replicates. All data were presented as mean ± SD. Student's *t*-test, One-way analysis of variance (ANOVA) and multiple comparison between the groups were performed by using SNK method, in which $*p < 0.05$, $**p < 0.01$ represented as the difference significance.

Results

CircRNA_009934 Was Conspicuously Increased in Mature osteoclasts

For osteoclast differentiation, BMMs isolated from mouse femur were cultured with RANKL and M-CSF to generated osteoclasts, following confirmed by qRT-PCR analysis of osteoclastogenesis specific genes (Figure 1A). Western blot results revealed that the expression of osteoclastic genes, such as MMP9, TRAP, CTSK and DC-STAMP was significantly increased after 72 h incubation (Figure 1B). By bioinformatics analysis of a circRNA expression data of osteoclasts at different stages during osteoclastogenesis (0 h, 24 h and 72 h), we screened out circRNA_009934, which has high specific expression in mature osteoclasts (Figure 1C). We then performed qRT-PCR analysis for further validation. The results indicated that the expression of circRNA_009934 was significantly upregulated in matures osteoclasts compared with BMMs ($p<0.05$) (Figure 1D).

CircRNA_009934 Expression is Associated with Osteoclast Differentiation and Bone Resorption Function

To explore the functions of circRNA_009934 in osteoclasts, lentiviral circRNA_009934 was constructed and subsequently transfected into BMMs and circRNA_009934 expression was then detected. qRT-PCR analysis showed that circRNA_009934 expression was remarkably downregulated after lentiviral circRNA_009934 transfection (Figure 2A). The CCK-8 assay was performed and showed that overexpressing circRNA_009934 significantly increased BMMs proliferation (Figure 2B), whereas the inhibition of circRNA_009934 significantly suppressed the cell proliferation activity after 1 d and 3 d (Figure 2C). CircRNA_009934-inhibited BMMs were stimulated with RANKL and M-CSF for 3 days for osteoclast differentiation, which showed a poor efficiency of osteoclastogenesis compared with control BMMs (Figure 2D), indicating that circRNA_009934 plays an important role in the regulation of osteoclastogenesis. The number of mature osteoclasts was decreased after inhibition of circRNA_009934 (Figure 2E). qRT-PCR analy-

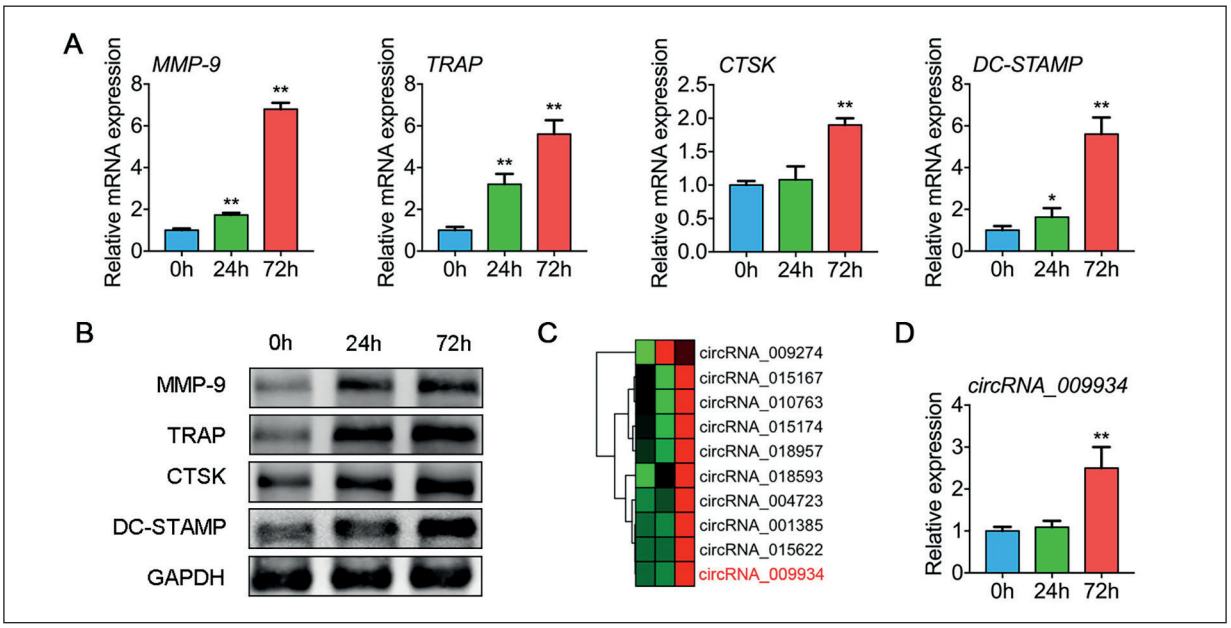

Figure 1. CircRNA_009934 and its co-expressed mRNA TRAF6 was highly expressed in osteoclasts. **A**, Relative mRNA expression levels of CTSK, DC-STAMP, MMP-9, TRAP of osteoclasts at different stages during osteoclastogenesis (0 h, 24 h and 72 h). **B**, The protein expression levels of CTSK, DC-STAMP, MMP-9 and TRAP were measured by Western blot of osteoclasts at different stages. **C**, The cluster heat map of expression profiles of 10 circRNAs at different stages during osteoclastogenesis. **D**, Relative expression of circRNA_009934 at different stages during osteoclastogenesis (0 h, 24 h and 72 h) assessed by qRT-PCR. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

sis was also performed to detect osteoclastogenesis related genes between circRNA_009934-inhibited cells and control cells after osteoclast induction for 3 days. The results showed a significant downregulation of osteoclast differentiation associated genes, such as TRAF6, c-FOS and TRAP and bone resorption function related genes, including CTSK, MMP9 and DC-STAMP in osteoclasts after circRNA_009934 inhibition (Figure 2F, 2H). Meanwhile, on the protein level, Western blot revealed the same results (Figure 2G, 2I). Together, these results suggested that circRNA_009934 expression is important for osteoclastogenesis and bone resorption function, inhibition of circRNA_009934 leads to a decreased of osteoclastogenesis and dysfunction of bone resorption.

MiR-5107 Was Downregulated in Osteoclasts and Negatively Correlated with CircRNA_009934

In order to investigate whether circRNA_009934 was correlated miRNA, we used StarBase 2.0 to predict the target miRNA of circRNA_009934 and found that miR-5107 is one of the target miRNAs of circRNA_009934. Therefore, we used qRT-PCR analysis to detect the miR-5107 expressions of osteoclasts. Results showed that miR-5107 was downregulated in osteoclasts compared with BMMs (Figure 3A). We, then, used correlation analysis to further explore the relationship between circRNA_009934 and miR-5107; the results showed that miR-5107 was significantly negatively correlated with circRNA_009934, indicating that miR-5107 might be regulated by circRNA_009934 (Figure 3B). These results suggested that miR-5107 was low expressed in osteoclasts and negatively correlated with circRNA_009934.

CircRNA_009934 Could Directly Bind With MiR-5107 in Osteoclasts

Previous studies demonstrated that circRNAs might act as a competing sponge in regulating the biological functions of miRNAs. As discussed above, miR-5107 was negatively correlated with circRNA_009934, so we hypothesized that circRNA_009934 regulated osteoclast differentiation and bone resorption through interaction with miR-5107. To further detect the relationship between miR-5107 and circRNA_009934, circRNA_009934-wt Luciferase reporter vector and circRNA_009934-mut 3'UTR Luciferase re-

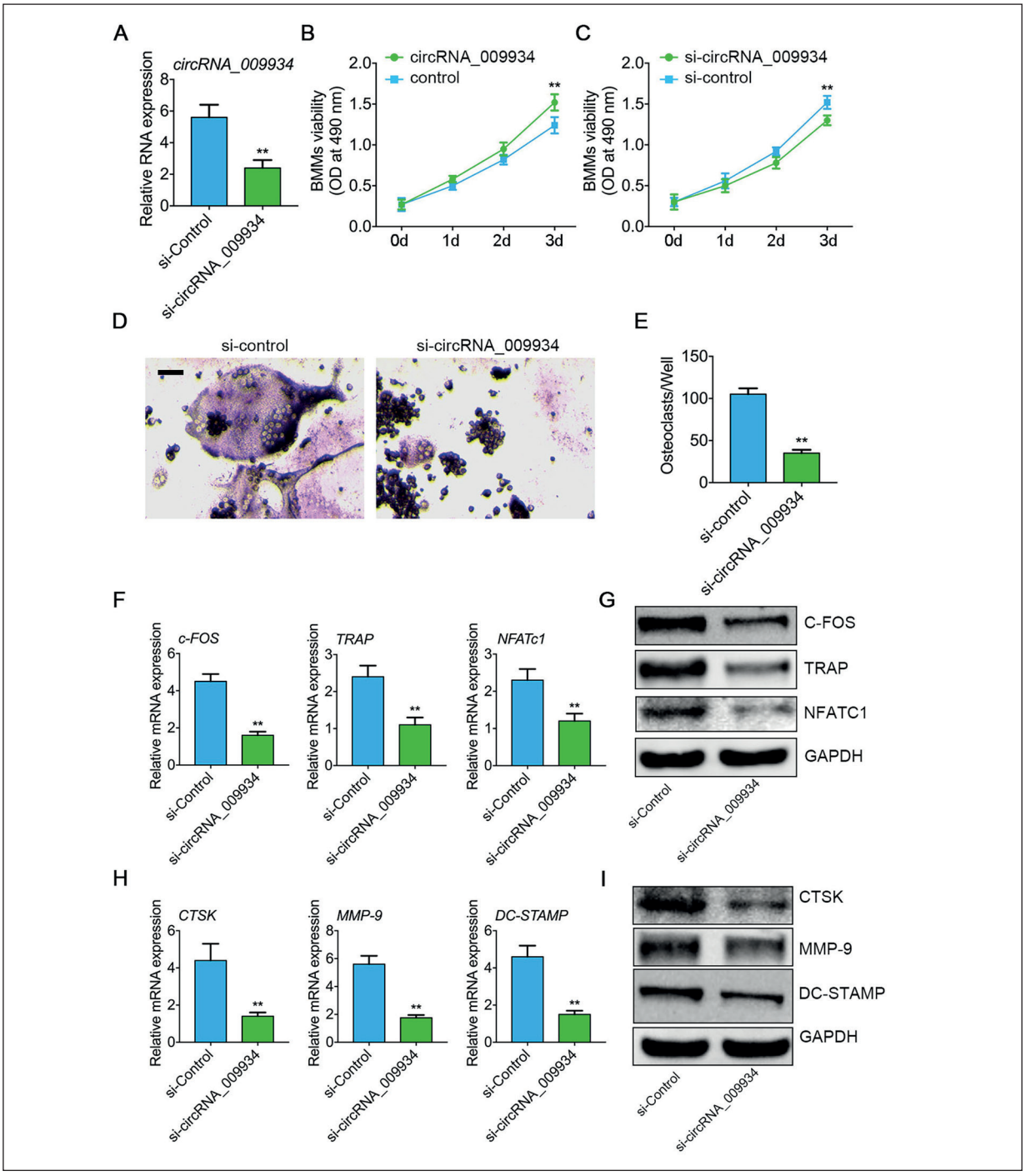

Figure 2. CircRNA_009934 expression is associated with osteoclastogenesis and bone resorption function. **A,** Relative mRNA expression levels of circRNA_009934 in osteoclasts transfected with si-control and si-circRNA_009934. **B,** Absorption at 490 nm of BMMs treated with circRNA_009934 detected by CCK-8 assay at 1 d, 2 d and 3 d. **C,** Absorption at 490 nm of BMMs treated with si-circRNA_009934 detected by CCK-8 assay at 1 d, 2 d and 3 d. **D,** TRAP staining assay of osteoclast transfected with si-circRNA_009934, magnification:×200). **E,** Quantification of osteoclasts in each well. **F,** Relative mRNA expression levels of NFATc-1, c-FOS and TRAP in osteoclasts transfected with si-control and si-circRNA_009934 detected by qRT-PCR. **G,** The protein expression level of NFATc-1, c-FOS and TRAP in osteoclasts after transfection with si-control and si-circRNA_009934 were measured by WB. **H,** Relative mRNA expression levels of CTSK, MMP-9 and DC-STAMP in osteoclasts transfected with si-control and si-circRNA_009934 detected by qRT-PCR. **I,** The protein expression levels of CTSK, MMP-9 and DC-STAMP in osteoclasts after transfection with si-control and si-circRNA_009934 were measured by WB. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

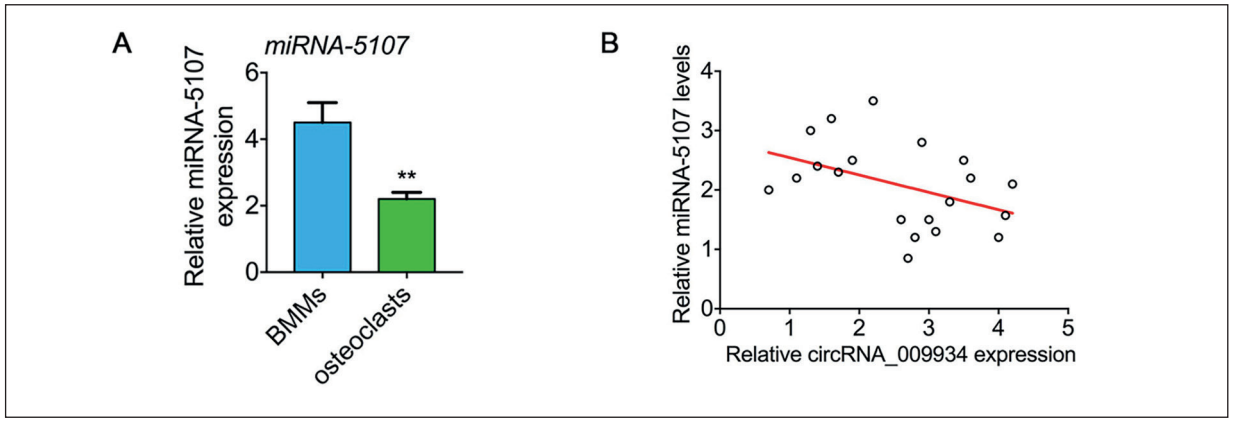

Figure 3. MiR-5107 was downregulated in osteoclasts and negatively correlated with circRNA_009934. **A,** Relative miR-5107 expression in BMMs and osteoclasts detected by qRT-PCR. **B,** Correlation analysis was performed to evaluate the relationship between miR-5107 and circRNA_009934. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

porter vector were synthesized (Figure 4A) and Luciferase reporter assay was performed. Compared with the control, the Luciferase activity of BMMs that co-transfected with miR-5107 mimic and circRNA_009934-wt was significantly decreased ($p<0.05$), and it was reversely increased in circRNA_009934-mut compared with circRNA_009934-wt ($p<0.01$) (Figure 4B). These results demonstrated that miR-5107 could directly bind to circRNA_009934. Besides, circRNA_009934 overexpression suppressed miR-5107 expression and circRNA_009934 inhibition reversely facili-

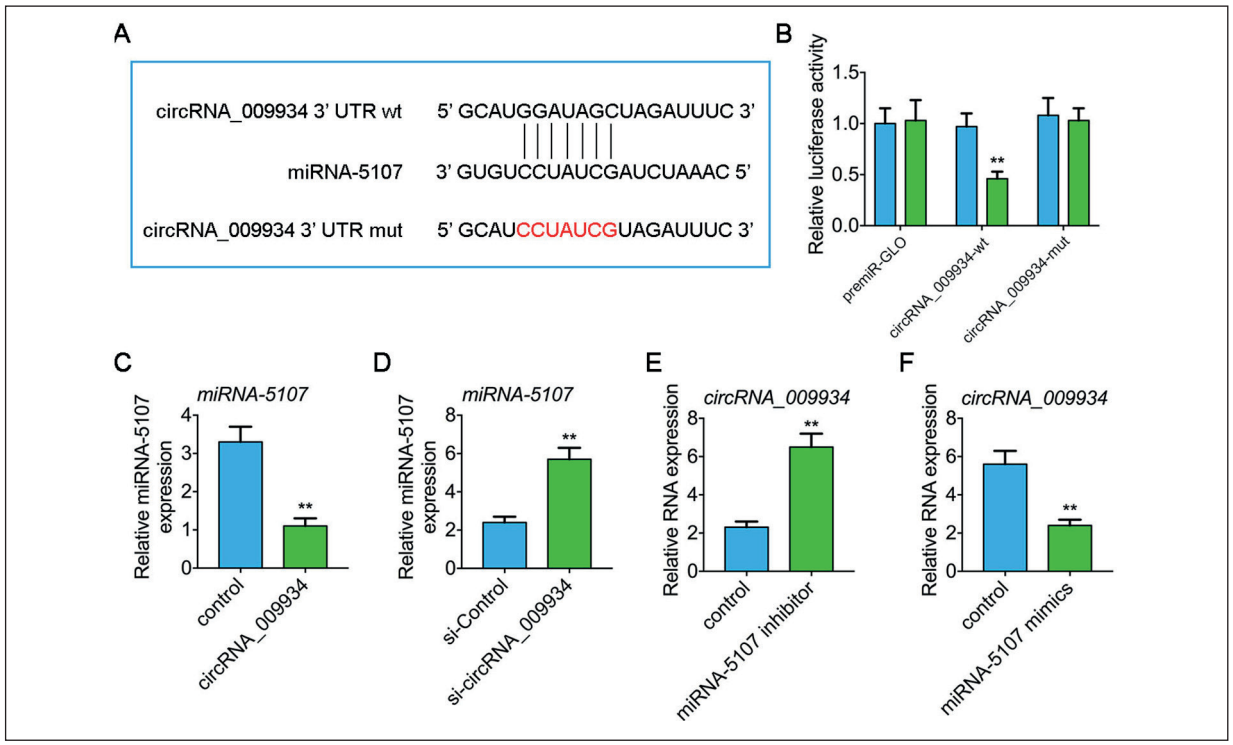

Figure 4. CircRNA_009934 could directly bind with miR-5107 in osteoclasts. **A,** Schematic illustration of the predicted miR-5107 binding sites and mutant sites in circRNA_009934. **B,** Relative Luciferase activity of BMMs. **C-D,** qRT-PCR analysis of miR-5107 expression level in BMMs transfected with lentiviral circRNA_009934 and si- circRNA_009934. **E-F,** Relative circRNA_009934 expression was detected in BMMs after miR-5107 mimic and miR-5107 inhibitor by RT-PCR. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

tated miR-5107 expression in BMMs (Figure 4C, 4D). Additionally, we also transfected miR-5107 mimic and miR-5107 inhibitor into BMMs; the results revealed that miR-5107 mimic inhibited circRNA_009934 expression and miR-5107 inhibitor increased circRNA_009934 expression (Figure 4E, 4F). All above, these results suggested that miR-5107 directly bound to circRNA_009934 at the recognition sites.

CircRNA_009934 Serves as a Molecular Sponge for MiR-5107 to Control the Expression TRAF6

TRAF6 is one of the most important transcription factors regulating osteoclastogenesis. To test whether miR-5107 targets the TRAF6, we performed qRT-PCR analysis for TRAF6 in the presence of miR-5107 mimics or inhibitor. We observed that the mRNA expression level of TRAF6 was strongly suppressed after transfection of miR-5107 mimics (Figure 5A). The Western blot revealed that TRAF6 expression was decreased when BMMs were treated with the miR-5107 mimics, indicating that miR-5107 downregulated TRAF6 expression (Figure 5B). To validate this mechanism, we cloned the mice TRAF6 3′-UTR into the Luciferase reporter vector and constructed miR-5107 binding mutants in which the putative miR-5107 binding sites CAGCAU in the TRAF6 3′-UTR were mutated into GUCGUA (Figure 5C). As expected, Dual-Luciferase report results showed that miR-5107 mimics significantly downregulated the TRAF6 expression whereas point mutations in the TRAF6 3′-UTR abrogated the suppressed effect of miR-5107 (Figure 5D). Next, we validated whether circRNA_009934 can regulate TRAF6 expression *via* competition for miR-5107 bind-

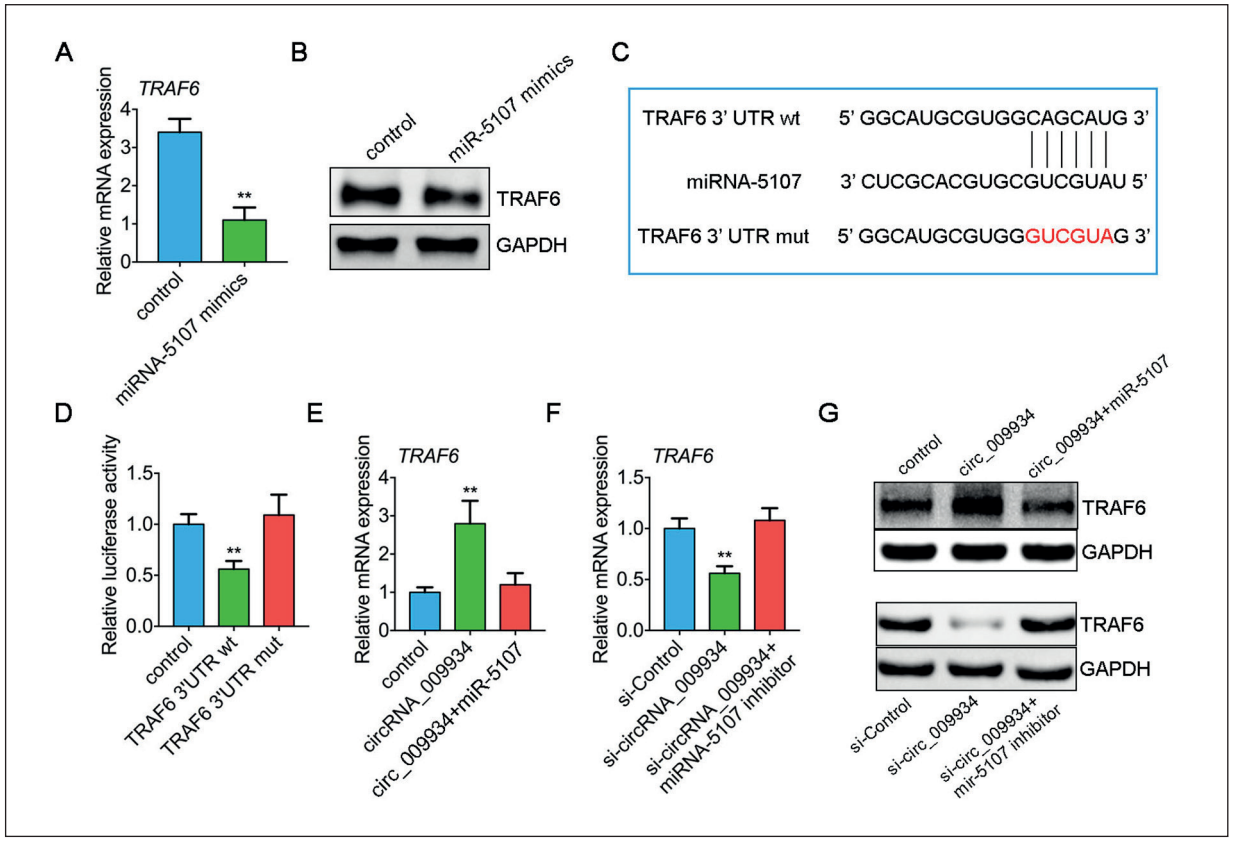

Figure 5. CircRNA_009934 acts as a molecular sponge for miR-5107 and controls the miR-5107 target, TRAF6. **A,** qRT-PCR analysis of TRAF6 mRNA expression level in BMMs treated with the miR-5107 mimics. **B,** Western blot analysis of TRAF6 expression level. **C,** Schematic illustration of the predicted TRAF6 binding sites and mutant sites in miR-5107. **D,** Relative Luciferase activity of BMMs. **E,** mRNA expression levels of TRAF6 in BMMs transfected with circRNA_009934 and circRNA_009934+miR-5107. **F,** mRNA expression levels of TRAF6 in BMMs transfected with si-circRNA_009934, si-circRNA_009934 and miR-5107 inhibitor by qRT-PCR. **G,** The protein expression level of TRAF6 measured by WB. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

ing. The results showed that circRNA_009934 could significantly increase TRAF6 expression; however, mutagenesis of the miR-5107 recognition element in circRNA_009934 abrogated effectively the function (Figure 5E). Conversely, the inhibition of miR-5107 overcame the suppression of TRAF6 by circRNA_009934 knockdown (Figure 5F). On the protein level, Western blot revealed the same results (Figure 5G). Taken together, these data indicate that by interacting with miR-5107, circRNA_009934 acts as a molecular sponge for the miR-5107 target TRAF6, to partly modulate the inhibitory effect of miR-5107 on TRAF6.

Discussion

Osteoclastogenesis is a highly regulated process which is important for bone health[28,29]. The expression of genes of osteoclasts undergoes great changes during osteoclastogenesis, resulting in differences in cells shape and function[28,30-32]. Therefore, the intervention and regulation of several osteoclast-associated genes during osteoclastogenesis is of great significance for regulating osteoclastogenesis[33-35].

The study of lncRNAs in skeletal system is generally rare. Pioneering studies[36] were performed on the expression profile of circRNA during osteoclast differentiation. Meng et al[37] have manifested that circRNA functions as crucial gene regulators by their post-transcriptional modification, such as binding miRNA, assembling RNA-binding proteins, and modulating transcription factors. CircRNA contains miRNA-binding site and usually behaves as a miRNA sponge to negatively regulate expression of target mRNAs[38]. Current studies have shown circRNAs to be involved in the occurrence and development of multiple diseases (such as atherosclerosis, myotonic dystrophy, and prion disease) as miRNA sponges, and were closely related to nervous system diseases (such as Alzheimer's disease and Parkinson's disease)[26,27]. CircRNAs play important roles in tumor development and drug resistance, and they are expected to be suitable molecular markers for tumor diagnosis and therapeutic targets[39-41]. However, the regulatory role of circRNA in bone remodeling, especially in the regulation of osteoclastogenesis, has been poorly reported. In this study, we found circRNA_009934 plays a role in osteoclast differentiation. Further studies showed that circRNA_009934 exerts its regulatory roles *via* harboring miR-5107 to reduce the expression of TRAF6, suggesting that circRNA_009934 served as ceRNA for miR-5107 to regulate TRAF6 expression.

Conclusions

The present study verified the high expression of circRNA_009934 in osteoclasts and found its important role in osteoclastogenesis and bone resorption function. Through target prediction, we found that miR-5107 was one of the target miRNAs of circRNA_009934 and validated the combination relationship of circRNA_009934 and miR-5107 using Luciferase reporter assay. Next, we found that miR-5107 can bind with circRNA_009934 co-expression gene TRAF6 and downregulate the expression of TRAF6. CircRNA_009934 could significantly upregulate TRAF6 gene expression; however, mutagenesis of the miR-5107 recognition element in circRNA_009934 abrogated the function effectively. Therefore, we proved that circRNA_009934 served as ceRNA of miR-5107 to upregulate TRAF6 expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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